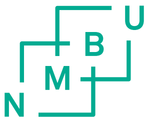

Norwegian University of Life Sciences
Faculty of Biosciences
Department of Animal and Aquacultural Sciences

Philosophiae Doctor (PhD)
Thesis 2020:76

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Økt utnyttelse av fytinsyrebundet fosfor i fôr til fjørfe og gris gjennom økt fytaseaktivitet i fremre del av fordøyelsessystemet

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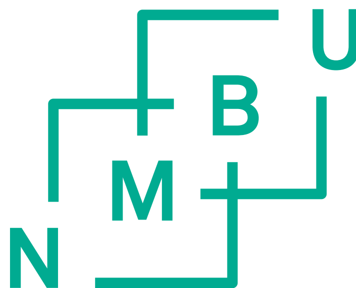

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Supervisors

Prof. Birger Svihus
Department of Animal and Aquacultural Sciences
Norwegian University of Life Sciences
Postboks 5003 NMBU
N-1432 Ås, Norway

Ass. Prof. Nils Petter Kjos
Department of Animal and Aquacultural Sciences
Norwegian University of Life Sciences
Postboks 5003 NMBU
N-1432 Ås, Norway

Dr. Torger Gjefsen
Felleskjøpet Rogaland Agder
Postboks 208 Sentrum
N-4001 Stavanger, Norway

Evaluation Committee

Prof. Øystein Ahlstrøm
Department of Animal and Aquacultural Sciences
Norwegian University of Life Sciences
Postboks 5003 NMBU
N-1432 Ås, Norway

Prof. Damian Józefiak
Poznan University of Life Sciences
Department of Animal Nutrition
Ul. Wołyńska 33
60-637 Poznań, Poland

Dr. Hilde Schøyen
Fiskå Mølle
Fiskåvegen 1010
N-4120 Tau, Norway

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Abbreviations

P	Phosphorus
Ca	Calcium
Mg	Magnesium
Fe	Iron
Zn	Zinc
Cu	Cobber
InsP	Inositol phosphate
DM	Dry matter
FI	Feed intake
AJD	Apparent jejunal digestibility
AID	Apparent ileal digestibility
ATTD	Apparent total tract digestibility
CON	Control diet
PHY	Diet with phytase added
ACID	Diet with acid added
PHYA	Diet with phytase and acid added.

Summary

The majority of phosphorus (P) in feedstuffs is present as phytate-P. Phytate is the storage form of P in seeds and grains and consists of an inositol ring with six phosphate groups bound to it. The phytate-P is only partially utilised by monogastric animals. The undigested P is excreted in the animal manure and may then lead to drainage of P from agricultural land and subsequently environmental pollution. To reduce the amount of P in faeces exogenous phytase is routinely added to the diet of monogastric animals. However, even with phytase added, the degradation of phytate is not complete. The efficacy of phytase is dependent on pH levels in addition to the retention time in the segments of the digestive system with the optimal pH. The most important site for exogenous phytase activity is in anterior digestive tract, due to the pH levels there.

To test the effect of acid addition to the diet on the efficacy of an exogenous phytase, and whether the efficacy would be further improved by an increased retention time in the anterior digestive tract, three animal experiments were carried out. In all animal experiments, a wheat-based diet with a high phytate-P content was used. The diet had either no addition (CON), formic acid added (ACID), 500 FTU *C. braakii*-derived phytase added (PHY) or both acid and phytase added (PHYA). In addition, an *in vitro* experiment where the efficacy of four commercial phytase products at selected pH levels and incubation times was tested, and a field assessment of the crop usage in commercial broiler chickens was carried out.

The results from the *in vitro* experiment described in Paper I was used to determine which phytase and pH levels to use in the experimental diets. In experiment I, 800 broiler chickens in 80 different pens were either *ad libitum* or intermittently fed the experimental diets from 15 to 36 days of age. From one bird per pen, tibia and contents from jejunum and ileum were collected. Crop contents were collected from intermittently fed birds 80, 160 and 240 min after the start of the feeding. The results showed that there was a positive effect of phytase on P digestibility and bone mineralisation. Already after 80 minutes retention time in the crop, there was an effect of acid addition on phytase efficacy, with a considerably enhanced phytate degradation and formation of lower inositol phosphate-isomers. A prolonged retention time in the crop further increased this degradation. Phytase increased P digestibility, intermittent feeding increased apparent jejunal digestibility (AJD) of P and P retention.

In Paper II, a field assessment was carried out to map the extent of crop usage and thus retention time in *ad libitum* fed commercial broiler chickens. The assessment showed that *ad libitum* fed birds use the crop for storage to a higher degree than previously assumed. In the chicken experiment (experiment II), the short-term effect of acid addition on phytase efficacy in the crop was studied. At 20 and 21 days of age, starved birds were fed for one hour, and crop and gizzard contents were collected every 20 minutes until 140 minutes after the start of the feeding. In the birds euthanised 60 and 140 minutes after the start of the feeding, contents from jejunum and ileum were collected as well. Phytase improved P retention, and the PHYA diet increased the AJD of P. All diets reduced the concentration of phytate in the crop with time. However, the PHYA diet resulted in a higher reduction than the other two diets from 20 minutes and onwards. Simultaneously, the concentration of the lower inositol phosphate isomers increased.

In Paper III, 32 piglets with a mean weight of 21.1 kg were distributed in eight pens with recording of individual feed intake. Equal amounts of the experimental diets were fed twice daily. Performance was recorded, pH in the stomach, jejunum and ileum was measured and content from the jejunum and ileum was collected for assessment of P digestibility. Faeces was collected for total digestibility measurements, and left third and fourth metacarpal were analysed for bone mineralisation. Phytase addition increased growth, AJD of P, apparent total tract digestibility (ATTD) of P and Ca in addition to bone mineralisation. Acid addition improved growth, feed efficacy and ATTD of Mg, Fe and Ca, however no interaction effect between acid and phytase was found.

Even though acid addition reduced the stomach pH in pigs no indications of an increased phytase activity with acid addition was found. In chickens, an increased phytate degradation in the anterior digestive tract and improved AJD of P was found with acidification of the diet containing phytase. The effect of acid addition on phytase activity in the crop in broiler chicken was found already after a short retention typically could be observed with *ad libitum* feeding, and was increased with increased retention time in the crop. However, no additional effect of acid addition was found in the apparent ileal digestibility (AID) and the total tract digestibility of P in any of the experiments.

Sammendrag

Størstedelen av fosfor (P) i fôrvarer finnes i form av fytat-P. Fytat er lagringsformen for P i frø og korn og består av en inositolring med seks fosfatgrupper bundet til den. Fytat-P er bare delvis tilgjengelig for enmagede dyr. Ufordøyd P skilles ut i gjødsla og kan deretter føre til avrenning av P fra jordbruksmark og dermed forurensning. For å redusere mengden P i gjødsla blir derfor eksogen fytase rutinemessig tilsatt fôret til enmagede dyr. Selv med tilsatt fytase er ikke nedbrytningen av fytat fullstendig. Effekten av fytase er avhengig av pH-nivået i tillegg til oppholdstiden i de delene av fordøyelseskanalen med optimal pH. Det viktigste stedet for eksogen fytaseaktivitet er i den fremre delen av fordøyelseskanalen på grunn av pH-nivået der.

For å teste effekten av syretilsetning i fôret på effektiviteten til en eksogen fytase, og om denne effekten ville forbedres ytterligere ved økt oppholdstidtid i den fremre delen av fordøyelseskanalen, ble det utført tre dyreforsøk. I alle forsøkene ble det brukt et hvetebasert fôr med et høyt innhold av fytat-P. Fôret var enten uten tilsetninger (CON), tilsatt maursyre (ACID), tilsatt 500 FTU C. braakii-derivert fytase (PHY) eller tilsatt både syre og fytase (PHYA). I tillegg ble det gjort et *in vitro* forsøk hvor effektiviteten av fire kommersielle fytaser ved gitte pH-nivåer og inkubasjonstider ble testet, og det ble gjort en kartlegging av bruken av kro i kommersielle slaktekyllinger.

Resultatene fra *in vitro* forsøket som er beskrevet i artikkel I ble brukt til å avgjøre hvilken fytase og hvilket pH-nivåer som skulle brukes i fôret i dyreforsøkene. I forsøk I ble 800 slaktekyllinger fordelt på 80 forskjellige binger enten fôret *ad libitum* eller med måltidsfôring fra 15 til 36 dagers alder. Fra en kylling i hver binge ble tibia og innholdet fra jejunum og ileum samlet. Innholdet i kro ble samlet fra måltidsfôrede kyllinger 80, 160 og 240 minutter etter starten av fôringen. Resultatene viste at fytase økte fosforfordøyeligheten og mineralisering av bein. Allerede etter 80 minutter oppholdstid i kroa var det en effekt av syretilsetning på fytase-effektivitet, med en betydelig forbedret nedbrytning av fytat og danning av lavere inositol fosfat-isomerer. Lengre oppholdstid i kroa økte denne nedbrytningen ytterligere. Fytase økte fordøyeligheten av P og måltidsfôring økte apparent jejunal fordøyelighet (AJD) av P og P retensjon.

I artikkel II ble det gjennomført en kartlegging av omfanget av bruken av kro, og dermed oppholdstid, i *ad libitum* fôrede kommersielle slaktekyllinger. Kartleggingen viste at *ad libitum*-fôrede kyllinger bruker kroa til lagring i større grad enn tidligere antatt. I kyllingforsøket (forsøk II) ble effekten av kort oppholdstid og syretilsetning på fytaseeffektivitet i kro undersøkt. Ved 20 og 21 dagers alder ble fastende kyllinger gitt tilgang på fôr i en time, og innholdet i kro og krås ble samlet hvert tjuende minutt til 140 minutter etter starten av fôringen. Hos kyllingene som ble avlivet 60 og 140 minutter etter starten av fôringen ble innhold fra jejunum og ileum også samlet. Fytase økte P retensjon, og PHYA-fôret økte AJD av P. Alle fôrene reduserte konsentrasjonen av fytat i kro over tid. PHYA-fôret resulterte imidlertid i en større reduksjon enn de andre to fôrene fra 20 minutter og utover. Samtidig økte konsentrasjonen av lavere inositol fosfat-isomerer.

I artikkel III ble 32 smågris med en gjennomsnittsvekt på 21,1 kg fordelt i åtte binger med individuell registrering av fôropptak. Grisene ble tildelt like store mengder fôr to ganger daglig. Produksjonsresultater ble beregnet, pH i magesekken, jejunum og ileum ble målt og innhold fra jejunum og ileum ble samlet for beregning av fordøyelighet av P. Gjødsel ble samlet for beregning av total fordøyelighet og tredje og fjerde metakarpal ble samlet for beregning av mineralisering av bein. Tilsetning av fytase økte tilveksten, AJD av P, og apparent total fordøyelighet (ATTD) av P og Ca i tillegg til mineraliseringen av bein. Tilsetning av syre økte tilveksten, fôrutnyttelsen og total fordøyelighet av Mg, Fe og Ca, men ingen samspillseffekt mellom syre og fytase ble funnet.

Selv om tilsetning av syre reduserte pH i magesekken hos gris, ble det ikke funnet indikasjoner på økt fytaseaktivitet med syretilsetning. Hos kyllinger ble det funnet en økt nedbrytning av fytat i den fremre delen av fordøyelseskanalen og økt jejunal fordøyelighet av P med tilsetning av syre til fôret med fytase. Det ble også funnet en effekt av syretilsetning på fytaseaktivitet allerede etter kort oppholdstid i kroa hos slaktekylling, som typisk kan bli sett med *ad libitum* fôring, og ble økt med lengre oppholdstid i kroa. Imidlertid ble det ikke funnet noen tilleggseffekt av syretilsetning på apparent ileal fordøyelighet (AID) og total fordøyelighet av P i noen av forsøkene.

List of publications

This thesis is based on the three papers listed below:

Paper I: S. Kristoffersen, K. Itani, A. Benzertiha, B. Kierończyk, N. P. Kjos, and B. Svihus. Effect of crop retention time and acidification of the feed on phytase efficacy in broiler chickens. (Under revision for publication in British Poultry Science)

Paper II: S. Kristoffersen, Z. Wiśniewska, S. Kaczmarek, T. Gjefsen, N. P. Kjos, A. Cowieson and B. Svihus. Assessment of crop usage in *ad libitum* fed birds and short-term phytase efficiency as affected by acid addition. (Submitted November 2020)

Paper III: S. Kristoffersen, T.Gjefsen, N. P. Kjos, and B. Svihus. The effect of reduced feed pH, phytase addition and their interaction to improve mineral utilization in pigs. (Submitted October 2020)

1. Introduction

The majority of P in feedstuffs are present as phytate-P (Selle and Ravindran 2008) and this P is only partially available for absorption by monogastric animals. Theoretically, the amount of P in feed ingredients is adequate to cover the requirement of P for monogastric animals. However, as the phytate-P is partly unavailable for digestion, P from a mineral source is added, often with safety margins, to the diet to cover the P requirement. Excessive and undigested phytate-P will be excreted in the animal manure. This may contribute to environmental pollution as drainage of P from agricultural land can cause eutrophication of water (Singh 2008). A reduction of the amount of P in animal manure is important to reduce this pollution and this is especially important in areas with intensive animal farming. To reduce the P in manure from monogastric animals a high utilisation of phytate-P is essential.

1.1 Phytate

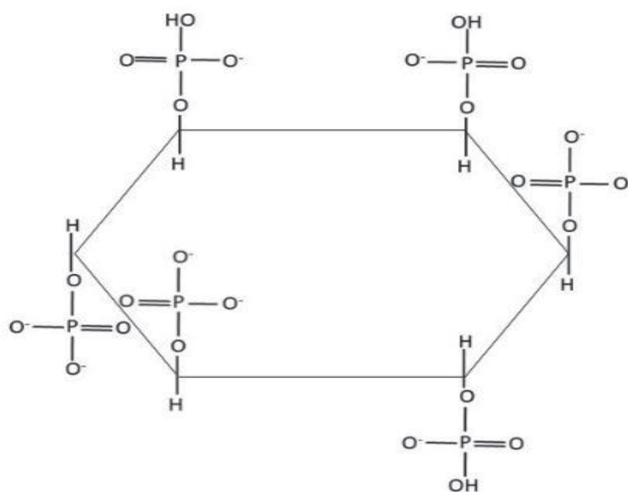

Figure 1 Structure of a phytic acid at neutral pH based on the Anderson model described by Erdman (1979)

Phytic acid is the free form of myo-inositol hexakisphosphate ($InsP_6$) (Figure 1). This consists of an inositol ring with six phosphate groups bound to it. However, phytic acid is unstable and occurs mainly in the form of salt in plants and under physiological

conditions, this salt is called phytate. Phytate is the storage form of P and other minerals in seeds (Humer *et al.* 2015).

The total amount of P and the percentage of P bound in phytate varies both between different plants, and between different parts of the plants. The portion of total P occurring as phytate-P varies from 45 % (soybeans) to 88 % (oat, corn, wheat bran) in the commonly used feedstuffs listed in Table 1. There are in general a higher content of total P and phytate-P in cereal by-product than in whole grains.

Table 1 Phosphorous, phytate and phytase activity in common feedstuffs. The range are the average values from Viveros et al. (2000) and Selle et al. (2003).

Feedstuff	Total P (%)	Phytate-P (%)	Proportion (%)[1]	Phytase activity (FTU/kg)
Oat	0.24-0.29	0.17-0.21	59-88	38-84
Wheat	0.29-0.31	0.22-0.23	71-79	503-1637
Barley	0.27- 0.31	0.19	61-70	348-1016
Corn	0.23-0.24	0.18-0.21	78-88	25-70
Wheat bran	0.80-1.16	0.70-0.88	76-88	2173-4624
Oat bran[2]	0.83	0.68	82	25
Corn gluten meal	0.42-0.50	0.29-0.42	69-84	45-173
Soybean	0.56-0.73	0.31-0.33	45-55	32-40
Soybean meal[3]	0.67	0.45	67	42
Rapeseed meal	0.88-1.05	0.67-0.76	72-76	5-41

[1]Calculated as phytate-P/total P

[2]From Viveros *et al.* (2000) only

[3]From Selle *et al.* (2003) only

The phytate bound P is not available for absorption in the intestine of monogastric animals. The phosphate groups need to be liberated from the inositol ring in order to be available for absorption, and this process is catalysed by the enzyme phytase.

Phytate has an anti-nutritive effect by forming chelates with other molecules like protein, carbohydrates, and cations like Ca, Fe, Zn, Mg, Mn and Cu (Figure 2). These chelates makes

the phytate-bound molecules less accessible for digestion and may make the phytate more resistant to hydrolysis by enzymes (Zyla *et al.* 1995; Maenz *et al.* 1999). The potential for forming chelate with other molecules is dependent of the pH, a higher pH increases the binding potential (Maenz *et al.* 1999; Vieira *et al.* 2018). A degradation of the phytate whereby phosphate groups are released from the inositol ring reduces the binding capacity to other molecules, as there will be fewer potential binding sites left (Vieira *et al.* 2018).

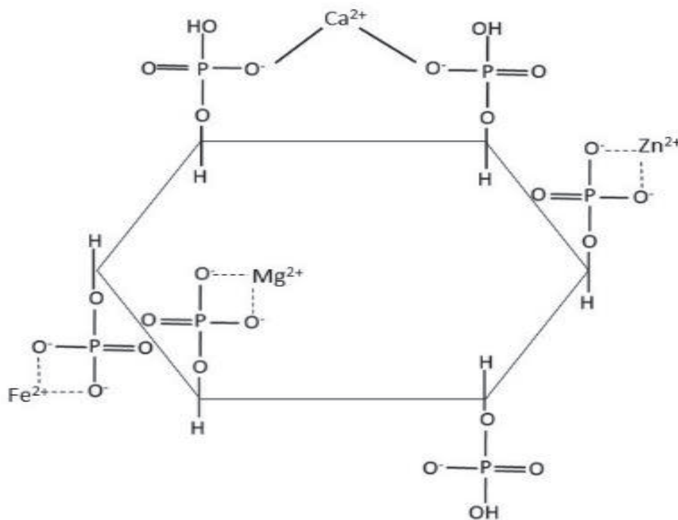

Figure 2 Phytate chelated with several cations at neutral pH (Erdman 1979)

1.2 Phytase

Phytases are the enzymes that catalyses the stepwise hydrolysis from $InsP_6$ to inorganic phosphate and myo-inositol and they are categorised as phosphatases. The hydrolysis generates a series of lower InsP-isomers via a stepwise reaction illustrated in Figure 3, and the potential end products of this reaction is one myo-inositol and six free phosphate groups.

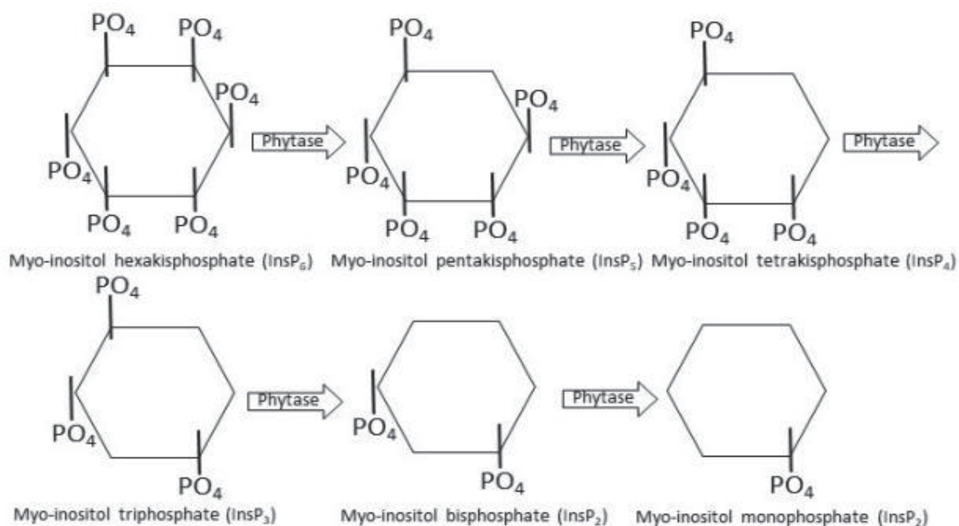

Figure 3 Stepwise degradation of phytate by phytase

There are four potential sources for phytases present in monogastric animals: intrinsic phytase from the feedstuffs, mucosal phytase and other phosphatases from the small intestine, phytase produced by the microflora in the intestine and exogenous enzymes included in the diet (Selle and Ravindran 2007, 2008).

The amount of intrinsic plant phytase varies widely between different feedstuffs (Table 1). High temperature reduces the stability of intrinsic phytase (Esmaeilipour *et al.* 2012). Thus, phytases from feedstuff are mostly inactivated during the production of commercial feed mixtures for monogastric animals due to the high temperatures used. The commonly used processes of steam-pelleting or extruding, almost eliminated the intrinsic phytase activity in the feedstuffs (Jongbloed and Kemme 1990; Schlemmer *et al.* 2001). Therefore, the intrinsic plant phytase has no or very limited effect on phytate degradation in ordinary commercial diets, which for other good reasons are either pelleted or extruded.

The mucosal phytase activity has been regarded to be of little importance in monogastric animals (Selle and Ravindran 2007). However, poultry and pigs have very effective phytases and other phosphatases, both mucosal and bacterial, in the intestine. A degradation of $InsP_6$ from the jejunum to the duodenum without exogenous phytase addition has been observed in chickens (Zeller *et al.* 2015a, 2015b), indicates some

mucosal phytase activity. Tamim *et al.* (2004) found a considerable ileal phytate degradation (69 %) in broiler chickens without exogenous phytase or Ca added in the diet. However, when Ca was added to the diet this degradation was significantly reduced to 25 %. This demonstrate that the commonly used dietary Ca levels in commercial broiler diets have a substantial negative influence the phytate degradation. In pigs there has been found a mucosal phosphatase activity in the small intestine (Hu *et al.* 1996; Schlemmer *et al.* 2001) which might influence phytate degradation. The effect of the mucosal phosphatases and phytases are limited because of the low solubility of phytate at the pH-levels found in the intestine. In addition, the high dietary concentration of Ca reduces the solubility of the phytate by forming insoluble phytate complexes in the intestine (Selle *et al.*, 2009). Microflora in the hindgut also produces phytases. This phytases could contribute to a near complete degradation of InsP6 in both pigs and poultry (Selle and Ravindran 2007, 2008). However, as the main absorption of P occurs before the last two-thirds of the ileum in chickens (Rodehutscord *et al*. 2012) and a negligible part of the P absorption occurs after the ileum in pigs (Baumgärtel *et al*. 2008; Rutherfurd *et al*. 2014), the phytate-P released in the hindgut will not be absorbed.

Exogenous phytase is isolated from bacteria, yeast or fungi and added to diets for monogastric animals to improve degradation of phytate. Phytases can be categorised and grouped based on the pH optimum of the phytase. Acidic phytases have a pH optimum from 3.0 to 5.5, and alkaline phytases have a pH optimum between 7.0 and 8.0 (Humer *et al.* 2015). For most new-generation exogenous phytases the pH for optimum efficacy is between pH 4.0 and 5.0 (Menezes-Blackburn *et al.* 2015; Vieira *et al.* 2018). However, Menezes-Blackburn *et al.* (2015) showed that the optimal pH for seven different commercial phytases varies considerably. The range where 80 % of the maximum phytase activity for each phytase was found varied from pH 3.0 to pH 5.5. Hence, a relatively high phytase activity could be found both with low and higher pH-values depending on the phytase.

The first governmental regulation aimed at limiting pollution due to excessive supplies of P to the environment came in the Netherlands in 1991. As a response to this, the first commercial phytase was introduced to the market (Selle and Ravindran 2007, 2008). Since then there have been a lot of research and development on exogenous phytases and today there are numerous different phytases available commercially. Exogenous phytase

is today routinely added to the diets of monogastric animals to improve phytate degradation and utilisation of phytate-P (Wilcock and Walk 2016).

1.3 Place for exogenous phytase activity

Both pigs and chickens are monogastric animals. The pig has a “simple” digestion system with a single-chambered stomach where gastric juices are secreted. The stomach is followed by the small intestine and then large intestine where a considerable fermentation activity takes place.

The crop in poultry is a distensible sack-formed expansion on the oesophagus. When the feed has entered the crop it will be gradually moisturised (Svihus *et al.* 2010), mostly by drinking water. The crop is not important in the chemical digestion of the feed, because no secretion of digestive juices takes place there. However, exogenous enzymes are activated by moisturising in the crop (Svihus 2014; Classen *et al.* 2016). If there are any intrinsic enzymes in the feed, these may also start to exert their effect in the crop. The stomach in birds consist of two chambers, proventriculus and gizzard. The crop functions as a buffer for the feed flow to the gizzard, and the use of the crop is regulated by the feed content in the gizzard. The proventriculus is a glandular stomach, where gastric juices are secreted. The gizzard follows the proventriculus and is a muscular stomach without any secretion of digestive juices.

Phytate degradation by exogenous enzymes primarily takes place in the anterior part of the digestive system, the crop, proventriculus and gizzard of chicken and the stomach of pigs (Selle and Ravindran 2007, 2008; Classen *et al.* 2016). Up to 71 % degradation of $InsP_6$ has been observed in the crop (Zeller *et al.* 2015a, 2016; Sommerfeld *et al.* 2018). The main reason that the exogenous phytase activity is highest in the forestomach is that the pH level there is more beneficial for phytase activity and phytate solubility than in the intestine. An early onset of the phytate degradation is beneficial because most of the P absorption takes place in the anterior part of the intestine. A degradation of phytate in the anterior digestive tract also reduces the anti-nutritive effects of phytate (Vieira *et al.* 2018).

Chicken feed normally has a pH between 6.0 (Ao *et al.* 2008) and 6.5 (Sacranie *et al.* 2017), and the pH in the crop is normally at the same level when the feed enters the crop.

Therefore, the pH in the crop is usually somewhat higher than the optimal pH for exogenous phytase activity. Pig feed have been reported to have a pH between 6.3 and 7.3 (Radcliffe *et al.* 1998; Kemme *et al.* 1999; Omogbenigun *et al.* 2003; Blank *et al.* 2012). When the feed enters the stomach of pigs and the proventriculus and gizzard of chickens, gastric juices with a low pH will be mixed with the feed and reduce the pH of the digesta. The pH in the stomach of pigs has been reported to be between 1.8 and 4.8 with an average pH of 3.5 (Dersjant-Li *et al.* 2001; Omogbenigun *et al.* 2003; Eberhard *et al.* 2007; Lee *et al.* 2018b). The pH in the gizzard is between 1.2 and 3.6 with an average of pH 2.2 (Sacranie *et al.* 2012; Lee *et al.* 2017; Lee *et al.* 2018b; Lee *et al.* 2018a). Even though the pH in the gizzard is somewhat lower than the pH for optimal phytase activity, it is still considered to be an important site for phytase activity.

When the digesta enters the intestine, the pH is quickly elevated to a higher pH due to the mixing with pancreatic juices. In the intestine, the pH normally is between 6.3 and 7.5 in chickens (Ao *et al.* 2008; González-Alvarado *et al.* 2008; Smulikowska *et al.* 2009), and between 5.5 and pH 6.8 in pigs (Dersjant-Li *et al.* 2001; Omogbenigun *et al.* 2003; Eberhard *et al.* 2007). Because of the high pH in the intestine, and hence the reduced solubility of the phytate, this part of the digestive tract is not considered to be an important site of exogenous phytase activity. However, there may be a considerable exogenous phytase activity in the intestine as well, and different phytases have different levels of activity in the intestine (Onyango *et al.* 2005).

The time the feed stays in the favourable environment in the anterior digestive tract is important for the phytase activity (Tamim *et al.* 2004). Retention time in the crop is dependent on feeding regime for the chickens. Observations of *ad libitum* fed broilers have shown that they eat small amounts of feed many times throughout the day (Nielsen 2004; Svihus *et al.* 2010, 2013). This eating pattern will limit the use of the crop for storage and hence it is assumed that *ad libitum* fed birds do not use the crop to a large extent (Svihus 2014). The crop content of *ad libitum*-fed birds also reflects this. Svihus *et al.* (2010) found that 78 % of *ad libitum*-fed chickens had less than five g of dry matter in the crop. Intermittent feeding will in contrast increase the use of the crop and the retention time of the feed in that part of the digestive tract (Svihus 2014). As much as 50 g DM has been found in the crop of intermittently fed birds and this content gradually disappears within a period of up to 5 hours after termination of the last feeding bout

(Sacranie *et al.* 2017). Thus, stimulating retention of feed in the crop could possibly increase phytate degradation when exogenous phytase is being used. Svihus *et al.* (2013) were not able to demonstrate an effect of intermittent feeding on phytase efficacy. However, Sacranie *et al.* (2017) found a higher P digestibility and $InsP_6$ digestibility with intermittent feeding than with *ad libitum* access to feed. Svihus *et al.* (2010) did find a 50 % reduction in concentration of $InsP_6$ in the crop with 100 minutes retention time. Retention time in crop is also associated with a considerable fermentation activity by *lactobacilli* and consequently a production of lactic acid that will reduce the pH in crop over time (Cutler *et al.* 2005; Jozefiak *et al.* 2006).

The retention time of the feed in the stomach of pigs and the flow of digesta from the stomach is influenced by the meal size and the physical state of the diet (Gregory *et al.* 1990). According to Blaabjerg *et al.* (2011), more than half of the DM eaten by pigs leave the stomach before the gastric pH becomes as low as pH 3.5, which is the pH optimum for commercial phytases with the lowest pH optimum. With an increased retention time in the stomach, the degradation of phytate will be improved (Blaabjerg *et al*., 2011).

1.4 Limitations in phytate degradation

The degradation of phytate by exogenous phytase is not complete. In a review by Slominski (2011) it was established that the release of P from phytate in broiler chicken was increased from an average of 19 % without phytase addition to 38 % with phytase addition. In pigs, exogenous phytase addition increased the phytate-P digestibility from 39 % without phytase addition to 65 % with phytase (Rosenfelder-Kuon *et al.* 2020). The incomplete degradation found in this review is an indication that there is a potential for improving the utilisation of phytate-P when phytase is added to the diet. If the phytase efficacy is improved, the utilisation of phytate-P could be increased further. This will be of significance to the industry since it may reduce the amount of P in the manure from farm animals and consequently reduce the environmental pollution.

1.5 Acid and phytase

It has been shown that the addition of organic acid to the feed may reduce the pH in the crop of chicken and in the stomach of pigs (Kim *et al.* 2015; Suiryanrayna and Ramana 2015). The phytase activity in the crop may be increased if the pH is lowered to a pH closer

to the optimum of the phytase. In pigs, it has been shown that a reduced pH in the stomach will reduce the emptying rate (Van der Aar *et al*. 2017). In addition, an reduction in the pH in the stomach by acid addition in the feed may lead to an increased degradation of phytate because the pH is immediately lowered to a more beneficial pH for phytase activity and phytate solubility.

In pigs, there are conflicting results regarding the effect of acid addition on phytase efficacy. Several experiments have shown that the addition of organic acids have a positive effect on P digestibility in pigs (Kemme *et al.* 1999; Jongbloed *et al.* 2000). However, other experiments have failed to show an interaction between organic acid and phytase efficacy (Radcliffe *et al.* 1998; Omogbenigun *et al.* 2003). In chickens there is also conflicting results on this topic, a meta-analysis by Vieira *et al.* (2017) showed that the combination of citric acid and phytases improved performance and bone mineralisation in broiler chickens. Emami *et al.* (2013) and Woyengo *et al.* (2010) found an increased ileal P digestibility by combining organic acid with phytase, however in the review by Vieira *et al. (*2018) few of the cited experiments found an additional effect of acid on phytase efficacy.

As there is a potential for better utilisation of phytate-P in monogastric animals, more knowledge about the effect of acidification of the diet on phytase efficacy in the anterior digestive tract is required. Together with the question if this effect would be improved by an increased retention time in the anterior part of the digestive tract, and how this effects the total tract digestibly this was the basis for the objectives in this study.

2. Aims of the thesis

The main aim of this thesis was to contribute to an improved phytate-P degradation and P utilisation by improving the efficacy of phytase. The aim was derived from a target of reducing the amount of P in manure from monogastric animals.

In order to achieve this aim, the following hypotheses were tested:

- Acidification of the diet will increase exogenous phytase efficacy through increased phytate degradation in the anterior digestive tract.
- An increased phytate degradation in the anterior digestive tract will improve overall P digestibility.

In addition, this specific hypothesis for chickens was tested:

- The effect of acid addition to the diet on phytase efficacy will be further improved by an increased crop retention time due to intermittent feeding.

3. Methodology

Working with this thesis, the hypotheses was approached by an *in vitro test*, a field assay and three animal experiments. The *in vitro* study was carried out to investigate the effect of acid and retention time on different commercial phytases. A field assessment to map the crop content in broiler chickens from commercial farms was conducted. Two broiler chicken experiments, one with chickens in pens, and one with chicken in individual cages, were carried out to assess information about the effect of acid addition and retention time in the crop on phytase efficacy. One pig experiment was carried out to assess the effect of acid addition on phytase efficacy in pigs.

3.1 *In vitro* test

A simple *in vitro* procedure simulating the crop was used to test the efficacy of four commercial phytase products at selected pH levels and incubation times. One g (±0.01 g) of the diet was mixed with 5.0 ml deionised water, 0 or 500 FTU phytase and 0.0, 4.0, 13.0 or 50.0 µl formic acid to reach the pH-levels of 6.7, 5.5, 4.5 and 3.5 in a test tube. The test tubes were vortexed well before incubation in a 40°C water bath, where they were shaken by hand every 5 minutes. After an incubation of 10, 20, 30, 45 or 60 min, the enzymatic reaction was terminated by adding 5.0 ml 4% trichloroacetic acid (TCA). Thereafter the test tubes were centrifuged, followed by collection of the supernatant and analysis for free phosphate as described in Paper I. The phytase with the largest response to a reduced pH was the *C. braakii*-derived phytase, and this was chosen together with a pH level of 4.5 to be used in the diets for the *in vivo* experiments.

3.2 Experiment I

Effect of acid addition on phytase efficacy in intermittently and *ad libitum* fed chickens

Eight hundred one-day-old male Ross 308 chickens were randomly assigned to 80 pens with 10 birds in each. The pens were arranged in the middle of an environmentally controlled broiler house with 9,000 loose-housed birds hatched at the same time surrounding the experimental pens. The chickens were *ad libitum* or intermittently fed either a high phytate-P diet (CON), or the same diet with formic acid added (ACID), 500 FTU *C. braakii*-derived phytase added (PHY) or both added (PHYA) in a 2 x 2 x 2 factorial

design from day 15 to 36 of age. The a*d libitum* fed birds had two 4-hour dark periods with 2 hour light in between. The intermittently fed birds had access to feed between the hours of 08:00 to 09:00, 12:00 to 13:00, 16:30 to 17:30, 21:00 to 22:00 and from 02:00 to 04:00.

On day 36, one bird from each *ad libitum* fed pen was killed for collection of content from the crop, the jejunum and the ileum in addition to the left tibia. On day 37, one bird per pen of intermittently fed birds was killed for collection of the crop content 80, 160 and 240 minutes after the start of the feeding. In addition, content from the jejunum and the ileum and the left tibia were collected 240 minutes after the start of the feeding. Diet production, sample preparation, analytical procedures and more details on the sampling procedure are described in Paper I.

3.3 Field assessment

A field assessment was carried out to map the extent of crop usage and thus retention time in commercial broiler chickens. At 10, 20 and 30 days of age, 40 (±1) chickens in each of four farms were killed. Feeding and management procedures on the farms are described in detail in paper II. The crop content was collected quantitatively immediately after killing and thereafter frozen at -20 °C. After thawing, pH was measured, and DM content was determined by drying at 104 °C overnight.

3.4 Experiment II

Assessment of short-term phytase efficiency as affected by acid addition

The second chicken experiment was designed to investigate the degradation of phytate in the crop shorter time after the start of the feeding and more frequently than in experiment I. At 11 days of age, 120 male Ross 308 chickens were placed in individual cages. The birds were intermittently fed a diet high in phytate-P. Access to feed was given between the hours of 08:00 to 09:00, 12:00 to 13:00, 16:30 to 17:30, 21:00 to 22:00 and from 02:00 to 04:00. As there was no effect of only acid addition in experiment I, the ACID diet was omitted from the second chicken experiment. The diets were either with no addition (CON), with 500 FYT *C. braakii*-derived phytase added (PHY) or with both phytase and formic acid added (PHYA). At 19 days of age, excreta were collected for assessment of P retention. At 20 and 21 days of age, birds that had been without feed for eight hours were

fed for one hour. The crop and gizzard content was collected immediately before the start of the feeding and thereafter every 20 minutes until 140 minutes after the start of the feeding. At 60 and 140 minutes, also contents from jejunum and ileum were collected. Diet production, sample preparation and analytical procedures are described in Paper II.

3.5 Experiment III

Effect of acid addition on phytase efficacy in pigs

Thirty-two pigs were fed a high phytate-P diet (CON), or the same diet with formic acid added (ACID), 500 FTU C. braakii-derived phytase added (PHY) or both added (PHYA) in a 2 x 2 factorial design. At 52 days of age and an average initial body weight of 21.06 kg, the pigs were equally distributed by weight and randomly distributed in groups of four. They were kept in eight different concrete-floored, partially slatted pens, with individual feeding stations. The individual feeding stations enabled recording of individual feed intake and that all four dietary treatments were represented in all pens. The pigs were fed equal amounts twice daily at 08:00 and 14:00 based on an estimated feed intake of 3 % of the live body weight, and leftovers were recorded. Individual faecal samples were collected at experiment days 24 to 27. Dissection was carried out on day 28 and 29, starting two hours after the morning feeding. Stomach, jejunum and ileum pH were measured. Intestinal content from the jejunum and the ileum and the left third and fourth metacarpal from each pig were collected. Diet production, sample preparation and analytical procedures are described in Paper III.

4. Main results and discussion

The main aim of this thesis was to consider ways to contribute to a better phytate-P utilisation by improving the efficacy of phytase. This was done by reducing the pH and prolonging the retention time in the anterior digestive tract. In this section, the main results presented in Papers I, II and III are discussed with respect to this aim.

The work carried out for this thesis confirmed that phytase addition improved P digestibility as expected, with an increased P retention in the chicken experiments and an improved ATTD of P in the pig experiment (Selle and Ravindran 2007, 2008). In addition, an improved bone mineralisation by phytase addition was found. These results are in concurrence with previous knowledge (Selle and Ravindran 2007; Singh 2008; Torres-Pitarch *et al.* 2017).

In the pig experiment, even though acid addition reduced the stomach pH no increased P digestibility was found. An effect of pH-reduction and increased retention time in the anterior digestive tract of chickens for the efficacy of phytase was demonstrated. There was an increased phytate degradation with the PHYA diet compared to the PHY diet even with short retention times, however the effect was amplified with a prolonged retention time. The increased phytate degradation led to an improved AJD of P. However, there was no additional effect of the PHYA diet compared to the PHY diet on the AID of P or the P retention, this suggests that the level of pH and the degradation of phytate in the anterior parts of the digestive system is not limiting for P retention in intermittently fed chickens.

Both a reduced pH and an early onset of the degradation of phytate reduces the chelating capacity of the phytate to cations, and hence reduce the negative effects of phytate on the digestibility of these minerals (Yu *et al.* 2012). Therefore, the effect of phytase and acid addition on the digestibility of Ca, Zn, Fe, Cu and Mg was examined in experiment III. Acid addition increased the total tract digestibility of Ca, Mg and Fe, which could indicate that smaller amounts of these minerals were bound to the phytate when pH in the stomach was reduced. The ATTD of Ca was also increased by phytase addition. As Ca it the most likely mineral to be bound to phytate due to the high level of Ca in the diet (Humer *et al.* 2015), this effect on Ca digestibiilty was expected. This increased Ca digestibility with acid addition is indicating that the phytate solubility is increasing when acid was added.

4.1 Retention time and pH in the anterior digestive tract

The *in vitro* test in Paper I was carried out to study how the phytase efficacy of four different commercial phytases was influenced by a reduction in pH and an increased incubation time. All phytases had an increased phytase efficacy with a reduced pH. The phytase with the greatest response to a reduced pH was the *C. braakii*-derived phytase, and this was chosen together with a pH level of 4.5 to be used in the diets for the *in vivo* experiments. An increased incubation time increased the degradation of phytate for all phytases, in accordance with Tamim *et al.* (2004). Based on this, together with the results presented by Svihus *et al.* (2010) where the phytate degradation was increased with increased retention time in the crop, intermittent feeding was used in order to increase the retention time of the feed in the crop in the chicken experiments in this thesis.

The DM content of the crop was gradually reduced for the intermittently fed birds in experiment I and II. In both experiments, three hours passed between most of the feeding bouts, and the total retention time was approximately similar to the time from the start of one feeding bout to the start of the next. In experiment I, there was 2.5 g DM left in the crop 4 hours after the start of the feeding. The estimated total retention time in experiment II, based on the crop content and assumed passage rate, was 3 hours and 51 minutes. This confirms that intermittent feeding stimulates storage of large quantities of feed in the crop in similarity with previous reports (Svihus 2014; Sacranie *et al.* 2017).

The crop content of *ad libitum* fed birds in experiment I reflected that they use the crop less actively than intermittently fed birds. This was expected since observations of *ad libitum* fed broilers have shown that they eat semi-continuously (Nielsen 2004; Svihus *et al.* 2010, 2013). However, in experiment I, no difference in the crop content between *ad libitum* fed birds and intermittently fed birds 160 minutes after the start of the feeding was found. This was contradictory to the observations of Sacranie *et al.* (2017), who found that intermittently fed birds had a significantly higher crop content than *ad libitum* fed birds 180 minutes after the start of the feeding. The lack of difference in crop content was probably due to a higher crop content in *ad libitum* fed birds than what has been observed earlier, e.g. by Svihus *et al.* (2010). Both the experiment of Svihus *et al.* (2010) and Sacranie *et al.* (2017) were performed with few animals in each cage and little or no visual contact between the birds with differet trreatments. Experiment I was carried out in a

commercial chicken house with loose-housed birds surrounding the pens, and ten birds in each pen. The surrounding birds and "competitive" effect between could have led to the *ad libitum* fed birds consuming larger meals, and hence reduced the difference in amount of crop content between *ad libitum* and intermittently. Thus, it was hypothesised that in a commercial setting the *ad libitum* fed birds use their crop more than what the assumption has been earlier.

In the field assessment in Paper II, the hypothesis that *ad libitum* fed birds also use the crop for storage was confirmed by the high percentage of chickens with more than the expected average hourly FI in the crop. More than the expected hourly FI was found in the crop of 86 % of the 10-day old chickens, while for 20 and 30-day old chickens this percentage was 53 and 47, respectively. The expected average hourly FI was calculated based on table values for daily FI of mixed-sex Ross 308 (Aviagen 2019). From the crop content and the expected average hourly FI, an average retention time in the crop at 10, 20 and 30 days was estimated to be 168, 82 and 84 minutes, respectively. Hence, these results indicates that *ad libitum* fed birds use the crop for storage to a higher degree than previously assumed, and that a substantial retention time in the crop may be observed in *ad libitum* fed birds.

The pH-value of 4.5 in the experimental diets with acid added was chosen based on the response of reduced pH in the *in vitro* experiment for the selected phytase. The pH in the crop was significantly reduced with acidification of the feed in both chicken experiments to 4.4 (experiment I) and 4.1 (experiment II) in accordance with previous knowledge (Kim *et al.* 2015). However, the pH in the gizzard was not affected by acidification. These observed reductions of the pH were expected to increase the phytase activity as the maximum phytase activity for the phytase used was reported to be at pH 4.0 (Menezes-Blackburn *et al.* 2015). There was not found any reduction in pH without acid addition with 160 and 140 minutes retention time in experiment I and II, respectively. However, 4 hours after the start of the feeding in experiment I there was found a reduced pH in the crop of intermittently fed birds, in concurrence with the findings of Sacranie *et al.* (2017). This reduction in pH may be expected as a result of the fermentation activity by *lactobacilli* and their production of lactic acid (Cutler *et al.* 2005; Jozefiak *et al.* 2006).

In experiment III, the pH in the pig stomach was reduced to 4.1 by acid addition as expected (Suiryanrayna and Ramana 2015), and this reduction is of the same magnitude

as to what was observed in the chicken experiments and was also here expected to increase the phytase activity. No planned manipulation or estimation of the retention time in the stomach was carried out. However, a lower pH in the stomach of pigs due to addition of organic acid has been associated with a reduced emptying rate (Van der Aar *et al.* 2017). Therefore, acid addition to the feed might increase the retention time in the stomach of pigs.

4.2 Phytate degradation and P digestibility

As there was a higher degradation of $InsP_6$ and a higher content of both $InsP_4$ and $InsP_3$ with the PHYA diet than with the PHY diet already after 80 minutes retention time in the crop in experiment I, the second chicken experiment was set up to study the effect of acid addition on shorter retention times in the crop. In experiment II, the PHYA diet reduced the concentration of $InsP_6$ compared to the other two diets already 20 minutes after the start of the feeding. The PHYA diet gave a linear increase in concentration of $InsP_5$ and there was found $InsP_{3\&4}$ from 60 minutes in contrast to what was observed with the other two diets. As the effect of acid addition occurred with the short retention time in the crop, it seems reasonable to assume that this effect of acid addition on phytase efficacy might be found with *ad libitum* feeding. The reduction in $InsP_6$ and $InsP_5$ isomers led to higher concentrations of $InsP_4$ and $InsP_3$ with than without phytase addition in experiment I. In experiment II, the degradation of $InsP_6$ increased the concentration of $InsP_5$ and $InsP_{3\&4}$. The increased amount of lower isomers as a result of the degradation of $InsP_6$ is in accordance with what has been described earlier (Zeller *et al.* 2016; Sommerfeld *et al.* 2018). The significant interaction between acid addition and feeding regime on AJD of P in experiment I, where acid addition increased AJD of P in *ad libitum* fed birds, but not in intermittently fed birds, supports the hypothesis that acid addition might be beneficial for the phytase activity in *ad libitum* fed birds. It may be speculated that this interaction of acidification was caused by the positive effect on acid addition in the *ad libitum* fed bird because of the shorter retention time in crop and less time with a lowered pH without acidification. With intermittent feeding, the reduced pH with longer retention times in the crop also without acid in may compensate for the lack of instant pH reduction.

There was an increased degradation of $InsP_6$ with increased retention time in the crop in both experiment I and II. This is in accordance with Svihus *et al.* (2010) who also found a

reduced concentration of $InsP_6$ with increased retention time. The effect of acid addition on phytase efficacy increased with time as well, as a number of results in experiment I and II indicates. There was a significant interaction effect of acid and phytase addition on degradation of $InsP_6$ 160 minutes but not 80 minutes after the start of the feeding in experiment I. In addition, an increased number of significant interactions between acid and phytase was found when the retention time was increased from 80 to 160 minutes, where acid addition increased the concentration of the lower InsP-isomers ($InsP_3$ and $InsP_4$). In experiment II there was an increased formation of the lower isomers ($InsP_5$ and $InsP_{3\&4}$) with the PHYA diet compared to the other two diets from 60 minutes retention time and onwards. There was also found an effect of a prolonged retention time in the crop on the P utilisation in the anterior digestive tract independent of phytase addition. In experiment II, the AJD of P was higher 140 minutes after the start of the feeding than after 60 minutes for all treatments. The AJD of P was increased with intermittent feeding compared to *ad libitum* feeding in experiment I.

There was not a difference between the CON diet and the PHY diet regarding the concentration of both $InsP_6$ and $InsP_5$ in the crop in experiment II. The PHYA diet increased the AJD of P compared to the other two diets as well. With 80 minutes retention time in the crop in experiment I, the same tendency of no effect with only phytase addition on the degradation of $InsP_6$ was found. This is surprising, as the crop is the main site of exogenous phytase activity (Selle and Ravindran 2007) and previous experiments with a shorter retention times in the crop than the current experiments have shown a significant effect on phytase addition on the degradation of $InsP_6$ in the crop (Zeller *et al.* 2015a, 2016; Sommerfeld *et al.* 2018). The lack of phytase effect on degradation of phytate was also reflected in the lack of difference in the AJD of P between the CON diet and PHY diet in experiment II. The lack of effect by only phytase may be due to the high pH in the crop without acid addition, the high pH reduces the solubility of the phytate, making it less accessible for degradation by phytase (Humer *et al.* 2015). As the optimal pH for the phytase used was lower than the observed pH in the crop (Menezes-Blackburn *et al.* 2015) this might also have contributed to the lack of effect of only phytase addition.

In the pig experiment (experiment III), the degradation of phytate was not investigated. However, the increased AJD of P with phytase addition indicates that phytase addition increased the degradation of phytate in the anterior digestive tract. The findings of

Blaabjerg *et al.* (2011) and Kemme *et al.* (2006) supported this, as in both experiments there was found an increased degradation of $InsP_6$ with phytase addition in the stomach and duodenum digesta, respectively. Blaabjerg *et al.* (2011) found that the phytase activity in the stomach was greatest the first hour after feeding, as the gastric pH was closest to the optimal pH for the phytase during this period of time. In experiment III, the pH in the stomach was closest to the optimal pH for the phytase when acid was added, and hence a higher phytase activity was expected with acid addition. There was, however, no indications of an increased phytase activity in the stomach with acid addition in experiment III, as no difference in AJD of P between the PHY and the PHYA diet was found.

The difference in P digestibility between diets with and without phytase addition was reduced from jejunum to ileum in all experiments. In experiment I, phytase addition significantly increased AID of P, but the effect of phytase addition was reduced. Moreover, in experiment II and III, there was not observed any effect of phytase on AID of P, in contrast to previous results with phytase (Zeng *et al.* 2011; Ravindran *et al.* 2000). These results indicate that a considerable phytate degradation takes place both in the anterior and the posterior parts of the digestive system for diets without exogenous phytase added. This is in accordance with earlier observations in both pigs and broiler chickens (Schlemmer *et al.* 2001; Zeller *et al.* 2015a, 2015b), and may be caused of a lower Ca level in the diets than the common level in broiler diets (Appelgate *et al.* 2003). In pigs it has been reported that exogenous phytase addition may reduce the mucosal phytase activity (Selle and Ravindran 2008), and this may have contributed to the lack of difference between treatments.

4.3 Limitations and future perspectives

In all experiments, attempts were made to reduce the difference between diets with regard to other factors than phytase and pH of the diets. However, the addition of both acid and phytase on top of the pelleted diet in experiment I led to an unintentional reduction in phytase activity in the PHYA diet (385 FYT/kg feed) compared to the PHY diet (520 FYT/kg feed). Therefore, in the feed production for experiment II and III, acid was added before pelleting to distribute the acid throughout the pellet and then the phytase was added on top of the pellet after pelleting. This method was successful, and the difference found in the phytase activity in the first experiment din not occur in the last two.

The inclusion of Ca in the diets was lower than the common inclusion level of Ca in the diets used in this thesis, in experiment II the inclusion was particularly low. The Ca:P ratio was kept constant in the diet formulation, and therefore the Ca level was reduced concurrently with the reduction in P level. This did not hamper the result in any of the experiments. However, it is worth noticing that if the Ca level in the diets was higher (around 8-9 g/kg), a different result might have been found. Because the low Ca inclusion could increase the phytate solubility in the intestine and hence reduce the importance of retention time and reduced pH in the crop.

The phytase used in the animal experiments was chosen based on that it responded positive to reduction of pH. Therefore, it must be taken into account when considering future use of these results that the phytase activity of another phytase with a different pH optimum may show a different effect when acid is added.

The effect on phytate degradation when phytase and acid was combined indicates that there is a considerable potential for improving the efficacy of exogenous phytases by optimising the conditions in which the phytases are used. More research is needed to clarify the potential of such efforts both in pigs and broiler chickens. Subjects as, for example, the dietary phytate and cation concentration, the effect of the pH in drinking water and higher phytase doses combined with an increased retention time in the anterior digestive system should be assessed. In future studies, analyses of InsP-isomers in other segments of the digestive system as the gizzard for chicken, stomach for pigs and different segments of the intestine may give us useful information on this topic.

5. Concluding remarks

It can be concluded from the experiments carried out in the present thesis that:

- Even though acid addition reduced the stomach pH in pigs, no indications of an increased phytase activity in the stomach with acid addition was found.
- The increased ATTD of Ca with acid addition suggest that acid addition is beneficial for the phytate solubility.
- The results from the crop DM measurements show that there will be an increased retention time in the crop with intermittent feeding of chickens. However, *ad libitum* fed birds could also have a substantial retention time of the feed in the crop.
- Acidification of the diet containing phytase increased phytate degradation in the anterior digestive tract of chickens, and this improved the AJD of P.
- The effect of acid addition on phytase activity in the crop in broiler chicken was found already after a short retention typically observed with *ad libitum* feeding. This effect was increased with increased retention time in the crop.
- The phytate degradation and P digestibility in the anterior digestive tract does not appear to be limiting for P retention under intermittent feeding situations of chickens.

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Paper I

Effect of crop retention time and acidification of the feed on phytase efficacy in broiler chickens.

Siril Kristoffersen, Faculty of Biosciences, Norwegian University of Life Sciences, P.O. Box 5003, N-1432 Aas, Norway and Felleskjøpet Rogaland Agder, Sandvikveien 21, N-4002 Stavanger, Norway. Email: siril.kristoffersen@fkra.no (corresponding author)

Khaled Itani, Faculty of Biosciences, Norwegian University of Life Sciences, P.O. Box 5003, N-1432 Aas, Norway. Email: kwi01@hotmail.com

Abdelbasset Benzertiha, Department of Animal Nutrition and Feed Management, Poznań University of Life Sciences, Wołyńska 33, 60-637 Poznań, Poland. Email: basset@up.poznan.pl

Bartosz Kierończyk, Department of Animal Nutrition and Feed Management, Poznań University of Life Sciences, Wołyńska 33, 60-637 Poznań, Poland. Email: bartosz.kieronczyk@up.poznan.pl

Nils Petter Kjos, Faculty of Biosciences, Norwegian University of Life Sciences, P.O. Box 5003, N-1432 Aas, Norway. Email: nils.kjos@nmbu.no

Birger Svihus, Faculty of Biosciences, Norwegian University of Life Sciences, P.O. Box 5003, N-1432 Aas, Norway. Email: birger.svihus@nmbu.no

Abstract

1. One *in vitro* test to study the effect of pH reduction and incubation time on four commercial phytases was conducted. Both pH and time had a positive effect ($P<0.005$) on phytase efficacy but the magnitude of the effects varied between phytases.

2. Based on the result from the *in vitro* experiment, the effect of intermittent feeding, addition of 500 FYT *C. braakii*-derived phytase and 1% formic acid to reduce the feed pH to 4.5 were tested in a 2x2x2 factorial arrangement in a broiler experiment. Ten pens containing 10 birds each were fed each of the treatment combinations from 15 to 36 days of age. *Ad libitum* fed birds had two 4-h dark periods with 2-h light in-between, while intermittently fed birds in addition had restricted access to feed through four 1-h feeding bouts.

3. In addition to assessing performance, excreta were collected on pen basis. From one bird per pen, tibia and contents from jejunum and ileum were collected. In addition, crop contents were collected from intermittently fed birds 80, 160 and 240 min after start of feeding.

4. Phytase improved performance, ileal and jejunal P digestibility, P retention and tibia ash and P concentration ($P<0.001$). Intermittent feeding increased jejunal P digestibility and P retention ($P<0.001$), but ileal P digestibility increased only in intermittently fed birds compared to *ad libitum* fed without phytase addition ($P=0.025$). Acidification increased jejunal P digestibility only in *ad libitum* fed birds ($P=0.019$). There was a considerable inositol hexakisphosphate degradation to lower isomers in the crop after 80 min for diets with phytase ($InsP_{3\ \mathrm{and}\ 4}$:$P<0.001$), and acidification further increased this degradation ($InsP_4$:$P=0.007$)). After 160 minutes retention time the effect of phytase and acidification was even higher with more significant ($P<0.05$) interactions.

5. The current data show that a prolonged retention time in the crop combined with feed acidification increased phytase efficacy by improving the phytate degradation.

Keywords: phosphorus, phytate, intermittent feeding, performance, inositol phosphates, anterior digestive tract

49 **Introduction**

50 A large part of the phosphorus (P) in grains and legumes is found in the form of phytic acid,
51 which cannot be degraded by enzymes secreted by chickens (Selle and Ravindran 2007). To
52 increase P utilisation and avoid environmental pollution, exogenous phytase is used as a feed
53 additive in poultry nutrition. However, even with phytase addition, the degradation of phytate
54 is incomplete, as indicated by Slominski (2011), who found that liberation of P from phytate
55 was increased from an average of 19% without phytase addition to 38% with phytase addition.

56 The efficacy of phytase is dependent on pH level (Menezes-Blackburn *et al.* 2015; Tamim *et*
57 *al.* 2004; Vieira *et al.* 2018). As for most enzymes, there is an optimal pH range, which for most
58 new-generation phytases is between 4.0 and 5.0 (Vieira *et al.* 2018). In addition, the phytate
59 substrate may be resistant to hydrolysis by phytase when pH is raised above 4.0 due to the
60 formation of mineral-phytate complexes (Angel *et al.* 2002; Selle and Ravindran 2007).
61 Although gizzard is considered an important site for phytase activity, the pH is usually too low
62 here at between 2.0 to 4.0 (Svihus 2011) for phytases to exert optimal activity. Likewise, the
63 pH in the small intestine is too high at between 6.5 and 7.5 (Svihus 2011). Due to this, the crop
64 has been considered a major site for exogenous phytase activity (Sommerfeld *et al.* 2018; Zeller
65 *et al.* 2015).

66 Exogenous enzymes that are added to the diet will be activated by the moisturising in the crop
67 and start to exert their effect, as demonstrated by Svihus *et al.* (2010) and Zeller *et al.* (2015).
68 However, the pH in the crop is normally at the same level as in the feed at the time the feed
69 enters the crop. Thus, since chicken feeds normally have a pH between 6.0 (Ao *et al.* 2008) and
70 6.5 (Sacranie *et al.* 2017), this will possibly be a limiting factor for phytase efficacy. Retention
71 time in crop is associated with a considerable fermentation activity by lactobacilli and
72 production of lactic acid that reduces the pH in crop over time (Cutler *et al.* 2005; Jozefiak *et*

73 *al.* 2006). Furthermore, to reduce pH by the addition of an acid, as demonstrated by Kim *et al.*
74 (2015), could be an effective way to improve phytase activity.

75 Previous research have shown that simultaneous addition of organic acid and phytase improve
76 phytase efficacy. Emami *et al.* (2013) and Woyengo *et al.* (2010) found an increased ileal P
77 digestibility by combining organic acid with phytase. In addition, Vieira *et al.* (2017) performed
78 a meta-analysis that showed that adding both phytase and citric acid did improve weight gain
79 and ash content in tibia.

80 Phytase efficacy is also dependent on the retention time in the favourable crop environment
81 (Tamim *et al.* 2004). Svihus *et al.* (2010) have previously found an increased degradation of
82 myo-inositol hexakisphosphate ($InsP_6$) in the crop over time. Depending on the feeding
83 regimen, the retention time in the crop may be considerable (Sacranie *et al.* 2017; Svihus *et al.*
84 2010). Birds adapted to intermittent feeding are able to store large quantities of feed in their
85 crop (up to 50 gram DM), and this content gradually disappears from the crop within a period
86 of up to 5 hours (Sacranie *et al.* 2017) after termination of the last feeding bout. In contrast,
87 Svihus *et al.* (2010) found that 78% of *ad libitum* fed birds had under 5 gram DM of feed in the
88 crop.

89 Thus, stimulating retention of feed in the crop could possibly increase phytate degradation when
90 phytase is used. Svihus *et al.* (2013) were not able to demonstrate an effect of intermittent
91 feeding on phytase efficacy. However, Sacranie *et al.* (2017) found a higher P digestibility and
92 $InsP_6$ digestibility in duodenum/jejunum with intermittent feeding.

93 Therefore, an experiment was designed to test the hypothesis that acidification of the diet would
94 improve the efficacy of an exogenous phytase, and that the efficacy would further be improved
95 by increased crop retention time due to intermittent feeding.

96

97 **Materials and methods**

98 ***In vitro test***

99 A simple *in vitro* procedure simulating the crop based on the work of Zyla *et al.* (1995) was
100 used to test four commercial phytase products at selected pH levels and incubation times. The
101 phytases came from the following donor organisms: *Escherichia coli, Buttiauxella* sp.,
102 *Aspergillus niger and Citrobacter braakii*, arbitrary denoted phytase A, B, C and D. A high-
103 phytate diet (Table 1) was mixed in a 6-litre twin-shaft paddle mixer (Forberg, Sandefjord,
104 Norway) from pre-ground raw materials. The feed had the same vitamin, mineral and enzyme
105 content as a commercial chicken feed, with no phytase added. Formic acid (98%) (Merck,
106 Darmstadt, Germany) was used to adjust pH, and the level of formic acid needed to achieve the
107 desired pH levels was determined through a pilot trial with incremental addition of formic acid
108 to the experimental diet, followed by pH measurement, 0 and 5 min after acid addition. The pH
109 was measured using a pH meter (pH 100, VWR International, Radnor, PA, USA). In both the
110 pilot trial and the *in vitro* test, 1.0 g (±0.01 g) of the diet was added to a test tube containing 5.0
111 ml deionised water. The amount of formic acid added was 0, 4.0, 13.0 and 50.0 µl to reach pH
112 6.7, 5.5, 4.5 and 3.5, respectively. Phytase (0 or 500 FTU) and the specified amount of formic
113 acid were added in the test tube followed by vortexing. The test tubes were incubated in a 40°C
114 water bath, and were shaken by hand every 5 min during incubation. After an incubation of
115 respectively 10, 20, 30, 45 or 60 min, the enzymatic reaction was terminated by adding 5.0 ml
116 4% trichloroacetic acid (TCA) (Sigma-Aldrich, St. Louis, MO, USA). The test tubes were
117 centrifuged at 3000 rpm for 15 min, followed by collection and freezing of the supernatant at -
118 20°C until analysis for free phosphate. Each treatment combination had four replicates, except
119 for the negative control without phytase, which only had duplicates.

120

121

122 ***Broiler experiment***

123 According to the Polish law and the EU directive (no 2010/63/EU) the experiments conducted
124 within this study do not require approval of the Local Ethical Committee for Experiments on
125 Animals in Poznań, Poland. However, all activities complied with the guidelines of the
126 Committee with respect to animal experimentation and care of the animals under the study.

127 ***Birds, housing and management***

128 Eight hundred one-day-old male Ross 308 chickens were randomly assigned to eight different
129 treatments in 80 pens. The pens were arranged in the middle of an environmentally controlled
130 broiler house (PIAST PASZE Sp. z o.o., Experimental Unit no. 0616, Olszowa, Poland) with
131 9,000 loose-housed birds hatched at the same time not included in the experiment surrounding
132 the pens. The birds were fed a diet with or without phytase and with or without formic acid *ad*
133 *libitum* or intermittently in a 2 x 2 x 2 factorial design with 10 replicate pens per treatment
134 combination. The pens (100 cm x 100 cm) were made of wire, so birds had visual contact with
135 surrounding birds. The pens with the *ad libitum* fed birds were located along one drinking line
136 and the intermittently fed bird were located along a second drinking line to minimise
137 behavioural influence between the treatments. Straw was used as bedding, and a temperature
138 of 33ºC was maintained during the first week and then reduced weekly by 2-3ºC to 21ºC on day
139 28. The birds were maintained on a commercial pelleted diet produced by Piast Pasze feed mill
140 (Lewkowiec, Poland) until day 15. From day 11, the intermittently fed birds had access to feed
141 between the hours of 08:00 to 09:00, 12:00 to 13:00, 16:30 to 17:30, 21:00 to 22:00 and from
142 02:00 to 04:00. The feeders were removed from the cages between feedings, except from 22:00
143 to 02:00 and 04:00 to 08:00 when the light was turned off. All birds were given the experimental
144 diets from day 15 to 36. All birds had *ad libitum* access to water throughout the experimental
145 period.

146 ***Experimental diet***

147 Based on the response to reduced in the *in vitro* test the *Citrobacter braakii*-derived phytase
148 and the desired pH of the acidified feed was set to 4.5. A high-phytate diet (Table 1)
149 containing 5 g/kg titanium dioxide (TiO_2) as a digestibility marker was produced at the Center
150 for Feed Technology (Ås, Norway). Half of the soy oil was added to the mash before
151 pelleting, while the remaining was added after pelleting. The two diets with acid were added
152 1.0 % of 85 % formic acid (POCH, Avantor Performance Materials, Poland). For the two
153 diets with phytase, 500 FYT phytase (RONOZYME® HiPhos, DSM, Denmark) was added
154 per kg feed. The phytase was diluted with a small amount of water that did not influence
155 water content of the diet, to ensure even distribution and sprayed on the pellet before soy oil
156 was added. Due to an error, too little acid was added during feed processing. The analysed
157 phytase activity was 520 FYT/kg feed with phytase added and 385 FYT/g feed with both
158 phytase and acid added, while phytase activity for the diets without phytase added was below
159 detection level. Diet pH was measured in samples taken from the feeders in the chicken
160 house, with the same method as in the *in vitro* test. The feed pH in the negative control (NC),
161 phytase added (NC+phy), formic acid added (NC+acid) and formic acid and phytase added
162 (NC+phy+acid) was 6.7, 6.6, 5.1 and 4.9, respectively. The level of the different inositol
163 phosphate (InsP) isomers per g DM feed was 26.33 µmol $InsP_6$, 1.1 µmol $Ins(1,2,4,5,6)P_5$, 0.7
164 µmol $Ins(1,2,3,4,5)P_5$, 0.4 µmol $Ins(1,2,3,4,6)P_5$ and 0.15 µmol $InsP_4$.

165 ***Performance and sample collection***

166 Body weight (BW) and feed intake (FI) were recorded weekly on a pen basis. Excreta was
167 collected on day 28 by placing paper sheets on pen floors at the start of feeding at 12:00,
168 followed by repeated manual collection of excreta without contamination during the next 4
169 hours. The excreta from each pen was pooled and mixed before a representative sample was
170 taken.

171 On day 36, one random bird from each pen of the *ad libitum* fed birds was killed by a blow to
172 the head followed by cervical dislocation. Thereafter a zip tie was tightened around the neck to
173 avoid loss of crop content. The start of killing was four hours after the light came on at 08:00
174 to give the birds sufficient feeding time. On day 37, one randomly selected intermittently-fed
175 bird from each pen was killed 80, 160 and 240 min after start of feeding. On day 36, the feeding
176 was adjusted 10 min between groups, to ensure that all intermittently fed birds were killed at
177 the same time interval after feeding. The crop was emptied for all birds and pH was measured
178 in the crop content by inserting the electrode of a pH meter (pH 100, VWR International,
179 Radnor, PA, USA) into the sampling container. For *ad libitum* and intermittently fed birds killed
180 after 240 min, gizzard pH was measured by placing the pH electrode directly into the gizzard.
181 In addition, the left tibia and content of jejunum and last 2/3 of ileum were collected. Tibias
182 were frozen at -20°C and kept frozen until analyses. Other samples were immediately put on
183 dry ice and kept frozen at -20°C until lyophilised. Due to insufficient crop contents, pH could
184 not be measured in 2, 10 and 22 crops after 80, 160 and 240 min, respectively, with
185 approximately equal number of missing values between treatments. A minimum of 0.5 g DM
186 was required for the InsP analysis. Thus, the InsP values could not be determined in 4, 15 and
187 27 crops after 80, 160 and 240 min, respectively.

188 ***Chemical analyses***

189 Free phosphate was quantified by using a modification of the ammonium molybdate method
190 (Heinonen and Lahti 1981). Briefly, a solution with ascorbic acid, sulphuric acid and water, and
191 a second solution with ammonium molybdate and water was mixed with water in the ratio
192 5:1:10 to form a colour reagent. Thirty µl of supernatant was mixed with 240 µl colour reagent
193 in microtiter plates, shaken on a microplate shaker and incubated at 37°C for 60 min. After
194 incubation, the absorbance at 820 nm was measured on a SpectraMax M2e, (Molecular Devices,
195 San Jose, CA, USA) and g P released per kg of feed was calculated.

196 All digesta and excreta samples were lyophilised and homogenised. Lyophilised DM content
197 was used in all calculations. Crude protein in the feed was determined by the Kjeldahl N method
198 with a Kjel-Foss Automatic 16210 (Foss Electric, Hillerød, Denmark), according to AOAC
199 (2005) no. 976.05. The excreta, digesta and feed were analysed for TiO_2 content as described
200 by Short *et al.* (1996). P in diets and digesta was analysed by adding a solution of $HClO_4$, HNO_3
201 and H_2SO_4 to the samples, this was mineralised until the solution became colourless. Thereafter
202 an ammonium molybdate solution was added and the samples were read on a Marcel Media
203 spectrophotometer (Marcel S.A., Zielonka, Poland) at 720 nm. Ileal digesta were analysed
204 enzymatically for starch content based on the use of thermostable α-amylase and amylo-
205 glucosidase (McCleary *et al.* 1994). Phytase activity in the feed was determined according to
206 the internal method of the enzyme producer (assay at pH 5.5 and 37°C). Soft tissue from tibia
207 were removed by hand, and dry matter and ash content were then determined after drying for
208 16 h at 104°C and 16 h ashing at 550°C, respectively. P was analysed in the tibia according to
209 the method of FAO (2011). Briefly, HCl was added to the samples and the solution was
210 mineralised until it became colourless. Thereafter an ammonium molybdate solution was added
211 and the samples were read on a MaxMat PL II Multi-analyser (MaxMat, France) at 340 nm.
212 Inositol phosphates were extracted using the method described by Zeller *et al.* (2015). Briefly,
213 samples were extracted with a solution containing 0.2 M EDTA and 0.1 M NaF using a rotary
214 shaker. The extracts were filtered through a 0.2-µm cellulose acetate filter (VWR, Darmstadt,
215 Germany) into a Microcon® filter and centrifuged at 14,000 *g* for 30 min. Filtrates were
216 analysed using high-performance ion chromatography and UV detection at 290 nm after post-
217 column derivatisation using an ICS-3000 system (Dionex, Idstein, Germany). InsPs with
218 different degrees of phosphorylation ($InsP_3$ to $InsP_6$) and their positional isomers were separated
219 without enantiomer differentiation on a Carbo Pac PA 200 column and corresponding guard
220 column. $Fe(NO_3)_3$ solution in $HClO_4$ was used as the reagent for derivatisation in accordance

221 with (Phillippy and Bland 1988). The elution order of InsPs was established using commercial
222 standards if available.

223 ***Calculations***

224 The P retention and ileal and jejunal digestibility coefficient of P and starch were calculated
225 by the following formula:
226 Nutrient digestibility coefficient/retention = 1 – [([TiO_2]diet/[TiO_2] digesta) ×
227 ([nutrient]digesta/[nutrient]diet)]

228 ***Statistical analyses***

229 For statistical analyses the general linear model procedure in SAS software 9.4 (SAS Inst. Inc.,
230 Cary, NC, USA) was used with the Ryan–Einot–Gabriel–Welsh F-test to investigate differences
231 ($P<0.05$) between the different treatment groups. P-values between 0.05 and 0.1 were
232 considered tendencies. The square root of means square error (√MSE) was used as a measure
233 of random variation. Results within each phytase in the *in vitro* test was subjected to a two-way
234 ANOVA with pH and incubation time as main effects and each replicate as one experimental
235 unit. Performance, digestibility and tibia parameters were subjected to a three-way ANOVA
236 with phytase, acid and feeding regimen as main effects. Pen was used as the experimental unit.
237 Crop pH in intermittently fed birds was subjected to a two-way ANOVA with time and
238 acidification as main effects. Crop dry matter content in intermittently fed birds and crop pH in
239 *ad libitum* fed birds were subjected to a simple one-way ANOVA with time and acid as effects,
240 respectively. Concentration of InsP isomers and $InsP_6$ degradation were subjected to a two-way
241 ANOVA with acid and phytase addition as main effects.

242

243

244

Results

In vitro test

The amount of P in the form of free phosphate in the feed was 0.61 mg/g. The highest amount of P released without phytase addition ($P<0.05$) occurred at pH 5.5, followed by pH 4.5, 6.7 and 3.5 (Table 2). For all four phytases, significant (Phytase A; $P=0.001$, phytase B; $P=0.002$, phytase C; $P=0.041$, Phytase D; $P<0.001$) interactions between pH and time were observed. The highest amount of P released with phytase A was observed at pH 4.5 after 60 min incubation time. For phytase B, D and C the highest amounts of P released occurred at pH 3.5 after 45, 45 and 60 min incubation time, respectively. Even without phytase addition, the amount of P released was improved with increased incubation times ($P<0.001$) for all pH levels. Similarly, increased incubation time increased the amount of P released from phytase C at pH 6.7 ($P<0.001$), from phytase B at pH 6.7 and 3.5 ($P<0.001$) and from phytase A at pH 4.5 and 3.5 ($P<0.001$). For phytase D, there was an increase in the amount of P released with increasing time of incubation for all pH levels ($P<0.001$). An increase in P released from phytase B ($P<0.001$) was seen when the pH was reduced from 6.7 independent of incubation time. For phytase A and C there was an increase in amount of P released (Phytase A; $P<0.001$, phytase C; $P=0.004$) when the pH was reduced from 6.7 at certain times. For phytase D, there was an increase in amount of P released when the pH was reduced to 4.5 for all incubation times ($P<0.001$). In addition, for 10 min incubation time, there was also an increase in the amount of P released when the pH was reduced from 4.5 to 3.5.

Broiler experiment

Intermittent feeding resulted in a lower FI and a lower body weight gain (BWG) ($P<0.001$) than *ad libitum* feeding (Table 3). Acidification of the feed tended to reduce FI ($P=0.059$) and BWG ($P=0.090$). A tendency for an interaction ($P=0.057$) between phytase and formic acid was observed for BWG, where reduced BWG with acidification was only observed when no phytase

270 was used. Phytase increased FI, BWG and feed conversion ratio (FCR) (P<0.001). A tendency
271 for an interaction (P=0.063) between phytase and acid addition was also observed for FCR,
272 where reduced FCR with acidification was only observed when phytase was used. The mortality
273 (data not shown) was 4.25% and treatment had no impact on mortality.

274 Dry matter content (data not shown) in the crop for intermittently fed birds was reduced
275 (P<0.001) from 16.4 g 80 min after start of feeding to 7.3 and 2.5 g after 160 min and 240 min,
276 respectively. In *ad libitum* fed birds, the average dry matter content in the crop was 8.8 g.

277 For intermittently fed birds given feed without acid, crop pH was higher (P<0.001) 80 min after
278 start of feeding than 240 min after feeding (Table 4). The average crop pH for *ad libitum* fed
279 birds (data not shown) given feed with acid was significantly lower (P=0.019) at 4.3 (measured
280 in 17 birds) compared to the crops from birds fed diets without acid, where pH was 4.9
281 (measured in 20 birds). The pH in the gizzard (data not shown) was reduced (P=0.004) from
282 2.3 to 2.0 with intermittent feeding and increased (P=0.041) from 2.0 to 2.2 with phytase
283 addition. A tendency (P=0.063) of increased pH in the gizzard from 2.0 to 2.2 with addition of
284 acid was also seen.

285 As shown in Table 5, phytase addition increased (P<0.001) P digestibility coefficient (PDC) in
286 the jejunum and ileum and P retention in addition to g ash per kg tibia and g P in tibia per kg
287 body weight (BW). Intermittent feeding increased jejunal PDC and P retention (P<0.001), but
288 did not influence PDC in the ileum. Acidification of the feed tended to (P=0.061) increase ileal
289 PDC. An interaction (P=0.025) between phytase and feeding regimen was observed on ileal
290 PDC, where intermittent feeding increased ileal PDC only when phytase was not added. An
291 interaction (P=0.019) between feeding and acid was observed on jejunal PDC, where
292 acidification of the feed increased jejunal PDC only in *ad libitum* fed birds. A tendency
293 (P=0.078) for an interaction between acid and phytase was observed, where acidification of the

294 feed increased jejunal PDC only when phytase was used. There was no difference (P>0.05) in
295 ileal starch digestibility coefficient between the different treatments.

296 The figure shows the $InsP_6$ degradation in crop 80, 160 and 240 min after start of feeding.
297 Phytase addition increased (P<0.001) $InsP_6$ degradation in the crop of intermittently fed birds
298 80 and 160 min after start of feeding (P-values not shown in the figure). After 80 min,
299 acidification tended (P=0.078) to increase $InsP_6$ degradation, this effect being significant
300 (P=0.028) after 160 min. A tendency (P=0.079) for an interaction between acid and phytase
301 was observed after 80 min, where acid supplementation increased $InsP_6$ degradation only in
302 phytase-containing diets. This effect was significant (P=0.034) after 160 min. After 240 min,
303 only three samples from phytase-containing diets contained enough material for analysis of
304 InsP-isomers. The average degradation of $InsP_6$ in these samples was 98.6%, however the low
305 number of samples precluded a proper evaluation of the significance of these data.

306 80 min after feeding, the concentration of Ins(1,2,3,4,5)P_5 in the crop was higher with
307 acidification than without (P=0.007) (Table 6). The other $InsP_5$ isomers were not affected by
308 acidification at 80 min. Acid addition tended to reduce the concentration of Ins(1,2,3,4,6)P_5
309 after 160 min. The concentration of Ins(1,2,4,5,6)P_5 and Ins(1,2,3,4,6)P_5 was lower with
310 phytase addition both after 80 and 160 min (P<0.001) compared to no phytase addition. With
311 phytase addition, the concentration of Ins(1,2,3,4,5)P_5 was lower (P=0.006) after 160 min than
312 without. At 160 min, a significant interaction between acid and phytase was seen for
313 Ins(1,2,3,4,6)P_5 (P=0.046) and Ins(1,2,4,5,6)P_5 (P=0.05), where reduced concentration of the
314 isomers was found with acidification when phytase also was used, and no effect of acidification
315 was seen in the absence of phytase. The concentration of $InsP_4$ was increased by phytase
316 (P<0.001) and acidification after both, 80 min (P=0.010) and 160 min (P<0.001). A significant
317 interaction between acid and phytase after 80 min (P=0.007) and 160 min (P<0.001) was
318 observed, where acidification increased concentration of $InsP_4$ only with phytase containing

319 diets. At both times, considerable quantities of $InsP_3$ were only found when phytase was present
320 (P<0.001). A tendency for increased concentration of $InsP_3$ (P=0.057) with acidification
321 compared to no acidification after 80 min was observed, which was significant (P<0.001) after
322 160 min. A tendency of interaction (P=0.057) between acid and phytase after 80 min and a
323 significant interaction after 160 min (P<0.001) was observed, where acidification increased
324 concentration of $InsP_3$ only when phytase was present.

325 **Discussion**

326 As expected, with the high phytate diet in this experiment, phytase increased performance, PDC
327 and g ash per kg tibia. Phytase starts to hydrolyse phytate already in the crop, as demonstrated
328 in previous research (Sommerfeld *et al.* 2018; Svihus *et al.* 2010). However, the current
329 experiment showed inconsistent results regarding the interaction between increased retention
330 time and acidification on the efficacy of phytase.

331 The reduced feed intake and BWG with intermittent feeding in the current experiment is in
332 consistence with results obtained by Sacranie *et al.* (2017) where the intermittently fed birds
333 only had access to feed 5 hours per day. However, other experiments where intermittently fed
334 bird had 6 hours access to feed per day, as in the current, no difference in FI and BWG was
335 found between intermittent and *ad libitum* feeding (Sacranie *et al.* 2012; Svihus *et al.* 2010).

336 The difference in amount of crop content between *ad libitum* and intermittently fed bird was
337 smaller than expected based on previous research. Sacranie *et al.* (2017) found the crop content
338 of intermittently fed birds to be significantly higher than *ad libitum* fed birds 180 min after start
339 of feeding, while in the current experiment, there was no difference in content between *ad*
340 *libitum* and intermittent feeding after 160 min. The amount of feed in the crop for intermittently
341 fed birds after 240 min was also higher in the experiment of Sacranie *et al.* (2017) than in the
342 current experiment. The low amount of feed in the crop of intermittently fed birds in the current

343 experiment might have contributed to a small difference in the crop content between
344 intermittently and *ad libitum* fed birds. The amount of crop content in *ad libitum* fed birds was
345 also higher than previously found. Svihus *et al.* (2010) found that 78% of *ad libitum* fed birds
346 (age 31 to 39 days) had less than 5 gram of dry matter in their crop, whereas in the current
347 experiment this number was 49%. A different number of chickens in each pen compared to
348 previous experiments, in addition to the fact that a commercial flock surrounded the pens, could
349 have influenced the feeding behaviour and led to this difference.

350 Despite the small difference in crop content between feeding regimens, a higher jejunal PDC
351 was seen with intermittent feeding, as also demonstrated by Sacranie *et al.* (2017). The
352 increased $InsP_6$ degradation in the crop from 80 to 160 min with phytase addition in the current
353 experiment was in accordance with the finding of Svihus *et al.* (2010). This suggests that the
354 degradation of phytate in the anterior digestive tract is higher for the intermittently fed birds
355 due to an increased retention time. Similarly, the increased P retention with intermittent feeding
356 may indicate that even an increased retention time alone without phytase addition may be
357 favourable for total P retention.

358 The loss of difference in P digestibility between *ad libitum* and intermittently fed birds from
359 jejunum to ileum when phytase was used suggest that retention time in the crop does not seem
360 to be a limiting factor for phytate degradation by exogenous phytase. Compensation of the
361 lesser phytate degradation in the foregut seems to take place in the intestine when exogenous
362 phytases are used. It may be speculated that the higher ileal PDC without phytase addition for
363 intermittently fed birds compared to *ad libitum* fed birds is due to the prolonged time in the
364 crop and thus the intrinsic phytase will have enough time in the crop to degrade the phytate. In
365 the current *in vitro* test, the amount of released P increased with time (up to 60 min) even when
366 no phytase was added. A 22% disappearance of $InsP_6$ was also found in a wheat-based diet
367 without phytase added in an *in vitro* trial by Sommerfeld *et al.* (2017). A possible explanation

368 for the high phytate degradation *in vitro* without the addition of exogenous phytase could be a
369 high intrinsic phytase activity in wheat (>1000 U kg^{-1}) (Eeckhout and De Paepe 1994). The
370 intrinsic phytase activity in corn is low (<100 U kg^{-1}) (Eeckhout and De Paepe 1994) and hence
371 the hypothesis is supported by the lack of $InsP_6$ degradation in a corn-based diet without phytase
372 addition (Sommerfeld *et al.* 2017). However, in the current experiment, the lack of increase in
373 $InsP_6$ degradation from 80 to 160 min without exogenous phytase indicates that there is no more
374 available $InsP_6$ for degradation to lower isomers after 80 min. The difference in effect without
375 phytase addition between the current *in vivo* and *in vitro* trials could partly be explained by the
376 differences in incubation time where the maximum time in the *in vitro* experiment was 60
377 minutes, and the measurements in the *in vivo* experiment started 80 minutes after the start of
378 the feeding. In addition, the feed used in the *in vitro* trial was not heat-treated, hence no
379 inactivation of intrinsic phytase had taken place. Contrary, in the diet used in the *in vivo*
380 experiment no phytase activity was detected during enzyme activity analysis because of the
381 assumed inactivation of intrinsic phytase with heat treatment (Esmaeilipour, Van Krimpen *et*
382 *al.* 2012).

383 Contrary to what was intended, a slight reduction in phytase was detected in the feed containing
384 both formic acid and phytase. One possible explanation for this lowered phytase activity could
385 be the addition of the acid together with the phytase on top of the pellet, which may have
386 resulted in acidic degradation and hence inactivation of the phytase in the pellet. This
387 hypothesis is reinforced by phytase activity analyses, which was carried out on the feed before
388 acid addition, where the phytase activity was 544 FYT/kg feed. The results of acidification and
389 interaction between acid and phytase must be interpreted in consideration of the difference in
390 phytase activity.

391 The tendency for reduced FI and BWG with acidification in the current study has also been
392 reported in other experiments, where high dosage of citric acid in feed decreased FI (Brenes *et*

393 *al.* 2003; Esmaeilipour, Moravej *et al.* 2012). A possible explanation for this could be a reduced
394 palatability of the feed with a high amount of acid added. In addition, a lowered gizzard pH
395 could reduce the gastric emptying rate because a larger amount of pancreatic juice is needed to
396 neutralize the stomach content that reach the duodenum, this effect is well known in pigs (Van
397 der Aar *et al.* 2017). The lowered emptying rate is causing a reduced feed intake, in addition to
398 an improved efficacy of the digestion (Vieira *et al.* 2018). This relationship between
399 acidification and improved digestive efficacy could be the reason why acidification did not
400 reduce growth when both acid and phytase was added in the feed, as the tendency when only
401 acid was added implied. Conversely, there was no reduction in gizzard pH with acidification of
402 the feed in the current experiment. The lack of reduction in gizzard pH is in accordance with
403 data described in a review by Kim *et al.* (2015). However, a lower gizzard pH for intermittently
404 fed birds at an earlier time after feeding than 240 min cannot be ruled out, as acidification of
405 the feed did not reduce the pH in the crop after 240 min either.

406 The increased P release with reduced pH and phytase addition in the current *in vitro* test and
407 other *in vitro* experiments (Menezes-Blackburn et al. 2015; Tamim et al. 2004), made an
408 expectation of an increased efficacy of phytase with acidification. Some results in the current
409 experiment is indicating that this effect was present in the current experiment. The increased
410 degradation of $InsP_6$ in the crop after 160 min by acid and phytase addition compared to only
411 phytase addition may have led to the observed tendency of increased jejunal P digestibility by
412 acid, only when used together with phytase. However, no effect on ileal P digestibility with
413 acid addition was seen, contradictory to Emami *et al.* (2013) and Woyengo *et al.* (2010) who
414 found an increased ileal P digestibility with organic acid and phytase addition.

415 A reduction in the crop pH for intermittently fed birds over time without acid addition was also
416 found by Sacranie *et al.* (2017) and could be the cause for the tendency for an interaction
417 between feeding regimen and acid, where an increased jejunal PDC with acidification was seen

418 only in *ad libitum* fed birds. It may be speculated that acidification had a positive effect on the
419 *ad libitum* fed bird because of the presumed shorter crop retention time and less time with a
420 lowered pH without acidification and that the reduced pH with the longer retention time in the
421 crop for intermittently fed birds will compensate for the lack of instant pH reduction.

422 The decrease in the concentrations of $InsP_6$ and two of the $InsP_5$ isomers in the crop with phytase
423 addition leading to higher concentrations of $InsP_4$ and $InsP_3$ isomers have been demonstrated
424 previously by Zeller *et al.* (2016) and Sommerfeld *et al.* (2018). Ins(1,2,3,4,5)P_5 is the main
425 $InsP_5$ isomer in the degradation pathway for the *C. braakii* phytase (Pontoppidan *et al.* 2012),
426 and the large degradation of insP_6 with both acid and phytase added might have led to the high
427 concentration of Ins(1,2,3,4,5)P_5 and hence no phytase effect after 80 min was seen for this
428 isomer. The increased number of significant interactions from 80 to 160 min, where all isomers
429 except Ins(1,2,3,4,5)P_5 had a significant interaction, indicates that acidification and an
430 increased retention time in the crop improved phytase activity. A more rapid passage from the
431 crop of some of the isomers cannot be ruled out, but selective retention of crop content has not
432 been found in the literature.

433 The lack of interaction effects between acidification and prolonged retention time in crop with
434 phytase on performance, PDC and bone mineralisation are implying that the exogenous phytase
435 activity is not dependent on these factors. The only tendency of a three-way interaction in the
436 current experiment was for P in tibia/kg BW, where this interaction is suggesting that
437 acidification increased P mineralization of the bone only when no phytase was used in *ad*
438 *libitum* fed birds. This tendency might simply be a result of a slightly reduced BW for *ad libitum*
439 fed birds without phytase compared to birds fed with phytase. However, the lack of three-way
440 interactions could also be due to the low effect of feed manipulations in the current experiment.
441 The amount of feed in the crop for the intermittently fed birds was relatively low, hence a
442 shorter retention time than expected was seen. In addition, there was no difference between the

443 crop pH at 240 min for birds with acid and without acid, which is surprising. The lower phytase
444 activity in the feed with acid addition could also be a possible explanation why a lack of effect
445 of simultaneous acid and phytase addition was seen.

446 In conclusion, the current data showed that there was an effect of acidification of a diet
447 containing phytase on degradation of $InsP_6$ and further reduction to lower isomers after both 80
448 and 160 min in the crop. This together with the effect on jejunal PDC, indicate that acid addition
449 to the diet may be beneficial for the phytase activity in the anterior digestive system.

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571 Enzymic Dephosphorylation of Phytate in Maize-Soyabean Feeds for Turkey Poults."
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573

574 Table 1. Composition and nutrient content of the basal diets used in in vitro trial and broiler
575 experiment (g/kg as fed unless otherwise stated).

Ingredients	*In vitro*	Broiler experiment
Wheat	371.8	361
Wheat bran	350	310
Soybean meal (466 g/kg CP)	147	121
Rape seed meal (340 g/kg CP)	-	120
Maize gluten	37	-
Soy oil	-	58
Rapeseed oil	63	-
Limestone	12.5	12
Sodium chloride	-	2
Titanium dioxide	-	5
Mineral and vitamin premix	5.3	4[1]
L-threonine	1.3	1
DL-methionine	2.2	2
L-lysine	4.3	3
Sodium bicarbonate	5	0.9
Cholin chloride	0.4	-
Enzyme	0.2	0.05[2]
Nutrient composition		
Calculated gross energy MJ/kg		12.01
Calculated Phytate P		4.0
Analysed Crude protein		186.4
Analysed Starch		224.1
Analysed Total P		5.29
Analysed Ca		7.36

576 [1] Mineral and vitamin premix provided the following per kg diet: 2.57 mg retinol, 0.13 mg
577 cholecalciferol, 56.1 mg tocopherol, 3.6 mg menadione, 2.4 mg thiamine, 9 mg riboflavin, 36 mg
578 niacin, 7.2 mg pyridoxine, 0.012 mg cobalamin, 36 mg pantothenic acid, 1.8mg folic acid, 0.24mg
579 biotin, 85.7 mg Mn, 49.4 mg Zn, 45.4 mg Fe, 6.76 mg Cu, 0.32 mg Se, 0.86 mg I.
580 [2]Enzyme Rovabio® Excel AP, Adisseo, France, provided xylanase and *ß*-glucanase obtained from a
581 fermentation broth of *Penicillium funiculosum.*
582

Table 2. Released phosphorus (mg/g feed) from test feed in the negative control with no exogenous phytase added and with four different commercial phytases, at four different pH levels and five different incubation times (in vitro trial).

Incubation (minutes)	pH	Negative control	Phytase A[1]	Phytase B[1]	Phytase C[1]	Phytase D[1]	Average[2]
10	6.7	0.9^{k}	4.2^{c}	3.1^{g}	3.6^{f}	1.8^{i}	3.2^{f}
20	6.7	1.2^{ij}	4.3^{bc}	3.3^{fg}	3.7^{ef}	2.2^{h}	3.4^{ef}
30	6.7	1.5^{gh}	4.6^{abc}	3.5^{f}	4.1^{bcde}	2.4^{h}	3.6^{def}
45	6.7	1.8^{f}	4.5^{abc}	3.6^{ef}	4.0^{cdef}	2.9^{g}	3.7^{cde}
60	6.7	2.0^{e}	4.4^{bc}	3.6^{ef}	3.9^{fde}	3.0^{g}	3.7^{cde}
10	5.5	1.3^{hi}	4.6^{ab}	3.9^{de}	4.1^{bcde}	3.5^{f}	4.0^{bcd}
20	5.5	1.9^{ef}	4.5^{abc}	4.1^{bcd}	4.5^{ab}	3.8^{e}	4.2^{abc}
30	5.5	2.2^{d}	4.6^{abc}	4.0^{cd}	4.3^{abcd}	3.9^{de}	4.2^{abc}
45	5.5	2.8^{b}	4.8^{a}	4.1^{bcd}	4.5^{ab}	4.0^{de}	4.4^{ab}
60	5.5	3.1^{a}	4.5^{abc}	4.1^{bcd}	4.3^{abcd}	4.2^{cd}	4.3^{ab}
10	4.5	1.1^{jk}	4.6^{abc}	4.1^{bcd}	4.3^{abcd}	4.2^{cd}	4.3^{ab}
20	4.5	1.6^{g}	4.4^{bc}	4.3^{bc}	4.4^{abc}	4.5^{abc}	4.4^{ab}
30	4.5	1.9^{ef}	4.6^{abc}	4.3^{bc}	4.4^{abc}	4.6^{ab}	4.5^{ab}
45	4.5	2.4^{c}	4.8^{a}	4.1^{bcd}	4.4^{abcd}	4.7^{ab}	4.5^{ab}
60	4.5	3.0^{ab}	4.9^{a}	4.3^{bc}	4.4^{abcd}	4.7^{ab}	4.5^{ab}
10	3.5	0.8^{l}	4.4^{bc}	4.3^{bc}	4.4^{abc}	4.4^{bc}	4.4^{ab}
20	3.5	0.9^{kl}	4.6^{ab}	4.1^{bcd}	4.3^{abcd}	4.5^{abc}	4.4^{ab}
30	3.5	1.0^{jk}	4.8^{a}	4.4^{abc}	4.6^{ab}	4.6^{ab}	4.6^{a}
45	3.5	1.4^{h}	4.6^{abc}	4.7^{a}	4.6^{ab}	4.8^{a}	4.6^{a}
60	3.5	1.6^{g}	4.6^{ab}	4.5^{ab}	4.7^{a}	4.7^{ab}	4.6^{a}
√MSE[3]		0.05	0.15	0.15	0.19	0.15	0.45
pH							
6.7		1.5^{c}	4.4^{b}	3.4^{d}	3.9^{c}	2.5^{c}	3.5^{c}
5.5		2.3^{a}	4.6^{a}	4.0^{c}	4.3^{b}	3.9^{b}	4.2^{b}
4.5		2.0^{b}	4.6^{a}	4.2^{b}	4.4^{b}	4.5^{a}	4.4^{a}
3.5		1.1^{d}	4.6^{a}	4.4^{a}	4.5^{a}	4.6^{a}	4.5^{a}

Table 2 (Continued)

Incubation (minutes)						
10	1.0^{e}	4.5^{b}	3.8^{c}	4.1^{b}	3.5^{c}	4.0^{b}
20	1.4^{d}	4.5^{b}	3.9^{bc}	4.3^{ab}	3.8^{b}	4.1^{ab}
30	1.7^{c}	4.6^{a}	4.0^{ab}	4.3^{a}	3.9^{b}	4.2^{a}
45	2.1^{b}	4.7^{a}	4.1^{a}	4.3^{a}	4.0^{a}	4.3^{a}
60	2.4^{a}	4.6^{a}	4.1^{a}	4.3^{a}	4.1^{a}	4.3^{a}
p-value						
pH	<0.001	<0.001	<0.001	0.004	<0.001	<0.001
Time	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Time x pH	<0.001	0.001	0.002	0.041	<0.001	0.903

[1]The phytases had the following donor organisms: *Escherichia coli, Buttiauxella sp., Aspergillus niger* and *Citrobacter braakii*

[2]Average of all phytases used

[3]√MSE: square root of means square error in the analysis of variance

[a-k] Means within column without common letters are significantly different at $P < 0.05$.

596 Table 3. Effects of intermittent feeding, formic acid and phytase addition on performance of
597 broilers from day 11 to 36

Feeding regimen	Phytase	Acid	FI[1]	BWG[2]	FCR[3]
Ad libitum	No	No	4031	2343[b]	1.72[ab]
Intermittent	No	No	3836	2248[bc]	1.71[abc]
Ad libitum	No	Yes	3968	2280[b]	1.74[a]
Intermittent	No	Yes	3754	2178[c]	1.72[ab]
Ad libitum	Yes	No	4115	2457[a]	1.68[bc]
Intermittent	Yes	No	3944	2335[b]	1.69[abc]
Ad libitum	Yes	Yes	4118	2467[a]	1.67[bc]
Intermittent	Yes	Yes	3863	2334[b]	1.66[c]
$\sqrt{MSE}$[4]			130.2	80.9	0.042
Feeding regimen					
Ad libitum			4058[a]	2388[a]	1.70
Intermittent			3849[b]	2274[b]	1.69
Phytase					
No			3897[b]	2262[b]	1.72[a]
Yes			4010[a]	2398[a]	1.67[b]
Acid					
No			3981	2346	1.70
Yes			3926	2315	1.70
p-value					
Feeding regimen			<0.001	<0.001	0.413
Phytase			<0.001	<0.001	<0.001
Acid			0.059	0.090	0.853
Feeding x phytase			0.890	0.410	0.329
Feeding x acid			0.380	0.811	0.509
Phytase x acid			0.569	0.057	0.063
Feeding x phytase x acid			0.580	0.950	0.579

598 [1]Feed intake d 11-36
599 [2]Body weight gain d 11-36
600 [3]Feed conversion ratio, calculated as feed:gain
601 [4]Square root of means square error in the analysis of variance
602 [a,b,c] Means within column without common letters are significantly different at $P<0.05$.
603

604 Table 4. The pH in the crop of intermittently fed chickens with or without formic acid
605 addition to the feed 80, 160 and 240 minutes after start of feeding.

Time after start of feeding	Acid	pH
80 minutes[1]	No	5.6[a]
80 minutes[1]	Yes	4.5[b]
160 minutes[1]	No	5.3[a]
160 minutes[1]	Yes	4.5[b]
240 minutes[1]	No	4.2[b]
240 minutes[1]	Yes	4.1[b]
√MSE[2]		0.64
Time		
80		5.0[a]
160		4.9[a]
240		4.1[b]
Acid		
No		5.2[a]
Yes		4.4[b]
p-value		
Time		<0.001
Acid		<0.001
Time x acid		0.054

606 [1]The pH values were based on 20 crops with acid and 18 without acid at 80 minutes, 15 with acid and
607 15 without acid at 160 minutes, and 11 with acid and 7 without acid at 240 minutes.
608 [2]Square root of means square error in the analysis of variance
609 [a,b]Means within column without common letters are significantly different at P<0.05.
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Table 5. Effects of intermittent feeding, formic acid and phytase addition on bone mineralisation and P and starch digestibility coefficients in selected segments of the intestine.

Feeding regimen	Phytase	Acid	Tibia ash g/kg[1]	g P in tibia/kg BW	Jejunal P digestibility	Ileal P digestibility	P retention	Ileal starch digestibility
Ad libitum	No	No	382.8	0.126[d]	0.21[d]	0.34[c]	0.25	0.92
Intermittent	No	No	397.6	0.14[bcd]	0.44[b]	0.46[bc]	0.36	0.93
Ad libitum	No	Yes	390.1	0.14[bcd]	0.26[cd]	0.39[c]	0.26	0.91
Intermittent	No	Yes	390.9	0.13[cd]	0.38[bc]	0.45[bc]	0.37	0.92
Ad libitum	Yes	No	434.4	0.16[ab]	0.48[b]	0.60[ab]	0.32	0.94
Intermittent	Yes	No	444.2	0.16[a]	0.68[a]	0.60[ab]	0.41	0.93
Ad libitum	Yes	Yes	433.4	0.15[abc]	0.65[a]	0.72[a]	0.30	0.91
Intermittent	Yes	Yes	426.2	0.16[ab]	0.69[a]	0.65[a]	0.46	0.93
√MSE[2]			2.75	0.020	0.121	0.128	0.052	0.045
Feeding regimen								
Ad libitum			410.1	0.142	0.40[b]	0.51	0.28[b]	0.92
Intermittent			414.7	0.147	0.55[a]	0.54	0.40[a]	0.93
Phytase								
No			390.3[b]	0.129[b]	0.32[b]	0.41[b]	0.31[b]	0.92
Yes			434.5[a]	0.161[a]	0.63[a]	0.64[a]	0.37[a]	0.93

616 617 618 619 620 621 622 623 624 625 626 627 628 629 630 631 632 633 634 635 636 637 638 639 640 641 642 643 644 645 646

Table 5 (Continued)

Acid						
No	414.7	0.144	0.45	0.50	0.33	0.93
Yes	410.1	0.145	0.49	0.55	0.34	0.92
p-value						
Feeding regimen	0.459	0.230	<0.001	0.318	<0.001	0.591
Phytase	<0.001	<0.001	<0.001	<0.001	<0.001	0.502
Acid	0.459	0.913	0.121	0.061	0.142	0.274
Feeding x phytase	0.599	0.913	0.243	0.025	0.314	0.641
Feeding x acid	0.213	0.275	0.019	0.298	0.489	0.288
Phytase x acid	0.426	0.230	0.078	0.226	0.832	0.876
Feeding x phytase x acid	0.903	0.083	0.667	0.946	0.117	0.773

[1]g/kg of tibia dry matter

[2]Square root of means square error in the analysis of variance

[a-d]Means within column without common letters are significantly different at $P<0.05$.

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Table 6. Effect of acid and phytase addition on concentration of different inositol phosphate isomers (InsPs) (µmol/g DM) in the crop digesta of intermittently fed birds 80 and 160 minutes after start of feeding

		80 minutes after start of feeding[1]					
Phytase	Acid	$InsP_3$[2]	$InsP_4$[3]	$Ins(1,2,3,4,6)P_5$	$Ins(1,2,3,4,5)P_5$	$Ins(1,2,4,5,6)P_5$	$InsP_6$
No	No	0.0^c	0.5^c	0.7	1.1	1.0	23.7^a
No	Yes	0.0^c	0.3^c	0.7	1.3	1.1	23.6^a
Yes	No	1.6^b	3.2^b	0.3	0.7	0.7	17.7^a
Yes	Yes	3.0^a	7.4^a	0.1	1.3	0.5	10.3^b
√MSE[4]		1.09	2.22	0.13	0.46	0.28	6.03
Phytase							
No		0.0^b	0.4^b	0.7^a	1.2	1.1^a	23.6^a
Yes		2.4^a	5.6^a	0.2^b	1.1	0.6^b	13.6^b
Acid							
No		0.7	1.6^b	0.5	0.9^b	0.9	21.2
Yes		1.4	3.7^a	0.5	1.3^a	0.9	17.3
p-value							
Phytase		<0.001	<0.001	<0.001	0.453	<0.001	<0.001
Acid		0.057	0.010	0.236	0.007	0.794	0.078
Phytase x acid		0.057	0.007	0.107	0.160	0.161	0.079

Table 6 (Continued)

Phytase	Acid	160 minutes after start of feeding[1]					
		$InsP_3$[2]	$InsP_4$[3]	$Ins(1,2,3,4,6)P_5$	$Ins(1,2,3,4,5)P_5$	$Ins(1,2,4,5,6)P_5$	$InsP_6$
No	No	0.2^{c}	0.8^{bc}	0.9^{a}	1.2^{ab}	1.0^{a}	23.7^{a}
No	Yes	0.0^{c}	0.5^{c}	0.9^{a}	1.7^{a}	1.1^{a}	23.5^{a}
Yes	No	1.5^{b}	3.3^{b}	0.4^{b}	0.8^{b}	0.6^{b}	13.4^{b}
Yes	Yes	6.6^{a}	11.2^{a}	0.0^{c}	0.8^{b}	0.2^{b}	3.9^{c}
√MSE[4]		0.83	1.82	0.22	0.49	0.27	4.87
Phytase							
No		0.1^{b}	0.7^{b}	0.9^{a}	1.4^{a}	1.1^{a}	23.6^{a}
Yes		4.3^{a}	7.7^{a}	0.2^{b}	0.8^{b}	0.4^{b}	8.1^{b}
Acid							
No		0.6^{b}	1.6^{b}	0.7	1.1	0.9	20.3^{a}
Yes		2.6^{a}	4.7^{a}	0.6	1.3	0.8	16.0^{b}
p-value							
Phytase		<0.001	<0.001	<0.001	0.006	<0.001	<0.001
Acid		<0.001	<0.001	0.060	0.313	0.207	0.028
Phytase x acid		<0.001	<0.001	0.046	0.302	0.050	0.034

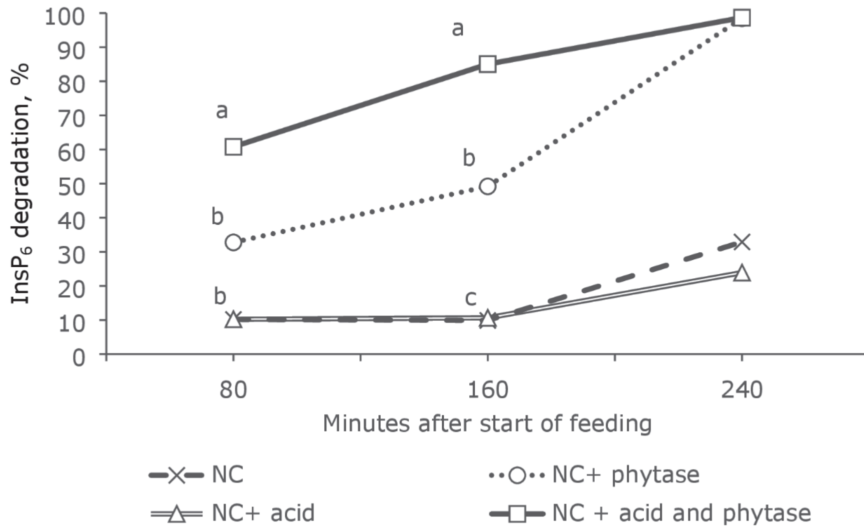
[1]The InsP values were based on 10 NC, 7 NC+phy, 10 NC+acid and 9 NC+phy+acid crops at 80 minutes and 8 NC, 4 NC+phy, 8 NC+acid and 5 NC+phy+acid crops at 160 minutes.

[2]At least one of the following isomers: $Ins(1,2,6)P_3$, $Ins(1,4,5)P_3$, $Ins(2,4,5)P_3$, $Ins(1,4,6)P_3$ and $Ins(2,3,5)P_3$

[3]At least one of the following isomers $Ins(1,2,3,4)P_4$ and $Ins(1,2,5,6)P_4$

[4]Square root of means square error in the analysis of variance

[a,b,c] Means within column without common letters are significantly different at $P<0.05$.



696

697 Figure. $InsP_6$ degradation (%) in the crop digesta 80, 160 and 240 minutes after start of feeding. The
698 InsP values were based on 10 NC, 7 NC+phy, 10 NC+acid and 9 NC+phy+acid crops at 80 minutes, 8
699 NC, 4 NC+phy, 8 NC+acid and 5 NC+phy+acid crops at 160 minutes, and 3 NC, 1 NC+phy, 7
700 NC+acid and 2 NC+phy+acid crops at 240 minutes. Treatment means within time with different letters
701 are significantly different ($P<0.05$), $\sqrt{MSE}$ is 22.89 for 80 and 18.52 for 160 minutes. The $InsP_6$ values
702 for 240 minutes have not been included in the statistics.

Paper II

1 **Assessment of crop usage in *ad libitum* fed birds and short-term**
2 **phytase efficiency as affected by acid addition.**

3 Siril Kristoffersen, Faculty of Biosciences, Norwegian University of Life Sciences, P.O. Box
4 5003, N-1432 Aas, Norway and Felleskjøpet Rogaland Agder, Sandvikveien 21, N-4002
5 Stavanger, Norway. Email: siril.kristoffersen@fkra.no (corresponding author)

6 Zuzanna Wiśniewska, Department of Animal Nutrition and Feed Management, Poznań
7 University of Life Sciences, Wołyńska 33, 60-637 Poznań, Poland. Email:
8 Zuzanna.wisniewska@up.poznan.pl

9 Sebastian Kaczmarek, Department of Animal Nutrition and Feed Management, Poznań
10 University of Life Sciences, Wołyńska 33, 60-637 Poznań, Poland. Email:
11 sebastian.kaczmarek@up.poznan.pl

12 Torger Gjefsen, Felleskjøpet Rogaland Agder, Sandvikveien 21, N-4002 Stavanger, Norway.
13 Email: tgjefsen@gmail.com

14 Nils Petter Kjos, Faculty of Biosciences, Norwegian University of Life Sciences, P.O. Box
15 5003, N-1432 Aas, Norway. Email: nils.kjos@nmbu.no

16 Aaron J. Cowieson, DSM Nutritional Products, Wurmisweg 576, 4303 Kaiseraugst,
17 Switzerland Email: aaron.cowieson@dsm.com

18 Birger Svihus, Faculty of Biosciences, Norwegian University of Life Sciences, P.O. Box
19 5003, N-1432 Aas, Norway. Email: birger.svihus@nmbu.no

20

21 ## Abstract

22 1. A field assessment was performed to map the extent of crop usage and thus retention time in
23 broiler chickens. In addition, a chicken experiment was carried out to study the short-term effect
24 of acid addition on phytase efficacy in the crop.

25 2. In the field assessment, the crop content of 40 *ad libitum* fed broiler chickens from 4 different
26 farms were sampled at 10, 20 and 30 days of age. The dry matter (DM) content varied from
27 zero to 32 g. The average crop retention time at 30 days of age was estimated to 84 minutes.

28 3. From 11 days of age, 120 individually caged chickens were intermittently fed a high phytate-
29 P diet with either no addition, 500 FYT *C. braakii*-derived phytase added or both phytase and
30 1.4 % formic acid added. Excreta were collected for assessment of phosphorus (P) retention.
31 At 20 and 21 days of age, starved birds were fed for one hour, and thereafter crop and gizzard
32 contents were collected every 20 minutes until 140 minutes after start of the feeding. At 60 and
33 140 minutes, also contents from jejunum and ileum were collected.

34 4. All diets reduced the concentration of phytate in the crop, however the diet with a
35 combination of acid and phytase resulted in a higher ($P<0.05$) degradation than the other diets
36 from 20 minutes after start of feeding. Simultaneously, the concentration of the lower inositol
37 phosphate isomers such as inositol-5-phosphate increased ($P<0.05$). Phytase increased
38 ($P=0.026$) P retention, and the combination of acid and phytase increased the jejunal
39 digestibility of P ($P=0.018$) compared to the other diets.

40 5. These results indicate that a lowered pH in the crop due to acid addition improve phytase
41 efficacy and increases P digestibility in the anterior digestive tract even with short retention
42 times. However, the lack of effect on P retention indicates that these are not limiting factors for
43 phytate degradation during intermittent feeding.

44 Keywords: intermittent feeding, inositol phosphates, anterior digestive tract, broiler chicken

45 Introduction

46 A large part of the phosphorus (P) in grains and legumes is found in the form of phytate
47 ($InsP_6$), which is poorly available for monogastric animals. Exogenous phytase is added to
48 diets for broiler chicken to increase the degradation of phytate as the contribution of phytases
49 and phosphatases secreted in the chickens on the degradation of phytate has been considered
50 negligible (Selle and Ravindran 2007). However, the phytate degradation is not complete
51 even with phytase addition. Slominski (2011) found that the release of P from phytate was
52 increased from 19 % without phytase addition to 38 % with phytase addition.
53
54 The crop is considered an important site for exogenous phytase activity (Classen *et al.* 2016).
55 Up to 71 % degradation of $InsP_6$ has been observed in the crop (Zeller *et al.* 2015a, 2016;
56 Sommerfeld *et al.* 2018). In the intestine there are a secretion of phytase and other
57 phosphatases, however, the pH-level in this segment and dietary Ca-levels reduces the
58 solubility of the phytate and hence reduce the phytase efficacy here (Selle *et al.*, 2009). The
59 efficacy of phytase is dependent on pH, and most new-generation phytases has a pH-optimum
60 between 3.0 and 5.0 (Tamim *et al.* 2004; Menezes-Blackburn *et al.* 2015; Vieira *et al.* 2018).
61 When the feed enters the crop, the pH there is usually the same as in the feed (Svihus 2014).
62 Since broiler diets have been reported to have a pH between 6.0 and 6.7 (Ao *et al.* 2008;
63 Sacranie *et al.* 2017; Kristoffersen *et al.* 2020), the pH in the crop is normally higher than the
64 optimal pH for exogenous phytase activity. Addition of organic acid to the feed can reduce
65 the pH in the crop (Kim *et al.* 2015; Kristoffersen *et al.* 2020) and may be a method to
66 improve conditions for phytase activity. In addition, retention time in the crop is associated
67 with *lactobacilli* fermentation activity, which would further reduce the pH in the crop over
68 time due to lactic acid production (Cutler *et al.* 2005; Jozefiak *et al.* 2006). A long retention

69 time in the crop may also improve the efficacy of exogenous phytase. Svihus *et al*. (2010)
70 observed a gradual reduction of $InsP_6$ to 50 % after 100 minutes retention time in the crop.
71 Similarly, Kristoffersen *et al.* (2020) found an increase in $InsP_6$ degradation from 80 to 160
72 minutes retention time. However, a considerably higher degradation of $InsP_6$ and a higher
73 content of both $InsP_4$ and $InsP_3$ was seen with a diet containing both phytase and acid than
74 with a diet with phytase but without acid already after 80 minutes retention time in the crop
75 (Kristoffersen *et al.* 2020).

76 The retention time in the crop is dependent on feeding regime. A high amount of feed in the
77 crop for intermittently fed birds leads to a long retention time (Svihus 2014). Observations of
78 broilers on *ad libitum* feeding have shown that they eat in a semi-continuous manner (Nielsen
79 2004; Svihus *et al.* 2010, 2013). This eating pattern will most likely reduce the use of the crop.
80 Svihus *et al.* (2010) found that 78 % of *ad libitum* fed 31 to 39 day old chickens had less than
81 5 g of dry matter (DM) in the crop. In contrast, as much as 50 g DM has been found in the crop
82 of intermittently fed birds (Sacranie *et al.* 2017). This leads to a presumption of a short retention
83 time due to a limited use of the crop in *ad libitum* fed chickens, although this has been scantily
84 studied.

85 Since most birds are fed *ad libitum* in practice, data are needed to clarify the interaction of acid
86 addition and phytase after shorter retention times in the crop. Based on the results from
87 Kristoffersen *et al.* (2020), a chicken experiment to study the short-term effect of acid addition
88 on phytase efficacy in the crop was carried out. In addition, a field assessment was carried out
89 to map crop retention time in *ad libitum* fed broilers.

90

91

92

93 # Material and methods

94 **Field assessment**

95 All chicken farms contributing in this study followed the laws and regulations concerning
96 animal welfare and chicken meat production in Norway (the Animal Welfare Act of June 19,
97 2009 and the regulations on keeping chickens and turkeys of December 12, 2001).

98 Four commercial chicken houses in Rogaland, Norway, with Ross 308 chickens were selected
99 based on the age of birds in the period the experiment was done. On all farms, birds were fed
100 a commercial pelleted diet *ad libitum* (Felleskjøpet Rogaland Agder, Stavanger, Norway),
101 with only minor differences in energy concentration. All diets contained 0.3 % buffered
102 formic acid ((Amasil NA®) BASF, Ludwigshafen, Germany), and the average pH was 5.4. At
103 10, 20 and 30 days of age, 40 (±1) chickens in each house were killed and crop content
104 collected. However, at 10 days of age only 32 chickens were collected in one of the houses. In
105 all four houses, there was 2 x 4 hour dark periods with 8 hours in between. The collection of
106 crop samples was carried out in the middle of the 8-hour light period. The birds were killed by
107 a blow to the head followed by cervical dislocation. The crop content was immediately
108 collected quantitatively and thereafter frozen at -20 °C. After thawing, pH measurements were
109 carried out and DM content was determined by drying at 104 °C overnight.

110 **Chicken experiment**

111 All the experimental procedures complied with the guidelines of the Local Ethical Committee
112 for Experiments on Animals in Poznan regarding animal experimentation and animal care
113 under study (European Union (EU) Directive 2010/63/EU for animal experiments).

114 Day-old male Ross 308 chickens were reared in groups of 4 animals in 1.2 × 0.8 m floor pens
115 with wood-shaving litter until day 11, and were fed a balanced maize and soybean-based diet
116 produced at the experimental plant of Animal Nutrition Experimental Station, Gorzyń,

117 Poland. The diet was formulated to meet Aviagen recommendation for Ross 308 and was
118 presented in crumbled form. The temperature was kept at 32 °C during the first week, and it
119 was gradually reduced until it reached 23 °C at the end of the experiment. At 11 days of age,
120 120 birds were placed in individual cages (width 40 cm × depth 50 cm × height 50 cm). The
121 average start weights of the birds were 215 g (± 18.6 standard deviation). The cages had wire-
122 mesh floor and a collection tray for excreta. Forty randomly selected birds were fed either a
123 control diet (CON), a control diet with phytase added (PHY) or a control diet with phytase
124 and formic acid added (PHYA). The birds had access to feed between the hours of 08:00 to
125 09:00, 12:00 to 13:00, 16:30 to 17:30, 21:00 to 22:00 and from 02:00 to 04:00. The feeders
126 were removed from the cages between feedings. The light was off from 22:00 to 02:00 and
127 from 04:00 to 08:00. All birds had *ad libitum* access to water throughout the experimental
128 period.

129 **Experimental diet**

130 The diet was wheat based, with a high content of phytate-P (Table 1). The pelleted diet (4
131 mm) was produced in two batches in a commercial feed plant (Felleskjøpet Rogaland Agder,
132 Stavanger, Norway), and contained 5.0 g/kg titanium dioxide (TiO_2) as a digestibility marker.
133 One batch was produced without added formic acid, and another batch was produced with 1.4
134 % formic acid (85 %) (ADDCON Nordic, Porsgrunn, Norway) added. The amount of formic
135 acid needed to achieve the desired pH at 4.5 was determined by gradually adding formic acid
136 to 1.0 g (±0.01 g) of the non-acidified diet mixed with 5.0 ml deionised water until the desired
137 pH was obtained. For the two diets with phytase, 500 FYT phytase (RONOZYME® HiPhos,
138 DSM, Denmark) was added per kg feed at the Center for Feed Technology (Ås, Norway). The
139 phytase was mixed with 0.4 % water to ensure even distribution and sprayed on the pellet in
140 a twin-shaft paddle mixer-vacuum-coater (Dinnissen, Sevenum, the Netherlands). To adjust
141 the pellet size to the eating capability of chicken, the pellets were crumbled in a roller mill

142 (DT900-12, CPM, Roskamp, Waterloo, IA, USA) with 2.8 mm distance between the rolls.
143 The feed was stored in a freezer at -20 °C for 9 months before the experiment started. The
144 analysed phytase activity prior to the broiler experiment was 564 FYT/kg feed with phytase
145 added and 574 FYT/g feed with both phytase and acid added, while phytase activity for the
146 diets without phytase added was below detection level. Samples taken from the buckets used
147 for feed storage in the chicken room were used to measure diet pH. The pH in the CON, PHY
148 and PHYA diets was 5.8, 5.7 and 4.3, respectively.

149 **Sample collection**

150 Body weight (BW) and feed intake (FI) were recorded weekly for each bird. Excreta were
151 collected on day 19 by using trays put under the cages for total excreta collection during a four-
152 hour period. The birds were killed at day 20 and 21 by stunning followed by cervical
153 dislocation. A plastic zip tie was tightened around the neck immediately after killing, in order
154 to prevent loss of crop content. The birds were starved for eight hours before start of the last
155 feeding to make sure that the digestive system was as empty as possible. The first five birds
156 from each diet were killed right before feeding (time zero), and then five birds for each diet
157 were killed at 20, 40, 60, 80, 100, 120 and 140 minutes after start of the feeding. The last feeding
158 lasted 60 minutes, and the FI of this meal was measured. The crop and the gizzard of all birds
159 were emptied and the pH was measured immediately after sampling by inserting the electrode
160 of a pH meter (CP-411, Elmetron, Zabrze, Poland) into the container with crop content, and by
161 placing the pH electrode (pH 100, VWR International, Radnor, PA, USA) directly into the
162 gizzard. All crops were empty at time zero hence no crop pH was measured at that time. In
163 addition to collection of crop content, the contents of jejunum from the end of the duodenal
164 loop until Meckel's diverticulum and the last 2/3 of ileum were collected for all birds killed 60
165 and 140 minutes after the start of feeding. All samples were immediately frozen in liquid
166 nitrogen and were kept at -20 °C until lyophilisation.

167 **Chemical analyses**

168 All samples of digesta and excreta were homogenised using a Stomacher homogeniser
169 (Interscience, France), and then freeze-dried (Christ Epsilon-10D LSC plus, Medizinischer
170 Apparatebau, Osterode/Harz, Germany) and ground, in order to pass through a 0.5 mm sieve
171 (Retsch, Ultra Centrifugal Mill ZM 200, Haan, Germany). Lyophilised DM content was used
172 in all calculations. The diets, digesta and excreta were analysed for TiO_2 content as described
173 by Short *et al.* (1996). P in diets, digesta and excreta was analysed according to the method of
174 FAO (2011). Briefly, a solution of $HClO_4$, HNO_3 and H_2SO_4 was added to the samples and this
175 was mineralised until the solutions became colourless. Thereafter an ammonium molybdate
176 solution was added, and the values of P were read on a Marcel Media spectrophotometer
177 (Marcel S.A., Zielonka, Poland) at 720 nm. Phytase activity in the diet was determined
178 according to the recommended method of the enzyme producer (assay at pH 5.5 and 37 °C).
179 Crude protein in diets (Kjeldahl-nitrogen × 6.25) was determined according to the methods
180 described by the European Commission Regulation (EC) (No 152/2009). The quantitative
181 determination of isomers of inositol in the crop and the diet samples were analysed using High
182 Performance Liquid Chromatography, ThermoFisher ICS-5000+ (Thermo Fisher Scientific,
183 Waltham, MA, USA) by the Research Center for Animal Nutrition and Health of DSM
184 Nutritional products (Village Neuf, France), and was based on the method described in
185 Skoglund *et al.* (1997). To assess the daily and long-term laboratory performance of the method,
186 dedicated standard samples were analysed with unknown samples to ensure the accuracy and
187 precision of the method. Data acquisition, integration and quantification were performed by
188 Chromeleon® software (Thermo Fisher Scientific, Waltham, MA, USA).

189 **Statistics**

190 As the field assessment could not be carried out with independent replications, no ANOVA was
191 performed on these results. For the chicken experiment ANOVA and regression analysis in

192 SAS software 9.4 (SAS Inst. Inc., Cary, NC, USA) was used. For jejunal and ileal P digestibility
193 a factorial ANOVA was performed to determine the effect of two fixed times and three diets.
194 For P retention, a one-way ANOVA was performed to determine the effect of diet. For crop
195 pH, gizzard pH and concentration of InsPs a one-way ANOVA was performed to determine the
196 effect of diet within each time. In addition, the random effect of time was determined with a
197 regression analysis to assess linear and quadratic time effects for crop pH, gizzard pH
198 concentration of InsPs and crop and gizzard DM. Effects were considered significant when $P <$
199 0.05, and P-values between 0.05 and 0.1 were considered tendencies. As a measure of random
200 variation, $\sqrt{MSE}$ was used.

201 **Calculations**

202 The ileal and jejunal digestibility coefficient of P and P retention were calculated by the
203 following formula:

204 Nutrient digestibility coefficient/retention = 1 – [([TiO_2]diet/[TiO_2] digesta(excreta)) ×
205 ([P]digesta(excreta)/[P]diet)]

206 Results

207 **Field assessment**

208 At all ages, zero to 4 g of crop DM content was the most common amount observed, although
209 some birds had a much higher content (Table 2). From 10 to 20 days of age weight and
210 distribution of crop DM did not change considerably, but at 30 days of age, the average crop
211 content weight was higher due to a larger amount of crops with more than 10 g DM. The average
212 hourly intake of 10-day old chickens based on table values for daily FI of mixed-sex Ross 308
213 (Aviagen 2019) is 2.04 g/h, 4.24 g/h for 20-day old chickens and 6.67 g/h for 30-day old
214 chickens. A higher DM content in crop than this average hourly intake was found in 66 % of
215 the chickens. It can be estimated from the crop content that the average retention time in the
216 crop at 10, 20 and 30 days would be 168, 82 and 84 minutes, respectively. More than 300 g/kg

217 water in the crop content was found in 87 % of the chickens and 41 % of the chickens had more
218 than 450 g/kg water in their crop contents. Most of the chickens had a pH in the crop between
219 4.5 and 5.0, which was somewhat lower than the reported pH for the diet.

220 **Chicken experiment**

221 The significantly lower FI (P<0.001) of the diet with both acid and phytase (687 g) compared
222 to the other two diets (768 g) resulted in a lower body weight gain (BWG) (P<0.001) with 552
223 g for the diet with acid and phytase compared 624 g BWG for to the other two diets (data not
224 shown). However, no difference in FCR (P>0.05) was seen between diets.

225 Prior to the start of the last feeding, all chicken crops were empty and the average weight of
226 DM in the gizzards was 0.9 g (data not shown). The average FI for the birds at the last feeding
227 with 20, 40 and 60 minutes access to feed before killing was 30.2, 31.8 and 32.9 g, respectively
228 (data not shown). However, no difference (P>0.05) in FI between the different durations of the
229 last feeding was found. Due to a gradual reduction in crop content after the feed was taken away
230 at 60 minutes, the crop DM weight decreased linearly with increasing time after start of the
231 feeding (P<0.001) (Table 3). However, 38 % of the FI was still present in the crop 140 minutes
232 after the start of the feeding. The DM content of the gizzard did not change (P>0.05) during the
233 entire 140 minutes period. Average DM content in the jejunum was 1.4 g after 60 minutes and
234 1.8 g after 140 minutes (data not shown). In the ileum, the DM content was 0.6 g after 60
235 minutes and 1.4 g after 140 minutes.

236 The average level of pH in the gizzard before the start of the feeding was 1.71 (data not shown).
237 No difference in gizzard pH between the diets at different times after feeding was observed
238 (P>0.05) (Table 3). Gizzard pH was linearly reduced with increasing time after feeding
239 (P<0.05) for all diets. The crop pH for the birds fed the diet with phytase and acid was lower

240 than for the two other diets (P<0.05) for all times. No effect of time on crop pH was found,
241 except for the diet with both acid and phytase, where pH increased over time (P<0.05).

242 The apparent jejunal digestibility (AJD) of P was increased (P<0.001) from 60 minutes after
243 feeding to 140 minutes after feeding (Table 4). The diet with both acid and phytase had a
244 significantly higher (P=0.018) AJD of P than the negative control and the diet with phytase
245 only. There was also a tendency (P=0.058) of an interaction between diet and time, where
246 increased AJD of P from 60 to 140 minutes was seen only for the control diet and not for the
247 two diets with phytase added. There was no difference in apparent ileal digestibility (AID) of
248 P between diets or time (P>0.05). P retention was significant higher (P=0.026) with the two
249 diets containing added phytase than with the control diet (Table 4).

250 For all three diets there was a significant reduction in the concentration of $InsP_6$ (P<0.001) in
251 the crop with increased retention time (Figure). After 140 minutes retention time in the crop
252 12.2 % of the $InsP_6$ was degraded with the CON diet, 26.8 % of the $InsP_6$ was degraded using
253 the PHY diet and for the PHYA diet, 37.1 % was degraded. The concentration of $InsP_6$ was
254 significantly (P<0.05) lower for the diet with both acid and phytase than the other two diets for
255 all times except 40 and 120 minutes after feeding, and only significantly lower than the control
256 feed at 140 minutes retention time. The diet with both phytase and acid resulted in an increased
257 concentration of $InsP_5$ (P<0.001) with increased retention time, while no time effect was seen
258 for the other two diets. A significantly higher (P<0.05) $InsP_5$ concentration for the diet with both
259 acid and phytase compared to the other two diets was seen from 60 minutes. No $InsP_{3\&4}$ were
260 found in the crop with the control diet. $InsP_{3\&4}$ were detected from 80 minutes after start of
261 feeding for the diet with both phytase and acid added. For the diet with phytase, $InsP_{3\&4}$ were
262 only found after 140 minutes retention time. The concentration of $InsP_{1\&2}$ was increased
263 (P<0.001) with increased retention time for the control diet as well as for the diet with both
264 phytase and acid added.

265 ## Discussion

266 The results from the current experiment establish that the degradation of phytate in crop and
267 AJD of P was increased with acidification of a diet with phytase even with a short retention
268 time in the crop. In addition, the degradation of phytate was further increased with increased
269 retention time in the crop. The performance was not the subject of the current experiment, but
270 the reduced FI and BWG with acid addition was expected as it has been shown in other
271 experiments (Brenes *et al.* 2003; Esmaeilipour *et al.* 2012).

272 The fact that all birds except those killed at 20 and 40 minutes had 60 minutes feed availability
273 reduces the accuracy of estimated retention time in the crop. However, in accordance with
274 Svihus *et al.* (2010) the feed was consumed mainly within the first 20 minutes of the feeding
275 bout and therefore no significant differences in FI between the different durations of the last
276 feeding was found.

277 The average crop retention time of more than one hour in the field assessment and the high
278 percentage of chickens with more than the average hourly FI in the crop is contradictory to
279 previous assumptions about *ad libitum* fed birds. The established perception is that *ad libitum*
280 fed birds largely omits the use of the crop as a storage organ. This is based on observation of
281 *ad libitum* fed birds, which show that they eat small amounts of feed many times throughout
282 the day (Svihus *et al.* 2010, 2013). In addition, the crop has previously been found to contain
283 less than 5 g DM in two-thirds (Svihus *et al.* 2010) or half of the birds (Kristoffersen *et al.*
284 2020) under *ad libitum* feeding.

285 The high average water content of the crop content in the field assessment indicates that water
286 was not a limited factor for phytase activity, since Denstadli *et al.* (2006) showed that a water
287 content of 450 g/kg increased the phytase activity compared to 250 or 350 g/kg. Svihus *et al.*

288 (2013) observed that more than half of the birds were drinking within 8 minutes after finishing
289 an eating bout, and this could explain the water content in the crop. DM content

290 Acid addition increases the degradation of $InsP_6$ regardless of retention time in the crop, as seen
291 by the lower concentration of $InsP_6$ for the PHYA diet than the other two diets for most of the
292 retention times. In addition, there was a higher AJD of P 60 minutes after start of feeding for
293 the PHYA diet compared to the other two diets, possibly as a consequence of the higher
294 degradation of phytate in the crop. A tendency to lower $InsP_6$ concentration after 80 minutes
295 for a diet with both acid and phytase compared with a diet with only phytase has previously
296 been observed (Kristoffersen *et al.* 2020). As this effect of acid addition is occurring with the
297 short retention time in crop, it seems reasonable to assume that this effect could also be found
298 with *ad libitum* feeding. There is, however, an increased formation of the lower isomers ($InsP_5$,
299 $InsP_4$ and $InsP_3$) with the PHYA diet compared to the other two diets from 60 minutes retention
300 time and onwards. This indicates that the effect of acidification is increased with prolonged
301 retention time in the crop. Intermittent feeding has previously resulted in an increased P
302 digestibility compared to *ad libitum* feeding (Sacranie *et al.* 2017; Kristoffersen *et al.* 2020).
303 Therefore, crop retention time is important for phytate-P utilisation, and a feeding regime that
304 increases the crop retention time will be beneficial for improving the utilisation of P.

305 The lack of reduction in crop pH with time without acid addition is in accordance with the
306 results of Kristoffersen *et al.* (2020). However, the surprising lack of difference between the
307 CON diet and the PHY diet on concentration of $InsP_6$ and $InsP_5$ in the crop may be caused by a
308 too high pH in the crop without acid addition, as the pH optimum for the phytase used is lower
309 than the pH in crop without acid addition (Menezes-Blackburn *et al.* 2015). In addition, the
310 phytate solubility is decreased with higher pH levels and hence the potential for degradation is
311 reduced (Vieira *et al.* 2018). Even though the same pattern was found in the experiment of
312 Kristoffersen *et al.* (2020) with a short retention time in crop, there is usually a substantial effect

313 of phytase on degradation of $InsP_6$ in the crop regardless of acid addition in the feed (Zeller *et*
314 *al.* 2015a, 2016; Sommerfeld *et al.* 2018; Svihus *et al.* 2010).

315 In the current experiment, there was no difference in ileal digestibility between diets, which
316 indicates that there is also a considerable phytase activity and phytate degradation later in the
317 digestive system in addition to that in the crop. The intestine has not been considered an
318 important site of exogenous phytase activity due to the high pH here (Selle and Ravindran
319 2007). In addition, it has been shown that different phytases have different levels of activity in
320 the intestine (Onyango *et al.* 2005). However, the presumed phytase activity in the intestine in
321 the current experiment is in accordance with observations in earlier experiments (Zeller *et al.*
322 2015a, 2015b), the low Ca inclusion in the diet may have contributed to this (Appelgate *et al.*
323 2003). The increased P retention with phytase addition, but no further effect of acid also
324 supports this presumed intestine phytase activity. The lack of effect of acid on phytase activity
325 in the posterior digestive system demonstrates that acid addition only enhances phytase efficacy
326 in the anterior digestive system.

327 The results in the current experiment show clearly that the reduced pH in the crop due to acid
328 addition increases phytate degradation and improves P digestibility in the anterior digestive
329 tract even with such short retention times as is expected in *ad libitum* fed birds. However, these
330 differences were not present at the excreta level. This indicates that the degradation of phytate
331 in the anterior digestive system is not a limiting factor for the P retention during intermittent
332 feeding. The interaction between retention time in the crop and dietary phytate concentration,
333 higher phytase doses, drinking water pH and dietary cation concentrations need further
334 exploration.

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449 *Table 1 Composition and nutrient content of the experimental diets (g/kg as fed unless otherwise*
450 *stated).*

Ingredient	CON and PHY[1]	PHYA[2]
Wheat	42.40	42.00
Wheat bran	19.90	19.60
Pea starch	10.00	9.70
Rape seed meal (CP 34.4 %)	8.00	7.90
Soy protein (CP 56 %)	9.30	9.20
Soy oil	2.00	2.00
Pea protein (CP 48.6 %)	1.80	1.70
Corn gluten (CP 60.6 %)	1.10	1.00
Animal fat	2.00	2.00
Limestone	0.70	0.70
Sodium chloride	0.57	0.59
L-lysine	0.55	0.53
Mineral and vitamin premix[3]	0.56	0.56
L-threonine	0.20	0.20
Methionine analogue	0.20	0.20
Choline chloride	0.08	0.08
L-Tryptophan	0.06	0.06
L-valine	0.04	0.04
Enzyme[4]	0.02	0.02
Taste enhancer	0.02	0.02
Titanium dioxide	0.50	0.50
Formic acid	—	1.40
Nutrient composition		
Calculated		
MJ/kg DM	10.9	10.8
Phytate-P (%)	0.32	0.32
Determined		
Crude protein	187.0	180.0
Starch	366.0	330.0
Crude fat	64.8	65.1
Total P	5.0	4.6
Ca	5.4	5.1

451 [1]CON, PHY: Control diet, with and without phytase added
452 [2]PHYA: Diet with phytase and acid added
453 [3]Supplied per kilogram of diet: 2.41 mg retinol, 0.038 mg cholecalciferol, 188 mg tocopheryl
454 acetate, 6.0 mg menadione, 105 mg ascorbic acid, 4.0 mg thiamine, 12.1 mg riboflavin, 60.25
455 mg niacin, 12.05 mg pyridoxine, 0.04 mg cyanocobalamin, 30.12 mg pantothenic acid, 3.2
456 mg folic acid, 0.4 mg biotin, 72.3 mg Mn ($MnSO_4$), 108.4 mg Zn (ZnO), 144.6 mg Fe
457 ($FeSO_4$), 26.5 mg Cu ($CuSO_4$), 0.45 mg Se (Na_2SeO_3), 0.72 mg I.
458 [4]Enzyme Rovabio® Excel LC 2, Adisseo, France, provided xylanase and *ß*-glucanase obtained
459 from a fermentation broth of *Penicillium funiculosum.*
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462 *Table 2 Percentage of birds in different intervals plus average and range for crop dry matter content,*
463 *water content and pH in commercial chickens at 10, 20 and 30 days of age*

Dry matter content	0-4 g	4-7 g	7-10 g	10-15 g	> 15 g	Min.	Average	Max.
Age (days)			in %					
10	46.7	28.3	15.1	9.9	0.0	0.13	5.02	13.78
20	41.9	27.5	23.1	7.5	0.0	0.09	5.13	14.61
30	34.2	20.5	12.4	32.9	17.4	0.00	8.21	31.63
All	40.8	25.4	16.9	16.9	5.9		6.14	
Water content	**< 450 g/kg**	**> 450 g/kg**				**Min.**	**Average**	**Max.**
Age (days)	in %							
10	80	20				140	380	490
20	54	46				130	430	620
30	45	55				120	420	630
All	59	41					420	
pH	**< pH 4.0**	**pH 4.0-4.5**	**pH 4.5-5.0**	**pH 5.0-5.5**	**> pH 5.5**	**Min.**	**Average**	**Max.**
Age (days)			in %					
10	1.4	23.0	68.9	6.8	0.0	3.87	4.66	5.42
20	0.0	0.0	66.9	33.1	0.0	4.54	4.92	5.41
30	0.0	4.3	65.9	29.0	0.7	4.05	4.87	5.58
All	0.5	9.2	67.3	22.9	0.2		4.82	

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Table 3 Effect of time after start of feeding on contents in crop and gizzard, and the effect of time and diet on pH in crop and gizzard of intermittently fed chickens.

Time (minutes)	Crop DM (g)[1]	Gizzard DM (g)[1]	Crop pH				Gizzard pH[2]			
			CON[3]	PHY[3]	PHYA[3]	√MSE[4]	CON	PHY	PHYA	√MSE
20	21.3	2.2	5.54^{a}	5.50^{a}	4.09^{b}	0.150	2.85	3.42	2.47	0.635
40	21.2	2.9	5.47^{a}	5.46^{a}	3.96^{b}	0.225	2.19	2.14	2.09	0.526
60	20.1	2.7	5.30^{a}	5.46^{a}	4.10^{b}	0.163	2.22	2.44	1.83	0.484
80	20.4	2.4	5.54^{a}	5.49^{a}	4.10^{b}	0.142	2.03	1.94	2.09	0.407
100	17.9	2.7	5.58^{a}	5.46^{a}	4.21^{b}	0.100	1.73	2.15	1.73	0.559
120	16.8	2.5	5.22^{b}	5.54^{a}	4.18^{b}	0.303	1.91	2.12	1.78	0.542
140	12.4	2.5	5.48^{a}	5.50^{a}	4.23^{b}	0.105	2.23	1.87	2.11	0.551
√MSE	6.49	1.08	0.242	0.091	0.192		0.507	0.682	0.483	
P-values										
Linear	<0.001	0.975	0.488	0.399	0.039		0.020	0.004	0.039	
Quadratic	0.410	0.482	0.680	0.608	0.877		0.002	0.020	0.877	

[a,b]Crop pH means within row without common letters are significantly different at $P < 0.001$

[1]Average dry matter content for all diets

[2]No significant differences between gizzard pH means was found

[3]CON: control diet, PHY: diet with phytase added, PHYA: diet with both phytase and acid added.

[4]Square root of means square error in the analysis of variance

474 *Table 4 Effect of time and diet on jejunal and ileal P digestibility and the effect of diet on P*
475 *retention*

Diet	Time	AJD of P[1]	AID of P[2]	P retention
CON[3]	60	0.30[c]	0.71	
PHY[3]	60	0.45[bc]	0.62	
PHYA[3]	60	0.54[ab]	0.75	
CON	140	0.63[ab]	0.49	
PHY	140	0.54[ab]	0.60	
PHYA	140	0.68[a]	0.67	
√MSE[4]		0.147	0.112	0.054
Diet				
CON		0.47[b]	0.60	0.48[b]
PHY		0.49[b]	0.61	0.51[a]
PHYA		0.61[a]	0.71	0.52[a]
Time				
60		0.43[b]	0.69	
140		0.62[a]	0.59	
P-values				
Diet		0.018	0.209	0.026
Time		<0.001	0.069	
Diet*time		0.058	0.327	

476 [a,b,c]Means within column without common letters are significantly different at $P<0.05$.
477 [1]Apparent jejunal phosphorus digestibility
478 [2]Apparent ileal phosphorus digestibility
479 [3]CON: control diet, PHY: diet with phytase added, PHYA: diet with both phytase and acid added.
480 [4]Square root of means square error in the analysis of variance
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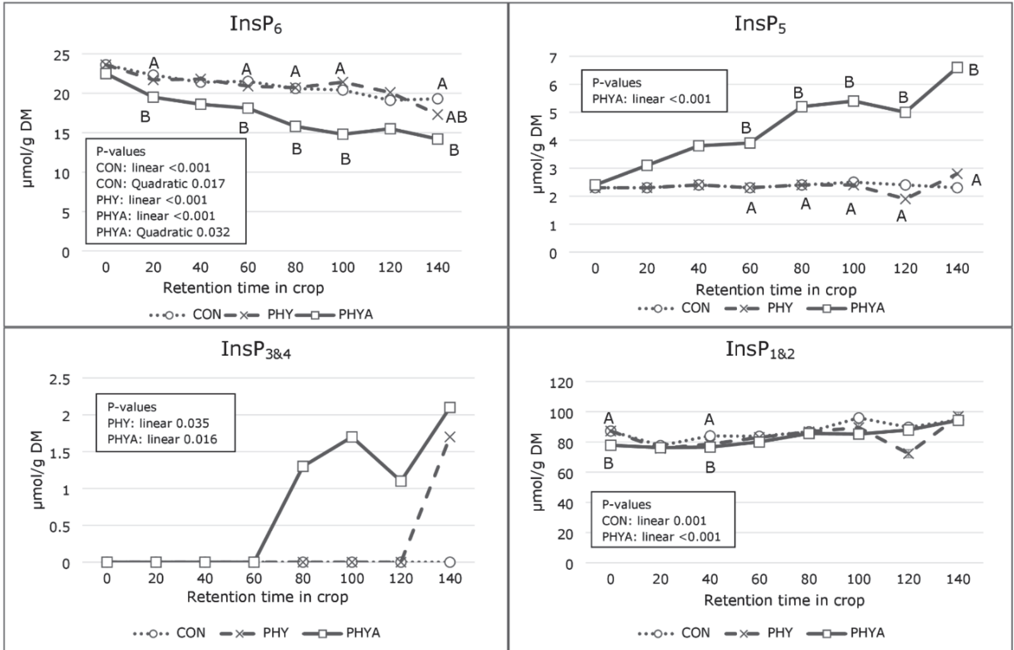

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484 Figure. Concentrations of inositol phosphates ($InsP_6$, $InsP_5$, $InsP_{3\&4}$, and $InsP_{1\&2}$) in feed (time zero)
485 and crop content 20 to 140 minutes after start of feeding.

486 Only significant (P<0.05) linear and quadratic regressions are showed. Treatment means within time
487 with different letters are significantly different at (P<0.05). CON: Control diet, PHY: Diet with
488 phytase, PHYA: Diet with both phytase and acid added. √MSE: CON: $InsP_6$ 1.28, $InsP_5$ 0.16, $InsP_{1\&2}$
489 6.94. PHY: $InsP_6$ 1.82, $InsP_5$ 0.47, $InsP_{3\&4}$ 0.95, $InsP_{1\&2}$ 15.97. PHYA: $InsP_6$ 3.59, $InsP_5$ 1.89, $InsP_{3\&4}$
490 1.82, $InsP_{1\&2}$ 8.46.
491

Paper III

The effect of reduced feed pH, phytase addition and their interaction on mineral utilization in pigs

Siril Kristoffersen[1,2] Email: siril.kristoffersen@fkra.no (corresponding author)

Torger Gjefsen[2] Email: tgjefsen@gmail.com

Birger Svihus[1], Email: birger.svihus@nmbu.no

Nils Petter Kjos[1], Email: nils.kjos@nmbu.no

[1]Faculty of biosciences, Norwegian University of Life Sciences, P.O. Box 5003, N-1432 Aas, Norway.

[2]Felleskjøpet Rogaland Agder, Sandvikveien 21, N-4002 Stavanger, Norway.

23

Abstract

24 A 2x2 factorial experiment was carried out to assess the effect of reduced feed pH and
25 addition of phytase and their interactions on performance, mineral retention (Ca, P, Mg, Zn,
26 Fe and Cu) and bone mineralization. A wheat-based diet with a high phytate-P content and no
27 inorganic P added, or the same diet with either 1.4 % formic acid, 500 FTU *C. braakii*-
28 derived phytase or both added, were used. Thirty-two piglets, with a mean weight of 21.06 ±
29 0.83 kg were distributed in eight pens. Individual feed intake was recorded, and all treatments
30 were represented in all pens. The experimental period was 28 days. Performance was
31 recorded, pH in stomach, jejunum and ileum was measured and content from jejunum and
32 ileum was collected for assessment of P digestibility. In addition, feces were collected for
33 total digestibility measurements and left third and fourth metacarpal were analyzed for bone
34 mineralization. Phytase addition increased growth (P=0.046), jejunal P digestibility
35 (P=0.004), total tract digestibility of P (P<0.001) and Ca (P<0.001) and bone mineralization
36 (P<0.001). Acid addition improved growth (P=0.002) and FCR (P=0.033) in addition to total
37 tract digestibility of Mg (P=0.04), Fe (P<0.001) and Ca (P=0.001).
38 The experiment confirmed that phytase addition improved P digestibility. However, no
39 increased phytase efficacy was seen with acid addition.

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Keywords

41 Phosphorus, phytate, formic acid, digestibility

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48 ## Introduction

49 Phosphorus (P) from plant sources in pig diets occurs primarily in the form of phytate, which
50 cannot be degraded by enzymes secreted by the pig. To reduce the amount of P in feces and
51 hence environmental pollution, exogenous phytase is routinely added to pig diets (Wilcock
52 and Walk, 2016). However, phytase does not degrade the phytate completely. Phytase
53 addition increased the phytate-P digestibility from 39 % to of 65 % (Rosenfelder-Kuon et al.,
54 2020).
55
56 As for most enzymes, phytase has an optimal pH range. For most new-generation phytases,
57 this optimum pH is between 3.0 and 5.0 (Menezes-Blackburn et al., 2015; Vieira et al., 2018).
58 The main site for exogenous phytase activity in pigs is the stomach (Selle and Ravindran,
59 2008). However, the pH in stomach varies, and is reported to be between 2.7 and 4.8
60 (Dersjant-Li et al., 2001; Omogbenigun et al., 2003; Eberhard et al., 2007; Lee et al., 2018). A
61 pH in the lower range of this interval will be beneficial to promote phytase activity and
62 efficacy for the phytases that have a low pH optimum. The pH of the stomach may be lowered
63 with addition of organic acid to the feed (Suiryanrayna and Ramana, 2015). A reduced pH in
64 the stomach could also reduce the stomach emptying rate (Van der Aar et al., 2017). An
65 increased degradation of phytate could be seen because of the increased retention time in the
66 favorable low-pH environment (Blaabjerg et al., 2011). Organic acids may improve growth
67 and feed conversion ratio (FCR) (Partanen and Mroz, 1999). This is the main reason, together
68 with the beneficial anti-microbial effect (Suiryanrayna and Ramana, 2015), why organic acid
69 is routinely added to pig feed today (Tugnoli et al., 2020).
70
71 Many minerals like calcium (Ca), zinc (Zn), iron (Fe), copper (Cu) and magnesium (Mg) are
72 bound to phytate in mineral-phytate complexes that reduce phytase degradation, thus

73 decreasing both mineral digestibility and phytate-P availability (Maenz et al., 1999). The
74 effect of phytase addition on mineral digestibility is not consistent. Arredondo et al. (2019)
75 found an increased apparent total tract digestibility (ATTD) of Mg and Zn with phytase
76 addition, but no improvement in ATTD of Cu, Fe and Mn. The affinity of the minerals to
77 phytate is dependent on pH level, so that a reduction in the inhibitory effects of minerals is
78 seen when pH is lowered (Maenz et al., 1999). Therefore, organic acids are expected to
79 increase mineral absorption by lowering stomach pH and reducing the binding of these
80 minerals to phytate in addition to the increased phytase efficacy by lowered pH (Jongbloed et
81 al., 2000).
82
83 Several experiments have shown that organic acids have a positive effect on phytase efficacy
84 (Kemme et al., 1999; Jongbloed et al., 2000). However, other experiments have shown no
85 interaction between organic acid and phytase (Radcliffe et al., 1998; Omogbenigun et al.,
86 2003). As organic acid is routinely added to pig feed and conflicting results on the effect on
87 phytase efficacy have been reported, it is interesting to investigate the effect of phytase, acid
88 addition and their interaction effects. Therefore the hypothesis that acidification of the feed in
89 combination with addition of microbial phytase will increase phytase efficacy was tested. In
90 addition, the effect on other phytate-bound minerals was examined.
91

92 Material and methods

93 All the animals were handled in accordance with the applicable laws and regulations
94 controlling experiments with live animals in Norway (the Animal Welfare Act of 28th of
95 December 2009 and the local legislation derived from the directive 2010/63 EU of the
96 European Parliament and Council of 22nd September 2010 on the protection of animals used
97 for scientific purposes). The experiment was performed at the Center for Livestock

98 Production, Norwegian University of Life Sciences, Ås, Norway, from April to May 2019,
99 and lasted for twenty-eight days.
100
101 Thirty-two pigs were fed a diet with or without phytase and with or without formic acid in a 2
102 x 2 factorial design, with eight pigs per treatment combination. Seven sows (Norwegian
103 Landrace × Yorkshire) inseminated with Duroc semen provided the piglets for this
104 experiment. The piglets were weaned at approximately 5 weeks of age. At 52 days of age, and
105 an average initial body weight of 21.06 kg ± 0.80 standard deviations, the pigs were equally
106 distributed by weight and randomly assigned to one of the four dietary treatments with three
107 barrows and five gilts on each diet. All pigs in one pen were from the same litter.
108
109 The pigs were distributed in groups of four and kept in eight 3.35 m × 2.25 m concrete-
110 floored, partially slatted pens, with individual feeding stations of 0.37 m × 1.35 m. The
111 individual feeding stations enabled recording of individual feed intake and all four dietary
112 treatments to be represented in all pens. A rubber mat of approximately 90 cm × 100 cm and
113 wood shavings was used on the pen floor. The pens were equipped with activity enrichment
114 toys. The room temperature was 18 °C, with 11 h of light and 13 h dark. During the dark
115 hours, only a night light was used.
116
117 The pigs were fed equal amounts twice daily at 08:00 and 14:00 in the individual feeding
118 stalls for approximately 30 min, and leftovers were recorded. Feed was provided during these
119 periods based on an estimated feed intake of 3 % of the live body weight, which was assumed
120 to be close to *ad libitum*. Water was accessible *ad libitum* via nipple drinkers. All pigs were
121 healthy at the start of the experiment and the clinical health status of the pigs was monitored
122 daily.

123

124 The feed was wheat based, with a high content of phytate-P and no inorganic P added (Table
125 1). The feed was produced in two batches in a commercial feed plant (Felleskjøpet Rogaland
126 Agder, Stavanger, Norway), and contained 5.0 g/kg titanium dioxide (TiO_2) as a digestibility
127 marker. One batch was produced without added formic acid, and the second batch was
128 produced with 1.4 % formic acid (85 %) (ADDCON Nordic, Porsgrunn, Norway) added. The
129 amount of formic acid needed to achieve the wanted pH level of 4.5 was determined by
130 gradually adding formic acid to 1.0 g (±0.01 g) of the non-acidified diet mixed with 5.0 ml
131 deionized water until the wanted pH level was obtained. For the two diets with phytase, 500
132 FYT phytase (RONOZYME® HiPhos, DSM, Denmark) was added per kg feed at the Center
133 for Feed Technology (Ås, Norway). The phytase was mixed with 0.4% water to ensure even
134 distribution and sprayed on the pellet in a twin-shaft paddle mixer-vacuum-coater (Dinnissen,
135 Sevenum, the Netherlands) to ensure even distribution.

136

137 The analyzed phytase activity was 660 FYT/kg feed with phytase added and 692 FYT/g feed
138 with both phytase and acid added, while phytase activity for the diets without phytase added
139 was below detection level. Samples taken from wheelbarrows used in feeding were used to
140 measure feed pH. The pH in the control diet, diet with phytase added, diet with formic acid
141 added and the diet with both formic acid and phytase added was 5.7, 5.9, 4.2 and 4.2,
142 respectively.

143

144 ## Sampling

145 Body weight (BW) and feed intake (FI) were recorded weekly. However, the first two weeks
146 of the experimental period was regarded as an adaption period, and therefore body weight
147 gain (BWG), FCR and FI only from the last two weeks have been used to determine

148 differences between treatments. However, performance results from the first period is also
149 presented. Individual fecal samples were collected once per day from the floor immediately
150 after defecation or from rectum at experiment days 24 to 27. The fecal samples were frozen at
151 -20 °C between each sampling and samples from each pig were pooled. Dissection was done
152 on day 28 and 29. The feeding time of the pigs was adjusted between pens the day before
153 dissection to ensure that all pigs had two hours from start of feeding to start of euthanizing.
154 The animals were euthanized with a captive bolt pistol followed by exsanguination. Intestinal
155 content from the last meter of jejunum and the last 1.5 meter of ileum was collected
156 immediately after slaughter. The stomach content was emptied into a container and mixed
157 well before pH was measured. All pH-values were measured by inserting the pH meter (pH
158 100, VWR International, Radnor, PA, USA) into the container with the samples. In addition,
159 the left third and fourth metacarpals from each pig were collected for determination of bone
160 ash. The digesta samples were immediately frozen in liquid nitrogen after pH measurements,
161 and stored at -20 °C.
162

163 ## Chemical analyses

164 The diets were analyzed in duplicate for dry matter (DM), starch, crude protein (CP), P, Ca,
165 Cu, Mg, Zn and Fe. Fecal, jejunal and ileal samples were freeze-dried and homogenized.
166 Jejunal samples were analyzed in duplicate for P and titanium. Ileal samples were analyzed in
167 duplicate for P, titanium, starch, and CP. Fecal samples were analyzed for titanium, P, Ca, Cu,
168 Mg, Zn and Fe. The DM used in calculations was the lyophilized DM content. Crude protein
169 (Kjeldahl-nitrogen × 6.25) was determined according to the methods described in
170 the European Commission Regulation (EC) (No 152/2009). Titanium content was determined
171 by following the procedure described by Short et al. (1996). P analysis was done according to
172 the method of FAO (2011). Briefly, HCl was added to the samples and the solutions were

173 mineralized until they became colorless. Thereafter an ammonium molybdate solution was
174 added, and the samples were read on a MaxMat PL II Multi-analyser (MaxMat, France) at
175 340 nm. Starch was hydrolyzed with α-amylase and amyloglucosidase-enzymes to glucose,
176 and glucose concentration was determined using a spectrophotometer (MaxMat PL II
177 Multianalyzer, France) as described by McCleary et al. (1994). Ca, Cu, Mg, Fe and Zn was
178 analyzed according to the method described in European Commission Regulation (EC) (No
179 152/2009), with the modification that Application Note PRO-AG-02; Dried Plant Tissue
180 (Milestone Srl) was used for decomposition, and analyzed spectrophotometric in a MP-AES
181 4200 (Agilent Technologies Inc, Santa Clara, USA). Phytase activity in the feed was
182 determined according to the internal method of the enzyme producer (assay at pH 5.5 and 37°
183 C). For metacarpal analyses, soft tissues from the bones were removed by hand after boiling
184 the pig's trotters. DM was determined by drying the bones for 16 h at 104° C, thereafter bones
185 were ashed at 550° C for 16 h to determine and percentage.

186

187 Calculations

188 Feed conversion ratio (FCR) was calculated as feed/gain.

189

190 Apparent digestibility of nutrients was calculated by the following formula:
191 Nutrient digestibility coefficient/retention = 1 – [([TiO_2]diet/[TiO_2]digesta) ×
192 ([nutrient]digesta/[nutrient]diet)]

193

194 Statistical analyses

195 For statistical analyses the general linear model procedure in SAS software 9.4 (SAS Inst.
196 Inc., Cary, NC, USA) was used with the Ryan–Einot–Gabriel–Welsh F-test to investigate
197 differences ($P<0.05$) between the different treatment groups. P-values between 0.05 and 0.1

were considered tendencies. The square root of means square error (√MSE) was used as a measure of random variation. Performance, digestibility, and bone parameters were subjected to a two-way analysis with phytase and acid as effects. Pig was used as the experimental unit.

Results

The pigs were healthy, and no diarrhea or other illness were registered during the experimental period. For the first two weeks in the experiment (adaption period), no difference in performance between treatments was found (data not shown). An increased weight gain in the last 14 days of experimental period was seen with phytase (P=0.046) and acid addition (P=0.002) (Table 2). In addition, acid reduced FCR (P=0.033).

Acid addition reduced pH in stomach (P<0.001), but did not influence pH in the jejunum or ileum (P>0.05) (Table 3). Ileal pH was reduced by phytase addition (P=0.014). Table 4 show that phytase addition more than doubled the apparent jejunal digestibility (AJD) of P (P=0.004) and increased ATTD of P with 70 % and Ca with 15 % (P<0.001). In addition, phytase tended to improve apparent ileal digestibility (AID) of P (P=0.086). Acid addition increased ATTD of Ca (P=0.001), Mg (P=0.040) and Fe (P<0.001) and reduced the ATTD of Zn (P<0.001). In addition, a tendency of interaction (P=0.062) between acid and phytase was seen on ATTD of Fe where acidification of the feed only improved Fe digestibility when no phytase was added. In addition, phytase increased ash content and concentration of P in bone (P<0.001), as shown in Table 5.

Discussion

The increased growth with phytase addition is in accordance with previous experiments (Torres-Pitarch et al., 2017). However, the lack of any effect on feed intake and FCR is

223 contrary to the conclusion in the same meta-analysis. Though it should be noted, that in 11 of
224 36 studies included in this meta-analysis, an unchanged or reduced FCR was observed. The
225 increase in BWG and improved FCR with acidification during the last two weeks of the
226 experimental period was in accordance with the results of Jongbloed et al. (2000). A
227 prolonged retention time in the stomach is expected to increase the digestibility of protein and
228 energy (Partanen and Mroz, 1999), and hence increase growth. Lowering of pH in stomach by
229 adding organic acids to the feed is associated with a reduced gastric emptying rate (Van der
230 Aar et al., 2017). However, in the current experiment no improvement in ileal digestibility of
231 protein or starch was observed. Therefore, the increased weight gain and improved FCR with
232 acid addition seems not to be caused by an improved energy digestibility. A possible
233 explanation for the improved growth could be other effects of the acid supplementation, such
234 as acting as an energy source or an improved total tract digestibility (Partanen and Mroz,
235 1999; Suiryanrayna and Ramana, 2015).
236
237 The more than doubled AJD of P with phytase addition compared to no phytase addition,
238 implies that the main site of exogenous phytase activity is in the anterior digestive system, as
239 previously described (Selle and Ravindran, 2008). The difference in P digestibility between
240 diets with phytase and diets without phytase was reduced from jejunum to ileum. A possible
241 explanation for this lack of difference between treatments could be that the phytate
242 degradation potential of the phytase was already used before the feed reached jejunum.
243 Exogenous phytase may also reduce the mucosal phytase activity (Selle and Ravindran,
244 2008), and hence there could be less total phytase activity in the ileum for the diets with
245 phytase added. Previous studies where pigs with cannulas in the distal ileum where used,
246 showed a significant higher AID of P with phytase addition (Lindberg et al., 2007; Zeng et al.,
247 2011). The methodology in the current experiment where digesta from the last 1.5 meters of

248 ileum were used to determine AID of P could be a possible explanation for the lack of finding
249 a significant effect of phytase on AID of P. However, there was a clear effect of phytase
250 addition on ATTD of P in the current experiment concurrent with previous knowledge (Selle
251 and Ravindran, 2008; Torres-Pitarch et al., 2017). Phytase also improved bone mineralization
252 in accordance with previous results (Torres-Pitarch et al., 2017).
253
254 The variation in stomach pH is large even between pigs at the same age, and the effect of acid
255 addition on stomach pH is not consistent (Partanen and Mroz, 1999). Acid addition reduced
256 the pH in stomach in the current experiment to 4.1. This reduction in pH was expected to
257 increase phytate-P digestibility, as the maximum phytase activity for the phytase used was
258 observed with pH 4.0 (Menezes-Blackburn et al., 2015). The surprising lack of effect of acid
259 addition may be due to a too small pH reduction. However, experiments with a larger pH
260 difference in the stomach between different treatments than in the current experiment
261 (Radcliffe et al., 1998; Omogbenigun et al., 2003), did not either show an increased phytase
262 effect with acid addition. Another explanation for the unexpected lack of interaction effects
263 between acid and phytase could be because the phytase was utilized to its full capacity even
264 without acid addition. However, in experiments with a higher effect of phytase alone, there
265 was still an additional effect of organic acid addition on phytase efficacy (Kemme et al., 1999;
266 Jongbloed et al., 2000). In addition, the P digestibility was increased throughout the whole
267 digestive system for both diets with phytase addition, indicating that there is phytase activity
268 also in the intestine from exogenous phytase. Thus, the lack of an additional acid effect could
269 not be explained by a high phytase efficacy without acid addition and the reason for the lack
270 of effect of acid addition remains unexplained.
271

272 Lowered stomach pH by organic acids is expected to increase mineral absorption by reducing
273 the binding of minerals to phytate (Jongbloed et al., 2000). In the current experiment, all
274 minerals except available P were formulated to meet the requirements of the animal. The
275 major mechanism for regulation of Cu, Fe and Zn levels in the body is rate of absorption from
276 the intestine (Windisch, 2002), and ATTD of these minerals will therefore not be increased
277 more than the requirement of the animal. A surplus of Mg and Ca could be absorbed despite
278 regulatory mechanisms and the potential surplus in blood and body tissues is regulated by
279 urine secretion (De Baaij et al., 2015; Sjaastad et al., 2016). Because of this regulation
280 mechanism, it could be more likely to see an effect of phytase addition on ATTD of Ca and
281 Mg. The digestibility of Ca was increased by both phytase and acid addition. Because of the
282 regulation mechanism, and since Ca is the most abundant mineral in the diet of the minerals
283 examined and thus most likely to be bound to phytate (Humer et al., 2015) this was expected.
284 A surprisingly decreased digestibility of Zn with acid addition was found, However, Blank et
285 al. (2012) also found a decrease in ATTD of Zn with formic acid addition. In the absorption
286 Cu and Zn are antagonists (Bikker et al., 2012), and this could influence absorption of both
287 Cu and Zn as the digestibility is dependent of the level of inclusion in the diet and solubility
288 of the minerals.
289
290 In the current experiment, it was confirmed that phytase increases P and Ca digestibility in
291 addition to improving bone mineralization. The addition of formic acid improved growth,
292 FCR and ATTD of Ca, Fe and Mg. However, even though acid addition reduced stomach pH,
293 no interaction between phytase and acidification was seen.

294 Conflict of interest statement

295 The authors declare that they have no conflict of interest

296

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300

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408

409 *Table 1 Composition and nutrient content of the experimental diets (g/kg as fed unless otherwise*
410 *stated).*

Ingredient	C[1]	C+acid[2]
Wheat	42.4	42.0
Wheat bran	19.9	19.6
Pea starch	10.0	9.7
Rape seed meal (CP 34.4%)	8.0	7.9
Soy protein (CP 56%)	9.3	9.2
Soy oil	2.0	2.0
Pea protein (CP 48.6%)	1.8	1.7
Corn gluten (CP 60.6%)	1.1	1.0
Animal fat	2.0	2.0
Limestone	0.7	0.7
Sodium chloride	0.57	0.59
L-lysine	0.55	0.53
Mineral and vitamin premix[3]	0.56	0.56
L-threonine	0.20	0.20
Methionine analogue	0.20	0.20
Choline chloride	0.08	0.08
L-Tryptophan	0.06	0.06
L-valine	0.04	0.04
Enzyme[4]	0.02	0.02
Taste enhancer	0.02	0.02
Titanium dioxide	0.5	0.5
Formic acid	-	1.4
Nutrient composition		
Calculated MJ/kg DM	10.9	10.8
Calculated Phytate P (%)	0.32	0.32
Analyzed CP	187	180
Analyzed Starch	366	330
Analyzed Fat	64.8	65.1
Analyzed Total P	5.0	4.6
Analyzed Ca	5.38	5.07
Analyzed Mg	2.18	2.14
Analyzed Cu	0.02	0.03
Analyzed Fe	0.26	0.30
Analyzed Zn	0.17	0.14

411 [1]C: Control feed, with and without phytase added
412 [2]C+acid: Control feed with acid added, with and without phytase added
413 [3]Supplied per kilogram of diet: 8030 IU vitamin A, 1506 IU cholecalciferol, 188 mg tocopheryl
414 acetate, 6.0 mg menadione, 105 mg ascorbic acid, 4.0 mg thiamine, 12.1 mg riboflavin, 60.25 mg
415 niacin, 12.05 mg pyridoxine, 0.04 mg cyanocobalamin, 30.12 mg pantothenic acid, 3.2 mg folic acid,
416 0.4 mg biotin, 72.3 mg Mn ($MnSO_4$), 108.4 mg Zn (ZnO), 144.6 mg Fe (FeSO4), 26.5 mg Cu
417 ($CuSO_4$), 0.45 mg Se (Na_2SeO_3), 0.72 mg I.
418 [4]Enzyme Rovabio® Excel LC 2, Adisseo, France, provided xylanase and *β*-glucanase obtained from a
419 fermentation broth of *Penicillium funiculosum.*
420

421 *Table 2 Effect of phytase and formic acid addition on performance in growing pigs from experimental*
422 *day 15 to 28.*

Phytase	Acid	BWG[1]	FI[2]	FCR[3]
No	No	9.9[b]	17.7	1.79
Yes	No	10.7[ab]	18.6	1.75
No	Yes	11.1[a]	18.2	1.64
Yes	Yes	11.5[a]	19.2	1.68
√MSE[4]		0.84	1.86	0.138
Phytase				
Yes		11.1[a]	18.9	1.71
No		10.5[b]	17.9	1.72
Acid				
Yes		11.3[a]	18.7	1.66[b]
No		10.3[b]	18.2	1.77[a]
p-value				
Phytase		0.046	0.130	0.977
Acid		0.002	0.410	0.033
Acid x phytase		0.444	0.950	0.399

423 [1]Body weight gain (kg) day 15-28
424 [2]Total feed intake (kg) day 15-28
425 [3]Feed conversion ratio, calculated as feed:gain, day 15-28
426 [4]Square root of means square error in the analysis of variance
427 [a-d]Means within column without common letters are significantly different at $P<0.05$
428
429

430 *Table 3 Effect of phytase and formic acid addition on pH in stomach, jejunum and ileum in growing*
431 *pigs*

Phytase	Acid	Stomach pH	Jejunal pH	Ileal pH
No	No	4.75[a]	6.17	7.01
Yes	No	4.86[a]	6.50	7.15
No	Yes	4.10[b]	6.40	6.95
Yes	Yes	4.02[b]	6.43	7.09
√MSE[1]		0.255	0.337	0.15
Phytase				
Yes		4.44	6.46	7.12[a]
No		4.42	6.28	6.98[b]
Acid				
Yes		4.06[b]	6.41	7.02
No		4.80[a]	6.34	7.08
p-value				
Phytase		0.855	0.152	0.014
Acid		<0.001	0.535	0.249
Acid x phytase		0.286	0.206	0.991

432 [1]Square root of means square error in the analysis of variance
433 [a-d]Means within column without common letters are significantly different at $P<0.05$.
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Table 4 Effect of phytase and formic acid addition on the apparent jejunal and ileal digestibility and apparent total tract digestibility in growing pigs.

Phytase	Acid	AJD[1] P	AID[2] P	AID Starch	AID CP[3]	ATTD[4] P	ATTD Ca	ATTD Cu	ATTD Mg	ATTD Zn	ATTD Fe
No	No	0.15	0.33	0.98	0.78	0.36^{b}	0.62^{c}	0.13	0.31	0.24^{a}	-0.04^{c}
Yes	No	0.29	0.43	0.97	0.73	0.58^{a}	0.70^{b}	0.12	0.28	0.26^{a}	0.003^{bc}
No	Yes	0.10	0.34	0.97	0.67	0.35^{b}	0.69^{bc}	0.19	0.35	0.11^{b}	0.16^{a}
Yes	Yes	0.29	0.44	0.99	0.71	0.65^{a}	0.79^{a}	0.12	0.33	0.09^{b}	0.09^{ab}
√MSE[5]		0.130	0.153	0.025	0.118	0.063	0.061	0.054	0.061	0.073	0.082
Phytase											
Yes		0.29^{a}	0.43	0.98	0.72	0.61^{a}	0.75^{a}	0.12	0.31	0.17	0.05
No		0.12^{b}	0.33	0.97	0.72	0.36^{b}	0.65^{b}	0.16	0.33	0.17	0.06
Acid											
Yes		0.19	0.39	0.98	0.69	0.50	0.74^{a}	0.15	0.34^{a}	0.10^{b}	0.12^{a}
No		0.23	0.39	0.98	0.76	0.47	0.66^{b}	0.13	0.30^{b}	0.25^{a}	-0.02^{b}
p-value											
Phytase		0.004	0.086	0.587	0.967	<0.001	<0.001	0.060	0.369	0.912	0.735
Acid		0.654	0.849	0.791	0.141	0.202	0.001	0.168	0.040	<0.001	<0.001
Acid x phytase		0.573	0.987	0.277	0.288	0.163	0.648	0.115	0.961	0.548	0.062

[1]Apparent jejunal digestibility

[2]Apparent ileal digestibility

[3]Crude protein

[4]Apparent total tract digestibility

[5]Square root of means square error in the analysis of variance

[a-d]Means within column without common letters are significantly different at $P<0.05$.

444 *Table 5 Effect of phytase and formic acid addition on bone mineralization of the left third and fourth*

445 *metacarpal in growing pigs*

Phytase	Acid	Ash %[1]	mg P/g[2]
No	No	37.0[b]	40.7[b]
Yes	No	41.6[a]	46.3[a]
No	Yes	37.7[b]	40.9[b]
Yes	Yes	41.1[a]	47.2[a]
√MSE[3]		1.78	2.85
Phytase			
Yes		41.3[a]	46.8[a]
No		37.34[b]	40.8[b]
Acid			
Yes		39.4	44.1
No		39.3	43.5
p-value			
Phytase		<0.001	<0.001
Acid		0.839	0.556
Acid x phytase		0.354	0.720

446 [1]ash % of bone DM

447 [2]g/kg of bone DM

448 [3]Square root of means square error in the analysis of variance

449 [a-d]Means within column without common letters are significantly different at $P<0.05$.

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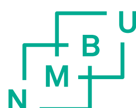
Fiskå Mølle AS
Fiskåvegen 1010
4120 Tau
www.fiska.no

Felleskjøpet Rogaland Agder SA
Sandvikveien 21
4016 Stavanger
www.fkra.no



Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no

108793 / ANDVORDGRAFISK.NO