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MEASUREMENTS OF ZINC SHOW PHYTATE EFFECTS

Use of Zn concentration in the gastrointestinal tract as a measure of phytate susceptibility to the effect of phytase supplementation in broilers

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ABSTRACT

14 Zinc (Zn) is the most vulnerable cation to complexation with phytate. An experiment
15 was conducted to evaluate the potential of measurements of Zn concentration in the
16 gastrointestinal tract as a marker to assess the anti-nutritional impact of phytate and
17 susceptibility of phytate to phytase in broilers. Ross 308 broilers (n = 180) were fed one of 5
18 experimental diets with differing phytase activity levels, analyzed at 605, 1150, 1804, 3954
19 and 5925 U/kg. Broiler performance and Zn concentration, pH and amount of phytate
20 hydrolyzed in the gizzard, duodenum and ileum were analyzed at d21 post hatch. Phytate
21 susceptibility to phytase degradation was determined *in vivo* and *in vitro* by measuring total
22 phytate-P hydrolyzed in the tract or in conditions that mimicked the tract, respectively. Phytase
23 activity level had a significant ($P < 0.05$) impact on Zn concentration and phytate hydrolyzed
24 in the gizzard and ileum, but not in the duodenum. Strong relationships were observed between
25 the amount of phytate hydrolyzed and Zn concentration in the gizzard in birds fed the diets
26 with 1804 U/kg or higher levels of phytase. Phytate and phytase effects could therefore
27 potentially be evaluated by measuring Zn concentration in the gizzard. Susceptible phytate
28 levels measured *in vivo* and *in vitro* were almost identical in the diet with phytase activity of
29 5925 U/kg, but in the diets with lower phytase activity levels the *in vitro* assay overestimated
30 the amount of P released. There were strong relationships between *in vivo* susceptible phytate
31 level and pH and amount of phytate hydrolyzed in the gizzard, duodenum and ileum and Zn
32 concentration in the gizzard and ileum. This illustrates that phytate susceptibility directly
33 effects mineral availability in the gastrointestinal tract. Measurements of Zn concentration in
34 the gastrointestinal tract, particularly in the gizzard, can potentially be used as a marker to
35 assess the anti-nutritional impact of phytate and susceptibility of phytate to phytase in broilers.

36 **Key words:** broiler, zinc, phytate, phytase

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INTRODUCTION

38 The current commonly used methods for measuring phytate concentration and phytase
39 activity in poultry diets and digesta are colorimetric, whereby the color formation between
40 molybdate and released inorganic orthophosphate is measured. There is however large
41 variation and poor reproducibility using these methods. This is because free or water-soluble P
42 results in high background and hinders degradation of phytate, the absorbance readings are
43 often unstable as the background color of the blank is strong (and thus the color contribution
44 by phytase hydrolysis is reduced) and the samples do not mix very well as the presence of fat
45 and carbohydrate causes an oil layer to form (Kim, 2005). High performance liquid
46 chromatography (HPLC) is also widely used for phytate analysis (Cowieson et al., 2006), but
47 each analysis requires expensive and time-consuming anion-exchange purification. Alternative
48 methods for measuring phytate concentration include using measurements of turbidity as an
49 indicator of phytate complex hydrolysis, based on the principle that stable protein-phytate
50 complexes form turbid solutions (Tran et al. 2011), heating samples with a standardized amount
51 of FeCl_3 and measuring the amount of precipitated ferric phytate (Wheeler and Ferrel, 1971),
52 electrophoresis (Blatny et al. 1995), paper chromatography (Agostinho et al. 2016) and nuclear
53 magnetic resonance (Leytem et al. 2008). The flaws with these methods are that they are non-
54 specific to phytate and require a large volume of sample. The most sensitive method for phytate
55 analysis is the use of metal dye detection reagents (Mayr, 1988) but the extreme sensitivity of
56 this method makes it complex and expensive. Alternative methods for measuring phytate and
57 phytase activity in poultry diets and digesta which are sensitive, low cost and require small
58 sample size are therefore required.

59 This study explores the hypothesis that it may be advantageous to measure the impact
60 of phytate and phytase on mineral availability and the gastrointestinal environment as opposed
61 to measuring phytate and phytase directly. Zinc (Zn) is the most vulnerable of the essential

62 cations to complexation with phytate (Park et al., 2013). Zn deficiency in broilers occurs only
63 when high concentrations of phytate are present (Kornegay, 2001; Oberleas and Harland, 1996);
64 phytate binds to the positively charged Zn resulting in the formation of insoluble Zn-protein-
65 phytate complexes (especially in the small intestine) that cannot be absorbed in the digestive
66 tract (Schlegel et al., 2010). This suggests that the degree of Zn deficiency, due to the
67 vulnerability of Zn to phytate, could be used as an indicator of the chelating capacity and hence
68 anti-nutritional impact of phytate. The presence of calcium aggravates the effect of phytate on
69 Zn utilization (Linares et al. 2007), which may be because it increases pH and insoluble
70 calcium-phytate-Zn complexes form which prevent Zn absorption.

71 Measuring the total phytate-P concentration of a diet following phytase addition may
72 be misleading because the total amount of phytate in the diet does not necessarily represent the
73 quantity of phosphorus available for hydrolysis. This is because insoluble phytate-mineral
74 precipitates and soluble mineral-phytate complexes may be resistant to phytase hydrolysis
75 (Maenz et al., 1999). Measurements of the susceptibility of phytate to phytase may give a better
76 indication of the impact of phytate and phytase and should potentially be taken into account
77 when formulating diets with phytase. Phytate susceptibility is determined *in vitro* by exposing
78 samples to conditions that mimic the bird gastrointestinal tract and then measuring phytate-P
79 released. This is then assumed to be the proportion of total phytate that is susceptible to phytase
80 degradation. In this study, the relationship between *in vitro* and *in vivo* phytate susceptibility
81 and Zn is investigated to examine if measurements of gastrointestinal Zn levels could be used
82 as a tool to indicate phytate susceptibility to phytase. This was carried out by observing if there
83 was a direct relationship between level of Zn and level of susceptible phytate; in the presence
84 of phytase the higher the level of Zn suggests the more phytate has been degraded as more Zn
85 has been released from phytate, and hence the more susceptible the phytate is to the effects of
86 phytase. This would be advantageous as enables predictions and assessments of phytase

87 success and extent of the impact of phytate in different ingredients and combinations to be
88 carried in a relatively cheap and simple way.

89 Dose response curves for the effects of phytase on Zn utilisation are not as clearly
90 understood as they are for Ca and P and there is lack of understanding and consistency amongst
91 studies about the extent to which phytase improves bioavailability and digestibility of Zn. Some
92 studies have found that phytase improves Zn digestibility and retention. For example, Yi et al.
93 (1996) found that approximately 0.9 mg of Zn was released per 100 U of phytase over the range
94 of 150 to 600 U of phytase. Also, Thiel and Weigand (1992) found that the addition of 800
95 U/kg phytase to a diet containing 27mg Zn/kg significantly increased Zn retention and reduced
96 Zn excretion in chicks. Thiel et al. (1993) reported that Zn content of chick femurs was similar
97 between birds fed diets containing 30mg Zn/kg with 700U/kg phytase and those fed diets
98 containing 39mg Zn/kg without supplemental phytase. Biehl et al. (1995) found that phytase
99 and 1,25-dihydroxycholecalciferol supplementation in chicks increased growth rate and tibia
100 Zn to the same degree. Some studies however have found that there is a negative relationship
101 between Zn and phytase, for example Augspurger et al. (2004) reported that 800mg/kg of Zn
102 supplementation in chick diets reduced the phosphorus-releasing efficacy of phytase, resulting
103 in decreased tibia ash (14%) in the presence of phytase. This study illustrates a potent inhibitory
104 effect of Zn^{2+} on phytate hydrolysis by phytase. Furthermore, Roberson and Edwards (1994)
105 did not observe consistent improvements in Zn absorption when broilers were fed 600-750U/kg
106 phytase in diets containing 32mg Zn/kg. When dietary Zn level is greater than the requirement
107 for growth, plasma and tibia concentration, at approximately 45mg/kg (Mohanna and Nys,
108 1999), it is excreted, which has negative implications both economically and environmentally,
109 as excess Zn can cause phytotoxicity in soil. The findings from these studies suggest a lot more
110 investigation is required into the relationship between phytate and phytase and Zn in poultry.

136 there was no significant difference in BW by pen across diets. Birds were allowed ad libitum
137 access to the treatment diets and water for the duration of the trial. The room was
138 thermostatically controlled to produce an initial temperature of 32°C on d1 and reduced in steps
139 of 0.5°C per d, reaching 21°C by day 14. The lighting regimen used was 24 hours light on d1,
140 with darkness increasing by 1 hour a day until 6 hours of darkness was reached, which was
141 maintained throughout the remainder of the study. All birds sampled were euthanized by
142 cervical dislocation on d21 post-hatch. This occurred after at least 6 hours of light, to ensure
143 maximal gut fill. Total pen weight and feed intake (**FI**) was determined on d21 post-hatch and
144 was used to calculate feed conversion ratio (**FCR**). Mortality was recorded daily, and any birds
145 culled or dead were weighed. Institutional and national guidelines for the care and use of
146 animals were followed and all experimental procedures involving animals were approved by
147 the University's College of Science ethical review committee.

148

149 *Dietary Treatments*

150 Diets were formulated to produce total phytase levels of approximately 400, 700, 2000,
151 4000 and 6000 U/kg. This study was conducted primarily to examine a novel variety of high
152 phytase wheat (*Triticum aestivum L.*) and has been reported elsewhere (Brinch-Pedersen et al.,
153 2012). The control diet, with a predicted phytase activity of 400 FTU/kg, had no supplemental
154 phytase added (Table 1). The diet with a predicted phytase activity of 700 FTU/kg was the
155 control diet with added supplemental phytase (Quantum Blue™, AB Vista Feed Ingredients).
156 The diets formulated to have predicted phytase levels of 2000, 4000 and 6000 U/kg were the
157 control diet with a proportion of the wheat replaced with wheat that had higher intrinsic phytase
158 content. A P-adequate control diet was used in this study so direct comparisons could be made
159 between the endogenous phytase activity provided by the novel wheat compared to standard
160 wheat. The conventional wheat had a measured phytase level of 1060 U/kg and the higher

161 phytase wheat had a measured phytase level of 6196 U/kg. These treatments also enabled
162 differences between phytase effects from microbial sources compared to plant sources to be
163 assessed. The measured phytase levels of the diets were 605 U/kg for the control diet and 1150,
164 1804, 3954 and 5925 U/kg for the treatment diets. The diets had total phytate levels ranging
165 from approximately 10-12g/kg DM (Table 2), dietary Ca levels of approximately 7.80g/kg DM
166 (as dicalcium phosphate and limestone) (Table 1) and non-phytate-P levels of approximately
167 2.50 g/kg DM (Table 2). There were five treatments with each treatment replicated by 9 pens
168 of 3 birds each (27 birds/dietary treatment). Diets were mixed in house and fed as mash. They
169 were analyzed for gross energy by bomb calorimetry (Latshaw and Moritz, 2009), dry matter
170 and protein content (calculated as nitrogen multiplied by 6.25) by the AOAC standard methods
171 (930.15 and 990.03, respectively). Zn, P and Ca content of the diets were analyzed by
172 inductively coupled plasma-optical emission spectroscopy (ICP-OES) following an aqua regia
173 digestion step (AOAC 985.01, Thomas and Ravindran, 2010). Titanium dioxide (TiO₂) was
174 added at a rate of 0.5% to act as an inert marker for evaluation of dietary phytate hydrolyzed
175 and the dietary content of TiO₂ was quantified by ICP-OES following aqua regia digestion, by
176 the method of Morgan et al. (2014). Total phytic acid content was analyzed by a K-Phyt assay
177 kit (MegazymeTM, Wicklow, Ireland, UK). This assay quantitatively measured available
178 phosphorus released from the samples. Briefly, inositol phosphates were acid extracted
179 followed by treatment with a phytase specific for IP₆-IP₂ and alkaline phosphate added to
180 ensure release of the final phosphate from myo-inositol phosphate (IP₁). The total phosphate
181 released was measured using a modified colorimetric method and given as grams of phosphorus
182 per 100 g of sample material. *In vitro* susceptible phytate content was analysed by adding
183 warmed acetate buffer (2.5M acetic acid and 2.5M sodium acetate, pH 4.5) to the diet samples,
184 incubating and centrifuging the solution, neutralizing the supernatant with 0.25M NaOH, and
185 subsequently using the K-PhytTM assay to analyze phytate content. Phytase activity was

186 analyzed according to the method of Engelen et al. (2001). Diet composition and analyzed GE,
187 CP and mineral values for the diets are shown in Table 1. Analyzed phytase, phytate and
188 susceptible phytate values for each of the diets are shown in Table 2.

189 ***Response Variables***

190 Two birds per pen were individually weighed and marked with a colored pen for
191 identification purposes post-euthanasia. Gizzard, duodenum and ileum pH of these two birds
192 was determined by inserting a spear tip piercing pH electrode (Sensorex, CA) with digital pH
193 meter (Mettler-Toledo, Leicester, UK) directly into the digesta in the gut lumen as soon as they
194 had been excised. Readings were repeated three times per section of gut per bird (ensuring the
195 probe did not touch the gut wall) and average pH was calculated.

196 Gizzard, duodenum and ileum digesta contents from 3 birds per pen were then collected
197 by gentle digital pressure into one pot per section of tract per pen, and stored at -20°C prior to
198 freeze drying. Once freeze dried, the samples were ground to a fine powder with a pestle and
199 mortar. Zn and titanium dioxide content of the digesta was determined by ICP-OES following
200 aqua regia digestion as previously discussed for the diets. Total phytate content of the gizzard,
201 duodenum and ileum digesta samples were analyzed by a K-Phyt assay as previously discussed
202 and the amount of dietary phytate hydrolyzed was calculated using the equation:

$$203 \text{ Dietary phytate (g/kg DM)} * (1 - (\text{digesta phytate (g/kg DM)} * \text{TiO}_2 \text{ diet (g/kg DM)}) / (\text{TiO}_2 \text{ digesta} \\ 204 \text{ (g/kg DM)} * \text{dietary phytate (g/kg DM)})$$

205 These values were used to calculate the *in vivo* susceptible phytate level by calculating the
206 proportion of total phytate that had been successfully hydrolyzed in the gizzard, duodenum and
207 ileum.

208 ***Data Analysis***

209 All data were analyzed using IBM SPSS statistics version 22. After Kolmogorov–
210 Smirnov testing to confirm normality, one-way ANOVA was used to test the equality of the

211 means and multiple ANOVA was used to test interactions. When means were significantly
212 different, Duncan post-hoc tests were conducted to differentiate between them. Following
213 polynomial contrast tests to test polynomial patterns in all of the data, correlations between
214 measured factors were analyzed by bivariate correlations using Pearson product- moment
215 correlation coefficient. Interpretations of the strength of the relationships between the factors
216 were based on determining a very strong relationship as $r = 0.800$ to 1.000 , strong as $r = 0.600$
217 to 0.790 , moderate as $r = 0.400$ to 0.590 , weak as $r = 0.200$ to 0.390 and very weak as $r = 0.000$
218 to 0.190 . Statistical significance was declared at $P < 0.05$.

219 **RESULTS AND DISCUSSION**

220 Table 2 shows that the susceptible phytate levels measured *in vitro* and *in vivo* were
221 almost identical in the diets with phytase activity of 5925 FTU/kg, but at the lower dietary
222 phytase levels the *in vitro* assay used in this study overestimated how much of the total dietary
223 phytate was susceptible to the effects of phytase. This was particularly the case in the diet with
224 phytase activity of only 605 FTU/kg, in which it was predicted that approximately 5% more
225 phytate would be hydrolyzed than was actually observed *in vivo*. Similar findings of observed
226 stronger relationships between *in vitro* and *in vivo* methods in diets with phytase levels of 5000
227 FTU/kg or higher compared to diets with lower phytase levels were also observed by Morgan
228 *et al.* (2014b). This is likely to be because the *in vitro* assay was not able to completely mimic
229 conditions in the tract. This is because the *in vitro* assay was a closed system in which P ions
230 released by phytate hydrolysis were not removed, so minor phytase effects appear to be more
231 influential than would be observed *in vivo*. This suggests that the *in vitro* assay was successful
232 at detecting P release when there was near complete phosphate hydrolysis, which likely
233 occurred at this high phytase activity level, but was not sensitive enough to measure only partial
234 phytate hydrolysis when low levels of P were released. The 6 phosphate groups on the inositol
235 ring of phytate are hydrolysed in sequential order, with 6-phytases, such as the ones featured

236 in this study, starting at position 6, then position 1, 2, 3, 4 and 5. Degrading IP₆ to IP₄ eliminates
237 most of the anti-nutritional effects of phytate, but does not generate the full phosphorus
238 hydrolyzing capacity. In the diets with lower phytase activity the partial phytate hydrolysis
239 would result in a higher presence of lower inositols (namely IP₄ and IP₃) and hence a lower
240 concentration of IP₆ for the phytase to act on. IP₄ and IP₃ form weaker mineral complexes and
241 bind to fewer minerals than IP₅ and IP₆. It may be possible therefore that the impact of the
242 alkaline phosphatase was more pronounced in the *in vitro* assay due to the higher presence of
243 lower inositols and the degradation of these would occur more rapidly compared to degradation
244 of IP₅ and IP₆, resulting in overestimation by the *in vitro* assay. In the *in vivo* assay however
245 the effects of the alkaline phosphatase addition were less extreme as the phytate had already
246 been exposed to both phytase and alkaline phosphatase in the bird's gastrointestinal tract.
247 Further work is needed to improve the quality of the *in vitro* assay, especially at lower levels
248 of dietary phytase. It was noted that the 3954 FTU diets recorded the lowest phytate
249 susceptibility which may be because these diets had the highest total phytate content, resulting
250 in the phytate and Zn dissociating to a greater extent in these diets (Schlegel et al. 2010).

251 Table 3 shows that as phytase activity level increased more phytate was hydrolyzed and
252 more Zn was released from phytate-Zn complexes. In this study there were strong correlations
253 between the amount of phytate hydrolyzed and Zn concentration in the gizzard in birds fed the
254 diets with 1804 FTU/kg ($r = 0.689$, $P = 0.040$), 3954 FTU/kg ($r = 0.632$, $P = 0.048$) and 5925
255 FTU/kg ($r = 0.735$, $P = 0.032$). Phytate readily seeks cations, chiefly Zn, to complex with, so
256 in the presence of phytate the rate of Zn secretion into the duodenum via the pancreas exceeds
257 the rate at which dietary Zn is consumed. This suggests that measuring Zn level could be used
258 as a tool to indicate the chelating capacity of phytate and phytate susceptibility to phytase,
259 because any Zn bound to phytate would be excreted, so the higher the phytate presence the
260 lower the amount of free Zn present in the GIT. Phytase activity had a significant effect on the

261 amount of phytate hydrolyzed and Zn concentration in both the gizzard and ileum but not in
262 the duodenum. Previously it was hypothesized that the duodenum may be the most appropriate
263 area of the tract to determine the relationship between phytate and Zn, but findings from this
264 study suggest that the gizzard gives the optimum location in the gastrointestinal tract for
265 assessing the relationship between phytate and Zn, despite the upper part of the small intestine
266 being the main site of Zn absorption (Schlegel et al. 2010). This is potentially because protein-
267 phytate-mineral complexes, which are difficult to hydrolyze, do not readily form at gizzard pH
268 but form at higher pH, meaning phytate is most susceptible to the effects of phytase in the
269 gizzard (Amerah et al. 2014). This is advantageous because there is significantly more digesta
270 sample in the gizzard, resulting in better homogeneity of the sample, allowing greater
271 replication and potentially reducing the number of birds required for sample collection. In the
272 duodenum and ileum however there were weak and non-significant relationships between the
273 amount of phytate hydrolyzed and Zn concentration (Table 3). Further investigation is required
274 to assess the impact of method of euthanization on Zn and phytate levels in the different
275 sections of the tract, as it is possible that cervical dislocation resulted in issues such as mixing
276 of digesta between segments due to peristalsis and mucosal shedding, which may have reduced
277 the accuracy of the measurements in the digesta. Additionally, the observed effects in birds fed
278 the diets with 1804, 3954 and 5925 U/kg phytase but not at the lower phytase levels may be
279 partly because the phytase in these diets is from a plant source as opposed to a microbial source,
280 but this requires further investigation.

281 The lack of strong correlations between gizzard pH and phytate hydrolysis in this study
282 illustrates that gizzard pH was sufficiently low in all diets to allow for phytate hydrolysis (Table
283 4). This study was not designed to distinguish performance differences between the diets and
284 P-adequate dietary treatments were fed, hence why significant performance effects were not
285 observed, although FCR was numerically improved in the presence of phytase (Table 5).

286 Strong relationships were observed between pH and phytate susceptibility *in vivo* and
287 *in vitro* in the gizzard ($r = 0.749$ $P = 0.045$ and $r = 0.966$ $P = 0.007$ respectively) and ileum (r
288 $= 0.664$ $P = 0.021$ and $r = 0.861$ $P = 0.041$ respectively), and between duodenum pH and
289 phytate susceptibility measured *in vivo* ($r = 0.609$ $P = 0.026$) (Table 6). This may be because
290 it is addition of H⁺ ions to the weak acid phosphate groups of phytate that convert it from being
291 resistant to susceptible to the effects of phytase. This study was in agreement with the work of
292 Schlegel et al. (2010) who found that lower gizzard pH directly related to higher Zn solubility
293 and that phytase was efficient in increasing digestive Zn and improving Zn status in the bird.
294 Zn-phytate complexes dissociate as soon as pH is below 4 (Ellis et al. 1982), so the results
295 presented for pH (Table 4) and phytate hydrolyzed (Table 3) for the 605 FTU/kg phytase diet
296 suggest that the low pH in the gizzard allowed for Zn-phytate complexes to dissociate, even in
297 the presence of very low levels of phytase. At higher pH, such as in the duodenum and ileum,
298 phytate must first be hydrolyzed by phytase before Zn can be released. In this study the level
299 of Zn was the same in each treatment in order to determine the direct effect of phytate and
300 phytase on intestinal Zn levels, as opposed to the impact of dietary Zn presence on any of the
301 parameters studied, but it would be advantageous in future studies to observe the effects of
302 different Zn concentrations and define the optimum level of Zn for a successful marker.

303 In the gizzard, *in vivo* phytate susceptibility correlated moderately with Zn
304 concentration ($r = 0.583$ $P = 0.025$) and strongly with phytate hydrolyzed ($r = 0.733$ $P = 0.014$)
305 (Table 6). This suggests that measuring Zn level in the gizzard could be used as an indicator of
306 phytate susceptibility to phytase and hence used to predict the anti-nutritional effect of phytate
307 and the ability of phytase to combat its effects. In this study over 80% of the dietary zinc was
308 utilized in the presence of phytase and as phytase level increased the strength of the relationship
309 between phytate hydrolysis and zinc increased, highlighting heightened zinc release from
310 phytate. This suggests that Zn deficiency could potentially be almost completely eliminated by

311 supplementing diets with high levels of phytase (Linares et al., 2007; Adams et al, 2002). A
312 further advantage is that the ICP-OES assay is less time consuming and more sensitive than
313 the colorimetric assay for measuring phytate and Zn can be measured concurrently with other
314 mineral analysis. Zn availability is dependent on solubility in the digesta which is dictated by
315 pH and dietary Zn concentration as discussed, but also the source of the supplemented Zn and
316 interactions between diet Zn and other components in the diet (Schlegel et al., 2010). Further
317 investigation is therefore needed into the interaction between phytate and phytase with different
318 Zn sources and diet combinations. These findings also illustrate that additional phytase
319 delivered in the form of an endogenous enzyme in wheat was effective at increasing zinc
320 availability to the bird.

321 It can be concluded that measurements of Zn concentration in the gastrointestinal tract,
322 particularly in the gizzard, have the potential to be used as a marker to assess the anti-nutritional
323 impact of phytate and phytate susceptibility to the effects of phytase in broilers. Phytate
324 susceptibility to phytase effects has a direct effect on Zn availability in the gastrointestinal tract
325 and hence bird performance. It was hypothesized that measurements in the duodenum would
326 give the best indication of phytate susceptibility to phytase and the anti-nutritional impact of
327 phytate on Zn bioavailability, but it was found that that measurements of Zn in the gizzard
328 provided the best indicator of phytate and phytase effects. This study also illustrated that
329 additional phytase delivered in the form of an endogenous enzyme in wheat is effective at
330 increasing Zn availability. Further work is needed to improve the quality of the susceptible
331 phytate assay and to assess the relationship between Zn and phytate in diets with differing
332 ingredient combinations and hence differing Zn and phytate levels.

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REFERENCES

341 Adams, C.L., M. Hambridge, V. Raboy, J.A. Dorsch, L. Sian, J.L. Westcott and N.F. Krebs.
342 2002. Zinc absorption from a low-phytic acid maize. *Am. J. Clin. Nutr.* 76: 556-559.

343 Agostinho, A.J., W. de Souza Oliveira, D.S. Anunciação, J. C.C. Santos. 2016. Simple and
344 sensitive spectrophotometric method for phytic acid determination in grains. *Food Anal.*
345 *Methods.* 9:2087-2096.

346 Augspurger, N.R., J.D. Spencer, D.M. Webel and D.H. Baker. 2004. Pharmacological zinc
347 levels reduce the phosphorus-releasing efficacy of phytase in young pigs and chickens. *J.*
348 *Anim. Sci.* 82:1732–1739.

349 Blatny, P., F. Kvasnicka and E. Kenndler. 1995. Determination of phytic acid in cereal grains,
350 legumes and feeds by capillary isotachopheresis. *J. Agric. Food. Chem.* 43: 129-133.

351 Brinch-Pedersen, H., Madsen, C. K., Dionisio, G., & Holm, P. B. (2012). New mutant cereal
352 plant, useful for manufacturing composition, which is useful as animal fodder, p. 1-55,
353 WO2012146597-A1

354 Cohen, J. 1988. *Statistical power analysis for the behavioural sciences.* 2nd ed. Erlbaum,
355 Hilssdale, NJ.

356 Cowieson, A.J., T. Acamovic and M.R. Bedford. 2006. Phytic Acid and Phytase: Implications
357 for protein utilization by poultry. *Poult. Sci.* 85: 878–885.

358 Ellis, R., E.R. Morris and A.D. Hill. 1982. Bioavailability to rats of iron and zinc in calcium-
359 iron-phytate and calcium-zinc-phytate complexes. *Nutr. Res.* 2:319-322.

360 Engelen A.J., F.C. Van der Heeft, P.H. Randsdorp, W.A. Somers, J. Schaefer and B.J. Van der
361 Vat. 2001. Determination of phytase activity in feed by colorimetric enzymatic method:
362 collaborative interlaboratory study. *J. AOAC Int.* 84: 629-633.

363 Harland, B.F. and D.A. Oberleas. 1986. Anion exchange method for determination of phytate
364 in foods: Collaborative study. *J. Assoc. Off. Anal. Chem.* 69: 667-670.

365 Kim, J. C., Simmins P. H., Mullan B. P., Pluske J. R. 2005. The digestible energy value of
366 wheat for pigs, with special reference to the post-weaned animal. *Anim. Feed Sci. Technol.*
367 122:257–287.

368 Kornegay, E. T. 2001. Digestion of phosphorus and other nutrients: the role of phytases and
369 factors influencing their activity. Pages 237-272 in *Enzymes in Farm Animal Nutrition.*
370 CABI Publishing, New York, NY.

- 371 Latshaw, J.D. and J.S. Moritz. 2009. The portioning of metabolizable energy by broiler
372 chickens. *Poult. Sci.* 88: 98-105.
- 373 Leytem, A.B., B.P. Willing and P.A. Thacker. 2008. Phytate utilization and phosphorus
374 excretion by broiler chickens fed diets containing cereal grains varying in phytate and
375 phytase content. *Anim. Feed. Sci. Tech.*146: 160-168.
- 376 Linares, L.B., J.N. Broomhead, E.A. Guaiume, D.R. Ledoux, T.L. Veu and V. Raboy. 2007.
377 Effects of low phytate barley (*Hordeum vulgare* L.) on zinc utilization in young broiler
378 chicks. *Poult. Sci.* 86:299-308.
- 379 Maenz D.D., C.M. Engele-Schaan, R.W.Newkirk and H.L. Classen. 1999. The effect of
380 minerals and mineral chelators on the formation of phytase-resistant and phytase-
381 susceptible forms of phytic acid in solution and in a slurry of canola meal. *Anim. Feed.*
382 *Sci. Tech.*81:177-192.
- 383 Mayr, G.W. 1988. A novel metal-dye detection system permits isomolar-range H.P.L.C.
384 analysis of inositol polyphosphates from non-radioactively labeled cell or tissue
385 specimens. *Biochem. J.* 254:585-591.
- 386 Mohanna, C. and Y. Nys, 1999. Effect of dietary zinc content and sources on the growth, body
387 zinc deposition and retention, zinc excretion and immune response in chickens. *Br. Poult.*
388 *Sci.* 40:108-114.
- 389 Morgan, N.K., D.V. Scholey and E.J. Burton. 2014a. A comparison of two methods for
390 determining titanium dioxide marker content in broiler digestibility studies. *Animal.* 1-5.
- 391 Morgan, N.K., C.L Walk, M.R. Bedford and E.J. Burton. 2014b. In vitro versus in situ
392 evaluation of the effect of phytase supplementation on calcium and phosphorus solubility
393 in soya bean and rapeseed meal broiler diets. *Br. Poult. Sci.* 55:238-245.
- 394 Oberleas, D. and B. F. Harland. 1996. Impact of phytic acid on nutrient availability. Pages 77–
395 84 in *Phytase in Animal Nutrition and Waste Management*. BASF Corp., Mount Olive,
396 NJ.
- 397 Park, K.H., B. Park, D.S. Yoon, S.H. Kwon, D.M. Shin, J.W. Lee, H.G. Lee, J-H. Shim, J.H.
398 Park and J.M. Lee. 2013. Zinc inhibits osteoclast differentiation by suppression of Ca²⁺-
399 Calcineurin-NFATc1 signaling pathway. *J. Cell Commun. Signal.* 11:74.
- 400 Schlegel, P., Y. Nys and C. Jondreville. 2010. Zinc availability and digestive zinc solubility in
401 piglets and broilers fed diets varying in their phytate contents, phytase activity and
402 supplemented zinc source. *Animal.* 4:200-209.
- 403 Tran, T.T., R. Hatti-Kaul, S. Dalsgaard, and S. Yu, 2011. A simple and fast kinetic assay for
404 phytases using acid-protein complex as substrate. *Anal. Biochem.* 410:177-184.
- 405 Thiel, U. and E. Weigand. 1992. Influence of dietary zinc and microbial phytase
406 supplementation on zinc retention and zinc excretion in broiler chicks. XIX World's
407 Poultry Congress, Amsterdam, The Netherlands, 20-24 September.
- 408 Thiel, U., E. Weignad F.J. Schoner and P.P. Hoppe. 1993. Zinc retention of broiler chicken as
409 affected by dietary supplementation of zinc and microbial phytase. In: Anke, M., D.

- 410 Meissner and C.F., Mills (eds). Proceedings of 8th International Symposium, Trace
411 elements in man and animal (TEMA). Dresden, Gersdorf, 658-659.
- 412 Thomas, D.V. and V. Ravindran. 2010. Mineral retention in young broiler chicks fed diets
413 based on wheat, sorghum or maize. *Asian-Aus. J. Anim. Sci.* 23(1): 68-73.
- 414 Wheeler, E.L. and R.E. Ferrel. 1971. A method for phytic acid determination in wheat and
415 wheat fractions. Presented at the American association of Cereal Chemists 54th Annual
416 Meeting. April-May 1969.
- 417 Yi, Z., E.T. Kornegay and D.M. Denbow. 1996. Supplemental microbial phytase improves zinc
418 utilization in broilers. *Poult. Sci.* 75:540-546.
- 419 Yu, Y., L. Lu, R.L. Wang, L. Xi, X.G. Luo and B. Liu. 2010. Effects of zinc source and
420 phytate on zinc absorption by in situ ligated intestinal loops of broilers. *Poult. Sci.* 89:
421 2157-2165.

Table 1. Composition of control diet

Ingredient	Phytase, U/kg ¹				
	605	1150	1804	3954	5925
Standard wheat, 10% CP	57.21	57.21	38.11	19.11	0
Higher phytase wheat	0	0	19.10	38.10	57.21
Soyabean meal, 48% CP	35	35	35	35	35
Soy oil	3.78	3.78	3.78	3.78	3.78
Limestone	1.28	1.28	1.28	1.28	1.28
Salt	0.17	0.17	0.17	0.17	0.17
Sodium bicarbonate	0.26	0.26	0.26	0.26	0.26
Monocalcium phosphate HCl	1.23	1.23	1.23	1.23	1.23
Lysine HCl	0.21	0.21	0.21	0.21	0.21
Methionine	0.32	0.32	0.32	0.32	0.32
Threonine	0.13	0.13	0.13	0.13	0.13
Econase XT	0.01	0.01	0.01	0.01	0.01
Quantum Blue Phytase	0	0.01	0	0	0
Vitamin/Mineral Premix ¹	0.40	0.40	0.40	0.40	0.40
Titanium Dioxide	0.50	0.50	0.50	0.50	0.50
Analyzed composition					
CP, %	26.73	26.92	27.28	27.45	27.69
Gross energy, kcal/kg	4687	4686	4842	4905	4886
Total Zn, mg/kg	314.93	359.90	329.04	338.01	355.23
Total P, %	0.58	0.57	0.52	0.56	0.59
Total Ca, %	0.78	0.80	0.77	0.78	0.78

¹Supplied per kilogram of diet: 10g calcium, 4.5g phosphorus, 1.5g sodium, 4.00g potassium, 1.5g chloride, 0.6g magnesium, 60mg manganese, 50mg Zn, 80mg iron, 6mg copper, 0.5mg iodine, 0.2mg molybdenum, 0.15mg selenium, 2.25mg retinol, 37.5µg cholecalciferol, 10mg tocopherol, 3.0mg menadione, 3.0mg thiamine, 5.0mg riboflavin, 10mg pantothenic acid, 4.0mg pyroxidine, 30mg niacin, 10µg cobalamin, 1.5mg folic acid, 0.15mg biotin, 1.4g choline, 125mg amprolium, 125mg antioxidant.

Table 2. Proximate phytate and phytase composition of experimental diets and *in vitro* and *in vivo* evaluation of phytate susceptibility to phytase on a dry matter basis

	Phytase, U/kg ¹				
	605	1150	1804	3954	5925
Phytate, %	1.01	1.01	1.02	1.21	1.19
Phytate-P, % ²	0.29	0.29	0.29	0.34	0.34
Non-phytate-P, % ³	0.30	0.28	0.24	0.22	0.21
<i>In vitro</i> Susceptible Phytate, %	0.56	0.57	0.58	0.64	0.65
<i>In vitro</i> Susceptible Phytate (% of phytic acid)	55.25	56.14	56.96	52.89	54.71
<i>In vivo</i> ⁴ Susceptible Phytate, %	0.51	0.54	0.56	0.59	0.64
<i>In vivo</i> ⁴ Susceptible Phytate (% of phytic acid)	50.20	53.37	55.29	48.84	54.12

¹Total phytase activity was analysed by a colorimetric enzymatic method and calculated as (net optical density at 415nm*dilution volume)/(slope of standard curve*mass*incubation time) (Engelen et al. 2001).

² Phytate-P was calculated as 28.2% of phytate (Harland and Oberleas, 1986).

³ Non-phytate P was calculated as the difference between total P and phytate-P.

⁴ *In vivo* means represent the average response of 27 birds/treatment.

Table 3. Effect of phytase concentration on amount of dietary phytic acid hydrolyzed and remaining after hydrolysis (g/kg dry matter) and Zn concentration in the gizzard, duodenum and ileum (mg/kg dry matter) and correlation between the amount of phytate hydrolyzed and Zn concentration at d21¹

		Phytase, U/kg ¹					SEM	P-value
		605	1150	1804	3954	5925		
Gizzard	Remaining	5.24 ^b	4.98 ^b	4.89 ^b	6.17 ^a	6.08 ^a	0.24	<0.001
	Hydrolyzed	3.68 ^c	3.96 ^{bc}	4.19 ^{abc}	4.44 ^{ab}	4.75 ^a	0.16	0.030
	Zn	35.61 ^c	37.13 ^c	41.28 ^b	41.48 ^b	46.34 ^a	1.68	<0.001
	Correlation ²	0.138	0.634	0.689	0.632	0.735		
	P-value	0.053	0.066	0.040	0.048	0.032		
Duodenum	Remaining	4.85	4.56	4.44	5.81	5.49	0.24	0.202
	Hydrolyzed	4.07	4.38	4.64	4.80	5.34	0.19	0.063
	Zn	58.00	61.41	60.27	60.61	62.51	0.67	0.897
	Correlation ²	0.079	0.413	0.234	0.297	0.301		
	P-value	0.841	0.269	0.544	0.438	0.998		
Ileum	Remaining	3.85 ^b	3.55 ^b	3.44 ^b	4.70 ^a	4.38 ^a	0.22	<0.001
	Hydrolyzed	5.07 ^c	5.39 ^{bc}	5.64 ^b	5.91 ^b	6.44 ^a	0.21	<0.001
	Zn	88.49 ^c	94.36 ^{bc}	98.36 ^{abc}	103.09 ^{ab}	106.81 ^a	2.88	0.021
	Correlation ²	0.013	0.188	0.317	0.005	0.084		
	P-value	0.973	0.387	0.529	0.990	0.276		

^{a-c} Means within the same column with no common superscript differ significantly ($P \leq 0.05$). 2-way ANOVA and Duncan Post-Hoc test were used to differentiate between means.

¹Means represent the average response of 27 birds/treatment.

²Strength of the relationship between the amount of phytate hydrolyzed and Zn concentration in the gizzard, duodenum and ileum.

Table 4. Effect of phytase concentration on gizzard, duodenum and ileum pH and correlation between gastrointestinal pH² and the amount of phytate hydrolyzed² in broilers from d 0 to 21

		Phytase, U/kg ¹					<i>P</i> -value
		605	1150	1804	3954	5925	
Gizzard	pH	2.83	2.59	2.57	3.05	2.89	0.099
	Correlation ³	0.276	0.190	0.292	0.351	0.108	
	<i>P</i> -value	0.472	0.625	0.446	0.141	0.782	
Duodenum	pH	6.19	6.18	6.20	6.17	6.24	0.826
	Correlation ³	0.482	0.148	0.269	0.719	0.349	
	<i>P</i> -value	0.189	0.703	0.484	0.029	0.357	
Ileum	pH	6.91	7.08	6.92	7.96	7.10	0.583
	Correlation ³	0.011	0.627	0.303	0.092	0.086	
	<i>P</i> -value	0.978	0.041	0.428	0.815	0.825	

¹Means represent the average gastrointestinal pH of 18 birds/treatment.

²Means represent the average response of 27 birds/treatment.

³Strength of the relationship between the amount of phytate hydrolyzed and pH in the gizzard, duodenum and ileum.

Table 5. Effect of phytase concentration on bird performance in broilers from d 0 to 21¹

	Phytase, U/kg ¹					SEM	<i>P</i> -value
	605	1150	1804	3954	5925		
FI (g)	1238.01	1162.25	1139.00	1124.24	1073.92	24.03	0.070
BWG (g)	764.53	722.19	737.75	726.91	790.41	11.47	0.268
FCR	1.63	1.57	1.54	1.49	1.46	0.03	0.317

¹Means represent the average response of 36 birds/treatment

Table 6. Strength of the relationship between susceptible phytate levels measured *in vitro* and *in vivo* (g/kg) and gastrointestinal Zn concentration (g), phytate hydrolyzed (g) and pH in broilers from d 0 to 21

	Susceptible Phytate Method	Zn		Phytate Hydrolyzed		pH	
		Correlation	P- value	Correlation	P- value	Correlation	P- value
Gizzard	<i>In vitro</i>	0.244	0.692	0.430	0.470	0.966	0.007
	<i>In vivo</i>	0.603	0.025	0.733	0.014	0.749	0.045
Duodenum	<i>In vitro</i>	0.087	0.890	0.335	0.582	0.188	0.762
	<i>In vivo</i>	0.484	0.409	0.607	0.034	0.609	0.026
Ileum	<i>In vitro</i>	0.435	0.465	0.395	0.511	0.861	0.041
	<i>In vivo</i>	0.750	0.197	0.681	0.253	0.664	0.021

