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1 PHYTATE ESTERS ON BROILER GROWTH AND NUTRIENT USE

2
3 **Hydrolysis of phytate to its lower esters can influence the growth performance and**
4 **nutrient utilization of broilers with regular or super doses of phytase**

5
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17 **ABSTRACT**

18 The aim of the study was to observe the effects of dietary available phosphorus (**aP**) and
19 calcium (**Ca**), with regular or super doses of phytase, on phytate hydrolysis and subsequent
20 influences on broiler growth performance and nutrient utilization. In a 2×3 factorial design,
21 384 Ross-308 broilers were allocated to one of 6 dietary treatments with 8 replicates in a
22 randomized complete block design for 21 days. Diets were nutritionally adequate (positive
23 control, **PC**) or marginally deficient in aP and Ca (negative control, **NC**), with 0, 500 or
24 1,500 FTU/kg phytase. Bird and feed weights were recorded on days 0 and 21, excreta were
25 collected on days 19 and 20, and gizzard and ileal contents collected on day 21. Body weight
26 gain ($P < 0.01$) increased linearly with phytase in the PC and quadratically in the NC. There
27 was an interactive effect on ileal DM, N and P utilization, increasing quadratically with
28 phytase supplementation in the NC, but no phytase influence in the PC ($P < 0.05$). Phytase
29 linearly increased copper ($P < 0.001$) and linearly decreased Ca ($P < 0.05$) utilization in the
30 ileum. Phytase decreased ileal (**IPx**, inositol x-phosphate) IP6 and IP5 and increased inositol
31 (quadratic, $P < 0.001$) but had no effect on IP4 or IP3. The influence of the dietary aP was
32 more apparent on the hydrolysis of phytate and phytate esters after the ileum, with increasing
33 (linear and quadratic, $P < 0.05$) IP4 and IP3 content in the excreta of birds fed the NC or PC
34 when phytase was added. Phytate hydrolysis improves the growth potential of birds fed NC
35 diets, allowing them to match the growth performance of birds fed PC diets and improve
36 nutrient utilization. These results indicate that dietary Ca and aP concentrations can be
37 reduced when phytase is supplemented. It may also be beneficial to apply the enzyme
38 nutrient matrix to other nutrients in the diet to maintain an optimal balance of nutrients in the
39 digesta.

40 **Key words:** phytase; super-doses; growth performance; nutrient utilization; phytate
41 hydrolysis

INTRODUCTION

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It is well documented that non-ruminant species, particularly poultry and pigs, are unable to sufficiently utilize phosphorus (**P**) from plant-based feed ingredients due to its binding with phytate, and the animals' inability to produce sufficient endogenous phytase. Exogenous phytase has been supplemented to commercial poultry and pig diets for many years, with the aim of improving growth performance in P deficient diets (Biehl et al., 1995; DeLaune et al., 2004), as well as reducing environmental P pollution (Angel et al., 2002). Regular doses of phytase have been reported to improve body weight gain, efficiency of feed conversion, nutrient utilization and bone mineralization in broilers fed diets containing low available P (**aP**; Leske and Coon, 1999; Olukosi and Adeola, 2008; Selle et al., 2012). Super-dosing is the addition of phytase at levels around or above 2,500 FTU/kg (Adeola and Cowieson, 2011), however 1,500 FTU/kg is often considered a super-dose as it is much higher than the regular- doses used commercially of 500 to 1,500 FTU/kg. Improvements in growth performance observed are likely to be partly a result of an increase in availability of the nutrients which had previously been bound with phytate, including protein, and minerals such as calcium, zinc and sodium (Adeola and Cowieson, 2011; Applegate et al., 2003; Maenz, 2001; Ravindran et al., 2001). The 'extra-phosphoric effects' of super-doses of phytase are benefits resulting from more than just an increase in P availability and may be a result of changes occurring within the gut and the overall health of the animal. Increased phytate hydrolysis may improve overall performance and efficiency of nutrient utilization, thereby increasing the efficiency of production for poultry farmers. It is the aim of the current experiment to consider the effect of the inositol phosphate esters (**IPs**) produced during phytate hydrolysis on the growth performance and nutrient utilization of broilers fed diets containing different levels of aP, calcium and phytase.

MATERIALS AND METHODS

67

68 *Animals, Diets and Housing*

69 A total of 384, one-day-old Ross 308 broilers were used for the study and housed in modified
70 raised-floor pens with water and experimental diets provided on an *ad libitum* basis, for 21
71 days. There were 48 cages each containing 8 birds. Birds and feed were weighed on days 0
72 and 21 for determination of growth performance.

73 Birds were monitored at least twice daily, ensuring good health and that feed and
74 water supplies were clean and adequate, and were wing-tagged at day 8 for individual
75 identification. Diets were in mash form and fed *ad libitum* throughout. The house temperature
76 was as detailed in the Ross broiler manual (Ross 308 Specifications, 2007), where ambient
77 temperature (measured at chick height) was 30°C, litter temperature 28-30°C and humidity at
78 60-70%. At day 3, the temperature was decreased at a rate of 1°C per day, so that on day 21,
79 the temperature was 22°C. For the first 7 days, the lighting regime was set to 23:1 light: dark
80 hours, with 30-40 lux intensity. All procedures were approved by the SRUC Animal
81 Experiment Committee prior to commencement.

82 On arrival birds were randomly allocated to 6 treatments with 8 replicates each in a
83 Randomised Complete Block Design and a 2 × 3 factorial arrangement. The factors included
84 two levels of dietary aP (0.50% and 0.35%) and three levels of phytase supplementation (0,
85 500 FTU/kg and 1,500 FTU/kg; Quantum Blue, E. coli phytase, AB Vista, Marlborough,
86 UK). The positive control (**PC**) diet was formulated to meet Ross 308 energy and nutrient
87 requirements (Ross 308 Specifications, 2007). The negative control diet was formulated to be
88 deficient from the PC by 0.15% aP and 0.16% calcium (**Ca**), in accordance to the enzymes
89 nutritional matrix specifications for 500 FTU/kg inclusion rate. Therefore, the dietary
90 treatments were: (1) Positive control, **PC**; (2) PC + 500 FTU/kg phytase; (3) PC + 1,500
91 FTU/kg phytase; (4) Negative control, **NC**; (5) NC + 500 FTU/kg phytase; (6) NC + 1,500

92 FTU/kg phytase. Titanium dioxide was used as an indigestible marker for digestibility
93 calculations. Table 1 shows the ingredient composition of the diets.

94

95 ***Sample Collection***

96 Birds and feed were weighed on days 0 and 21 for determination of growth performance. On
97 day 21, six birds per pen (the 2 remaining birds, with body weights close to the pen average,
98 were used for another study) were euthanized by cervical dislocation and used for collection
99 of the entire content of the gizzard and ileum. Excreta were collected from each cage
100 on days 19 and 20 to determine total tract nutrient retention. Ileal digesta was collected from
101 the terminal ileum, by flushing, for determination of ileal nutrient utilization. Ileal and
102 gizzard contents were analyzed for phytate P and inositol esters of phytate.

103

104 ***Processing of Samples***

105 ***Chemical analyses of the diets and digesta.*** Diets and digesta were dried in a forced
106 draft oven at 80°C, for a minimum of 48 hours or until a constant dry weight was reached,
107 and ground through a 0.5mm sieve. Nitrogen, DM, minerals, and gross energy were analyzed
108 using AOAC procedures (955.04, 968.04). Inositol phosphate esters and inositol were
109 determined by high-performance ion chromatography-based techniques, similar to those used
110 by Blaabjerg et al. (2010), refined by the University of East Anglia. Phytate-P was predicted
111 by NIR (ESC Standard Analytical Method, SAM120; AB Vista). Non-phytate P was
112 calculated by subtracting phytate-P from total P. Phytase was analysed by ELISA specific for
113 Quantum Blue (ESC Standard Analytical Method, SAM099; AB Vista), in a method similar
114 to that described by Engelen et al. (2001). One unit of phytase is defined as “the quantity of

115 enzyme that will liberate 1 mol inorganic ortho-phosphate per minute under the conditions of
116 the assay” (Engelen et al., 2001).

117 ***Titanium digestion.*** Determination of titanium concentration in diet, excreta and
118 digesta samples were performed as described by Short et al. (1996). Each sample was
119 analyzed in duplicate and the absorbance measured using the Jenway 6300 spectrophotometer
120 (63-Zero, software version 1.2.3008.30151), at an absorbance of 410 nm. The determined
121 values were then used for calculation of utilization of the various nutrients.

122

123 ***Calculations and Statistical Analysis***

124 Feed disappearance and BW were recorded to calculate feed intake and BW gain. Feed
125 conversion ratio was calculated from the body weight gain and feed intake data. Feed intake
126 was corrected for mortality through calculation of bird-days. Statistical analysis of mortality
127 % was performed following the application of $1/(\sqrt{X+0.1})$ to allow for analysis of zero
128 mortality.

129 ***Nutrients.*** In the ileum, nutrient digestibility reflects the nutrients utilized, whereas in
130 the excreta this reflects the overall nutrients retained from the diet. Nutrient digestibility was
131 calculated using the following equation:

$$\text{Nutrient digestibility (\%)} = \left[1 - \left(\frac{\text{Ti in diet}}{\text{Ti in sample}} \right) \times \left(\frac{\text{Nutrient in sample}}{\text{Nutrient in diet}} \right) \right] \times 100$$

132 Dry matter digestibility was calculated by:

$$\text{Dry matter digestibility (\%)} = 1 - \left(\frac{\text{Ti in diet}}{\text{Ti in excreta}} \right)$$

133 Means (for each pen) were pooled for each of the diets to allow comparison of the treatment
134 effects. Statistical analysis of the data was done using the General ANOVA function of
135 Genstat (14th Edition, VSN International Ltd.). Data was checked for normality and suitably

136 transformed when required and analyzed as a 2×3 factorial where the model included aP
137 level, phytase and the interaction. Statistical significance was considered where $P \leq 0.05$.
138 When the main effect of phytase was significant, orthogonal polynomial contrasts were used
139 to assess linear and quadratic treatment effects. Contrasts were adjusted for unequal spacing
140 of phytase doses, with the linear contrast being -0.61, -0.15, 0.76 and the quadratic contrast
141 being 0.53, -0.80, 0.27.

142

143

RESULTS

144 *Chemical Analyses of the Diets*

145 The analyzed chemical compositions of the diets are presented in Table 2. Analyzed Ca of the
146 diets was higher than formulated and consequently this raised the Ca:P ratio above
147 anticipated (formulated 1.4:1 in PC and 1.5:1 in NC, analyzed as 1.5 to 1.7: 1 in PC and 1.8:1
148 in NC). The phytase activity of the diets was greater than the expected levels, being < 50 , 715
149 to 744 and 1,880 to 1,960 FTU/kg in diets formulated to have 0, 500 and 1,500 FTU/kg
150 respectively, but still reflecting regular- and super-doses.

151

152 *Growth Performance*

153 Day 21 growth performance data is presented in Table 3. There were no treatment effects on
154 initial body weight (**IBW**) therefore this was not required to be added as a co-factor in the
155 statistical model. There was aP \times phytase interaction ($P < 0.01$) on BW gain (**BWG**) with
156 phytase increasing BWG in birds fed the NC (quadratic, $P < 0.001$) and PC (quadratic, $P <$
157 0.01). Feed conversion ratio (**FCR**) was improved (quadratic, $P < 0.01$) with the addition of
158 phytase. There were no treatment effects on feed intake (**FI**) or mortality.

159

160 ***Nutrient Utilization***

161 ***Ileal Nutrient Utilization.*** There was significant aP × phytase interaction on ileal DM,
162 nitrogen (N) and P (P < 0.05) digestibility (Table 4). The digestibility of DM, N and P were
163 increased with the addition of phytase in the NC diets (quadratic, P < 0.01), but there was no
164 effect of phytase on DM, N, or P digestibility in birds fed the PC. The digestibility of Ca
165 increased as the dietary aP was reduced (P < 0.001) but decreased with the addition of
166 phytase (linear, P < 0.001).

167

168 ***Total Tract Nutrient Retention.*** The total tract nutrient retention (TTR) data are
169 presented in Table 5. There was significant aP × phytase interaction on the TTR of DM, N
170 and P (P < 0.05). The TTR of DM and N was increased (quadratic, P < 0.05) when phytase
171 was added to the NC treatments but there was no effect of phytase addition on the TTR of
172 DM or N in birds fed the PC. The TTR of P increased (quadratic, P < 0.05) in birds fed the
173 NC and there was no effect of phytase dose on the TTR of P in birds fed the PC. The TTR of
174 Ca was greater in birds fed the NC compared with birds fed the PC diets (P < 0.01). At the
175 highest level of inclusion of phytase in the NC, retention reached or exceeded that of the PC
176 for most nutrients.

177

178 ***Phytate Hydrolysis***

179 The treatment effects on IP content of the gizzard are shown in Table 6. IP3 and IP2 were not
180 detected in the gizzard. There was an aP × phytase interaction on the gizzard content of IP4
181 (P < 0.01). Whereas the addition of phytase to the PC had no effect on IP4 content, there was
182 a quadratic increase in IP4 content when phytase was added to the NC (P < 0.001). There was
183 a quadratic decrease (P < 0.001) in IP6 and IP5 content as phytase dose increased, whereas
184 there was a tendency for a quadratic increase (P = 0.070) in inositol content with phytase

185 supplementation. The inositol content of the gizzard was greater in the NC than PC diets ($P <$
186 0.05) but there was no effect on aP on IP6 or IP5 content in the gizzard.

187 In the ileum (Table 7), there was no significant aP \times phytase interaction on the IP
188 content, however both the aP and phytase levels had a significant main effect. There was aP \times
189 phytase interaction on the disappearance of IP6 at the ileum ($P < 0.01$). Disappearance of IP6
190 increased quadratically in both the PC and NC diets ($P < 0.001$) and was numerically
191 comparable between the PC and NC with 1,500 FTU/kg, but greater in the NC + 500 FTU/kg
192 than when the same phytase dose was added to the PC diet (95% disappearance vs. 90%). In
193 the absence of phytase, phytate disappearance was greater in the NC than PC.

194 There was a decrease in IP6 and IP5 (quadratic, $P < 0.05$) and increase in inositol ($P <$
195 0.001) content in the ileum as phytase dose increased. IP6 ($P < 0.01$), IP5 and IP4 ($P < 0.05$)
196 were lower, and inositol ($P < 0.001$) higher in the ileum of birds fed the NC compared with
197 birds fed the PC. There was no effect of diet on IP3 and phytase dose had no effect on IP4
198 content in the ileum.

199 There was significant aP \times phytase interaction on excreta IP5, IP4, IP3 and inositol
200 content (Table 8). IP5 content in the excreta was reduced quadratically in birds fed the PC
201 diet and in birds fed the NC ($P < 0.05$). However, in birds fed the NC diets, 500 FTU/kg had
202 increased excreta IP5 to a greater extent than birds fed the PC (13% compared with 1%,
203 respectively) and 1,500 FTU/kg decreased IP5 to a greater extent in the excreta of birds fed
204 the PC compared with birds fed the NC (43% compared with 24%, respectively). The content
205 of IP4 increased with the addition of phytase in the NC (quadratic, $P < 0.001$) and PC (linear,
206 $P < 0.001$), as did IP3 in both the PC (quadratic, $P < 0.001$) and NC (linear, $P < 0.001$). The
207 inositol content was higher in the NC than PC, and did not increase significantly in the NC
208 with phytase supplementation, however, in the PC the increase was linear ($P < 0.001$). The

209 IP6 content was influenced by phytase ($P < 0.01$), tending to decrease as the level of
210 supplementation increased (quadratic, $P = 0.055$).

211

212

DISCUSSION

213 *Growth performance*

214 There are many reports that the addition of phytase has the benefit of improving growth
215 performance, in particular BWG and FCR, with the greatest improvements seen in P deficient
216 diets (Akyurek et al., 2011; Cowieson et al., 2011; Liu et al., 2008; Sebastian et al., 1996).
217 This study shows that phytase supplementation in diets low in available P is able to prevent
218 the P deficiency symptoms and associated depression in growth performance, allowing the
219 birds to match the growth performance of those birds fed Ca and P adequate diets. Overall,
220 the specific benefits of phytase supplementation to nutrient deficient diets (NC) may vary
221 between studies, according to the level of aP (and other nutrient) deficiency, ultimately
222 giving different responses to phytase and the dose effects.

223 For BWG in the current study, the dietary level of aP had an important influence on
224 the efficacy of phytase. The additional release of nutrients following phytate hydrolysis may
225 lead to further interactions between nutrients and phytate within the digesta, and reduce the
226 efficacy of phytase and potential for improvements in growth performance. In the NC diets
227 where aP was limiting, subsequent Ca release from phytate hydrolysis can have detrimental
228 effects on performance (Shafey, 1993) and may influence the Ca:aP ratio, which is important
229 for phytase efficacy and performance (Cowieson et al., 2011; Rao et al., 2006; Shafey et al.,
230 1990). The Ca:aP ratio is deemed more critical than absolute Ca and aP levels, with higher
231 ratios having a detrimental effect on phytase efficacy (Cowieson et al., 2011; Shafey et al.,
232 1990). Qian et al. (1997) reported that a greater Ca:aP ratio resulted in a reduction in growth
233 performance, with the negative influence of a wide ratio being more apparent at the lower

234 levels of phytase supplementation. Olukosi and Fru (2014b) reported that a wide Ca:aP ratio
235 had a muting effect on the efficacy of phytase on performance parameters. The authors also
236 reported that a wide Ca:aP ratio was more detrimental in reduced Ca and aP diets. In the
237 current study, the analyzed dietary Ca content was higher than formulated, which following
238 the release of Ca from phytate hydrolysis, may have had the effect of further widening the
239 Ca:aP ratio, leading to the observed difference in phytase effects between nutrient adequate
240 PC and NC diets which are marginally deficient in aP (and often Ca).

241

242 *Nutrient utilization*

243 Ravindran et al. (2006) reported a phytase mediated increase in Ca digestibility, regardless of
244 the dietary phytate content. In the current study, ileal Ca digestibility was also influenced by
245 phytase dose and not dietary aP level, whereas Ca digestibility decreased with 1,500 FTU/kg
246 phytase. This effect was likely a consequence of increased Ca released into the digesta
247 following phytate hydrolysis. As well as potentially influencing the gut pH, absorption may
248 already be saturated due to the higher than anticipated dietary Ca levels and this is confirmed
249 by the lower Ca retention in birds fed the PC compared with birds fed the NC with reduced
250 dietary aP and Ca levels. High Ca concentrations following release from phytate hydrolysis
251 and thus a widening of the Ca:aP ratio can reduce the availability of other nutrients, primarily
252 divalent minerals, in the digesta for absorption and increase their excretion (Shafey, 1993).
253 The ratio between aP and Ca is important for nutrient utilization. Shafey et al. (1990)
254 reported that the retention of P was not influenced over a wide range of dietary aP
255 concentrations, provided adequate Ca was also provided to maintain the balance. However,
256 when dietary Ca levels are high, P availability is observed to decrease (Tamim and Angel,
257 2003). High dietary Ca levels have also been reported to form insoluble Ca-phosphate
258 complexes, which subsequently reduces the absorption of both Ca and P (Underwood, 1999).

259 Nutrient release following phytate hydrolysis can result in antagonism between the
260 minerals in the digesta, reducing or preventing their absorption (Sebastian et al., 1996), with
261 nutrient solubility and absorption being dependant on gut pH. Wilkinson et al. (2011)
262 reported an inverse relationship between dietary Ca levels and P, Mg and K digestibility.
263 However in the current study we observed a positive correlation between Ca and P
264 digestibility ($r^2 = +0.68$, $P < 0.001$). Sebastian et al. (1996) observed an increase in TTR of P
265 and Ca when phytase was added to low P diets and Olukosi and Fru (2014a) reported an
266 increase in Ca and P TTR with the addition of phytase to diets with a 2:1 Ca:aP ratio, but not
267 when the ratio was increased to 2.5:1. This study by Olukosi and Fru (2014a) highlights the
268 importance of the Ca:aP ratio in addition to consideration of the overall dietary Ca and aP
269 concentrations as the authors also concluded that the negative influence of a wide Ca:aP ratio
270 was more pronounced in low aP and Ca diets. In the current study, increasing the aP level
271 reduced Ca and P TTR with a tendency for an increase in phytase dose to decrease P TTR
272 (quadratic response). These results may indicate the levels of Ca in the NC, particularly with
273 phytase supplementation, and also in the PC may have been much greater than the birds
274 requirement resulting in excretion of both Ca and P.

275 High doses of phytase were able to improve the digestibility of protein and P when
276 phytate levels are low, as reported by Ravindran et al. (2006). Although in the current study
277 the overall dietary phytate was comparable between treatments, by the time the digesta
278 reached the ileum, there were differences in the IP6 content. Consequently, this may have
279 then influenced the digesta environment, ileal nutrient utilization and TTR, due to its
280 potential interaction and chelation with other nutrients in the digesta. Phytase increased N
281 digestibility in the NC diets but not the PC, with digestibility being greater than the NC when
282 500 FTU/kg phytase was added, but not with 1,500 FTU. This difference in phytase dose
283 response compared to a study by Ravindran et al. (2006) may be due to the additional release

284 of Ca and P with 1,500 FTU/kg, influencing the Ca: aP ratio, thus changing the gut
285 conditions (e.g. pH) for phytase action and nutrient absorption. The additional Ca present in
286 the PC diets (further increased by phytate hydrolysis) may have bound to the phytate,
287 preventing the hydrolysis of phytate and release of bound N. Similarly, the greater Ca content
288 may have influenced the digesta pH such that the protein/ N did not precipitate from the
289 phytate-protein complexes in the digesta for its utilisation.

290

291 *Phytate hydrolysis*

292 As digesta flowed from the ileum through the remainder of the digestive tract, it appears that
293 dietary aP had more of an influence on the phytate and the IP esters than it did in the gizzard.
294 Li et al. (2016) reported that the proventriculus and gizzard are the most active sites of
295 phytate hydrolysis by exogenous phytase and may be associated with the most soluble
296 location for IP6 in the GIT, which is important for its interaction with phytase as suggested
297 by Zeller et al. (2015). Inositol phosphate esters must be soluble in the digesta to be able to
298 interact with phytase. Although IP3, IP2 and IP1 were below the limits of detection in the
299 gizzard, this does not mean that they were not present, and will have some contribution to the
300 overall IP content in the gizzard. It may be that IP2 was below the limits of detection, or may
301 have contributed to the IP1/ inositol values. The decrease in IP6 and IP5 and increase in
302 inositol in the gizzard in the presence of phytase reflects the hydrolytic action of the phytase
303 occurring in the gizzard. Some of the IP3, IP2 and IP1 will be hydrolysed and accounted for
304 in the inositol content. It is unlikely that any of the esters will have been absorbed in the
305 gizzard, however, due to the solubility of the lower esters, during the process of the removal
306 of water from the gizzard to allow its function, these water-soluble lower esters may have
307 passed out of the gizzard and are thus not detected on analysis. The relatively high IP6
308 content of the ileal digesta in the diets without phytase shows that little endogenous phytate

309 hydrolysis had occurred up to this point. The phytate disappearance data shows that almost
310 10% greater ileal IP6 disappearance in the NC than PC diets. The higher ileal IP6 content in
311 the PC than the NC (both without phytase) suggests that some endogenous phytase activity
312 was occurring, but that in the PC diet there may have been phytate-interaction with other
313 nutrients (i.e. minerals such as Ca), preventing its binding with endogenous phytase for
314 hydrolysis.

315 It appears that the majority of the dietary IP6 may be hydrolysed (step-wise) through
316 to IP4 and IP3, with the IP5 and IP4 esters produced by phytase hydrolysis being more
317 susceptible to hydrolysis than the IP5 and IP4 esters occurring in the feed. Li et al. (2016)
318 reported the accumulation of IP6 in the distal ileum regardless of dietary factors, suggesting
319 that in the absence of phytase, there are few dietary influences on the disappearance of IP6.
320 When Li et al. (2016) supplemented phytase, there were no observed differences in the ability
321 of phytase to hydrolyse phytate at different levels of dietary Ca, aP and phytate-P, however
322 the low dietary Ca levels used were generally higher than the 'high dietary Ca' treatments in
323 many other studies. Ca is regularly reported as having a negative effect on phytase activity
324 when present at high levels, with phytate-P hydrolysis reported to decrease with increasing
325 dietary Ca (Tamim and Angel, 2003; Tamim et al., 2004). In the study reported by Li et al.
326 (2016) the chelation sites for IP6 with Ca may have already been full at the 'low' Ca level.
327 As a result of which no differences would have been observed by increasing the Ca levels
328 further, either through dietary manipulation or following phytate hydrolysis. As there were
329 treatment effects in the current study on the excreta content of IP5, IP4 and IP3, but not in the
330 ileum (with numerically greater concentrations in the excreta than the ileum, even in the
331 treatments where phytase was absent), some IP6 hydrolysis appears to have occurred in the
332 caecum. Phytate hydrolysis in the caecum may be a result of bacterial action, as the phytase
333 enzyme would be expected to be completely degraded by this point (Zeller et al., 2015).

334 Perhaps the greater IP4 content of the phytase supplemented diets may reflect the inability of
335 the caecal bacteria to hydrolyse exogenous phytase-produced IP4, which may have a different
336 structure to the IP4 present in the feed (Bedford, 2015, personal communication). Zeller et al.
337 (2015) reported different enantiomers of the IPs produced during phytate hydrolysis.

338 Additional observation of the ratios between IP6 and IP4 contents of the ileal digesta
339 and excreta suggests this may be the case, as highlighted by the identification of a negative
340 correlation between excreta IP6 and IP4 content. In the ileum and excreta, the ratio of IP6:IP4
341 decreased as phytase increased, in both the PC and NC diets, reaching 1:1 in the excreta when
342 high doses of phytase had been used. In the absence of phytase in the ileum, the IP6 content
343 was greater than the IP4. Strong negative correlations between excreta IP4 and IP6 content
344 were identified ($r^2 = -0.82$; $P < 0.001$). As the phytase dose increased, the ratio between IP6
345 and IP4 decreased as IP6 was hydrolysed and IP4 content marginally increased. Differences
346 were more apparent in the excreta, where the ratio decreased as phytase dose increased.
347 Hydrolysis of IP6 led to increased IP4 content, which without the addition of the super-dose
348 of 1,500 FTU/kg phytase would have accumulated in the digesta. Instead, the IP6:IP4 ratio
349 came closer to 1:1 as IP4 was cleared by hydrolysis. These results suggest that IP4 is more
350 susceptible to hydrolysis post-ileum in the control diets than the phytase supplemented diets.
351 This may be explained by a difference in structure between the IP4 produced by endogenous
352 and exogenous phytase sources and the ability of bacteria in the caeca to hydrolyse these
353 molecules. However, the use of super-doses of phytase is more likely to hydrolyse the IP4 to
354 the lower esters, as indicated by the IP6: IP4 ratio decreasing to 1:1 with 1,500 FTU/kg
355 phytase.

356 The interactive effect between dietary aP and phytase appeared to become irrelevant
357 for the inositol content in the excreta. As expected, inositol content increased with increasing
358 doses of phytase, indicating that high levels of phytase are more effective at increasing

359 inositol concentrations than regular levels. Inositol content of the excreta is numerically
360 lower than the content in the ileum, which suggests potential uptake of inositol in the terminal
361 region of the ileum, or by bacteria residing in the caeca.

362

363

CONCLUSION

364 Phytase supplementation was able to prevent the depressions in growth performance
365 observed in the NC diet without phytase, being comparable to the PC, with or without
366 phytase. There was no interaction on phytate hydrolysis in the ileum, with phytase decreasing
367 IP6 and increasing inositol contents. Nutrient imbalance may be mediated by nutrient release
368 from phytate hydrolysis and have further downstream effects on hydrolysis of the inositol
369 phosphate esters and gut characteristics.

370

371

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377

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541 **Table 1.** Ingredient composition of the experimental diets

Description of diets	PC ¹	PC500	PC1,500	NC ²	NC500	NC1,500
Phytase, FTU/kg	0	500	1,500	0	500	1,500
Ingredients, g/kg						
Corn	346.5	346.5	346.5	382.9	382.9	382.9
Wheat	200	200	200	200	200	200
Soybean meal	302	302	302	295	295	295
Soybean oil	50	50	50	28	28	28
Di-calcium phosphate ³	21.0	21.0	21.0	12.6	12.6	12.6
Limestone ⁴	9.7	9.7	9.7	11.3	11.3	11.3
Titanium-dioxide premix ⁵	25.0	25.0	25.0	25.0	25.0	25.0
CGM ⁴ for enzyme	30.0	20.0	0.0	30.0	20.0	0.0
Enzyme premix ⁶	0.0	10.0	30.0	0.0	10.0	30.0
Vitamin-mineral premix ⁷	5.0	5.0	5.0	5.0	5.0	5.0
Methionine	1.9	1.9	1.9	1.9	1.9	1.9
Lysine	3.6	3.6	3.6	3.8	3.8	3.8
Threonine	0.7	0.7	0.7	0.4	0.4	0.4
Salt NaCl	3.1	3.1	3.1	3.1	3.1	3.1
NaHCO ₃	1.5	1.5	1.5	1.0	1.0	1.0
Total	1,000	1,000	1,000	1,000	1,000	1,000

542

543 ¹PC: Positive Control.

544 ²NC: Negative control.

545 ³Di-calcium phosphate, 25.7% Ca, 17.5% P.

546 ⁴Limestone, 38.9% Ca.

547 ⁵3.5kg TiO₂ mixed with 13.8kg corn gluten meal.

548 ⁶Corn gluten meal forms the basis of the enzyme premix and is used as a filler in the place of
549 phytase.

550 ⁷Phytase premix is mixed with corn and has an activity of 150 FTU/g.

551 ⁸Premix supplies the following per kg diet: Vit. A, 5484 IU; Vit. D3, 2643 ICU; Vit E, 11 IU;
552 Menadione sodium bisulfite, 4.38 mg; Riboflavin, 5.49 mg; d-pantothenic acid, 11 mg;
553 Niacin, 44.1 mg; Choline chloride, 771 mg; Vit B12, 13.2 µg; Biotin, 55.2 ug; Thiamine
554 mononitrate, 2.2 mg; Folic acid, 990 µg; Pyridoxine hydrochloride, 3.3 mg; I, 1.11 mg; Mn,
555 66.06 mg; Cu, 4.44 mg; Fe, 44.1 mg; Zn, 44.1 mg; Se, 300 µg.

556 **Table 2.** Calculated and analyzed nutrient and energy content of the experimental diets

Description of diets	PC ¹	PC500	PC1,500	NC ²	NC500	NC1,500
Phytase, FTU/kg	0	500	1,500	0	500	1,500
Calculated nutrients and energy:						
Protein, g/kg	230.5	230.5	230.5	230.3	230.3	230.3
ME, kcal/kg	3,020	3,020	3,020	2,941	2,941	2,941
Ca, g/kg	10.0	10.0	10.0	8.4	8.4	8.4
P, g/kg	6.9	6.9	6.9	5.5	5.5	5.5
Available P, g/kg	5.0	5.0	5.0	3.5	3.5	3.5
Ca:P	1.4	1.4	1.4	1.5	1.5	1.5
Na	1.8	1.8	1.8	1.7	1.7	1.7
K	7.9	7.9	7.9	7.9	7.9	7.9
Cl	2.2	2.2	2.2	2.2	2.2	2.2
Mg	1.5	1.5	1.5	1.5	1.5	1.5
Total amino acids, g/kg						
Lys	14.0	14.0	14.0	14.1	14.1	14.1
Met	5.0	5.0	5.0	5.0	5.0	5.0
Cys	4.0	4.0	4.0	4.0	4.0	4.0
Phe + Tyr	17.5	17.5	17.5	17.5	17.5	17.5
Analyzed nutrient content (g/kg):						
Ca	12.2	11.1	10.7	10.8	10.4	10.2
P	7.5	6.7	7.1	6.1	5.8	5.7
Ca:P	1.6	1.6	1.5	1.8	1.8	1.8
Phytate-P	1.7	2.4	2.2	1.7	2.0	1.8

Crude protein ³	3.54	3.55	3.45	3.44	3.58	3.66
IP6 (mg/kg)	11.54	11.53	11.56	11.07	11.14	11.93
IP5 (mg/kg)	0.558	0.574	0.595	0.629	0.665	0.661
Na	1.8	1.7	1.5	1.7	1.6	1.6
K	9.0	8.5	9.7	9.0	8.9	8.5
Mg	1.4	1.3	1.5	1.4	1.4	1.3
Phytase ^{4,5,6} (FTU/kg)	< 50	715	1,880	< 50	744	1,960

557

558 ¹PC: Positive Control.

559 ²NC: Negative control.

560 ³Crude protein: Nitrogen analyses × 6.25

561 ⁴The enzyme used was Quantum Blue, provided by AB Vista, with an initial activity level of
562 5,000 FTU/kg. The premix was made to 150 FTU/kg and added at the rate of 10 g/kg or 30
563 g/kg to give activity levels of 500 or 1,500 FTU/kg, respectively.

564 ⁵Dietary phytase quantification determined using ELISA method by AB Vista on final diets.

565 ⁶Enzyme premix quantified at 56 FTU/kg using Quantum method by AB Vista.

566

567 **Table 3.** Mortality, initial body weight (IBW, d0) and body weight gain (BWG, d21), feed
568 intake (FI) and feed conversion ration (FCR) of broilers fed diets adequate or deficient in aP
569 and Ca, with 0, 500 or 1,500 FTU/kg phytase, for 21 days

Diet		IBW, g	BWG, g	FI, g	FCR	Mortality ² %
Simple effect means						
Negative control (NC)		41.1	729	1184	1.624	3.1
NC + 500 FTU/kg		40.9	852	1215	1.428	1.6
NC + 1,500 FTU/kg		41.2	849	1192	1.407	1.6
Positive control (PC)		41.8	840	1242	1.487	4.7
PC + 500 FTU/kg		41.4	874	1201	1.377	1.6
PC + 1,500 FTU/kg		41.3	882	1256	1.426	3.1
	SEM	0.35	14.2	31.8	0.040	2.1
Main effect means						
aP, %	0.35	41.1	810	1197	1.486	2.1
	0.50	41.5	865	1233	1.430	3.1
	SEM	0.20	8.2	18.4	0.023	1.2
Phytase ¹	0	41.4	7814	1213	1.555	3.9
	500	41.1	863	1208	1.402	1.6
	1,500	41.3	865	1224	1.417	2.3
	SEM	0.25	10.0	22.5	0.028	1.5
P values for main effects						
aP, %		0.153	< 0.001	0.174	0.095	0.702
Phytase		0.749	< 0.001	0.879	< 0.001	0.673
aP × phytase		0.725	0.007	0.391	0.167	0.899

P values for orthogonal contrasts

Phytase linear	< 0.001	0.006
Phytase quadratic	< 0.001	0.005
PC linear	0.043	
NC linear	< 0.001	
NC quadratic	< 0.001	

570

571 ¹FTU/kg phytase.

572 ²Statistical analysis of mortality % was performed following the application of $1/(\sqrt{X+0.1})$

573 to allow for analysis of zero mortality.

574 8 replications per diet.

575 **Table 4.** Ileal nutrient digestibility of broilers fed diets adequate or deficient in aP and Ca,
 576 with 0, 500 or 1,500 FTU/kg phytase, for 21 days

		Ileal digestibility coefficients			
		DM	N	P	Ca
		Simple effect means			
	Negative control (NC)	0.537	0.513	0.417	0.466
	NC + 500 FTU/kg	0.626	0.623	0.524	0.464
	NC + 1,500 FTU/kg	0.590	0.586	0.410	0.295
	Positive control (PC)	0.583	0.589	0.431	0.397
	PC + 500 FTU/kg	0.569	0.569	0.371	0.287
	PC + 1,500 FTU/kg	0.572	0.562	0.418	0.242
	SEM	0.012	0.018	0.032	0.033
		Main effect means			
aP, %	0.35	0.584	0.574	0.450	0.408
	0.50	0.574	0.573	0.407	0.309
	SEM	0.007	0.010	0.019	0.019
Phytase ¹	0	0.560	0.551	0.424	0.431
	500	0.598	0.596	0.478	0.376
	1,500	0.581	0.574	0.414	0.269
	SEM	0.009	0.013	0.023	0.033
		P values for main effects			
aP, %		0.328	0.956	0.107	< 0.001
Phytase		0.016	0.058	0.560	< 0.001
aP × phytase		< 0.001	0.003	0.022	0.131
		P values for orthogonal contrasts			

Phytase linear				< 0.001
Phytase quadratic				0.824
PC linear	0.598	0.311	0.972	
NC linear	0.032	0.036	0.504	
NC quadratic	< 0.001	< 0.001	0.010	

577

578 ¹FTU/kg phytase.

579 8 replications per diet.

580 N: Nitrogen.

581 P: Phosphorus.

582 Ca: Calcium.

583 **Table 5.** Total tract nutrient retention of broilers fed diets adequate or deficient in aP and Ca,
 584 with 0, 500 or 1,500 FTU/kg phytase, for 21 days

Diet		Total tract retention coefficients			
		DM	N	P	Ca
		Simple effect means			
Negative control (NC)		0.702	0.626	0.617	0.511
NC + 500 FTU/kg		0.734	0.679	0.703	0.569
NC + 1,500 FTU/kg		0.737	0.698	0.678	0.536
Positive control (PC)		0.729	0.699	0.581	0.513
PC + 500 FTU/kg		0.717	0.678	0.534	0.456
PC + 1,500 FTU/kg		0.718	0.674	0.568	0.446
SEM		0.007	0.011	0.023	0.029
		Main effect means			
aP, %	0.35	0.724	0.668	0.666	0.539
	0.50	0.721	0.683	0.561	0.472
SEM		0.004	0.006	0.013	0.017
Phytase ¹	0	0.716	0.663	0.599	0.512
	500	0.725	0.678	0.618	0.512
	1,500	0.728	0.686	0.623	0.491
SEM		0.005	0.008	0.016	0.020
		P values for main effects			
aP, %		0.554	0.092	< 0.001	0.008
Phytase		0.181	0.114	0.558	0.704
aP × phytase		0.002	< 0.001	0.025	0.125
		P values for orthogonal contrasts			

PC linear	0.336	0.147	0.903
PC quadratic	0.320	0.360	0.147
NC linear	0.002	< 0.001	0.155
NC quadratic	0.025	0.046	0.030

585

586 ¹FTU/kg phytase.

587 8 replications per diet.

588 N: Nitrogen.

589 P: Phosphorus.

590 Ca: Calcium.

591 **Table 6.** Inositol phosphate ester content of the gizzard of broilers fed diets adequate or
 592 deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase

Gizzard		mg/g DM ²			
		IP6 ¹	IP5 ¹	IP4	Inositol
Simple effect means					
Negative control (NC)		3.58	0.50	0.55	0.09
NC + 500 FTU/kg		0.59	0.11	0.94	0.12
NC + 1,500 FTU/kg		0.52	0.07	0.67	0.14
Positive control (PC)		3.15	0.47	0.72	0.08
PC + 500 FTU/kg		0.65	0.12	0.83	0.11
PC + 1,500 FTU/kg		0.42	0.04	0.78	0.11
SEM		0.11	0.02	0.04	0.008
Main effect means					
aP, %	0.35	1.56	0.22	0.72	0.12
	0.50	1.41	0.21	0.78	0.10
SEM		0.06	0.01	0.03	0.005
Phytase ³	0	3.37	0.48	0.63	0.09
	500	0.62	0.11	0.89	0.12
	1,500	0.47	0.05	0.73	0.13
SEM		0.08	0.01	0.03	0.006
P values for main effects					
aP, %		0.474	0.243	0.107	0.018
Phytase		< 0.001	< 0.001	< 0.001	< 0.001
aP × phytase		0.606	0.129	0.005	0.315
P values for orthogonal contrasts					
Phytase linear		< 0.001	< 0.001	0.322	< 0.001
Phytase quadratic		< 0.001	< 0.001	< 0.001	0.070
PC linear				0.488	
NC linear				0.374	
NC quadratic				< 0.001	

- 593 ¹Log transformation of IP6 and IP5 prior to analysis.
- 594 ²IP2 and IP3 not detected.
- 595 ³FTU/kg.
- 596 8 replications per diet.
- 597 IP6-4: Inositol phosphate ester with 6- 4 bound phosphate molecules.

598 **Table 7.** Inositol phosphate ester content of the ileum of broilers fed diets adequate or
 599 deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase, for 21 days

Ileum		IP6 ^{1,2} disappearance	mg/g DM				
		IP6 ¹	IP5 ¹	IP4	IP3	Inositol	
Simple effect means							
Negative control (NC)		0.788	8.4	1.89	1.61	0.62	1.72
NC + 500 FTU/kg		0.938	3.6	0.55	1.04	0.67	2.26
NC + 1,500 FTU/kg		0.951	2.4	0.41	1.31	0.49	2.86
Positive control (PC)		0.671	14.9	2.29	1.69	0.44	1.23
PC + 500 FTU/kg		0.902	5.9	0.98	1.72	0.58	1.90
PC + 1,500 FTU/kg		0.953	2.5	0.45	1.48	0.63	2.07
SEM		0.0177	0.930	0.16	0.178	0.121	0.135
Main effect means							
aP, %	0.35	0.892	4.79	0.95	1.32	0.59	2.28
	0.50	0.842	7.79	1.24	1.63	0.55	1.73
SEM		0.0102	0.537	0.094	0.126	0.070	0.078
Phytase ³	0	0.730	11.67	2.09	1.65	0.53	1.47
	500	0.920	4.77	0.77	1.38	0.62	2.08
	1,500	0.952	2.44	0.43	1.39	0.56	2.47
SEM		0.0125	0.658	0.12	0.126	0.086	0.095
P values for main effects							
aP, %		0.001	0.008	0.035	0.041	0.670	< 0.001

Phytase	< 0.001	< 0.001	<0.001	0.257	0.734	< 0.001
aP × phytase	0.003	0.105	0.106	0.199	0.410	0.276
P values for orthogonal contrasts						
Phytase linear		< 0.001	< 0.001			< 0.001
Phytase quadratic		0.013	< 0.001			0.102
PC linear	< 0.001					
PC quadratic	< 0.001					
NC linear	< 0.001					
NC quadratic	< 0.001					

600

601 ¹Log transformation prior to analysis.

602 ²IP6 disappearance, coefficient of

603 ³FTU/kg.

604 No IP2 detected.

605 8 replications per diet.

606 IP6-3: Inositol phosphate ester with 6- 3 bound phosphate molecules.

607 **Table 8.** Inositol phosphate ester content of the excreta of broilers fed diets adequate or
 608 deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase, for 21 days

Total tract		mg/g DM				
		IP6 ¹	IP5	IP4 ¹	IP3	Inositol
Simple effect means						
Negative control (NC)		35.6	3.60	1.83	0.17	1.21
NC + 500 FTU/kg		20.4	4.14	4.18	0.22	1.41
NC + 1,500 FTU/kg		12.9	3.14	7.05	0.24	1.46
Positive control (PC)		39.5	4.70	3.04	0.24	0.37
PC + 500 FTU/kg		20.7	4.76	4.93	0.18	0.89
PC + 1,500 FTU/kg		8.8	2.73	7.30	0.26	1.32
SEM		1.687	0.173	0.389	0.010	0.130
Main effect means						
aP, %	0.35	22.92	3.63	4.35	0.21	1.36
	0.50	23.01	4.07	5.09	0.23	0.86
	SEM	0.974	0.100	0.225	0.006	0.075
Phytase ²	0	37.55	4.15	2.43	0.21	0.79
	500	20.55	4.45	4.55	0.20	1.15
	1,500	10.81	2.94	7.17	0.25	1.39
	SEM	1.193	0.12	0.275	0.007	0.092
P values for main effects						
aP, %		0.715	0.004	< 0.001	0.033	< 0.001
Phytase		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
aP × phytase		0.162	< 0.001	0.018	< 0.001	0.034
P values for orthogonal contrasts						

Phytase quadratic	0.055	< 0.001	< 0.001	0.067	0.357
PC linear		< 0.001	< 0.001	0.077	< 0.001
PC quadratic		0.002	0.060	< 0.001	0.212
NC linear		0.016	< 0.001	< 0.001	0.231
NC quadratic		0.003	< 0.001	0.110	0.485

609

610 ¹Log transformation of IP4 and IP6 prior to analysis.

611 ²FTU/kg.

612 8 replications per diet.

613 IP6-3: Inositol phosphate ester with 6- 3 bound phosphate molecules.