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Scotland's Rural College

The interplay of dietary nutrient level and varying calcium to phosphorus ratios on efficacy of bacterial phytase: 2. Ileal and total tract nutrient utilization

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ABSTRACT A 14-d broiler experiment was conducted to assess the effects of two dietary variables on efficacy of a bacterial 6-phytase expressed in *Aspergillus oryzae* on nutrient and phytate phosphorus (PP) utilization. Diets were formulated with or without nutrient matrix values (matrix) for phytase as negative control (NC) or positive control (PC), respectively and with two Ca:tP levels (2:1 or 2.5:1). The diets were supplemented with 0, 1,000 or 2,000 FYT/kg phytase thus producing a 2×2×3 factorial arrangement. Excreta were collected on d 19 to 21 and ileal digesta on d 21. There was no three-way interaction on digestibility of any nutrient. There was matrix × phytase (P < 0.01) interaction for Ca and DM digestibility and Ca:tP × phytase interaction (P < 0.05) for acid hydrolyzed fat, Ca and P digestibility. Pre-cecal flow of Mn, Zn and Na was greater (P < 0.05) in NC diets whereas phytase increased (P < 0.05) pre-cecal flow of Mg, Fe, Mn, and Zn but decreased (P < 0.05) pre-cecal Na flow. Total tract PP disappearance and total tract Ca retention increased (P < 0.05) with phytase supplementation in diets with 2:1 Ca:tP whereas there was no effect of phytase supplementation on PP disappearance or Ca retention in diets with 2.5:1 Ca:tP. Total P and Ca retention were reduced (P < 0.05) in PC and NC diets when Ca:tP increased to 2.5:1 but the depression was more pronounced in the NC diet. In addition, PP disappearance decreased (P < 0.05) with increasing Ca:tP in the PC diets but there was no effect of widening Ca:tP on PP disappearance in NC diets. It was concluded from the current study that the effect of phytase supplementation on P utilization is reduced when diets contain adequate P as exemplified in the PC diets and that the negative impact of wide Ca:tP is more pronounced in diets with phytase matrix allowance as exemplified in the NC diets.

31   **Key words:** broilers, calcium:phosphorus, nutrient utilization, phytase matrix

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INTRODUCTION

The use of phytase in non-ruminant diets and the effects of different nutritional variables on the efficacy of phytase have been well studied in recent years (Adeola and Cowieson, 2011). However, there is continued interest in understanding the various factors that mitigate the efficacy of phytase or that may improve its effect in poultry diets because of the sheer amount of phytate present in typical poultry diets. A typical corn-soybean meal diet for poultry formulated using conventional (i.e. non low-phytate varieties) may contain up to 4 g/kg phytate-P (Selle and Ravindran, 2007) which are largely unavailable to birds without the action of phytase (endogenous and exogenous). Liberation of 60% of the P tied up in phytate will be a considerable saving in terms of reducing both the cost for inorganic P supplementation and environmental impact of P excretion. Therefore it is imperative to understand factors that may hinder or enhance the efficacy of phytase.

The negative effect of wide Ca:P on phytase efficacy is well known (Tamim et al., 2004; Adeola et al., 2006) and this is related to the formation of recalcitrant calcium-phytate (Taylor, 1965, Nelson and Kirby, 1987) or Ca-phosphate complexes (Long et al., 1984). In addition it is common practice to reduce dietary levels of inorganic P, Ca, Na, energy and some digestible amino acids (phytase matrix) in phytase-supplemented diets (Shelton et al., 2004). It would seem that supplementation of phytase to diets that already meet birds' requirements for these minerals can be both wasteful and counterproductive. However, supplementation of phytase at high levels (Shirley and Edwards, 2003; Cowieson et al., 2006) or to diets that already meet nutrient requirements of broilers has produced improvement in animal performance presumably via mitigation of anti-nutritive effects of phytate rather than supply of limiting nutrients (Walk et al., 2013).

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56   The current study examines the interplay of the variation in Ca:tP (tp, **total P**) and dietary

57   nutrient levels on efficacy of phytase added at low and high doses. There have been considerable

58   amount of investigations on the former and much less on the latter. Therefore, the objective of

59   the current experiment was to investigate how the use of a nutrient replacement values for

60   phytase (phytase matrix) affects phytase efficacy on nutrient utilization (with particular focus on

61   utilization of Ca, tP and phytate P), and especially within the context of variable dietary Ca:tP.

62   The companion article considers how these dietary factors influence growth performance and

63   bone mineralization in broilers.

MATERIALS AND METHODS

65   All the animal experimentation procedures used in the current study were approved by

66   the Scotland's Rural College's Animal Experimentation Committee.

Diets and experimental design

68   A total of 576 birds were used for the 14-d experiment to study the influence of nutrient

69   specification and Ca:tP on efficacy of phytase on nutrients and minerals utilization in broilers.

70   The birds were brooded together in a floor pen for the first 7 days of age during which they

71   received a standard diet that meets NRC (1994) nutrient requirement for broilers. On day 7, the

72   birds were weighed and allocated to 12 dietary treatments in a randomized complete block

73   design and a 2×2×3 factorial arrangement of treatments. Each treatment had 8 replicate cages

74   and 6 birds per replicate cage. The factors were two levels of nutrient specifications (explained

75   below), two levels of Ca:tP (2:1 and 2.5:1) and three levels of phytase supplementation (0, 1,000

76   and 2,000 FYT/kg). Excreta were collected on d 19 to 21 of the birds' age and ileal digesta were

77   collected on d 21 after euthanasia of the birds.

78   The composition of the experimental diets is presented in Table 1. Nutrient specification

79   was used to define the diets that were formulated to meet all the nutrient requirements for

80   broilers (full nutrient specification without phytase matrix or positive control, **PC**) and another

81   set of diets with reduced nutrient specification formulated to be deficient in P, Ca, crude protein

82   (**CP**), amino acids, and energy (down specification or negative control, **NC**). The nutrients and

83   energy levels in the NC diets were reduced relative to the PC diets on the basis of the amount of

84   nutrients and energy that the phytase was expected to release (nutrient matrix values for

85   phytase). The matrix values used per kg feed for 1,000 FYT were approximately, 75 kcal ME,

86   1.5 g for available P, 1.8 g for Ca, 0.26 g for CP, 0.11, 0.07, 0.04, and 0.07 g for digestible

87   lysine, total sulphur amino acids, methionine, and threonine, respectively. One phytase (FYT)

88   unit is defined as the activity that releases 1 μmol inorganic phosphate from 5.0 mM phytate per

89   minute at pH 5.5 and 37°C.

90   *Chemical analysis*

91   Diets, ileal digesta and excreta were analyzed for dry matter, N, gross energy, Ti, and

92   minerals. Dry matter was determined by drying the samples in a drying oven (Uniterm, Russel-

93   Lindsey Engineering Ltd., Birmingham, England, UK) at 105°C for 24 hours (AOAC Method

94   934.01; AOAC, 2006). Total N content was determined by the combustion method (Method

95   968.06; AOAC, 2006). Gross energy was determined in an adiabatic bomb calorimeter (Model

96   6200, Parr Instruments, Moline, IL) using benzoic acid as an internal standard. Titanium

97   concentration in the samples was determined using the method of Short *et al*. (1996). Minerals

98   content was determined using Inductively Coupled Plasma – Optical Emission Spectroscopy

99   (AOAC Method 990.08; AOAC, 2006) following digestion, in turn, in concentrated HNO_3 and

100   HCl. Free fat was determined using extraction by petroleum ether in a Soxhlet apparatus for six

101   hours whereas acid hydrolyzed fat (**AHF**) was determined by acid hydrolysis using 30% HCl

102   followed by ether extraction.

103   *Statistical analysis*

104   The data were analyzed by the MIXED procedure of SAS as appropriate for a

105   randomized complete block design and a factorial treatment arrangement. For ease of reference,

106   the two types of control diets (NC and PC) were coded as matrix (i.e. nutrient matrix for phytase)

107   in the factorial arrangement with PC (as diets without phytase matrix) and NC (as diets with

108   phytase matrix). The three-way interactions were investigated first in the analysis. Where the 3-

109   way interactions were not significant they were dropped from the model and the data re-

110   analyzed. Non-significant interactions were dropped for more thorough investigation of the main

111   effects means. Because the two-way interactions were significant for most of the responses even

112   though the three-way interactions were not, the simple effects means are presented in the tables.

113   Because of the hierarchical arrangement of main effects and interactions, only the interactions

114   are discussed for responses in which all the two-way interactions are significant, whereas main

115   effects means are also discussed in cases where one or more of the two-way interactions are not

116   significant.

117   **RESULTS**

118   The analyzed nutrients compositions of the experimental diets are shown in Table 2 and

119   show that expected nutrient compositions were met despite some slightly higher recoveries of the

120   phytase.

121   The data on ileal nutrient digestibility response to the dietary treatments are presented in

122   Table 3. There were no three-way interaction effects on digestibility of any of the nutrients.

123   There were matrix $\times$ Ca:tP ($P < 0.05$) interaction for DM and AHF digestibility explained by

124   lower ($P < 0.01$) DM and AHF digestibility in the NC diets with narrow Ca:tP whereas there

125   was no effect of Ca:tP on DM and AHF digestibility in the PC diets. There was also matrix $\times$

126   phytase ($P < 0.01$) interaction for Ca and DM digestibility with lower ($P < 0.01$) DM and Ca

127 digestibility (drastic reduction observed for Ca digestibility) in phytase-supplemented NC diets

128 whereas such effect was not observed in the PC diets. Ca:tP × phytase interaction was observed

129 (P < 0.05) for AHF, Ca and P digestibility with phytase at 2,000 FYT/kg increasing (P < 0.05)

130 AHF digestibility in the diets with narrow Ca:tP whereas phytase supplementation had no effect

131 on AHF digestibility in diets with wide Ca:tP.   The Ca:tP × phytase interaction for Ca

132 digestibility was characterized by drastic and stepwise reduction (P < 0.05) in Ca digestibility

133 with increasing phytase supplemental level in the diets with wide Ca:tP but a less drastic

134 reduction in Ca digestibility at 1,000 FYT/kg in diets with narrow Ca:tP. For P digestibility, the

135 Ca:tP × phytase interaction was manifested in reduced (P < 0.05) P digestibility at 1,000 FYT/kg

136 and an increase (P < 0.05) at 2,000 FYT/kg in diets with narrow Ca:tP but a reduction (P < 0.05)

137 in P digestibility at both 1,000 and 2,000 FYT/kg in diets with wide Ca:tP.

138   The data on pre-cecal flow of micro-minerals in response to the dietary treatments are

139 presented in Table 4. Pre-cecal flow of Na, Mn and Zn was greater (P < 0.05) in NC diets; in

140 addition pre-cecal flow of K and M n was greater (P < 0.05) in diets with wide Ca:tP. On the

141 other hand, phytase supplementation increased (P < 0.05) pre-cecal flow of Mg, Fe, Mn, and Zn,

142 decreased (P < 0.01) flow of Na and had no effect on K flow. There were significant Ca:tP ×

143 phytase interactions (P < 0.05) for pre-cecal flow of Mg and Mn with a decrease in the pre-cecal

144 flow of the minerals with phytase supplementation in diets with narrow Ca:tP. On the other hand

145 there was an increase in pre-cecal flow of the minerals with phytase supplementation in diets

146 with wide Ca:tP. Ca:tP × matrix interaction was significant (P < 0.01) for pre-cecal flow of Na

147 and Mn. Generally pre-cecal flow of Na and Mn was greater (P < 0.01) in PC diets with wide

148 Ca:tP whereas Ca:tP had no effect on flow of the minerals in NC diets. Matrix × phytase

149 interaction was observed (P<0.01) for pre-cecal Na and K flow. Phytase supplementation

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150   decreased ($P < 0.05$) pre-cecal Na flow but increased ($P < 0.05$) K flow in NC diets however

151   phytase supplementation had no effect on pre-cecal Na flow but decreased ($P < 0.05$) pre-cecal K

152   flow in PC diets.

153   The effects of the treatments on total tract nutrient retention are shown in Table 5. There

154   were Ca:tP $\times$ phytase interaction ($P < 0.05$) for total tract retention of DM, fat, AHF, and N, as

155   well as AME. Dry matter and N retention as well as AME increased ($P < 0.05$) with phytase

156   supplementation in diets with narrow Ca:tP but DM retention decreased ($P < 0.05$) whereas

157   AME, N and fat retention were unaffected by phytase supplementation in diets with wide Ca:tP.

158   The interaction of Ca:tP $\times$ matrix was significant ($P < 0.05$) for retention of DM, AHF, N, and

159   AME. In PC diets, retention of DM, AHF and N decreased ($P < 0.05$) whereas AME increased ($P

160   < 0.05$) with widening of Ca:tP. In NC diets, AHF and N retention increased ($P < 0.05$) whereas

161   there was no change in DM retention and AME with widening of Ca:tP.

162   The effect of the dietary treatments on total tract retention of Ca, P and PP are shown in

163   Table 6. Widening Ca:tP to 2.5:1 decreased ($P < 0.05$) total tract retention of Ca and tP as well as

164   PP disappearance. Ca:tP $\times$ phytase interaction was significant ($P < 0.05$) for total tract retention

165   of Ca, tP and PP. In the diets with narrow Ca:tP, only 2,000 FYT phytase increased ($P < 0.05$) tP

166   retention. In the diets with wide Ca:tP, phytase supplementation at 1,000 FYT/kg improved tP

167   retention. Total tract PP disappearance and Ca retention increased ($P < 0.05$) with phytase

168   supplementation in diets with narrow Ca:tP but no effect was observed in diets with wide Ca:tP.

169   There was significant Ca:tP $\times$ matrix ($P < 0.05$) on total tract retention of tP and Ca as well as PP

170   disappearance. Total P and Ca retention were reduced ($P<0.05$) in both NC and PC with

171   widening of Ca:tP to 2.5:1 but the depression in P and Ca retention due to widening of Ca:tP was

172   more pronounced in the NC diets. In addition, PP disappearance decreased ($P<0.05$) with

173 widening Ca:tP in the PC diets but there was no effect of widening Ca:tP on PP disappearance in

174 the NC diets. Matrix × phytase was significant ($P < 0.05$) only for total tract P retention with

175 phytase supplementation increasing P retention only at 2,000 FYT/kg in PC diets and only at

176 1,000 FYT/kg in NC diets.

177   **DISCUSSION**

178   There is a preponderance of information on the effects of phytase on nutrient utilization

179 (Selle and Ravindran, 2007; Adeola and Cowieson, 2011) as well as the effect of Ca:P on

180 phytase efficacy (Qian et al, 1997; Selle et al., 2009). In addition, it is a usual practice to reduce

181 nutrient specification in phytase-supplemented diets or provide a nutrient matrix values for

182 phytase (Shelton et al., 2004; Silversides and Hruby, 2009). Therefore the objective of the

183 current experiment was to study the interactivity of varying Ca:tP in PC and NC diets on the

184 efficacy of phytase at low and high doses in promoting nutrient utilization in broilers. Although

185 the effects of the treatments in the current experiment were observed on energy and a large

186 number of nutrients, the main responses that will subsequently be focused on are phytate and

187 total P as well as Ca in view of their association with phytic acid.

188 *Effects of use of nutrient matrix for phytase on phytase efficacy*

189   The use of a phytase matrix in phytase-supplemented feed enables a reduction in nutrient

190 specification, reduces nutrient excretion and increases the chance of being able to observe

191 phytase effects. Two lines of evidence are presented here to show that the use of a phytase matrix

192 differently affects phytase effects on PP and tP.

193   At the ileal level, the efficacy of phytase in promoting PP disappearance was the same in

194 both PC and NC diets but at the total tract level, phytase supplementation of PC diet marginally

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195　reduced PP disappearance whereas phytase supplementation increased PP disappearance in the

196　NC diets. The PP level was virtually the same across all diets (average of 0.22%) and hence the

197　lower PP disappearance in PC diet without phytase suggests that the higher dietary non-phytate P

198　(**nPP**) level (providing greater quantities of readily available P) in the diet may provide a

199　feedback mechanism inhibiting the degradation of phytic acid. It is not clear if such a mechanism

200　exists, however others have similarly observed reduced PP disappearance in diets with high

201　levels of nPP (Ballam et al. 1982; Olukosi et al., 2013).

202　　　　Ravindran et al. (2000) observed that hydrolysis of PP increases with an increase in

203　dietary PP level. This is intuitive, up to a point, as higher PP provides more substrate for phytase.

204　But it is also of interest to consider how PP hydrolysis is affected by nPP levels in diets with the

205　same content of PP. In the current study, ileal PP disappearance was similar in both PC and NC

206　diets supplemented with 2,000 FYT/kg even though ileal PP disappearance in the diets without

207　phytase was five percentage units greater in the NC diet. This shows that the effect of phytase on

208　PP disappearance, relative to the control, was greater in the PC diet. The observation that PP

209　disappearance was the same in the diets supplemented with 2,000 FYT/kg in both PC and NC

210　diets indicates that effect of phytase supplementation on PP disappearance did not depend on

211　dietary level of nPP as also observed by Plumstead et al. (2008).

212　　　　Phytate P disappearance at the total tract level in response to phytase supplementation

213　was greater in both PC and NC diets compared with the disappearance at the ileal level. However

214　the difference in PP disappearance at both levels in diets supplemented with 2,000 FYT/kg was

215　greater for NC diet (11 %) compared with PC diet (5%). Phytase did not improve total tract PP

216　disappearance in the PC diet but improved PP disappearance in the NC diet.　Increased PP

217　disappearance in the excreta compared with the ileal level is an indication of either that the

218 phytase continued to be effective post-ileal or of the possible effects of microorganisms on

219 phytic acid hydrolysis. Overall the observation on PP disappearance and P utilization indicate

220 that reducing the level of nPP is beneficial in promoting greater PP and total P utilization.

221   Phytase supplementation did not increase ileal P digestibility in both the NC and PC diets

222 but increased total tract P retention by 11% in the NC and had no effect in the PC diets. The

223 numerical increase of 4.3 percentage units for total tract P retention in phytase-supplemented PC

224 diet decreased retained P by 190 mg/kg. On the other hand, phytase supplementation of NC diet

225 increased P retention by 11 percentage units and increased retained P by 830 mg/kg. In spite of

226 the greater retained P in phytase-supplemented NC compared with PC diet, the total retained P at

227 2,000 FYT/kg was 3.74 and 3.62 g/kg for PC and NC diets, respectively. The hydrolyzed PP at

228 the total tract level for PC and NC diets supplemented with 2,000 FYT/kg were 1.66 and 1.84

229 g/kg, respectively. Taken together therefore, the data imply that the high nPP content of the PC

230 diets "hinders" phytase from exerting its full effect on phytate. Although the total retained P in

231 PC diet supplemented with 2,000 FYT/kg phytase was greater than retained P in comparable NC

232 diet, this extra retained P could have resulted from the higher dietary P in the PC diet because the

233 amount of PP hydrolyzed was actually lower in the phytase-supplemented PC diet.

234 ***The interplay of Ca:tP and dietary nutrient levels (phytase matrix)***

235   The use of a phytase matrix in diet formulation enables a reduction in nutrient content of

236 phytase-supplemented diets. This may be a necessary dietary intervention in phytase-

237 supplemented diets in order to optimize phytase effect and maximize reduction in nutrient

238 excretion (Shelton et al., 2004; Silversides et al., 2009). In the current study, at both the ileal and

239 total tract levels, widening Ca:tP decreased P and Ca digestibility in both PC and NC diets but

240   the decrease produced by the wider Ca:tP was more pronounced in NC diets and the depression

241   in Ca utilization due to wide Ca:tP was greater at the total tract level. The decreased digestibility

242   values were also reflected in decreased digestible and retained Ca and P in the diets in response

243   to widening the Ca:tP. Two scenarios emerging from these observations are: 1) a decrease in Ca

244   and P utilization with a widening of Ca:tP irrespective of whether it was in PC or NC diet and, 2)

245   a more pronounced negative effect of widening Ca:tP in NC diets.

246   In the first scenario, the decreased Ca utilization with increased Ca:tP can be associated

247   with increased relative dietary concentration of Ca in the diets with wide Ca:tP because the

248   analyzed Ca in these diets was 27% higher than in diets with narrow Ca:tP. This greater dietary

249   Ca content produced correspondingly higher Ca intake and hence reduced Ca retained as a

250   percentage of intake. Similar observations have been reported in rats (Hoek et al., 1988), pigs

251   (Qian et al., 1996) and chickens (Qian et al., 1997). Thus it seems that the decreased Ca

252   utilization in diets with wide Ca:tP can be largely explained by the presence of an abundance of

253   Ca in the intestine, than can be utilized by the birds, leading to excessive Ca excretion or reduced

254   efficiency of Ca absorption.

255   The decrease in P utilization in the diets with wide Ca:tP ratio is also primarily driven by

256   dietary Ca content because tP content was similar in diets with wide and narrow Ca:tP. Hoek et

257   al. (1988) similarly observed high P excretion in rats receiving diets with high Ca level. This

258   reduced P utilization in diet with wide Ca:tP can be explained by the fact that high concentration

259   of Ca relative to P increases the possibility for negative interaction of Ca and P, leading to

260   greater chances for formation of calcium phosphate (Hurwitz and Bar, 1971). Al-Masri (1995)

261   observed that P digestibility, absorption and endogenous excretion in chickens decreased with

262   increasing Ca:P ratio. Similar effect has been reported by Edwards and Veltmann (1983) and

263   Qian et al. (1997). Clearly, an increase in Ca:tP increases the concentration of Ca relative to P

264   and hence increases the chances of more Ca being chemically bound and becoming indigestible.

265   It has been suggested that another way by which high Ca:P reduces P and Ca utilization is

266   by the formation of recalcitrant Ca-phytate complex (Wise, 1983; Maenz et al., 1999). The effect

267   of Ca:tP on PP disappearance was not consistently observed in the current study. It was only at

268   the total tract level that high Ca:tP decreased PP disappearance in the PC diet. The analyzed PP

269   was the same in all diets in the current experiment and the only differences among diets were Ca

270   and P levels. In addition, the depressed PP disappearance observed in the current study was not

271   dependent on Ca:tP per se but rather on dietary concentration of both Ca and P, i.e. the diet with

272   high contents of both Ca and P had depressed PP disappearance.

273   For the second scenario, it is possible that the reason for the decrease in Ca and P

274   utilization in NC diets with wide Ca:tP relative to similar diets in PC diets was due to the lower

275   Ca and P contents of the NC diets compared with PC diets. Phytate P made up a greater

276   proportion of total P in NC compared with the PC (additional P in the PC diet was supplied by

277   dicalcium phosphate) and hence the P will be less digestible in NC than the more readily

278   digestible P in the inorganic P sources used in the PC diet. Consequently the current data show

279   that the dietary content of Ca and P, not just the ratio, need to be considered in interpretation of

280   the effect of Ca:tP.

281   In light of the observations in the current experiment, it can be concluded that the effects

282   of wide Ca:tP are more likely to be severe in diets in which nutrient matrix for phytase is used

283   (as exemplified by the NC diets in this experiment) especially as it relates to Ca utilization; and

284 that the negative effect of high Ca:tP on P and Ca utilization could be mediated via mechanisms

285 independent of phytic acid degradation.

286

287 **ACKNOWLEDGEMENTS**

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Table 1. Ingredient composition (g/kg) of the experimental basal diets

Basal diet	1	2	3	4
Ca:tP[1]	2:1		2.5:1	
Control (phytase matrix)	Positive	Negative	Positive	Negative
Corn	482.6	477.4	466.6	499.4
Wheat	-	50.0	-	-
Soybean meal	397.5	382.5	400.5	394.5
Soybean oil	58.0	40.0	60.0	45.0
Corn Starch	15.0	15.0	15.0	15.0
Dicalcium phosphate	17.5	9.0	17.5	10.0
Limestone	17.0	15.5	28.0	24.0
Titanium dioxide	0.5	0.5	0.5	0.5
L-Lysine·HCl	1.0	0.4	1.0	0.7
DL-Methionine	2.8	1.9	2.8	2.8
Threonine	0.6	0.3	0.6	0.6
Vitamin-mineral premix[2]	2.5	2.5	2.5	2.5
Salt	5.0	5.0	5.0	5.0
Phytase premix[3]	To 1,000	To 1,000	To 1,000	To 1,000
Total	1,000	1,000	1,000	1,000
Calculated nutrients and energy, %				
Metabolizable energy, kcal/kg	3,185	3,125	3,154	3,127
Crude protein	22.9	22.8	22.9	22.9
Total P	0.71	0.56	0.70	0.57
Non-phytate P	0.45	0.30	0.45	0.31
Ca	1.06	0.82	1.43	1.13

[1]Ca:tP based on analyzed chemical composition

[2]Supplied the following per kilogram of diet: vitamin A, 5,484 IU; vitamin D3, 2,643 ICU; vitamin E, 11 IU; menadione sodium bisulfite, 4.38 mg;riboflavin, 5.49 mg; d-pantothenic acid, 11 mg; niacin, 44.1 mg; choline chloride, 771 mg; vitamin B12, 13.2 μg; biotin, 55.2 μg; thiamine mononitrate,2.2 mg; folic acid, 990 μg; pyridoxine hydrochloride, 3.3 mg; I, 1.11 mg; Mn, 66.06 mg; Cu, 4.44 mg; Fe, 44.1 mg; Zn, 44.1 mg; Se, 300 μg.

[3]Phytase premix containing 100 phytase units (FYT)/g replaced corn starch to provide 1,000 or 2,000 FYT/kg.

Table 2. Analyzed nutrient composition (%, dry matter basis) and phytase activity in the experimental diets

Diets	1	2	3	4	5	6	7	8	9	10	11	12
Ca:tP				2:1						2.5:1		
Matrix		Positive Control			Negative Control			Positive Control			Negative Control	
GE, kcal/kg	4,452	4,681	4,652	4,672	4,619	4,603	5,720	5,782	4,610	4,641	4,572	4,580
Ether extract	10.2	10.3	8.80	7.11	7.83	6.84	11.4	11.3	8.94	7.67	8.26	7.95
Acid hydrolysed fat	11.1	9.49	9.80	8.03	7.96	8.03	12.1	12.9	9.70	8.30	9.37	8.37
N	3.99	3.86	4.13	3.84	3.90	3.80	5.11	4.70	4.15	4.04	3.73	3.88
Ca	1.37	1.63	1.40	1.31	1.18	1.17	2.23	2.24	1.87	1.06	1.47	1.38
P	0.73	0.81	0.71	0.65	0.61	0.57	0.93	0.91	0.74	0.48	0.60	0.62
Phytate P	0.26	0.24	0.23	0.25	0.23	0.24	0.28	0.28	0.29	0.24	0.23	0.29
non-phytate P[1]	0.47	0.57	0.48	0.40	0.38	0.33	0.65	0.63	0.45	0.24	0.37	0.33
Na	0.23	0.26	0.24	0.26	0.25	0.23	0.24	0.28	0.23	0.17	0.23	0.19
Mg	0.18	0.18	0.16	0.18	0.18	0.16	0.23	0.22	0.18	0.15	0.18	0.19
Cu, mg	11.3	15.8	14.7	12.5	10.2	10.2	28.2	18.2	12.4	10.2	13.6	15.8
Fe, mg	82.4	87.0	79.2	90.7	78.5	71.3	111.3	106.5	83.3	56.6	81.4	79.0
Mn, mg	81.3	94.9	87.1	100.9	84.2	83.8	107.1	105.1	83.3	61.1	83.7	86.9
Zn, mg	79.1	96	99.5	92.9	84.2	81.5	101.4	99.5	83.3	58.9	75.7	79.0
K	1.16	1.14	1.00	1.16	1.19	0.96	1.47	1.43	1.14	0.97	1.15	1.28
Phytase, FTY/kg[2]	BD	1,121	2,977	BD	1,406	2,400	BD	1,717	2,537	BD	1,011	2,640

[1]non-phytate phosphorus level was determined by difference (total P – phytate P)

[2]BD – below detection limit

Table 3. Simple effects means for ileal nutrient digestibility response to varying levels of phytase supplementation, dietary Ca:total P broiler diets with or without nutrient matrix values for phytase[1]

Diet[2,3]	1	2	3	4	5	6	7	8	9	10	11	12		P-values for interactions[2,3]		
Ca:tP			2:1						2.5:1							
M[3]		Positive Control			Negative Control			Positive Control			Negative Control		SEM	Ca:tP × M	Ca:tP × Ph	M × Ph
Ph[3]	0	1000	2000	0	1000	2000	0	1000	2000	0	1000	2000				
DM	69.9	67.8	68.6	66.1	62.7	68.5	66.2	65.4	65.4	68.2	66.3	63.1	0.897	0.003	0.635	0.001
EE[3]	89.5	88.3	87.2	83.2	83.6	83.0	87.2	88.0	86.7	85.5	86.2	85.0	1.12	0.058	0.794	0.821
AHF[3]	82.0	79.6	82.4	76.4	74.4	78.0	81.3	81.8	81.0	78.1	81.9	78.4	1.31	0.043	0.003	0.768
N	76.9	73.8	77.1	73.9	69.9	75.0	73.5	71.4	74.8	73.8	70.3	70.0	1.12	0.636	0.156	0.447
Ca	42.8	32.5	35.9	61.7	45.9	44.9	34.0	31.9	25.8	53.5	34.8	25.9	2.56	0.062	0.029	0.001
P	43.8	38.7	47.2	48.5	37.9	46.9	34.0	32.3	33.0	37.6	31.4	30.2	1.97	0.358	0.005	0.071
PP[3]	48.5	42.7	58.7	57.7	43.3	65.4	36.0	44.1	58.6	39.3	51.1	51.7	6.03	0.493	0.088	0.784

[1]Means were obtained from 8 replicate cages of 6 birds per replicate cage

[2]The main effect for Ca:tP was significant ($P < 0.05$) for all nutrients except EE and PP; The main effect for nutrient matrix was significant for all nutrients except P and PP; The main effect for phytase was significant for all nutrients except EE and AHF; The three-way interaction was not significant for any nutrient

[3]M = nutrient matrix for phytase; Ph = phytase; EE = crude fat; AHF =acid hydrolysed fat; PP = phytate phosphorus

Table 4. Simple effects means for pre-cecal flow (g/100 g dry matter intake) of micro-minerals in response to varying levels of phytase supplementation, dietary Ca:total P broiler diets with or without nutrient matrix values for phytase[1]

Diet	1	2	3	4	5	6	7	8	9	10	11	12		P-values for interactions[2,3]		
Ca:tP			2:1							2.5:1			SEM			
M[3]	Positive Control			Negative Control			Positive Control			Negative Control				Ca:tP × Ph	Ca:tP × M	M × Ph
Ph[3]	0	1000	2000	0	1000	2000	0	1000	2000	0	1000	2000				
Na	0.271	0.235	0.232	0.409	0.346	0.292	0.257	0.242	0.260	0.362	0.290	0.294	0.017	0.119	0.032	0.008
Mg	0.155	0.166	0.158	0.168	0.190	0.157	0.164	0.178	0.171	0.156	0.171	0.186	0.006	0.003	0.096	0.620
Fe, mg	65.8	73.6	74.5	64.7	70.9	86.9	73.3	75.3	85.5	71.7	78.0	86.6	7.55	0.965	0.804	0.717
Mn, mg	88.0	92.7	84.5	94.0	99.4	89.4	92.6	96.9	94.2	91.6	91.9	101.0	2.20	< 0.001	0.028	0.309
Zn, mg	76.7	85.2	77.1	84.2	89.0	83.1	80.6	86.6	81.9	79.8	87.7	88.7	3.45	0.448	0.418	0.676
K	0.158	0.153	0.146	0.141	0.158	0.142	0.191	0.149	0.169	0.161	0.174	0.172	0.023	0.152	0.747	0.006

[1]Means were obtained from 8 replicate cages of 6 birds per replicate cage

[2]Phytase matrix effect only significant (P < 0.05) for Na, Mn and Zn; Ca:tP effect only significant for Mn and K; Phytase effect significant (P < 0.05) for all except K; The three-way interaction was not significant for any nutrient

[3]M = nutrient matrix for phytase; Ph = phytase

Table 5. Simple effects means for total tract nutrient retention response to varying levels of phytase supplementation, dietary Ca:total P broiler diets with or without nutrient matrix values for phytase[1]

Diet	1	2	3	4	5	6	7	8	9	10	11	12		P-values for interactions[2,3]		
Ca:tP			2:1							2.5:1						
													SEM	Ca:tP × Ph	Ca:tP × M	M × Ph
M[3]	Positive Control			Negative Control			Positive Control			Negative Control						
Ph[3]	0	1000	2000	0	1000	2000	0	1000	2000	0	1000	2000				
DM	71.0	68.8	71.4	67.5	66.8	70.2	69.1	67.5	66.2	68.8	70.1	67.2	0.581	0.001	0.020	0.001
AME	3,321	3,557	3,535	3,516	3,469	3,458	3,515	3,540	3,519	3,514	3,453	3,455	5.81	0.001	0.001	0.001
EE	91.7	90.4	90.0	86.7	86.5	86.4	90.6	88.7	89.7	89.0	90.3	88.4	0.636	0.001	0.063	0.876
AHF	89.5	84.4	87.2	83.4	81.8	83.3	86.1	85.0	84.9	85.9	87.8	83.5	0.818	0.001	0.014	0.001
N	63.7	57.7	65.2	53.0	54.8	58.3	62.8	58.2	60.0	56.8	59.0	57.3	1.07	0.001	0.001	0.001

[1]Means were obtained from 8 replicate cages of 6 birds per replicate cage

[2]Matrix (M) effect was significant for all nutrients except DM; Ca:tP effect was significant for all nutrients except AHF and N; Phytase (Ph) effect was significant for all nutrients except DM and AME; P-values for three-way interaction was not significant for any of the nutrients

[3]M = nutrient matrix for phytase; Ph = phytase; EE = crude fat; AHF =acid hydrolysed fat

Table 6. Simple effects means for total tract retention response of calcium, total and phytate phosphorus to varying levels of phytase supplementation, dietary Ca:total P broiler diets with or without nutrient matrix values for phytase[1]

Diet	1	2	3	4	5	6	7	8	9	10	11	12		P-values for interactions[2,3]		
Ca:tP			2:1						2.5:1							
													SEM	Ca:tP × Ph	Ca:tP × M	M × Ph
M[3]	Positive Control			Negative Control			Positive Control			Negative Control						
Ph[3]	0	1000	2000	0	1000	2000	0	1000	2000	0	1000	2000				
P	52.1	49.6	56.8	56.1	61.1	69.1	42.4	46.5	46.5	42.5	54.6	52.5	1.32	< 0.001	0.004	$<.0001$
PP[3]	68.4	68.6	68.6	63.6	60.7	71.8	64.7	62.7	58.5	62.9	66.8	67.2	2.41	0.018	0.019	0.096
Ca	39.9	36.5	42.1	42.3	38.3	46.4	25.2	22.1	22.0	17.8	25.2	16.1	1.59	< 0.001	0.002	0.067

[1]Means were obtained from 8 replicate cages of 6 birds per replicate cage

[2]Phytase matrix effect only significant for P; Ca:tP effect significant for all the nutrients; Phytase effect only significant for P only; P-values for three-way interaction was not significant for any of the nutrients

[3]M = nutrient matrix for phytase; Ph = phytase; PP = phytate phosphorus