

1                                   INTESTINAL PHYTASE ACTIVITY IN YOUNG BROILERS

2    **Contribution of intestinal and cereal derived phytase activity on phytate degradation in**  
3                                   **young broilers**

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## ABSTRACT

18 There is little consensus as to the capability of poultry to utilize dietary phytate without  
19 supplemental phytase. Therefore, an experiment was conducted to examine the extent to which  
20 endogenous phytase of intestinal and cereal origin contributes to phytate degradation in birds  
21 aged 0-14 d post hatch. Ross 308 broilers (n=720) were fed one of four experimental diets with  
22 differing dietary ingredient combinations and approximate total phytate levels of 10 g/kg,  
23 dietary phytase activity analyzed at 460 U/kg, dietary calcium (Ca) levels of 11 g/kg and non-  
24 phytate-phosphorus levels of 4 g/kg. Broiler performance, gizzard, duodenum, jejunum and  
25 ileum pH, Ca and phosphorus (P) digestibility and solubility and amount of dietary phytate  
26 hydrolyzed in the gizzard, jejunum and ileum Ca and P digestibility and ileal digesta phytase  
27 activity were analyzed at d 4, 6, 8, 10, 12 and 14 post hatch. Intestinal endogenous phytase  
28 activity increased significantly ( $P < 0.001$ ) between d4 and d6, resulting in increased phytate  
29 hydrolysis in the gizzard ( $P = 0.003$ ), jejunum ( $P < 0.001$ ) and ileum ( $P < 0.001$ ). Phytase  
30 activity and phytate hydrolysis continued to increase with age, with a greater phytase activity  
31 and associated increase in phytate hydrolysis and mineral utilization between d 10 and 12.  
32 Gizzard and jejunum Ca and P solubility and ileal Ca and P digestibility increased significantly  
33 ( $P < 0.001$ ), and gastrointestinal pH decreased significantly ( $P < 0.001$ ) between d 4 and 6. By  
34 d 14, phytase activity recovered in the ileum was approximately 45 U/kg. There were strong  
35 correlations between phytase activity measured in the ileum and phytate hydrolyzed in the  
36 gizzard ( $r = 0.905$ ,  $P < 0.001$ ), jejunum ( $r = 0.901$ ,  $P = 0.023$ ) and ileum ( $r = 0.938$ ,  $P = 0.042$ ).  
37 This study shows intestinal and dietary derived endogenous phytase activity is responsible for  
38 phytate-P hydrolysis in broilers.

39 **Key words:** broiler, endogenous phytase, mineral digestibility, pH

## INTRODUCTION

40

41           It has been well documented that phytate-phosphorus (**P**) is largely unavailable for  
42 utilization due to a lack of effective phytase from either intestinal bacteria and mucosa or from  
43 dietary cereals themselves, commonly collectively referred to as endogenous phytase  
44 (Cowieson et al., 2006). Phytase and phosphatase activity has however been detected in the  
45 intestinal mucosa, predominantly in the duodenum (Maenz and Classen, 1998), and in the liver  
46 and blood (Cowieson et al., 2011). Its effects are thought to be negligible because of poor  
47 solubility of phytate in the small intestine, largely due to high luminal cation, particularly  
48 calcium (**Ca**), concentration coupled with a relatively high pH, but there is very little  
49 information in the literature about its precise contribution to phytate hydrolysis. Estimations of  
50 total phytase activity in the gastrointestinal tract of poultry may therefore be flawed when based  
51 on exogenous supplemental phytase alone, as such measures do not account for background  
52 interference caused by presence of intestinal and dietary endogenous phytase (Yu et al., 2004).

53           Intestinal endogenous phytase presence and activity has been shown to increase with  
54 bird age (Marounek et al., 2010), potentially due to increased gut maturity and greater small  
55 intestine mucosal surface area. There is little consensus among studies as to the capability of  
56 poultry to utilize dietary phytate without supplemental phytase. For example in broilers,  
57 Mohammed et al. (1991) found that phytate digestibility ranged from 32-54%, whereas  
58 Edwards (1993) found it ranged from 56-63% and Applegate et al. (2003), Leske and Coon  
59 (2002) and Plumstead et al. (2008) identified apparent ileal phytate hydrolysis of approximately  
60 40%, 32% and 20% respectively. In these studies there was no differentiation between dietary  
61 supplemented phytase and phytase from cereal or intestinal origin.

62           The aim of this study was to investigate the contribution of endogenous phytase derived  
63 from cereals and the intestinal tract to degradation of phytate in young broilers. A two factorial

64 design study was run to examine the extent to which intestinal and dietary endogenous phytase  
65 enhances phytate degradation between age d 0-14 post-hatch, in birds fed a variety of  
66 commercial based diet combinations to produce differing gastrointestinal environments.

## 67 **MATERIALS AND METHODS**

### 68 ***Birds and Husbandry***

69 Ross 308, male broilers (n = 720) from a 43-week-old breeder flock were obtained from a  
70 commercial hatchery at day of hatch. Chicks were randomized by weight and placed in 0.64  
71 m<sup>2</sup> floor pens in groups of 15, bedded on clean wood shavings. Two pens were considered as  
72 one replicate, classified as a plot. Twenty seven birds per plot were sampled in the study, with  
73 three spare birds per plot. Birds were allowed ad libitum access to the treatment diets and water  
74 for the duration of the trial. The room was thermostatically controlled to produce an initial  
75 temperature of 32°C on d1 and reduced in steps of 0.5°C per d, reaching 21°C by day 14. The  
76 lighting regimen used was 24 hours light on d 1, with darkness increasing by 1 hour a day until  
77 6 hours of darkness was reached, which was maintained throughout the remainder of the study.  
78 All birds sampled were euthanized by cervical dislocation. This occurred at the same time each  
79 sampling day; after at least 6 hours of light, to ensure maximal gut fill. Institutional and national  
80 guidelines for the care and use of animals were followed and all experimental procedures  
81 involving animals were approved by the University's College of Science ethical review  
82 committee.

### 83 ***Dietary Treatments***

84 Experimental diets were formulated to be as nutritionally similar as possible but with  
85 different combinations of plant-based feed ingredients. Each diet had approximate total phytate  
86 levels of 10 g/kg, dietary phytase activity analyzed at 460 U/kg, dietary Ca levels of 11 g/kg  
87 and non-phytate-P levels of 4 g/kg (as dicalcium phosphate and limestone) (Table 1). There  
88 were four treatments with each treatment replicated by 12 pens of 15 birds each (n=6 plots of

89 30 birds, 180 birds/dietary treatment). The treatments were a combination of ground corn, soy  
90 meal, ground wheat, rice bran meal, ground rye or rapeseed meal and designed by their grain  
91 type. For example, diet one contained 20% rapeseed (Rapeseed), diet two contained 8% rice  
92 bran (Rice Bran), diet three contained 30% wheat (Wheat) and diet four contained 7.5% rye  
93 (Rye; Table 1). Diets were fed in mash form, mixed in house, and were analyzed for gross  
94 energy by bomb calorimetry (Robbins and Firman, 2006), dry matter and protein content  
95 (calculated as nitrogen multiplied by 6.25) by the AOAC standard methods (930.15 and 990.03,  
96 respectively). Phosphorus and Ca content of the diets were analyzed by inductively coupled  
97 plasma-optical emission spectroscopy (ICP-OES) following an aqua regia digestion step  
98 (AOAC 985.01, Leytem et al. 2006). Titanium dioxide was added at a rate of 0.5% to act as an  
99 inert marker for evaluation of ileal Ca and P digestibility and dietary phytate hydrolyzed and  
100 the dietary content quantified by ICP-OES following aqua regia digestion (Morgan et al., 2014).  
101 Total phytate content was analyzed by a K-Phyt assay kit (Megazyme™, Wicklow, Ireland,  
102 UK). This assay quantitatively measured available phosphorus released from the samples.  
103 Briefly, inositol phosphates were acid extracted followed by treatment with a phytase specific  
104 for IP<sub>6</sub>-IP<sub>2</sub> and then treatment with alkaline phosphate to ensure release of the final phosphate  
105 from myo-inositol phosphate (IP<sub>1</sub>). The total phosphate released was measured using a  
106 modified colorimetric method and given as grams of phosphorus per 100 g of sample material.  
107 Phytase activity was analyzed according to the method of Engelen et al. (2001). Calculated and  
108 analyzed values for each diet are shown in Table 1.

### 109 ***Response Variables***

110 On arrival birds were individually weighed and allocated to a pen. Pen allocation was  
111 randomized across the room. Total pen weight and mean chick body weight (**BW**) were  
112 calculated, and diet allocation was arranged to ensure there was no significant difference in

113 BW by pen across diets. Total pen weight and feed intake (**FI**) was determined on d 4, 6, 8, 10,  
114 12 and 14 post-hatch and was used to calculate feed conversion ratio (**FCR**).

115         Sampling was carried out at the same time each sampling day. On d 4, 10 birds per plot  
116 (5 birds from each pen per plot) were euthanized, on d 6, 5 birds per plot were euthanized (3  
117 birds from one pen and 2 from the other pen per plot), and on d 8, 10, 12 and 14, 3 birds per  
118 plot were euthanized (2 birds from one pen and 1 from the other pen per plot). The pen weight  
119 and intake was divided by the number of birds in the pen to determine individual bird BW and  
120 FI. Mortality was recorded daily, and any birds culled or dead were weighed. FCR was  
121 corrected by mortality.

122         Immediately post-euthanasia, two of the euthanized birds per plot, one per pen, were  
123 individually weighed and marked with a colored pen for identification purposes. Gizzard,  
124 duodenum, jejunum and ileum pH of these two birds was determined by inserting a spear tip  
125 piercing pH electrode (Sensorex, California, USA) with digital pH meter (Mettler-Toledo, UK)  
126 directly into the digesta in the gut lumen as soon as they had been excised. Readings were  
127 repeated three times per section of gut per bird (ensuring the probe did not touch the gut wall)  
128 and average pH was calculated. Gizzard, jejunum and ileum digesta contents from all the birds  
129 sampled per plot were then collected by gentle digital pressure into one pot per section of tract  
130 per plot, and stored at -20°C prior to freeze drying. Once freeze dried the samples were ground  
131 to a fine powder with a pestle and mortar. Titanium dioxide content of the digesta was  
132 determined by ICP-OES following aqua regia digestion as previously discussed for the diets.

133         For each plot, total and soluble Ca and P and phytate content was determined in the  
134 freeze-dried gizzard, jejunum and ileum digesta. Total Ca and P was determined by ICP-OES  
135 following aqua regia digestion as discussed previously. Soluble Ca and P was determined by  
136 mixing the samples with ultra-pure water and centrifuging before measuring Ca and P content  
137 of the supernatant by ICP-OES. Solubility coefficients were obtained using this equation:

138  $(\text{Mineral (g/kg DM)})_{\text{digesta supernatant}}/(\text{Mineral (g/kg DM)})_{\text{diet}}$ .

139 Apparent ileal Ca and P digestibility coefficients were obtained using this equation:

140  $[(\text{nutrient/TiO}_2 \text{ (g/kg DM)})_{\text{diet}} - (\text{nutrient/TiO}_2 \text{ (g/kg DM)})_{\text{ileum digesta}}]/(\text{nutrient/TiO}_2 \text{ (g/kg}$   
141  $\text{DM)})_{\text{diet}}$

142 Total phytate content of the gizzard, jejunum and ileum digesta samples was analyzed by a

143 K-Phyt assay as previously discussed and the amount of dietary phytate hydrolyzed was

144 calculated using the equation:

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

147 Total phytase activity in the ileal digesta samples was analyzed in triplicate according to the

148 method of Engelen et al. (2001).

#### 149 *Data Analysis*

150 All data were analyzed using IBM SPSS statistics version 21. After Kolmogorov–  
151 Smirnov testing to confirm normality, ANOVA was conducted to determine 2-way interactions  
152 between bird age and diet. When means were significantly different, Duncan post-hoc tests  
153 were conducted to differentiate between them. Multiple comparison tests between treatments  
154 were conducted when there were significant interactions. Correlations between measured  
155 factors were analyzed by bivariate correlation using Pearson product- moment correlation  
156 coefficient. Interpretations of the strength of the relationships between the factors were based  
157 on guidelines by Cohen (1988); weak relationship  $r = 0.10$  to  $0.29$ , medium relationship  $r =$   
158  $0.30$  to  $0.49$  and strong relationship  $r = 0.50$  to  $1.0$ . Statistical significance was declared at  $P$   
159  $< 0.05$ .

## 160 **RESULTS AND DISCUSSION**

161 The analyzed dietary Ca, P and phytate were within acceptable ranges and in agreement  
162 with formulated values when mixing and assay variation were considered (Table 1). Intestinal

163 endogenous phytase activity increased significantly ( $P < 0.001$ ) between d4 and d6, resulting  
164 in increased phytate hydrolysis in the gizzard ( $P = 0.003$ ), jejunum ( $P < 0.001$ ) and ileum ( $P$   
165  $< 0.001$ ). Intestinal endogenous phytase activity increased gradually with bird age, and was  
166 associated with increased phytate hydrolysis ( $P < 0.001$ ) and mineral utilization ( $P = 0.001$  and  
167  $P < 0.001$  for Ca and P respectively) between d 10 and d 12 (Table 2). There were strong  
168 correlations between phytase activity measured in the ileum and phytate hydrolyzed in the  
169 gizzard ( $r = 0.905$ ,  $P < 0.001$ ), jejunum ( $r = 0.901$ ,  $P = 0.023$ ) and ileum ( $r = 0.938$ ,  $P = 0.042$ ),  
170 suggesting that endogenous intestinal and dietary derived phytase activity is responsible for  
171 phytate-P hydrolysis.

172 Strong correlations between ileal phytase activity and P solubility (Table 6) in the  
173 gizzard and ileum ( $r = 0.989$ ,  $P < 0.001$  and  $r = 0.921$ ,  $P < 0.001$  respectively) potentially  
174 illustrate that as the intestinal endogenous phytase levels increased substantially more phytate  
175 was hydrolyzed, resulting in increased P solubility and digestibility (Table 4 and 6). This study,  
176 and a study conducted by Zeller *et al.* (2015), showed that phytate is hydrolyzed in the small  
177 intestine as well as in the gizzard, and the efficacy of intestinal phytase is dictated by conditions  
178 in both the gastric and intestinal phases. In this study, it is however possible that phytase further  
179 hydrolyzed phosphate during the extraction process for analysis of soluble Ca and P, as phytase  
180 activity was not inhibited during extraction and centrifugation. This means that the observed  
181 correlation between phytase activity and P solubility may be because there was greater release  
182 of phosphate from samples with higher phytase activity during sample extraction.

183 The onset of intestinal endogenous phytase activity (Table 2) likely instigated the  
184 observed increase in Ca ( $P < 0.001$ ) and P ( $P = 0.003$ ) utilization and FI ( $P < 0.001$ ) between  
185 d4 and d6. This may also be representative of a combination of the shift to dependence on  
186 dietary intake for nutrients, as supplies from the yolk last only 4-5 days (Selle *et al.*, 1991), and  
187 due to increased gut maturity. The transition to nutrient supplies from feed causes increased



188 intestinal weight and hence increased intestinal brush border surface area and heightened  
189 intestinal phytase production (Maenz and Classen, 1998). This increase in intestinal weight  
190 occurs more rapidly than the body mass of the whole bird (Sklan, 2001). Intestinal phytase  
191 activity may be subject to regulation in response to dietary P status of the bird, as illustrated by  
192 strong correlations between ileal phytase activity and P solubility in the gizzard and ileum  
193 ( $r=0.989$ ,  $p<0.001$  and  $r=0.921$ ,  $p<0.001$  respectively). At d 10 villus volume in the jejunum  
194 and ileum reaches its peak (Noy and Sklan, 1997). This potentially explains why phytate  
195 hydrolysis in the jejunum increased significantly between d10 and d12 post-hatch (Table 2)  
196 and feed intake and BWG increased significantly between d8 to d10 (Table 3), as there was  
197 increased intestinal brush border surface area and nutrient absorption in relation to bird size.  
198 Birds younger than d4 post-hatch were not analyzed in this study due to the large number  
199 required to obtain sufficient digesta.

200 Mucosal alkaline phosphatase is not secreted into the gut lumen until approximately  
201 d5 (Sabatakou et al., 2007); the pH optimum for alkaline phosphatase lowers as the  
202 concentration of phytate lowers, due to presence of phytase. This partly explains why apparent  
203 ileal digestibility of Ca (Table 5) increased between d4 and d6. There were no significant  
204 correlations between ileal phytase activity or phytate hydrolysis and pH in any section of the  
205 tract measured. Duodenum, jejunum and ileum pH was however lowest at d6 and closest to the  
206 optimum for phytase activity in the small intestine (pH 5.5-6) (Maenz and Classen, 1998). It is  
207 possible that the onset of intestinal phytase activity caused this pH reduction by increasing Ca  
208 absorption and reducing the amount of Ca was present in the gut lumen to influence pH, as  
209 shown by correlations between gizzard Ca solubility and jejunum and ileum pH ( $r=0.251$ ,  
210  $p=0.025$  and  $r=0.283$ ,  $p<0.001$  respectively) at this age. From d 8 onwards pH increased  
211 possibly because there was increased dietary limestone ingested with increased feed intake.  
212 The decrease in gizzard pH between d8 and d10 in birds fed the rapeseed diet may suggest

213 selective Ca consumption of the mash diets or modified diet consumption based on Ca  
214 requirements (Wilkinson et al., 2011). The significant decrease in gizzard pH observed between  
215 d 4 and 6 in all diets coincides with an increase in phytate hydrolysis (Table 2) and Ca  
216 digestibility (Table 5), which is surprising since the pH optimum of cereal (Afify et al., 2011),  
217 mucosal (Angel et al., 2002) and bacterial (Elkhalil et al., 2007) phytases is considerably higher  
218 than this. The lower pH at d 6 may have played a role in keeping more phytate soluble such  
219 that there was more substrate for the enzyme to attack. The lack of diet effect observed at d 4  
220 on pH in the gizzard is likely due to the very small quantities of feed consumed (Table 7).

221 By d 14, ileal phytase activity levels were approximately 45 U/kg (Table 2). Further  
222 investigation is however required to differentiate between how much of this was intestinal  
223 endogenous phytase or dietary endogenous phytase activity, and to assess the sensitivity of the  
224 phytase assay used for analysis of the digesta samples. Ileal Ca digestibility was higher at d 14  
225 compared to younger birds and there were strong correlations between ileal phytase activity  
226 and Ca and P solubility in both the gizzard ( $r = 0.698$ ,  $P < 0.001$  and  $r = 0.888$ ,  $P = 0.002$   
227 respectively) and jejunum ( $r = 0.288$ ,  $P < 0.001$  and  $r = 0.281$ ,  $P < 0.001$  respectively) at this  
228 age, illustrating the impact that intestinal and dietary endogenous phytase had on mineral  
229 utilization (Table 4 and 5). It is likely that the phytase produced in the intestine alone is  
230 responsible for these findings as dietary phytase levels remained constant per gram with bird  
231 age. The observed significant decrease in gizzard pH of birds fed the rapeseed and rice bran  
232 diets at d 14 compared to birds fed the wheat and rye diets may be because these diets had  
233 lower limestone content, so the buffering capacity was comparatively lower.

234 Feed conversion and BWG were highest in birds fed the rice bran diet which may be  
235 due to its higher protein content and because it had the highest P content which may have  
236 increased skeletal weight. P digestibility was highest in birds fed the rapeseed meal diet at bird  
237 age d4, 6, 10 and 14 possibly because it had a Ca:P ratio of 1.49:1 which was the closest to the

238 optimum of 1.5:1 for P digestibility (Mitchell and Edwards, 1996) and gastrointestinal pH in  
239 all sections of the tract was lowest in birds fed this diet, suggesting the phytate-complexes were  
240 more soluble and ternary phytate-protein-mineral complexes were less likely to form (Table 1).

241 It can be concluded that endogenous phytase activity is quantifiable in the intestine  
242 and seems to be correlated with phytate-P hydrolysis and Ca and P digestibility. Ileal phytase  
243 activity continues to increase with age to d 14, reaching approximately 45 U/kg, with associated  
244 increased phytate hydrolysis in the terminal ileum. Further investigation is required to  
245 determine the extent of the impact of intestinal and dietary endogenous phytase in older birds.

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**Table 1.** Composition and nutrient content of experimental diets

Item	Rapeseed	Rice Bran	Wheat	Rye
Ingredient, %				
Maize	51	53	27	54
Rye			5	8
Wheat			30	
Rapeseed Extruded	20	5		
Soybean meal 46	18	26	29	30
Rice Bran	1.50	8.00	0.00	1.00
Soy oil	4.49	2.62	3.71	2.57
Salt	0.46	0.46	0.44	0.46
Valine	0.10	0.10	0.10	0.10
DL Methionine	0.26	0.33	0.36	0.36
Lysine HCl	0.38	0.35	0.36	0.36
Threonine	0.09	0.11	0.14	0.13
L-Arginine HCl	0.18	0.09	0.11	0.11
Isoleucine	0.11	0.08	0.08	0.08
Limestone	0.73	0.63	1.07	0.94
Dicalcium Phos	1.78	1.84	1.74	1.91
Coccidiostat (Coban - monensin)	0.02	0.02	0.02	0.02
Vitamin premix*	0.40	0.40	0.40	0.40
Titanium Dioxide	0.50	0.50	0.50	0.50
Calculated composition				
CP, %	20.70	20.65	20.80	20.47
Gross energy, kcal/kg	4,660	4,660	4,660	4,660
Total P, %	0.84	0.88	0.74	0.77
Total Ca, %	1.00	1.00	1.00	1.00
Lys, %	1.33	1.33	1.33	1.33
Met, %	0.61	0.65	0.66	0.67
Total sulphur amino acids %	0.88	0.88	0.89	0.89
Sodium, %	0.20	0.20	0.20	0.20
Potassium, %	0.79	0.91	0.86	0.87
Chloride, %	0.39	0.39	0.38	0.38
Phytate, %	1.12	1.12	0.82	0.88
Phytate-P g/kg	3.16	3.16	2.31	2.48
Analyzed composition				
CP, %	19.95	21.64	21.42	20.01
Gross energy, kcal/kg	4,535	4,619	4,662	4,581
Total P, %	0.80	0.81	0.75	0.72
Total Ca, %	1.20	1.18	1.15	1.02
Phytate %	1.20	1.06	0.99	0.86
Phytate-P g/kg	3.38	2.99	2.79	2.43
Endogenous phytase, U/kg	407	396	470	472

333 <sup>1</sup>Supplied per kilogram of diet: manganese, 100 mg; zinc, 80 mg; iron (ferrous sulphate),  
334 20 mg; copper, 10 mg; iodine, 1 mg; molybdenum, 0.48 mg; selenium, 0.25 mg; retinol,  
335 13.5 mg; cholecalciferol, 3 mg; tocopherol, 25 mg; menadione, 5.0 mg; thiamine, 3 mg;

336 riboflavin, 10 mg; pantothenic acid, 15 mg; pyroxidine, 3.0 mg; niacin, 60 mg; cobalamin,  
337 30 µg; folic acid, 1.5 mg; vitamin E, 100mg; vitamin A, 13.5mg; vitamin D3, 5mg;  
338 vitamin B1, 3mg; vitamin B2, 10mg; vitamin B6, 3mg; vitamin B12, 30mg; and biotin,  
339 125 mg.

340 **Table 2.** Influence of bird age on dietary phytate hydrolyzed (g/kg DM) by the gizzard,  
 341 jejunum and ileum and phytase activity (U/kg) in the ileum in broilers from d 0 to 14

Age, d	Dietary phytate hydrolyzed (g/kg DM)			Ileal phytase activity (U/kg)
	Gizzard	Jejunum	Ileum	
4 <sup>1</sup>	1.20 <sup>c</sup>	2.00 <sup>c</sup>	3.12 <sup>d</sup>	22 <sup>e</sup>
6 <sup>2</sup>	1.33 <sup>b</sup>	3.21 <sup>b</sup>	3.39 <sup>c</sup>	38 <sup>d</sup>
8 <sup>3</sup>	1.44 <sup>b</sup>	3.20 <sup>b</sup>	3.46 <sup>c</sup>	40 <sup>cd</sup>
10 <sup>3</sup>	1.46 <sup>b</sup>	3.32 <sup>b</sup>	3.61 <sup>bc</sup>	41 <sup>bc</sup>
12 <sup>3</sup>	1.51 <sup>a</sup>	3.41 <sup>a</sup>	3.79 <sup>ab</sup>	43 <sup>ab</sup>
14 <sup>3</sup>	1.52 <sup>a</sup>	3.47 <sup>a</sup>	3.96 <sup>a</sup>	44 <sup>a</sup>
SEM	0.04	0.21	0.11	1.68
P-values				
Age	0.003	<0.001	<0.001	<0.001
Diet	0.846	0.544	0.786	0.770
Age x Diet	0.059	0.070	0.690	0.759

342 <sup>a-c</sup> Means within the same column with no common superscript differ significantly (P ≤  
 343 0.05). 2-way ANOVA and Duncan Post-Hoc test were used to differentiate between  
 344 means.

345 <sup>1</sup>Means represent the average response of 12 pens, 6 plots (120 birds/ treatment).

346 <sup>2</sup>Means represent the average response of 12 pens, 6 plots (60 birds/ treatment).

347 <sup>3</sup>Means represent the average response of 12 pens, 6 plots (36 birds/ treatment).



348 **Table 3.** Influence of bird age and diet on growth performance of broilers from d 0 to 14

Age, d	Feed intake, g	Individual BW gain, g	FCR <sup>4</sup>
0-4 <sup>1</sup>	40 <sup>g</sup>	32 <sup>h</sup>	1.24 <sup>c</sup>
4-6 <sup>2</sup>	54 <sup>f</sup>	38 <sup>gh</sup>	1.41 <sup>ab</sup>
6-8 <sup>3</sup>	53 <sup>f</sup>	45 <sup>g</sup>	1.19 <sup>c</sup>
8-10 <sup>3</sup>	81 <sup>e</sup>	60 <sup>f</sup>	1.35 <sup>b</sup>
10-12 <sup>3</sup>	91 <sup>e</sup>	63 <sup>f</sup>	1.45 <sup>a</sup>
12-14 <sup>3</sup>	111 <sup>d</sup>	82 <sup>e</sup>	1.35 <sup>b</sup>
SEM	9.27	6.35	0.04
Diet			
Rapeseed	422 <sup>ab</sup>	301 <sup>b</sup>	1.36 <sup>b</sup>
Rice Bran	448 <sup>a</sup>	354 <sup>a</sup>	1.27 <sup>c</sup>
Wheat	439 <sup>a</sup>	311 <sup>b</sup>	1.41 <sup>a</sup>
Rye	408 <sup>b</sup>	310 <sup>b</sup>	1.36 <sup>b</sup>
SEM	5.33	10.21	0.03
<i>P</i> -value			
Diet	0.013	<0.001	0.003
Age	<0.001	<0.001	0.017
Diet x age	0.642	0.662	0.394

349 <sup>a-c</sup> Means within the same column with no common superscript differ significantly ( $P \leq$   
350 0.05). 2-way ANOVA and Duncan Post-Hoc test were used to differentiate between means.

351 <sup>1</sup>Means represent the average response of 12 pens, 6 plots (120 birds/ treatment).

352 <sup>2</sup>Means represent the average response of 12 pens, 6 plots (60 birds/ treatment).

353 <sup>3</sup>Means represent the average response of 12 pens, 6 plots (36 birds/ treatment).

354 <sup>4</sup>FCR = feed conversion ratio, corrected for mortality.

355 **Table 4.** Influence of bird age and diet on apparent ileal  
 356 P digestibility<sup>1</sup> in broilers from d 0 to 14

Age, d	P Digestibility			
	Rapeseed	Rice Bran	Wheat	Rye
4 <sup>2</sup>	0.67 <sup>c</sup>	0.63 <sup>c</sup>	0.64 <sup>c</sup>	0.66 <sup>c</sup>
6 <sup>3</sup>	0.76 <sup>b</sup>	0.73 <sup>b</sup>	0.73 <sup>b</sup>	0.72 <sup>bc</sup>
8 <sup>4</sup>	0.76 <sup>b</sup>	0.76 <sup>b</sup>	0.76 <sup>b</sup>	0.77 <sup>b</sup>
10 <sup>4</sup>	0.82 <sup>a</sup>	0.75 <sup>b</sup>	0.77 <sup>b</sup>	0.70 <sup>b</sup>
12 <sup>4</sup>	0.79 <sup>ab</sup>	0.82 <sup>a</sup>	0.71 <sup>b</sup>	0.69 <sup>b</sup>
14 <sup>4</sup>	0.79 <sup>ab</sup>	0.77 <sup>ab</sup>	0.74 <sup>b</sup>	0.73 <sup>b</sup>
SEM	0.02	0.02	0.05	0.01
<i>P</i> -value				
Age	<0.001			
Diet	0.017			
Diet x Age	0.001			

357 <sup>a-c</sup> Means within the same column and same row with  
 358 no common superscript differ significantly ( $P \leq 0.05$ ).  
 359 2-way ANOVA and Duncan Post-Hoc test were used to  
 360 differentiate between means.  
 361 <sup>1</sup>Digestibility coefficients obtained using the equation:  
 362  $[(\text{nutrient}/\text{TiO}_2)_{\text{diet}} -$   
 363  $(\text{nutrient}/\text{TiO}_2)_{\text{ileum}}]/(\text{nutrient}/\text{TiO}_2)_{\text{diet}}$ .  
 364 <sup>2</sup>Means represent the average response of 12 pens, 6  
 365 plots (120 birds/ treatment).  
 366 <sup>3</sup>Means represent the average response of 12 pens, 6  
 367 plots (60 birds/ treatment).  
 368 <sup>4</sup>Means represent the average response of 12 pens, 6  
 369 plots (36 birds/ treatment).

370 **Table 5.** Influence of bird age on apparent ileal Ca  
 371 digestibility<sup>1</sup> in broilers from d 0 to 14

Age, d	Ca Digestibility	
4 <sup>2</sup>	0.56 <sup>c</sup>	373
6 <sup>3</sup>	0.63 <sup>b</sup>	374
8 <sup>4</sup>	0.61 <sup>bc</sup>	375
10 <sup>4</sup>	0.62 <sup>b</sup>	376
12 <sup>4</sup>	0.67 <sup>b</sup>	
14 <sup>4</sup>	0.72 <sup>a</sup>	377
SEM	0.02	
<i>P</i> -value		378
Age	0.001	379
Diet	0.209	
Diet x Age	0.617	380

381 <sup>a-c</sup> Means within the same column with no  
 382 common superscript differ significantly ( $P \leq 0.05$ ).  
 383 2-way ANOVA and Duncan Post-Hoc test were  
 384 used to differentiate between means.

385 <sup>1</sup>Digestibility coefficients obtained using the  
 386 equation:

$$387 \frac{[(\text{nutrient}/\text{TiO}_2)_{\text{diet}} - (\text{nutrient}/\text{TiO}_2)_{\text{ileum}}]}{(\text{nutrient}/\text{TiO}_2)_{\text{diet}}}$$

388 <sup>2</sup>Means represent the average response of 12 pens,  
 389 6 plots (120 birds/ treatment).

391 <sup>3</sup>Means represent the average response of 12 pens,  
 392 6 plots (60 birds/ treatment).

393 <sup>4</sup>Means represent the average response of 12 pens,  
 394 6 plots (36 birds/ treatment).

395 **Table 6.** Influence of bird age on Ca and P solubility coefficients<sup>1</sup> in the  
 396 gizzard, jejunum and ileum

Age, d	Gizzard		Jejunum		Ileum	
	Ca	P	Ca	P	Ca	P
4 <sup>2</sup>	0.41 <sup>b</sup>	0.38 <sup>b</sup>	0.22 <sup>b</sup>	0.27 <sup>c</sup>	0.14	0.19
6 <sup>3</sup>	0.61 <sup>a</sup>	0.51 <sup>a</sup>	0.29 <sup>a</sup>	0.33 <sup>b</sup>	0.15	0.21
8 <sup>4</sup>	0.55 <sup>a</sup>	0.51 <sup>a</sup>	0.30 <sup>a</sup>	0.34 <sup>ab</sup>	0.16	0.23
10 <sup>4</sup>	0.60 <sup>a</sup>	0.51 <sup>a</sup>	0.30 <sup>a</sup>	0.36 <sup>ab</sup>	0.17	0.23
12 <sup>4</sup>	0.57 <sup>a</sup>	0.52 <sup>a</sup>	0.30 <sup>a</sup>	0.38 <sup>ab</sup>	0.17	0.23
14 <sup>4</sup>	0.61 <sup>a</sup>	0.55 <sup>a</sup>	0.32 <sup>a</sup>	0.39 <sup>a</sup>	0.18	0.25
SEM	0.03	0.02	0.01	0.02	0.01	0.01
<i>P</i> -value						
Age	<0.001	<0.001	<0.001	<0.001	0.576	0.574
Diet	0.945	0.368	0.410	0.146	0.258	0.281
Diet x Age	0.962	0.981	0.843	0.720	0.951	0.224

397 <sup>a-c</sup> Means within the same column with no common superscript differ  
 398 significantly ( $P \leq 0.05$ ). 2-way ANOVA and Duncan Post-Hoc test were  
 399 used to differentiate between means.

400 <sup>1</sup>Solubility coefficients obtained using the equation:

401  $(\text{Mineral})_{\text{supernatant}}/(\text{Mineral})_{\text{diet}}$ .

402 <sup>2</sup>Means represent the average response of 12 pens, 6 plots (120 birds/  
 403 treatment).

404 <sup>3</sup>Means represent the average response of 12 pens, 6 plots (60 birds/  
 405 treatment).

406 <sup>4</sup>Means represent the average response of 12 pens, 6 plots (36 birds/  
 407 treatment)

408 **Table 7.** Influence of bird age and diet on gizzard pH in broilers<sup>1</sup>  
 409 from d 0 to 14

Diet	Rapeseed	Rice Bran	Wheat	Rye
Age, d				
4	2.91 <sup>a</sup>	2.90 <sup>a</sup>	2.74 <sup>a</sup>	2.75 <sup>a</sup>
6	2.38 <sup>b</sup>	2.50 <sup>b</sup>	2.69 <sup>b</sup>	2.42 <sup>b</sup>
8	2.74 <sup>a</sup>	2.94 <sup>a</sup>	2.61 <sup>b</sup>	3.01 <sup>a</sup>
10	2.59 <sup>b</sup>	2.93 <sup>a</sup>	2.58 <sup>b</sup>	2.81 <sup>a</sup>
12	2.62 <sup>ab</sup>	2.78 <sup>a</sup>	2.85 <sup>a</sup>	2.33 <sup>b</sup>
14	1.99 <sup>c</sup>	1.67 <sup>c</sup>	2.48 <sup>b</sup>	2.30 <sup>b</sup>
SEM	0.12	0.18	0.05	0.11
<i>P</i> -value				
Age			0.025	
Diet			0.046	
Diet x Age			0.037	

410 <sup>a-c</sup> Means within the same column and same row with no  
 411 common superscript differ significantly ( $P \leq 0.05$ ). 2-way  
 412 ANOVA and Duncan Post-Hoc test were used to differentiate  
 413 between means.

414 <sup>1</sup> Means represent the average response of 12 pens, 6 plots per  
 415 age (12 birds/ treatment per age)

**Table 8.** Influence of bird age on duodenum, jejunum and ileum pH in broilers<sup>1</sup> from d 0 to 14

	Duodenum	Jejunum	Ileum
Age, d			
4	6.10 <sup>a</sup>	6.03 <sup>b</sup>	6.74 <sup>b</sup>
6	5.99 <sup>b</sup>	5.90 <sup>c</sup>	6.19 <sup>c</sup>
8	6.10 <sup>a</sup>	6.13 <sup>ab</sup>	7.24 <sup>a</sup>
10	6.11 <sup>a</sup>	6.08 <sup>ab</sup>	7.33 <sup>a</sup>
12	6.08 <sup>a</sup>	6.08 <sup>ab</sup>	7.08 <sup>a</sup>
14	6.13 <sup>a</sup>	6.15 <sup>a</sup>	7.24 <sup>a</sup>
SEM	0.02	0.03	0.16
<i>P</i> -value			
Age	0.020	<0.001	<0.001
Diet	0.613	0.660	0.840
Diet x Age	0.670	0.233	0.166

<sup>a-c</sup> 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.

<sup>1</sup> Means represent the average response of 12 pens, 6 plots per age (12 birds/ treatment per age)