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

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ABSTRACT

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

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

INTRODUCTION

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

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

The aim of this study was to investigate the contribution of endogenous phytase derived 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^2 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

30 birds, 180 birds/dietary treatment). The treatments were a combination of ground corn, soy meal, ground wheat, rice bran meal, ground rye or rapeseed meal and designed by their grain type. For example, diet one contained 20% rapeseed (Rapeseed), diet two contained 8% rice bran (Rice Bran), diet three contained 30% wheat (Wheat) and diet four contained 7.5% rye (Rye; Table 1). Diets were fed in mash form, mixed in house, and were analyzed for gross energy by bomb calorimetry (Robbins and Firman, 2006), dry matter and protein content (calculated as nitrogen multiplied by 6.25) by the AOAC standard methods (930.15 and 990.03, respectively). Phosphorus and Ca content of the diets were analyzed by inductively coupled plasma-optical emission spectroscopy (ICP-OES) following an aqua regia digestion step (AOAC 985.01, Leytem et al. 2006). Titanium dioxide was added at a rate of 0.5% to act as an inert marker for evaluation of ileal Ca and P digestibility and dietary phytate hydrolyzed and the dietary content quantified by ICP-OES following aqua regia digestion (Morgan et al., 2014). Total phytate content was analyzed by a K-Phyt assay kit (Megazyme^{TM}, Wicklow, Ireland, UK). This assay quantitatively measured available phosphorus released from the samples. Briefly, inositol phosphates were acid extracted followed by treatment with a phytase specific for IP_6-IP_2 and then treatment with alkaline phosphate to ensure release of the final phosphate from myo-inositol phosphate (IP1). The total phosphate released was measured using a modified colorimetric method and given as grams of phosphorus per 100 g of sample material. Phytase activity was analyzed according to the method of Engelen et al. (2001). Calculated and analyzed values for each diet are shown in Table 1.

Response Variables

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

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

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

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

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

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

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

140 [(nutrient/TiO_2 (g/kg DM))$_{\text{diet}}$ − (nutrient/TiO_2 (g/kg DM))$_{\text{ileum digesta}}$]/(nutrient/TiO_2 (g/kg

141 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 Dietary phytate (g/kg DM)*(1-(digesta phytate (g/kg DM) *$TiO_{2\ \text{diet}}$ (g/kg DM)) / (TiO_2

146 digesta (g/kg DM)* 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

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

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

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

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

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

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

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

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

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

It can be concluded that endogenous phytase activity is quantifiable in the intestine and seems to be correlated with phytate-P hydrolysis and Ca and P digestibility. Ileal phytase activity continues to increase with age to d 14, reaching approximately 45 U/kg, with associated increased phytate hydrolysis in the terminal ileum. Further investigation is required to 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

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

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

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

	Dietary phytate hydrolyzed (g/kg DM)			Ileal phytase activity (U/kg)
Age, d	Gizzard	Jejunum	Ileum	
4[1]	1.20[c]	2.00[c]	3.12[d]	22 [e]
6[2]	1.33[b]	3.21[b]	3.39[c]	38[d]
8[3]	1.44[b]	3.20[b]	3.46[c]	40[cd]
10[3]	1.46[b]	3.32[b]	3.61[bc]	41[bc]
12[3]	1.51[a]	3.41[a]	3.79[ab]	43[ab]
14[3]	1.52[a]	3.47[a]	3.96[a]	44[a]
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

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

[1]Means represent the average response of 12 pens, 6 plots (120 birds/ treatment).

[2]Means represent the average response of 12 pens, 6 plots (60 birds/ treatment).

[3]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[4]
0-4[1]	40[g]	32[h]	1.24[c]
4-6[2]	54[f]	38[gh]	1.41[ab]
6-8[3]	53[f]	45[g]	1.19[c]
8-10[3]	81[e]	60[f]	1.35[b]
10-12[3]	91[e]	63[f]	1.45[a]
12-14[3]	111[d]	82[e]	1.35[b]
SEM	9.27	6.35	0.04
Diet			
Rapeseed	422[ab]	301[b]	1.36[b]
Rice Bran	448[a]	354[a]	1.27[c]
Wheat	439[a]	311[b]	1.41[a]
Rye	408[b]	310[b]	1.36[b]
SEM	5.33	10.21	0.03
P-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 [a–c] 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 [1]Means represent the average response of 12 pens, 6 plots (120 birds/ treatment).
352 [2]Means represent the average response of 12 pens, 6 plots (60 birds/ treatment).
353 [3]Means represent the average response of 12 pens, 6 plots (36 birds/ treatment).
354 [4]FCR = feed conversion ratio, corrected for mortality.

Table 4. Influence of bird age and diet on apparent ileal P digestibility[1] in broilers from d 0 to 14

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

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

[1]Digestibility coefficients obtained using the equation: $[(\text{nutrient}/TiO_2)_{diet} - (\text{nutrient}/TiO_2)_{ileum}]/(\text{nutrient}/TiO_2)_{diet}$.

[2]Means represent the average response of 12 pens, 6 plots (120 birds/ treatment).

[3]Means represent the average response of 12 pens, 6 plots (60 birds/ treatment).

[4]Means represent the average response of 12 pens, 6 plots (36 birds/ treatment).

Table 5. Influence of bird age on apparent ileal Ca digestibility[1] in broilers from d 0 to 14

Age, d	Ca Digestibility
4[2]	0.56[c]
6[3]	0.63[b]
8[4]	0.61[bc]
10[4]	0.62[b]
12[4]	0.67[b]
14[4]	0.72[a]
SEM	0.02
P-value	
Age	0.001
Diet	0.209
Diet x Age	0.617

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

[1]Digestibility coefficients obtained using the equation: $[(\text{nutrient}/TiO_2)_{diet} - (\text{nutrient}/TiO_2)_{ileum}]/(\text{nutrient}/TiO_2)_{diet}$.

[2]Means represent the average response of 12 pens, 6 plots (120 birds/ treatment).

[3]Means represent the average response of 12 pens, 6 plots (60 birds/ treatment).

[4]Means represent the average response of 12 pens, 6 plots (36 birds/ treatment).

Table 6. Influence of bird age on Ca and P solubility coefficients[1] in the gizzard, jejunum and ileum

	Gizzard		Jejunum		Ileum	
Age, d	Ca	P	Ca	P	Ca	P
4[2]	0.41[b]	0.38[b]	0.22[b]	0.27[c]	0.14	0.19
6[3]	0.61[a]	0.51[a]	0.29[a]	0.33[b]	0.15	0.21
8[4]	0.55[a]	0.51[a]	0.30[a]	0.34[ab]	0.16	0.23
10[4]	0.60[a]	0.51[a]	0.30[a]	0.36[ab]	0.17	0.23
12[4]	0.57[a]	0.52[a]	0.30[a]	0.38[ab]	0.17	0.23
14[4]	0.61[a]	0.55[a]	0.32[a]	0.39[a]	0.18	0.25
SEM	0.03	0.02	0.01	0.02	0.01	0.01
P-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

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

[1]Solubility coefficients obtained using the equation: $(\text{Mineral})_{\text{supernatant}}/(\text{Mineral})_{\text{diet.}}$

[2]Means represent the average response of 12 pens, 6 plots (120 birds/ treatment).

[3]Means represent the average response of 12 pens, 6 plots (60 birds/ treatment).

[4]Means represent the average response of 12 pens, 6 plots (36 birds/ treatment)

Table 7. Influence of bird age and diet on gizzard pH in broilers[1] from d 0 to 14

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

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

[1] Means represent the average response of 12 pens, 6 plots per age (12 birds/ treatment per age)

Table 8. Influence of bird age on duodenum, jejunum and ileum pH in broilers[1] from d 0 to 14

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

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

[1] Means represent the average response of 12 pens, 6 plots per age (12 birds/ treatment per age)