

## Phosphorus bioavailability, amino acid digestibility and metabolizable energy of broiler chick diets supplemented with low-oil distiller's dried grains with solubles

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### Abstract

Phosphorus (P) bioavailability, crude protein (CP), amino acid (AA) digestibility, and apparent metabolizable energy (AME<sub>P</sub>) were determined in broiler chick diets, supplemented with one of two low-oil distiller's dried grains with solubles (oil contents 6.54% and 5.35% for DDGS A and B, respectively). Diets were provided *ad libitum* in a mash form, from days 8 to 21 of age. In Experiment 1, one day old male broiler chicks (n=210) were assigned to one of 7 treatments, with 3 replicates of 10 birds each. Animals in treatment 1 (T1) were fed a basal diet formulated to be deficient in nonphytate P (0.14%). Birds in T2 and T3 received the basal diet supplemented with 0.05% or 0.10% of P from monocalcium phosphate (MCP), respectively. Phosphorus from DDGS was added to diets in T4 (0.05% from DDGS A), T5 (0.1% from DDGS A), T6 (0.05% from DDGS B) and T7 (0.1% from DDGS B). Percent of inclusion of DDGS was dictated by total P content as determined by chemical analyses. Growth performance and P intake were assessed in all birds. Phosphorus bioavailability was determined using the slope ratio assay, with tibia ash as the response variable. For Experiment 2, one-day-old male broiler chicks (n=200), were assigned to 5 treatments with 4 replicates of 10 birds each. A sorghum-soya control diet was given to animals in T1. Treatments 2 and 3 included this same diet supplemented with either 5% or 10% of DDGS A. Birds in T4 and 5 received the control diet with either- 5% or 10% of DDGS B. Amino acid digestibility was measured from ileal contents in 7 euthanized birds from each group at day 21. Apparent metabolizable energy was determined in oven dried feces from all animals during the last 3 days of the experiment. Weight gain and feed consumption

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Additional information and declarations  
can be found on page 11

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increased ( $P < 0.001$ ) with larger inclusion of dietary P in birds from experiment 1. Relative P bioavailability for DDGS A and B was 72% and 86% respectively, from a P total content of 0.85% and 0.94%. Growth performance was similar for all treatment groups in experiment 2 ( $P > 0.05$ ). The average apparent ileal amino acid digestibility coefficient for DDGS A 76.5% and B was 76.4%. No differences were found for essential AA coefficients between the two DDGS ( $P > 0.05$ ). The  $AME_n$  values on a dry matter basis were 2828 and 2854 kcal/kg for DDGS A and B, respectively.

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**Keywords:** Amino acids, Ileal digestibility, Apparent metabolizable energy, Available phosphorus, Low-oil DDGS

## Introduction

More than 142.7 million tons of corn were directed to ethanol production worldwide in 2018. Derived from this process, thirty million tons of distiller's dried grains with solubles (DDGS), which are the nutrient rich co-product of dry-milled ethanol production, reached the trading market for animal feeding.<sup>1</sup> Concurrently, Mexico imported close to 22% of the total DDGS production from the USA. At present, most ethanol production plants are removing oil from DDGS by spinning the soluble portion at the end of the fermentation process. This oil is then used for biodiesel, and the remaining co-product is known as oil-extracted or low-oil DDGS.<sup>2</sup> These low-oil DDGS have close to 50% less oil than conventional DDGS, and a somewhat higher protein content.<sup>2</sup> However, assessment of the nutritional value of oil extracted DDGS for use in livestock feed is scarce. Kim *et al.*<sup>3</sup> reported a phosphorus (P) bioavailability of 60% and 56% for DDGS containing 10% and 2.9% of oil respectively. Conversely, P bioavailability for conventional DDGS has been reported as being close to 75%. Moreover, amino acid (AA) digestibility, has been shown to be similar between low-oil and conventional DDGS, with a lysine digestibility of 69% in DDGS samples ranging from 9 to 13.2% of oil content.<sup>4-6</sup> Lastly, few studies have assessed the energy value of low-oil DDGS. Therefore, the objective of the current study was to evaluate P bioavailability, crude protein content (CP), AA ileal digestibility, and apparent metabolizable energy (AMEn) of two low-oil DDGS samples added to sorghum-soybean meal-based diets for broiler chicks.

## Materials and methods

Experiment 1 was designed to evaluate P bioavailability of low-oil DDGS (relative to monocalcium phosphate -MCP-), when added to broiler chick diets. Experiment 2 aimed to determine AA digestibility and AME<sub>n</sub> of two oil extracted DDGS samples, and to evaluate their feeding value at increasing practical levels of inclusion.<sup>7</sup>

## Laboratory analyses

The chemical composition of the two low-oil DDGS used in this study is presented in Table 1. Crude protein (CP) and total AA content in diets were determined in duplicates according to the 982.30 AOAC<sup>8</sup> method. Tryptophan was measured by alkaline hydrolysis with sodium hydroxide, followed by high performance liquid chromatography (HPLC). To assess methionine and cysteine, samples were previously subjected to oxidation with performic acid. The remaining AA were analyzed by acid hydrolysis (110 °C- HCl, 6N) for 24 h, and further subjected to ionic exchange chromatography.

## Poultry husbandry

Animal handling, housing, and euthanasia were approved by the Bioethics and Animal Welfare Committee of the Faculty of Veterinary Medicine of the National Autonomous University of Mexico. One day old chicks were obtained from a commercial hatchery and vaccinated against Marek's disease using eye drops. At 10 days of age, broilers were also vaccinated against Newcastle by subcutaneous injection.

**Table 1.** Chemical composition of two low-oil distiller's dried grain with solubles (DDGS).

Analyses	DDGS A (%)	DDGS B (%)
Dry matter	95.45	95.05
Moisture	4.55	4.95
Crude protein	28.05	27.02
Ether extract	6.54	5.39
Ash	5.40	5.26
Crude fiber	8.05	8.43
Nitrogen free extract	47.41	48.96
Calcium	0.12	0.05
Total phosphorus	0.85	0.94
Phytate phosphorus	0.27	0.31
Nonphytate P	0.58	0.63
<b>Gross energy, kcal/kg</b>	<b>4532</b>	<b>4394</b>

Battery cages with wire floors, fitted with individual drinkers and feeders, were used for animal housing. All birds received a sorghum-soybean meal-based diet from days 1 to 7 of age, formulated to comply with nutritional the requirements specified by the Ross 308 manual,<sup>9</sup> and according to the primary breeder recommendations before starting Experiment 1.

## Experimental design

### Experiment 1

Two hundred and ten one-day-old Ross 308 male broiler chicks were individually weighed and sorted to have a similar animal weight distribution in every replicate. Birds were assigned to 7 dietary treatments, from days 8 to 21 of age, with 3 replicates of 10 birds each. Feed was provided *ad libitum* in a mash form. Cellulose was replaced in experimental diets by P from MCP or DDGS (A or B). Chicks in treatment 1 were given a basal diet formulated to be deficient in nonphytate P (0.14%) (table 2). Birds in treatments 2 and 3 received this same basal diet supplemented with 0.05% or 0.10% of P from MCP, respectively. Basal diets in treatments 4 and 5 were supplemented with 0.05% or 0.10% of P from DDGS-A, respectively. In treatments 6 and 7, 0.05% or 0.10% of P from DDGS-B were, respectively, added to the control diet. Percentage of inclusion of both DDGS was dictated by their respective total P content, as determined by chemical analyses.

Feed consumption, weight gain, and feed conversion (measured as the ratio of feed consumed:weight gained) were measured daily throughout the experimental period. At d 21 of age, 6 birds per pen were euthanized by CO<sub>2</sub> asphyxiation. The left tibias were subsequently collected, fat-extracted, dried, and processed for bone ash determination (method 932.16 AOAC).<sup>8</sup>

### Experiment 2

Two hundred one day old male Ross 308 broiler chicks were randomly assigned to 5 dietary treatments, with 4 replicates of 10 birds each. Feed was provided *ad libitum* from 8 to 21 d of age in a mash form. Treatments consisted of a control sorghum-soybean meal diet (T1), or this same diet supplemented with either 5%

**Table 2.** Ingredients and nutrient content of the sorghum-soya based control diet formulated to be deficient in nonphytate phosphorous (P) (0.14%) used in Experiment 1.

Ingredients	Inclusion, %
Sorghum <sup>a</sup>	38.050
Soybean meal <sup>a</sup>	38.720
Soybean oil	8.000
Calcium carbonate	2.500
Cellulose	11.573
Salt	0.382
DL-methionine	0.314
L-lysine HCl	0.146
Vitamin and mineral premix <sup>b</sup>	0.200
Choline chloride 60%	0.100
Antioxidant BHT	0.015
Nutrient	Calculated analysis
Protein	22.00
Digestible lysine	1.39
Digestible methionine + cysteine	0.92
Calcium	1.02
Nonphytate P	0.14
ME, kcal/kg	2950

<sup>a</sup> Analyzed total phosphorus values were 0.35% and 0.70% for sorghum and soybean meal ingredients, respectively. Calculated nonphytate phosphorus values were 0.12% and 0.24% for these same ingredients, respectively.

<sup>b</sup> Provides: vitamin A, 6 000 000 UI; vitamin D<sub>3</sub>, 1,500,000 UI; vitamin E, 12,000 UI; vitamin K<sub>3</sub>, 2.0 g; riboflavin, 8 g; vitamin B<sub>12</sub>, 0.120 g; pyridoxine, 6.0 g; calcium pantothenate, 26.0 g; niacin, 50 g; biotin, 0.126 g; choline chloride, 500 g; selenium, 0.2 g; cobalt, 0.1 g; iodine, 0.3 g; copper, 10 g; zinc, 50 g; iron, 100 g; manganese, 100 g; excipient qs 2,000 g.

or 10% of DDGS A (T2 and T3 respectively) or DDGS B (T4 and T5 respectively). Composition of diets is presented in Table 3. Measured values for crude protein and total amino acids, as determined by chemical analyses in both DDGS, were used for diet formulation.

Titanium dioxide (TiO<sub>2</sub>, 0.2%) was added to diets as an indigestible marker<sup>10</sup> to assess raw protein and AA digestibility. Its presence in diets and within ileal contents was analyzed according to the method described by Lomer *et al.*<sup>11</sup> and read on a Varian inductively coupled plasma mass spectrometer (Varian Inc., Palo Alto, CA). Digestibility coefficients were calculated by the following formula:

$$AD_{AA} = 100 - [100 \times \frac{(AA) \text{ content} \times (TiO_2) \text{ diet}}{(AA) \text{ diet} \times (TiO_2) \text{ content}}$$

Where:

$AD_{AA}$  = individual AA apparent digestibility (percentage)

$[AA]_{\text{content}}$  = AA concentration in ileal contents

$[AA]_{\text{diet}}$  = AA concentration in diet

$[TiO_2]_{\text{content}}$  = Titanium dioxide concentration in ileal contents

$[TiO_2]_{\text{diet}}$  = Titanium dioxide concentration in diet

**Table 3.** Nutrient content and chemical analyses of diets used in Experiment 2.

Ingredient	Control	5% DDGS-A	10% DDGS-A	5% DDGS-B	10% DDGS-B
Sorghum	593.985	568.117	542.246	566.073	538.158
Soybean meal	339.214	314.745	290.276	314.934	290.654
DDGS	-----	50.000	100.000	50.000	100.00
Soybean oil	22.797	23.197	23.598	25.041	27.285
Monocalcium phosphate	20.230	19.068	17.907	18.938	17.647
Calcium carbonate	12.256	12.859	13.463	13.013	13.770
Salt	3.788	3.834	3.881	3.836	3.884
DL-methionine	2.546	2.481	2.416	2.480	2.415
L-lysine HCl	2.034	2.549	3.063	2.535	3.037
Vitamin premix <sup>a</sup>	1.000	1.000	1.000	1.000	1.000
Mineral premix <sup>a</sup>	0.500	0.500	0.500	0.500	0.500
Choline chloride 60%	1.000	1.000	1.000	1.000	1.000
Zinc bacitracin 10%	0.300	0.300	0.300	0.300	0.300
Titanium dioxide	0.200	0.200	0.200	0.200	0.200
Antioxidant BHT	0.150	0.150	0.150	0.150	0.150
Total	1000.0	1000.0	1000.0	1000.0	1000.0
Chemical analysis					
Protein, %	22.0	22.0	22.0	22.0	22.0
ME, kcal/kg	3000	3000	3000	3000	3000
Digestible methionine + cysteine, %	0.85	0.85	0.85	0.85	0.85
Digestible lysine, %	1.20	1.20	1.20	1.20	1.20
Calcium, %	1.00	1.00	1.00	1.00	1.00
Nonphytate P, %	0.50	0.50	0.50	0.50	0.50

<sup>a</sup> Provides: vitamin A, 6,000,000 IU; vitamin D<sub>3</sub>, 1,500,000 IU; vitamin E, 12,000 IU; vitamin K<sub>3</sub>, 2.0 g; riboflavin, 8 g; vitamin B<sub>12</sub>, 0.120 g; pyridoxine, 6.0 g; calcium pantothenate, 26.0 g; niacin, 50 g; biotin, 0.126 g; choline chloride, 500 g; selenium, 0.2 g; cobalt, 0.1 g; iodine, 0.3 g; copper, 10 g; zinc, 50 g; iron, 100 g; manganese, 100 g; excipient qs, 2,000 g.

At 21 d of age, 7 birds per treatment group were euthanized by CO<sub>2</sub> asphyxiation and used for ileal digesta collection. Briefly, an intestinal segment between Meckel's diverticulum and the ileocecal junction was dissected, ileal contents were removed, stored in a plastic bag, and immediately frozen until analyzed. For CP and AA determination, digesta samples were freeze-dried and analyzed at the Evonik Industries laboratory, by ionic exchange chromatography with post-column ninhydrin derivatization.<sup>12</sup>

Apparent metabolizable energy was determined in oven dried feces (65 °C for 72 hours) from all animals during the last three days of the experiment. The Leeson and Summers procedure was used,<sup>10</sup> correcting at 0% nitrogen retention, and using the following formula:

$$\text{AMEn} = \text{ME control diet} - \frac{(\text{ME control diet} - \text{ME diets with DDGS})}{\% \text{ DDGS}}$$

Apparent ileal digestibility coefficients for CP and AA were also calculated using the Leeson and Summers<sup>10</sup> formula. Diet raw energy was measured in both feed and feces samples with Parr's calorimetric pump (Parr Instruments, Moline, IA), and nitrogen content was assessed by the Kjeldahl procedure (AOAC standardized method 982.30).<sup>8</sup>

## Statistical analyses

### Experiment 1

Growth performance data was analyzed by ANOVA for a completely randomized design and the Tukey's multiple comparison test. Phosphorus consumption and tibia bone ash content were fitted into a linear regression model. Phosphorus Bioavailability was assessed by the slope ratio methodology,<sup>13</sup> using tibia bone ash as the dependent variable (Y) and P consumption as the independent variable (X). The MCP consumption was used as the standard line ( $\beta_1X_1$ ) and P consumption from each DDGS source was the tested response ( $\beta_2X_2+\beta_3X_3$ ).

### Experiment 2

Body weight gain, feed consumption, feed conversion, AA digestibility and AMEn were analyzed by ANOVA for a completely randomized design and the Tukey's post hoc test.

All statistical analyses were conducted using the SPSS statistical package for Windows version 17 SPSS Inc.<sup>13</sup> Statistical differences were set at  $P < 0.05$ .

## Results

### Experiment 1

Weight gain, feed consumption, P consumption, and tibia bone ash are presented in Table 4. Body weight gain and feed consumption increased as dietary P increased ( $P < 0.05$ ). The highest weight gain and feed consumption were observed in the birds fed the diets with 0.10% of supplemented P. Phosphorus consumption also increased ( $P < 0.01$ ) as dietary P increased from MCP or DDGS (A or B). Tibia bone ash content was greater when diets were supplemented with 0.10% P.

Tibia bone ash as an indicator of P consumption can be explained by the following equation:  $Y = 27.614 + 0.007x_1 + 0.005x_2 + 0.006x_3$ ; where  $X_1$  relates to P consumed from MCP (T1, T2, and T3), whereas  $X_2$  and  $X_3$  represent P intake from diets containing DDGS A or DDGS B respectively. Phosphorus bioavailability was estimated as 72% and 86% for DDGS A and B respectively, when compared to MCP (100%) (Figure 1).

### Experiment 2

Weight gain, feed consumption and feed conversion were similar between groups ( $P > 0.05$ ) (Table 5). Total amino acid content and digestibility coefficients were similar ( $P > 0.05$ ) between diets supplemented with DDGS (Table 6).

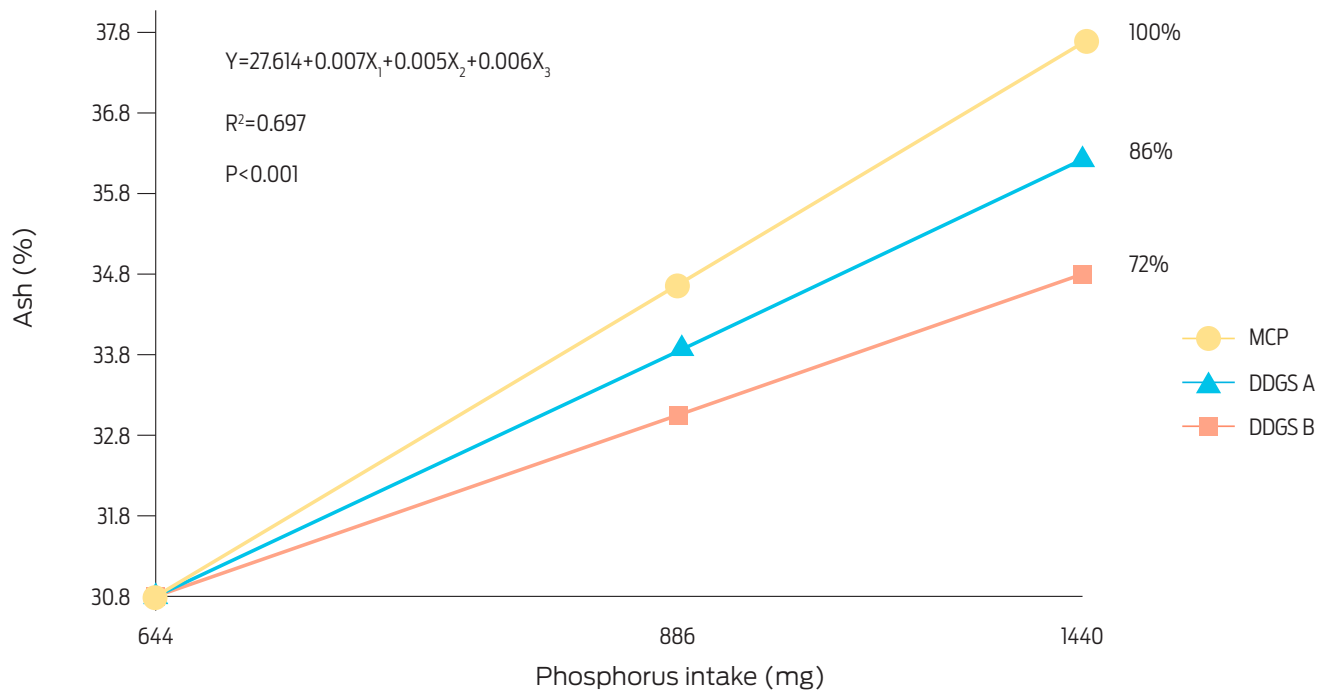
## Discussion

Weight gain of broiler chicks in experiment 1 increased when P was added to a sorghum-based diet, formulated to be deficient in nonphytate. This result agrees with those of previous studies where corn-soybean meal based diets were supplemented with Peither from either MCP or DDGS.<sup>3,4,5,14</sup> Moreover, tibia bone ash and P consumption increased with greater P inclusion in diets (from MCP or oil

**Table 4.** Weight gain (g/bird), feed intake (g/bird), feed efficiency (g:g), phosphorus consumption (mg/bird), and tibia bone ash (%) of broiler chicks fed 0.05% or 0.1% of phosphorus from either monocalcium phosphate or one of two DDGS samples from d 8 to 21 of age (Experiment 1).

Treatment	Phosphorus content, %	Phosphorus consumption (mg)	Weight gain (g)	Feed consumption (g)	Feed efficiency (g:g)	Tibia bone ash (%)
1. Basal diet	0.140	644 ± 40.2 <sup>a</sup>	285 ± 5.19 <sup>a</sup>	460 ± 40.6 <sup>a</sup>	0.68 ± 0.04 <sup>a</sup>	30.8 ± 0.88 <sup>a</sup>
2. +0.05% from MCP	0.190	886 ± 27.6 <sup>b</sup>	331 ± 28.7 <sup>a,b</sup>	479 ± 21.1 <sup>a</sup>	0.82 ± 0.06 <sup>a</sup>	34.5 ± 0.99 <sup>a,b,c</sup>
3. +0.10% P from MCP	0.240	1440 ± 35.7 <sup>c</sup>	447 ± 10.8 <sup>c</sup>	626 ± 21.9 <sup>b,c</sup>	0.79 ± 0.04 <sup>a</sup>	36.6 ± 1.05 <sup>b,c</sup>
4. +0.05% P from DDGS A	0.190	1008 ± 27.7 <sup>b</sup>	343 ± 16.5 <sup>a,b</sup>	534 ± 20.7 <sup>a,b</sup>	0.65 ± 0.01 <sup>a</sup>	32.4 ± 0.93 <sup>a,b</sup>
5. +0.10% P from DDGS A	0.240	1337 ± 49.7 <sup>c</sup>	405 ± 22.5 <sup>b,c</sup>	562 ± 29.5 <sup>a,b,c</sup>	0.74 ± 0.01 <sup>a</sup>	36.9 ± 1.06 <sup>b,c</sup>
6. +0.05% P from DDGS B	0.190	1026 ± 10.2 <sup>b</sup>	339 ± 4.16 <sup>ab</sup>	518 ± 7.37 <sup>ab</sup>	0.67 ± 0.01 <sup>a</sup>	34.7 ± 1.00 <sup>a,b,c</sup>
7. +0.10% P from DDGS B	0.240	1641 ± 27.9 <sup>c</sup>	472 ± 6.43 <sup>c</sup>	657 ± 15.8 <sup>c</sup>	0.73 ± 0.01 <sup>a</sup>	38.0 ± 1.09 <sup>c</sup>

<sup>a-c</sup>Means with different superscript within a column differ significantly (P < 0.01).



**Figure 1.** Tibia bone ash content (%) in 21-day old broiler chicks fed increasing phosphorus levels from monocalcium phosphate or one of two DDGS samples from 8 to 21 days of age.



**Table 5.** Weight gain (g), feed consumption (g/bird), and feed conversion (g/g) of broiler chicks fed a sorghum based control diet or this same diet supplemented with either 5% or 10% of two low-oil DDGS from days 8 to 21 of age (Experiment 2).<sup>a</sup>

Treatments	Weight gain (g)	Feed consumption (g)	Feed conversion (g/g)
1. Control	369 ± 17.4	741 ± 10.0	2.01 ± 0.98
2. 5% DDGS A	333 ± 11.2	732 ± 10.6	2.20 ± 0.91
3. 10% DDGS A	336 ± 3.25	732 ± 23.8	2.17 ± 0.53
4. 5% DDGS B	337 ± 6.58	746 ± 7.54	2.21 ± 0.44
5. 10% DDGS B	339 ± 4.64	738 ± 5.63	2.17 ± 0.27

<sup>a</sup> Means ± SEM.

extracted DDGS), as previously observed by other authors.<sup>5,4,3,14</sup> However, these studies used conventional DDGS to supplement corn-soybean meal based diets.

There were no differences in mean weight gain of chicks between treatments in experiment 2, showing that inclusion of low-oil DDGS in feed does not have a negative impact on this productive parameter. Similarly, Guney *et al.*<sup>15</sup> supplemented broiler chick diets with either 10% or 20% of low-oil DDGS (7.52% and 6.74% of oil content, respectively), and found no negative effects on growth performance of birds. Further, an 8% inclusion of conventional DDGS in feed did not affect weight gain of chicks from 0 to 14 d of age, but it did however negatively affect feed conversion.<sup>16</sup> In contrast, other studies found that a 16% or 24% inclusion of conventional DDGS in starter chick diets improves weight gain of birds.<sup>17</sup> Conversely, Lumpkins *et al.*<sup>18</sup> show that an 18% supplementation of conventional DDGS, resulted in a decrease in growth rate.

Phosphorus bioavailability for each DDGS used to supplement diets in this work was similar to that reported in previous studies (75%-80%).<sup>4,14</sup> Moreover, inclusion percentages of P in diets formulated for this study are similar to those used by Martinez *et al.*<sup>5</sup> who established that these values were higher than those needed to comply with broiler chick diet requirements published by the NRC<sup>19</sup> (based both on total and non-phytate P). However, there are other studies that report a lower P bioavailability rate (66%) for low-oil DDGS.<sup>20</sup>

Data for apparent ileal CP and AA digestibility in this study agree with other work where AA digestibility was determined in conventional DDGS, with percentages ranging from 54.8-77% for lysine and 77.5%-86.9% for methionine.<sup>6,21-25</sup> To date, few studies have assessed AA digestibility in low-oil DDGS, however, Wamsley *et al.*<sup>20</sup> found a 65% digestibility for lysine in Cobb 500 chickens fed with a low-oil DDGS supplemented diet and 88%, respectively.

Average apparent metabolizable energy values obtained from excreta of birds in this study (2828 and 2854 kcal/kg for DDGS-A and B respectively), were similar to those found in other work with either sorghum or corn-soybean based diets, supplemented with varying percentages of low oil or conventional DDGS.<sup>21,24,26-28</sup> Moreover, Adeola and Zhai<sup>28</sup> found ileal digestible energy values of 2841 and 2659 kcal/kg in corn-soybean meal diets containing 30% or 60% of conventional DDGS respectively.

**Table 6.** Total amino acid content (%), digestibility coefficients (%) and AMEn on a dry matter basis (Kcal/kg), of diets supplemented with two low-oil distillers dried grains with solubles (DDGS). Assessed in ileal content of 21 d old broiler chicks.

Amino acid, %	DDGS-A		DDGS-B	
	Total, %	Digestibility coefficient, %	Total, %	Digestibility coefficient, %
Methionine	0.55	85.3	0.57	84.7
Cystine	0.48	76.6	0.49	75.0
Lysine	1.00	65.4	0.98	64.6
Threonine	0.93	63.2	0.96	64.1
Arginine	1.17	77.0	1.11	77.7
Leucine	3.15	86.7	3.34	84.0
Isoleucine	1.07	77.8	1.08	77.8
Valine	1.11	74.4	1.16	77.3
Phenylalanine	1.35	81.9	1.34	81.7
Histidine	0.80	77.0	0.83	77.4
Protein	28.05	76.5	27.02	76.4
AME <sub>N</sub> , kcal/kg <sup>a</sup>	2828		2854	

<sup>a</sup>Dry matter basis.

## Conclusion

Bioavailability of P in the low oil DDGS samples used in this study (DDGS A 6.54% and DDGS B 5.39% of oil content) was 72% and 86%, respectively. Apparent ileal AA digestibility coefficients (methionine, cysteine, lysine, threonine, arginine, leucine, isoleucine, valine, phenylalanine, and histidine) were similar for the two assessed DDGS. The average apparent ileal amino acid digestibility coefficient for DDGS A 76.5% and B was 76.4%. The AME<sub>N</sub> values on a dry matter basis were 2828 and 2854 kcal/kg for DDGS A and B, respectively.

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## Conflicts of interest

Authors declare that there is no conflict of interest regarding publication of this article.

## Author contributions

C. C. A.: experiment design, field work, statistical analysis, final document preparation; J. A.: statistical analysis of data. E. A.: statistical analysis of data, final document preparation; C. L.: experiment design, statistical analysis.

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