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Comparative effects of dietary carbohydrases without or with protease on the ileal digestibility of energy and amino acids and AME_n in young broilers

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ABSTRACT

Four digestibility trials using broilers from 12 to 21 d or 7 to 21 d of age were conducted to evaluate the nutrient digestibility responses of two enzyme combinations when supplemented to maize–soybean meal (SBM) based diets without (trials 1 and 3) or with (trials 2 and 4) 70 or 100 g/kg maize dried distillers grain with solubles (DDGS). Apparent ileal digestible energy (AIDE), apparent ileal digestibility (AID) of amino acids (AA), total tract apparent retention (TTAR) of nitrogen (N) and N-corrected apparent metabolisable energy (AME_n) were assessed. In each trial, a control diet and the control diet supplemented with an enzyme complex containing xylanase and amylase (XA) or one containing protease, xylanase and amylase (PXA) were tested. Trials 1 and 2 had six replicate cages per treatment, with six birds per cage; and trials 3 and 4 had eight replicate cages per treatment, with five birds per cage. Data were analysed by ANOVA for each trial, as well as a combined dataset, which model included the main effects of maize-DDGS inclusion, dietary enzyme and trial site and all two-way interactions. Across trials, both XA (13.61 MJ/kg) and PXA (13.70 MJ/kg) increased the AIDE ($P<0.05$) compared to the control diets (13.29 MJ/kg). XA increased ($P<0.05$) AME_n (12.89 MJ/kg) compared to the control diets (12.61 MJ/kg), and PXA further increased it (13.00 MJ/kg) compared to XA. Supplementation with XA had no effect on the AID of AA, whereas PXA increased the AID of nitrogen and all AA ($P<0.05$) except methionine. The AA with the greatest response to PXA were cysteine (+5.4%), threonine (+4.4%), glycine (+3.6%) and valine (+3.3%). The least responsive AA to PXA inclusion were methionine (+1.0), glutamine (+2.0), lysine (+1.9%) and arginine (+2.2%). Irrespective of the AA, PXA increased AID of AA as a proportion of the ileal undigested fraction of AA in the control diets by 12–13%. Supplemental xylanase and amylase increased AIDE and AME_n and the addition of protease on top of carbohydrases further increased AID of AA and AME_n in young broilers fed maize–SBM diets without or with maize-DDGS.

Abbreviations: AA, amino acids; AME, apparent metabolisable energy; AME_n, nitrogen corrected apparent metabolisable energy; AID, apparent ileal digestibility; AIDE, apparent ileal digestible energy; DDGS, dried distillers grain with solubles; N, nitrogen; NSPs, non-starch polysaccharides; PXA, protease, xylanase and amylase; TTAR, total tract apparent retention; XA, xylanase and amylase.

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1. Introduction

Carbohydrase enzymes such as xylanases, amylases and glucanases are being adopted by the poultry industry worldwide. In wheat-based diets, these enzymes are used to ameliorate the adverse effects of non-starch polysaccharides (NSPs) on digesta viscosity, nutrient digestibility and bird performance (Choct, 2006). Exogenous carbohydrases are now increasingly used in maize-based diets for broilers (Cowieson, 2010) despite low concentrations of soluble NSP in these diets. Economic benefits from the use of these enzymes are obtained through a reduction of feed cost that occurs when improvements on apparent metabolisable energy (AME) and, to a lesser extent, amino acids (AA) digestibility are accounted for in diet formulations. Possible mechanisms of action of carbohydrases in poultry diets include: improved access of endogenous enzymes to cell contents due to hydrolysis of cell wall arabinoxylans (Cowieson, 2005), augmentation of digestive enzymes in young animals, particularly of amylase (Ritz et al., 1995; Gracia et al., 2003), modulation of intestinal microflora (Fernandez et al., 2000) and reduction of endogenous AA losses, particularly through changes on pancreatic amylase (Jiang et al., 2008) and mucin secretion (Cowieson and Bedford, 2009). In general, improvements of AME due to exogenous xylanase and amylase appear to be a combination of two mechanisms, namely, increased digestion and absorption of the undigested portion of starch and fat of the diet and down-regulation of various digestive secretions.

Exogenous proteases of microbial origin are being increasingly used in broiler diets (Adeola and Cowieson, 2011). The economic benefit of the use of exogenous proteases is through improvements in the digestibility of dietary AA. The primary mechanism for this increment appears to be the augmentation of dietary protein hydrolysis and increased protein solubility (Caine et al., 1998). Conclusive evidence is not available supporting a positive or negative change in endogenous secretions caused by exogenous proteases. However, increments of AME caused by exogenous proteases in conjunction with xylanases and amylases are suggestive of the ability of exogenous proteases to disrupt protein–starch interactions in cereal grains (McAllister et al., 1993; Belles et al., 2000). Nonetheless, the additional effect of protease on top of carbohydrases on energy metabolisability and apparent ileal digestibility (AID) of AA has not been fully assessed. That is particularly relevant for modern broiler diets containing non-traditional protein sources such as maize dried distillers grain with solubles (DDGS), where the effects of proteases and carbohydrases may differ in importance compared to traditional maize–soybean meal (SBM) diets. The current study aimed to assess the effect of an exogenous bacterial serine protease in combination with a xylanase and an amylase *versus* xylanase and amylase only in broilers fed either a maize–SBM, or a maize–SBM-DDGS based diet, in terms of apparent ileal digestible energy (AIDE), AID of AA, total tract apparent retention (TTAR) of nitrogen (N) and N-corrected AME (AME_n). The results reported herein were from four independent trials with similar design performed at two research sites.

2. Materials and methods

2.1. Exogenous enzymes

Two enzyme products were used: a prototype enzyme preparation containing endo-1,4-beta-xylanase (EC 3.2.1.8) and alpha-amylase (EC 3.2.1.1); and a commercial enzyme preparation (Avizyme 1502; Danisco Animal Nutrition DuPont Industrial Biosciences, Marlborough, UK) containing endo-1,4-beta-xylanase (EC 3.2.1.8), alpha-amylase (EC 3.2.1.1) and an alkaline serine protease (EC 3.4.21.62). The xylanase originated from *Trichoderma reesei*, the amylase from *Bacillus amyloliquifaciens* and the protease from *Bacillus subtilis*.

2.2. Experimental design

Four digestibility trials were conducted to evaluate AIDE, AID of AA and AME_n of broilers fed maize–SBM diets without (two trials) or with (two trials) maize-DDGS inclusion, supplemented with an enzyme complex containing xylanase and amylase (XA), or one containing protease, xylanase and amylase (PXA), as compared to a control diet. In each trial, three dietary treatments (control, XA and PXA) were evaluated in diets for broilers from hatch to 21 d of age. Trials 1 and 2, conducted at Massey University (Palmerston North, New Zealand) consisted of six replicates per treatment, with six birds per replicate cage. Trials 3 and 4, conducted at the University of Illinois (Urbana, USA), consisted of eight replicates per treatment, with five birds per replicate cage.

2.3. Experimental diets

Ingredients and calculated diet composition are presented in Table 1. The diets were based on maize and SBM, with slight variations in ingredient composition between the trial sites. Diets were provided in mash form. In each research site, one trial was performed without and one with maize-DDGS inclusion. Maize-DDGS was included at 100 g/kg in diets of trial 2 and 70 g/kg in trial 4. Calculated AME values were constant within site. Titanium dioxide (3.0 g/kg) was added to all diets as an indigestible marker. Diets were manufactured in one batch for each trial and then subdivided in three experimental diets, two of them containing enzymes. Concentrates of the test enzymes were sprayed into a wheat carrier and added to the respective diets in dry form at 0.5 g/kg, after being premixed with 5 g of maize/kg from the diets.

Table 1
Composition and analysis of basal diets of four trials using maize–soybean meal based diets without (trials 1 and 3) or with (trials 2–4) 70 or 100 g/kg maize-DDGS.

	Trial 1	Trial 2	Trial 3	Trial 4
Ingredients (g/kg)				
Maize	606.0	548.4	577.0	539.7
Soybean meal (480 g/kg)	337.9	289.1	372.1	339.0
Maize-DDGS	0.0	100.0	0.0	70.0
Soy oil	15.0	22.1	12.4	13.1
Dicalcium phosphate	13.8	12.1	13.5	12.5
Limestone	11.7	12.9	11.7	12.1
Sodium bicarbonate	2.7	1.6	0.0	0.0
Salt	2.0	2.3	3.5	3.5
L-Lysine HCl (780 g/kg)	1.8	2.7	0.5	1.0
DL-Methionine (990 g/kg)	2.6	2.2	2.3	2.1
L-Threonine, (980 g/kg)	0.0	0.1	0.0	0.0
Broiler premix[a,b]	3.0	3.0	3.5	3.5
Titanium dioxide	3.0	3.0	3.0	3.0
Starch or enzyme	0.5	0.5	0.5	0.5
Determined composition (g/kg)				
Dry matter	894	890	886	892
AME_n (MJ/kg)	11.3	11.3	11.0	11.3
Crude protein	198	200	195	201
Lysine	12.7	11.6	11.7	11.7
Methionine	6.3	6.4	4.9	4.8
Methionine + cysteine	9.0	9.6	8.0	7.9
Calculated nutrient composition (g/kg)				
AME (MJ/kg)	12.5	12.5	12.4	12.4
Crude protein	217	217	230	229
Calcium	8.5	8.5	8.5	8.5
Available phosphorus	3.8	3.8	3.8	3.8
Lysine	13.0	13.0	13.0	13.0
Methionine	6.0	5.9	5.8	5.7
Methionine + cysteine	9.5	9.5	9.5	9.5

[a] Trials 1 and 2. The premix supplied per kilogram of diet: biotin, 0.2 mg; D-Ca-pantothenate, 12.8 mg; cholecalciferol, 60 μg; cyanocobalamin, 0.017 mg; folic acid, 5.2 mg; menadione, 4 mg; niacin, 35 mg; pyridoxine, 10 mg; trans-retinol, 3.33 mg; riboflavin, 12 mg; thiamine, 3.0 mg; DL-α-tocopheryl acetate, 60 mg; choline chloride, 638 mg; Co, 0.3 mg; Cu, 3 mg; Fe, 25 mg; I, 1 mg; Mn, 125 mg; Mo, 0.5 mg; Se, 200 μg; Zn, 60 mg; and antioxidant, 100 mg.
[b] Trials 3 and 4. The premix supplied per kilogram of diet; D-Ca-pantothenate, 10 mg; cholecalciferol, 25 μg; cyanocobalamin, 0.01 mg; menadione sodium bisulphite, 2.33 mg; retinyl acetate, 4400 IU; riboflavin, 4.41 mg; DL-α-tocopheryl acetate, 11 IU; niacin, 22 mg; Cu, 5 mg; Fe, 75 mg; Mn, 75 mg; Se, 0.1 mg; Zn, 75 mg; I, 0.75 mg.

Enzyme activities in feed samples (200 g) were measured at the Danisco Innovation Laboratories (Brabrand, Denmark; Table 2) in duplicate. One xylanase unit was defined as the amount of enzyme that releases 0.48 μmol of reducing sugar as xylose from wheat arabino xylan per minute at pH 4.2 and 50 °C. Azurine cross linked arabinoxylan isolated from wheat (Megazyme International Ireland Ltd., Bray, Ireland) was used as substrate. Sample extracts (100 μl) were incubated at 50 °C for 60 min, mixed with a vortex and centrifuged at 960×*g* for 10 min, after which absorbance of the supernatant was measured with a spectrophotometer at 590 nm against a blank sample and units were calculated in reference to a calibration curve.

One unit of amylase was defined as the amount of the enzyme catalysing the hydrolysis of 1 μmol glucosidic linkage per minute. Amylase activity was measured based on the method described by McCleary and Sheehan (1989) and Barnes and Blakeney (1974). Briefly, 400 μl sample extracts were incubated at 37 °C for 30 min with phadebas tablets (Megazyme International Ireland Ltd., Bray, Ireland), mixed with a vortex and centrifuged at 960×*g* for 10 min, after which absorbance was measured with a spectrophotometer at 620 nm against a reagent blank and units were calculated in reference to a calibration curve.

One protease unit was defined as the amount of enzyme that releases 1.0 μg of phenolic compound, expressed as tyrosine equivalents, from a casein substrate per minute at pH 7.5 and 40 °C. Briefly, sample extracts were incubated at 50 °C for 60 min with a protazyme tablet (Megazyme International Ireland Ltd., Bray, Ireland) using pH 10 Tris/HCl buffers, centrifuged at 960×*g* for 10 min, after which the absorbance of supernatants was measured with a spectrophotometer at 590 nm against the blank and units were calculated in reference to a calibration curve.

2.4. Birds and management

At Massey University, day-old male broilers (Ross 308) were obtained, reared in floor pens and fed unsupplemented control diets prior to the introduction to cages, which were housed in an environmentally controlled room. The same procedures were followed at the University of Illinois using Ross 308 birds, except that the birds were kept in battery cages from the first day. Birds received a 20-h light programme and were allowed free access to feed and water. Birds were

Table 2
Expected and measured enzyme activities in samples of the experimental diets in four independent broiler digestibility trials.

Trial	Xylanase[a]		Amylase[b]		Protease[c]	
	Expected	Measured	Expected	Measured	Expected	Measured
Trial 1						
Control	0	–	0	–	0	–
XA	2000	2238	1800	2519	0	–
PXA	1000	1139	1800	2112	4000	3432
Trial 2						
Control	0	–	0	–	0	–
XA	2000	1800	1800	2011	0	–
PXA	1000	1056	1800	2108	4000	4482
Trial 3						
Control	0	–	0	–	0	–
XA	2000	1478	1800	1366	0	–
PXA	1000	941	1800	1505	4000	2899
Trial 4						
Control	0	–	0	–	0	–
XA	2000	1521	1800	2054	0	–
PXA	1000	912	1800	1771	4000	3597

[a] One xylanase unit was defined as the amount of enzyme that releases 0.48 μmol of reducing sugar as xylose from wheat arabino xylan per minute at pH 4.2 and 50 °C.
[b] One unit of amylase was defined as the amount of the enzyme catalysing the hydrolysis of 1 μmol glucosidic linkage per minute.
[c] One protease unit was defined as the amount of enzyme that releases 1.0 μg of phenolic compound, expressed as tyrosine equivalents, from a casein substrate per minute at pH 7.5 and 40 °C.

randomly allocated to cages and treatments at 12 d of age in trials 1 and 2 and at 7 d of age in trials 3 and 4. Experimental diets were fed from 12 to 21 d in trials 1 and 2 and from 7 to 21 d of in trials 3 and 4. The studies were conducted under approved animal use protocols that complied with the New Zealand Code of Practice for the Care and Use of Animals for Scientific Purposes (trials 1 and 2) and the Institutional Animal Care and Use Committee (trials 3 and 4), respectively.

2.5. AME_n and AID measurements

In trials 1 and 2, AME_n measurement was performed by total excreta collection. Feed intake and total excreta output were measured quantitatively per cage over four consecutive days starting at day 17. In trials 3 and 4, representative samples of excreta were collected daily on trays under each cage from 17 to 21 d of age. In all trials, excreta collections were pooled within a cage, mixed in a blender and sub-sampled. Each sub-sample was lyophilised, ground to pass through a 0.5 mm sieve and stored in airtight plastic containers at −4 °C pending analysis of dry matter, gross energy and nitrogen (N). Excreta samples from trials 3 and 4 were additionally analysed for titanium.

For assessment of AIDE and AID of AA and N, birds were euthanised by intracardial injection of sodium pentobarbitone in trials 1 and 2, or carbon dioxide asphyxiation in trials 3 and 4 and contents of the lower half of the ileum were obtained by gentle flushing with distilled water. The ileum was defined as the portion of the small intestine extending from the Meckel's diverticulum to a point approximately 40 mm proximal to the ileo-caecal junction (Ravindran et al., 2005). Digesta from birds within a cage were pooled and frozen immediately after collection, lyophilised and processed. Samples of digesta and diets were analysed for dry matter, N, AA, titanium and gross energy.

In trials 1 and 2, AME_n and TTAR of N were calculated as reported by Ravindran et al. (2008). In trials 3 and 4, AME_n and TTAR of N were calculated based on concentration of the indigestible marker in the diets using equations reported by Ravindran et al. (2005). Appropriate corrections were made for differences in moisture content. AME_n was determined for zero N retention by multiplication with 34.4 kJ/g of N retained in the body (Hill and Anderson, 1958). Calculation of AID coefficients based on the concentration of indigestible marker was performed as reported by Ravindran et al. (2005).

2.6. Chemical analysis

Dry matter content was determined using standard procedures (method 930.15; AOAC International, 2005). Gross energy was determined using adiabatic bomb calorimeter standardised with benzoic acid in all trials. N content was determined by the combustion method (method 968.06; AOAC International, 2005) using a CNS-2000 carbon, N and sulphur analyser. In trials 1 and 2, titanium content was measured on a UV spectrophotometer following the method of Short et al. (1996). AA, including cysteine and methionine, were determined in a Waters ion-exchange high performance liquid chromatographic system, as described by Ravindran et al. (2009). In trials 3 and 4, AA were analysed using the methods of AOAC International (2005). The samples were hydrolysed under N with 6 N HCl for 24 h at 110 °C before analyses were performed by automated cation-exchange chromatography. Analysis of cysteine and methionine involved performic acid oxidation before hydrolysis. Titanium analyses were conducted using procedures described by Myers et al. (2004).

Table 3
Effects of enzyme supplementation on apparent ileal digestible energy (AIDE), coefficient of apparent ileal digestibility (AID) of nitrogen (N), total tract apparent retention (TTAR) of N and N-corrected apparent metabolisable energy (AME_n) in young broiler chickens in four independent trials.[a,b]

Trial	Enzyme[c]	Maize-DDGS inclusion (g/kg)	AIDE (MJ/kg dry matter)	AID of N	TTAR of N (g/kg dry matter intake)	AME_n (MJ/kg dry matter)
Trial 1	Control	0	13.30	0.806^{y}	23.3	12.66^{y}
	XA	0	13.50	0.817^{xy}	23.5	12.78^{x}
	PXA	0	13.57	0.829^{x}	23.9	12.81^{x}
	SEM		0.09	0.005	0.20	0.04
	Probability		0.13	0.012	0.21	0.035
Trial 2	Control	100	13.38	0.793	23.5^{y}	12.68
	XA	100	13.74	0.810	24.3^{x}	12.82
	PXA	100	13.79	0.812	24.4^{x}	12.81
	SEM		0.14	0.008	0.26	0.05
	Probability		0.11	0.18	0.045	0.16
Trial 3	Control	0	13.39^{y}	0.822	18.8^{y}	12.45^{y}
	XA	0	13.64^{xy}	0.821	20.0^{xy}	12.98^{x}
	PXA	0	13.73^{x}	0.833	20.4^{x}	13.17^{x}
	SEM		0.11	0.007	0.45	0.08
	Probability		0.10	0.40	0.06	<0.001
Trial 4	Control	70	13.11^{y}	0.780	21.4^{y}	12.66^{z}
	XA	70	13.59^{x}	0.784	20.9^{y}	13.01^{y}
	PXA	70	13.73^{x}	0.800	22.3^{x}	13.22^{x}
	SEM		0.16	0.011	0.25	0.07
	Probability		0.031	0.43	0.002	<0.001

[x,y,z]Means with no common superscripts within column and sub-grouping are different at P<0.05.

[a] Trials 1 and 2 consisted of six replicates per treatment, with six birds per replicate cage. Trials 3 and 4 consisted of eight replicates per treatment, with five birds per replicate cage.

[b] Ileal digesta or excreta samples were pooled by cage. Cage was considered as experimental unit.

[c] Negative control diets were supplemented with an enzyme complex containing xylanase and amylase (XA), or one containing protease, xylanase and amylase (PXA).

2.7. Statistical analysis

Results from each of the trials were analysed separately using an ANOVA (Proc Mixed; SAS, 1997) that included the fixed effect of dietary treatment. Results of the four trials were then combined in a single data set and analysed using a model (Proc Mixed; SAS, 1997) that included the main effects of dietary enzyme, maize-DDGS inclusion and trial site as well as all the two-way interactions. Due to the heterogeneity of variances between sites, a heterogeneous variance model by trial site was specified using the Variance Components structure of Proc Mixed (SAS, 1997), which maximised fit. Least-squares-mean separation was made through multiple *t*-tests. Significance was assessed at P<0.05.

Linear regressions were performed to calculate the slope of the effect of enzymes on AID of AA compared to the control diets as a function of the amount of undigested AA in the control diets, using the REG Procedure of SAS (1997). Regressions were carried out separately for each of the enzyme treatments using the change in AID of AA for each AA compared to the control diets as independent variable. This change was calculated as the difference between the mean digestible AA in response to the enzyme and the mean digestible AA of the control treatment expressed in g AA/kg feed. The ileal undigested AA, calculated as the dietary AA minus the mean ileal digested AA of the control treatments and expressed in g AA/kg feed, was the dependent variable. No intercept was considered under the assumption that the improvement of digestibility at zero undigested AA was zero.

3. Results

Initially, differences in AIDE, AID of N, TTAR of N and AME_n due to enzyme supplementation were analysed for each of the four trials separately (Table 3). In trial 1, PXA increased (P<0.05) the AID of N while both XA and PXA increased (P<0.05) the AME_n compared to the control treatment. AIDE and TTAR of N were unaffected by the enzyme treatments. In trial 2, both XA and PXA increased (P<0.05) the TTAR of N compared to the control treatment, but had no effects on the AIDE and N and AME_n. In trial 3, PXA increased (P<0.05) the AIDE and TTAR of N; and both XA and PXA increased (P<0.05) AME_n compared to the control treatment. Enzymes, however, had no effect on the AID of N. In trial 4, both XA and PXA increased (P<0.05) the AIDE and PXA increased the TTAR of N compared to the control treatment, whereas XA and PXA increased (P<0.05) AME_n in a stepwise manner.

Given the similarities in the design and diets and the fact that diets without and with DDGS were used in each research site, combined analyses of the four trials were also performed, which increased the sensitivity of the analyses and allowed

Table 4
Effects of enzyme supplementation and maize-DDGS inclusion on apparent ileal digestible energy (AIDE), total tract apparent retention (TTAR) of nitrogen (N) and N-corrected apparent metabolisable energy (AME_n) in young broilers.[a,b,c].

Enzyme[d]	Maize-DDGS inclusion[e] (g/kg)	AIDE (MJ/kg dry matter)	TTAR of N (g/kg dry matter intake)	AME_n (MJ/kg dry matter)
Control		13.29[y]	21.8[y]	12.61[z]
XA		13.61[x]	22.2[y]	12.89[y]
PXA		13.70[x]	22.8[x]	13.00[x]
SEM		0.015	0.16	0.007
	0	13.51	21.7[y]	12.80
	70–100	13.55	22.8[x]	12.86
	SEM	0.012	0.13	0.006

Effect[f]	Probability		
	AIDE	TTAR of N	AME_n
Enzyme	<0.001	0.001	<0.001
Maize-DDGS	0.61	<0.001	0.11
Enzyme×maize-DDGS	0.47	0.97	0.74
Site	0.84	<0.001	<0.001
Site×enzyme	0.73	0.28	<0.001
Site×maize-DDGS	0.05	<0.001	0.27

[x,y,z] Means with no common superscripts within column and sub-grouping are different at P<0.05.
[a] Data from four independent trials with 21-d old broiler chickens, two without and two with dietary inclusion of maize-DDGS, were pooled.
[b] Trials 1 and 2 consisted of six replicates per treatment, with six birds per replicate cage. Trials 3 and 4 consisted of eight replicates per treatment, with five birds per replicate cage.
[c] Ileal digesta or excreta samples were pooled by cage. Cage was considered as experimental unit.
[d] Negative control diets were supplemented with an enzyme complex containing xylanase and amylase (XA), or one containing protease, xylanase and amylase (PXA).
[e] Diets from trials 1 and 3 did not contain maize-DDGS, whereas trial 2 used 100 g/kg and trial 4 used 70 g/kg maize-DDGS.
[f] A heterogeneous variance model by site was specified using the Variance Components structure of Proc Mixed (SAS, 1997).

studying the interaction between enzyme and diets. Due to heterogeneity of variance between trial sites, a heterogeneous variance model by trial site was specified in the mixed model, which improved fit statistics.

AIDE increased (P<0.05) with both enzyme combinations compared to the control diets (Table 4). The XA combination increased AIDE by 0.33 MJ/kg, whereas the PXA combination increased it by 0.41 MJ/kg compared to the control diet. AIDE of diets without and with maize-DDGS was similar (P>0.05). No interaction was evident between enzyme addition and maize-DDGS inclusion on the AIDE. The AME_n followed a similar trend compared to that of AIDE, but there was a difference (P<0.05) between XA and PXA. XA increased AME_n by 0.28 MJ/kg, while PXA increased AME_n by 0.39 MJ/kg compared to the control diets. Increments of AME_n due to enzymes were similar in diets without or with maize-DDGS, as indicated by the lack of enzyme×maize-DDGS interaction. TTAR of N increased (P<0.05) with PXA supplementation *versus* the control treatment (Table 4), whereas XA supplementation did not cause a significant change from the control diet. There were significant effects of trial site on TTAR of N and AMEn, which were expected due to differences in ingredient sources. No significant interactions of enzyme and site were evident for AIDE and TTAR of N, but this interaction was significant for AME_n. The effect of XA and PXA on AME_n compared to the control diets differed only in one of the sites and not in the other.

Significant (P<0.05–0.001) effects of enzymes on AID were evident for all analysed AA (Table 5). However, XA did not exhibit significant differences on the digestibility of any of the evaluated AA compared to the control diets, whereas PXA increased (P<0.05) the AID of N and all AA with the exception of methionine. The average increment in the AID of AA from the addition of PXA combination was 2.8%. There were significant effects of trial site on AID of AA for seven of the 17 analysed AA. Generally, no interactions between enzyme and trial site were present with the exception of asparagine, serine and threonine.

4. Discussion

Analyses of each of the four trials separately demonstrated more consistent effects of enzymes at the total tract level than the ileal level, both for AME_n and TTAR of N, although significant effects on AIDE and AID of N due to XA and PXA supplementation were present in one and two of the trials, respectively. This pattern may indicate that effects of these enzymes on nutrient digestibility were not limited to the small intestine, but the magnitude of the digestibility response increased during caecal digestion. Combined analyses of these four trials increased the statistical power and the ability to discern some of the main trends, in particular in terms of AID.

Increments of energy digestibility in response to dietary carbohydrases without and with proteases have been previously reported in broilers fed maize-based diets (Meng and Slominski, 2005; Cowieson and Ravindran, 2008), although some reports have not found effects in response to these enzyme combinations (Olukosi et al., 2007). Variability in energy responses to enzymes in poultry appears to depend on a number of factors, of which NSP structure and concentration are of greatest importance (Adeola and Cowieson, 2011). This study demonstrated increments in AIDE and AME_n in maize-based diets in

Table 5
Effects of enzyme supplementation and maize-DDGS inclusion on the apparent ileal digestibility (AID) coefficients of nitrogen (N) and amino acids in young broiler chickens.[a,b,c].

	Enzyme[d]				Maize-DDGS inclusion[e] (g/kg)			Probability[f]					
	Control	XA	PXA	SEM	0	70–100	SEM	Enzyme	Maize-DDGS	Enzyme×maize-DDGS	Site	Site×enzyme	Site×maize-DDGS
N	0.800^{y}	0.808^{xy}	0.818^{x}	0.004	0.821^{x}	0.797^{y}	0.003	0.006	<0.001	0.79	0.31	0.51	0.007
Asparagine	0.795^{y}	0.802^{y}	0.819^{x}	0.004	0.825^{x}	0.785^{y}	0.003	<0.001	<0.001	0.73	0.27	0.036	0.27
Threonine	0.733^{y}	0.739^{y}	0.765^{x}	0.005	0.765^{x}	0.726^{y}	0.004	<0.001	<0.001	0.68	0.030	0.024	0.08
Serine	0.792^{y}	0.792^{y}	0.816^{x}	0.004	0.813^{x}	0.787^{y}	0.003	<0.001	<0.001	0.47	0.06	0.003	<0.001
Glutamine	0.868^{y}	0.872^{y}	0.885^{x}	0.003	0.885^{x}	0.865^{y}	0.003	<0.001	<0.001	0.98	0.32	0.16	0.09
Proline	0.818^{y}	0.825^{y}	0.840^{x}	0.004	0.833^{x}	0.823^{y}	0.003	<0.001	0.022	0.94	0.08	0.41	<0.001
Glycine	0.756^{y}	0.765^{y}	0.783^{x}	0.005	0.784^{x}	0.753^{y}	0.004	<0.001	<0.001	0.75	0.05	0.08	0.014
Alanine	0.821^{y}	0.825^{y}	0.843^{x}	0.004	0.838^{x}	0.821^{y}	0.004	0.001	<0.001	0.86	0.97	0.17	0.043
Valine	0.792^{y}	0.801^{y}	0.818^{x}	0.005	0.819^{x}	0.788^{y}	0.004	0.001	<0.001	0.98	0.58	0.29	0.08
Isoleucine	0.814^{y}	0.821^{y}	0.836^{x}	0.005	0.839^{x}	0.807^{y}	0.004	0.003	<0.001	0.97	0.27	0.31	0.92
Leucine	0.837^{y}	0.840^{y}	0.860^{x}	0.004	0.851^{x}	0.840^{y}	0.003	<0.001	0.017	0.92	0.13	0.35	0.07
Tyrosine	0.829^{y}	0.834^{y}	0.852^{x}	0.004	0.853^{x}	0.823^{y}	0.003	<0.001	<0.001	0.74	0.65	0.17	0.83
Phenylalanine	0.844^{y}	0.847^{y}	0.864^{x}	0.004	0.861^{x}	0.843^{y}	0.003	<0.001	<0.001	0.87	0.008	0.25	0.25
Histidine	0.821^{y}	0.824^{y}	0.842^{x}	0.004	0.839^{x}	0.819^{y}	0.003	<0.001	<0.001	0.88	<0.001	0.14	0.08
Lysine	0.849^{y}	0.846^{y}	0.865^{x}	0.005	0.874^{x}	0.833^{y}	0.004	0.024	<0.001	0.91	0.019	0.09	0.45
Arginine	0.870^{y}	0.873^{y}	0.889^{x}	0.004	0.891^{x}	0.864^{y}	0.003	0.003	<0.001	0.81	0.001	0.09	0.59
Cysteine	0.705^{y}	0.714^{y}	0.743^{x}	0.006	0.740^{x}	0.702^{y}	0.005	<0.001	<0.001	0.70	<0.001	0.22	0.005
Methionine	0.917^{xy}	0.914^{y}	0.926^{x}	0.003	0.934^{x}	0.904^{y}	0.003	0.036	<0.001	0.98	0.003	0.34	0.42

^{x,y,z}Means with no common superscripts within a column are different at P<0.05.

[a] Data from four independent trials with 21-d old broiler chickens, two with and two without dietary inclusion of maize-DDGS, were pooled.

[b] Trials 1 and 2 consisted of six replicates per treatment, with six birds per replicate cage. Trials 3 and 4 consisted of eight replicates per treatment, with five birds per replicate cage.

[c] Ileal digesta or excreta samples were pooled by cage. Cage was considered as experimental unit.

[d] Negative control diets were supplemented with an enzyme complex containing xylanase and amylase (XA), or one containing protease, xylanase and amylase (PXA).

[e] Diets from trials 1 and 3 did not contain maize-DDGS, whereas trial 2 used 100 g/kg and trial 4 used 70 g/kg maize-DDGS.

[f] A heterogeneous variance model by site was specified using the Variance Components structure of Proc Mixed (SAS, 1997).

response to carbohydrases without or with protease. AIDE did not differ between XA and PXA fed broilers, but AME_n differed by 0.11 MJ/kg, suggesting that the presence of protease in PXA increased the digestion in the hind gut of broilers compared to XA. The lack of interaction between enzymes and diets on energy digestibility and TTAR of N indicated that responses of both enzyme combinations were positive and in the same order of magnitude irrespective of the presence of maize-DDGS.

Improvements of AID of AA due to combinations of carbohydrases in poultry diets have been previously reported (Rutherfurd et al., 2007), although other reports have not found positive effects (Boguhn and Rodehutscord, 2010). It has been suggested that part of the improvements in AA digestibility due to carbohydrases are explained by a reduction of mucin secretion caused by the enhanced digestion of fibre and reductions in fibre components that irritate the gut lining (Cowieson and Bedford, 2009). Nonetheless, evidence to support that hypothesis in chickens is not conclusive (Sharma et al., 1997; Fernandez et al., 2000). Amylase activity may also contribute to decreasing endogenous AA losses. Because starch is the major energy-yielding substrate in poultry diets, endogenous alpha-amylase secretions constitute an important part of the demands of AA and energy for digestion. In turn, pancreatic alpha-amylase expression has been demonstrated to be down-regulated as a feedback mechanism to the presence of exogenous amylase activity in the feed (Jiang et al., 2008). Reduced expression of amylase would ultimately result in increments of AID of AA and net energy.

Increments of AID of AA in broilers fed combinations of protease, xylanase and amylase have also been previously demonstrated (Cowieson and Ravindran, 2008). Nonetheless, the differential value of protease when applied in combination with xylanase and amylase had not been assessed. The current data indicate that the addition of protease was largely responsible for the observed increments in the coefficients of AID of AA. Even though XA did not exhibit significant effects on AA digestibility in the combined analysis of the four trials, it produced increments in AA digestibility in one of the trials (trial 1; data not shown). However, in the combined analyses, no evidence of interaction between trial site and enzymes was present with the exception of the AID of asparagine, threonine and serine. It may be hypothesised that the mechanism of action of the protease on AA digestibility is more dependent on the quality of protein in the diet, whereas effects of carbohydrases are more dependent on changes on endogenous secretions and the intestinal microbial environment. Although these aspects were not measured in the present studies, they might explain the variable responses to supplemental carbohydrases in different trials.

The reasons for the use of exogenous proteases in maize–SBM diets have been, first, to directly increase protein digestibility through the hydrolysis of dietary protein (Caine et al., 1998) and second, to disrupt protein–starch interactions in cereals, particularly maize (McAllister et al., 1993; Belles et al., 2000), which would improve the digestibility of starch. Under the conditions of the present studies, the effect of protease on top of carbohydrase had a clear influence on AA digestibility in the small intestine. There was no effect on AIDE, but an effect on the AME_n was present. These findings suggest that the direct hydrolysis of protein was more important in the small intestine and disruption of interactions of protein and other nutrients affected fermentation in the caecum. It appears, however, that post-ileal effects were not a function of the amount of NSP in the diet because there was no enzyme interaction with the presence of maize DDGS, which supplied additional insoluble NSPs.

The AA with the greatest digestibility response to PXA (Table 5) were cysteine (+5.4%), threonine (+4.4%), glycine (+3.6%) and valine (+3.3%). Threonine and cysteine have previously been reported as the most responsive to improvements in digestibility due to the PXA enzyme combination (Cowieson and Ravindran, 2008). These authors found improvements in AID of AA of +5.2% for threonine and +9.1% for cysteine when they included PXA on maize–SBM based diets. Similarly, Cowieson et al. (2006) reported increments on AID of +8.6% for threonine and +11.4% for cysteine with the inclusion of PXA and phytase in broilers fed maize–SBM diets. These AA, as well as valine, are particularly interesting to be included in matrix values of exogenous enzymes in diet formulations because they are limiting AA in maize–SBM broiler diets.

In contrast, the least responsive AA to PXA inclusion were methionine (+1.0), glutamine (+2.0), lysine (+1.9%) and arginine (+2.2%). Cowieson and Ravindran (2008) reported methionine (+0.5%), arginine (+0.8%) and glutamine (1.2%) as the AA with the lowest response to PXA, whereas lysine presented an intermediate response (+1.8%). Part of the low responsiveness of lysine and methionine in this trial may be explained by the inclusion of crystalline AA, which are highly digestible. The hydrolytic activity of exogenous proteases is not expected to affect crystalline AA. If it is assumed that proteolytic activity only affected the digestibility of the intact protein in the vegetable ingredients, the increment of AA digestibility of PXA would be underestimated in diets with inclusion of L-lysine. HCl and DL-methionine. Although the effect of PXA on lysine and methionine digestibility of the vegetable ingredients may be greater than it appears, this also means that improvements in digestibility of these two AA in practical diets are likely to be limited. Therefore, the effects of each of the most limiting essential AA should be considered independently when formulating diets supplemented with exogenous enzymes to avoid overestimating the contribution of the enzymes on the most limiting AA, particularly when they are supplemented with crystalline sources.

To further explore the reasons for the divergence in the digestibility response to PXA of different AA, the response relative to undigested fractions of each AA in the control diets was analysed (Fig. 1). It was previously suggested that the digestibility of nutrients is a major determinant of the digestibility responses to added exogenous enzymes in poultry diets (Cowieson, 2010). Analysing the data from 19 peer-reviewed studies, Cowieson (2010) reported a negative nonlinear relationship between improvements in the AID of AA due to supplemental xylanase and the AID of respective control diets, which suggests that a greater responses should be expected for AA with lower digestibility. Interestingly, the current study found that the best variable to explain the AA digestibility response to enzymes was not the digestibility coefficient, but the absolute amount of undigested AA of control diets at the ileal level. The amounts of ileal undigested AA were calculated by

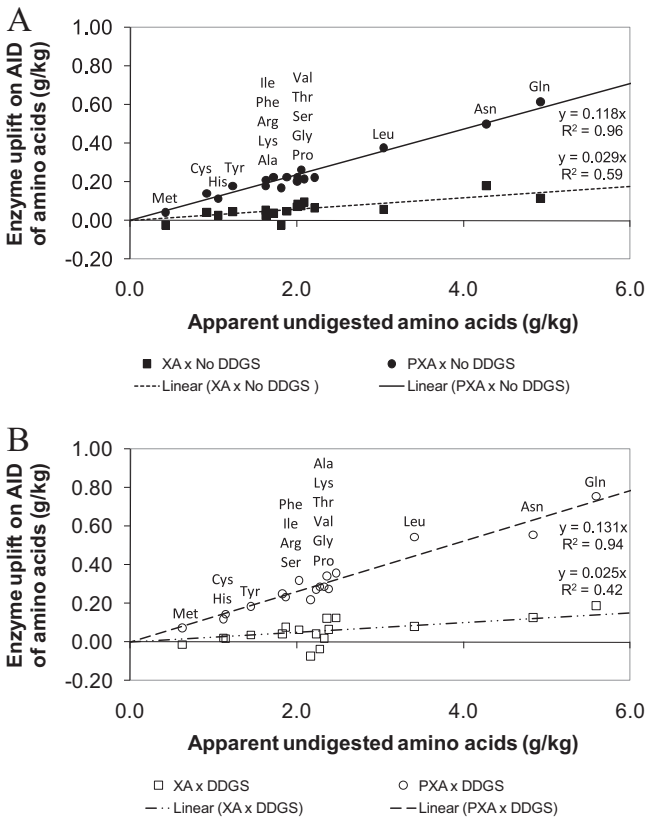

Fig. 1. Change in apparent ileal digestibility (AID) of amino acids of 21-d broiler chickens in response to enzymes relative to the amounts of ileal undigested amino acids of the control diets without (A) or with (B) 7–10% maize-DDGS inclusion (XA = xylanase and amylase; PXA = protease, xylanase and amylase).

subtracting the apparent digestible AA (g/kg feed) of the control treatment from the AA concentrations of the control diet for each AA (g/kg feed).

Irrespective of the AA, a strong linear relationship between the amount of undigested AA and the response to enzymes was evident for PXA (R^2 = 0.94 and 0.96 for maize–SBM and maize–SBM–maize-DDGS diets, respectively; P<0.0001), but not for XA (R^2 = 0.42–0.59 for maize–SBM and maize–SBM–maize-DDGS diets, respectively; P<0.01). These data indicate that PXA increased the AID of the undigested AA fraction by 12–13%. The difference in the relationship for PXA *versus* XA supplementation suggests that protein hydrolysis catalysed by the exogenous protease was responsible for the improvements in digestibility of AA. This effect of the protease was non-specific among AA. It is important to recognise that the apparently undigested fraction contains both undigested AA of dietary origin, as well as endogenous secretions. However, if a reduction of endogenous AA losses had occurred, specific AA that are important constituents of mucin, *i.e.* threonine, serine, aspartic acid, glutamic acid and glycine (Lien et al., 1996), would have increased in a greater proportion compared to the rest of AA in response to the PXA and XA treatments. It is possible that the effects of carbohydrases, *i.e.* xylanase, on mucin secretion, are less predictable because they depend on interactions with the gut wall and the intestinal microbiota. The direct effect of protease on dietary protein digestibility appears to be more predictable. This relationship did not significantly change in the diets tested in this study, although it is possible that the digestibility effects of these enzymes on the undigested fraction of other feed ingredients varies according to protein quality, which needs to be confirmed in future studies.

Results of the present studies were derived from broiler chickens fed mash diets, which may limit direct inferences to pelleted diets. Changes in chemical and structural characteristics of ingredients, particle size and chicken digestive development with pelleted diets (Amerah et al., 2007) may change the magnitude of the response of energy digestibility to different enzymes. Nonetheless, in reference to AA digestibility, pelleted diets may be expected to have a high potential for the action of exogenous enzymes, because poor gizzard development due to pelleting may not be beneficial to gastric digestion.

The present findings may allow the development of accurate AA matrices of exogenous enzymes for diet formulation, as long as reliable estimations of the undigested AA fraction in feed ingredients are available. Increments of energy and AA digestibility due to the use of exogenous carbohydrases and protease have the potential to reduce feed cost and contribute to reductions on the environmental footprint of poultry production systems.

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