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Original Research Article

Xylanase, protease and superdosing phytase interactions in broiler performance, carcass yield and digesta transit time

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ABSTRACT

The interaction of xylanase, protease and superdosing (1,500 FTU/kg) phytase in a 2 × 2 × 2 factorial arrangement was studied in broilers fed sorghum-based diets. A total of 2,800 one-day-old unsexed Ross 308 chicks were housed in 56 pens with 50 birds per pen, with or without inclusion of xylanase, protease and phytase, totaling 8 treatments and 7 replicates per treatment. Body weight (BW) and feed intake (FI) were measured at 21 and 42 days of age, and mortality corrected feed conversion ratio (FCR) was calculated for each period and cumulatively. Tibia ash and carcass yield were determined in 2 birds per replicate at 21 and 42 days of age, respectively. Digesta transit time was determined at 21, 28, 35 and 42 days of age using 5 birds per replicate. Results showed that superdosing phytase increased BW and FI at 42 days of age ($P < 0.05$) and xylanase improved FCR ($P < 0.05$). Xylanase and phytase also positively influenced carcass yield and breast weight, respectively. Overall, inclusion of superdosing phytase increased transit time when included in a diet containing xylanase, and no change with protease inclusion. In conclusion, the beneficial effects of xylanase, protease and superdosing phytase in broiler performance were not additive. This limitation is likely not related to the lack of efficacy of any one of the individual enzymes but to a limitation of the bird to respond additively to successive additions of enzymes.

1. Introduction

Sorghum is used in certain regions of the world as a main cereal in animal feeds. However, one of the constraints on the utilization of sorghum in the feed is the occurrence of some anti-nutritional factors, including phytic acid and tannins. Phytic acid and tannins have the ability to form complexes with proteins, carbohydrates and mineral nutrients, making them unavailable for digestion and absorption (Taylor, 2005; Selle et al., 2010). The elimination of such anti-nutritional compounds should improve the nutritional quality of sorghum and effectively utilize its full potential in feeds.

Enzymes became commercially available for use in monogastric nutrition at the end of the nineteen eighties, with use continually increasing to the present day. The most common enzyme in monogastric diets is phytase, used to increase the hydrolysis phytate and thus release phosphorus, reducing the need for the addition of expensive inorganic phosphorus sources to the diet. The second most common group is carbohydrase, initially used in viscous diets with high wheat, barley and rye inclusion and subsequently in corn and sorghum based diets, with the objective of improving nutrient absorption and animal performance (Masey O'Neill et al., 2012). Recently other enzymes types and applications have been developed, such as the use of high phytase inclusion rates to reduce the anti-nutritional effects of phytate rather than focusing simply on the release of phosphorous. This is referred to as superdosing (Walk et al., 2013). Inclusion of higher doses of phytase in broiler feed improves meat yield, reduces feed

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conversion and improves body weight gain (BWG) due to the restoration of a balance between minerals, reduction of endogenous losses caused by phytate, and provision of inositol (Cowieson et al., 2011). The dosage of phytase needed to achieve this response will vary according to the source of the phytase used (dos Santos et al., 2013; Manobhavan et al., 2016; Shirley and Edwards, 2003). Other enzymes have also become commercially available, such as proteases (Freitas et al., 2011), to improve protein digestibility and consequently animal performance.

Development of such enzymes are usually done in isolation without the presence of other enzymes (Masey O'Neill et al., 2014), but commercially more than one enzyme are often used together. The impact that one enzyme has on the response to a second or third enzyme has seldom been evaluated. Although each of the enzymes included may have activity focused on a different substrate, enzyme response may not be additive. Cowieson and Bedford (2009) hypothesized that animal response to carbohydrase addition depends on the indigestible fraction of the diet. Therefore, if an enzyme is included and reduces the indigestible fraction of the diet, it will also reduce the possible response when a second enzyme is included in the diet. Masey O'Neill et al. (2014) proposed that the correct way to evaluate the impact of different enzymes added to the same diet was through a factorial arrangement where each of these enzymes where included in isolation and in conjunction. Enzyme treatment has been demonstrated to improve digestibility of phosphorus in sorghum (Schons et al., 2011). Although the simultaneous application of phytase, xylanase and protease has not yet been reported in broiler chickens fed this grain.

The objective of this study was to investigate the interaction of xylanase, protease and superdosing phytase on broiler performance, carcass yield, bone ash content and digesta transit time in broilers fed sorghum-based diets.

2. Materials and methods

2.1. Birds and experimental design

A total of 2,800 one-day-old unsexed Ross 308 broilers were sourced specifically for the experiment and housed in 56 pens with 50 birds per pen. Pen dimensions were 2.0 m × 2.5 m, with birds stocked at 10 birds/m^2 at the beginning of the trial onto rice hull bedding. Birds had access to water drinkers and manual feeders, with both water and feed provided *ad libitum*. Treatments consisted of a 2 × 2 × 2 factorial arrangement with or without inclusion of xylanase, protease and phytase at a 'superdose' level (1,000 FTU/kg on top of the control diet that already contained 500 FTU/kg phytase following the nutritional matrix provided by the supplier: AvP 0.15%; Ca 0.165%; Na 0.035%; DLys 0.017%; DMet + DCys 0.039%; DThr 0.033%; AME 52 kcal/kg). Experimental designed comprised 8 dietary treatments with 7 replicates per diet (Table 1).

Table 1
Treatments description.

Treatment	Phytase, g/t	Xylanase, g/t	Protease, g/t
CON	100	–	–
XYL	100	100	–
PRO	100	–	200
SD PHY	300	–	–
XYL + PRO	100	100	200
XYL + SD PHY	300	100	–
PRO + SD PHY	300	–	200
ALL	300	100	200

CON = control; XYL = xylanase; PRO = protease; PHY = phytase; SD PHY = superdosing phytase.

2.2. Diets and experimental products

Ingredients were analyzed for protein, fiber, minerals and fat content prior to formulation. Diets were formulated as described in Table 2. Energy and amino acid levels were formulated marginally below requirement (NRC, 1994), so that improvements in digestibility and the reduction in anti-nutritional effects with enzyme inclusion could be translated into performance improvement. If the energy, amino acid and minerals matrixes attributed to protease, xylanase and phytase were included; all nutrients would be above requirements (Freitas et al., 2011; Masey O'Neill et al., 2012; Walk et al., 2013).

Starter diet was provided from 0 to 21 days of age; grower diet from 22 to 35 days of age and finisher from 36 to 42 days of age. An entire batch of each feed was produced and then divided for addition of the relevant enzyme to produce the different treatments. While re-mixing each diet, 3 samples of each feed were collected, mixed and a sub-sample taken for enzyme activity determination. Enzyme inclusions were made following the supplier's recommendations. The control diet was formulated with the inclusion of phytase at 100 g/ton (500 FTU/kg), taking into consideration its nutritional matrix as recommended by the supplier. For other treatments, xylanase was included at 100 g/ton (16,000 BXU/kg), protease at 200 g/ton (7,500 PROT/kg) and superdosing phytase at a further 200 g/ton (1,000 FTU/kg) at the expense of sorghum. No matrices were taken for these subsequent additions of enzyme. Maximum feed dilution caused by enzyme inclusion was 500 g/ton and was not considered to significantly interfere with overall nutrient level of the diets. Enzyme products used in this experiment were xylanase (Econase XT 25P, AB Vista,

Table 2
Diet formulation for control diet.[1]

Item	Starter	Grower	Finisher
Ingredient, g/kg			
Sorghum (9.5% CP)	640.4	656.1	692.8
Soybean meal (46% CP)	308.3	282.0	234.2
Soybean oil	15.3	29.0	40.8
Monodicalcium phosphate	10.3	9.40	7.48
Limestone	10.6	11.3	11.5
NaCl	3.05	3.10	2.60
DL-Methionine	2.94	2.15	2.26
Lysine·HCl	3.57	2.13	3.16
Choline chloride	1.00	1.00	1.00
L-threonine	0.90	0.24	0.69
Sodium bicarbonate	1.00	1.00	1.00
Phytase[2]	0.10	0.10	0.10
Vitamin/Mineral premix[3]	2.50	2.50	2.50
Calculated nutritional value, %			
Crude protein	22.0	20.0	18.0
Metabolizable energy, kcal/kg	3,000	3,100	3,200
Crude fiber	2.40	2.31	2.18
Calcium	0.90	0.90	0.85
Phosphorous	0.56	0.54	0.50
Available phosphorous	0.46	0.44	0.40
Sodium	0.20	0.20	0.18
Total lysine	1.24	1.05	1.00
Total methionine + cysteine	0.87	0.76	0.72
Total threonine	0.79	0.68	0.65

[1] Enzymes were included in test diets at the expense of sorghum.
[2] Quantum Blue 5G, AB Vista formulated considering a nutrient equivalence of 52 kcal/kg metabolizable energy; 0.421% crude protein; 0.017% lysine; 0.039% methionine + cysteine; 0.033% threonine; 0.035% sodium; 0.15% available phosphorus and 0.165% calcium.
[3] Supplied per kilogram diet: iron (Ferrous sulfate), 60 mg, manganese (Manganese sulfate and manganese oxide), 120 mg; zinc (Zinc oxide), 100 mg; iodine (Calcium iodate), 1 mg; copper (Copper sulfate), 8 mg; selenium (Sodium selenite), 0.3 mg, vitamin A, 9,600 IU; vitamin D_3 3,600 IU; vitamin E, 18 mg; vitamin B_{12}, 15 μg; riboflavin, 10 mg; niacin, 48 mg; D-pantothenic acid, 18 mg; vitamin K, 2 mg; folic acid, 1.2 mg; vitamin B_6, 4 mg; thiamine, 3 mg; d-biotin, 72 μg.

Marlborough, UK; 160,000 BXU/g), protease (Ronozyme ProAct, Royal DSM, Heerlen, the Netherlands; 75,000 PROT/g) and phytase (Quantum Blue 5G, AB Vista, Marlborough, UK; 5,000 FTU/g).

2.3. Enzyme activity determination

Activity of xylanase and phytase was determined using the reference method of analysis recommended by the supplier (at Enzyme Services Consultancy, Ystrad Mynach, UK). Protease activity, due to a laboratory restriction, had to use a different method than the recommended by the supplier, in this case enzyme activity was performed originally in the product and the expected activity in the feed calculated based on the recommended inclusion level used at feed formulation. The authors understand that is not the optimal evaluation process but understand that the good correlation between expected and analyzed activity in the feed and the absence of protease activity in feed samples without protease inclusion supports that the inclusion rate of the enzyme and its distribution in the feed was done appropriately. Phytase activity was determined at pH 5.5 and 37 °C, using sodium phytate as substrate (Gizzi et al., 2008), xylanase activity was determined at pH 5.3 and 50 °C, using birchwood xylan as a substrate (Bailey et al., 1992) and protease activity was determined at pH 7.5 and 50 °C, using casein as a substrate (NFIA, 1991).

2.4. Sample collection

Birds were weighed by pen at 0, 21 and 42 days of age, to measure mean BW and calculate BWG for each period and cumulatively (0 to 42 days of age). Feed intake (FI) was measured by period, and mortality corrected FCR calculated for each period and cumulatively. Mortality and room temperature were recorded daily, with culled and dead birds weighed daily. At 21 days of age, 2 birds per replicate were euthanized by cervical dislocation and the left tibia collected for bone ash determination. Tibia samples were cleaned, de-fatted and weighed prior to ash determination (Garcia and Dale, 2006). At 42 days of age, 2 male broilers per replicate were selected and euthanized by cervical dislocation for carcass yield determination. Birds were weighed for original body weight determination, followed by feather, head, feet, viscera and abdominal fat extraction. Carcass was weighed and carcass yield calculated as the proportion of the carcass against the original BW. Breast meat was separated from the carcass and weighed. Breast yield was calculated as the proportion of the original BW.

2.5. Digesta transit time

Digesta transit time was determined at 21, 28, 35 and 42 days of age. At each of these ages, 5 male birds per replicate were separated and allocated to cages. Animals were provided with the same diets as provided to the original replicates but including 2 kg/ton of ferric oxide as an indigestible marker. Animals had restricted access to feed for 30 min before being offered feed with the marker. Transit time was determined as the period between when feed with added ferric oxide was provided to the animals until the time when the red color was first visible in the faeces. Birds that had red color in the faeces were returned to the pen from which they were originally collected. Results were the average time for the 5 animals in each cage.

2.6. Statistical analysis

The performance, bone ash and digesta transit time data were subjected to ANOVA using the GLM models for completely randomized design procedure of Minitab. Percent livability data were arc sine transformed before analysis, but are reported untransformed to facilitate understanding. Pen served as the experimental unit for FI, BWG, FCR and livability, and cage as experimental unit for digesta transit time. When the effects were found to be significant, treatment means were separated using Tukey's Honest Significant Difference test. Statistical significance was accepted at $P < 0.05$ and trends were discussed at $P < 0.10$. As liveability was affected by treatments at 42 days of age ($P < 0.05$), feed conversion for that period was not corrected for mortality.

3. Results

Analyzed enzyme activities in feed samples are listed in Table 3. Enzyme activities of all samples were close to expected.

3.1. Animal performance and tibia ash

At 21 days of age, FI and BWG were higher ($P < 0.05$) in birds fed superdosing phytase, and FCR improved ($P < 0.05$) by protease inclusion (Table 4). A 3-way interaction was observed for tibia ash % at 21 days of age ($P < 0.05$); birds fed the diet with all enzymes had a lower tibia ash concentration than those fed the diet with xylanase and superdosing phytase. Tibia ash contents for all treatments were similar to the control diet. At 42 days of age, both FI and BWG were increased ($P < 0.05$) by superdosing phytase (Table 5). Animals fed diets with xylanase had improved FCR ($P < 0.05$). Livability was reduced in birds fed protease plus superdosed phytase when compared with birds fed only superdosed phytase.

3.2. Carcass yield

Carcass yield was improved by xylanase ($P < 0.05$) and a similar trend ($P < 0.10$) was observed for superdosing phytase; no interaction between enzymes was observed (Table 6). Protease inclusion tended to reduce breast meat yield ($P < 0.10$). Although no differences were observed in the weight of the animals used for carcass and breast meat yield determination, birds fed the xylanase had a heavier carcass ($P < 0.05$), while birds fed superdosed phytase tended to have heavier carcass ($P < 0.10$) and had heavier breast weights ($P < 0.05$).

3.3. Transit time

As expected, digesta transit time increased as animals got older (Table 7). Birds at 42 days of age had longer transit time when compared with all other ages, and birds at 35 days of age had longer transit time when compared with birds at 21 and 28 days of age. No differences between the control group and enzyme treated groups were observed at 21 and 28 days of age. At 35 days of age, birds fed superdosed phytase or the combination of xylanase and protease had shorter transit time than the control group, while at 42 days of age birds fed superdosed phytase had longer transit time than those of the control group. Overall, inclusion of superdosed phytase increased transit time when included in a diet containing xylanase, with no further change with the further addition of protease.

4. Discussion

The use of enzymes for broilers has become routine in commercial production worldwide, although studies regarding possible synergies or limitations in the use of more than one enzyme are still uncommon (Adeola and Cowieson, 2011), even less so in experiments with full factorial arrangements where all enzymes are tested in all combinations. Furthermore, the information in the literature regarding enzyme supplementation of sorghum-based diets is limited. The objective of the present trial was to investigate the

Table 3
Analyzed enzyme activity in feed samples.

Treatment	Phytase, FTU/kg[1]			Xylanase, BXU/kg[2]			Protease, U/kg[3]		
	Starter	Grower	Finisher	Starter	Grower	Finisher	Starter	Grower	Finisher
CON[4]	403	559	651	2,860	2,520	2,930	nd[8]	nd	nd
XYL[5]	551	382	463	21,800	22,800	20,700	nd	nd	nd
PRO[6]	577	483	352	2,870	3,580	2,830	9,000	7,400	7,000
SD PHY[7]	2,030	1,220	1,680	2,390	2,950	2,740	nd	nd	nd
XYL + PRO	534	499	730	23,700	23,700	21,700	7,500	6,300	7,000
XYL + SD PHY	1,790	1,280	1,540	23,500	25,500	17,800	nd	nd	nd
PRO + SD PHY	1,650	1,770	1,450	1,960	2,690	2,920	7,500	6,000	6,000
ALL	1,970	1,580	2,020	20,200	26,700	19,200	7,500	6,700	6,000

CON = control; XYL = xylanase; PRO = protease; PHY = phytase; SD PHY = superdosing phytase.
[1] One FTU is defined as the amount of enzyme required to release 1 μmol of inorganic P per min from sodium phytate at 37 °C and pH 5.5.
[2] One BXU is defined as the amount of enzyme that produces 1 nmol reducing sugars from birchwood xylan in 1 s at 50 °C and pH 5.3.
[3] One U is defined as the amount of enzyme that produces small peptides and amino acids from casein in 1 min at 50 °C and pH 7.5.
[4] Expected activity 500 FTU/kg.
[5] Expected activity 16,000 BXU/kg.
[6] Expected activity 6,000 U/kg, based on product analysis and inclusion rate.
[7] Expected activity 1,500 FTU/kg.
[8] Below detection limit (2,000 U/kg).

Table 4
Effect of xylanase, protease and superdosing phytase on feed intake (FI), body weight gain (BWG), feed conversion ratio (FCR), livability (Liv) and tibia ash (TA) of broilers at 21 days of age.[1]

Treatment		FI, g	BWG, g	FCR, g/g	Liv, %	TA, %
CON		1,011	647	1.551	97.71	52.28[ab]
XYL		1,015	653	1.543	98.57	51.49[ab]
PRO		1,006	652	1.537	98.86	52.29[ab]
SD PHY		1,037	665	1.560	98.86	51.32[ab]
XYL + PRO		1,003	655	1.526	98.00	52.27[ab]
XYL + SD PHY		1,042	668	1.559	98.57	52.60[a]
PRO + SD PHY		1,034	671	1.527	97.43	52.32[ab]
ALL		1,024	669	1.521	97.43	50.62[b]
Std dev		28	20	0.037	1.88	1.69
XYL	+	1,021	661	1.537	98.14	51.75
	−	1,022	659	1.544	98.22	52.05
PRO	+	1,017	662	1.528[a]	97.93	51.88
	−	1,026	658	1.553[b]	98.43	51.92
SD PHY	+	1,034[a]	668[a]	1.542	98.07	51.72
	−	1,009[b]	652[b]	1.539	98.29	52.08
P-value						
XYL		0.886	0.594	0.474	0.887	0.498
PRO		0.133	0.472	0.008	0.354	0.917
SD PHY		0.001	0.004	0.785	0.704	0.420
XYL × PRO		0.364	0.708	0.791	0.510	0.222
XYL × SD PHY		0.805	0.708	0.743	0.904	0.833
PRO × SD PHY		0.895	0.979	0.281	0.143	0.328
XYL × PRO × SD PHY		0.726	0.936	0.963	0.357	0.042

CON = control; XYL = xylanase; PRO = protease; PHY = phytase; SD PHY = superdosing phytase.
[a,b] Means within the same column with different superscripts are different ($P < 0.05$).
[1] Means represent 50 birds per replicate pen and 7 replicates per treatment for FI, BWG, Liv and FCR, and 2 birds per replicate and 7 replicates per treatment for TA.

Table 5
Effect of xylanase, protease and superdosing phytase on feed intake (FI), body weight gain (BWG), feed conversion ratio (FCR) and livability (Liv) of broilers at 42 days of age.[1]

Treatment		FI, g	BWG, g	FCR, g/g	Liv, %
CON		4,467	2,506	1.783	95.71
XYL		4,400	2,513	1.751	96.86
PRO		4,393	2,508	1.752	97.43
PHY		4,474	2,509	1.784	98.00
XYL + PRO		4,430	2,512	1.764	97.14
XYL + PHY		4,479	2,549	1.758	97.43
PRO + PHY		4,495	2,534	1.774	96.57
ALL		4,483	2,563	1.749	95.14
Std dev		70	44	0.029	2.31
XYL	+	4,448	2,534	1.756[b]	96.64
	−	4,457	2,514	1.773[a]	96.93
PRO	+	4,450	2,529	1.760	96.57
	−	4,455	2,519	1.769	97.00
SD PHY	+	4,483[a]	2,539[a]	1.766	96.79
	−	4,423[b]	2,510[b]	1.763	96.79
P-value					
XYL		0.603	0.071	0.011	0.630
PRO		0.774	0.373	0.191	0.488
SD PHY		0.001	0.011	0.575	0.985
XYL × PRO		0.209	0.756	0.102	0.349
XYL × SD PHY		0.752	0.185	0.241	0.248
PRO × SD PHY		0.325	0.366	0.996	0.024
XYL × PRO × SD PHY		0.090	0.857	0.123	0.824

CON = control; XYL = xylanase; PRO = protease; PHY = phytase; SD PHY = superdosing phytase.
[a,b] Means within the same column with different superscripts are different ($P < 0.05$).
[1] Means represent 48 birds per replicate pen and 7 replicates per treatment.

interaction of xylanase, protease and high levels of phytase (without considering its additional matrix; superdosing) on performance, carcass yield, bone ash content and digesta transit time.

Overall bird performance was below expectation for the genetic line (Aviagen, 2012). The marginal amino acid content of the rations are one explanation and these were implemented in order to give the enzymes employed an opportunity to demonstrate their potential. Coupled with this, the relatively low initial weight of the birds at d 0 may have played a role, as initial weight at hatch may affect subsequent animal performance. dos Santos et al. (2010) observed that smaller day-old chicks (from lighter eggs) had lower BWG during all growing periods up to 42 days of age, with a 3 g difference at 0 day of age resulting in a 55 g difference at 42 days of age. In the present trial, birds were 7 g lighter than expected (average weight 35 g), and this may have affected the final performance at 42 days of age.

Xylanases reduce digesta viscosity by reducing the molecular weight of soluble xylan (Annison, 1992), thereby increasing contact time of endogenous enzymes with substrate nutrients. The oligosaccharides resulting from the action of xylanases in the gut can also modulate the microbiota in the hindgut (Bedford and Cowieson, 2012). Such oligosaccharides have been shown to improve FCR in broilers (Courtin et al., 2008). It is possible that the fermentation of these oligosaccharides, particularly in the caeca, influences the release of gut hormones which are implicated in gut health and gastric retention (Goodlad et al., 1987; Singh et al., 2012), and thus, potentially, digestibility.

Protease improves protein digestibility and amino acid absorption (Angel et al., 2011), and can improve performance in broilers

Table 6
Effect of xylanase, protease and superdosing phytase on carcass and breast weight and yield of broilers at 42 days of age.[1]

Treatment		Live weight, g	Carcass, g	Carcass, %	Breast, g	Breast, %
CON		2,679	2,079	77.59	639	30.75
XYL		2,706	2,114	78.14	657	31.06
PRO		2,699	2,112	78.24	650	30.74
PHY		2,691	2,107	78.31	662	31.42
XYL + PRO		2,687	2,119	78.84	643	30.32
XYL + PHY		2,709	2,150	79.36	685	31.90
PRO + PHY		2,670	2,111	79.05	647	30.64
ALL		2,713	2,149	79.24	663	30.84
Std dev		46	46	1.25	32	1.30
XYL	+	2,704	2,133[a]	78.90	662	31.03
	−	2,685	2,102[b]	78.30	650	30.89
PRO	+	2,692	2,123	78.84	651	30.64
	−	2,696	2,113	78.35	661	31.28
SD PHY	+	2,696	2,129	78.99[a]	664[a]	31.20
	−	2,693	2,106	78.20[b]	647[b]	30.72
P-value						
XYL		0.143	0.012	0.070	0.123	0.669
PRO		0.753	0.402	0.133	0.190	0.052
SD PHY		0.826	0.055	0.019	0.039	0.148
XYL × PRO		0.796	0.496	0.533	0.319	0.442
XYL × SD PHY		0.368	0.416	0.945	0.377	0.552
PRO × SD PHY		0.723	0.462	0.575	0.294	0.413
XYL × PRO × SD PHY		0.222	0.613	0.484	0.596	0.732

CON = control; XYL = xylanase; PRO = protease; PHY = phytase; SD PHY = superdosing phytase.
[a,b] Means within the same column with different superscripts are different ($P < 0.05$).
[1] Means represent 2 birds per replicate pen and 7 replicates per treatment.

Table 7
Effect of xylanase, protease and superdosing phytase on digesta transit time (min) of broilers at 21, 28, 35 and 42 days of age.[1]

Treatment		21 days of age	28 days of age	35 days of age	42 days of age
CON		182	194	279	218
XYL		149	184	252	205
PRO		244	164	238	268
PHY		198	182	185	339
XYL + PRO		203	177	186	264
XYL + PHY		188	217	238	331
PRO + PHY		158	234	298	222
ALL		200	218	246	285
Std dev		33	34	44	52
XYL	+	185	199	231	271
	−	196	194	250	262
PRO	+	201[a]	198	242	260
	−	179[b]	194	239	273
SD PHY	+	186	213[a]	242	294[a]
	−	195	180[b]	239	239[b]
P-value					
XYL		0.179	0.597	0.061	0.251
PRO		0.007	0.694	0.747	0.106
SD PHY		0.274	0.003	0.825	0.001
XYL × PRO		0.169	0.475	0.003	0.025
XYL × SD PHY		0.002	0.713	0.046	0.039
PRO × SD PHY		0.001	0.032	0.001	0.001
XYL × PRO × SD PHY		0.065	0.077	0.058	0.069

CON = control; XYL = xylanase; PRO = protease; PHY = phytase; SD PHY = superdosing phytase.
[a,b] Means within the same column with different superscripts are different ($P < 0.05$).
[1] Means represent 7 replicates per treatment, each replicate with 5 birds.

(Freitas et al., 2011). These effects can be greater in situations where protein digestibility is naturally impaired. Peek et al. (2008) reported that protease supplementation improved broiler performance, but this was more prominent when the enzyme was fed to coccidiosis-challenged broilers.

Inclusion of higher levels of phytase without considering the full mineral matrix (superdosing), which targets the reduction of the anti-nutritional effects of phytate, has been proposed as a possible tool to improve performance of monogastrics (Cowieson et al., 2011), and positive effects have already been reported (dos Santos et al., 2013; Walk et al., 2013). The improvement in performance of broilers fed diets with high phytase inclusion but not limited in phosphorus is attributed to the reduction in anti-nutritional effects of phytate, leading to an improvement in nutrient absorption and inositol formation in the gut.

In the present trial, protease inclusion improved FCR during the starter phase, xylanase improved FCR and tended to increase BWG from 0 to 42 days of age and superdosing phytase increased FI and BWG at both 21 and 42 days of age. The current results supports some of the hypotheses above, as younger birds general digest and absorb amino acids less efficiently (Cowieson et al., 2010), enabling protease to show performance improvement in the starter phase, whereas any effect on the modulation of lower gut microbiota, such as those possibly influenced by xylanase, could take longer to show an effect. The ability of both superdosing phytase and xylanase to improve nutrient digestibility is supported by the effects observed at processing, where superdosing phytase improved carcass yield and breast weight and tended to increase carcass weight while xylanase improved carcass weight and tended to increase carcass yield.

In the present trial, digesta transit time was similar at 21 and 28 days of age, then increasing to 35 days of age and further to 42 days of age. Superdosing phytase in diets which contained xylanase increased digesta transit time but no further change was observed when protease was added to this combination. A longer transit time may allow for more complete nutrient digestion and absorption and consequently better performance efficiency, provided the intake of digestible nutrients on a daily basis is not compromised by associated reductions in intake. The effects of these enzymes on transit appear to be more apparent in older animals, when transit time naturally increases. Kras et al. (2013) provided diets with high fiber (wheat bran and oat hulls) contents to broilers and observed no difference in digesta transit time with younger birds (19 days of age) compared to birds fed a regular corn/soybean meal diet; however, at 38 days of age birds fed a regular diet had longer digesta transit time than those fed the high fiber diet. The fact that some enzymes take time to influence digesta transit times of broilers may be one of the reasons why improvements in performance sometimes only appear later in the life cycle.

Measuring tibia ash at 21 days of age is a reliable method to determine the P and Ca requirement of broilers. Tibia ash determination in the present trial was done to evaluate if the control treatment had sufficient levels of minerals to sustain animal performance and bone development. As some of the treatments had a high inclusion of phytase, an enzyme that has a direct effect in the digestibility of these minerals, it was important to assure that any improvement observed in these treatments with extra phytase was related to a reduction in anti-nutritional effects of phytate and not due to the control diets being formulated below requirements. Although a 3-way interaction was observed, tibia ash content in birds fed the control diet was not different from any other treatment, indicating that P and Ca were not limiting bird performance. This suggested that the improvement in FI and BWG observed in the superdosing phytase treatment was not related to a higher P absorption, but to a reduction in the anti-nutritional effects of phytate (dos Santos et al., 2013; Walk et al., 2013).

5. Conclusion

Although positive effects have been observed following enzyme inclusion in the present trial with sorghum-based diets, few

additive effects have been observed, as shown by the lack of further improvements in performance when enzymes were used in combination. These results support the hypothesis that the presence of an enzyme that improves nutrient absorption will reduce the possible response to a second or third enzyme (Adeola and Cowieson, 2011). In the present trial, all enzyme inclusions had some beneficial effects on at least one parameter during bird development, but a lack of any additive response, with further improvements when all enzymes were used together, shows that enzyme response may be limited not by a substrate limitation, as all enzymes used in this trial had activity against different substrates, but by the inability of the animal to further improve performance. An important point to make here, however, is the significant benefits of xylanase and protease when there was already a standard dose of phytase (and associated calcium/phosphorous matrices) present in the diet. Commonly, phytases are left out of experiments with 'secondary' enzymes.

Conflict of interests

All authors declare no conflicts of interest.

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