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E-mail: editorijps@gmail.com

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Research Article
Effect of an Exogenous Protease in Association with Carbohydrases in Broilers Infected with Coccidia

[1,2]J.V. Caldas, [1,3]M.A. Sabir, [1,4]M. Putsakum, [1]J.A. England and [1]C.N. Coon

[1]Center of Excellence for Poultry Science, University of Arkansas, Fayetteville, AR 72701, USA
[2]Cobb-Vantress, P.O. Box 1030, Siloam Springs, Arkansas, USA
[3]Sabir's Group Companies, 10 Shershah Block, New Garden Town, 54600 Lahore, Pakistan
[4]School of Agriculture and Cooperatives, 11120 Nonthaburi, Thailand

Abstract

Background: Live coccidiosis vaccines given to broilers at hatch generally decrease body weight gain during the early feeding phases but the effect could reverse after the development of immunity. This study intended to determine if diets supplemented with exogenous protease and carbohydrases can improve Body Weight Gain (BWG) and Feed Conversion Ratio (FCR) after coccidia infection. **Methodology:** Two thousand three hundred and four male chicks were randomly divided into six diets: Negative Control (NC), Positive Control (PC) and 4 multi-enzyme composites (MEC), all enzyme diets had protease but different carbohydrase combinations for 42 days study. The ANOVA test was utilized. **Results:** Three different MEC decreased the *E. coli* population in the ileum. Diets with MEC provided an additional apparent metabolizable energy corrected by nitrogen (AMEn) from 91-236 kcal kg^{-1} compared to the NC and improved digestibility of Amino Acids (AA) from 0.86-5.53% for 3 of the MEC. Cystine, threonine and serine digestibility were each increased >2.8% with MEC compared to the NC. Proteins in mucins contain high quantities of these AA, so enzymes may be providing more of these AA. The FCR for NC broilers was worse than PC ($p \leq 0.05$). The FCR, tended to be improved with three of the MEC, however one composite did not achieve better FCR. **Conclusion:** The MEC improved nutrient utilization with tendency to improve FCR. However, more time may be required to achieve compensatory BWG using MEC with a coccidia infection. The present study also opens the door to study the interaction of MEC and microflora population in the gastrointestinal tract of chickens.

Key words: Coccidia, broiler, protease, carbohydrase, MEC

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Corresponding Author: C.N. Coon, Center of Excellence for Poultry Science, University of Arkansas, Fayetteville, AR 72701, USA
Tel: 479-575-4134 Fax: 479-575-3026

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The global meat consumption is projected to grow 1.4% annually during the decade 2015-2024 according to OECD. and FAO.[1]. Poultry meat is estimated to be half of this growth, increasing from 111.9 Mt (million tons) in 2015 to 133.8 Mt in 2024. The expected increase in poultry meat production will demand producers provide poultry meat more efficiently in time and cost. Broilers can attain market weight of 2.8 kg in 6 weeks[2] but any type of disease or gastrointestinal challenge will change performance expectations. Coccidiosis is considered the disease with the greatest economic impact on the poultry industry. Coccidiosis is a parasite existing in most broiler houses around the world. Broiler integrators presently utilize coccidiosis vaccines in a yearly rotation system with chemical and ionophore coccidiostats in the feed in order to minimize coccidia resistance in the field. A review of the economic impact of coccidiosis is outlined by Amerah and Ravindran[3]. In brief, the researchers reports coccidiosis as an expensive disease that cost the US poultry industry 3.2 billion USD per year. Clinical coccidiosis is usually harmful when the birds are 21 days and older so vaccines are applied at hatch or the 1st week of age as suggested by Chapman[4]. The oocysts' shedding generally peaks from 6-9 days after vaccinating day old broiler chicks with coccidia[4]. Coccidia infection negatively affects the digestion and absorptive capacities in the small intestine because of the intestinal villi damage[5,6]. Adams *et al*.[5] inoculated broilers with *Eimeria* oocysts as an infection model to study nutrient digestion and retention and proved that there is an increase in energetic costs caused by coccidiosis because of a greater epithelial turnover in chickens infected with coccidia compared to epithelial turnover in chickens not infected with coccidia. Fernando and McCraw[7] reported the same findings. Even though a live coccidiosis vaccine is essential to develop an immune response against coccidiosis, the coccidiosis challenge through the vaccine will interfere with optimum BWG and FCR in broilers[4]. Mathis[8] showed broilers had a compensatory growth when vaccinated at hatch. Birds decreased BWG with a poorer FCR at 21 days but this poorer performance was reversed from 29-42 days after infection at hatch and showed an accelerated rate of gain and improvement in FCR following the development of immunity. The coccidiosis vaccines may increase the opportunity of using protease and carbohydrase enzymes in the feed to supply additional amino acids and energy during times of low feed intake that may limit protein and energy digestion (Teeter in 2012 personal communication). Chicks fed corn-soybean meal diets and infected with coccidia have poorer fat digestibility[5,6], protein digestibility, Metabolizable Energy (ME) and amino acid digestibility[9]. The reductions in nutrient digestibility are proposed to be caused by a reduction of endogenous maltase, sucrase and protease[10] activities in the digesta. The objective of the present study was to determine if broilers inoculated with live coccidia show an improvement in nutrient digestibility, BWG, FCR when exogenous protease associated with carbohydrases are added to the diets during 1-42 days of grow out.

MATERIALS AND METHODS

All management practices and procedures were approved by the University of Arkansas Institutional Animal Care and Use Committee (IACUC) #12041.

Birds and housing: Male chicks (2304) of a commercial strain[2] were obtained from a local hatchery, where they were vaccinated in ovo for Marek's disease. The chicks were placed in 48 floor pens. Pen area (4.5 m^2) contained 48 chicks per pen. Each pen was equipped with 10 nipples per line, two hanging type feeders with a round pan that provided 208 cm of feeder space per pen. The broilers were raised on cement floors with new litter (softwood shavings). The lighting program was 23 h light: 1 h dark. The broiler chicks were weighed on 1, 14, 21, 28 and 42 days. The initial Body Wight (BW) (1 day) was the same for all the treatments (Mean 38.6 g±SD 0.55). Feed was weighed back at the end of each dietary phase 1-14, 15-21, 15-28 and 29-42 days. Feed Conversion Ratio (FCR) was calculated as feed intake/BWG. Mortality and weight of deceased broilers were recorded daily and the FCR at 42 days was corrected by mortality weight. On 15 days, five broilers per floor pen (48 pens) were moved to metabolic cages (48 cages) for a week evaluation after the chicks were weighed and fed the coccidia inoculum. Two hundred and forty broilers in total were used with 8 replications per treatment to evaluate microorganism profile, viscosity, grower diet ileal amino acid digestibility, nitrogen, fat, phosphorus, starch and Neutral Detergent Fiber (NDF) digestibility and apparent metabolizable energy corrected for nitrogen (AMEn). Broilers selected for metabolic evaluation had the same BW as the broilers in the pens. Since the BW at 14 days was not significantly different between treatments, the broilers were selected to have a mean 290±SD 35 g for all the treatments. The broiler chicks in the metabolic cages underwent 4 days of adaptation (15-18 days) before the excreta and ileal content were collected in the last 3 days of evaluation (19-21 days).

Table 1: Dietary treatments

Treatments	Abbreviation	Description	Minimum content (U kg^{-1} feed)	Dose (g Mt^{-1})
Negative control	NC	3100 kcal AMEn grower/19% protein	-	-
Positive control	PC	120% NC, PC and amino acids	-	-
NC+protease+carbohydrases 1	PG	Protease	15,000 PROT	200
		β-glucanase	7.5 FBG	150
NC+protease+carbohydrases 2	PAX	Protease	15,000 PROT	200
		α-amylase	25.6 kNU	160
		Endo-xylanase	0.3 FXU	40
NC+protease+carbohydrases 3	PX	Protease	15,000 PROT	200
		endo-1,4 β-xylanase	26.0 U	110
NC+protease+carbohydrases 4	PXA	Protease	15,000 PROT	200
		α-amylase	6.4 kNU	40
		Endo-xylanase	0.9 FXU	160

Experimental diets: The broilers were fed a starter diet from 1-14 days, a grower diet from 15-28 days and a finisher diet from 29-42 days. Six dietary treatments with 8 replicates each were used in the feeding study including a Negative Control (NC) and a Positive Control (PC). The PC had 20% more protein and amino acids compared to NC. Four different enzyme composites were added on-top of the NC basal to produce the respective dietary treatments: T1 (NC), T2 (PC), T3 (NC+protease 200 g/Mt+glucanase 150 g/Mt) (PG), T4 (NC+protease 200 g/Mt+amylase 160 g/Mt+xylanase 40 g/Mt) (PAX), T5 (NC+protease 200 g/Mt+xylanase 110 g/Mt) (PX) and T6 (NC+protease 200 g/Mt+xylanase 160 g/Mt+amylase 40 g/Mt) (PXA). The xylanase in T5 is produced by a different microorganism than the xylanase in T4 and T6 (Table 1). The dose level of the enzymes for each treatment were the same for starter, grower and finisher. The protease that was common in T3, T4, T5 and T6 is a granulated serine protease with chymotrypsin specificity from *Nocardiopsis prasina* (donor microorganism) expressed in *Bacillus licheniformis* (host or production microorganism). The glucanase in T3 comes from a multi-component enzyme produced by fermentation of *Aspergillus aculeatus*, however, it has been standardized only for endo-1,3(4)β-glucanase. This multi-component enzyme has also hemicellulase and pectinase activities[11]. The α-amylase in T4 and T6 is produced by fermentation of *Bacillus amyloliquefaciens*. The xylanase in T4 and T6 is produced from *Thermomyces lanuginosus* expressed and produced by fermentation of *Aspergillus oryzae*. The other xylanase in T5 is part of a multi-component enzyme from *Trichoderma longibrachiatum*. This microorganism also produces endo-1,4-β-glucanase but only the main enzyme is cited in treatments (Table 1). Diets consisted of a corn-soybean meal basal formulated to provide the Cobb 500 nutrient specs[2] (Table 2). Titanium dioxide 0.5% was added as a marker in the grower diets for nutrient digestibility analysis and apparent metabolizable energy corrected for nitrogen (AMEn). Major ingredients such as corn and soybean meal and minor ingredients such as wheat middlings and distiller's dried grain with solubles (DDGS) were analyzed with Near Infrared Reflectance (NIR). The diets were formulated with NIR predicted AMEn, digestible amino acids, calcium and total phosphorus from ingredients using Brill formulation (Feed management system) software. All diets were fed in mash form.

Coccidia inoculation: To enhance coccidia inoculation through feed consumption, at the beginning of the 15th day, tube feeders containing grower feed were elevated in height to be out of the reach of chicks. The chicks were weighed and 1 kg of the grower feed removed from the respective diet. *Eimeria acervulina*, *E. maxima* and *E. tenella* were sprayed on to the feed to provide 50,000, 20,000 and 30,000 oocysts $bird^{-1}$, respectively. The inoculation dose for each of the three strains of *Eimeria* was previously reported by Teeter *et al.*[12] to challenge the birds without killing them. The chicks in each pen were fasted for 3 h and then allowed to consume the inoculated feed. The inoculated feed with oocysts was rapidly eaten by the chicks. After the chicks consumed the inoculated feed, the tube feeders were lowered in each pen to allow *ad libitum* feed access. One broiler per floor pen replicate, 8 broilers per treatment were humanely euthanized using CO_2 inhalation 7 days post inoculation to determine the coccidia lesion score in three regions of the gastrointestinal tract (duodenum, jejunum and ceca). The duodenum was considered the area from the junction with the gizzard to the cystic duct, while the jejunum was the area from the cystic duct to the vitelline diverticulum and finally the ceca was defined as a pair of tubular structures lying caudally along the ileum from the ileo-cecal-colic junction[13]. Each segment was cut open longitudinally and the intestinal contents were removed. The coccidia lesions in each of the intestinal sections were scored on a scale of 0 (none) to 4 (severe) based on the methodology of Johnson and Reid[13] and Mathis *et al.*[14].

Table 2: Composition and nutrient calculations (g/100 g as fed) of the basal diet

	Starter 1-14 days		Grower 15-28 days		Finisher 29-42 days	
Ingredients (%)	Negative control	Positive control	Negative control	Positive control	Negative control	Positive control
Corn 8.8% CP	54.00	48.91	59.30	52.49	61.26	52.38
Soybean meal 46.4% CP	30.48	34.34	25.18	30.74	22.86	30.46
Wheat middlings	5.00	5.00	5.00	5.00	5.00	5.00
Corn DDGS	4.00	4.00	4.00	4.00	4.00	4.00
Poultry fat	2.45	3.30	3.04	4.10	3.83	5.14
DL-methionine	0.22	0.36	0.19	0.30	0.11	0.17
L-lysine HCl	0.12	0.28	0.16	0.24	0.05	0.02
L-threonine	0.02	0.11	0.04	0.09	0.02	0.00
Limestone	1.39	1.30	1.23	1.22	1.19	1.18
Dicalcium phosphate	1.27	1.29	0.94	0.91	0.79	0.75
Salt	0.52	0.52	0.38	0.38	0.35	0.35
Vitamin and mineral premix*	0.52	0.52	0.52	0.52	0.52	0.52
Phytase[#]	+					
Calculated composition (%)						
ME (kcal kg^{-1})	3000	3000	3100	3100	3176	3176
Crude protein	21.0	22.60	19.0	21.10	18.0	20.80
Calcium[$]	1.00	1.00	0.85	0.85	0.80	0.80
Non-phytate phosphorus	0.50	0.50	0.43	0.43	0.40	0.40
Digestible lysine	1.10	1.32	1.00	1.20	0.85	1.02
Digestible methionine	0.53	0.69	0.48	0.61	0.39	0.49
Digestible methionine+cysteine	0.83	1.00	0.76	0.91	0.65	0.78
Digestible threonine	0.73	0.88	0.67	0.80	0.62	0.71
Choline (mg kg^{-1})	2335	2409	2224	2333	2172	2325
Analyzed composition (%)						
Gross energy (kcal kg^{-1})			4093	4230		
Crude fat			5.82	7.76		
Crude protein	22.1	23.10	19.4	21.60	18.2	21.80
Digestible lysine			1.18	1.37		
Digestible methionine+cysteine			0.90	1.25		
Digestible threonine			0.72	0.80		
Digestible phosphorus			0.45	0.45		

*Supplied per kilogram of diet: Antioxidant 200 mg, 15,432 UI vitamin A, 11,023 IU vitamin D_3, 110 UI vitamin E, 3 mg menadione, 13 mg riboflavin, 20 mg pantothenic acid, 77 mg niacin, 2 mg folic acid, 0.03 mg vitamin B_{12}, 6 mg pyridoxine, 0.2 mg biotin, 3 mg thiamine, 1200 mg of choline chlorine, 100 mg Mn, 27 mg Mg, 100 mg Zn, 50 mg Fe, 10 mg Cu, 1 mg I and 0.20 mg Se, [#]Ronozyme HiPhos, DSM, Nutritional Products LLC, Parsippany, NJ. The enzyme was included at a rate of 50 g Mt^{-1} to supply a guaranteed minimum of 500 FTY kg^{-1} of feed, [$]Includes contribution from phytase of 0.10% Ca and 0.10% digestible P

Microorganism profile: On 21 days, the ileal content of 1 bird per metabolic cage (8 birds per treatment) was taken for analysis of the colonization of *E. coli* and facultative anaerobic Gram-positive bacteria into colony forming units (CFU, log CFU per gram digesta dry weight). The colonization was measured as described by Hubener *et al.*[15].

Nutrient digestibility analysis: The excreta samples from 3 days were pooled and mixed within a cage and a subsample of 120 g was stored at -20°C. The broilers were humanely sacrificed by CO_2 inhalation to obtain the ileal content. The ileum was defined as the portion of the small intestine extending from the vitelline diverticulum to a point 40 mm proximal to the ileo-cecal junction. The ileal digesta from 4 broilers per cage were collected by gently flushing with distilled water into plastic containers. Digesta samples were pooled within a cage. Excreta and ileal contents were freeze dried and ground with a commercial grinder to pass through a 0.5 mm sieve before analysis. Energy, dry matter, nitrogen, fat, phosphorus and Neutral Detergent Fiber (NDF) were analyzed. The Gross Energy (GE) was determined in a bomb calorimeter (Parr 6200 bomb calorimeter, Parr Instruments Co., Moline, IL.). Dry matter analyzed by method of AOAC International[16] 934.01, nitrogen by method of AOAC International[17] 990.03, fat by method of AOAC International[18] 920.39C, phosphorus by method of AOAC International[19] 968.08 adapted to an inductively coupled plasma, ICP and Neutral Detergent Fiber (NDF) analysis were conducted by batch procedures as outlined by Ankom Technology Corp. (Fairport, NY) using an Ankom 200 fiber analyzer. All analysis were conducted at the Central Analytical Laboratory, University of Arkansas and Center of Excellence for Poultry Science. The extraction and hydrolysis of starch was based on the colorimetric methods of Varns and Sowokinos[20]. Briefly, 20 mg of ileal content or feed were added to a plastic resistant tube and diluted with 1 mL of 80% of ethanol to be

placed on water heat bath at 90°C for 3 min. Tube contents were centrifuged at 10000×g for 3 min and the supernatant discarded. This ethanol procedure was repeated two more times. The starch extraction was performed with water and NaOH. Tube pellet contents were suspended with 1 mL of distilled deionized water, placed in hot water bath at 96°C for 5 min and centrifuged at 15000×g for 3 min to recover the supernatant in new plastic test tubes. Tube pellet contents were suspended with 1 mL of 0.5 N NaOH and treated similar to the water extraction. The alkaline extraction was repeated but centrifuged at 27000×g. The starch hydrolysis was performed by adding 0.36 mL of 6 N HCl to starch extractions and placed in hot water bath at 96°C for 2.5 h. The hydrolytic solution was neutralized with 0.3 mL of 10 N NaOH and utilized for glucose determination by the dinitrosalicylic acid method[21]. Amino acid analysis was determined for diets and ileal content by HPLC. The amino acids were analyzed in triplicate utilizing the standard Amino Acid (AA) procedure, AOAC[22] 982.30 and procedure for cystine/methionine, AOAC[23] 985.28. The ileal nutrient digestibility was determined with titanium dioxide as a digestible marker added to the feed in a dose level of 0.5% following the methodology of Myers *et al.*[24]. Ileal digestibility of nutrients (DN) and percentage digestibility of nutrients (DN%) were calculated as follows:

$$DN = N_{diet} - N_{ileal} \times \frac{TiO_2 diet}{TiO_2 ileal}$$

$$DN\% = \frac{dN}{N_{diet}} \times 100$$

The AMEn was determined following the equation:

$$AMEn\ (kcal\ kg^{-1}) = GE_{diet} - GE_{excreta}\left(\frac{TiO_2 diet}{TiO_2 excreta}\right) - 8.22 \times \left(N_{diet} - N_{excreta} \times \left(\frac{TiO_2 diet}{TiO_2 excreta}\right)\right)$$

Where:

GE = Gross energy (kcal)
TiO_2 = Titanium dioxide (%)
N = Nitrogen (%)

Viscosity: On 21 days, *in vitro* viscosity of the jejunum content (jejunum defined from the cystic duct to the vitelline diverticulum) of the same chicks (4) used for ileal content collection described in the previous section were pooled within a cage and centrifuged at 3000 rpm for 10 min. An aliquot of 0.5 mL was used to measure viscosity in centipoise (cp = 1/100 dyne sec cm^{-2}) with a Brookfield viscometer (Model DV-II+viscometer) utilizing laboratory conditions of 20°C during 2 h following sampling.

Statistical analysis: Data analysis was performed by using JMP pro11 statistical analysis software[25]. A completely randomized design was used. Data analyzed by ANOVA is presented as mean with overall SEM and p-value reported. When the effects were significant, means were separated using Tukey HSD test at $p \leq 0.05$. Data for microflora population was converted to logarithmic numbers before ANOVA test. A contrast analysis between negative control and other treatments were determined at $p \leq 0.05$ for performance and nutrient digestibility data. When $p \leq 0.10$, the results are mentioned as a tendency.

RESULTS AND DISCUSSION

Coccidia lesion score: There were no significant differences ($p \geq 0.05$) between treatments in lesion scores in the duodenum, jejunum and ceca (Table 3) indicating all dietary treatments were equally challenged with coccidia. Mean lesion score in the ceca is higher (1.89) compared to duodenum (0.57) and jejunum (0.17). The genus *Eimeria* parasite develops in different segments of the gastrointestinal tract. For example, the three most prevalent species found in broilers are *E. acervulina* which is found in the duodenum, *E. maxima* in the mid-intestine and *E. tenella* develops in the ceca[26]. Since lesions in the ceca are known to be caused by *E. tenella*, the present study shows that *E. tenella* was more prevalent compared to the other species of *Eimeria* used to infect the birds.

Table 3: Coccidia lesion score* per-treatment#

Treatments	Duodenum	Jejunum	Ceca
NC	0.50	0.38	1.88
PC	0.25	0.00	1.50
PG	1.00	0.19	2.00
PAX	0.44	0.00	1.63
PX	0.75	0.44	2.19
PXA	0.50	0.06	2.13
Mean	0.57	0.18	1.89
SEM	0.18	0.17	0.45
p-value	0.074	0.300	0.862

*Coccidia lesions in each of the intestinal sections were scored on a scale of 0 (none) to 4 (severe) based on the methodology of Johnson and Reid[13] and Mathis *et al.*[14], #NC: Negative control, PC: Positive control, PG: NC+protease+glucanase, PAX: NC+protease+amylase+xylanase, PX: NC+protease+xylanase, PXA: NC+protease+xylanase+amylase. Means with no common superscripts within a column are different at $p \leq 0.05$, SEM: Pooled standard error mean

Microorganisms in the ileum: There is a significant reduction in *E. coli* in the ileum for the enzyme treatments PG (protease+glucanase), PAX (protease+amylase+xylanase) and PXA (protease+xylanase+amylase) compared to the NC ($p \leq 0.001$) (Fig. 1). The addition of xylanase in wheat/rye diets has been previously shown to lower CFU of entero-bacteria from ileal mucosal tissues[15]. The present study is showing that xylanase combined with protease and amylase also decreased entero-bacteria which may be due to a lower substrate for entero-bacteria to grow in the ileum when these enzymes are present. A normal microflora colonization in the intestinal tract of chicks occurs right after the eggs are hatched[27] and two weeks after hatch the microflora is established with Gram-positive bacteria being the majority (65-85%) in the duodenum, ileum and cecum[28]. In the present study, the enzyme treatments with significantly lower *E. coli* colony counts (PG, PAX and PXA) follow the trend of keeping the ratio of more Gram-positive: *E. coli* (Fig. 1). This finding opens the door to study the interaction of exogenous enzymes and microflora population in the gastrointestinal tract of chickens, although, no differences for facultative Gram-positive bacteria anaerobes between treatments were found.

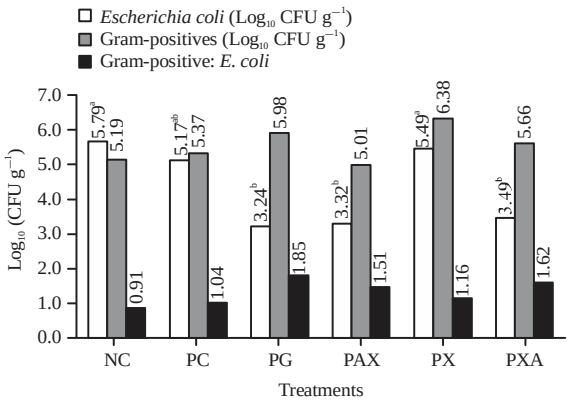

Fig. 1: *Escherichia coli* and Gram-positive bacteria in the ileum at 21 days of age, least squares means for microorganism in the ileum, NC: Negative control, PC: Positive control, PG: NC+protease+glucanase, PAX: NC+protease+amylase 80%+xylanase 20%, PX: NC+protease+xylanase, PXA: NC+protease+xylanase 80%+amylase 20%. Means with no common superscripts (a, b) are different for *E. coli* ($p \leq 0.033$), SEM 0.46 and for Gram-positive bacteria ($p<0.072$), SEM 0.33, G-positive: *E. coli* ($p<0.061$), SEM 0.229, SEM: Pooled standard error mean

Apparent metabolizable energy corrected for nitrogen (AMEn): Overall, dietary treatment ME values were each lower compared to the formulated (calculated) energy value (3100 ME, kcal kg^{-1}) possibly because of a coccidia challenge during the time period when AMEn was determined in the test diets. All dietary treatments containing enzyme composites increased the AMEn compared to the NC ($p \leq 0.001$) but were lower than the PC. Broilers fed the PC showed a higher AMEn energy value than other treatments ($p \leq 0.001$). The PC AMEn was 325 kcal kg^{-1} higher than NC. Broilers fed PG, PAX, PX and PXA had 118, 158, 91 and 236 kcal more AMEn compared with NC, respectively ($p \leq 0.001$) (Table 4). Carbohydrases such as pectinase, xylanase and glucanase are designed to break down complex polysaccharides such as pectin, xyloglucans and β-glucans, respectively, liberating nutrients which could yield energy[29]. However, it is unlikely that exogenous carbohydrases break down complex polysaccharides to monomers but they can help degrade part of these complexes allowing pancreatic enzymes access to nutrients trapped within the cell[30]. During coccidia infection, extra dietary energy from multi-enzymes may improve broiler performance during the compensatory period. Research with multi- enzyme composites have shown a 183 kcal kg^{-1} increase in AMEn[31] whereas, other research groups[29,32,30] have only shown 72, 74 and 118 kcal kg^{-1}. Other researchers have showed no difference in dietary energy using multi-enzymes[33] compared to the NC. Consistently obtaining a positive energy response with multi-enzymes composites is difficult because of differences in nutrient profile, level of enzymes, source of ingredients and environment.

Nutrient digestibility: Nitrogen digestibility was not different between dietary treatments ($p>0.05$). Fat digestibility was significantly higher in a pair-pair comparison for PC vs NC ($p \leq 0.015$), PG vs NC ($p \leq 0.022$) and PAX vs NC ($p \leq 0.025$) (Table 4). Fat digestibility was improved with the use of enzyme composite PG (+8.2%) containing protease+glucanase and PAX (+8.0%) which included protease+amylase+xylanase, compared to NC. Fat digestibility was not improved by the PX enzyme composite (protease+xylanase) and PXA (protease+xylanase+amylase) ($p \geq 0.05$) (Table 4). The improvement in fat digestibility may have produced the observed increase in AMEn (Table 4). Glucanase alone has been reported to improve fat digestibility in barley based diets[34], however, there is limited research reported on the effect of multi-enzyme composites on fat digestibility. Juanpere *et al.*[35] has reported a higher fat digestibility when using phytase+galactosidase. Slominski *et al.*[36] also reported a 14% increase in fat

Table 4: AMEn (kcal kg^{-1}) and ileal nutrient digestibility (%) at 21 days of age

Treatments[1]	AMEn (kcal kg^{-1})	Nitrogen	Fat	Phosphorus	Starch	NDF
		(%)				
NC	2575^{e}	88.3	67.6	76.4	80.4	33.3^{b}
PC	2899^{a}	89.9	76.6	75.4	78.9	44.6^{a}
PG	2692^{cd}	89.0	75.8	79.6	83.1	42.1^{a}
PAX	2732^{c}	89.1	75.6	77.5	82.1	40.0^{ab}
PX	2665^{d}	89.1	73.3	77.7	81.0	38.3^{ab}
PXA	2810^{b}	88.6	70.7	78.1	81.0	39.1^{ab}
SEM	13.38	0.73	2.45	0.99	1.01	1.01
p-value	<0.001	0.803	0.102	0.052	0.130	0.002
Contrast analysis						
p-value						
NC vs PC	<0.001	0.188	0.015	0.459	0.337	<0.001
NC vs PG	<0.001	0.589	0.022	0.023	0.091	<0.001
NC vs PAX	<0.001	0.565	0.025	0.413	0.256	0.008
NC vs PX	<0.001	0.585	0.146	0.386	0.702	0.042
NC vs PXA	<0.001	0.938	0.407	0.259	0.664	0.037

NC: Negative control, PC: Positive control, PG: NC+protease+glucanase, PAX: NC+protease+amylase+xylanase, PX: NC+protease+xylanase, PXA: NC+protease+xylanase+amylase. [a-e]Means with no common superscripts within a column are different at p≤0.05, NDF: Neutral detergent fiber, SEM: Pooled standard error mean

digestibility with a multi-carbohydrase enzyme composite in adult roosters when tested with flaxseed. Other researchers adding dietary amylase+xylanase+protease have found 5.5% and 2.1% more fat digestibility in wheat and corn based diets, respectively[33]. The increased fat digestibility with the use of carbohydrases may be attributed to a decrease in viscosity of intestinal fluids associated with passing digesta but the mechanism of how exogenous protease improves fat digestibility is unknown[32]. The viscosity in the present study was not different between treatments suggesting fat digestibility improvement is a complex mechanism when multi-enzyme composites are used. The overall comparison of phosphorus (P) digestibility between treatments showed a trend (p≤0.052) of being significantly different when contrast analysis was performed against NC. The P digestibility was higher for PG vs NC (p≤0.023). Woyengo *et al.*[31] found that multi-enzymes (protease, pectinase, glucanase, amylase and xylanase) from the same enzyme source used in the present study improved P ileal digestibility by 10.4% when using multi-enzymes in diets with phytase. The 10.4% increase in P digestibility produced by the Canadian group is similiar to observed improvement with enzyme treatment PG (protease+glucanase) in the present study, however, the Canadian group didn't introduce a coccidia challenge and included more enzymes in the blend. Multi-carbohydrases are known to improve P digestibility because they expose the phytate encapsulated within the vegetable cells, so the phytase is more prone to catalyze the reaction. The NC in the present study had the same amount of phytase in all treatments but only the PG produced more P digestibility suggesting the combination protease+glucanase was a good combination for improvement in P digestibility. Starch digestibility was not different between treatments and the values were low compared to other researchers[31]. Most of the study with starch indicates a high starch digestion capacity in chickens[31], however, starch digestibility values below 90% (measured as ileal digestibility or both) have also been reported. Maisonnier *et al.*[37] has reported 82-85% starch digestibility in corn based diets. Svihus[38] has found a large variability 57-99% of starch digestibility in different wheat varieties. Carre[39] has reported the reasons for variation in digestibility of starch in different feedstuffs. In the present study, starch digestibility was evaluated 1 week following inoculation with coccidia. The coccidia infection may have caused the decrease of the starch digestibility in all treatments. The overall ME values were also low in the present experiment. Since starch is the highest source of energy for broilers, the results of lower AMEn energy validate the low starch digestibility. The overall comparison of Neutral Detergent Fiber (NDF) digestibility was improved for PC and PG treatments compared to NC (p≤0.002) (Table 4). The NDF digestibility was improved with all the enzyme complexes when compared by contrast analysis to the NC (p≤0.05). Improvements in NDF digestibility with enzyme treatments compared to NC were +8.8% PG, +6.7% PAX, +5.0% PX and +5.8% PXA. Neutral detergent fiber is a common method for expressing the fiber content in diets. The NDF refers to the insoluble part of non-starch polysaccharides (NSP). Waititu *et al.*[33] reported a tendency to produce an increase in apparent total tract retention of NDF with multi-enzyme blends. Most of the study with exogenous enzymes measures the NSP digestibility instead of NDF. Since,

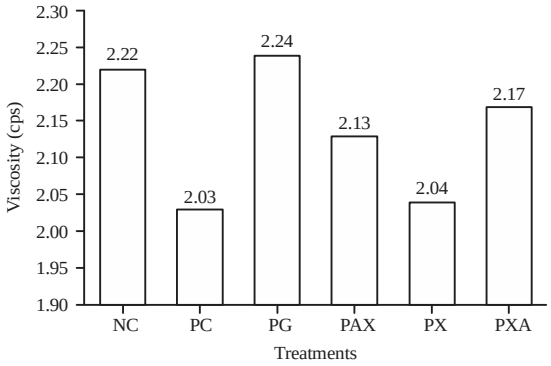

Fig. 2: Viscosity in the jejunum content of broilers at 21 days of age, Least squares means for viscosity. NC: Negative control, PC: Positive control, PG: NC+protease+glucanase, PAX: NC+protease+amylase 80%+xylanase 20%, PX: NC+protease+xylanase, PXA: NC+protease+xylanase 80%+amylase 20%. Means were not different (p<0.183), SEM 0.06, SEM: Pooled standard error mean

NSP digestibility may be a better indicator of fiber hydrolysis with exogenous carbohydrases[40] there are limited studies for NDF digestibility comparisons with different exogenous enzymes. Viscosity was not different between treatments which is expected in corn and soybean diets (Fig. 2). Even though the diets in present study contained 4% DDGS and 5% wheat middlings, the inclusion amounts may be insufficient to create a viscous digesta. As cited by Bedford and Walk[41], viscosity is more relevant for grains such as wheat and barley.

Amino Acid (AA) digestibility: The PC showed higher digestibility compared to NC for Lys, Met, Cys, Thr, Arg, Val, Iso, Phe and Tyr ($p \leq 0.05$) (Table 5) but not for Leu, Gly, Ser, His, Ala, Asp and Glu. The increase in digestibility for the key essential AA in the PC diet may be due to the 20% increase in quality protein and AA that was mainly provided from increased amounts of soybean meal and three synthetic amino acids (Met, Lys and Thr). The PC was also higher in Lys digestibility compared to PX treatment but not higher than PG, PAX and PXA treatments ($p \leq 0.001$). Thr digestibility was higher with the multi-enzyme PG compared to other treatments. The PC produced a higher AA digestibility for more than half of the AA evaluated compared to the NC, however, the AA digestibility for most amino acids for the PC broilers was not different from digestibility of AA for broilers fed the multi-enzyme treatments suggesting multi-enzyme composites improved AA digestibility during coccidia infection. Further analysis of pair-pair contrast of enzyme treatments compared to the NC for every AA showed that PG improved the digestibility of all 16 AA evaluated in the study ranging from 1.1% (Met) to 5.53% (Cys) ($p \leq 0.05$), PAX increased digestibility all AA ranging from 0.86% (Glu) to 4.13% (Cys) ($p \leq 0.05$), PX improved the digestibility of 7 AA compared to NC (Cys, Thr, Arg, Tyr, Gly, Ser, His and Asp) from 1.13% (Asp) to 3.14% (Cys) and PXA improved the digestibility of most of the AA from 1.03 (glutamic acid) to 3.96% (Cys) but only showed a tendency for improvement of Met ($p \leq 0.052$) and His ($p \leq 0.074$) (Table 5). Cystine was the AA improved the most with enzymes (3.14-5.53%), followed by Thr (1.47-4.17), Ser (1.50-4.03%) and Gly (1.54-2.97%). The enzyme treatment PG produced the highest amino acid digestibility response compared to NC ($p \leq 0.001$), followed by PAX, PXA and finally PX. Lobley *et al.*[42] reported the principal proteins in the intestinal secretory mucins are composed of high amounts of Cys, Thr, Pro and Ser. Since the AA digestibility was measured 7 days after coccidia inoculation in the present study, it is probable that the broilers required more of these AA for the turnover of the mucins and the exogenous enzymes provided directly or indirectly the extra AA. Romero *et al.*[43] reported the combination of added protease, xylanase and amylase (PXA) produced an increased apparent digestibility of 5.4% for Cys, 4.4% for Thr, 3.6% for Gly and 3.3% for valine. The PAX and PXA treatments improved the digestibility of Cys, Thr, Gly and Valine by 4.13, 3.96, 2.51, 1.47, 2.11, 1.74 and 1.77 and 1.75%, respectively. The PAX and PXA multi-enzyme composites in present study produced a similar trend for improving AA digestibility compared to the results of Romero *et al.*[43]. Cowieson and Ravindran[44] also reported an increased amino acid digestibility when using multi-enzyme blends (protease+xylanase+amylase) comparable to the present study. These researchers reported improvements which ranged from only 0.44% for Met to over 9% for Cys digestibility in corn-soy based diets for broilers, however, their results were achieved under normal growth conditions and not under coccidia infection as in the present study.

Growth performance: Live BW was the same until 21 days across treatments (Table 6), however, there was a tendency of higher BW for the PC in a pair-pair comparison to NC ($p \leq 0.09$). Body weight was higher for the PC ($p \leq 0.027$) at 28 days compared to all treatments. The final broiler BW at 42 days was heavier for the PC compared to NC ($p \leq 0.006$). The BWG in the phase 15-28 days was higher for the PC compared to PXA treatment only ($p \leq 0.026$), however, in a pair-pair comparison against NC and PC was also heavier to NC ($p \leq 0.016$). The enzyme treatments show no difference compared to NC ($p > 0.05$) in BWG from 15-28 and 29-42 days. Feed intake was lower with PG compared only to NC ($p \leq 0.04$)

Table 5: Apparent amino acid digestibility (%) at 21 days of age

Treatments	Lys	Met	Cys	Thr	Arg	Val	Leu	Iso	Phe	Tyr	Gly	Ser	His	Ala	Asp	Glu
NC	91.4^{c}	94.9^{b}	81.4^{b}	85.4^{c}	89.5^{b}	87.9^{c}	89.8^{b}	88.5^{b}	88.9^{b}	89.0^{b}	86.4^{c}	87.4^{c}	90.1^{b}	90.2^{b}	87.9^{b}	92.2^{b}
PC	93.2^{a}	96.3^{a}	85.4^{a}	87.4^{b}	91.4^{a}	89.7^{ab}	90.6^{ab}	90.4^{a}	90.5^{a}	91.0^{a}	87.5^{bc}	88.4^{bc}	91.5^{ab}	91.2^{ab}	89.4^{ab}	92.9^{ab}
PG	93.2^{a}	96.1^{ab}	86.7^{a}	89.6^{a}	91.0^{ab}	90.3^{a}	91.8^{a}	91.1^{a}	91.2^{a}	91.8^{a}	89.4^{a}	91.4^{a}	92.3^{a}	91.9^{a}	90.7^{a}	93.6^{a}
PAX	92.7^{ab}	95.9^{ab}	85.5^{a}	87.9^{b}	90.7^{ab}	89.7^{ab}	90.9^{ab}	90.4^{a}	90.5^{a}	91.2^{a}	88.50^{ab}	89.8^{ab}	91.7^{a}	91.20^{ab}	89.90^{a}	93.1^{ab}
PX	92.0^{bc}	95.5^{ab}	84.5^{ab}	87.0^{b}	90.11^{a}	88.8^{bc}	90.2^{ab}	89.6^{ab}	89.6^{ab}	90.4^{ab}	87.90^{ab}	88.9^{bc}	91.1^{ab}	90.5^{b}	89.40^{ab}	92.5^{ab}
PXA	92.6^{ab}	95.7^{ab}	85.3^{a}	86.9^{b}	90.8^{ab}	89.6^{ab}	91.2^{ab}	90.5^{a}	90.7^{a}	90.9^{ab}	88.10^{ab}	89.7^{ab}	91.0^{ab}	91.3^{ab}	90.10^{a}	93.3^{ab}
SEM	0.245	0.231	0.711	0.301	0.371	0.320	0.393	0.334	0.367	0.430	0.340	0.472	0.367	0.280	0.346	0.230
p-value	<0.001	0.0125	0.0010	<0.001	0.0205	0.0002	0.0224	0.0013	0.0020	0.0027	<0.001	<0.001	0.0047	0.0078	<0.001	0.0238
Contrast analysis																
Estimate (%) (Difference TRT-NC)																
PC vs NC	1.80	1.38	4.02	2.01	1.85	1.83	0.84	1.58	1.66	1.99	1.10	1.06	1.43	0.97	1.43	0.66
PG vs NC	1.77	1.11	5.53	4.17	1.48	2.44	1.99	2.28	2.33	2.80	2.97	4.03	2.23	1.74	2.73	1.38
PAX vs NC	1.25	0.94	4.13	2.51	1.20	1.77	1.15	1.56	1.63	2.16	2.11	2.44	1.66	1.00	1.91	0.86
PX vs NC	0.63	0.51	3.14	1.61	0.60	0.89	0.43	0.80	0.75	1.33	1.54	1.50	1.09	0.30	1.13	0.31
PXA vs NC	1.19	0.78	3.96	1.47	1.30	1.75	1.43	1.71	1.84	1.83	1.74	2.29	0.92	1.11	2.11	1.03
p-value																
PC vs NC	<0.001	0.001	<0.001	<0.001	0.002	<0.001	0.129	0.003	0.003	0.003	0.030	0.123	0.010	0.038	0.005	0.104
PG vs NC	<0.001	0.013	<0.001	<0.001	0.013	<0.001	0.002	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.003
PAX vs NC	<0.001	0.017	<0.001	<0.001	0.023	<0.001	0.035	0.002	0.003	<0.001	<0.001	<0.001	0.002	0.024	<0.001	0.031
PX vs NC	0.065	0.198	0.007	<0.001	0.255	0.059	0.435	0.106	0.161	0.036	0.003	0.026	0.036	0.493	0.028	0.429
PXA vs NC	<0.001	0.052	<0.001	0.002	0.017	<0.001	0.013	<0.001	<0.001	0.005	0.001	0.001	0.074	0.015	<0.001	0.013

NC: Negative control, PC: Positive control, PG: NC+protease+glucanase, PAX: NC+protease+amylase+xylanase, PX: NC+protease+xylanase, PXA: NC+protease+ xylanase+amylase. $^{a-c}$Means with no common superscripts within a column are different at p≤0.05, SEM: Pooled standard error mean, TRT: Treatments

Table 6: Body weight, body weight gain, feed intake, feed conversion ratio and mortality of broilers from 1-42 days

	Body weight (g)				Body weight gain (g)				Feed intake (g)				FCR					Mortality (%)		
	14	21	28	42	1-14	15-21	15-28	29-42	1-14	15-21	15-28	29-42	1-14	15-21	15-28	29-42	1-42	1-14	15-28	29-42
Treatments	(Days)																			
NC	289	590	1023^{b}	2263	250	301	734^{ab}	1245	475	560	1490	2451	1.869	1.863^{a}	2.033	1.928^{a}	1.919^{a}	0.78	1.48	0.83
PC	294	614	1061^{a}	2362	256	323	770^{a}	1312	472	560	1497	2414	1.911	1.737^{c}	1.949	1.839^{b}	1.851^{b}	1.04	2.21	0.42
PG	292	587	1022^{b}	2284	253	297	730^{ab}	1270	461	526	1452	2414	1.888	1.766^{bc}	2.001	1.914^{a}	1.880^{ab}	0.26	2.22	0.39
PAX	291	589	1011^{b}	2307	252	298	728^{ab}	1289	458	549	1455	2421	1.860	1.842^{a}	2.018	1.893^{ab}	1.881^{ab}	0.78	2.21	0.00
PX	288	588	1015^{b}	2305	249	300	728^{ab}	1290	477	546	1468	2447	1.883	1.822^{ab}	2.017	1.881^{ab}	1.878^{ab}	1.30	3.27	0.89
PXA	288	582	1011^{b}	2273	250	294	723^{b}	1261	468	547	1475	2421	1.813	1.863^{a}	2.041	1.921^{a}	1.907^{ab}	0.00	2.32	0.00
SEM	4.13	10.01	11.80	23.31	4.08	6.88	10.29	19.3	10.12	10.42	16.4	18.2	0.04	0.02	0.03	0.02	0.015	0.56	1.01	0.40
p-value	0.906	0.059	0.027	0.071	0.901	0.053	0.026	0.227	0.730	0.154	0.313	0.566	0.638	0.005	0.312	0.012	0.023	0.596	0.901	0.476
Contrast analysis																				
p-value																				
NC vs PC	0.408	0.090	0.021	0.006	0.366	0.032	0.016	0.023	0.839	0.974	0.746	0.701	0.473	<0.001	0.051	0.001	0.002	0.743	0.614	0.467
NC vs PG	0.639	0.829	0.645	0.551	0.650	0.731	0.805	0.409	0.330	0.040	0.113	0.403	0.723	0.005	0.472	0.604	0.079	0.516	0.610	0.440
NC vs PAX	0.685	0.969	0.491	0.188	0.713	0.778	0.670	0.119	0.232	0.481	0.143	0.237	0.878	0.523	0.731	0.172	0.067	1.000	0.613	0.149
NC vs PX	0.831	0.859	0.656	0.208	0.880	0.915	0.689	0.111	0.937	0.358	0.340	0.468	0.791	0.197	0.713	0.063	0.055	0.516	0.221	0.927
NC vs PXA	0.949	0.571	0.487	0.773	1.000	0.454	0.459	0.561	0.604	0.400	0.515	0.501	0.316	0.992	0.851	0.770	0.593	0.332	0.564	0.149

NC: Negative control, PC: Positive control, PG: NC+protease+glucanase, PAX: NC+protease+amylase+xylanase, PX: NC+protease+xylanase, PXA: NC+protease+xylanase+amylase. $^{a-c}$Means with no common superscripts within a column are different at p≤0.05, SEM: Pooled standard error mean

in the first part of the grower phase 15-21 days but feed intake remained the same across treatments during the starter and finisher feeding periods. The FCR was not different between treatments from 1-14 days, FCR was significantly improved from 15-21 days for PC (1.737 vs 1.863 NC) ($p \leq 0.001$) and PG (1.766 vs 1.863 NC) ($p \leq 0.005$). The improvement in FCR by PC is the result of an increase in BWG and PG produced a lower feed intake during this period. In the overall grower period (15-28 days) the FCR tended to be better only for the PC ($p \leq 0.051$) when compared to the NC. The FCR during the last phase (29-42 days) was improved for PC broilers compared to NC, PG and PXA ($p \leq 0.012$) but there was no difference between the PC group and PAX and PX. The PC had a significantly better FCR compared only to NC (1.839 vs 1.928 NC) ($p \leq 0.001$). Treatment PX had a tendency to improve FCR (1.881 vs 1.928 NC) ($p \leq 0.063$). Overall FCR from 1-42 days was better with PC compared to the NC ($p \leq 0.023$) as expected but not different from the enzyme treatments (Table 6). Three enzyme treatments tended to improve 1-42 days FCR compared to NC, PX (1.878 vs 1.912 NC) ($p \leq 0.055$), PG (1.880 vs 1.912 NC) ($p \leq 0.079$) and PAX (1.881 vs 1.912 NC) ($p \leq 0.067$). Total mortality in present study was below 5% which is considered normal in the broiler industry. There was no difference in mortality between treatments. Morgan and Bedford[45] inoculated broilers with coccidia and observed a reduction in viscosity and improved FCR when the broilers were fed diets containing a carbohydrase. In addition, Teeter *et al.*[12] suggested the positive response to amylase, xylanase and protease was much stronger during the later stages of grow-out when the deleterious effects of the coccidia inoculation were the highest. In the present study, the FCR tended to improve with enzyme composite PG, PX and PAX. Even though, the treatment PXA showed improvements in AMEn and amino acid digestibility, the FCR was not better when compared to the control, which could mean a longer time-period after inoculation is needed to show significant differences. Mathis[8] reported broilers vaccinated at 1 day were affected through 3 weeks of age and then started showing signs of recovery on weekly basis. The vaccinated broilers reported by Mathis[8] had the same overall performance as non-vaccinated broilers at 42 days of age. The study indicates that it takes at least 4 weeks after coccidia exposure prior to initiating compensatory responses and 6 weeks after initial exposure to obtain the same overall performance. In the present study, the birds were infected on 15 days, so when the study was finished at 42 days, the PC broilers and broilers fed enzymes were beginning to show recovery and added performance compared to NC broilers from the infection. If the broiler study had been conducted for 2 additional weeks, the broiler performance fed diets with added enzyme composites may have improved since all the enzyme composites produced a better AMEn and nutrient digestibility. Girgis *et al.*[46] reported a compensatory mechanism that may lead to recovery is increased villus height in jejunum and ileum in pullets that were observed 14 days after coccidia inoculation.

CONCLUSION

In conclusion, the enzyme composites: PG (protease+glucanase), PAX (protease+amylase 80%+xylanase 20%) and PXA (protease+xylanase 80%+amylase 20%) reduced the *E. coli* colonization suggesting the enzymes affect the microflora population in the ileum of young birds by removing the carbohydrate and other nutritional substrates. However, the enzyme composite PX produced no effects compared to controls. All the enzyme composites evaluated in the present experiment improved the AMEn (kcal kg^{-1}) when compared to the NC but not to PC. The amino acid digestibility was partially improved by the enzyme composites. Enzyme treatments PG, PAX and PXA improved all 16 amino acids evaluated in this study in different levels when compared to the NC. Enzyme treatment PX improved only seven amino acids, these improvements provide a potential nutrient matrix for the formulation of diets using the enzyme composites in corn-soybean based diets under coccidia challenge. Fat was improved with only two enzyme composites (PG and PAX), phosphorus digestibility improvement occurred with only one enzyme composite (PG) and NDF was improved with all enzyme composites. There was no significant improvement for dietary treatments for nitrogen and starch digestibility. The FCR was improved with most of the enzyme composites: PG, PAX and PX but not with PXA. The lack of improvement in FCR for some dietary treatments may be because more time was needed for compensatory growth after coccidia inoculation. The added energy and digestible amino acids produced from multi-enzyme composites should be added to the nutrient matrix when formulating diets to maximize the return on investment. Additional broiler research is needed to show control with no coccidia infection alongside inoculated broilers to evaluate the potential compensatory performance advantages of feeding exogenous enzymes.

SIGNIFICANCE STATEMENT

- Coccidiosis is a problem in the poultry industry and with companies going antibiotic free (ABF), some coccidiostats are been excluded from the market, therefore, coccidiosis vaccines are important for the poultry industry along with feed additives to maintain or improve productive performance
- Broiler diets contain multiple non digestible nutrients that will end up in the ceca and be excreted. This becomes a challenge under coccidia risk and the need for exogenous enzymes becomes important to target multiple substrates
- Proteases in combination with carbohydrases have shown partial improvements in studies with and without coccidia challenge. This creates opportunities to test the best combinations of exogenous enzymes for the various challenges the broiler industry faces at this time

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