Comparative effects of two multi-enzyme combinations and a *Bacillus* probiotic on growth performance, digestibility of energy and nutrients, disappearance of non-starch polysaccharides, and gut microflora in broiler chickens

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ABSTRACT The efficacy of two exogenous enzyme combinations and a multi-strain *Bacillus* probiotic (DFM) on the growth performance, nutrient digestibility, disappearance of non-starch polysaccharides (NSP) and gut microbial composition was investigated in broilers. One-day old Ross 308 chicks were assigned to 36 pens with 22 birds/pen and 6 pens/treatment (Experiment 1) or 36 cages with 8 birds/cage and 6 cages/treatment (Experiment 2). Treatment additives were added to nutritionally complete corn/soy based starter (d 1 to 21) and finisher (d 22 to 42) diets. Treatments included 1) a control diet containing 500 FTU/kg phytase (CTL), 2) CTL + xylanase (2,000 U/kg) and amylase (200 U/kg; XA), 3) CTL+XA + protease (4000 U/g; XAP), 4) CTL+DFM (150,000 cfu/g of 3 strains of *Bacillus* spp), 5) CTL+DFM+XA, and 6) CTL+DFM+XAP. Supplementation with DFM increased BW, BWG, and FI compared with the CTL ($P < 0.05$); XAP, but not XA, resulted in increased final BW, BWG and FI compared to the control ($P < 0.05$). XA and XAP improved apparent ileal digestibility (AID) of starch and fat on d 22 to 42 with XAP improving AME_n (by ∼82 kcal) compared with CTL birds ($P < 0.01$). DFM+XAP improved apparent ileal digestible energy (AIDE), AID of fat and starch on d 22 to 42, and additionally had a greater than additive effect on AIDE and AME_n. Supplementation with DFM+XAP reduced the ileal and total tract flow of insoluble arabinose and additionally total tract flow of soluble and insoluble xylose and total galactose ($P < 0.05$); similar effects of XA+DFM were not seen or were lower in magnitude, suggesting that the protease component plays an important role in increasing the availability of NSP for hydrolysis. Supplementation with DFM alone did not affect gut bacterial populations, but XA and XAP reduced numbers of *Campylobacter* species (by > 2.5 log cfu/g; $P < 0.001$) and *Bacteroides* ($P < 0.02$) in the cecum compared with CTL birds.

Key words: broilers, performance, carbohydrase, probiotics, digestibility

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INTRODUCTION

In recent years, the increasing price and price volatility of poultry feed ingredients have led to more diets being formulated with high-fiber ingredients such as corn distillers' dried grains with solubles (**DDGS**), other cereal grains, milling by-products, and oilseed meals. These ingredients are more variable in their composition, and contain higher levels of non-starch polysaccharides (**NSP**), which reduce the digestibility of nutrients in the diets (Salim et al., 2010) and can result in poorer growth and performance of birds (Annison and Choct, 1991). Meanwhile, the use of sub-therapeutic antibiotics is restricted, both through regulatory action and consumer demand, in many geographies.

To address feed cost and variability, producers often turn to exogenous enzymes, including those targeted at the NSP fraction of the diet. Xylanase-supplemented birds have been shown to exhibit improved ileal digestibilities of nutrients and retention of components, with consequent improvements in apparent metabolizable energy (**AME_n**) and growth performance (Kiarie et al., 2014), effects that are evident in both wheat and corn based diets. Amylase targets the starch components of feed, improving starch digestibility via

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hydrolysis and releasing energy which can be utilized by the bird (Gracia et al., 2003). Proteases are perhaps less well utilized in poultry production, and their mode of action in the gastrointestinal tract is less clear than for other enzymes (Adeola and Cowieson, 2011). Nevertheless, research indicates that they can be effective in mediating the hydrolysis of proteins in the feed which both improves protein digestibility and reduces the presence of indigestible protein substrates for pathogenic bacteria in the gut. Evidence from a limited number of studies indicates that a combination of xylanase, amylase and protease can deliver greater improvements in outcomes such as AME_n (Romero et al., 2013, 2014; Adebiyi and Olukosi, 2015) nutrient utilization and solubilization of NSP in the gut (Olukosi et al., 2015), than proteases or NSP-hydrolyzing enzymes given alone in corn- and/or wheat-based poultry diets.

As the use of antimicrobials is restricted globally, probiotics (also known as direct-fed microbials [**DFMs**]) can offer an additional means to positively influence the health and performance of poultry. Their mode of action is fundamentally different to that of enzyme supplements, altering the gut environment, modulating the activation of the immune system, and promote the colonization of beneficial microorganisms and inhibit colonisation of potential pathogens (Lee et al., 2010). Recent studies of a commercial poultry probiotic based on 3 *Bacillus* strains have shown positive effects in terms of altered gut morphometry and reduced inflammatory markers (Lee et al., 2010), as well as lower mortality, increased body weight, and improved production efficiency in commercial conditions in poultry fed corn-based diets (Dersjant-Li et al., 2014).

Despite the frequency with which both NSP-hydrolyzing enzymes and probiotics are included in commercial poultry diets, little work to date has focused on the interaction between exogenous enzymes and probiotics in poultry diets—as enzymes are known to have a prebiotic effect in the broiler gut (Romero et al., 2013, 2014), it is reasonable to hypothesize that their inclusion in broiler diets might lead to changes in gut microbial composition, enhancing or negating the effects of the probiotics, and thus influence health and performance to a greater degree than enzymes or probiotics alone. Therefore, the present study aimed to investigate the comparative effect of a commercially available *Bacillus*-based probiotic product, given alone or in combination with either of 2 multi-enzyme supplements, on growth performance, digestibility of nutrients and energy, disappearance of NSP, and gut microbial composition in broilers fed a commercially relevant corn-based diet.

MATERIALS AND METHODS

The experimental procedures were approved by the Animal Ethics Committee of Massey University, Palmerston North, New Zealand and the DuPont Agriculture and Animal Ethics Committee.

Exogenous Enzymes and Probiotics

Two commercial multi-enzyme preparations were utilized: a combination of endo-1,4-β-xylanase (EC 3.2.1.8) originating from *Trichoderma reesei*, and α-amylase (EC 3.2.1.1) originating from *Bacillus licheniformis* (XA, Danisco Animal Nutrition, DuPont Industrial Biosciences, Marlborough, UK), formulated to provide 2,000 U/kg xylanase and 200 U/kg amylase, and; the same xylanase and amylase together with a serine protease (EC 3.4.21.62) originating from *Bacillus subtilis* (XAP, Danisco Animal Nutrition, DuPont Industrial Biosciences, Marlborough, UK), formulated to provide 2,000 U/kg feed xylanase, 200 U/kg amylase, and 4,000 U/kg protease. Enzyme activity levels in final feed samples (200 g) were measured at the DuPont Nutrition Biosciences Innovation Laboratories (Brabrand, Denmark) in duplicate, and reported as activity units. One xylanase unit is defined as the amount of enzyme that released 0.48 μmol of the reducing sugar xylose from wheat arabinoxylan per min at pH 4.2 and 50 °C. One amylase unit is defined as the amount of enzyme required to release, in the presence of excess α-glucosidase, 0.20 μmol of glucosidic linkages expressed as p-nitrophenol equivalents from a maltoheptaoside substrate per min at pH 8.0 and 40 °C. One protease unit is defined as the amount of enzyme that released 1.0 μg of phenolic compound, expressed as tyrosine equivalents, from a casein substrate per min at pH 7.5 and 40 °C.

The probiotic was a commercial preparation based on spores of a combination of 3 strains of *Bacillus amyloliquefaciens* (Danisco Animal Nutrition, DuPont Industrial Biosciences, Marlborough, UK). The probiotic was included in the diets at a rate of 1.5×10^5 cfu/g of feed.

Experimental and Control Diets

2 basal diets based on corn and soybean meal, with added cDDGS and wheat middlings, were formulated to meet the recommended requirements for nutrients of the birds during the starter (d 1 to 21) and finisher (d 22 to 42) phases (NRC, 1994; Table 1). These diets represented the experimental Control (CTL) treatments. All diets contained 500 FTU/kg of a commercial *E. coli* phytase, expressed in *Trichoderma reesei* (Phyzyme XP, Danisco Animal Nutrition, Marlborough, UK). Titanium dioxide was added to the basal diets of birds used in Experiment 2 as an indigestible marker. Five further experimental diets were prepared from each of the control diets (starter and finisher), by addition of XA (CTL+XA), XAP (CTL+XAP), probiotic (CTL+DFM), probiotic and XA (CTL+DFM+XA), or probiotic and XAP (CTL+DFM+XAP). Diets were provided to birds ad libitum in mash form. All analyzed enzyme activities were within 20% of target doses, and *Bacillus* recovery was within 1 log cfu/g of target dose.

Table 1. Ingredient and nutrient composition (% as fed) of the control (CTL) basal diets given in the starter (d 0–21) and finisher (d 21–42) phases.[1]

Item	Starter (d 0–21)	Finisher (d 22–42)
Ingredient, %		
Corn	46.22	46.73
Wheat middlings	6.73	10.00
Corn DDGS	7.00	7.00
Soybean meal (48% CP)	32.81	26.19
Corn/Wheat Starch[2]	0.30	0.30
Animal/Vegetable fat	3.00	5.75
L-Lysine HCL	0.27	0.30
DL-Methionine	0.30	0.28
L-Threonine	0.11	0.12
Titanium dioxide[3]	0.30	0.30
Salt	0.34	0.37
Limestone	1.12	1.14
Dicalcium phosphate	1.20	1.22
Poultry vitamin-mineral premix[4]	0.30	0.30
Calculated Nutrient Composition, %		
ME, kcal/kg	2952	3100
Crude Protein	23.00	20.40
Digestible Lysine	1.21	1.07
Digestible Methionine	0.62	0.57
Digestible Methionine + Cysteine	0.86	0.78
Digestible Threonine	0.76	0.68
Digestible Tryptophan	0.21	0.18
Total P	0.68	0.69
Available P	0.38	0.38
Ca	0.85	0.85
Na	0.18	0.19
Analyzed Nutrient Composition		
Dry Matter, %	91.70	90.78
Crude protein, % DM	24.95	22.60
Crude fat, % DM	6.87	9.44
Starch, % DM	32.26	35.34
GE, kcal/kg DM	4529.16	4746.65

[1]Both diets were top-dressed with phytase (Phyzyme XP, a 6-phytase from *E. coli*, expressed in *Trichoderma reesei* (Danisco Animal Nutrition, Marlborough, UK) to supply 500 FTU/kg feed.

[2]As a carrier for the enzyme and DFM premixes.

[3]Added to the diets used in Experiment 2 only (digestibility study), as an indigestible marker at the expense of corn.

[4]Supplied per kilogram of diet: antioxidant (ethoxyguin), 100 mg: biotin, 0.2 mg; calcium 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, 3.0 mg; Fe, 25 mg; I, 1 mg; Mn, 125 mg; Mo, 0.5 mg; Se, 200 μg; Zn, 60 mg.

Animals, Housing, and Experimental Design

One thousand and eighty Ross 308 male broiler chicks were obtained on day of hatch from a commercial hatchery, given a broiler coccidiosis vaccine (Immucox, Pacificvet, Christchurch, New Zealand) via drinking water, and assigned on the basis of body weight to floor pens (Experiment 1) or cohort cages (Experiment 2) so that pens and cages contained birds with approximately equal average bird weight.

In Experiment 1, a floor-pen trial to evaluate growth performance and gut microbiota composition was conducted. A total of 792 birds were allocated on d 1 to 36 pens with 22 birds/pen and 6 pens/dietary treatment in a random block arrangement. Pens were located in an environmentally controlled room where temperature was maintained at 32 ± 1 °C for the first 7 d and then gradually increased to 24 °C by d 21, under a 24 h fluorescent illumination cycle. Birds were given free access to diets and water. Body weight and feed intake (**FI**) were recorded on d 1, 21, 35, and 42. Mortality was recorded daily and used to correct calculations of feed conversion rate (**FCR**) at the end of the study (d 42). On d 11, 2 birds/pen (n = 72) birds were randomly selected, killed by cervical dislocation, and immediately dissected to obtain samples of ileal and cecal tissues. These were stored at −80 °C for subsequent microbial analysis by real-time polymerase chain reaction (**PCR**).

In Experiment 2, a cohort cage trial was conducted concurrently with the floor-pen trial to investigate the ileal digestibility of nutrients and energy resulting from the different dietary treatments, as well as their metabolizable energy content, and the ileal and total tract flow of sugar components of NSP. A total of 288 birds were allocated on d 1 to 36 cages with 8 birds/cage and 6 cages per dietary treatment in a random block arrangement. Cages were housed in an environmentally controlled room where temperature and light cycling conditions were identical to those in Experiment 1. Feed intake and total excreta output were measured quantitatively per cage on 4 consecutive days (d 17 to d 20) for the determination of AME and AME_n (via measurement of dry matter [**DM**], gross energy [**GE**], and nitrogen [**N**]). The daily excreta collections were pooled within a cage, mixed in a blender and subsampled. Each sub-sample was lyophilized, ground to pass through a 0.5 mm sieve and stored in airtight plastic containers at −4 °C pending analysis. On d 11 and 21, 4 birds per cage were euthanized by intracardial injection and the contents of the ileum expressed by gentle flushing with distilled water. Ileal digesta from birds within a cage were pooled, resulting in 6 samples per dietary treatment. Digesta samples were frozen immediately prior to analysis.

Chemical Analyses

Ileal digesta samples collected on d 11 (Experiment 2) were analyzed for titanium (**Ti**), N, DM, and GE. Ileal digesta samples collected on d 21 (Experiment 2) were analyzed for Ti, N, DM, GE, starch, and fat, as well as NSP components and digestion resistant oligosaccharides. Excreta samples collected on d 17 to 20 (Experiment 2) were analyzed for DM, GE and N, in order to calculate AME and AME_n, and additionally for NSP components and digestion resistant oligosaccharides. Digestion resistant oligosaccharides contain glycosidic bonds, often α(1,6)-linkages, which are less readily broken by intestinal enzymes, resulting in only partial digestion in the upper tract (Englyst et al., 1994).

Titanium content was measured by a UV spectrophotometer according to the method of Short et al., (1996). Nitrogen content was determined by the

combustion method (AOAC International, 2005, method 968.06) using a CNS-2000 carbon, nitrogen, and sulphur analyzer. DM content was determined using standard procedures (AOAC International, 2005; method 930.15). GE was determined using an adiabatic bomb calorimeter (Gallenkamp Autobomb, London, UK) standardized with benzoic acid. Starch content was determined using the Megazyme Total Starch Assay Procedure (Megazyme International Ireland Ltd, Wicklow, Ireland) based on thermostable α-amylase and amyloglucosidase. Fat content was determined following the Soxhlet extraction procedure (AOAC International, 2005; method 991.36). NSP components and digestion resistant oligosaccharides were analyzed using the methods of Englyst et al. (1994).

Calculations

AME was calculated according to the following formula, in accordance with Ravindran et al., (2008):

$$\text{AME} = [(\text{feed intake x gross energy}_{\text{diet}}) - (\text{excreta output x gross energy}_{\text{excreta}})]/\text{feed intake}$$

Total tract apparent retention of N was calculated according to the same formula by substitution of N_{diet} and $N_{excreta}$ in place of the respective GE values. Appropriate corrections were made for differences in moisture content. AME_n was calculated by multiplication of AME with 8.22 kcal/g of N retention as described by Hill and Anderson (1958).

The apparent ileal digestibility (AID) of N, energy (AIDE), starch, and fat were calculated according to the following formula, based on the determined concentration of titanium in the diet and in the digesta, in accordance with Ravindran et al. (2005):

$$\text{AME} = [(\text{feed intake x gross energy}_{\text{diet}}) - (\text{excreta output x gross energy}_{\text{excreta}})]/\text{feed intake}$$

where "nutrient" refers to either N, DM, starch, fat, or GE, $(\text{nutrient/Ti})_{\text{diet}}$ is the ratio of component and titanium in the experimental diet, and $(\text{nutrient/Ti})_{\text{ileal digesta}}$ is the ratio of component and titanium in ileal digesta.

Calculations of the flow of NSP components and of digestion resistant oligosaccharides were done using the concentrations of Ti and the respective NSP component in the diet as well as their concentrations in the ileal digesta (to determine ileal NSP/resistant oligosaccharide flow) or excreta (to determine total tract NSP/resistant oligosaccharide flow).

Real-Time PCR of Gut Microbiota

Genomic DNA was isolated from 200 mg (wet weight) of ileal and cecal mucosa samples using a commercial kit (Bioline DNA Kit, Cat no. BIO-52,038). Extracted DNA was cleaned using a PowerClean Clean-up kit (Geneworks, Cat no. 12,877–50) and stored at −20 °C prior to PCR analysis.

Quantitative real-time PCR was performed with a Light Cycler (Roche Diagnostics, Risch-Rotkreuz, Switzerland) using SYBR Green I Master (Roche Diagnostics, Risch-Rotkreuz, Switzerland) after generation of standard curves for each of 7 bacterial species using an appropriate species specific primer set (Table 2).

The composition of reaction mix per sample was: 3 μL PCR water, 1 μL of each primer (0.5 μM), 5 μL template DNA and 10 μL of SYBR Green I Master. The PCR program consisted of an initial denaturation and anti-Taq DNA polymerase antibody-inactivation step for 10 min at 95 °C, an amplification step (45 cycles of 15 s at 95 °C, 60 s at 63 °C and 10 s at 72 °C) and a melting-curve determination step (95 °C for 5 s, 65 °C for 60 S, 97 °C with continuous hold). Measurement of SYBR green fluorescence was performed at the end of each amplification step and continuously during the melt-curve-analysis.

For generation of each standard curve, a 10-fold dilution of an overnight grown culture was prepared in a particle-free sterile saline solution, after which triplicate 50 μL aliquots of each dilution were spread-plated on specific medium. Colonies were counted after 72 h of anaerobic incubation at 37 °C for anaerobes and overnight incubation for aerobes. Standard curves were generated in the range of 101 to 1010 bacterial cell numbers per mL from real-time PCR analysis of DNA extracted from the initial dilution series using 16SrRNA, with samples analyzed in duplicate. As a standard calibration point, each run included one DNA sample from the dilution series originally used to create the standard curve.

Results were reported as equivalent log 10 cfu per DNA concentration.

Statistical Analysis

Data on growth performance, digestibility of nutrients and energy, and flow of NSP components and digestion resistant oligosaccharides were based on a pen basis; data on microflora composition were based on individual birds. Data were analyzed by analysis of variance (ANOVA) using the Fit Model platform of JMP 11.0 (SAS Institute Inc., Cary, NC, 1989–2013) to investigate the effects of treatments, with dietary treatment included as a fixed effect. Means separation was achieved using Tukey's Honest Standard Difference test. Differences were considered significant at $P < 0.05$; $P < 0.01$ was considered a trend.

RESULTS

The influence of the multi-enzyme and probiotic combinations on broiler growth performance are given in

Table 2. Oligonucleotide primers used for real-time PCR of ileal and cecal microflora composition.

Bacteria	Species used to generate standard curve	Primer	Primer Sequence	Supporting reference
E. coli	*Escherichia coli*	IEC-UPf	CAA TTT TCG TGT CCC CTT	Khan et al., 2007
		IEC-DNr	GTT AAT GAT AGT GTG TCG AAA C	
Clostridium	*Clostridium rhamnosus* CDC 8179	CJF	CTG AAT TGG ATA CCT TAA GTG CAG C	Skanseng et al., 2006
		CJR	AGG CAC GCC TAA ACC TAT	
Campylobacter spp.	*Campylobacter jejuni* CCUG 11,284	R-campF2	CAC GTG CTA CAA TGG CAT AT	Lund et al., 2004
		R-campR2	GGC TTC ATG CTC TCG AGT T	
Bifidobacterium spp.	*Bifidobacterium breve* ATCC 15,700	Bif1-F	TCG CGT CYG GTG TGA AAG (Y = CT)	Rinttila et al., 2004
		Bif2-R	CCA CAT CCA GCR TCC AC (R = Ag)	
Bacteroides spp.	*Bacteroides thetaiotaomicron* ATCC 29,148	Allbac296F	GAG AGG AGG GTC CCC CA	Bernhard & Field, 2000
		Allbac412R	CGC TAC TTG GCT GGT TCA	
Lactobacillus spp.	*Lactobacillus acidophilus* ATCC 11,975	Lab-0159-F	GGA AAC AGR TGC TAA TAC CG	Collier et al., 2003
		UnivL-0515-R	ATC GTA TTA CCG CGG CTG CTG GCA	
Salmonella Spp.	*Salmonella typhimurium ATCC 14,028*	Sal-invA1-F	GTG AAA TTA TCG CCA CGT TCG GGC AA	Rahn et al., 1992
		Sal-invA2-R	TCA TCG CAC CGT CAA AGG ACC C	Eyigor et al., 2001

Table 3. Effect of multi-enzyme combinations XA (xylanase, amylase) and XAP (xylanase, amylase, and protease), and their combination with a *Bacillus* probiotic (DFM) on broiler growth performance (Experiment 1).

	Control (CTL)	NC + XA[1]	NC + XAP[2]	NC + DFM[3]	NC + DFM + XA	NC + DFM + XAP	SEM	*P*-value
Starter Phase, d 1–21								
BW gain (g/bird)	889.13[b]	880.42[b]	907.44[a,b]	953.19[a]	903.08[a,b]	928.62[a,b]	14.49	0.015
Feed intake (g/bird)	1141.01[a,b]	1110.85[b]	1141.63[a,b]	1209.65[a]	1149.92[a,b]	1169.53[a,b]	17.81	0.013
FCR	1.28	1.26	1.26	1.27	1.27	1.26	0.01	0.535
Finisher Phase, d 22–42								
BW gain (g/bird)	2240.13	2316.744	2378.54	2323.12	2326.33	2328.65	30.30	0.085
Feed intake (g/bird)	3820.83[b]	3923.83[a,b]	3987.97[a]	3980.73[a]	3897.82[a,b]	3941.91[a,b]	34.71	0.021
FCR	1.71	1.70	1.68	1.72	1.68	1.70	0.02	0.828
Overall, d 1 - 42								
Final BW (g/bird)	3166.54[b]	3234.61[a,b]	3323.48[a]	3313.78[a,b]	3266.83[a,b]	3294.73[a,b]	32.17	0.016
BW gain (g/bird)	3129.27[b]	3197.15[a,b]	3285.98[a]	3276.32[a]	3229.41[a,b]	3257.27[a,b]	32.18	0.017
Feed intake (g/bird)	4961.84[b]	5034.68[a,b]	5129.60[a,b]	5190.38[a]	5047.74[a,b]	5111.44[a,b]	46.45	0.025
FCRc[4]	1.64	1.62	1.60	1.62	1.60	1.60	0.01	0.132

[a–c]Means in the same row with no common superscripts are significantly different ($P < 0.05$)
[1]Supplied at a dose level which provided 2,000 units of xylanase per kg feed and 200 units of amylase per kg feed.
[2]Supplied at a dose level which provided 2,000 units of xylanase per kg feed, 200 units of amylase per kg feed, and 4000 units of protease per kg feed.
[3]Supplied at a dose level which provided 150,000 cfu/g of feed.
[4]Corrected for mortality

Table 3. Bodyweight gain (**BWG**) was increased in birds fed the probiotic alone (**DFM**) compared with those fed the control diet, both in the starter phase (d 1 to 21) (953.19 vs. 889.13 g/d; $P < 0.05$) and overall (d 1 to 42) (3,276.32 vs. 3,129.27 g/d; $P < 0.05$). There was no significant effect of XA or XAP on BWG, but XAP significantly improved BWG and final BW overall compared with control birds (BWG 3,285.98 vs. 3,129.27 g/d, Final BW 3,323.48 vs. 3,166.54 g; $P < 0.05$). Effects on FI were similar to those seen for BWG: in comparison with control birds, birds fed the probiotic exhibited increased FI at all 3 time points ($P < 0.05$), XA had no effect on FI, while XAP increased FI in the finisher period only ($P < 0.05$). Neither DFM+XA nor DFM+XAP affected BWG or FI significantly compared to control birds. No significant effects on FCR were observed for any of the dietary treatments.

None of the multi-enzyme/probiotic dietary treatment combinations gave rise to improvements in the AID of nitrogen (Table 4). However, there were significant improvements in all other variables: AIDE was increased among birds fed either XA or XAP in combination with the probiotic, compared with

Table 4. Effect of multi-enzyme combinations XA (xylanase, amylase) and XAP (xylanase, amylase, and protease), given with and without a *Bacillus* probiotic (DFM) on Ileal digestibility of nutrients and energy, nitrogen retention and metabolizable energy content in broilers (Experiment 2).

	Control (CTL)	NC + XA[1]	NC + XAP[2]	NC + DFM[3]	NC + DFM + XA	NC + DFM + XAP	SEM	*P*-value
Starter Phase, d 1–21								
Ileal digestibility of N (%)	83.47	85.11	84.79	84.04	84.72	84.45	0.63	0.497
Ileal digestibility of GE[4] (%)	68.20^{b}	71.66^{a,b}	72.16^{a,b}	70.35^{a,b}	72.58^{a}	72.58^{a}	0.96	0.020
AIDE[5] (kcal/kg DM)	3089.0^{b}	3246.0^{a,b}	3268.0^{a,b}	3186.0^{a,b}	3287.0^{a}	3287.0^{a}	43.51	0.020
Finisher Phase, d 22–42								
Ileal digestibility of N (%)	83.31	84.08	84.08	84.07	84.61	85.13	0.75	0.649
Ileal digestibility of Fat (%)	86.24^{c}	88.81^{a,b}	89.87^{a}	87.31^{b,c}	89.00^{a,b}	89.94^{a}	0.53	0.002
Ileal digestibility of Starch (%)	92.74^{c}	97.29^{a}	97.53^{a}	95.50^{b}	97.53^{a}	97.79^{a}	0.23	0.001
Ileal digestibility of GE (%)	70.48^{c}	72.16^{a-c}	72.12^{a-c}	71.24^{b,c}	73.25^{a,b}	74.15^{a}	0.61	0.001
AIDE (kcal/kg/DM)	3192.0^{c}	3268.0^{a-c}	3266.0^{a-c}	3226.0^{b,c}	3318.0^{a,b}	3358.0^{a}	27.40	0.002
Retention of N (%)	68.89^{b}	70.57^{a,b}	70.75^{a,b}	69.06^{b}	71.66^{a}	70.95^{a,b}	0.53	0.005
AME_n (Kcal)	2960.0^{c}	3016.0^{a-c}	3042.0^{a,b}	2995.0^{b,c}	3074.0^{a}	3092.0^{a}	16.76	0.001

$^{a–c}$Means in the same row with no common superscripts are significantly different ($P < 0.05$)

[1]Supplied at a dose level that provided 2,000 units of xylanase per kg feed and 200 units of amylase per kg feed.

[2]Supplied at a dose level that provided 2,000 units of xylanase per kg feed, 200 units of amylase per kg feed, and 4,000 units of protease per kg feed.

[3]Supplied at a dose level that provided 150,000 cfu/g of feed.

[4]GE- Gross Energy

[5]AIDE—Apparent ileal digestible energy

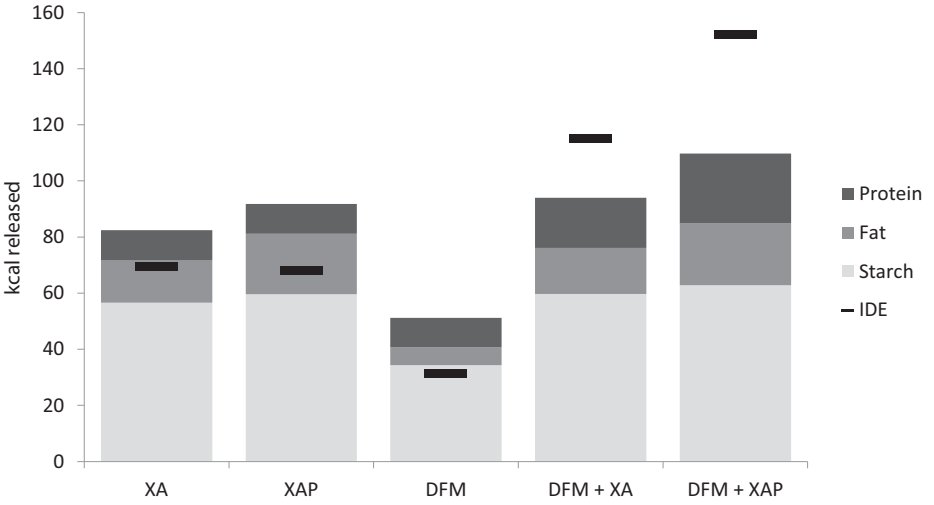

Figure 1. Improvements in the ileal digestibility of protein (nitrogen), fat, starch and energy (IDE) in broilers fed a corn-soybean based diet supplemented with enzymes (XA/XAP), a *Bacillus* probiotic (DFM), or both together, on d 21. Improvements in IDE are given by the black bars—the difference between the contributions of protein, fat and starch and overall IDE in the DFM+XA and DFM+XAP groups is hypothesized to be due to increased energy release from fiber.

control fed birds, at both time-points (AIDE on d 21 74.15% (DFM+XAP) vs. 70.48% (NC)) ($P < 0.05$). Although numerically higher, effects of the enzymes or probiotic given alone on AIDE were not significant. The size of the observed effects of the combined treatments (DFM+XA or DFM+XAP) on AIDE on d 21 were greater than the sum of the effect of the individual additives when given alone (by 0.33% in the DFM+XA group and by 1.27% (~58 kcal) in DFM+XAP birds). Improvements were also seen in AIDE on d 11, and to an even greater extent on d 21 with DFM+XA and DFM+XAP compared with the CTL group (3,318 kcal/kg DM (DFM+XA) or 3,358 kcal/kg DM (DFM+XAP) vs. 3,192 kcal/kg (NC)) ($P < 0.01$), but again these effects were not evident when the enzymes/probiotics were given alone. AID of fat on d 21 was increased by addition of XA and XAP, but not the probiotic, compared with the CTL group (88.81 vs. 86.24% and 89.87% vs. 86.24% respectively) ($P < 0.01$), and AID of starch was increased by all 3 of these treatments (XA, XAP, or DFM) ($P < 0.001$). Combining the enzymes and the probiotic together produced a similar positive effect on AID of fat and starch to that produced by the supplements given individually ($P < 0.01$). Figure 1 displays the relative improvements in the digestibility of protein (nitrogen), fat, starch, and energy in the enzyme and/or probiotic supplemented diets compared with the control diet, and the marked increase in AIDE in DFM+XAP supplemented birds is very apparent. AME_n was increased by

Table 5. Ileal flow (g/100 g DM intake) of components of non-starch polysaccharides in response to feeding diets supplemented with enzyme combinations XA (xylanase, amylase) and XAP (xylanase, amylase, and protease), with and without a *Bacillus* probiotic (DFM), in broilers (Experiment 2).

		Control (CTL)	NC + XA[1]	NC + XAP[2]	NC + DFM[3]	NC + DFM + XA	NC + DFM + XAP	SEM	*P*-value
Rhabinose	Soluble	0.04	0.03	0.03	0.05	0.02	0.03	0.01	0.826
	Insoluble	0.07	0.06	0.06	0.06	0.06	0.05	0.01	0.861
	Total	0.11	0.09	0.09	0.10	0.08	0.08	0.02	0.889
Fructose	Soluble	0.09	0.07	0.08	0.08	0.07	0.08	0.01	0.811
	Insoluble	0.04	0.05	0.03	0.04	0.04	0.04	0.01	0.559
	Total	0.13	0.12	0.12	0.12	0.11	0.12	0.01	0.940
Arabinose	Soluble	0.38	0.36	0.32	0.32	0.33	0.34	0.04	0.855
	Insoluble	1.90^{a}	1.77^{a,b}	1.83^{a,b}	1.91^{a}	1.74^{b}	1.69^{b}	0.05	0.024
	Total	2.29	2.13	2.15	2.23	2.08	2.03	0.06	0.057
Xylose	Soluble	0.29	0.22	0.25	0.22	0.20	0.22	0.04	0.773
	Insoluble	2.35	2.23	2.21	2.37	2.19	2.09	0.07	0.091
	Total	2.64	2.45	2.45	2.59	2.39	2.31	0.08	0.068
Mannose	Soluble	0.14	0.14	0.12	0.14	0.12	0.11	0.01	0.375
	Insoluble	0.15	0.14	0.16	0.16	0.15	0.15	0.02	0.980
	Total	0.29	0.29	0.29	0.30	0.27	0.26	0.02	0.874
Galactose	Soluble	0.59	0.58	0.56	0.55	0.57	0.53	0.04	0.890
	Insoluble	0.98^{a}	0.86^{a,b}	0.91^{a,b}	0.99^{a}	0.84^{b}	0.86^{a,b}	0.03	0.007
	Total	1.56^{a}	1.45^{a,b}	1.48^{a,b}	1.54^{a}	1.41^{b}	1.39^{b}	0.04	0.035
Glucose	Soluble	0.23	0.25	0.17	0.14	0.19	0.22	0.04	0.485
	Insoluble	2.61	2.44	2.53	2.60	2.42	2.34	0.07	0.083
	Total	2.84	2.69	2.70	2.75	2.62	2.56	0.08	0.271
Galacturonic Acid	Soluble	0.49	0.45	0.48	0.47	0.48	0.48	0.06	0.999
	Insoluble	0.45	0.42	0.44	0.45	0.39	0.38	0.04	0.766
	Total	0.94	0.87	0.92	0.92	0.87	0.86	0.05	0.805
Total	Soluble	2.02^{a}	1.76^{a,b}	1.71^{a,b}	1.85^{a,b}	1.58^{a,b}	1.56^{b}	0.10	0.041
	Insoluble	8.56^{a}	7.99^{a,b}	8.12^{a,b}	8.57^{a}	7.83^{a,b}	7.59^{b}	0.22	0.016
	Total	10.79	10.09	10.20	10.54	9.82	9.61	0.29	0.077

^{a,b}Means in the same row with no common superscripts are significantly different ($P < 0.05$)

[1]Supplied at a dose level that provided 2,000 units of xylanase per kg feed and 200 units of amylase per kg feed.

[2]Supplied at a dose level that provided 2,000 units of xylanase per kg feed, 200 units of amylase per kg feed, and 4,000 units of protease per kg feed.

[3]Supplied at a dose level that provided 150,000 cfu/g of feed.

XAP but not XA, compared with CTL birds (3,042 vs. 2,960 kcal) ($P < 0.001$), and was improved even more by DFM+XA and DFM+XAP (3,074 DFM+XA vs. 2,960 kcal CTL and 3,092 DFM+XAP vs. 2,960 kcal CTL respectively) ($P < 0.001$).

The ileal flow of NSP components in response to the dietary treatments is shown in Table 5. Administration of either XA, XAP or the probiotic alone did not affect ileal flow of NSP. Both DFM+XA and DFM+XAP reduced the ileal flow of insoluble arabinose ($P < 0.05$) relative to the CTL group. DFM+XAP additionally reduced the ileal flow of total galactose, and DFM+XA reduced ileal flow of both total galactose and insoluble galactose ($P < 0.01$) compared with CTL birds. Effects of the treatments on total tract flow of NSP (Table 6) were greater in magnitude, but otherwise generally mirrored effects on ileal flow of NSP, with the DFM+XAP treatment producing the most significant effects among the treatments, in both magnitude and number of NSP components affected. Total tract flow of total and insoluble arabinose, as well as total galactose, were reduced by DFM+XAP compared with the CTL group ($P < 0.001$, $P < 0.01$, and $P < 0.01$ respectively). In contrast, DFM+XA only significantly reduced the flow of total arabinose ($P < 0.001$). DFM+XAP additionally produced a reduction in the flow of soluble, insoluble and total xylose at the total tract level, compared with the CTL group ($P < 0.05$, $P < 0.001$, $P < 0.001$). This effect was not produced by DFM+XA.

The flow of total digestion resistant oligosaccharides (ROs) in the ileal tract was numerically lower among all treatments compared with the CTL group (Table 7), but this was only significant in XA fed birds ($P < 0.05$). Ileal flow of resistant fructose was reduced in the XAP treatment group compared with the CTL group ($P < 0.01$), but no other significant reductions in flow were detected among individual ROs at the ileal level. At the total tract level, no significant differences in total or individual flow of ROs were detected.

No differences in bacterial species levels in the ileum were apparent among the different treatment groups (Table 8). However, *Bacteroides* and *Campylobacter* species levels were both reduced in the cecum in the XA and XAP treatment groups compared with the CTL group ($P < 0.05$ and $P < 0.001$ respectively). The magnitude of the effect of XA and XAP on *Bacteroides* levels was relatively small (5.20 (XA) or 5.25 (XAP) vs. 5.58 log cfu/g (NC), $P = 0.028$) but effects on the levels of *Campylobacter* species were greater than 2.5 log cfu/g in both cases (3.43 (XA) or 2.54 (XAP) vs. 5.25 log cfu/g (NC), $P < 0.001$). Statistically significant effects were not detected among these species when the enzymes were combined with the

Table 6. Ileal and total tract flow of digestion resistant oligosaccharides and starch (g/100 g DM intake) in response to feeding diets supplemented with enzyme combinations XA (xylanase, amylase) and XAP (xylanase, amylase, and protease), with and without a *Bacillus* probiotic (DFM), in broilers (Experiment 2).

	Control (CTL)	NC + XA[1]	NC + XAP[2]	NC + DFM[3]	NC + DFM + XA	NC + DFM + XAP	SEM	*P* -value
Ileal flow								
Fructose	061[a]	050[a,b]	046[b]	058[a,b]	051[a,b]	052[a,b]	003	0007
Galactose	078	065	067	075	069	067	004	0072
Glucose	041	033	037	038	036	034	002	0249
Total NSP	180[a]	148[b]	152[a,b]	172[a,b]	158[a,b]	152[a,b]	007	0024
Total Starch	137	126	123	148	146	118	009	0140
Total Tract Flow								
Fructose	015	018	017	018	021	015	002	0502
Galactose	019	022	017	023	023	018	003	0456
Glucose	010	011	010	011	013	010	001	0617
Total NSP	044	051	043	052	055	042	006	0542
Total Starch	146	130	130	120	143	130	010	0514

[a,b]Means in the same row with no common superscripts are significantly different ($P < 0.05$)
[1]Supplied at a dose level which provided 2,000 units of xylanase per kg feed and 200 units of amylase per kg feed.
[2]Supplied at a dose level which provided 2,000 units of xylanase per kg feed, 200 units of amylase per kg feed, and 4,000 units of protease per kg feed.
[3]Supplied at a dose level which provided 150,000 cfu/g of feed.

Table 7. Total tract flow (g/100 g DM intake) of components of non-starch polysaccharides in response to feeding diets supplemented with enzyme combinations XA (xylanase, amylase) and XAP (xylanase, amylase, and protease), with and without a *Bacillus* probiotic (DFM), in broilers (Experiment 2).

		Control (CTL)	NC + XA[1]	NC + XAP[2]	NC + DFM[3]	NC + DFM + XA	NC + DFM + XAP	SEM	*P* -value
Rhabinose	Soluble	0.03	0.02	0.03	0.03	0.03	0.03	0.01	0.960
	Insoluble	0.06	0.07	0.07	0.06	0.06	0.05	0.01	0.451
	Total	0.09	0.09	0.10	0.09	0.09	0.08	0.01	0.522
Fructose	Soluble	0.06	0.06	0.06	0.07	0.05	0.06	0.01	0.653
	Insoluble	0.04	0.03	0.02	0.03	0.04	0.03	0.01	0.560
	Total	0.10	0.09	0.08	0.10	0.09	0.09	0.01	0.138
Arabinose	Soluble	0.27	0.26	0.26	0.29	0.19	0.23	0.03	0.167
	Insoluble	2.05[a]	1.92[a,b]	1.91[a,b]	2.03[a]	1.90[a,b]	1.82[b]	0.04	0.001
	Total	2.32[a]	2.17[a,b]	2.17[a,b]	2.32[a]	2.09[b]	2.05[b]	0.04	0.001
Xylose	Soluble	0.24[a]	0.15[b]	0.17[b]	0.20[a,b]	0.15[b]	0.16[b]	0.02	0.039
	Insoluble	2.51[a,b]	2.37[b,c]	2.36[b,c]	2.55[a]	2.38[b,c]	2.28[c]	0.04	0.001
	Total	2.76[a]	2.52[b]	2.54[b]	2.75[a]	2.52[b]	2.44[b]	0.04	0.001
Mannose	Soluble	0.11	0.13	0.12	0.12	0.11	0.13	0.01	0.788
	Insoluble	0.22	0.21	0.21	0.22	0.19	0.20	0.01	0.190
	Total	0.34	0.34	0.33	0.34	0.30	0.33	0.01	0.165
Galactose	Soluble	0.48	0.49	0.47	0.48	0.42	0.43	0.03	0.325
	Insoluble	1.09	1.01	1.01	1.12	1.02	1.00	0.03	0.084
	Total	1.57[a,b]	1.50[a-c]	1.48[a-c]	1.61[a]	1.44[b,c]	1.42[c]	0.03	0.002
Glucose	Soluble	0.29	0.13	0.15	0.16	0.15	0.13	0.04	0.089
	Insoluble	2.62	2.66	2.60	2.79	2.62	2.59	0.06	0.228
	Total	2.92	2.79	2.75	2.95	2.77	2.72	0.06	0.040
Galacturonic Acid	Soluble	0.52	0.52	0.45	0.49	0.48	0.41	0.04	0.368
	Insoluble	0.40	0.41	0.42	0.44	0.39	0.39	0.02	0.452
	Total	0.92	0.93	0.88	0.93	0.86	0.80	0.03	0.040
Total	Soluble	2.02[a]	1.76[a,b]	1.71[a,b]	1.84[a,b]	1.58[a,b]	1.56[b]	0.10	0.041
	Insoluble	8.99[a,b]	8.68[b,c]	8.61[b,c]	9.24[a]	8.59[b,c]	8.37[c]	0.12	0.001
	Total	11.01[a,b]	10.43[b,c]	10.32[c]	11.08[a]	10.17[c]	9.93[c]	0.15	0.001

[a–c]Means in the same row with no common superscripts are significantly different ($P < 0.05$)
[1]Supplied at a dose level that provided 2,000 units of xylanase per kg feed and 200 units of amylase per kg feed.
[2]Supplied at a dose level that provided 2,000 units of xylanase per kg feed, 200 units of amylase per kg feed, and 4,000 units of protease per kg feed.
[3]Supplied at a dose level that provided 150,000 cfu/g of feed.

probiotic, with the exception of DFM+XAP which produced a <1 log reduction in *Campylobacter* species levels compared with the CTL group ($P < 0.001$). There were no effects of the probiotic alone on levels of any of the species of bacteria studied in the ileum or cecum.

DISCUSSION

The inclusion of wheat middlings and corn-DDGS in grain-soybean meal poultry diets reduces their nutrient and energy digestibility (Jaroni et al., 1999; Salim et al., 2010). The major mechanism for this effect is thought

Table 8. Effect of dietary supplementation with enzyme combinations XA (xylanase, amylase) and XAP (xylanase, amylase, and protease), with and without a *Bacillus* probiotic (DFM), on ileal and cecal microflora composition (log cfu/g of wet digesta) of broilers on d 11 as determined by RT-PCR (Experiment 1).

	Control (CTL)	NC + XA[1]	NC + XAP[2]	NC + DFM[3]	NC + DFM + XA	NC + DFM + XAP	SEM	*P*-value
Ileum								
Bacteroides	5.98	6.12	6.08	6.07	5.84	6.01	0.07	0.054
Bifidobacteria	9.32	9.46	9.49	9.38	9.36	9.31	0.08	0.481
Campylobacter	5.86	5.98	5.34	5.82	4.13	5.64	0.58	0.222
Clostridium	4.08	4.81	4.73	3.99	3.96	4.02	0.31	0.174
E. Coli	3.28	3.88	4.31	4.14	4.41	3.91	0.32	0.152
Lactobacillus	9.61	9.69	9.62	9.53	9.21	9.46	0.16	0.363
Salmonella	3.87	3.83	4.02	4.07	4.10	4.10	0.10	0.252
Cecum								
Bacteroides	5.58^{a}	5.20^{b}	5.25^{b}	5.58^{a}	5.57^{a}	5.46^{a,b}	0.11	0.028
Bifidobacteria	9.16	9.08	9.05	9.13	9.15	9.14	0.07	0.860
Campylobacter	5.25^{a}	3.43^{b,c}	2.54^{c}	5.07^{a,b}	4.72^{a,b}	4.04^{b,c}	0.42	0.001
Clostridium	7.04	6.78	6.72	7.05	7.00	6.75	0.11	0.074
E. Coli	6.84	6.79	7.03	6.49	6.56	6.26	0.70	0.978
Lactobacillus	9.58	9.54	9.35	9.58	9.60	9.66	0.20	0.921
Salmonella	3.63	4.28	3.38	3.91	3.25	3.53	0.33	0.269

$^{a–c}$Means in the same row with no common superscripts are significantly different ($P < 0.05$)

[1]Supplied at a dose level that provided 2,000 units of xylanase per kg feed and 200 units of amylase per kg feed.

[2]Supplied at a dose level that provided 2,000 units of xylanase per kg feed, 200 units of amylase per kg feed, and 4,000 units of protease per kg feed.

[3]Supplied at a dose level that provided 150,000 cfu/g of feed.

to be associated with increased viscosity in the gut, mediated by higher levels of major NSP such as arabinoxylans and pentosans (Annison and Choct, 1991; Bedford and Schulze, 1998). These may reduce nutrient digestion in the small intestine via inhibition of the proper mixing of dietary components (Ward, 1996) and decreased flow-rate of metabolites along the gut (Fengler and Marquardt, 1988), leading to decreased absorption of nutrients. Increased viscosity may also alter the delicate balance of microflora in the gut, which may further impair digestion and absorption. The present study showed significant benefits of XA supplementation in terms of an improvement in the ileal digestibility of starch and of fat on d 22 to 42 compared to the control. Supplementation with XAP gave rise to similar improvements but, in addition, a significant improvement (of, on average, 82 kcal) in AME_n. These findings are in broad agreement with those of Romero et al. (2013, 2014), in which similar benefits of XA and XAP supplementation on AID of starch and of fat were observed. In addition, these authors reported a clear additional benefit of XAP over XA in terms of increased AID of nitrogen/protein and AA, and a greater increase in AID of energy and in AME_n (Romero et al., 2013, 2014). Olukosi et al. (2015) reported an improvement in AME in broilers fed a corn-based diet supplemented with protease. Similarly, the increased AME_n that we observed in the XAP supplemented group compared with the CTL group, which was absent in the XA treated group, suggests that the protease in the XAP may have resulted in beneficial effects in nutrient and energy utilization that were to some degree independent of protein digestion.

The combination of *Bacillus* probiotics and exogenous enzymes led to additional and more significant effects on the apparent ileal digestibility of nutrients and energy than their delivery alone. In particular, the magnitude of the improvements in AIDE and in AME_n in the DFM+XA and DFM+XAP treatments, when compared with treatments involving enzymes given alone and with the control diet, were suggestive of a beneficial interaction occurring between the probiotic and XA/XAP. The precise mechanisms for the interaction between enzymes and probiotic are as yet unclear. However, research indicates that these enzymes, in particular xylanases, can have a "prebiotic" effect in the poultry gut, via a combination of increasing the availability of substrates for beneficial bacteria due to their hydrolyzing effects on NSP, starch and protein (Bedford and Cowieson, 2012; Romero et al., 2013, 2014), and a reduction in indigestible protein and other substrates that favor the growth of pathogenic bacteria (Kiarie et al., 2013). XA and XAP supplementation, therefore, may have altered the substrates available to the probiotic *Bacillus* strains in the gut, resulting in more magnified effects on nutrient and energy utilization than were achieved by the probiotic or enzymes given alone.

The efficiency of digestion of starch, fat or protein in broiler diets is not necessarily comparable with that of NSP component sugars (Chwalibog, 2002). Therefore, it is potentially also helpful to look at the degree of NSP hydrolysis, potentially providing additional clues as to the contributing factors responsible for observed improvements in digestibility of energy and nutrients. It is expected that a greater degree of hydrolysis of NSP will result in a reduced level of NSP components being detectable in the DM. In the present study, combined supplementation of the enzymes (XA or XAP) with the probiotic led to reductions in the flow of insoluble arabinose (and galactose in the XA group)

compared with control fed birds, and these effects were apparent at both the ileal and total tract levels. The latter also showed a reduction in both soluble and insoluble fractions of xylose in the DFM+XAP supplementation group. This is broadly in line with previous studies, in particular Olukosi et al. (2015), who also reported a reduction in ileal flow of insoluble arabinose and in the post-cecal flow of insoluble xylose in XAP supplemented broilers on similar corn-based diets. Arabinose and xylose are important components of hemicellulose in corn and corn-DDGS, and the current results are indicative of an increase in the hydrolysis of arabinoxylan polymers in the small intestine of birds supplemented with DFM+XAP. These findings further suggest that DFM+XAP may deliver a greater benefit than DFM+XA in terms of reducing the flow of component NSP, particularly insoluble arabinose and xylose. As hypothesised by Olukosi et al. (2015), it is plausible that the joint actions of NSP-hydrolyzing enzymes and proteases disrupt the cell wall fiber-protein matrix, and thus may be responsible for the apparently greater effect of DFM+XAP compared with DFM+XA on NSP flow in our study.

The major ROs in corn-soybean meal based diets are raffinose and stachyose (Honig and Rakies, 1979), which make up 4 to 6% of the total oligosaccharides; β-mannans are also present in soybean meal to a lesser extent. These fermentable fibers have been ascribed both positive (Anderson et al., 2009) and negative (Coon et al., 1990) effects on various digestion and immunological related response measures associated with gut health in poultry. In a previous study, Olukosi et al. (2015) observed significant reductions in the post-cecal flow of glucose and galactose from the RO fraction in XAP supplemented broilers. In the present study, there were no effects on the flow of ROs at the total tract level. In the ileum, the responses were somewhat inconsistent, whereby flow of resistant fructose was reduced in XAP but not in DFM+XAP supplemented birds.

These changes in NSP flow through the gut are likely to be one of the primary mechanisms behind the observed reductions (>2.5 log) in *Campylobacter* presence in the ceca of birds supplemented with XA, XAP and DFM+XAP: the hydrolytic actions of the enzymes on the fiber-protein components of the feed may have reduced the levels of undigested materials which would otherwise form substrates for bacteria such as *Campylobacter,* thereby reducing their apparent levels in the cecum when compared with control fed birds, though this warrants further investigation. The effect of the NSP-hydrolyzing enzymes on *Campylobacter* is likely unrelated to the observed reductions in *Bacteroides* levels as the colonisation patterns of these 2 species in the gut are quite different, with *Bacteroides* predominantly colonising the small intestine and *Campylobacter* generally favoring the cecum (Newell and Fearnley, 2003; Callaway and Rickes, 2011).

As a consequence of these improvements in nutrient absorption and reductions in pathogen burden in the gut, this study found a beneficial effect of probiotic and enzyme supplementation on growth performance of the birds, in line with previous findings (Amerah et al., 2017; Wealleans et al., 2017a). In this study the combination of DFM+XAP lead to higher final bodyweights compared to XAP or the probiotic alone, but the difference was not significant; Flores et al. (2016) reported an improvement in FCR as well as reduced foot-pad lesion score and energy efficiency in DFM+XAP supplemented birds, but no effect on feed intake or BWG, while Wealleans et al. (2017b) found that performance improvements were greater in birds subjected to a coccidiosis vaccine program, rather than those raised with ionophores.

In conclusion, supplementation of a corn-soybean meal-based broiler diet with a combination of xylanase, amylase, and protease improved FI and BWG, while supplementation with xylanase and amylase alone did not. Both resulted in improvements in the ileal digestibility of fat, starch and energy, as well as in the total tract flow of soluble arabinose and a reduction in cecal populations of *Bacteroides* and *Campylobacter* species. Effects of XAP were generally greater in magnitude then effects of XA; this may be due to both independent and interactive effects of protease and NSP-hydrolyzing enzymes in digesting anti-nutritive NSP and in unlocking the complex protein-fiber matrices contained in the cell walls of the grains, as demonstrated by Romero et al. (2013). When XA or XAP were fed in combination with a multi-strain *Bacillus* probiotic, beneficial effects on growth performance, digestibility of nutrients and energy, NSP flow, and levels of potentially pathogenic *Campylobacter* species in the cecum were observed, supporting an emerging body of evidence that the combined actions of enzymes and probiotics may be beneficial in bringing about greater improvements in broiler growth performance than can be achieved by their use in isolation.

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