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Exogenous carbohydrases added to a starter diet reduced markers of systemic immune activation and decreased Lactobacillus in weaned pigs

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Abstract

Although the impact of carbohydrases on performance and nutrient utilization has been well studied, their effects on immune status and intestinal microbiota are less known in pigs. This study aimed to evaluate the impact of xylanase (X) and a carbohydrase enzyme blend (EB; cellulase, ß-glucanase, and xylanase) on the immune profile of the intestine and peripheral system as well as intestinal microbes and microbial metabolites of weaned pigs fed higher fiber diets. Pigs (n = 460; 6.43 ± 0.06 kg BW; F25 × 6.0 Genetiporc) were blocked by initial BW. Pens (n = 48; 12 per treatment; 9 or 10 pigs per pen) were randomly assigned to 1 of 4 dietary treatments, including a higher fiber control diet (CON) and the CON supplemented with 0.01% X, 0.01% EB, or both enzymes (X + EB), arranged in a 2 × 2 factorial. The diets were based on corn, soybean meal, corn distillers dried grains with solubles, and wheat middlings. After 7-d adaptation to the environment, pigs were fed experimental diets ad libitum for 28 d. Blood samples were collected from the same pig within each pen on days 0, 7, 14, and 28. Intestinal tissues and digesta were collected on day 28. Bacteria 16S rRNA gene copy numbers were quantified using qPCR. The mRNA levels of colonic *IL-17*, *occludin* (*OCLN*), and *claudin 3* (*CLDN3*) were greater in pigs fed diets with X + EB, but not X or EB, compared with those fed CON (P < 0.05). The EB in the diet reduced plasma IL-8 over the 28-d trial compared with diets without EB (P < 0.05). There was an X × EB interaction on plasma tumor necrosis factor α and IL-1ß (P < 0.05); their levels were decreased when X and EB were added together, but not individually, compared with CON. The EB decreased cecal propionate, butyrate, and total volatile fatty acids (P < 0.05). Pigs fed X had lower ileal *Lactobacillus* and greater ileal and cecal *Enterobacteriaceae* compared with those fed unsupplemented diets (P < 0.05). The EB decreased *Lactobacillus* (P < 0.05) and tended to decrease (P = 0.065) *Enterobacteriaceae* in the colon compared with diets without EB. In conclusion, the addition of X and EB together decreased systemic markers of immune activation, potentially diverting energy and nutrients towards growth. The EB reduced colonic *Lactobacillus* and cecal total volatile fatty acids, probably due to improved prececal fiber and starch degradation and thus reduced substrate availability in the large intestine. These data corroborated previously observed enhanced growth in pigs fed EB-supplemented diets.

Keywords

dietary enzymes, inflammation markers, microbiota, volatile fatty acids, swine

Disciplines

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Exogenous carbohydrases added to a starter diet reduced markers of systemic immune activation and decreased *Lactobacillus* in weaned pigs[1]

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ABSTRACT: Although the impact of carbohydrases on performance and nutrient utilization has been well studied, their effects on immune status and intestinal microbiota are less known in pigs. This study aimed to evaluate the impact of xylanase (**X**) and a carbohydrase enzyme blend (**EB**; cellulase, ß-glucanase, and xylanase) on the immune profile of the intestine and peripheral system as well as intestinal microbes and microbial metabolites of weaned pigs fed higher fiber diets. Pigs (n = 460; 6.43 ± 0.06 kg BW; F25 × 6.0 Genetiporc) were blocked by initial BW. Pens (n = 48; 12 per treatment; 9 or 10 pigs per pen) were randomly assigned to 1 of 4 dietary treatments, including a higher fiber control diet (**CON**) and the CON supplemented with 0.01% X, 0.01% EB, or both enzymes (X + EB), arranged in a 2 × 2 factorial. The diets were based on corn, soybean meal, corn distillers dried grains with solubles, and wheat middlings. After 7-d adaptation to the environment, pigs were fed experimental diets ad libitum for 28 d. Blood samples were collected from the same pig within each pen on days 0, 7, 14, and 28. Intestinal tissues and digesta were collected on day 28. Bacteria 16S rRNA gene copy numbers were quantified using qPCR. The mRNA levels of colonic *IL-17*, *occludin* (*OCLN*), and *claudin 3* (*CLDN3*) were greater in pigs fed diets with X + EB, but not X or EB, compared with those fed CON (P < 0.05). The EB in the diet reduced plasma IL-8 over the 28-d trial compared with diets without EB (P < 0.05). There was an X × EB interaction on plasma tumor necrosis factor α and IL-1ß (P < 0.05); their levels were decreased when X and EB were added together, but not individually, compared with CON. The EB decreased cecal propionate, butyrate, and total volatile fatty acids (P < 0.05). Pigs fed X had lower ileal *Lactobacillus* and greater ileal and cecal *Enterobacteriaceae* compared with those fed unsupplemented diets (P < 0.05). The EB decreased *Lactobacillus* (P < 0.05) and tended to decrease (P = 0.065) *Enterobacteriaceae* in the colon compared with diets without EB. In conclusion, the addition of X and EB together decreased systemic markers of immune activation, potentially diverting energy and nutrients towards growth. The EB reduced colonic *Lactobacillus* and cecal total volatile fatty acids, probably due to improved prececal fiber and starch degradation and thus reduced substrate availability in the large intestine. These data corroborated previously observed enhanced growth in pigs fed EB-supplemented diets.

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INTRODUCTION

Weaned pigs are not efficient at utilizing dietary fiber due to their limited physical gut capacity (Li and Patience, 2016) and fermentation

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capacity (Knudsen et al., 2012). Thus, nonstarch polysaccharides (**NSP**) degrading carbohydrases are currently supplemented to improve nutrient digestibility and growth performance of pigs (Adeola and Cowieson, 2011; Tsai et al., 2017). Additionally, carbohydrases (e.g., xylanase) degrade dietary NSP and liberate low molecular weight oligosaccharides (Lærke et al., 2015; Pedersen et al., 2015) and/or monosaccharides (Gill et al., 2000). The released oligosaccharides, especially arabinoxylan-oligosaccharides (**AXOS**), can then exert prebiotic effects by selectively modulating intestinal microbiota and altering host immune status through interaction with microbes or direct stimulation of epithelial cells and monocytes (Courtin et al., 2008; Chen et al., 2012; Mendis et al., 2016). Therefore, it is reasonable to hypothesize that carbohydrase supplementation in higher fiber diets (especially those abundant in arabinoxylan) may regulate intestinal microbiota and modulate immune status, either directly or indirectly, in pigs through fiber degradation and production of AXOS in the intestine.

It was previously found that a carbohydrase blend (**EB**; cellulase, ß-glucanase, and xylanase) enhanced growth rate of nursery pigs fed higher fiber diets, which may be partly due to improved barrier integrity and decreased inflammation markers of the small intestine (Li et al., 2018). This study is a further analysis of samples collected from the previous experiment to evaluate how the addition of xylanase (**X**) and/or EB affects the colonic and peripheral inflammatory cytokines as well as microbial populations in those pigs. It was hypothesized that supplementation with X and/or EB would down-regulate colonic and peripheral inflammatory status and beneficially alter the intestinal microbial population.

MATERIALS AND METHODS

All procedures in this experiment adhered to guidelines for the ethical and humane use of animals for research and were approved by the Institutional Animal Care and Use Committee at Iowa State University (#9-15-8097-S).

Animals and Experimental Design

Pigs used in this experiment and experimental design were previously described in Li et al. (2018). Briefly, 460 weaned pigs (6.43 ± 0.06 kg BW; 6.0 × F25 Genetiporc; PIC Inc., Hendersonville, TN) were blocked by initial BW and 48 pens (n = 12 per treatment) were randomly assigned to 1 of 4 dietary treatments, with 9 or10 pigs per pen. The 4 diets included a higher fiber control diet (**CON**) plus the CON supplemented with either X, EB, or X + EB, arranged in a 2 × 2 factorial: X (0 or 0.01%) and EB (0 or 0.01%). The inclusion rate of both enzymes was based on manufacturer's recommendations (Huvepharma, Peachtree City, GA). The X activity was 15,000 EPU xylanase/g and 1 g of EB contained 7,000 CU of cellulase, 5,000 U of β-glucanase, and 1,000 EPU of xylanase. Diets were based on corn, soybean meal, corn distillers dried grains with solubles (**DDGS**), and wheat middlings. At weaning, all pigs had a 7-d period of acclimation to the environment and were fed a common commercial starter diet that did not contain any antibiotics or zinc or copper at levels above their nutritional requirement. The experimental diets were then fed for 28 d in 2 phases, with days 0 to 14 as Phase 1 and days 15 to 28 as Phase 2 (Table 1). Phase 1 diets contained 5% corn DDGS and 5% wheat middlings, and phase 2 diets contained 10% corn DDGS and 10% wheat middlings.

Sample Collection

On day 0, 1 pig per pen, closest to pen average BW, was selected from which to collect repeated blood samples after being weighed and ear tagged again for identification. On days 7, 14, and 28, venous blood was collected from the same pigs by jugular venipuncture into 10-mL vacuum containers with sodium heparin (Becton Dickinson, Franklin Lakes, NJ) and placed on ice after collection. Harvested blood was centrifuged at 2,000 × g for 10 min at 4 °C, and the resulting plasma was aliquoted into 1.5-mL microcentrifuge tubes and stored at −80 °C for later analysis of cytokines, as markers of systemic inflammatory status.

On day 28, the same pig used for blood collection from each pen was euthanized by captive bolt stunning followed by exsanguination. Post-euthanasia, the abdomen was opened and the entire gastrointestinal tract was removed. Mid-colon tissues were collected, rinsed with ice-cold phosphate-buffered saline (**PBS**), snap-frozen in liquid nitrogen, and kept at −80 °C for later RNA extraction. Digesta samples (3 to 5 g) from distal ileum, cecum, and mid-colon were collected, snap-frozen in liquid nitrogen, and kept at −80 °C pending DNA extraction.

The pH of the digesta from the cecum and mid-colon was measured by directly inserting the

Table 1. Ingredients and chemical composition of the basal diet (as-fed basis)

Item	Phase 1	Phase 2
Ingredients, %		
Corn	48.11	49.80
Reduced-oil corn DDGS	5.00	10.00
Wheat middlings	5.00	10.00
Milk whey powder	7.50	–
Menhaden select fish meal	5.80	2.00
Hamlet protein, HP 300[1]	8.00	3.00
Soybean meal, 47.7	17.50	22.00
Soybean oil	0.60	0.60
Limestone	0.58	1.05
L-Lys HCl	0.42	0.48
DL-Met	0.14	0.08
L-Thr	0.09	0.11
L-Trp	0.02	0.02
Phytase[2]	0.0125	0.0125
Vitamin premix[3]	0.25	0.25
Trace mineral premix[4]	0.15	0.15
Tiamulin[5]	0.18	–
Chlortetracycline[6]	0.40	–
Carbadox[7]	–	0.20
Salt	0.25	0.25
Calculated nutrient levels, %		
ME, Mcal/kg	3.35	3.29
NE, Mcal/kg	2.43	2.37
CP	23.86	22.49
NDF	9.58	13.33
ADF	3.73	4.99
SID[8] Lys	1.45	1.31

Phase 1 = days 0–14; Phase 2 = days 14–28; 0.01% xylanase or a carbohydrase enzyme blend or both were mixed with premixes and then added to the basal diets

[1]Enzymatically treated soybean meal, Hamlet Protein Inc., Findlay, OH.

[2]OptiPhos 4000 G, Huvepharma Inc., Peachtree City, GA; assumed to release 0.15% standardized total tract digestible P in the diet based on manufacture's recommendation.

[3]Provided 6,614 IU vitamin A, 827 IU vitamin D, 26 IU vitamin E, 2.6 mg vitamin K, 29.8 mg niacin, 16.5 mg pantothenic acid, 5.0 mg riboflavin, and 0.023 mg vitamin B12 per kg of diet.

[4]Provided 165 mg Zn (zinc sulfate), 165 mg Fe (iron sulfate), 39 mg Mn (manganese sulfate), 17 mg Cu (copper sulfate), 0.3 mg I (calcium iodate), and 0.3 mg Se (sodium selenite) per kg of diet.

[5]Denagard 10 (tiamulin, 22 g per kg), Elanco Animal Health, Greenfield, IN.

[6]Aureomycin 50 (chlortetracycline, 110 g per kg), Zoetis Inc., Kalamazoo, MI.

[7]Mecadox 2.5 (carbadox, 5.5 g per kg), Phibro Animal Health Corp., Ridgefield Park, NJ.

[8]SID = standardized ileal digestible.

probe of a portable pH meter (Oakton Instruments, Vernon Hills, IL) into the content. Digesta samples (approximately 20 mL) were then collected into 50-mL tubes and immediately stored at −20 °C pending volatile fatty acids (VFA) analysis.

Analytical Methods

The procedures for RNA isolation and real-time quantitative PCR (**qPCR**) to determine gene transcript abundance in colonic tissue were previously described in Li et al. (2018). Briefly, total RNA was isolated from homogenized colonic tissues (50 to 100 mg) using Trizol (1 mL; Invitrogen, Carlsbad, CA). After RNA quantification, 1 mg of isolated RNA was used for cDNA synthesis using the QuantiTect Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany). The qPCR was performed in 20-µL reactions using iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA). Each reaction included 10 µL of SYBR Green Supermix, 1 µL of each forward and reverse primer, 2 µL of cDNA, and 6 µL of nuclease-free water. A no-reverse transcriptase negative control, a nuclease-free water control, and a pooled cDNA reference sample were included in each plate. Each sample was assayed in triplicate. The cycling conditions included 5-min initial denaturation at 95 °C followed by 40 PCR cycles (95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s) and a dissociation curve to verify the amplification of a single PCR product. Analyses of amplification plots were performed with the iQ5 Optical System Software version 2.0 (Bio-Rad Laboratories, Inc.) to obtain cycle threshold (**Ct**) values for each reaction. The mRNA abundance for each sample was normalized to a housekeeping gene (ribosomal protein—L19) and the pooled sample, and fold change was calculated using the $2^{-\Delta\Delta CT}$method (Livak and Schmittgen, 2001). Plasma samples were analyzed in duplicate for interferon (**IFN**)-α, IFN-γ, tumor necrosis factor alpha (**TNF**α), interleukin (**IL**)-1β, IL-4, IL-6, IL-8, and IL-10 using a multiplex ELISA following the manufacturer's recommendations (Aushon Biosystems, Billerica, MA).

The concentration of VFA was determined using Gas Chromatography (3800 Varian GC, Agilent Technologies, Santa Clara, CA). Digesta samples (1 g) were thawed and suspended in 2.5 mL of distilled water in a screw-capped tube. After being vortexed, 1 mL of the mixture was transferred into a 1.5-mL centrifuge tube and mixed with 0.2 mL of metaphosphoric acid and 0.1 mL of isocaproic acid as an internal standard (48.3 mM; Sigma-Aldrich, Saint Louis, MO). The tubes were then centrifuged at 15,000 × *g* at 4 °C for 20 min. Aliquots of the supernatant (1 mL) of the standard and digesta samples were transferred to 1.5-mL GC vials and 100 µL duplicates were injected into the GC for analysis. A standard curve was generated using 5

concentrations of acetate, propionate, butyrate, valerate, isobutyrate, and isovalerate (Sigma-Aldrich, Saint Louis, MO). A flame ionization detector was used with an oven temperature of 60 to 200 °C. The Nukol capillary column (15 m × 0.25 mm × 0.25 µm; Sigma-Aldrich, Bellefonte, PA) was operated with highly purified He, as the carrier gas, at 1 mL/min. Concentrations of acetate, propionate, butyrate, valerate, isobutyrate, and isovalerate were calculated using the ratio of the peak area of each compound to the internal standard and standard curve regression. Molar proportions of VFA (%) were calculated as the individual VFA/total VFA concentration × 100.

Microbial Quantification Using qPCR

Total genomic DNA was extracted from intestinal digesta (250 mg) using DNeasy PowerLyzer PowerSoil kit (Qiagen, Germantown, MD) according to manufacturer's instructions. Genomic DNA concentration and purity were measured using a ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE). All samples had 260:280 nm ratios above 1.7. Extracted DNA from the ileal, cecal, and colonic digesta was adjusted to 5, 10, and 20 ng/µL, respectively.

The primers used to amplify the bacterial 16S rRNA gene are shown in Table 2. *Lactobacillus* and *Bifidobacterium* were selected to indicate abundance of beneficial bacteria, and *Enterobacteriaceae* was selected as an indicator of potential opportunistic pathogens. The PCR amplicon size for the target 16S rRNA genes was checked by running 1% gel electrophoresis. Real-time qPCR was performed in 20-µL reactions using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). Each reaction was run in duplicate, including 10 µL of SYBR Green Supermix, 1 µL of each forward and reverse primer, 2 µL of DNA, and 6 µL of nuclease-free water. Duplicates of the no template negative control were included in each PCR run. All qPCR procedures were optimized and included: one cycle of predenaturation at 95 °C for 10 min; 40 cycles of denaturation at 95 °C for 15 s, at optimal annealing temperature for 30 s and extension at 72 °C for 60 s; one cycle of 95 °C for 1 min; and one cycle of 55 °C for 1 min. Data were collected at the extension step. Fluorescence of SYBR Green was quantified with the iQ5 Real Time PCR Detection System, and amplification plots were analyzed using the iQ5 Optical System Software version 2.0 (Bio-Rad Laboratories, Hercules, CA) to obtain the Ct values for each reaction. To determine the specificity of the amplification, melting curve analysis was performed by slow heating with an increment of 0.1 °C/s from 55 to 95 °C.

To quantify the 16S rRNA gene copy numbers of total bacteria and target bacteria, standard curves were generated using a series of 10-fold dilutions of purified PCR products or bacterial DNA extracted from pure cultures of specific bacteria. For total bacteria, PCR products from the amplifying microbial 16S rRNA gene using the universal primer set 27F-1492R (27F: AGAGTTTGATYMTGGCTCAG; 1492R: TACGGYTACCTTGTTACGACT) from digesta genomic DNA were purified with a PureLink PCR purification kit (Invitrogen Life Technologies, Carlsbad, CA) and used as standard DNA. Concentrations of standard DNA samples were quantified using Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Hanover Park, IL). The copy number of standard DNA molecules was calculated using the following equation: DNA (molecules/µL) = [6.02×10^{23} (molecules/mol) × DNA concentration (g/µL)]/

Table 2. Sequences of primers for quantification of total bacteria and target bacterial groups

Bacteria	Primer sequence, 5′–3′	Annealing temperature (°C)	Product size (bp)
Total bacteria			
Forward	CCTACGGGAGGCAGCAG	61	189
Reverse	ATTACCGCGGCTGCTGG		
Lactobacillus spp.			
Forward	AGCAGTAGGGAATCTTCCA	62	341
Reverse	CACCGCTACACATGGAG		
Bifidobacterium spp.			
Forward	TCGCGTCYGGTGTGAAAG	60	243
Reverse	CCACATCCAGCRTCCAC		
Enterobacteriaceae family			
Forward	CATTGACGTTACCCGCAGAAGAAGC	65	195
Reverse	CTCTACGAGACTCAAGCTTGC		

Primer sequences were from Metzler-Zebeli et al. (2013).

[DNA length (bp) × 660 (g/mol/bp)]. The standard DNAs were then serially diluted 10-fold (1×10^7 to 1×10^1 copies/μL) and qPCR reactions were run to build specific standard curves. Each standard curve was constructed by linear regression of the plotted points, and Ct values were plotted against the logarithm of template copy numbers.

The gene copy numbers of total bacteria and target bacterial groups in intestinal digesta DNAs were determined by relating the Ct values to respective standard curves. The final copy numbers of total bacteria and target bacterial groups per gram of wet digesta were calculated using the equation (QM × C × DV)/(S × V), according to Metzler-Zebeli et al. (2013), where QM is the quantitative mean of the copy number, C is the DNA concentration of each sample, DV is the dilution volume of extracted DNA, S is the DNA amount (ng) in each reaction, and V is the sample weight (g) used for DNA extraction. The amplification efficiency was confirmed to be between 80% and 110% for all reactions.

Statistical Analysis

Data were analyzed as a 2 × 2 factorial in a randomized complete block design using PROC GLIMMIX of SAS 9.4 (SAS Institute Inc., Cary, NC). The 2 factors were X (0 or 0.01%) and EB (0 or 0.01%). The UNIVARIATE procedure was used to check normality and equal variance of residuals, and to identify statistical outliers (>3 standard deviations from the mean). Xylanase, EB, and their interaction were fixed effects; block was considered a random effect. The individual pig from each pen from which samples were collected was the experimental unit.

Blood cytokine data were log-transformed before analysis, using repeated measurements with collection day as the repeated effect and a variance structure of auto-regressive 1 was applied. Because baseline (day 0) IFN-α was different and IL-8 tended to be different among treatments, their concentrations were used as a covariate in the model. If there was a treatment by day interaction, means of the main effect on each day were separated using the least square means statement and the slice = day option. The interactions between X and EB within each day were calculated using the lsmestimate statement. Bacterial 16S rRNA gene copies of total and specific bacteria data were $\log_{10}$-transformed for analysis. Least square means of treatments were reported. Differences were considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$.

RESULTS

Colonic Gene mRNA Abundance

Xylanase did not affect the mRNA abundance of *IL1B*; however, EB tended to decrease its abundance ($P = 0.054$; Table 3). A significant X × EB interaction ($P < 0.05$) was observed for colonic *IL-6* mRNA, with EB increasing *IL-6* mRNA in the presence of X, but not in the absence of X. The addition of X tended to decrease ($P < 0.10$) *IL-10* mRNA in the colon of pigs receiving diets with EB, but not in diets without EB. The mRNA abundance of *IL-17* tended to be greater in pigs fed diets with X, and *IL-22* tended to be decreased by EB ($P < 0.10$). A significant X × EB interaction was observed for colonic *IL-17*, *occludin* (***OCLN***), and *claudin-3* (***CLDN3***) mRNA ($P < 0.05$); their mRNA

Table 3. Effect of xylanase and a carbohydrase enzyme blend on the fold change of mRNA abundance of cytokines and tight junction protein genes in the colon

	Treatment[2]					*P*		
Gene name[1]	Control	X	EB	X + EB	SEM	X	EB	X × EB
IL-1B	3.58	2.91	2.35	0.87	0.89	0.245	0.054	0.491
IL-6	1.33^{ab}	0.75^{b}	0.84^{ab}	1.36^{a}	0.22	0.911	0.370	0.015
IL-10	1.65^{ab}	1.92^{ab}	2.04^{a}	0.83^{b}	0.48	0.199	0.874	0.055
IL-17	1.33^{b}	1.42^{ab}	1.13^{b}	1.74^{a}	0.15	0.072	0.668	0.015
IL-22	2.03	1.94	0.89	1.67	0.35	0.297	0.100	0.170
OCLN	1.08^{b}	1.20^{b}	0.68^{b}	1.53^{a}	0.21	0.052	0.305	0.014
CLDN3	1.63^{b}	1.22^{b}	1.17^{b}	2.40^{a}	0.27	0.309	0.059	0.013
ZO-1	0.41	0.45	0.44	0.67	0.11	0.148	0.075	0.212

n = 12 per treatment.

[1]*IL: interleukin; OCLN: occludin; CLDN3: claudin 3; ZO-1: zonula occludens-1.*

[2]X = xylanase, EB = enzyme blend, X + EB = combination of xylanase and enzyme blend. Xylanase activity was expected to be 15,000 EPU/g; one gram of EB was expected to contain 7,000 CU of cellulase, 5,000 U of β-glucanase, and 1,000 EPU of xylanase; the inclusion rate of both X and EB was 0.01% according to manufacturer's recommendation.

abundance was increased in pigs fed diets supplemented with X + EB, but not X or EB alone, compared with those fed unsupplemented CON diets ($P < 0.05$). Pigs consuming diets with X tended to have greater mRNA abundance of *OCLN,* and EB tended to increase mRNA abundance of *CLDN3* and *zonula occludens-1* (***ZO-1***; $P < 0.10$) compared with diets without enzyme supplementation.

Plasma Cytokines

More than half of the plasma samples were below the detection limit for IL-4, IL-6, IL-10, and IFNγ; thus, no statistical analysis was performed (data not shown). No X × EB × day interaction was observed for any blood cytokines. Because there was no X × day or EB × day interaction for IL-1ß and TNFα, the overall interaction effect of X × EB on IL-1ß and TNFα was shown. Plasma levels of IL-1ß and TNFα were significantly decreased only when X and EB were added together, but not individually, compared with CON ($P < 0.05$; Figure 1). The EB increased plasma IFNα on day 14 compared with diets without EB ($P < 0.05$); however, EB had no impact on IFNα on day 7 or over the 28-d period (Table 4). Neither X nor X × EB interaction affected plasma levels of IFNα or IL-8. Pigs fed diets with EB had lower concentrations of IL-8 on day 7 and over the 28-d period in the plasma compared with those receiving diets without EB ($P < 0.05$).

Volatile Fatty Acids and pH

In the cecum, there was no X × EB interaction or main effect of X on any VFA or on pH (Table 5). The EB decreased the concentration of propionate, butyrate, valerate, and total VFA ($P < 0.05$), but did not affect acetate concentration or pH in the cecum. The molar proportion (%) of cecal acetate was higher and butyrate was lower in EB-supplemented diets than those without EB ($P < 0.05$).

In the colon, the main effect of X did not affect the concentrations of any VFA (Table 6) except for isobutyrate and isovalerate, which were increased compared with diets without X ($P < 0.05$). Butyrate concentration tended to be lower in colon of pigs fed the EB-supplemented diet compared with pigs fed diets without EB ($P < 0.10$). There was a significant X × EB interaction for colonic propionate ($P < 0.05$); EB decreased its concentration when X was not present, but not when X was supplemented. A tendency for X × EB interaction was also observed for colonic total VFA ($P < 0.10$) such that EB decreased its concentration when added individually, but not together with X, compared with diets without EB. Xylanase tended to increase colonic pH when EB was not present ($P < 0.10$), but not when EB was present.

Microbial Populations in the Intestinal Digesta

The supplementation of X and EB, individually or together, did not alter 16S rRNA gene copies of total bacteria or *Bifidobacterium* spp. in all 3 intestinal segments (Table 7). Unexpectedly, X decreased *Lactobacillus* spp. in the ileum and increased the *Enterobacteriaceae* family in both ileum and cecum ($P < 0.05$). Similarly, EB tended to reduce the gene copies of *Lactobacillus* spp. in both ileum and cecum ($P < 0.10$) and significantly decreased *Lactobacillus* spp. gene copies in the colon compared with diets without EB ($P < 0.01$). The gene copies of the *Enterobacteriaceae* family tended to be lower in the colon of pigs fed EB-supplemented

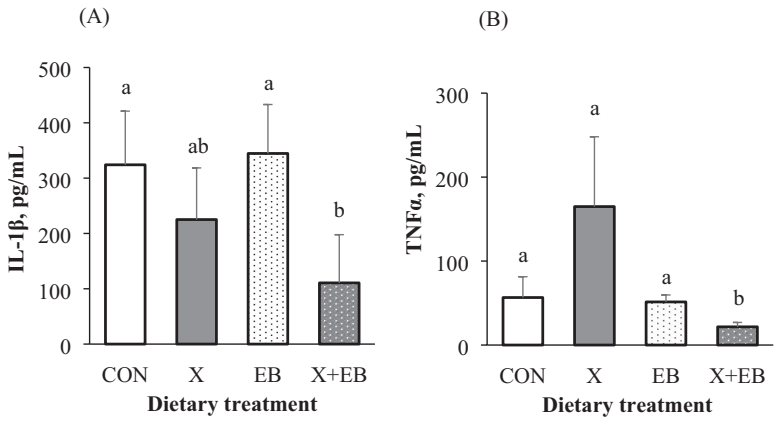

Figure 1. Effect of xylanase (X) and a carbohydrase enzyme blend (EB) on concentration of plasma cytokines in weaned pigs, pg/mL. (A): Interleukin (IL)-1β; (B) Tumor necrosis factor alpha (TNFα). X + EB: diets supplemented with both X and EB; [a,b]Means without a common superscript differ ($P < 0.05$); Data were average least square means across 4 collection days (days 0, 7, 14, and 28) and $n = 48$ per treatment.

Table 4. Effect of xylanase and a carbohydrase blend on plasma cytokine concentrations

	Treatment[2]					P[3,4]		
Item[1]	Control	X	EB	X + EB	SEM	X	EB	X × EB
IFNα, pg/mL						0.409	0.341	0.274
day 0	199.4	143.8	86.6	30.0	27.8	0.356	0.065	0.973
day 7	32.9	35.8	1.5	2.2	11.8	0.600	0.348	0.521
day 14	0.3	1.2	2.4	1.3	0.5	0.696	0.001	0.162
day 28	1.4	5.5	4.2	5.3	1.4	0.147	0.069	0.729
IL-8, pg/mL						0.543	0.046	0.727
day 0	86.4	14.0	31.9	10.7	17.6	0.008	0.239	0.997
day 7	19.2	19.3	8.7	7.3	3.5	0.846	0.042	0.344
day 14	4.5	7.4	3.7	4.1	0.8	0.529	0.328	0.787
day 28	10.7	10.9	9.5	13.7	1.9	0.489	0.899	0.600

n = 12 per treatment for individual collection day data and 48 for 4 collection days data; data were analyzed as repeated measurements with day as the repeated effect; P (day) < 0.01 and P (X × EB × day) > 0.10

[1]IFNα = interferon alpha; IL = interleukin.

[2]X = xylanase; EB = enzyme blend; X + EB = combination of xylanase and enzyme blend. Xylanase activity was expected to be 15,000 EPU/g; one gram of EB was expected to contain 7,000 CU of cellulase, 5,000 U of β-glucanase, and 1,000 EPU of xylanase; the inclusion rate of both X and EB was 0.01% according to manufacturer's recommendation.

[3]Baseline IFNα concentration on day 0 was different among treatments; thus, it was used as a covariate in data analysis.

[4]Baseline IL-8 concentration on day 0 tended to be different among treatments; thus, it was used as a covariate in data analysis.

Table 5. Effect of xylanase and a carbohydrase enzyme blend on volatile fatty acids and pH of digesta in the cecum

	Treatment[1]					P		
Item	Control	X	EB	X + EB	SEM	X	EB	X × EB
VFA[2] concentration, μmol/g								
Acetate	84.80	84.25	77.39	81.45	2.80	0.595	0.128	0.485
Propionate	39.53	38.15	32.53	34.26	1.05	0.907	0.001	0.300
Butyrate	16.50	17.54	12.74	13.11	0.84	0.556	0.002	0.780
Valerate	1.95	2.30	1.30	1.40	0.19	0.408	0.008	0.663
Isobutyrate	0.28	0.23	0.21	0.23	0.02	0.568	0.168	0.114
Isovalerate	0.30	0.26	0.27	0.28	0.02	0.717	0.865	0.514
Total	143.22	142.66	124.33	135.04	3.47	0.314	0.012	0.265
pH	5.69	5.64	5.72	5.74	0.04	0.758	0.282	0.568
VFA molar proportion, %[3]								
Acetate	59.36	59.01	62.13	62.03	0.80	0.808	0.005	0.896
Propionate	27.71	26.81	26.13	26.42	0.50	0.634	0.132	0.355
Butyrate	11.31	12.24	10.35	10.09	0.49	0.628	0.030	0.391
Valerate	1.32	1.64	1.08	1.13	0.15	0.349	0.062	0.480
Isobutyrate	0.19	0.16	0.17	0.19	0.02	0.789	0.968	0.199
Isovalerate	0.21	0.19	0.22	0.23	0.02	0.831	0.350	0.657

n = 12 per treatment.

[1]X = xylanase; EB = enzyme blend; X + EB = combination of X and EB. Xylanase activity was expected to be 15,000 EPU/g; one gram of EB was expected to contain 7,000 CU of cellulase, 5,000 U of β-glucanase, and 1,000 EPU of xylanase; the inclusion rate of both X and EB was 0.01% according to manufacturer's recommendation.

[2]VFA = volatile fatty acids.

[3]Calculated as the individual VFA concentration/total VFA concentration × 100%.

diets compared with those who received diets without EB (P < 0.10). No X × EB interaction was detected for *Enterobacteriaceae* in all 3 intestinal sections.

The relative abundance (%) of *Lactobacillus* spp. was decreased (P < 0.05) and *Bifidobacterium* spp. tended to be decreased (P < 0.10) in both ileum and cecum by the main effect of X (Table 8). The X also increased the relative abundance of *Enterobacteriaceae* in the ileum (P < 0.05). Pigs fed diets with EB tended to reduce the relative abundance of ileal *Lactobacillus* spp. (P < 0.10) and significantly reduced cecal and colonic relative abundance of *Lactobacillus* spp. compared with

Table 6. Effect of xylanase and a carbohydrase enzyme blend on volatile fatty acids and pH of digesta in the colon

	Treatment[1]					*P*		
Item	Control	X	EB	X + EB	SEM	X	EB	X × EB
VFA[2] concentration, µmol/g								
Acetate	93.60	86.44	85.16	88.48	3.95	0.640	0.437	0.207
Propionate	44.85[a]	36.64[b]	37.03[b]	38.13[b]	2.10	0.126	0.172	0.048
Butyrate	23.52	19.85	18.70	19.88	1.42	0.358	0.082	0.078
Valerate	3.48	3.19	2.61	3.05	0.28	0.853	0.203	0.352
Isobutyrate	0.83	1.18	0.89	1.15	0.10	0.038	0.904	0.745
Isovalerate	1.11	1.61	1.18	1.59	0.15	0.039	0.917	0.828
Total	167.40	148.91	145.57	152.27	7.16	0.395	0.186	0.075
pH	5.82	6.14	5.96	5.94	0.07	0.081	0.670	0.053
VFA molar proportion, %[3]								
Acetate	56.13	57.89	58.69	58.26	0.62	0.419	0.079	0.187
Propionate	26.78	24.58	25.17	24.79	0.45	0.049	0.275	0.158
Butyrate	13.90	13.26	12.72	12.99	0.48	0.762	0.233	0.451
Valerate	2.01	2.21	1.80	2.00	0.15	0.337	0.314	0.997
Isobutyrate	0.50	0.86	0.69	0.82	0.09	0.075	0.583	0.377
Isovalerate	0.68	1.20	0.93	1.14	0.14	0.072	0.616	0.434

n = 12 per treatment.

[1]X = xylanase; EB = enzyme blend; X + EB = combination of X and EB. Xylanase activity was expected to be 15,000 EPU/g; one gram of EB was expected to contain 7,000 CU of cellulase, 5,000 U of β-glucanase, and 1,000 EPU of xylanase; the inclusion rate of both X and EB was 0.01% according to manufacturer's recommendation.

[2]VFA = volatile fatty acids.

[3]Calculated as the individual VFA concentration/total VFA concentration × 100%

Table 7. Effect of xylanase and a carbohydrase enzyme blend on intestinal bacterial populations, $\log_{10}$ 16S rRNA gene copies/g wet digesta

	Treatment[1]					*P*		
Item	Control	X	EB	X + EB	SEM	X	EB	X × EB
Ileum								
Total	9.64	9.70	9.56	9.68	0.10	0.482	0.687	0.840
Lactobacillus spp.	8.66	8.41	8.46	7.86	0.15	0.045	0.074	0.391
Bifidobaterium spp.	8.05	7.58	7.43	7.63	0.26	0.710	0.440	0.366
Enterobacteriaceae family	4.43	5.34	4.64	5.01	0.20	0.012	0.813	0.266
Cecum								
Total	10.17	10.21	10.16	10.29	0.04	0.148	0.581	0.462
Lactobacillus spp.	8.90	8.89	8.71	8.61	0.08	0.644	0.061	0.712
Bifidobaterium spp.	8.30	7.90	7.86	8.03	0.20	0.683	0.575	0.302
Enterobacteriaceae family	6.01	6.52	6.13	6.50	0.15	0.046	0.818	0.761
Colon								
Total	9.97	10.09	9.98	9.96	0.05	0.323	0.256	0.199
Lactobacillus spp.	9.08	9.18	8.97	8.77	0.06	0.601	0.005	0.085
Bifidobaterium spp.	7.57	7.59	7.61	7.63	0.15	0.475	0.318	0.408
Enterobacteriaceae family	6.15	6.61	6.04	5.99	0.13	0.290	0.065	0.191

n = 12 per treatment.

[1]X = xylanase; EB = enzyme blend; X + EB = combination of X and EB. Xylanase activity was expected to be 15,000 EPU/g; one gram of EB was expected to contain 7,000 CU of cellulase, 5,000 U of β-glucanase, and 1,000 EPU of xylanase; the inclusion rate of both X and EB was 0.01% according to manufacturer's recommendation.

those receiving unsupplemented diets ($P < 0.05$). The main effect of EB and X × EB interaction had no impact on relative abundance of *Bifidobacterium* spp. and *Enterobacteriaceae*.

DISCUSSION

This study was a further analysis of samples collected during experiments conducted in previous work (Li et al., 2018). It was found that EB

Table 8. Effect of xylanase and a carbohydrase enzyme blend on relative abundance of intestinal bacteria

	Treatment[1]					*P*		
Item	Control	X	EB	X + EB	SEM	X	EB	X × EB
Ileum, %								
Lactobacillus spp.	13.25	10.57	10.75	3.39	2.48	0.007	0.075	0.289
Bifidobaterium spp.	4.76	2.62	3.93	1.64	0.92	0.098	0.489	0.954
Enterobacteriaceae family	0.001	0.018	0.003	0.031	0.01	0.033	0.939	0.225
Cecum, %								
Lactobacillus spp.	6.32	6.08	4.59	2.43	0.71	0.046	0.001	0.076
Bifidobaterium spp.	2.04	1.24	1.92	1.07	0.34	0.059	0.712	0.874
Enterobacteriaceae family	0.03	0.04	0.02	0.05	0.01	0.272	0.758	0.877
Colon, %								
Lactobacillus spp.	14.98	14.71	11.76	8.05	1.44	0.246	0.032	0.446
Bifidobaterium spp.	0.61	0.42	0.76	0.56	0.11	0.199	0.341	0.959
Enterobacteriaceae family	0.05	0.09	0.04	0.03	0.02	0.466	0.117	0.465

n = 12 per treatment.

[1]X = xylanase; EB = enzyme blend; X + EB = combination of X and EB. Xylanase activity was expected to be 15,000 EPU/g; one gram of EB was expected to contain 7,000 CU of cellulase, 5,000 U of β-glucanase, and 1,000 EPU of xylanase; the inclusion rate of both X and EB was 0.01% according to manufacturer's recommendation.

supplementation improved small intestinal barrier function and reduced markers of immune activation in the ileum, which concurred with an improved growth rate compared with diets without EB; yet, feed intake and digestibility of energy and nutrients were not improved by EB, except for ADF (Li et al., 2018). The current study was to examine the effects of EB on colonic and peripheral inflammatory status as well as microbial populations in the same pigs. Such work will provide further evidence for the mode of action of EB in improving the growth of weaned pigs fed higher fiber diets.

The current results that EB tended to increase *CLDN3* and *ZO-1* mRNA and decrease *IL-22* and *IL-1B* mRNA abundance in the colon agreed with previously reported findings in the ileum (Li et al., 2018). This may indicate that EB supplementation improves intestinal barrier function and reduces markers of inflammation not only in the small intestine but also the large intestine. Elevated levels of proinflammatory cytokines, such as IL-1β and TNFα, increase gut permeability through modulating tight junction proteins (Capaldo and Nusrat, 2009; Chen et al., 2013). Conversely, impaired barrier integrity in pigs caused by heat stress increases the translocation of lipopolysaccharide (Gabler et al., 2018), which is a potent immune stimulator to induce inflammation (Huntley et al., 2018). Thus, the reduced plasma IL-8 and colonic *IL-1B* mRNA in EB seemed consistent with previously observed improvements in small intestinal barrier integrity (Li et al., 2018) and the current upregulated tight junction proteins mRNA. The downregulation of *IL-1B* mRNA in the colon by EB may also be associated with decreased butyrate concentration. This is supported by Milo et al. (2002), who reported that VFA (acetate, propionate, and butyrate) supplementation increased ileal IL-1β and IL-6 protein abundance in pigs. Furthermore, IL-17 has been shown to stimulate the development of tight junctions in human intestinal epithelial cells, which was correlated with up-regulation of claudin-1 and claudin-2 mRNA levels (Kinugasa et al., 2000). These data partially support our findings that the greater mRNA abundance of colonic *OCLN* and *CLDN3* in diets with X + EB coincided with elevated colonic *IL-17* mRNA.

Neither X nor EB had an impact on plasma IFNα over the 28-d period, which may be explained by the fact that IFNα proteins are often associated with viral infection (Doyle, 2016). Interlukin-1ß, TNFα, and IL-8 are important inflammatory response mediators, levels of which are normally increased during an immune challenge to orchestrate host immune response (Huntley et al., 2018). However, increased amounts of proinflammatory cytokines generally have a negative influence on the growth and well-being of the animal (Elsasser et al., 1995; Huntley et al., 2018). Therefore, the lower plasma IL-8 in pigs fed EB and reduced plasma TNFα and IL-1ß in pigs fed diets with X + EB may suggest reduced systemic inflammation by carbohydrase supplementation. The observation that plasma TNFα and IL-1ß were decreased only by X + EB in comparison to CON indicates an additive impact of the 2 enzymes. Xylanase contained high xylanase activity and EB contained high cellulase and ß-glucanase activity, but low xylanase

activity. The combination of X and EB may be more effective in breaking down the NSP to release low molecular weight oligosaccharides, which then can reduce inflammation (Chen et al., 2012; Mendis et al., 2016). The decreased markers of inflammation by EB (plasma IL-8 and colonic *IL1B*) and X + EB (plasma TNFα and IL-1ß), as well as increased indicators for improved intestinal barrier integrity, may contribute to the previously observed improvement in ADG in EB-supplemented diets (Li et al., 2018).

Exogenous enzymes may regulate intestinal microbiota through modulating undigested substrates (e.g., starch and protein) and in situ release of soluble and more easily fermentable oligosaccharides in the intestine (Bedford and Cowieson, 2012; Kiarie et al., 2013). Previous research showed that AXOS derived from arabinoxylan degradation promoted the growth of some *Lactobacilli* and *Bifidobacteria* in chickens (Courtin et al., 2008) and humans (Kontula et al., 2000; Sanchez et al., 2009). Although there is a scarcity of information about xylanase-derived AXOS on intestinal microbiota in pigs, the current finding that X decreased *Lactobacillus* spp. tended to decrease *Bifidobacterium* spp. and increased *Enterobacteriaceae* family in the intestine is rather unexpected, and the reasons for those findings are unclear.

The 16S rRNA gene copies and relative abundance (%) of *Lactobacillus* spp. (amylolytic bacteria) tended to be decreased in the ileum and were significantly decreased in the colon of pigs fed EB-supplemented diets compared with unsupplemented diets. These data appear to be due to the accelerated removal of starch by EB (Bedford and Cowieson, 2012; Zhang et al., 2018). In accordance with the current experiment, Smith et al. (2010) showed that xylanase and ß-glucanase supplementation decreased *Lactobacilli* populations in the ileum compared with treatment without enzymes. Similarly, Vahjen et al. (2007) reported decreased *Lactobacillus* spp. in the stomach by adding either a multienzyme or a monoenzyme to a wheat-based diet in nursery pigs. The authors suspected that the inhibition of bacterial growth might be protective against generating more hydrogen peroxide by *Lactobacilli*, potentially leading to peroxidation and subsequently impaired growth performance. In contrast, Kiarie et al. (2007) and Zhang et al. (2014) reported that the addition of a blend of carbohydrases increased the *Lactobacilli* count in the ileum and feces in pigs.

Because EB improved growth rate and intestinal barrier integrity (Li et al., 2018), it seems that the decrease in *Lactobacillus* spp. by EB supplementation may not indicate disturbed intestinal microbial homeostasis. Xu et al. (2016) speculated that the decrease in *Lactobacillus* on day 8 post-weaning in the stomach and ileum of piglets supplemented with butyrate may not negatively affect gut health, as microbial diversity was increased and *Lactobacillus* was still the most predominant genus. Furthermore, pigs fed EB-supplemented diets tended to have reduced colonic *Enterobacteriaceae*, indicating less abundance of pathogenic bacteria, which agreed with Zhang et al. (2014) and Zhang et al. (2018). Collectively, the decreased *Lactobacillus* spp. abundance in the small and large intestine by X or EB may suggest reduced substrate availability, supported by Van der Meulen et al. (2001), who showed xylanase and cellulase activitity in the stomach and small intestine. Lower *Lactobacillus* abundance was reported in IL-22-deficient mice, with no detectable pathology or abnormal colon architecture (Zenewicz et al., 2013). Therefore, it is also possible that the decreased *Lactobacillus* abundance observed in EB is associated with an interaction with the host immune system (e.g., lower ileal *IL-22* mRNA reported in Li et al., 2018). The discrepancies in microbiota across experiments are probably associated with differences in initial gut microbial status and age of animals, type of carbohydrases used, dietary nutrient levels, and fibrous substrate concentration and characteristics [reviewed by Kiarie et al. (2013) and Mendis et al. (2016)]. Further studies are warranted to investigate the composition and function of microbial communities more broadly, in the context of carbohydrases as feed additives, to elucidate associated functions regarding intestinal health and immunity via molecular microbiology techniques, such as 16S rRNA sequencing, metagenomics, and metatranscriptomics.

As with reduction in *Lactobacillus* abundance, the lower total VFA in the cecum of pigs fed EB-supplemented diets compared with unsupplemented diets may reflect reduced substrate availability. It appears that the readily fermentable fiber was degraded by EB and the released degradation products were fermented by microbes in the small intestine, resulting in less degradable substrate entering the large intestine for microbial fermentation (Zeng et al., 2018). This theory is further confirmed by Clarke et al. (2018), who reported decreased total VFA in the colon accompanied with an improved ileal digestibility of gross energy in pigs receiving xylanase and β-glucanase supplemented diets. Energy absorbed as monosaccharides

(e.g., glucose) in the small intestine will be used by the pig with greater efficiency than as VFA produced by fermentation in the large intestine (Patience, 2017). Therefore, carbohydrases may improve energy utilization by shifting fiber degradation from the distal to proximal intestine. This agrees with the improved growth performance in EB-supplemented diets (Li et al., 2018). The X increased isobutyrate and isovalerate in the colon, suggesting increased fermentation of proteinaceous substrates, in agreement with Clarke et al. (2018). This is most likely due to increased degradation of easily fermentable fiber in X-supplemented diets before the colon, thus reducing substrate availability in the colon. The reduced available substrates, in turn, cause decreased fiber-utilizing bacteria and nitrogen utilization for protein synthesis by those bacteria, which consequently leads to increased proteinaceous substrates for fermentation (Clarke et al., 2018).

In conclusion, EB decreased IL-8 and the combination of X and EB decreased TNFα and IL-1ß in the plasma, possibly suggesting decreased systemic immune activation. The mRNA abundance of colonic tight junction protein genes was significantly increased. The reduced plasma proinflammatory cytokines and improved colonic tight junction protein mRNA levels by the combination of X + EB indicate additive effects of the 2 enzymes. Lower relative abundance of *Lactobacillus* spp. was observed in pigs fed X-supplemented diets compared with those fed diets without X. Supplementation of EB decreased the relative abundance of *Lactobacillus* spp. in the cecum and colon as well as total VFA in the cecum compared with diets without EB. These data may indicate improved fiber and starch degradation in the small intestine and hence decreased fermentable substrate availability for microbes in the large intestine. These data corroborated previously observed enhanced growth in pigs fed EB-supplemented diets. Further studies are warranted to investigate broader microbial population changes in response to dietary carbohydrase addition and mechanisms whereby carbohydrases alter immune response. In situ production of fiber degradation products in both small and large intestines should be considered to provide insight into the association between microbial activity and the amount as well as the type of specific NSP hydrolysis products.

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