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Original Research Article

Effects of xylanase, protease, and xylo-oligosaccharides on growth performance, nutrient utilization, short chain fatty acids, and microbiota in *Eimeria*-challenged broiler chickens fed high fiber diet

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ABSTRACT

A 21-d experiment was conducted to study the effect of xylanase, protease, and xylo-oligosaccharides on growth performance, nutrient utilization, gene expression of nutrient transporters, cecal short-chain fatty acids (SCFA), and cecal microbiota profile of broilers challenged with mixed *Eimeria* spp. The study utilized 392 zero-d-old male broiler chicks allocated to 8 treatments in a 4 × 2 factorial arrangement, as follows: corn-soybean meal diet with no enzyme (Con); Con plus xylanase alone (XYL); Con plus xylanase combined with protease (XYL + PRO); or Con plus xylo-oligosaccharides (XOS); with or without *Eimeria* challenge. Diets were based on a high-fiber (100 g/kg soluble fibers and 14 g/kg insoluble fibers) basal diet. At d 15, birds in challenged treatment were gavaged with a solution containing *Eimeria maxima*, *Eimeria acervulina*, and *Eimeria tenella* oocysts. At d 21, birds were sampled. *Eimeria* depressed ($P < 0.01$) growth performance and nutrient utilization, whereas supplementation had no effect. There were significant *Eimeria* × supplementation interactions for the sugar transporters *GLUT5* ($P = 0.02$), *SGLT1* ($P = 0.01$), *SGLT4* ($P < 0.01$), and peptide transporter *PepT1* ($P < 0.01$) in jejunal mucosa. *Eimeria* challenge increased the expression of *GM-CSF2* ($P < 0.01$) and *IL-17* ($P = 0.04$) but decreased ($P = 0.03$) *IL-1β* expression in the cecal tonsil. *Eimeria* × supplementation interactions for cecal acetate, butyrate, and total SCFA showed that concentrations increased or tended to be greater in the supplemented treatments, but only in non-challenged birds. Birds challenged with *Eimeria* spp. had higher concentrations of isobutyrate ($P < 0.01$), isovalerate ($P < 0.01$), and valerate ($P = 0.02$) in cecal content. *Eimeria* challenge significantly ($P < 0.01$) decreased the microbial richness and diversity, and increased ($P < 0.01$) the proportion of *Anaerostipes butyraticus*, *Bifidobacterium pseudolongum*, and *Lactobacillus pontis*. In conclusion, *Eimeria* infection depressed growth performance, nutrient utilization with regulating nutrient transporters. Furthermore, *Eimeria* challenge shifted the microbial profile and reduced microbial richness and diversity. On the other hand, enzyme supplementation showed limited benefits, which included increased concentrations of SCFA.

1. Introduction

Avian coccidiosis is currently the most important parasitic disease and cause of losses in the poultry industry. A recent calculation shows that coccidiosis results in losses of $14.5 billion annually in worldwide chicken production, mainly due to depressed growth performance caused by subclinical infection and the cost of treatment and prevention (Blake et al., 2020). *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella* are the three most prevalent species, targeting the upper intestine (duodenum), middle intestine

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(jejunum and ileum), and ceca of chickens, respectively (Allen and Fetterer, 2002).

The control of coccidiosis has become more challenging because of the global drive to reduce the use of antibiotics in livestock production, mainly due to the concern about antibiotic resistance. Ionophore anticoccidials, such as monensin, were added to poultry feed for more than fifty years and played crucial roles in controlling coccidiosis (Chapman et al., 2010). However, characterized as antibiotics by the Food & Drug Administration, ionophore anticoccidials cannot be used in antibiotic-free poultry production (Cervantes, 2015). Feed additives have been one of the potential approaches to compensate for feed ionophore restrictions.

One important mechanism of action of feed enzymes is in hydrolyzing anti-nutrients such as non-starch polysaccharides, which exist in relative abundance in high-fiber feedstuffs; thus, it is reasonable to expect that carbohydrases would be more effective in high fiber diets (Kiarie et al., 2013). Feed enzymes and prebiotics may alleviate *Eimeria*-induced performance losses, and this suggestion is mainly based on the anticipated mechanism of action that exogenous enzymes and prebiotics can beneficially alter the gut microbial composition. Coccidiosis negatively influences the intestinal microbiome and induces dysbiosis (Macdonald et al., 2017; Madlala et al., 2021), which is partly due to the increased content of undigested nutrients such as amino acids in the hindgut due to malabsorption in the foregut (Amerah and Ravindran, 2014). Prebiotics cannot be digested by chickens' endogenous enzymes, and thus reach the hindgut where they are utilized by microbiota, selectively promoting the growth of the bacteria that are able to derive their nutrients from degrading the prebiotics (Pourabedin and Zhao, 2015). In addition, feed enzymes hydrolyze anti-nutrients and the products from enzyme hydrolysis may fuel growth of beneficial gut microbiota. Previous works have demonstrated that feed additives, including enzymes and prebiotics, have the ability to affect intestinal microbiota by increasing beneficial and inhibiting detrimental bacteria (Józefiak et al., 2007; Pourabedin and Zhao, 2015; Munyaka et al., 2016). It has been reported that dietary supplementation of xylo-oligosaccharides increased the population of *Lactobacillus* spp. and cecal acetate production in broiler chickens (Pourabedin and Zhao, 2015). The increased acetate during xylo-oligosaccharides fermentation stimulates butyrate-producing bacteria through cross-feeding interaction, thus increasing butyrate production (De Maesschalck et al., 2015). Xylanases are hydrolytic enzymes targeting polysaccharides xylan and release xylo-oligosaccharides from arabinoxylans, benefiting the growth of carbohydrate-utilizing bacteria (Morgan et al., 2020). However, the effects of xylanase or xylo-oligosaccharides on intestinal microbiota in *Eimeria*-challenged birds require further investigation.

The current study was conducted to investigate and compare the potential and possible mechanisms of action of xylanase and xylo-oligosaccharides in alleviating the pathogenic effects of coccidiosis infection on growth performance and gut health in broiler chickens. The high fiber diets used in the current experiment was intended to maximize the effect of xylanase and is based on our previous study (Lin and Olukosi, 2021a). Protease was added in one treatment to simulate the combined use of the enzymes in the industry.

2. Materials and methods

2.1. Animal ethics statement

The experiment was performed at the Poultry Research Center at the University of Georgia. The Institutional Animal Care and Use Committee of the University of Georgia approved (A2018 08-026) the procedures in the current study.

2.2. Birds, diets, experimental design and Eimeria challenge

The experiment utilized 392 zero-d-old Cobb 500 (off-sex) male broiler chicks. High-fiber corn-soybean meal diets (Table 1) were formulated with supplemental phytase at 500 FTU/kg (Quantum Blue, AB Vista, Marlborough, UK). Wheat and wheat bran were added to increase the dietary fiber level. Birds were allocated to eight treatments in a 4 × 2 factorial arrangement, seven replicate cages per treatment, and seven birds per replicate cage. One factor was diet supplementation: basal diet without supplementation (Con); basal diet with 0.1 g/kg xylanase (AB Vista, Marlborough, UK) (XYL); basal diet with 0.1 g/kg xylanase and 0.2 g/kg protease (DSM, Pendergrass, GA, USA) (XYL + PRO); and basal diet with 0.5 g/kg

Table 1
Ingredients (as-fed basis), calculated and analyzed compositions (dry matter basis) of the experimental control diet.

Item	Content, g/kg
Ingredients	
Corn	339.7
Wheat	200.0
Wheat bran	100.0
Soybean meal	315.0
Soybean oil	13.0
Titanium dioxide	5.0
Di-calcium phosphate	8.0
Limestone	10.0
Lysine	1.2
Methionine	1.5
Threonine	0.2
$NaHCO_3$	0.8
Salt	3.0
Vitamin premix[1]	1.25
Trace minerals premix[2]	1.25
Phytase	0.1
Total	1000
Calculated nutrients and energy	
Crude protein	216
Metabolizable energy, kcal/kg	2804
Ca	7.2
Total P	6.4
Available P[3]	3.0
Met	4.8
Cys	3.5
Met + Cys	8.4
Lys	12.3
His	5.7
Trp	2.9
Thr	8.4
Arg	14.3
Analyzed composition	
Dry matter	895
Crude protein	221
Gross energy, kcal/kg	4027
Neutral detergent fiber	113
Acid detergent fiber	50
Insoluble fiber	100
Soluble fiber	14
Hemicellulose	63
Ca	7.47
Total P	6.27

[1] Supplemented per kilogram of diet: vitamin A, 5484 IU; vitamin D_3, 2643 IU; vitamin E, 11 IU; menadione sodium bisulfite, 4.38 mg; riboflavin, 5.49 mg, D-pantothenic acid, 11 mg; niacin, 44.1 mg, choline chloride, 771 mg; vitamin B_{12}, 13.2 μg; biotin, 55.2 μg; thiamine mononitrate, 2.2 mg; folic acid, 990 μg; pyridoxine hydrochloride, 3.3 mg.
[2] Supplemented per kilogram of diet: iodine, 1.11 mg; manganese, 66.06 mg; copper, 4.44 mg; iron, 44.1 mg; zinc, 44.1 mg; selenium, 300 μg.
[3] Available P level included the matrix for the phytase.

xylo-oligosaccharides (AIDP Inc., City of Industry, CA, USA) (XOS). The other factor was the presence or not of the *Eimeria* challenge. The protease and xylanase were produced from the fermentation of sporulation-deficient *Bacillus licheniformis* strain (Kalmendal and Tauson, 2012) and genetically modified *Trichoderma reesei* (Lin and Olukosi, 2021a), respectively. The xylo-oligosaccharides was obtained from non-genetically modified corn and was previously characterized (Silva et al., 2020; Yang et al., 2015). The *Eimeria* spp. oocysts were a mixed-species solution including 12,500 oocysts/mL of *E. maxima*, 12,500 oocysts/mL of *E. tenella*, and 62,500 oocysts/mL of *E. acervuline* for a mild infection as previously described (Teng et al., 2020).

On d 15, birds were gavaged with 1 mL mixed-species *Eimeria* oocysts solution or 1 mL water based on the treatments. In order to get blank blood samples as the standard reference for the gut permeability test, ten extra birds were raised (receiving basal diet) in a separate cage. The chickens were raised under temperature and lighting regimes based on the recommendation for Cobb 500. Tap water and feed were provided ad libitum.

2.3. Growth performance, intestinal permeability

Body weight gain (WG), feed intake (FI), and gain:feed ratio were measured and calculated from d 0 to 15 (pre-challenge phase) and d 15 to 21 (challenge phase).

An intestinal permeability test was performed on 5 d post-challenge at d 20 as described previously (Teng et al., 2020). At d 20 (5 d post-challenge), one bird from each challenged cage and unchallenged Con treatment were orally administrated with 1 mL of freshly prepared fluorescein isothiocyanate dextran (FITC-d, MW 4,000; Sigma–Aldrich, CA) solution (2.2 mg/mL). After 2 h of the administration, blood was collected from the birds' heart following cervical dislocation. Blank blood samples from extra birds were collected to dilute FITC-d for the standard curve preparation. Clotted blood was centrifuged at 1,000 × *g* for 12 min to separate serum, and the FITC-d concentration in serum was measured by spectrophotometer (Spectramax M5, Molecular Devices, San Jose, CA) at 485 nm excitation wavelength and 528 nm emission wavelength. A dark environment was used for the FITC-d procedures to avoid photolysis.

2.4. Sample collection

The excreta voided within 24 h from individual cages were collected at d 20 (5 d post-challenge) and oven-dried, ground, and later used for nutrient utilization measurements, including total tract retention of N. At d 21 (6 d post-challenge), 5 birds per cage were euthanized by carbon dioxide asphyxiation. Ileal digesta were collected from the 5 birds per cage and oven-dried for ileal digestibility measurement. Cecal contents were collected from 3 birds and stored at −20 °C for further analysis of short-chain fatty acids (SCFA), cecal protein and microbiota analysis. Jejunal mucosa and cecal tonsil were collected from 2 birds, snap-frozen in liquid nitrogen immediately and stored at −80 °C before further gene expression analysis. Three birds were used to score intestinal lesions based on a 0 to 4 (no lesion to severe lesion) scale grading. The upper (duodenum), middle (jejunum and ileum), and ceca sections of the digestive tract were scored separately (Johnson and Reid, 1970).

2.5. Oocyst shedding

Excreta voided within 24 h at d 21 (6 d post-challenge) were collected quantitatively from each cage and stored at 4 °C for further oocyst shedding measurement. After thorough mixing, approximately 5 g excreta samples from each cage were diluted with water in a 1:99 ratio. After the mixture was vortexed, 5 mL of diluted samples were mixed with 45 mL of saturated salt solution in a centrifuge tube. The mixture was vortexed again and the samples were thereafter loaded into a McMaster chamber and the number of oocysts was counted under a microscope.

2.6. Quantitative real-time PCR and 16S rRNA gene sequencing analysis

The mRNA quantities of target intestinal nutrient transporters in jejunum mucosa and immune-related genes in the cecal tonsil were analyzed using real-time PCR. Samples of jejunum mucosa or cecal tonsil were homogenized with QiAzol lysis reagent (QIAGEN, Hilden, North Rhine-Westphalia, Germany). The total RNA was extracted after homogenization according to the manufacturer's instructions. RNA quantity was measured by BioTek Synergy HTX spectrophotometer (Agilent, Santa Clara, CA, USA) and diluted to equal concentration. Extracted RNA was converted to cDNA by a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Waltham, MA, USA). The real-time PCR reaction was performed in Step One Plus real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA) with SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Samples were run in duplicate, and the results were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Primers used in the experiment are listed in Table 2.

QIAamp PowerFecal Pro DNA Kit (QIAGEN, Germantown, TN, USA) was used to extract the DNA from the cecal contents using a procedure combining mechanical and chemical methods, following the instructions of the manufacturer. DNA samples were sent to LC Sciences, LLC (Houston, TX, USA) for library preparation and 16S rRNA gene sequencing. Forward primer S-D-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) and reverse primer S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′) were used for PCR libraries. The Qiime2 was used for DNA sequence data analysis following previously described procedures (Akerele et al., 2022).

2.7. Chemical analysis

Oven-dried diets, excreta, and ileal digesta were ground through a 0.5-mm sieve to determine dry matter (DM) (Method 934.01, AOAC, 2006), N, gross energy (GE), and titanium dioxide. The determination of DM was achieved through the process of drying the samples in an oven at 100 °C for 24 h. Nitrogen concentration in the diets, ileal digesta, and excreta were determined using the combustion method (LECO, St. Joseph, MI, USA). Acid detergent fiber (ADF) was measured in the residue remaining after digesting with H_2SO_4 and hexadecyltrimethylammonium bromide (CTAB). Neutral detergent fiber (NDF) in diets was measured in the residue remaining after digesting in a detergent solution. The Ankom A200 Fiber Analyzer (Ankom Technology, Macedon, NY, USA) was used to measure ADF and NDF. Insoluble and soluble dietary fibers were measured by the Ankom TDF analyzer (Ankom Technology, Macedon, NY, USA) based on the AOAC method 991.43. Hemicellulose content was calculated as the difference between NDF and ADF quantities. Titanium concentration was determined according to the method of Short et al. (1996). Xylanase activity was analyzed by AB Vista analytical service with an enzyme-linked immunosorbent assay technology-based method (AB Vista, Plantation, FL, USA). Protease activity was analyzed by DSM technical analytical services. Diet oligosaccharides including $(Hex)_{3\text{-}6}$ and $(Pen)_{3\text{-}6}$ in diets were analyzed at the complex carbohydrate research center at the University of Georgia with matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) as previously described (Lin and Olukosi, 2021a).

Table 2
List of primers used for qPCR.

Gene symbol	Accession number	Full name	Function	Forward primer (5′to 3′)	Reverse primer (5′to 3′)
18S	MG967540.1	18S ribosomal RNA	Housekeeping gene	AGCCTGCGGCTTAATTTGAC	CAACTAAGAACGGCCATGCA
Beta-actin	NM_205518.1	Beta-actin	Housekeeping gene	CAACACAGTGCTGTCTGGTGGTA	ATCGTACTCCTGCTTGCTGATCC
GAPGH	NM_204305.2	Glyceraldehyde-3-phosphate dehydrogenase	Housekeeping gene	GAGGGTAGTGAAGGCTGCTG	CCACAACACGGTTGCTGTAT
PepT1 (SLC15A1)	KF366603.1	Peptide transporter 1	Peptide transporter	CCCCTGAGGAGGATCACTGTT	CAAAAGAGCAGCAGCAACGA
GLUT1 (SLC2A1)	NM_205209.1	Glucose transporter 1	Glucose transporter	CTTTGTCAACCGCTTTGG	CAGAATACAGGCCGATGAT
GLUT5 (SLC2A5)	XR_005855627.1	Glucose transporter 5	Glucose transporter	TTGCTGGCTTTGGGTTGTG	GGAGGTTGAGGGCCAAAGTC
SGLT1 (SLC5A1)	NM_001293240.1	Sodium glucose transporter 1	Glucose transporter	GCCGTGGCCAGGGCTTA	CAATAACCTGATCTGTGCACCAGT
SGLT4 (SLC5A9)	XM_040678521.2	Sodium glucose transporter 4	Glucose transporter	ATACCCAAGGTAATAGTCCCAAAC	TGGGTCCCTGAACAAATGAAA
ATP2B1	XM_046906440.1	ATPase plasma membrane Ca^{2+} transporting 1	Ca^{2+} transporter	TTAATGCCCGGAAAATTCAC	TCCACCAAACTGCACGATAA
CaSR	XM_040661543.2	Calcium sensing receptor	Calcium receptor	GCCAATCTGCTGGGACTCTT	CTGATGCTCGTCATTGGGGA
GM-CSF2	NM_001007078.2	Granulocyte-macrophage stimulating factor 2	Colony stimulating factor	CGCCCACCACAACATACTC	ACGATTCCGCTTTCTTCCT
IFN-γ	XM_031589614.1	Interferon-gamma	Macrophages primary activator	AGCTGACGGTGGACCTATTATT	GGCTTTGCGCTGGATTC
TGF-β1	XM_046937641.1	Transforming growth factor beta 1	Transforming growth factor	CGGGACGGATGAGAAGAAC	CGGCCCACGTAGTAAATGAT
IL-3	NM_001007083.2	Interleukin 3	Interleukins	CTCTGCCTGCTGCTGTCC	TTATCTGCTTTTTGCTGCTTTC
IL-1β	XM_026043102.1	Interleukin 1β	Interleukins	TGGGCATCAAGGGCTACA	TCGGGTTGGTTGGTGATG
IL-15	XM_046915461.1	Interleukin 15	Interleukins	TCTGTTCTTCTGTTCTGAGTGATG	AGTGATTTGCTTCTGTCTTTGGTA
IL-17	HM747027.1	Interleukin 17	Interleukins	CTCCGATCCCTTATTCTCCTC	AAGCGGTTGTGGTCCTCAT

In addition, diets and excreta samples were sent to the Central Analytical Laboratory of University of Arkansas for the measurement of gross energy and mineral profile. The determination of gross energy was carried out by utilizing an isoperibol bomb calorimeter (Model 6200, Parr Instruments, Moline, IL, US), with benzoic acid serving as the calibration standard. The determination of minerals was done using Spectro Analytical Instruments (Arcos OES ICP, Kleve, North Rhine-Westphalia, Germany) (method 968.08, AOAC), modified to use inductively-coupled plasma.

Cecal SCFA composition was analyzed by gas chromatography according to the previously described method (Lourenco et al., 2020). The cecal content sample (1 g) was diluted in 3 mL deionized water and centrifuged for 10 min at 10,000 × *g*. The resulting supernatant was combined with 25% meta-phosphoric acid, then frozen overnight before being thawed and mixed with ethyl acetate in a 1:2 ratio. After vortexing and settling, the uppermost layer of the mixture was transferred to a glass vial and analyzed via gas chromatograph.

2.8. Calculations and statistical analysis

The following index method equation was used to calculate total tract retention and apparent ileal digestibility of energy, DM, and crude protein:

$$\text{Digestibility} = 100 \times \left\{1 - \left[\left(\frac{C_i}{C_o}\right) \times \left(\frac{N_o}{N_i}\right)\right]\right\},$$

where C_i is the concentration of titanium in the diet, N_i is the nutrient content in the diet, C_o is the concentration of titanium in excreta or digesta, and N_o is the nutrient content in excreta or ileal digesta.

Apparent metabolizable energy (AME) was calculated by the following equation:

$$\text{AME} = \text{GE}_\text{i} - \left[\left(\frac{C_i}{C_o}\right) \times \text{GE}_\text{o}\right],$$

where GE_i is the gross energy of the diet, and GE_o is the gross energy of the excreta.

The mixed model procedure of JMP Pro 14.1.0 (SAS Institute Inc., Cary, NC, USA) was utilized for statistical analysis, as appropriate for a randomized complete block design and a factorial treatment arrangement. Except for lesion scores and microbiota data, two-way ANOVA was used for statistical analysis. The two factors were the *Eimeria* challenge (2 levels) and supplementations (4 levels). Means were separated using Tukey's honest significant difference test in case of a significant interaction. Intestinal lesion score, alpha diversity indices, relative bacterial richness, and comparisons of microbial composition between treatments were analyzed by Kruskal–Wallis nonparametric statistical method. Statistical significance was set at $P \leq 0.05$, and trends were set at $P \leq 0.10$.

3. Results and discussion

3.1. Diets, growth performance and nutrient utilization

The calculated and analyzed composition of diets are presented in Table 1. The xylanase activities were 17,400 and 15,800 active units in the XYL and XYL + PRO diets, respectively. The protease activity was 20,376 active units in the XYL + PRO diet. The total oligosaccharides including $(\text{Hex})_{3\text{-}6}$ and $(\text{Pent})_{3\text{-}6}$ are 53.9 μg/mg in the Con diet and 2055 μg/mg in the xylo-oligosaccharides supplemented diet.

There was no significant *Eimeria* × supplementation interaction on growth performance (Table 3). In the post-challenge phase, the *Eimeria* challenge significantly ($P < 0.01$) decreased WG, FI, and gain:feed ratio. There was no significant ($P < 0.01$) *Eimeria* × supplementation interaction on nutrient utilization (Table 4). *Eimeria* challenge significantly ($P < 0.01$) lowered ileal DM (−17.9 pp) and N (−21.2 pp), and also depressed ($P < 0.01$) AME and total tract retention of N. However, the supplements had no effects on growth performance or nutrient utilization.

When used individually or combined, the effects of exogenous xylanase, protease, and xylo-oligosaccharides on improving the growth and nutrient digestibility of non-challenged chickens have been widely demonstrated (Kalmendal and Tauson, 2012; De Maesschalck et al., 2015; Craig et al., 2020b). Mechanisms by which xylanase improves the growth performance have been described in the literature. Firstly, xylanase releases trapped

Table 3
Growth performance of the broiler chickens challenged or unchallenged with mixed *Eimeria* spp. in response to dietary supplementations.[1]

Treatment	*Eimeria*	Supplementation	Pre-challenge phase (d 0 to 15)			Challenge phase (d 15 to 21)		
			WG, g/bird	FI, g/bird	Gain:Feed, g/kg	WG, g/bird	FI, g/bird	Gain:Feed, g/kg
1	–	Con	393	537	707	359	443	809
2	–	XYL	402	573	702	396	471	841
3	–	XYL + PRO	395	573	690	407	478	850
4	–	XOS	402	598	676	391	475	835
5	+	Con	384	550	696	228	360	634
6	+	XYL	406	575	705	230	364	631
7	+	XYL + PRO	424	580	731	229	365	627
8	+	XOS	384	550	696	228	360	634
Pooled SEM			13.9	19.3	13.8	10.9	11.1	25.1
Means for main effect of *Eimeria* challenge								
	–					388	467	834
	+					228	363	627
Pooled SEM						5.4	5.6	12.5
P-values of *Eimeria* challenge						< 0.001	< 0.001	< 0.001
Means for main effect of supplementations								
		Con	389	544	701	294	401	722
		XYL	404	574	704	313	418	736
		XYL+PRO	410	576	711	318	422	739
		XOS	411	580	713	307	419	725
Pooled SEM			9.8	13.7	9.8	7.7	7.9	17.7
P-values of supplementations			0.332	0.166	0.846	0.499	0.499	0.635
P-values for interactions						0.608	0.608	0.687

WG = weight gain; FI = feed intake; Con = no supplementation; XYL = xylanase; XYL + PRO = xylanase and protease; XOS = xylo-oligosaccharides.
[1] $n = 7$ replicates for the simple effect; $n = 28$ replicates for the main effects of *Eimeria* challenge; $n = 14$ replicates for the main effects of each supplementation.

Table 4
Total tract nutrient retention and ileal digestibility (%, dry matter) for the broiler chickens challenged or unchallenged with mixed *Eimeria* spp. in response to dietary supplementations.[1]

Treatment	*Eimeria*	Supplementation	Ileal digestibility		Total tract retention	
			DM	Nitrogen	Nitrogen	AME, kcal/kg
1	–	Con	67.2	82.9	62.5	2654
2	–	XYL	68.7	79.9	62.5	2707
3	–	XYL + PRO	67.8	79.2	62.6	2725
4	–	XOS	65.2	78.2	61.0	2599
5	+	Con	51.0	60.4	24.0	1457
6	+	XYL	52.1	57.2	28.3	1710
7	+	XYL + PRO	50.3	58.9	28.1	1550
8	+	XOS	43.8	59.3	28.5	1576
Pooled SEM			3.39	3.36	3.61	92.2
Means for main effect of *Eimeria* challenge						
	–		67.2	80.1	62.2	2671
	+		49.3	58.9	27.2	1574
Pooled SEM			1.40	3.15	1.54	38.3
P-values for main effect of *Eimeria* challenge			<0.001	<0.001	<0.001	<0.001
Means for main effect of supplementations						
		Con	59.1	71.7	43.2	2056
		XYL	60.4	68.6	45.4	2209
		XYL + PRO	59.1	69.1	45.4	2138
		XOS	54.5	68.8	44.8	2087
Pooled SEM			2.07	1.97	1.86	44.8
P-values for main effect of supplementations			0.342	0.793	0.831	1.089
P-values for interactions			0.867	0.947	0.646	0.410

Con = no supplementation; XYL = xylanase; XYL + PRO = xylanase and protease; XOS = xylo-oligosaccharides; AME = apparent metabolizable energy; DM = dry matter.
[1] $n = 7$ replicates for the simple effect; $n = 28$ replicates for the main effects of *Eimeria* challenge; $n = 14$ replicates for the main effects of each supplementation.

nutrients by hydrolyzing the cell wall, increasing available nutrients for absorption (Annison, 1992). Secondly, xylanase hydrolyzes xylans and reduces the digesta viscosity, improving digestion efficiency (Lee et al., 2017). Thirdly, xylo-oligosaccharides, a xylan hydrolysis by-product, acts as a prebiotic which improves nutrient utilization by modulating gut microflora and SCFA profiles (Bedford and Partridge, 2022). The combination of protease and carbohydrases is widely reported in feed additive studies, however, the comparison between individual carbohydrase and combined with protease is not well documented. Kalmendal and Tauson (2012) noted that supplemental xylanase and protease improved the feed conversion, whereas the combination of the two enzymes was not superior to those supplied individually. The literature has shown that the scale of beneficial responses to xylanase and protease is widely variable, depending on ingredient and nutrient factors. In our previous study (Lin and Olukosi, 2021a), birds receiving diets rich in fibrous feedstuffs along with xylanase supplementation produce higher levels of digesta oligosaccharides and cecal SCFA, indicating that fibers maximize the beneficial effects of xylanase, by providing greater enzymatic hydrolysis products.

Moreover, the physicochemical properties of the fiber source play a critical role in xylanase efficacy. Xylanase perform better at digesting arabinoxylans in soluble but not insoluble fiber fraction (Hilhorst et al., 2002). Therefore, although similar fiber levels may be used, the differences in soluble fiber composition can be one of the factors causing variable xylanase responses. The diet analysis in this study showed a compraratively low soluble fiber content, which may partly explain the lack of growth performance and nutrient utilization responses to xylanase supplementation in the current study.

It has been demonstrated that the addition of xylanase, amylase, and protease provided a more pronounced improvement in feed conversion when chickens were fed high-fiber diets compared with low-fiber diets (Singh et al., 2017). Similarly, the effect of protease could be more observable in a diet with a high concentration of proteinaceous anti-nutrients such as trypsin inhibitors (Wedekind et al., 2020; Bedford and Partridge, 2022). Therefore, the variable effectiveness of the feed additives in the literature on growth performance and nutrient utilization may be partly explained by the possible insufficiency of anti-nutrients in the basal diet. In addition, *Eimeria* infection may be a factor influencing their effectiveness. Craig et al. (2020b) reported that xylo-oligosaccharides improved body weight gain in *Eimeria*-challenged broiler chickens (a vaccine model was used in the study). The efficacy of enzymes or prebiotics on growth performance and nutrient utilization under the disease model has not been consistently demonstrated (Craig et al., 2020a; Lin et al., 2022).

3.2. Intestinal permeability, lesion scores, and oocyst shedding

The gastrointestinal permeability response on d 20 (5 d post-challenge) is shown in Fig. 1. Birds challenged with mixed *Eimeria* spp. showed significantly ($P < 0.01$) higher serum FITC-d levels, whereas supplementations had no significant effect on intestinal permeability. Fig. 2 shows the results of intestinal lesion scores. Supplementations had no significant effect on intestinal lesion scores. *E. acervulina*, *E. maxima*, and *E. tenella* produced intestinal lesions in the upper intestine, middle intestine, and ceca, respectively. As shown in Fig. 3, oocysts were observed in the excreta samples from all *Eimeria*-challenged treatments, whereas supplementations had no effect on oocyst numbers.

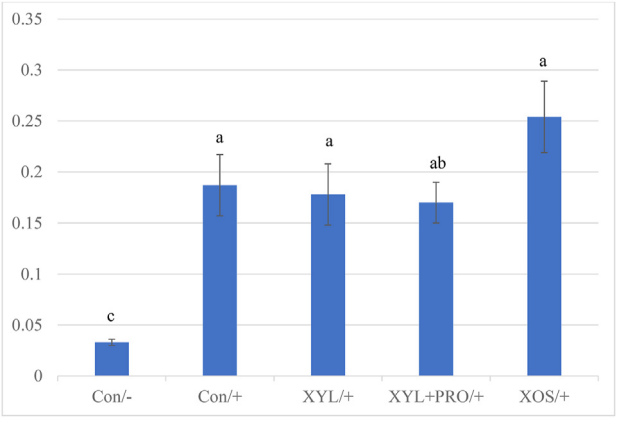

Fig. 1. Fluorescein isothiocyanate dextran concentration (FITC-d, μg/mL) in serum of *Eimeria*-challenged broiler chickens in response to dietary supplementations (5 d post-challenge). $n = 7$. Con/− = unchallenged and no feed additives; Con/+ = challenged and no supplementation; XYL/+ = challenged and supplemented with xylanase treatment; XYL + PRO/+ = challenged and supplemented with xylanase and protease; XOS/+ = challenged and supplemented with xylo-oligosaccharides. The error bars represent the SEM values. Bars with different superscripts are significantly different ($P < 0.05$).

Similar to the current study, previous studies have reported that *Eimeria*-caused lesions and oocyst shedding are unaffected by protease (Peek et al., 2009), xylanase, β-glucanase and xylo-oligosaccharides (Craig et al., 2020a). In contrast, Bozkurt et al. (2014) reported the combination of xylanase, amylase, protease, cellulose, β-glucanase, and mannanase, or the individual addition of mannan-oligosaccharides reduced lesion scores but had no effect on oocyst shedding. More studies with the individual rather than combined products should be done to further determine the effects of feed enzymes or prebiotics on oocyst shedding and lesion severity.

3.3. Gene expression of nutrients transporters and immune related genes

The expression of nutrient transporters in the jejunum mucosa in response to the *Eimeria* challenge and feed additives is shown in Table 5. There were significant *Eimeria* × supplementations interactions for sugar transporters glucose transporter 5 (*GLUT5*) ($P = 0.02$), sodium glucose transporter 1 (*SGLT1*) ($P = 0.01$), and sodium glucose transporter 4 (*SGLT4*) ($P < 0.01$), and peptide transporter 1 (*PepT1*) ($P < 0.01$). All the additives produced downward ($P < 0.01$) expression of *GLUT5* and *PepT1* in unchallenged treatments but had no effect in challenged treatments. *SGLT1* expression was numerically highest in the unchallenged with XOS treatment and lowest in the challenged with XYL treatment. In addition, the *Eimeria* challenge produced downward ($P < 0.01$) expression of the glucose transporter type 1 (*GLUT1*), Ca transporter ATPase plasma membrane Ca^{2+} transporting 1 (*ATP2B1*), and Ca sensing receptor (*CaSR*), whereas feed additives had no effect.

Consistent with the current study, the effect of *Eimeria* on downregulating nutrient transporters has been demonstrated and has been attributed to epithelial cell apoptosis and nutrient malabsorption (Su et al., 2014, 2015). It has been speculated that *Eimeria* infection downregulates nutrient transporters in the brush border such as *GLUT5* and *SGLT1*. Thus fewer nutrients are transported to epithelial cells, inducing nutritional depletion and apoptosis in epithelial cells (Paris and Wong, 2013; Su et al., 2014, 2015). Therefore, our observation regarding the effect of *Eimeria* challenge is not unexpected. In addition, similar observations of additive-supplemented diets in unchallenged treatments have been demonstrated in previous works. In agreement with the current study, individual xylanase, the combination of xylanase and protease or the individual xylo-oligosaccharides have been shown to downregulate the expressions of jejunal sugar transporters (*GLUT2* and *GLUT5*) and *PepT1* in unchallenged treatments but not in challenged treatments (Guo et al., 2014; Lin and Olukosi, 2021b; Lin et al., 2022). The mRNA response to feed additives can be inhibited by the damage in epithelial cells caused by *Eimeria* infection. Feed additive-induced SCFA increase may play an important role in lowering the expression of *GLUT2* and *GLUT5*. It has been demonstrated that elevated fatty acids concentration could reduce the expression of sugar transporters such as *GLUT2* (Gremlich et al., 1997). In addition, xylo-oligosaccharides improves mineral absorption including Ca by releasing minerals from the indigestible mineral complexes facilitated by lowering intestinal pH with increased SCFA (Lin et al., 2023; Scholz-Ahrens et al., 2001). It should be noted that the improved mineral absorption mainly takes place in ceca where SCFA are produced from microbial fermentation (Józefiak et al., 2004), explaining the lack of response on intestinal Ca transporter *CaSR* in this study, where the Ca transporter *CaSR* mRNA was measured in jejunal mucosa (Weaver, 2015).

Table 6 shows the effects of *Eimeria* infection and feed additives on immune-related gene expression in ceca tonsils. Additives

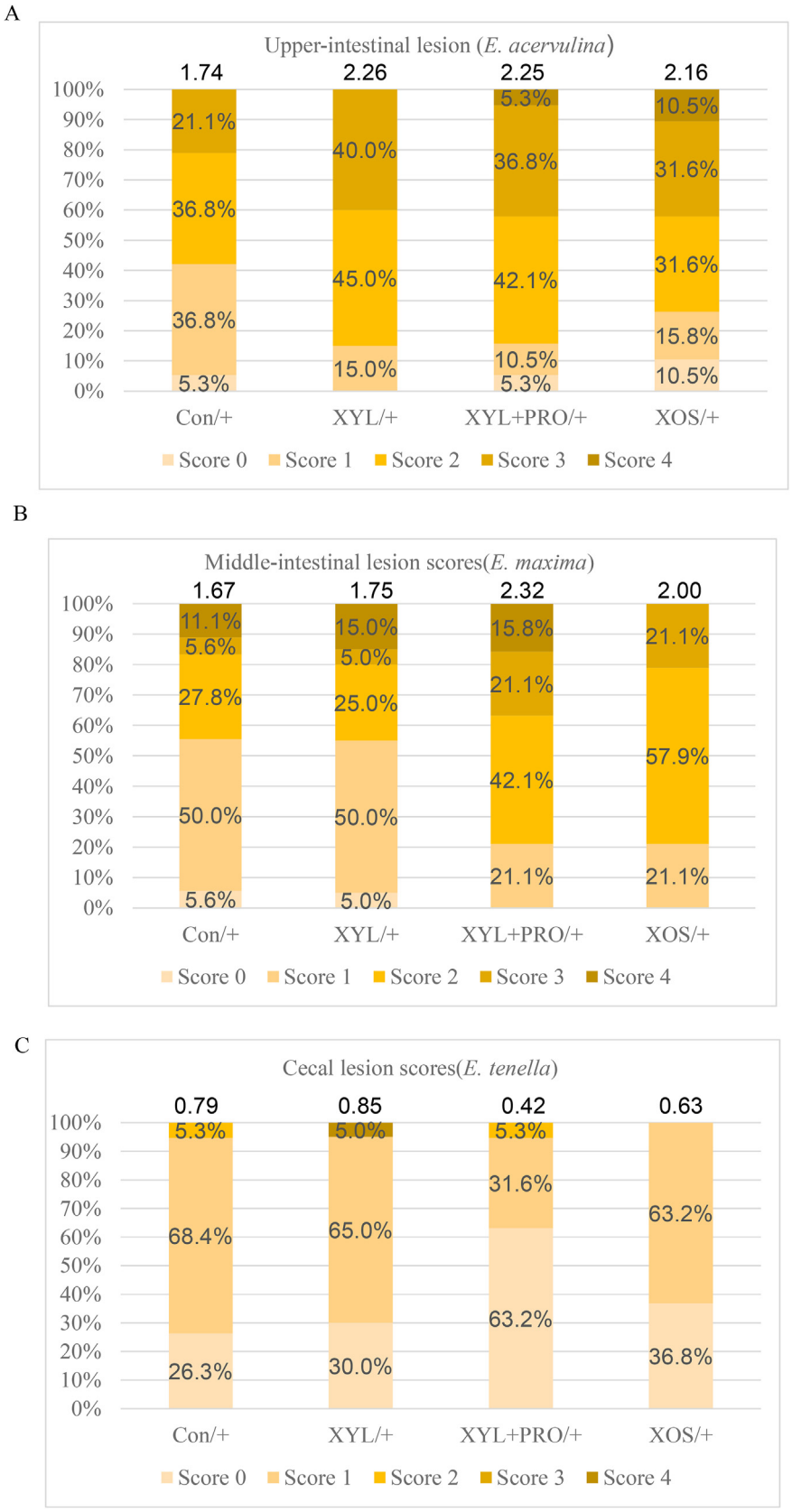

Fig. 2. Lesion scores in the upper intestine, middle intestine, and ceca of *Eimeria*-challenged broiler chicken in response to dietary supplementations (6 d post-challenge). Average scores of each treatment are present at the top of the bar. (A) Upper-intestine; (B) middle-intestine; (C) ceca. $n = 7$. Con/+ = challenged and no feed additives; XYL/+ = challenged and supplemented with xylanase; XYL + PRO/+ = challenged and supplemented with xylanase and protease; XOS/+ = challenged and supplemented with xylo-oligosaccharides treatment.

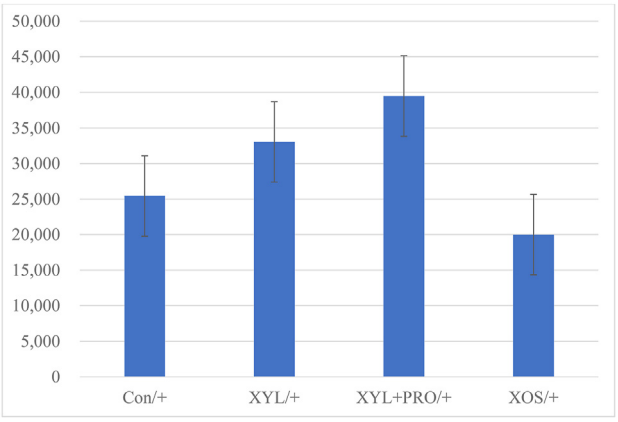

Fig. 3. Oocyst shedding (oocysts/g) of *Eimeria*-challenged broiler chickens in response to dietary supplementations (6 d post-challenge). $n = 7$. Con/+ = challenged and no feed additives; XYL/+ = challenged and supplemented with xylanase; XYL + PRO/+ = challenged and supplemented with xylanase and protease; XOS/+ = challenged and supplemented with xylo-oligosaccharides treatment. The error bars represent the SEM values.

supplementation had no effect on the probed genes. *Eimeria* challenge increased the expression of granulocyte-macrophage colony-stimulating factor *GM-CSF2* ($P < 0.01$) and interleukin (*IL)-17* ($P = 0.04$) but decreased ($P = 0.03$) *IL-1β* expression. *Eimeria* infection causes a strong immunogenic response in chickens. Bursectomy-treated chickens still show resistance to *Eimeria* reinfection, indicating the minimal function of antibodies to defend against coccidiosis (Rose and Long, 1970; Lillehoj, 1987). On the other hand, the protective immunity in response to the *Eimeria* challenge is mainly composed of cell-mediated immunity, including specific and nonspecific T lymphocytes and macrophages, with secreted cytokines and pro-inflammatory molecules (Dalloul and Lillehoj, 2006). The role of IL-17 which is elevated by *Eimeria* infection in this study has been highlighted in previous chicken coccidiosis studies. Produced by Th 17 $CD4^+$ T cells, increased IL-17 contributes to improving protective immunity against coccidiosis (Min et al., 2013). IL-17 stimulates the production of cytokines and chemokines such as IL-6, IL-8, and GM-CSF2, which is consistent with the upregulated expression of *GM-CSF2* in the current study (Pappu et al., 2012). GM-CSF2 is a Th2 cytokine shown to reduce the production of pro-inflammatory cytokines, inhibiting inflammatory responses (Hong et al., 2006). Therefore, the increase of GM-CSF2 and IL-17 is important for immunocompetence in chickens.

3.4. Cecal short chain fatty acids profile and protein concentration

The cecal short-chain fatty acids profile is shown in Table 7. The *Eimeria* × supplementations interaction for cecal butyrate showed that butyrate concentrations numerically increased in the supplemented treatments, but only in non-challenged birds. In addition, birds challenged with *Eimeria* spp. had higher concentrations of isobutyrate ($P < 0.01$), isovalerate ($P < 0.01$), and valerate ($P = 0.02$). Due to the infection, malabsorption and enteritis may occur in the chicken intestine, resulting in a significant shift in digesta substrates available for microbial fermentation as well as metabolites produced. Apart from propionate, SCFA concentrations influenced by *Eimeria* infection in this study were consistent with the literature. It has been shown that *Eimeria* infection decreased the concentration of SCFA produced from carbohydrate fermentation, such as acetate and butyrate, but increased the concentration of branched-chain fatty acids (BCFA), such as isobutyrate and isovalerate fermented from protein substrate (Lin and Olukosi, 2021b; Lin et al., 2022). Choi et al. (2021) observed a drop in cecal SCFA on 6 d post-challenge but an increase on 9 d post-challenge. Taken together, it can be proposed that *Eimeria* generally decreases the concentration of SCFA, followed by a transient but significant SCFA increase, before gradually returning to the normal level. The current study and a previous work indicated that the elevation in total SCFA content is in part due to the increased butyrate content (Hilliar et al., 2020), partly explained by the increase of butyrate

Table 5
Effects of mixed *Eimeria* spp. infection and supplementation on relative gene expression of nutrient transporters in the jejunum mucosa of broiler chickens on 6 d post-challenge.[1]

Treatment	*Eimeria*	Supplementation	*GLUT1*	*GLUT5*	*SGLT1*	*SGLT4*	*PepT1*	*ATP2B1*	*CaSR*
1	–	Con	1.000	1.000^{a}	1.000^{ab}	1.000^{ab}	1.000^{a}	1.000	1.000
2	–	XYL	0.999	0.523^{b}	1.176^{ab}	0.676^{ab}	0.385^{b}	1.150	1.785
3	–	XYL + PRO	0.891	0.628^{b}	0.712^{ab}	0.700^{ab}	0.410^{b}	1.819	0.769
4	–	XOS	1.538	0.597^{b}	1.221^{a}	0.626^{ab}	0.519^{b}	1.401	0.875
5	+	Con	0.262	0.329^{b}	0.819^{ab}	0.814^{ab}	0.335^{b}	0.706	0.489
6	+	XYL	0.243	0.262^{b}	0.571^{b}	0.585^{b}	0.312^{b}	0.707	0.305
7	+	XYL + PRO	0.309	0.340^{b}	0.942^{ab}	1.125^{a}	0.313^{b}	0.827	0.488
8	+	XOS	0.337	0.293^{b}	0.873^{ab}	1.005^{ab}	0.265^{b}	0.774	0.398
Pooled SEM			0.0571	0.0103	0.0514	0.0232	0.0050	0.0952	0.1122
Means for main effect of *Eimeria* challenge									
	–		1.107	0.687	1.027	0.750	0.578	1.342	1.107
	+		0.288	0.306	0.801	0.882	0.306	0.754	0.420
Pooled SEM			0.0503	0.1241	0.0829	0.0360	0.0255	0.0937	0.1163
P-values for main effect of *Eimeria* challenge			<0.001	<0.001	0.042	0.190	<0.001	<0.001	<0.001
Means for main effect of supplementations									
		Con	0.631	0.665	0.910	0.907	0.668	0.853	0.745
		XYL	0.600	0.484	0.827	0.912	0.361	1.323	0.629
		XYL + PRO	0.621	0.392	0.873	0.630	0.349	0.929	1.045
		XOS	0.937	0.445	1.047	0.816	0.392	1.087	0.637
Pooled SEM			0.1232	0.0511	0.1030	0.0754	0.0340	0.1539	0.1746
P-values for main effect of supplementations			0.320	0.022	0.482	0.047	<0.001	0.130	0.525
P-values for interactions			0.390	0.023	0.010	<0.001	<0.001	0.793	0.215

Con = no supplementation; XYL = xylanase; XYL + PRO = xylanase and protease; XOS = xylo-oligosaccharides; *GLUT1* = glucose transporter 1; *GLUT5* = glucose transporter 5; *SGLT1* = sodium glucose transporter 1; *SGLT4* = sodium glucose transporter 4; *PepT1* = peptide transporter 1; *ATP2B1* = ATPase plasma membrane Ca^{2+} transporting 1; *CaSR* = calcium sensing receptor.
Means along a column with different superscripts are significantly different ($P < 0.05$).
[1] $n = 7$ replicates for the simple effect; $n = 28$ replicates for the main effects of *Eimeria* challenge; $n = 14$ replicates for the main effects of each supplementation.

Table 6
Effects of mixed *Eimeria* spp. infection and supplementation on relative gene expression of immune response in ceca tonsils of broiler chickens on 6 d post-challenge.[1]

Treatment	*Eimeria*	Supplementation	*GM-CSF2*	*IFN-γ*	*TGF-β1*	*IL-3*	*IL-1β*	*IL-15*	*IL-17*
1	−	Con	1.000	1.000	1.000	1.000	1.000	1.000	1.000
2	−	XYL	0.628	0.830	1.040	0.597	0.708	0.821	0.984
3	−	XYL + PRO	0.409	0.518	0.823	0.692	0.709	0.751	0.753
4	−	XOS	0.715	0.622	0.763	0.646	0.953	0.927	0.836
5	+	Con	1.892	1.106	0.637	0.735	0.723	0.965	2.114
6	+	XYL	1.362	0.891	0.733	0.670	0.792	1.171	1.181
7	+	XYL + PRO	1.709	0.989	0.493	0.594	0.730	1.176	0.783
8	+	XOS	1.134	0.592	1.048	0.685	0.644	1.310	1.384
Pooled SEM			0.0832	0.0421	0.0441	0.0280	0.0436	0.0654	0.3401
Means for main effect of *Eimeria* challenge									
	−		0.688	0.743	0.906	0.734	0.842	0.875	0.893
	+		1.524	0.894	0.728	0.671	0.722	1.156	1.366
Pooled SEM			0.0920	0.0710	0.0712	0.0593	0.0365	0.0859	0.1315
P-values for main effect of *Eimeria* challenge			<0.001	0.255	0.517	0.586	0.028	0.100	0.038
Means for main effect of supplementations									
		Con	1.446	1.053	0.819	0.867	0.861	0.982	1.557
		XYL	0.995	0.861	0.886	0.633	0.750	0.996	1.083
		XYL + PRO	1.059	0.753	0.658	0.643	0.719	0.964	0.768
		XOS	0.925	0.607	0.905	0.666	0.798	1.119	1.110
Pooled SEM			0.1543	0.1083	0.1090	0.0892	0.1036	0.1111	0.3002
P-values for main effect of supplementations			0.180	0.100	0.517	0.339	0.898	0.762	0.477
P-values for interactions			0.326	0.517	0.235	0.639	0.796	0.575	0.644

Con = no supplementation; XYL = xylanase; XYL + PRO = xylanase and protease; XOS = xylo-oligosaccharides; *GM-CSF2* = granulocyte-macrophage stimulating factor 2; *IFN* = interferon; *TGF* = transforming growth factor; *IL* = interleukin.
[1] $n = 7$ replicates for the simple effect; $n = 28$ replicates for the main effects of *Eimeria* challenge; $n = 14$ replicates for the main effects of each supplementation.

Table 7
Short chain fatty acid profile (mM) in cecal content for the broiler chickens challenged or unchallenged with mixed *Eimeria* spp. in response to dietary supplementations.[1]

Treatment	*Eimeria*	Supplementation	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	Total SCFA
1	−	Con	64.9^{b}	2.38	0.406	9.0^{b}	0.538	0.820	78.1^{b}
2	−	XYL	91.7^{a}	6.01	0.149	16.4^{ab}	0.186	1.025	115.5^{a}
3	−	XYL + PRO	96.3^{a}	2.88	0.000	16.0^{ab}	0.000	0.433	115.7^{a}
4	−	XOS	96.9^{a}	2.72	0.057	17.2^{ab}	0.103	0.608	117.6^{a}
5	+	Con	72.6^{ab}	2.04	0.966	22.8^{a}	1.291	1.273	101.0^{ab}
6	+	XYL	70.8^{ab}	1.72	0.846	18.0^{ab}	1.197	1.027	93.6^{ab}
7	+	XYL + PRO	75.8^{ab}	1.88	0.845	22.3^{a}	1.159	1.253	103.3^{ab}
8	+	XOS	71.6^{ab}	1.90	0.928	17.6^{ab}	1.223	1.144	94.4^{ab}
Pooled SEM			5.91	1.453	0.1482	2.54	0.1960	0.2339	7.55
Means for main effect of *Eimeria* challenge									
	−		87.5	3.50	0.153	14.6	0.207	0.722	106.7
	+		72.7	1.89	0.896	20.2	1.217	1.174	98.1
Pooled SEM			3.32	0.713	0.0732	1.32	0.0973	0.1134	4.25
P-values for main effect of *Eimeria* challenge			0.013	0.132	<0.001	0.016	<0.001	0.019	0.309
Means for main effect of supplementations									
		Con	68.8	2.21	0.686	15.9	0.915	1.047	89.5
		XYL	81.3	3.86	0.497	17.2	0.692	1.026	104.6
		XYL + PRO	86.1	2.38	0.423	19.2	0.579	0.843	109.5
		XOS	84.3	2.31	0.492	17.4	0.663	0.876	106.0
Pooled SEM			4.86	1.032	0.1451	2.02	0.1950	0.1732	5.87
P-values for main effect of supplementations			0.011	0.640	0.337	0.581	0.344	0.759	0.024
P-values for interactions			0.015	0.526	0.703	0.028	0.709	0.376	0.004

Con = no supplementation; XYL = xylanase; XYL + PRO = xylanase and protease; XOS = xylo-oligosaccharides; SCFA = short-chain fatty acids.
Means along a column with different superscripts are significantly different ($P < 0.05$).
[1] $n = 7$ replicates for the simple effect; $n = 28$ replicates for the main effects of *Eimeria* challenge; $n = 14$ replicates for the main effects of each supplementation.

producers *Anaerostipes butyraticus* and *Bifidobacterium pseudolongum* as is subsequently discussed.

The *Eimeria* × additives supplementation interaction for cecal acetate ($P = 0.02$) and total SCFA ($P < 0.01$) concentration showed that the supplementation of all the additives increased the concentration of acetate (XYL, $P = 0.02$; XYL + PRO, $P < 0.01$; XOS, $P < 0.01$) and total SCFA ($P < 0.01$) in unchallenged treatments but not in challenged treatments. It has been demonstrated that the exogenous enzymes or prebiotics decreased the concentration of BCFA and increased the concentration of SCFA (Lin and Olukosi, 2021a, 2021b; Lin et al., 2022). The likely mechanism is suggested to be that by hydrolyzing carbohydrates, the enzymes are able to release more prebiotic oligosaccharides and preferentially promote the fermentation of the carbohydrates in the hindgut. The major products of carbohydrate fermentation are SCFA of which acetate is the predominant ceca SCFA (Shermer et al., 1998). However, the increase in cecal SCFA production in the current study did not translate into an improvement in growth performance. This is likely because the comparatively lower soluble fiber content in the diet limited the production of cecal SCFA, and the increased SCFA level did not reach the scale which can induce a considerable improvement in growth performance. The analyzed cecal protein concentration (Table 8) shows that XYL + PRO numerically decreased the cecal protein level in unchallenged birds. This likely resulted from

Table 8
Cecal protein concentration (μg/mg) in 21-d-old broiler chickens at 6 d post-challenge after receiving diets supplemented with protease, protease plus xylanase, or prebiotic oligosaccharides in diets formulated to be high in fiber. Birds were challenged, or not with mixed *Eimeria* spp.[1]

Treatment	*Eimeria*	Supplementation	Protein concentration
1	–	Con	48.3[ab]
2	–	XYL	47.6[ab]
3	–	XYL + PRO	45.0[b]
4	–	XOS	46.6[ab]
5	+	Con	48.5[ab]
6	+	XYL	49.7[a]
7	+	XYL + PRO	50.3[a]
8	+	XOS	47.6[ab]
Pooled SEM			0.96
Means for main effect of *Eimeria* challenge			
	–		46.9
	+		49.0
Pooled SEM			0.51
P-values			0.012
Means for main effect of supplementations			
		Con	48.4
		XYL	48.6
		XYL + PRO	47.7
		XOS	47.1
Pooled SEM			0.77
P-values for main effect of supplementations			0.312
P-values for interactions			0.030

Con = no supplementation; XYL = xylanase; XYL + PRO = xylanase and protease; XOS = xylo-oligosaccharides.
Means along a column with different superscripts are significantly different ($P < 0.05$).
[1] $n = 7$ replicates for the simple effect; $n = 28$ replicates for the main effects of *Eimeria* challenge; $n = 14$ replicates for the main effects of each supplementation.

reduced quantity of undigested protein, thus inhibiting cecal protein fermentation. However, XYL + PRO and XYL numerically increased the cecal protein level in challenged birds, thus, the beneficial effects of enzymes on the cecal substrate were more pronounced in unchallenged birds.

3.5. Cecal microbial profile

Microbial richness and alpha diversity for the birds in different treatments are shown in Table 9. Supplementation of the additives had neither effects on microbial profile richness nor diversities. The number of observed features was significantly lower ($P < 0.01$) in challenged birds, indicating a lower richness of the microbiome. Both Shannon diversity index ($P = 0.02$) and Faith's phylogenetic diversity index ($P < 0.01$) were decreased with the challenge, demonstrating the effect of coccidiosis on decreasing intestinal microbial diversity. In addition, there were 64.6% unique species in unchallenged chickens but only 3.4% unique species in challenged chickens, indicating the loss of unique bacterial units after infection (Fig. 4).

None of the supplemented additives showed any effects on microbial composition at the phylum level. The lack of response on microbial composition may be partly due to the low soluble fiber in the experimental diet. On the other hand, the microbial composition was significantly influenced by *Eimeria* infection (Fig. 5). *Eimeria* infection significantly ($P < 0.01$) decreased the abundance of Proteobacteria and Bacteroidota. On the other hand, the composition of Actinobacteriota was significantly ($P < 0.01$) higher in the challenged treatments. Bacterial species with significantly different abundances due to the challenge are shown in Table 10. *Eimeria* challenge decreased the abundance of the *Acaryochloris marina* ($P < 0.01$), *Akkermansia muciniphila* ($P = 0.04$), *Bacteroides barnesiae* ($P = 0.02$), *Bacteroides caecicola* ($P = 0.04$), *Campylobacter concisus* ($P = 0.02$), *Clostridium tyrobutyricum* ($P = 0.04$), *Desulfovibrio piger* ($P < 0.01$), *Haemophilus haemolyticus* ($P = 0.02$), *Haemophilus influenzae* ($P < 0.01$), *Haemophilus parahaemolyticus* ($P = 0.04$), *Lactobacillus brevis* ($P < 0.01$), *Plumaria plumosa* ($P = 0.02$), and *Prevotella melaninogenica* ($P < 0.01$), but increased the relative abundance of *A. butyraticus* ($P < 0.01$), *Bacteroides thetaiotaomicron* ($P = 0.04$), *B. pseudolongum* ($P < 0.01$), *Blautia hydrogenotrophica* ($P < 0.01$), *Eubacteriaceae bacterium* ($P = 0.04$), *Lachnoclostridium phocaeense* ($P = 0.02$), *Lactobacillus johnsonii* ($P < 0.01$) and *Lactobacillus pontis* ($P < 0.01$). Particularly, the abundance of *A. butyraticus* was increased 4.2-fold, *B. pseudolongum* was increased 8-fold, and *L. pontis* was increased 6.7-fold.

Anaerostipes is one of the important butyrate producers and has cross-feeding interactions with *Bifidobacterium* and *Lactobacillus* (De Maesschalck et al., 2015; Rivière et al., 2016; Biragyn and Ferrucci, 2018). *Lactobacillus* produces lactate, which is utilized by butyrate-producer *Anaerostipes* (De Maesschalck et al., 2015). In addition, acetate produced by *Bifidobacterium* can also be utilized

Table 9
Effect of *Eimeria* infection on richness and alpha diversity in cecal samples collected on 6 d post-challenge of 21-d-old broiler chickens after receiving diets supplemented with protease, protease plus xylanase, or prebiotic oligosaccharides in diets formulated to be high in fiber, and birds were challenged or not with mixed *Eimeria* spp.

Treatment	*Eimeria*	Supplementation	Observed features	Faith's phylogenetic diversity	Shannon index
1	–	Con	262	18.04	5.85
2	–	XYL	257	17.17	5.77
3	–	XYL + PRO	244	17.37	6.15
4	–	XOS	218	15.57	5.79
5	+	Con	128	8.00	5.31
6	+	XYL	132	8.12	5.34
7	+	XYL + PRO	165	10.26	5.82
8	+	XOS	146	8.60	5.75
Pooled SEM			24.0	1.530	0.196
P-values for treatments			<0.001	<0.001	0.085
Means for main effect of *Eimeria* challenge					
	–		245	17.04	5.89
	+		143	8.74	5.55
Pooled SEM			11.8	0.742	0.102
P-values for main effect of *Eimeria* challenge			<0.001	<0.001	0.023
		Con	195	13.02	5.58
		PRO	195	12.64	5.55
		PRO + XYL	205	13.82	5.98
		XOS	182	12.08	5.77
Pooled SEM			22.8	1.634	0.144
P-values for main effect of supplementations			0.919	0.897	0.141

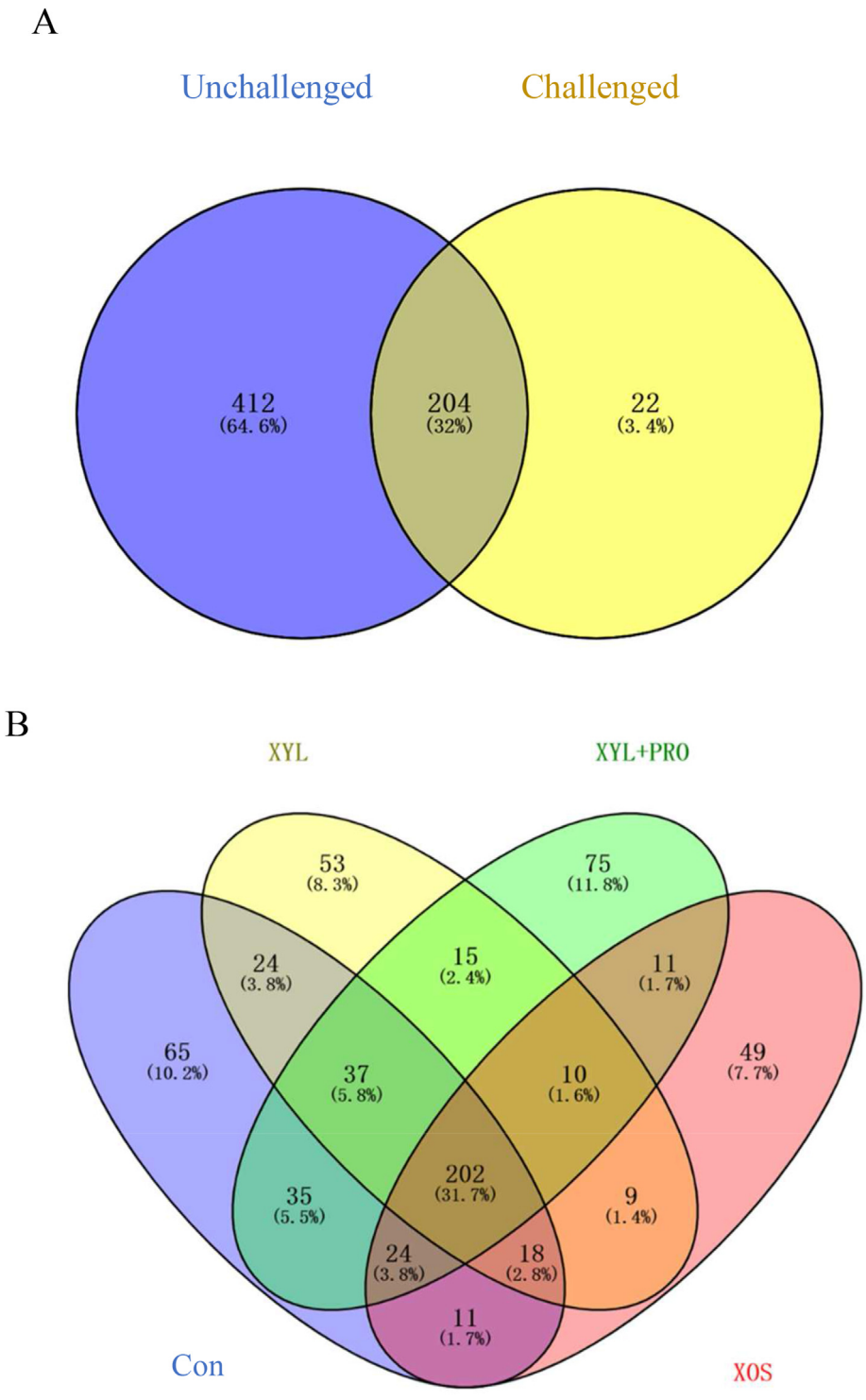

Fig. 4. (A) Venn diagram of bacterial species for challenge. Challenged with or without *Eimeria* spp. $n = 24$. (B) Venn diagram of bacterial species for feed additives. Con = no supplementation; XYL = supplemented with xylanase; XYL + PRO = supplemented with xylanase and protease; XOS = supplemented with xylo-oligosaccharides. $n = 12$.

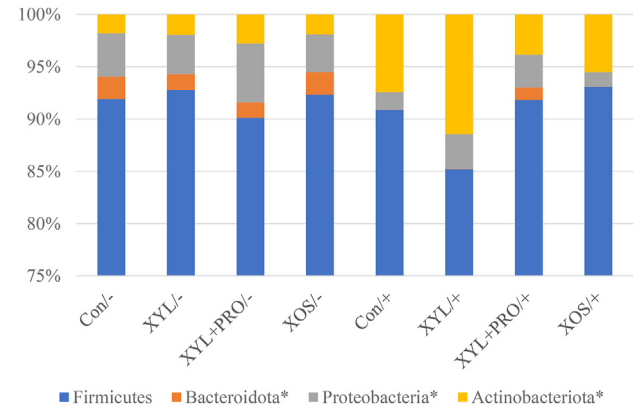

Fig. 5. Bar chart showing relative abundance of bacterial phyla in each treatment (6 d post-challenge). $n = 6$. Con/− = unchallenged and no supplementation treatment; XYL/− = unchallenged and supplemented with xylanase treatment; XYL + PRO/− = unchallenged and supplemented with xylanase and protease treatment; XOS/− = unchallenged and supplemented with xylo-oligosaccharides treatment; Con/+ = challenged-no supplementation treatment; XYL/+ = challenged and supplemented with xylanase treatment; XYL + PRO/+ = challenged and supplemented with xylanase and protease treatment; XOS/+ = challenged and supplemented with xylo-oligosaccharides treatment. Only phyla with relative abundances ≥1% in at least one sample type are shown. *Indicates a *P*-value ≤0.05 for the contrast: unchallenged versus challenge.

Table 10
Bacterial species with significantly different abundances (%) in cecal samples collected on 6 d post-challenge from21-d-old broiler chickens challenged, or not with mixed *Eimeria* spp.

Bacterial species	Unchallenged	Challenged	*P*-value
Acaryochloris marina	0.020	0.000	<0.001
Akkermansia muciniphila	0.092	0.047	0.040
Anaerostipes butyraticus	1.049	4.404	<0.001
Bacteroides barnesiae	0.088	0.007	0.018
Bacteroides caecicola	0.056	0.003	0.040
Bacteroides thetaiotaomicron	0.000	0.007	0.039
Bifidobacterium pseudolongum	0.648	5.173	<0.001
Blautia hydrogenotrophica	0.691	1.265	0.001
Campylobacter concisus	0.005	0.000	0.020
Clostridium tyrobutyricum	0.014	0.000	0.039
Desulfovibrio piger	0.083	0.000	0.005
Eubacteriaceae bacterium	0.001	0.009	0.042
Haemophilus haemolyticus	0.024	0.000	0.020
Haemophilus influenzae	0.057	0.000	0.005
Haemophilus parahaemolyticus	0.014	0.000	0.039
Lachnoclostridium phocaeense	0.267	0.430	0.019
Lactobacillus brevis	0.046	0.000	0.002
Lactobacillus johnsonii	0.431	1.558	<0.001
Lactobacillus pontis	0.099	0.662	<0.001
Plumaria plumosa	0.008	0.006	0.020
Prevotella melaninogenica	0.072	0.061	<0.001

by *Anaerostipes* (Rivière et al., 2016). It can be conjectured that the increased *Bifidobacterium* and *Lactobacillus* stimulated the growth of *Anaerostipes* by cross-feeding interactions, thus increasing the production of butyrate in the current study. This can be considered a possible protective action of the intestine trying to compensate for *Eimeria*-induced dysbiosis.

4. Conclusion

In conclusion, *Eimeria* infection significantly depressed chickens' growth performance and nutrient utilization. In addition, the infection detrimentally affected gut health with nutrients transporters downregulation, immunogenic stimulation and microbiota perturbation. All the supplemented additives improved the concentration of acetate and total SCFA in unchallenged birds, suggesting that improved SCFA is one of the potential mechanisms by which the additives supported chickens' gut health. However, the supplemented additives had no impact on the cecal microbiota.

Author contributions

Oluyinka A. Olukosi: Conceptualization, Methodology, Validation, Investigation, Data curation, Resources, Writing - Reviewing and Editing, Supervision, Project administration, Funding acquisition. **Yang Lin:** Conceptualization, Methodology, Investigation, Formal analysis, Software, Visualization, Writing - Original draft preparation. **Jeferson M. Lourenco:** Software, Visualization, Formal analysis, Resources, Writing - Reviewing and Editing.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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