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Article

Eimeria infection-related intestinal dynamics and microbiome, growth performance, and nutrient utilization in broiler chickens fed diets supplemented with multienzyme

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Abstract

Coccidiosis is an infectious parasitic disease. Increasing drug resistance and variable vaccine effectiveness have stimulated interests in developing alternate control strategies. Our research studied the effects of dietary supplementation of multienzyme (phytase, xylanase, β-glucanase, amylase, hemicellulase, and pectinase) in broiler chickens infected with coccidial pathogens by assessing (*i*) nutrient utilization, immunological response, and gut health-related indicators in small intestine; and (*ii*) intestinal microbiome modulations. One-day-old male Ross 708 chicks were assigned to 4 treatments in a 2 × 2 factorial arrangement comprising of 0 or 50 g·kg^{-1} multienzyme and oral challenge with phosphate buffer saline or mixed *Eimeria* spp. oocysts (250 000 *E. acervulina*, 50 000 *E. maxima*, and 50 000 *E. tenella*). Multienzyme reduced ($P < 0.05$) *Eimeria*-induced loss in feed efficiency and nutrient utilization, partially explained by reduced decrease of $b^{0,+}$ amino acid transporter in jejunum. Multienzyme suppressed ($P < 0.05$) the overexpression of interleukin-8 in duodenum and jejunum and ameliorated ($P = 0.05$) the decreased expression of antioxidant heme oxygenase 1 in ileum induced by *Eimeria* infection. Multienzyme facilitated ($P < 0.01$) the bloom of short-chain fatty acid-producing and fiber-degrading microbes. Multienzyme supplementation partly mitigated the adverse effects of *Eimeria* infection through multiple mechanisms: improving nutrient utilization, reducing local inflammation, and restoring microbial homeostasis.

Key words: Broiler chicken, *Eimeria* challenge, multienzyme, gut health, microbiota

Introduction

Avian coccidiosis, caused by protozoal *Eimeria* parasites, impairs the intestinal epithelial barrier, induces severe inflammatory responses, and gives rise to villous injury, which further influences performance and health of poultry. About $14 billion in financial loss is incurred worldwide annually due to coccidiosis in the poultry industry (Blake et al. 2020). Coccidiosis is also a well-known predisposing factor for the outbreak of enteric diseases, such as necrotic enteritis, *C. perfringens* (Timbermont et al. 2011), and *Salmonella* infections (Arakawa et al. 1981). Therefore, coccidiosis not only impacts poultry health, but also is a concern for food safety. Current approaches to control coccidiosis include chemoprophylaxis, vaccines, and natural compounds (Abbas et al. 2019). However, with increasing drug resistance and consumer demand for drug residue-free products, alternative strategies are being developed to control the occurrence of coccidiosis.

Diets of broiler chickens primarily composed of cereal grains contain significant amounts of non-digested components (like cellulose, peptic substance, arabinoxylan, β-glucan) and anti-nutritional factors (such as phytate), which not only suppress nutrient utilization and overall performance (Saki et al. 2011), but also intensify the pathogenicity of coccidia (Williams 1992). Exogenous carbohydrase and phytase have been extensively utilized in poultry feed to degrade non-starch polysaccharides and phytate complexes in cereal-derived components and maximize the growth potential in healthy animals. The potential mechanisms include reducing intestinal viscosity, releasing trapped nutrients, and enhancing nutrient availability (Wu et al. 2004). Other than nutritional impacts, these enzymes have been proposed to modulate microbiota, immune response, and free radicals generation (Pirgozliev et al. 2008; Craig et al. 2020).

Eimeria spp. infection elicits a variety of pathological and immunological responses in specific regions of the gastrointestinal tract. *Eimeria* species of *E. acervulina*, *E. maxima*, and *E. tenella* are considered the highly pathogenic species invading the duodenum, midgut, and ceca, respectively (Conway and McKenzie 2007). Coccidiosis is characterized by drastic reduction in the digestive and absorptive capacity of small intestine, and intestinal disorders, as evidenced in impaired gut morphology, downregulation of digestive enzymes (Walk

2009) and nutrient transporters, and dysbacteriosis in ceca (Zhou et al. 2017). *Eimeria* infection causes damage to intestinal barrier and enterocytes, induces local inflammation, perturbs local redox balance, and even results in severe bleeding in lumen (Chaudhari et al. 2020). During this process, serum release and increased endogenous loss are likely to provide substrates for the colonization and proliferation of pathogenic bacteria and disturb the microbial balance (Zhou et al. 2017). In the context of coccidiosis, cellular immunity mediated by T lymphocytes, natural killer cells, and macrophages has been considered the predominant protectors against coccidiosis (Mtshali and Adeleke 2020). The hypothesis of this study was that supplementation of multienzyme consisting of phytase, xylanase, β-glucanase, amylase, hemicellulases, and pectinases would ameliorate the negative effects of *Eimeria* infection in broiler chickens. Therefore, the objective of the current study was to evaluate the effects of dietary multienzyme supplementation on growth performance and further explain the impacts from following perspectives: (*i*) nutrient digestibility and health (immune profile and redox status) of the small intestine and (*ii*) microbial profiles in ceca of broiler chickens under *Eimeria* challenge.

Materials and methods

Ethics approval

Animal experimental protocol (1111000250) was reviewed and approved by the Purdue University Animal Care and Use Committee. The experiment followed the Federation of Animal Science Societies guidelines for the care and use of agricultural animals in agricultural research and teaching.

Experimental Diets and Animals

Newly hatched Ross 708 male chicks were procured from a commercial hatchery (Aviagen, Huntsville, AL). Birds were individually tagged with identification numbers, weighed, and reared in electrically heated battery brooders (model SB 4 T; Alternative Design Manufacturing and Supply, Siloam Springs, AR) during the experimental periods. The birds were then allotted to 2 diets consisting of 16 replicate cages and 10 birds per cage in a randomized complete block design with body weight (BW) as a blocking factor. The two diets (Table 1) were (1) control without multienzyme supplementation (CON) and (2) 50 g·kg^{-1} multienzyme (Ronozyme multicarbohydrase product, DSM Nutritional Products, Kaiseraugst, Switzerland) supplementation (ENZ). The enzyme recovery analysis in the supplemented diet was conducted by DSM Nutritional Products. On day 13 post hatching, birds were individually weighed and pooled within dietary treatment. Thereafter, 128 of 160 birds within each diet were selected and re-distributed to the additional 2 treatments (i.e., non-challenge (NCHA) or challenge (CHA)) with 8 replicate cages and 8 birds per cage in a randomized complete block design with BW as a blocking factor. Birds had free access to feed and water during the experimental period, and mortality was recorded daily.

Table 1. Ingredients and calculated nutrient composition of experimental diets, g·kg^{-1} as-fed basis[a].

Diets	CON	ENZ
Ingredient (g·kg^{-1})		
Wheat (Hard Red)	365.6	315.6
Barley	200.0	200.0
Soybean meal	225.0	225.0
Canola meal	100.0	100.0
Soybean oil	50.0	50.0
Ground limestone	15.3	15.3
Monocalcium phosphate	4.3	4.3
Salt	4.0	4.0
L-Lysine HCl	3.9	3.9
DL-Methionine	2.5	2.5
L-Threonine	1.4	1.4
Vitamin–mineral premix[b]	3.0	3.0
Multienzyme premix[c]	0.0	50.0
Chromic oxide premix[d]	25	25
Total	1000	1000
Calculated nutrient (g·kg^{-1})		
ME (kcal·kg^{-1})	3325	3325
CP	230	230
EE	66.4	66.4
Ca	8.0	8.0
P	5.8	5.8
Non-phytate P	3.0	3.0
SID AA		
Arg	12.1	12.1
His	4.9	4.9
Ile	8.0	8.0
Leu	13.8	13.8
Lys	12.7	12.7
Met	5.5	5.5
Met + Cys	9.1	9.1
Phe	9.1	9.1
Thr	8.1	8.1
Trp	2.5	2.5
Val	9.2	9.2

[a]AA, amino acid; CON, control without multienzyme additives; ENZ, multienzyme additives; EE, ether extract; SID, standardized ileal digestibility.
[b]Provided the following quantities per kg of complete diet: vitamin A, 5145 IU; vitamin D_3, 2580 IU; vitamin E, 17.1 IU; menadione, 4.4 mg; riboflavin, 5.5 mg; D-pantothenic acid, 11 mg; niacin, 44.1 mg; choline chloride, 771; vitamin B_{12}, 0.01 mg; biotin, 0.06 mg; thiamine mononitrate, 2.2 mg; folic acid, 0.99 mg; pyridoxine hydrochloride, 3.3 mg; I, 1.11 mg; Mn, 107.1 mg; Cu, 4.44 mg; Fe, 44.1 mg; Zn, 107.1 mg; and Se, 0.26 mg.
[c]Multienzyme premix was a mixture of phytase, xylanase, β-glucanase, amylase, hemicellulases, and pectinases. Multigrain premix was prepared with MultiGrain and wheat to contain 10 g·kg^{-1} diet. WX premix was prepared with WX and wheat to contain 10 g·kg^{-1} diet. VP premix was prepared with VP and wheat to contain 10 g·kg^{-1} diet. RumiStar premix was prepared with RumiStar and wheat to contain 10 g·kg^{-1} diet. Phytase premix was prepared with phytase and wheat to contain 10 g·kg^{-1} diet.
[d]Prepared as 5 g chromic oxide added to 20 g wheat.

Eimeria infection and sampling procedures

Individual BW of birds and feed consumption of cages were measured on days 7, 13, and 21 post hatching. On day 14 post hatching, each bird was orally gavaged with either 1 mL solution containing 3 selected *Eimeria* spp. sporulated oocysts

(*E. acervulina*, 250 000; *E. maxima*, 50 000; and *E. tenella*, 50 000) or 1 mL phosphate buffered saline (PBS; VWR International, Radnor, PA). Approximately 100 g of excreta samples were collected on days 19, 20, and 21 by placing waxed paper under the cages. On day 21 post hatching, all birds were euthanized by CO_2 asphyxiation. Six out of 8 birds within a cage were used to collect digesta from two-thirds of the distal ileum (a portion of the small intestine from Meckel's diverticulum to approximately 1 cm anterior to the ileocecal junction) by flushing the intact ileum with distilled water, which were then pooled and immediately stored at −20 °C for digestibility determination. Apparent ileal digestibility (AID) and apparent total tract utilization (ATTU) of dry matter (DM), nitrogen (N), and gross energy (GE) were calculated using the index method (Olukosi et al. 2007) with chromic oxide as the index marker.

Intestinal tissues were collected from two birds per cage based on BW closest to the cage median BW. Immediately after dissection, digesta samples in the ileum and ceca were collected from one of the selected birds, and 7 mL of homogenized ileal and cecal content was preserved in ice for microbial DNA extraction. Mucosa samples from the other selected bird were collected from mid- duodenum, jejunum, and ileum by flushing with ice-cold PBS solution and scrapping with a glass spatula. The mucosa samples were placed in 2 mL tubes containing Trizol reagent (Invitrogen, Grand Island, NY) and immediately stored at −80 °C until further analyses.

Oocysts shedding

Fresh excreta samples were collected from each cage on days 4, 5, and 6 post challenge (days 18, 19, and 20 post hatching). Prior to analysis, excreta samples from the same cage were pooled and mixed well. Approximately 200 g excreta sample of each cage was weighed and blended with 45 mL 3 M saturated Mg_2SO_4 solution. Following homogenization and sieving, excreta solution was placed in a McMaster chamber (Jorgensen Laboratories, Loveland, CO) and oocysts were counted under a microscope. Oocyst shedding was expressed as $\log_{10}$ (oocysts·g^{-1} feces).

RNA isolation and quantitative real-time PCR

Total RNA was extracted from duodenal, jejunal, and ileal mucosa using Trizol reagent. The concentration and integrity of extracted RNA were determined by NanoDrop 1000 (NanoDrop Technologies, Inc., Rockland, DE) and verified using 1% agarose gel electrophoresis, respectively. Extracted RNA in each 2-mg sample was reverse transcribed into cDNA by the Moloney murine leukemia virus reverse transcription system (Promega, Madison, WI). Real-time PCR was performed with a Bio-Rad CFX Connect machine (Bio-Rad, Hercules, CA) with the SYBR Green Master Mix (Qiagen, Valencia, CA) and the total reaction volume was 20 μL. The PCR programs were set as follows: 10 min at 95 °C; 40 cycles of 95 °C for 30 s, primer-specific annealing temperature for 30 s, and 72 °C for 30 s. The expression level of each gene was calculated with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) using the chicken β-actin gene as the housekeeping gene (Chaudhari et al. 2020) and the average of chickens in the CON–NCHA group as the calibrator sample. All primers used in this study, including housekeeping and target genes, are given in Table S1.

Extraction of bacterial DNA from digesta and 16S rRNA sequencing

Total DNA was extracted from ileal digesta and cecal contents using the FastDNA SPIN Kit for Soil (MP Biomedicals, Irvine, CA) following the manufacturer's protocol. The V4 region of microbial 16S rRNA genes was amplified using the 515R (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) primers and 2 × 250 paired-end sequencing was performed at Purdue Genomics Core Facility (MiSeq Illumina Inc., San Diego, CA). Overlapping paired-end reads were denoised using the DADA2 pipeline via open-source software Qiime2 (v.2021.2) (https://docs.qiime2.org/2021.2/). During this quality control process, the number of remaining sequencing reads in each step was recorded and is given in Table S2. The representative sequences were extracted with "feature-table" plugins, assigned by taxon via "feature-classifier" plugin, aligned to reference database Silva 138, and clustered into amplicon sequence variants (ASVs) with 99% sequence similarity. All the samples were rarefied to 49,143 and 31,628 sequencing reads for ileal and cecal samples, respectively (Fig. S1).

Statistical analysis

All the data, except microbial results, were analyzed using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC). Oocysts shedding data was subjected to logarithmic transformation prior to analyses. The main effects of enzyme supplementation and *Eimeria* challenge and their interaction were tested using two-way analysis of variance (ANOVA). If there was a significant interaction, Tukey's multiple comparison test was used to identify differences in simple effect means. The significance level was set at $P < 0.05$, and a trend level was set at $0.05 < P < 0.10$.

The statistical analysis of microbiota data was conducted using R 4.0.4 software. The *ggplot2* package was used to visualize alpha diversity. Shannon index was calculated to describe diversity (both richness and evenness), observed-features were measured to present richness, and faith-pd to estimate phylogenetic diversity. If the alpha metrics were normally distributed, an ANOVA test was applied to compare the difference of alpha diversity, otherwise the Kruskal–Wallis test was conducted. Principal coordinate analysis (PCoA) based on Bray-Curtis, weighted UniFrac, and unweighted UniFrac distances was performed in R to evaluate the dissimilarity of microbiota communities. The significance of microbial community composition was assessed in Qiime2 using permutational multivariate ANOVA (PERMANOVA) test and false discovery rate (FDR) adjusted *P* value was used for multiple comparisons. The *DESeq2* package in R was used to identify taxa with differential abundance the genus and ASV level. The FDR-adjusted *P* value below 0.01 was considered significant.

Table 2. Growth performance and total tract nutrient utilization of broiler chickens fed diets supplemented with 0 or 50 g·kg^{-1} multienzymes from day 1 to 13 post hatching[a].

	Diet			
Item	CON	ENZ	SD	*P* value
BW, g				
Day 1	49	49	3.1	0.99
Day 8	165	179	12.2	<0.01
Day 13	317	357	26.5	<0.01
Days 1–8				
BW gain, g·bird^{-1}	115.6	129.4	10.02	<0.01
Feed intake, g·bird^{-1}	146.1	156.2	10.59	0.01
G:F, g·kg^{-1}	791.6	827.7	30.77	<0.01
Days 8–13				
BW gain, g·bird^{-1}	151.2	177.8	15.96	<0.01
Feed intake, g·bird^{-1}	200.2	230.5	18.66	<0.01
G:F, g·kg^{-1}	755.2	771.4	37.33	0.23
Days 1–13				
BW gain, g·bird^{-1}	267.6	307.9	24.49	<0.01
Feed intake, g·bird^{-1}	346.3	386.4	26.86	<0.01
G:F, g·kg^{-1}	772.3	796.6	25.85	0.01
Day 13 total tract nutrient utilization, %				
DM, %	64.1	66	2.30	0.03
GE, %	68.7	70.4	2.18	0.03
N, %	64.9	67	2.92	0.05
Number of cages	16	16		

[a]CON, control without multienzyme additives; ENZ, multienzyme additives; BW, body weight; DM, dry matter; GE, gross energy; N, nitrogen.

Results

Analyzed enzyme activities of the supplemented diet were 1098 FYT·kg^{-1} phytase, 154 IU·kg^{-1} glucanase, 195 IU·kg^{-1} cellulase, and 303 ALU·kg^{-1} amylase.

Growth performance, ileal digestibility, total tract nutrient utilization, and oocysts shedding

The effects of multienzyme supplementation to a wheat and barley-based diet on growth performance and total tract nutrient utilization before the *Eimeria* challenge are presented in Table 2. Supplementing ENZ improved ($P < 0.05$) the BW gain, feed intake, and G:F ratio from day 1 to 13 post hatching. Similarly, the apparent total tract utilization (ATTU) of DM, GE, and N were increased ($P < 0.05$) due to ENZ supplementation (Table 2).

The day 13 BW (Table 2) and, therefore, the pre-challenge effects of diets were maintained at the time *Eimeria* challenge (Table 3). *Eimeria* infection severely reduced ($P < 0.01$) the growth performance, ileal digestibility, and total tract nutrient utilization regardless of dietary treatment. Adding ENZ improved ($P < 0.05$) ileal digestibility of DM, GE, and N and growth performance with higher ($P < 0.01$) average final BW, BW gain, and FI in comparison with CON regardless of *Eimeria* challenge. Interactions between *Eimeria* challenge and dietary enzyme supplementation were observed ($P < 0.05$) for G:F ratio and ATTU of DM and GE and a tendency ($P = 0.07$) for ATTU of N, with enzyme supplementation attenuating the impact *Eimeria* challenge (Table 3). Addition of ENZ did not influence total oocysts shedding (ENZ = 5.51 vs. CON = 5.59, $P = 0.18$, Table 3).

Gene expression in different intestinal segments

Genes encoding cytokines, tight junction proteins, nutrient transporters, and antioxidants were differentially expressed due to dietary enzymes and *Eimeria* challenge in the duodenum, jejunum, and ileum (Table 4). In the duodenum, *Eimeria* challenge suppressed ($P < 0.05$) the expression of nutrient transporters $b^{0,+}$AT and EAAT3, and antioxidant HMOX1. The interactions were observed for the expression of chemokine IL-8 ($P < 0.05$), and a tendency for cytokine IL-10 and tight junction protein JAM2 ($P = 0.06$). In the jejunum, birds subjected to CHA showed a reduction in the expression of all listed genes except for IL-8. Supplementation with ENZ led to higher expressions of $b^{0,+}$AT and EAAT3. Interactions between multienzyme and enteric challenge were observed ($P < 0.05$) on the level of IL8, $b^{0,+}$AT, and EAAT3 transcripts. In the ileum, mRNA encoding IL-6, $b^{0,+}$AT, EAAT3, SOD1, and HMOX1 were significantly downregulated ($P < 0.05$) due to *Eimeria* challenge. There was an interaction ($P < 0.05$) between *Eimeria* challenge and ENZ supplementation on the expression of the HMOX1 gene.

Structure and composition of gut microbiota

For cecal microbial communities, CHA significantly influenced the alpha diversity metrics related to diversity, richness, and evenness (Shannon, observed features, and evenness, $P < 0.001$; Fig. 1), and similar results were observed in ileal microbiota. However, there was no difference in alpha diversity metrics due to ENZ supplementation in ileum or ceca. The ileal and cecal microbiota separated into two clusters according to CHA treatment (PERMANOVA, pseudo $F = 15.514$, $P < 0.01$), but there was no significant difference between CON and ENZ groups based on the Bray–Curtis distances (Fig. 2). The major ASVs belonged to four main phyla, including *Firmicutes*, *Proteobacteria*, *Bacteroidota*, and *Actinobacteria* in ceca (Fig. S3). The relative abundance of *Proteobacteria* enriched after challenge. In general, 83 and 26 differentially abundant ASVs in the genus level were identified associated with CHA-NCHA and ENZ-CON in ceca (Fig. 3).

Discussion

During the pre-*Eimeria* challenge period, dietary multienzyme supplementation exerted beneficial impacts on animal growth in the current study. *Eimeria* challenge without ENZ treatment impaired the growth as evidenced by 33%, 64%, and 34% reduction in BW, BWG, and FI, respectively. Whereas, *Eimeria* challenge with dietary multienzyme supplementation improved performance by attenuating the decrease in gain to feed ratio. Dietary nutrients undergo digestion and absorption in small intestine and further microbial fermentation in hindgut to support animal maintenance and growth. From the perspective of nutrient-partitioning, birds exposed

Table 3. Growth performance, apparent ileal digestibilty, total tract nutrient utilization, and total oocysts shedding of broiler chickens fed diets supplemented with 0 or 50 g·kg^{-1} multienzymes with or without *Eimeria* challenge from day 13 to 21 post hatching[a].

	Diet									*P* value		
	CON		ENZ		Diet		Challenge					
Item	NCHA	CHA	NCHA	CHA	CON	ENZ	NCHA	CHA	SD	Diet	Challenge	Diet × challenge
BW, g												
Day 13	317	317	357	357	317	357	337	337	4.3	<0.01	0.99	0.98
Day 20	660	440	727	528	550	628	694	484	25.9	<0.01	<0.01	0.26
BW gain, g·bird^{-1}	342.9	122.9	369.5	170.9	232.9	270.2	356.2	146.9	25.40	<0.01	<0.01	0.25
Feed intake, g·bird^{-1}	453.1	295.0	504.0	385.4	374.1	444.7	478.6	340.2	38.08	<0.01	<0.01	0.16
G:F, g·kg^{-1}	759.3	419.6	730.8	444.3	589.5	587.6	745.1	432.0	30.48	0.87	<0.01	0.03
AID												
DM, %	64.9	56.7	70.2	59.7	60.8	64.9	67.5	58.2	3.65	<0.01	<0.01	0.40
GE, %	67.1	53.4	72.5	56.1	60.3	64.3	69.8	54.8	3.91	0.01	<0.01	0.37
N, %	72.3	56.7	77.9	58.2	64.5	68.1	75.1	57.5	4.15	0.04	<0.01	0.22
Total tract nutrient utilization												
DM, %	69.3	33.6	70.7	42.3	51.4	56.5	70.0	37.9	3.10	<0.01	<0.01	<0.01
GE, %	74.0	31.1	75.3	39.1	52.6	57.2	74.7	35.1	3.65	<0.01	<0.01	0.02
N, %	72.1	−17.8	71.6	−2.9	27.2	34.3	71.8	−10.4	11.19	0.09	<0.01	0.07
Total oocysts shedding[b]	ND	5.59	ND	5.51	5.59	5.51	ND	ND	0.113	0.18	–	–
Number of observations	8	8	8	8	16	16	16	16				

[a]AID, apparent ileal digestibility; CON, control without multienzyme additives; CHA, challenge; ENZ, multienzyme additives; GE, gross energy; N, nitrogen; NCHA, non-challenge; ND, not detected.
[b]Total oocyst shedding were expressed as $\log_{10}$ (oocysts·g^{-1} feces).

to *Eimeria* challenge impose considerable nutrient demand for the maintenance of immune function. Meanwhile, due to the drastic reduction in feed intake, limited nutrients are obtained from gastrointestinal tract. Therefore, the availability of nutrients to increase body mass is reduced and manifested as retarded growth. To further illustrate the impacts of multienzyme on broiler performance under challenge, the following discussion focused on (*i*) nutrient digestibility, utilization, and nutrient transporters in small intestine and (*ii*) immune response and indicators of gut health in small intestine. On the other hand, microbiota in gastrointestinal tract is critical for nutrient utilization, immune development, and exclusion of pathogens. Thus, cecal microbiota communities shed light on the impacts of dietary multienzyme and *Eimeria* challenge.

In the current study, the coccidiosis model was established by oral gavage with a mixture of live oocysts from three *Eimeria* species, including *E. acervulina*, *E. maxima*, and *E. tenella*. This enabled the infective oocysts to invade the whole segment of the gastrointestinal tract, because specific *Eimeria* species has been shown to infect specific segments of the gut. In general, *Eimeria* challenge decreased the digestion and absorption of energy and nutrients, attributable to damaged villus structure (data not shown) and amino acids (AAs) transporter, triggered inflammation, impaired paracellular seal, perturbed redox homeostasis, and dysbiosis of gut microbiota. These findings are in line with previous studies, and the adverse impacts could be further elucidated by elevated pathogen-specific endogenous loss, reduced endogenous enzyme secretion, and epithelial turnover (Su et al. 2015; Chaudhari et al. 2020; Teng et al. 2021). Although multienzyme supplementation did not influence the oocyst shedding in this study, it may exert beneficial effects by alleviating the deleterious influence of coccidiosis or facilitating host recovery from the disease.

A wheat and barley-based diet supplemented with multienzyme containing phytase, xylanase, β-glucanase, amylase, hemicellulases, and pectinases, showed some degree of protective effects on the retarded growth induced by *Eimeria* challenge. This could be directly explained by improved nutrient utilization and gene expression of AA transporters. The use of multienzyme increased the digestibility and utilization of energy and nutrients without *Eimeria* challenge. Moreover, multienzyme addition partially protected against the *Eimeria* challenge by ameliorating the reduction of total tract nutrient utilization and AA transporter $b^{0,+}$AT expression in the jejunum. However, there was no pronounced improvement in the AID of energy and nutrients, which is similar to the previous observation (Parker et al. 2007). However, divergent findings that a blend of xylanase and β-glucanase or phytase alone increased the digestibility of N, P, and several AAs were reported (Mathlouthi et al. 2002; Adedokun and Adeola 2016). Differences in diet composition, challenge dose, combination of enzymes, and efficacy of enzymes between ex-

Table 4. Intestinal gene expression for cytokines, tight junction protein, nutrient transporters, and antioxidants in broiler chickens fed with 0 or 50 g·kg^{-1} multienzyme under challenge or non-challenge conditions on day 21 post hatching[a].

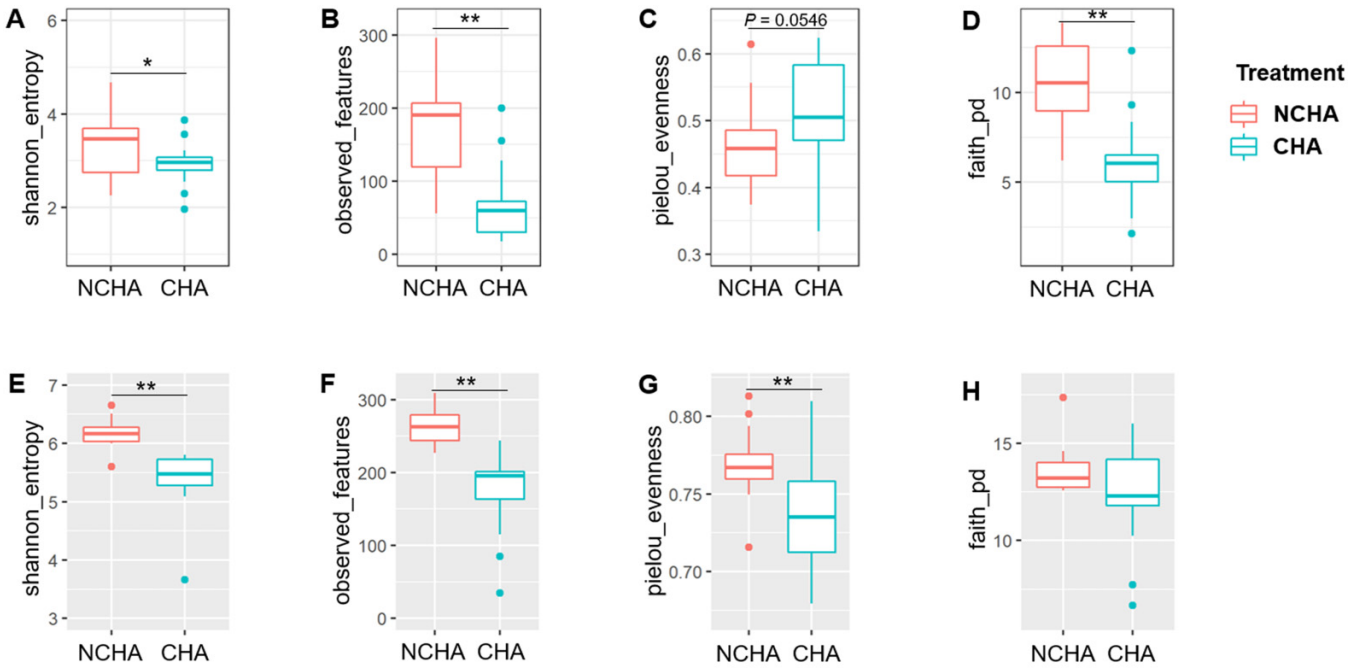
	Diet											
	CON		ENZ		Diet		Challenge			*P* value		
Item	NCHA	CHA	NCHA	CHA	CON	ENZ	NCHA	CHA	SD	Diet	Challenge	Diet × challenge
Duodenum												
IL-6	1.59	1.75	2.12	1.91	1.67	2.02	1.86	1.83	1.51	0.55	0.96	0.75
IL-8	1.44	5.22	3.43	2.74	3.33	3.09	2.44	3.98	2.56	0.79	0.11	0.02
IL-10	1.37	2.21	5.85	2.04	1.79	3.95	3.61	2.13	3.26	0.08	0.23	0.06
JAM2	1.30	1.97	4.65	1.85	1.64	3.25	2.98	1.91	2.44	0.08	0.24	0.06
Occludin	1.55	1.80	3.41	1.81	1.68	2.61	2.48	1.81	2.01	0.22	0.37	0.23
$b^{0,+}AT$	1.14	0.67	1.21	0.66	0.91	0.94	1.18	0.67	0.49	0.87	0.01	0.84
EAAT3	1.21	0.78	1.70	0.68	1.00	1.19	1.46	0.73	0.78	0.48	0.01	0.30
SOD1	1.10	0.73	1.06	1.01	0.92	1.04	1.08	0.87	0.37	0.38	0.14	0.25
HMOX1	1.27	0.39	0.90	0.46	0.83	0.68	1.09	0.43	0.39	0.31	<0.01	0.13
Jejunum												
IL-6	1.30	0.60	1.63	0.26	0.95	0.95	1.47	0.43	0.90	0.99	<0.01	0.33
IL-8	1.27	5.77	3.47	1.29	3.52	2.38	2.37	3.53	3.37	0.38	0.37	0.02
IL-10	1.52	1.05	1.48	0.58	1.29	1.03	1.50	0.82	0.86	0.44	0.05	0.53
JAM2	1.57	0.77	1.14	0.36	1.17	0.75	1.36	0.57	0.85	0.19	0.02	0.97
Occludin	1.74	0.64	1.18	0.27	1.19	0.73	1.46	0.46	1.00	0.23	0.01	0.80
$b^{0,+}AT$	1.09	0.25	1.96	0.35	0.67	1.16	1.53	0.30	0.43	0.00	<0.01	0.02
EAAT3	1.03	0.42	2.36	0.37	0.73	1.37	1.70	0.40	0.57	0.01	<0.01	0.00
SOD1	1.14	0.72	1.24	0.63	0.93	0.94	1.19	0.68	0.50	0.99	0.01	0.60
HMOX1	1.26	0.78	1.98	0.64	1.02	1.31	1.62	0.71	1.07	0.44	0.02	0.26
Ileum												
IL-6	1.23	0.55	1.40	0.36	0.89	0.88	1.32	0.46	0.76	0.96	<0.01	0.51
IL-8	1.67	2.10	1.86	1.01	1.89	1.44	1.77	1.56	1.48	0.41	0.69	0.24
IL-10	3.41	3.65	3.21	1.45	3.53	2.33	3.31	2.55	3.46	0.35	0.56	0.44
JAM2	1.62	2.69	2.02	1.23	2.16	1.63	1.82	1.96	1.87	0.46	0.84	0.19
Occludin	3.66	4.75	4.09	3.07	4.21	3.58	3.88	3.91	3.81	0.65	0.98	0.44
$b^{0,+}AT$	1.17	0.33	0.92	0.21	0.75	0.57	1.05	0.27	0.47	0.28	<0.01	0.69
EAAT3	1.13	0.45	0.87	0.25	0.79	0.56	1.00	0.35	0.41	0.14	0.00	0.83
SOD1	1.22	0.48	1.03	0.62	0.85	0.83	1.13	0.55	0.50	0.89	0.00	0.36
HMOX1	1.69	0.48	0.62	0.57	1.09	0.60	1.16	0.53	0.79	0.10	0.04	0.05
Number	8	8	8	8	16	16	16	16				

[a]$b^{0,+}AT$, $b^{0,+}$ amino acid transporter; CHA, challenge; CON, control without multienzyme additives; EAAT3, excitatory amino acid transporter 3; ENZ, multienzyme additives; HMOX1, heme oxygenase 1; IL, interleukin; JAM2, junctional adhesion molecule; NCHA, non-challenge; SOD1, superoxide dismutase 1.

periments could be responsible for the discrepant findings. Selle et al. (2003) pointed out that synergism existed across some specific enzymes, so the optimum combination of enzymes and the inclusion ratio need further investigation. In addition, considering the specific endogenous loss induced by coccidiosis (Teng et al. 2021), the impacts of multienzyme on true digestibility of nutrients might be confounded or hidden. Thus, the evaluation of endogenous loss and standardized ileal digestibility will broaden the understanding of the multienzyme under coccidiosis. The jejunum is the primary site of nutrient absorption, and the specialized transporters located on the brush border membrane (BBM) are responsible for absorption of various nutrients such as free AAs, short peptides, and monosaccharides. The EAAT3 is particularly essential for glutamate transport across BBM, which serves as the primary energy source for intestinal epithelial cells (IECs) during nutrient digestion and absorption (Su et al. 2015). The $b^{0,+}AT$ transports neutral and cationic AAs and has a high affinity with L-lysine (Torras-Llort et al. 2001). Multienzyme supplements increased molecular synthesis of jejunal transporters $b^{0,+}AT$ and EAAT3 in CON group, thus facilitating AA influx into enterocytes and fostering the absorption of protein building blocks. More importantly, multienzyme attenuated the decrease in jejunal $b^{0,+}AT$ that resulted from *Eimeria* challenge and consequently improved growth to some degree. Future studies should be directed at understanding the modulation of other nutrient transports, such as glucose, peptides, and mineral transporters.

During the critical phase of *Eimeria* infections, significant interactions occur between IECs and *Eimeria* oocysts. Thus, in-

Fig. 1. Alpha diversity metrics for ileal (A–D) and cecal (E–H) microbial structure in broiler chickens under challenge or non-challenge conditions at d 21 post hatching. (A and E) Diversity calculated by Shannon index, (B and F) richness measured by observed features, (C and G) evenness and (D and H) phylogenetic diversity estimated by faith_pd index. The boxplots indicate the median (line), 25th percentile (top of box), and 75th percentile (bottom of box). Significant differences are indicated by $^{*}P < 0.05$ and $^{**}P < 0.001$. CHA, challenge; NCHA, non-challenge.

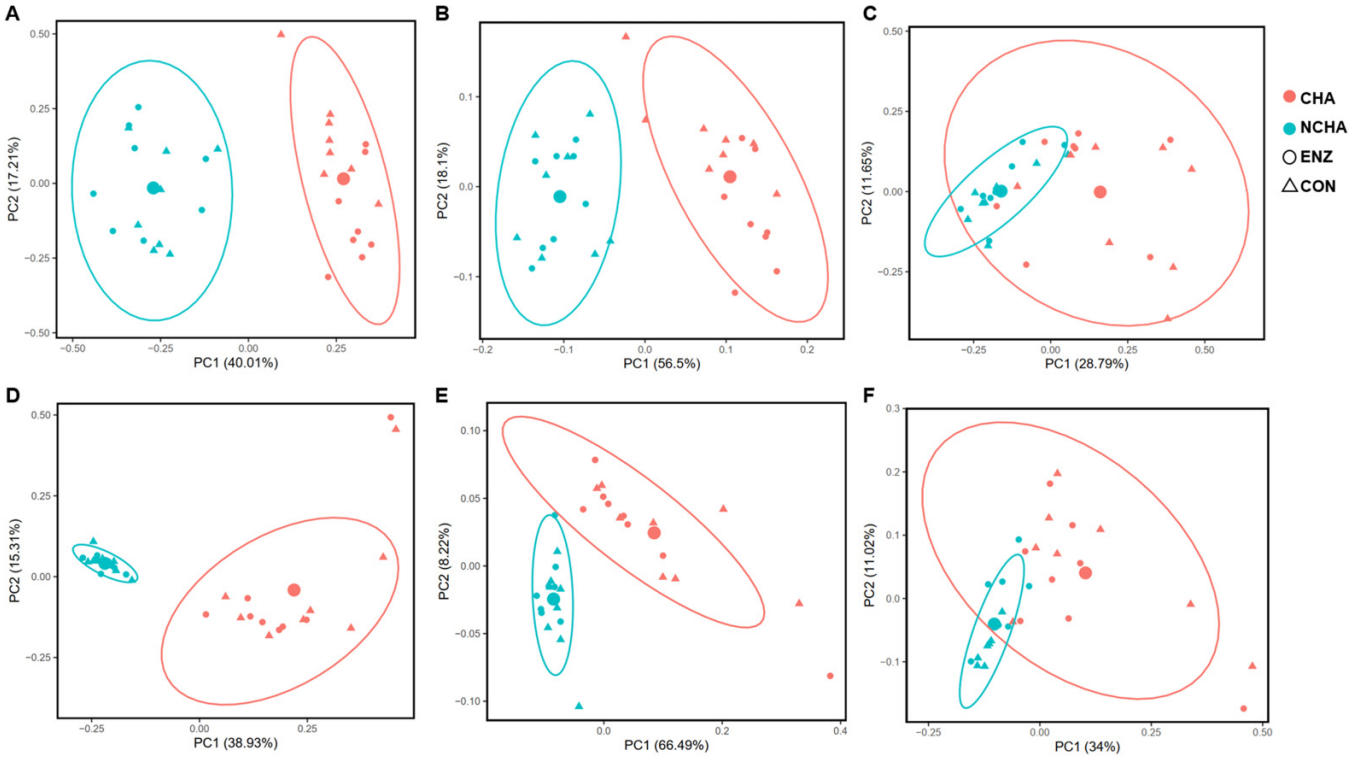


terventions that might reduce the viability of oocysts, meddle with, or obstruct the invasion process, and (or) enhance the opposition of IECs to *Eimeria* survival are potential strategies to alleviate or control coccidiosis. Innate immunity is the first line to defend against *Eimeria* challenge and the cells involve dendritic cells (DCs), macrophages, natural killer cells, and heterophils (avian analogue of mammalian neutrophils; Broom 2021). Heterophils/neutrophils are the most numerous and the first to be recruited by IL-8 to combat and ultimately eliminate infections by various pathways, including phagocytosis, reactive oxygen species, antimicrobial substances secretion, and neutrophil extracellular traps formation (Muñoz-Caro et al. 2016). As an early immunomodulatory cytokine, IL-10, functionally connects innate and adaptive immunity by regulating the Th1/Th2 cytokine balance (Haritova and Stanilova 2012). A recent study demonstrated that moderate IL-10 induction provided protective effects against *Eimeria* challenge. However, with excessive IL-10 produced by macrophages and DCs, the defensive Th1 response is terminated, bringing about disease prevalence (Haritova and Stanilova 2012; Bremner et al. 2021). Later, macrophages and cytotoxic T-cells are the major effectors during primary *Eimeria* infections. Following mixed *Eimeria* challenge, cytokines and chemokines undergo dynamic changes in different intestinal segments. In this study, *Eimeria* challenge suppressed the gene expression of IL-6 in jejunum and ileum, and IL-10 in jejunum. These results indicated the importance of sampling time and could be explained by the dynamic changes of cytokines reported by Hong et al. (2006). So future studies utilizing lower challenge doses and multiple sampling times should provide the details and dynamics of mucosal cytokines during coccidiosis. Furthermore, multienzyme supplementation decreased the elevated IL-8 expression in duodenum and jejunum that was stimulated by *Eimeria* challenge. The lower IL-8 expression in enzyme group might indicate a lower inflammatory response under the same *Eimeria* challenge in comparison with no-enzyme treatment. Multienzyme might support a sturdy immunity that helped with alleviating coccidiosis invasion and facilitating animal growth. Another possible explanation might be that multienzyme trigger a fast recovery from *Eimeria* infections.

Gastrointestinal parasitism is a primary stress factor resulting in dystrophy and delayed growth. Host cells initiate oxidative stress to combat *Eimeria* invasion by generating reactive oxygen species and nitric oxide (Idris et al. 2017). However, these free radicals break the oxidant/antioxidant balance and are cytotoxic agents resulting in intestinal tissue damage. This was consistent with the observations in the current study that *Eimeria* challenge drastically suppressed the expression of antioxidants SOD and HOMX1. Multienzyme alleviated the *Eimeria*-induced downregulation of antioxidant HMOX1 expression, which may help restore the redox balance. This may ultimately confer benefits on the immune system and gut barrier integrity and further contribute to the improved growth.

Well-balanced gut microbiota is critical for host health. Particularly, maintaining the superiority of obligate anaerobic bacteria in cecal is responsible for nutrient metabolism and

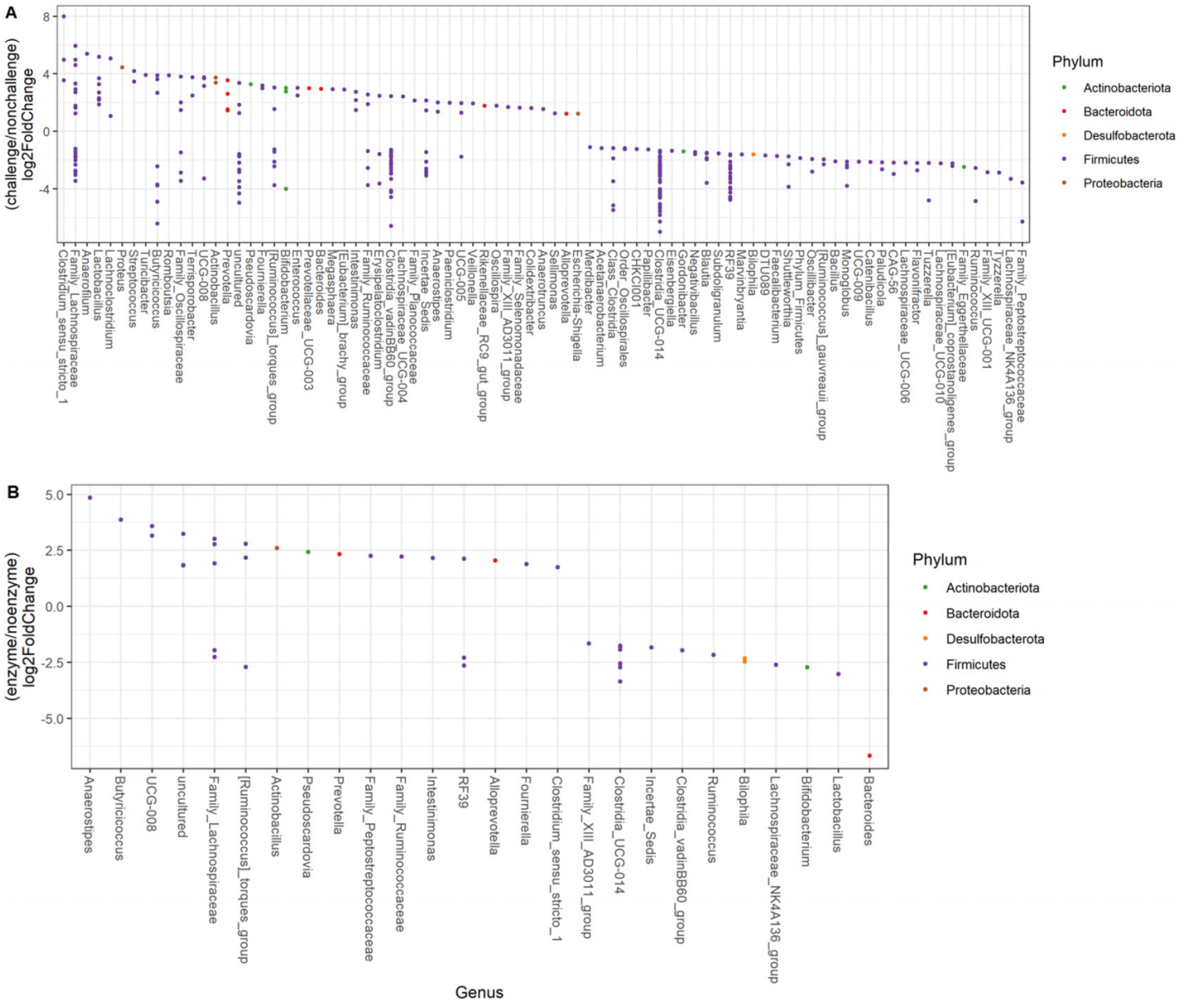
Fig. 2. Beta diversity shifted of broiler chickens ileal (A–C) and cecal (D–F) microbiota as affected by diet and challenge calculated by Bray–Curtis (A and D), weighted (B and E) and unweighted (C and F) UniFrac distance. Each point represents a different sample and the greater the distance between two points, the higher the dissimilarity between the two communities. Ellipses indicate the 95% confidence interval to contain all points from each indicated treatment group. CHA, challenge; CON, control without multienzyme additives; ENZ, multienzyme additives; NCHA, non-challenge.



gut homeostasis (Litvak et al. 2017). In this study, *Eimeria* challenge perturbed the structure and composition of cecal microbial communities. The abundance of facultative anaerobic *Proteobacteria* expanded, while that of obligate anaerobic *Firmicutes* reduced. Cecal microbiota shifting from obligate anaerobic to facultative anaerobic bacteria, especially the increase in *Proteobacteria*, is considered as a potent signature of gut dysbiosis (Litvak et al. 2017). The underlying mechanism or undergoing physiological processes could be explained based on the theories of Litvak et al. (2017). Cecal epithelial cells are hypoxic, and local inflammation triggered by *Eimeria* challenge increases oxygenation, interrupting anaerobiosis and promoting the growth of facultative anaerobic *Proteobacteria* via aerobic respiration. Thus, increased abundance of *Proteobacteria* may imply disturbed microbial communities and local inflammation in ceca. On the other hand, it may reflect a favorable protein fermentation resulting from increased endogenous loss and reduced nutrient utilization in small intestine. Moreover, further analysis confirmed that *Eimeria* challenge caused dysbiosis, as evidenced by the proliferation of opportunistic pathogenic microbiota of the genera *Actinobacillus*, *Clostridium sensu stricto 1*, *Escherichia*, *Enterococcus*, *Bacillus*, and *Streptococcus*, whereas there was a decline in potentially beneficial genera, such as *Bifidobacterium*, *Clostridia_UCG-014*, and *Facecalibacterium* in ceca. The findings of this research are in agreement with those of earlier studies in coccidiosis (Cui et al. 2017; Bortoluzzi et al. 2019). The reduction in the abundance of *Facecalibacterium*, as butyrate-producing bacteria, may impede the development of immune response and reduce the synthesis of butyrate utilized as an energy source (Chen et al. 2020). *Clostridia_UCG-014* was favorably associated with colitis relief and suppression of inflammation (Sheng et al. 2021), but enriched *Clostridium sensu stricto 1* was associated with increasing necrotic enteritis severity (Yang et al. 2019). Dysbiosis of gut microbiota and altered microbial communities are indicative of disease conditions that can modulate the immune response and further impact appetite and growth through the gut-brain axis.

In comparison with *Eimeria* challenge, multienzyme treatment showed limited impact on cecal microbiota at the phylum level. However, multienzyme exerted beneficial impact on restoring the gut homeostasis via promoting the proliferation of short-chain fatty acids (SCFA) generating genera, such as *Anaerostipes* spp., *Butyricicoccus*, *Intestinimonas*, *Pseudoscardovia*, and *Prevotella*. Intriguingly, a recent study validated that *Anaerostipes* spp. can be introduced as a therapeutic approach to promote host health and revealed underlying mechanisms (Bui et al. 2021). Two metabolic pathways have been identified, including (1) metabolizing inositol to generate propionate–acetate (Bui et al. 2021) and (2) conversion of lactate–acetate into butyrate (Shetty et al. 2020). Thus, the expansion of *Anaerostipes* spp. might be attributed to the phytase addition, which releases abundant inositol stereoisomers that can serve as substrates for *Anaerostipes* spp. to pro-

Fig. 3. Differentially abundant ASVs of cecal microbiota between two groups. Log2 fold changes calculated by DESeq2 in R for ASVs describe changes in the bacterial community in the ceca with or without corresponding treatment. Each dot represents an ASV with the classified taxonomic level (genus) shown on the *x* axis, and phylum indicated by color. (A) Ratio of challenge to non-challenge. (B) Ratio of enzyme to no-enzyme. A positive value indicates a significant increase of the specific ASV in challenge (A) (or enzyme, B) treatment relative to that of the non-challenge (A) (or no-enzyme, B) treatment, respectively. ASVs, amplicon sequence variants.



duce propionate and acetate (Bui et al. 2021). Also, this may result from enriched oligosaccharides resulting from carbohydrase addition and microbial cross-feeding in ceca. Furthermore, the prevalence of those butyrate producers *Butyricicoccus* and *Intestinimonas*, fiber-fermenting *Pseudoscardovia*, and SCFA-generating genus *Prevotella*, could modulate mucosal integrity, immunity, and energy supply via SCFA (van der Hee and Wells 2021). Thus, the alternation in microbial diversity could indirectly validate the potential of multienzyme on attenuating coccidiosis by restoring dysbiotic microbiome, especially enriching the population of SCFA producers. Another potential explanation is that multienzymes facilitate the early microbial establishment, drive the succession towards strictly anaerobic communities (Broom 2021), promote the generation of beneficial metabolites, and all of which help to alleviate the disturbance from pathogenic invasion.

In conclusion, the current study showed the beneficial effects of dietary multienzyme supplementation during *Eimeria* challenge. These benefits may occur by enzyme effect in improving feed efficiency, nutrient digestibility, intestinal immunity, intestinal barrier, and gut microbial homeosta-

sis. Therefore, multienzyme containing phytase, xylanase, β-glucanase, amylase, hemicellulases, and pectinases can serve as a potential strategy to abate the effects of coccidiosis in broiler chickens.

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Data availability

Data generated or analyzed during this study are provided in full within the published article and its supplementary materials.

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Competing interests

The authors declare there are no competing interests.

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Supplementary material

Supplementary data are available with the article at https://doi.org/10.1139/CJAS-2022-0046.

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