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Stimbiotics help improve intestinal immunity and positively modulate the gut microbiome in broilers with necrotic enteritis

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ABSTRACT

This experiment was conducted to investigate the effect of stimbiotic (STB) in broilers with necrotic enteritis (NE) on nutrient digestibility and gut health. A total of 200 one-day-old Arbor Acres (initial body weight of 44.03 ± 0.28 g) were used in this experiment for 28 days. All broilers were randomly allocated into four treatments, and each experimental group had 10 replicate cages with five broilers per cage. The experiment was conducted in a 2 × 2 factorial designs consisting of two levels of challenge (challenge and non-challenge) and two levels of STB (0 and 0.05 %). All broilers in challenged groups were orally challenged by overdosing with coccidia vaccines (× 10 recommended doses; Livacox® Q). The NE challenge significantly decreased ($P < 0.05$) nutrient digestibility, interferon-γ, heterophil levels in blood, and villus height:crypt depth (VH:CD) compared to the non-challenge group. Also, the NE challenge significantly lower ($P < 0.05$) *ZO-1* and higher *MUC2* gene expression than the non-challenge group. Supplementation of 0.05 % STB with NE challenge significantly increased ($P < 0.05$) gross energy digestibility and decreased ($P < 0.05$) the number of oocysts per gram of feces compared to the NE-challenged group. Supplementation of 0.05 % STB significantly increased ($P < 0.05$) the VH:CD in ileum compared to the non-supplementation group. Also, supplementation of 0.05 % STB is significantly lower ($P < 0.05$) *MUC2* and *TLR4* gene expression in ileum than the non-supplementation group. At the genus level, the supplementation of 0.05 % STB with NE challenge significantly decreased ($P < 0.05$) the abundance of *Muribaculaceae* compared to the NE-challenged group on d 21. In conclusion, supplementation of 0.05 % STB in a diet could positively regulate the cecal microflora and gene expression of tight junction protein and alleviate the decline in nutrient digestibility caused by NE.

Introduction

Stimbiotics (STB) are functional feed additives that can accelerate colonization of cellulose-degrading bacteria in the intestine and help maximize the utilization of dietary fiber from arabinoxylan in the diet (Ribeiro et al., 2018; González-Ortiz et al., 2019). The STBs are relatively new additives in powder form that consist of a combination of xylanase (XYL), a carbohydrate-degrading enzyme, and xylooligosaccharides (XOS) (Petry et al., 2021). Adding STB to a diet can increase the supply of XOS and stimulate fiber fermentation in the cecum (Veluri et al., 2024). The established microbial community in the cecum can release more XYL, further increasing the release and fermentation of fiber oligosaccharides (Veluri et al., 2024). The XYL is a carbohydrase that hydrolyzes the β−1, 4-glycosidic bond of arabinoxylan, so it can reduce the anti-nutritional effects of non-starch polysaccharides (NSP) and produce short-chain fatty acids to improve intestinal health and nutrient digestibility (Liu et al., 2021; Singh et al., 2021). Previous studies have reported that feeding a diet with STB supplementation to

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broilers and pigs can reduce intestinal inflammation caused by post-weaning diarrhea or necrotic enteritis (NE) and improve growth performance (Chang et al., 2024; Lee et al., 2022; Song et al., 2023).

Gut health depends on nutritional and health status of poultry, including the immune system, balanced gut microbiota, and intestinal mucosa (Rajput et al., 2020). It not only affects nutrient absorption, but also could play a role in preventing diseases caused by pathogens (Bailey, 2021; Stanley et al., 2012). Therefore, poor gut health can lead to pathogen invasion, accumulation of toxins and endotoxins, and damage to tight junction proteins, which can negatively affects the overall performance of broilers (Shojadoost et al., 2022; Zhao et al., 2022). NE caused by *Clostridium perfringens* is a representative enteric disease of broilers, causing significant economic losses by damaging the intestinal mucosa (Khalique et al., 2020). *C. perfringens* is generally found in intestines of healthy broilers (Shojadoost et al., 2012). However, when *C. perfringens* overgrows in the intestine due to coccidiosis and high animal protein or NSP content in the diet, it can secrete toxins such as NetB or α-toxin and lead to NE (Fathima et al., 2022). Therefore, new nutritional strategies such as STBs are needed to prevent NE and improve intestinal health of broilers.

Our previous study has shown that supplementation with 0.05 % STB in a diet could positively regulate fecal microflora and alleviate the decline in growth performance and nutrient digestibility caused by NE better than supplementation with 0.10 % STB (Chang et al., 2024). Accordingly, this study hypothesized that supplementing 0.05 % STB to NE-challenged broilers could improve intestinal barrier integrity and intestinal morphology, thereby enhancing nutrient digestibility. Therefore, this study was conducted to investigate effects of adding 0.05 % STB to an NE-challenged broiler diet on nutrient digestibility, oocyst excretion count, intestinal lesion score, intestinal morphology, expression of tight junction protein, blood profiles, and cecal microflora.

Materials and mehods

Ethics

The experimental protocols describing the management and care of animals were reviewed and approved by the Animal Care and Use Committee of Chungbuk National University (CBNUA-2276-24-01).

Animals, experimental design and diets

A total of 200 one-day-old Arbor Acres broilers (initial body weight of 44.03 ± 0.28 g) were obtained from a local hatchery (Cherrybro Co., Eumseong, Korea) and used in this experiment for 28 days. All broilers were randomly allocated into four treatments, and each experimental group had ten replicate cages with five broilers per cage (100 cm width, 40 cm depth, 45 cm height). The experiment was conducted in a 2 × 2 factorial design of treatments consisting of two levels of challenge (challenge and non-challenge) and two levels of STB (0 and 0.05 %). The experimental period was divided into four phases: pre-starter (0 to 7 days), starter (8 to 14 days), grower (15 to 21 days), and finisher (22 to 28 days). All diets were formulated to meet or exceed the National Research Council (NRC, 1994; Table 1). All broilers were given ad libitum access to diet and water throughout the experiments. The experiment initiation temperature was 32 ± 1°C, after that, the temperature was gradually lowered to maintain 25 ± 1°C.

NE challenge

On day 14, all broilers in challenged groups were orally challenged by overdosing with coccidia vaccines (× 10 recommended doses). Coccidia vaccine (Livacox® Q, Biopharm, Czech Republic) contained *Eimeria acervulina, E. tenella, E. maxima*, and *E. necatrix*. 1 mL vaccine contains 30,000 to 50,000 oocysts of each *E. acervulina, E. tenella*, and *E. maxima* and 10,000 oocysts of *E. necatrix*. On days 18 to 20, 3 mL of *C. perfringens* (1×10^7 CFU/mL) was orally challenged by dividing for 3 consecutive days. The *C. perfringens* was type A NCTC 8798 (NCTC, National Collection of Type Cultures, London, UK). The non-challenged group received the same dosage of sterile phosphate-buffered saline (PBS) via oral gavage.

Table 1
Ingredient composition of experimental diets[1].

Items	Pre-starter, d 0-7	Starter, d 8-14	Grower, d 15-21	Finisher, d 22-28
Ingredients, %				
Corn	36.59	41.17	44.73	47.82
Wheat fine	15.36	15.16	15.66	15.26
Rice pollards	2.49	2.59	2.59	2.69
Soybean meal, 45 % CP	26.98	21.04	17.73	15.57
DDGS	5.09	7.09	6.09	5.09
Tankage meal	6.05	5.37	5.55	5.42
Poultry offal	2.56	2.69	2.69	3.12
Animal fat	1.71	1.90	1.90	1.95
L-lysine	0.67	0.66	0.68	0.58
L-methionine	0.43	0.33	0.37	0.47
L-threonine	0.16	0.12	0.10	0.16
L-tryptophan	0.18	0.11	0.18	0.12
Salt	0.25	0.25	0.25	0.25
Limestone	0.58	0.62	0.58	0.60
MDCP	0.20	0.20	0.20	0.20
Liquid-Choline	0.10	0.10	0.10	0.10
Vitamin premix[2]	0.30	0.30	0.30	0.30
Mineral premix[3]	0.30	0.30	0.30	0.30
Total	100.0	100.0	100.0	100.0
Chemical composition				
AMEn, Kcal/kg	3000	3020	3070	3100
CP, %	23.30	21.37	20.24	19.10
Ether extract, %	5.59	5.90	5.78	5.74
Crude fiber, %	3.49	3.46	3.29	3.10
Crude ash, %	5.88	5.58	5.19	5.03
Calcium, %	0.85	0.80	0.75	0.73
Total phosphorus, %	0.67	0.62	0.62	0.58
Lysine, %	1.63	1.55	1.45	1.34
SAA, %	1.24	1.21	1.20	1.19

[1]Abbreviation: DDGS, Dried distiller's grains with soluble; MDCP, Monodicalcium phosphate; SAA, Sulfur amino acids; AMEn, nitrogen-corrected apparent metabolizable energy; CP, crude protein.
[2]Supplied per kg diet: vitamin A, 9000 IU; vitamin D_3, 3000 IU; vitamin E, 48 mg; vitamin K, 3 mg; thiamin, 1.8 mg; riboflavin, 6 mg; pyridoxine, 3 mg; vitamin B_{12}, 0.012 mg; niacin, 42 mg; folic acid, 1.2 mg; biotin, 0.24 mg; pantothenic acid, 12 mg.
[3]Supplied per kg of diet: manganese, 120 mg; zinc, 100 mg; iron, 80 mg; copper, 20 mg; iodine, 2 mg; selenium, 0.3 mg; cobalt, 0.5 mg.

Nutrient digestibility

During the second and final weeks of the experiment, 0.2 % chromium oxide (Cr_2O_3) was mixed as an indigestible marker in all broiler diets for analyzing apparent ileal digestibility (AID). On days 14 and 28, 10 broilers per treatment were euthanized with cervical dislocation, and ileal digesta was collected. At the same time, the diet was collected and immediately frozen at −20°C with ileal digesta. Before analysis, ileal digesta samples were dried at 70°C for 48 hours and then crushed through a 1 mm screen. The dry matter (DM; method 930.15) and crude protein (CP; method 984.13) were analyzed following AOAC methods (AOAC, 2007). The gross energy (GE) content was analyzed by using an adiabatic oxygen bomb calorimeter (Parr 6400 Bomb Calorimeter, Parr Instrument Co., Moline, IL, USA). Chromium levels were assessed using ultraviolet absorption spectrophotometry (UV-1201, Shimadzu, Kyoto, Japan). The AID was calculated using the formula: Digestibility (%) = [1 − {(concentration of nutrient in ileal digesta × concentration of Cr_2O_3 in the diet)/(concentration of nutrient in the diet × concentration of Cr_2O_3

in the ileal digesta)}] × 100.

Oocyst shedding

On days 19 to 21 and 28, clean plastic was placed under each cage and fresh feces were collected. After collecting feces in a sample bag from each cage, they were stored at 4°C until further processing. 5 g of fecal samples were diluted with 45 mL of tap water. Following adequate mixing, a 9 mL saturated salt solution was added to a 1 mL mixture. To allow the oocysts to float, the homogenized liquid stood for 30 seconds. The final samples were put into McMaster chambers (Jorgensen Laboratories, Loveland, CO) using a water dropper pipette (Thermo Fisher Scientific, Waltham, MA), and the number of oocysts was counted. The formula below was used to determine the number of oocysts per gram of feces (OPG): (Number of oocysts counted/0.15) × Dilution factor = OPG count, where 0.15 is the McMaster counting chamber's volume.

Blood profile

On days 14, 21, and 28, 10 broilers per treatment were used to analyze blood profiles. Blood samples were collected from the brachial wing vein. The samples were collected in K_3EDTA tube for complete blood count analysis and nonheparinized tubes for serum analysis, respectively. White blood cells (WBC), heterophils, and lymphocytes were analyzed using an automatic hematology analyzer (XE2100D, Sysmex, Kobe, Japan). Interleukin-10 (IL-10; P8000, R&D systems, Minneapolis, MN, USA) and interferon-γ (IFN-γ; DY985, R&D systems) were measured using commercially available ELISA kits.

Intestinal lesion score

On days 21 and 28, 10 broilers per treatment were euthanized after blood sampling, and the intestinal lesion score was measured according to the method of Dahiya et al. (2005) with slight modifications. Intestinal lesion score was measured in the jejunum and ileum, respectively. The score was as follows: 0 (apparently normal, no lesion), 1 (severely congested serosa and mesentery engorged with blood), 2 (thin-walled and friable intestines with small red petechiae), 3 (focal necrotic lesions), and 4 (patches of necrosis, 1 to 2 cm long).

Intestinal morphology

On days 21 and 28, 10 broilers per treatment were euthanized to analyze intestinal morphology. Intestinal tissues of about 5 cm from the jejunum (midpoint between the bile duct and Meckel's diverticulum) and ileum (close to the ileocecal junction) were collected. The samples were washed and stored with 10 % neutral buffered formalin (Sigma-Aldrich, St. Louis, MO, USA) until staining for analysis. After being dehydrated, the fixed intestine segments were embedded in paraffin. To measure intestinal morphology, 5 μm thick cross sections were cut out and stained with hematoxylin and eosin (H&E). An Olympus IX51 inverted phase-contrast microscope was used to view the slides. The mean values of six well-oriented villus and crypts were used to compute the villus height (VH), crypt depth (CD), and VH to CD ratio (VH:CD), respectively.

Real-time quantitative RT-PCR (qRT-PCR) analysis

On days 21 and 28, 10 broilers per treatment were euthanized after measuring intestinal lesion scores to collect the fresh jejunum and ileum mucosa. The total RNA extraction kit (iNtRON Biotechnology, Seongnam, Korea) was used to extract the RNA from the intestinal mucosa. The mRNA was converted to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). For cDNA synthesis, the mixed solution was heat treated at 25°C for 10 min, at 37°C for 2 h, and at 85°C for 5 min. Gene amplification was performed using Fast qPCR 2× SYBR Green Master Mix (Applied Biosystems). RT-qPCR was performed in two steps. The first step was an enzyme activation step, which was performed at 95°C for 2 minutes for 1 cycle. The second step was a denaturation step at 95°C for 15 seconds and an annealing/extend step at 56°C for 1 minute, repeating a total of 40 cycles to perform gene amplification. The target genes were zonula occludens-1 (*ZO-1*), claudin-1 (*CLDN-1*), mucin-2 (*MUC2*), toll-like receptor 4 (*TLR-4*), and *β-actin*. Primers used in the amplification are shown in Table 2. Normalization was performed using the reference gene *β-actin*. Relative gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Cecal 16S metagenome

On day 21, the cecal samples were collected in a conical tube from each pen's broiler and stored at −20°C until analysis. Using the QIIME2 next-generation microbiome bioinformatics methodology, cecal 16S rRNA sequencing data were examined. Sanigen (Anyang, South Korea) received the samples for microbial sequencing utilizing the 16S rRNA method. For use in subsequent processing, all raw data was converted into QIIME2 artifacts, which include details about the sources and types of data. The QIIME2 plugin's Divisive Amplicon Denoising Algorithm 2 (DADA2), which finds and fixes amplicon defects and eliminates possible base errors and chimeric sequences, was used to extract the amplicon sequence variations (ASVs) from raw sequence data (Bolyen et al., 2019). The QIIME2 plugins' collapse and features were used to construct the Relative Classification Frequency Table, which shows differential abundance testing at taxonomic levels. Alpha-diversity measurements and plots were estimated using R bioinformatics programs and the "diversity" QIIME2 plugin. The ASVs table, a higher-resolution counterpart of the conventional operational taxonomic unit database, was used as required input data in the construction of this microbial diversity study pipeline. Chao1, Shannon, and Simpson were used to evaluate the sampling depth while analyzing the variations in species richness and evenness scores. The V3-V4 hypervariable region of the bacterial 16S rRNA gene is estimated by each index. By comparing the average bacterial proportion and composition examined in each taxonomic ordering, a difference in relative abundance was examined. Furthermore, by comparing the taxonomy matching rate of each ASV taxonomy with the National Center for Biotechnology Information (NCBI) bacterial reference genome database at the family and genus level, the accuracy of the bacterial classification based on the various amplicon regions was cross-checked.

Statistical analysis

JMP Pro 16 (SAS Institute Inc., Cary, NC, United States) and GraphPad Prism (Version 9.1.0; GraphPad Software, San Diego, CA) were used for statistical analyses and graph visualization, respectively. All data, except cecal 16S metagenome data, was analyzed via two-way analysis of variance (ANOVA) using the Standard Least Squares model, with each pen as the experimental unit. The effects of STB

Table 2
Primer sequences used for the RT-qPCR analysis.

Gene	Primers	Sequence (5′–3′)
β-actin	Forward	GGCGCTTGACTCAGGATTAA
	Reverse	CACAGAGGCGAGTAACTTCC
ZO-1	Forward	GACAGCCAACAAGGCAAGTG
	Reverse	CTCAATGCCTCCTGTCCCTG
CLDN-1	Forward	ACTTGAGCTGGGTCACATGG
	Reverse	TCCCTGGCACAGGGTTAATG
MUC2	Forward	GTGTTCCCCTGTTGAGGGAG
	Reverse	GGTGGTGACATACTGCCAGA
TLR4	Forward	TTCCATGGCTTAACGTCGCT
	Reverse	GAGGAAAAGCTCAGGTGCCT

supplementation (0, 0.05 %), the NE challenge (-C, +*C*), and the interaction between NE and STB were included in the statistical model. To examine the link between categorical factors and the various combinations examined in this study, the intestinal lesion scores were calculated using contingency analysis. A Chi-square test was performed to determine if the different combinations affected the categorical variables' repartition with significance accepted at $P < 0.05$. Raw counts derived from Shannon estimators were used to compute the alpha diversity. Each treatment group served as the control group for quantitative beta diversity and PROC MIXED with Dunnett's post-hoc test was used to compare the treatment groups. Statistical significance was defined as a probability level of $P < 0.05$.

Results

Nutrient digestibility

On day 14, the supplementation of 0.05 % STB significantly increased ($P < 0.05$) the AID of DM, CP, and GE compared to the non-supplementation group (Table 3). On day 28, the NE challenge group significantly decreased ($P < 0.05$) the AID of DM, CP, and GE compared to the non-challenge group. The supplementation of 0.05 % STB significantly increased ($P < 0.05$) the AID of DM compared to the non-supplementation group. There was an interaction ($P < 0.05$) between the NE challenge and the supplementation of STB in CP and GE digestibility on day 28. The supplementation of 0.05 % STB with the NE challenge group was higher ($P < 0.05$) AID of GE than the non-supplementation with the NE challenge group.

Oocyst shedding

The NE challenge group significantly increased ($P < 0.05$) OPG count compared to the non-challenge group on days 19 to 21 and 28 (Table 4). The supplementation of 0.05 % STB significantly decreased ($P < 0.05$) OPG count compared to the non-supplementation group. There was an interaction ($P < 0.05$) between the NE challenge and the supplementation of STB in OPG count on all experimental days. The supplementation of 0.05 % STB with the NE challenge group was significantly lower ($P < 0.05$) OPG count than the non-supplementation with the NE challenge group.

Blood profile

On day 14, the supplementation of 0.05 % STB significantly increased ($P < 0.05$) lymphocyte levels compared to the non-supplementation group (Table 5). For IFN-γ, the supplementation of 0.05 % STB was significantly lower ($P < 0.05$) than that of the non-supplementation group. On day 21, the NE challenge group significantly increased ($P < 0.05$) WBC, heterophil, and IFN-γ levels compared to the non-challenge group. In contrast, the lymphocyte and IL-10 levels were significantly lower ($P < 0.05$) in the NE challenge group than in the non-challenge group. The supplementation of 0.05 % STB significantly decreased ($P < 0.05$) WBC, heterophil, and IFN-γ levels compared to the non-supplementation group. The IL-10 levels were significantly higher ($P < 0.05$) in the supplementation of 0.05 % STB than in the non-supplementation group. On day 28, the NE challenge group significantly increased ($P < 0.05$) IFN-γ levels compared to the non-challenge group. The supplementation of 0.05 % STB significantly increased ($P < 0.05$) lymphocyte and IL-10 levels and decreased ($P < 0.05$) IFN-γ levels compared to the non-supplementation group. There was an interaction ($P < 0.05$) between the NE challenge and the supplementation of STB in lymphocyte and IFN-γ levels on day 28.

Intestinal lesion score

On day 21 and 28, the NE challenge group significantly increased ($P < 0.05$) the jejunum and ileum lesion score compared to the non-challenge group (Figs. 1 and 2). There were no interactions between the NE challenge and the supplementation of STB in intestinal lesion score.

Intestinal morphology

Images of the morphology of the jejunum and ileum according to the STB 0.05 % feeding are shown in Figs. 3 and 4. On day 21, the NE challenge group significantly decreased ($P < 0.05$) the VH:CD of the jejunum and ileum compared to the non-challenge group (Table 6). The supplementation of 0.05 % STB significantly increased ($P < 0.05$) the VH of the jejunum and VH:CD of the ileum compared to the non-supplementation group. There was an interaction ($P < 0.05$) between the NE challenge and the supplementation of STB in the VH of the jejunum on day 21. The supplementation of 0.05 % STB with the NE challenge group was significantly higher ($P < 0.05$) in VH of jejunum than the non-supplementation with the NE challenge group. On day 28, the NE challenge group had significantly higher ($P < 0.05$) CD and lower ($P < 0.05$) VH:CD of ileum than the non-challenge group. The supplementation of 0.05 % STB significantly decreased ($P < 0.05$) the CD and increased ($P < 0.05$) the VH:CD of the ileum compared to the non-supplementation group.

Tight junction proteins, mucin, and toll-like receptor expression levels

On day 21, the NE challenge group significantly downregulated ($P < 0.05$) the *ZO-1* and upregulated the *MUC2* of the jejunum and ileum mucosa compared to the non-challenge group (Table 7). The jejunal *TLR4* was significantly upregulated ($P < 0.05$) in the NE challenge group compared to the non-challenge group. The ileal *CLDN-1* was significantly downregulated ($P < 0.05$) in the NE challenge group compared to the non-challenge group. The supplementation of 0.05 % STB significantly downregulated ($P < 0.05$) the *TLR4* of jejunum and ileum

Table 3
Effects of supplementation with 0.05 % stimbiotic (STB) on the nutrient digestibility of broilers challenged with necrotic enteritis (NE).

Items, %	-C		+*C*		SE	C		STB		*P*-value		
	0	0.05	0	0.05		-	+	0	0.05	C	STB	*C*×STB
d 14												
DM	69.25	69.88	68.28	69.76	0.098	69.57	69.02	68.77	69.82		<0.001	
CP	74.47	72.64	70.95	75.26	0.299	73.55	73.08	72.71	73.93		<0.001	
GE	74.02	75.58	72.64	75.98	0.161	74.80	74.31	73.33	75.78		<0.001	
d 28												
DM	70.88	71.15	70.37	70.83	0.165	71.02	70.60	70.63	70.99	0.017	0.033	0.576
CP	79.44[a]	78.39[ab]	77.10[b]	78.19[ab]	0.537	78.91	77.64	78.27	78.29	0.006	0.963	0.020
GE	82.91[a]	80.39[b]	77.48[c]	80.38[b]	0.394	81.65	78.93	80.20	80.39	<0.001	0.556	<0.001

-C, non-challenge; +*C*, NE challenge; 0, basal diet with non-supplementation of STB; 0.05, basal diet with supplementation of 0.05 % STB; DM, dry matter; CP, crude protein; GE, gross energy; SE, standard error.
a-c Means with different letters are significantly differ ($P < 0.05$).

Table 4
Effects of supplementation with 0.05 % stimbiotic (STB) on the oocysts per gram in feces (OPG) of broilers challenged with necrotic enteritis (NE).

Items, × 10^2	-C		+C		SE	C		STB		*P*-value		
	0	0.05	0	0.05		-	+	0	0.05	C	STB	C×STB
OPG count												
d 19	0.00^{c}	0.00^{c}	385.33^{a}	212.00^{b}	9.605	0.00	298.67	192.67	106.00	<0.001	<0.001	<0.001
d 20	0.00^{c}	0.00^{c}	145.33^{a}	101.33^{b}	3.963	0.00	123.33	72.67	50.67	<0.001	<0.001	<0.001
d 21	0.00^{c}	0.00^{c}	82.67^{a}	24.00^{b}	3.797	0.00	53.33	41.33	12.00	<0.001	<0.001	<0.001
d 28	0.00^{c}	0.00^{c}	34.67^{a}	16.00^{b}	1.721	0.00	25.33	17.33	8.00	<0.001	<0.001	<0.001

-C, non-challenge; +*C*, NE challenge; 0, basal diet with non-supplementation of STB; 0.05, basal diet with supplementation of 0.05 % STB; SE, standard error.
a-c Means with different letters are significantly differ ($P < 0.05$).

Table 5
Effects of supplementation with 0.05 % stimbiotic (STB) on the blood profile of broilers challenged with necrotic enteritis (NE).

Items	-C		+C		SE	C		STB		*P*-value		
	0	0.05	0	0.05		-	+	0	0.05	C	STB	C×STB
d 14												
WBC, 10^3/uL	25.88	26.44	24.70	25.35	0.755	26.16	25.03	25.29	25.89		0.432	
Heterophil, %	33.77	32.84	34.80	32.50	1.437	33.30	33.65	34.28	32.67		0.268	
Lymphocyte, %	51.58	53.94	51.06	53.41	0.858	52.76	52.24	51.32	53.68		0.009	
IL-10, pg/mL	30.61	35.41	35.15	37.79	3.678	33.01	36.47	32.88	36.60		0.319	
IFN-γ, pg/mL	66.49	55.57	68.94	51.58	4.181	61.03	60.26	67.72	53.57		0.002	
d 21												
WBC, 10^3/uL	26.06	23.74	27.33	25.40	0.672	24.90	26.36	26.69	24.57	0.036	0.003	0.777
Heterophil, %	34.94	32.49	37.73	35.73	0.874	33.72	36.73	36.34	34.11	0.002	0.015	0.795
Lymphocyte, %	52.82	51.40	48.50	49.70	0.798	52.11	49.10	50.66	50.55	<0.001	0.893	0.110
IL-10, pg/mL	32.89	35.85	21.53	29.28	1.887	34.37	25.40	27.21	32.57	<0.001	0.007	0.213
IFN-γ, pg/mL	68.35	57.73	91.96	84.34	3.587	63.04	88.15	80.15	71.03	<0.001	0.015	0.678
d 28												
WBC, 10^3/uL	22.89	21.89	22.21	21.21	1.077	22.39	21.71	22.55	21.55	0.534	0.357	0.997
Heterophil, %	32.26^{ab}	33.18^{a}	33.00^{a}	29.72^{b}	1.011	32.72	31.36	32.63	31.45	0.187	0.251	0.045
Lymphocyte, %	54.98^{b}	59.26^{ab}	48.74^{c}	63.08^{a}	1.574	57.12	55.91	51.86	61.17	0.447	<0.001	0.003
IL-10, pg/mL	32.20	36.83	35.60	39.32	1.824	34.52	37.46	33.90	38.07	0.115	0.028	0.802
IFN-γ, pg/mL	64.03^{a}	50.63^{b}	65.22^{a}	64.74^{a}	2.893	57.33	64.98	64.62	57.69	0.012	0.022	0.032

-C, non-challenge; +*C*, NE challenge; 0, basal diet with non-supplementation of STB; 0.05, basal diet with supplementation of 0.05 % STB; WBC, white blood cell; IL-10, interleukin-10; IFN-γ, interferon γ; SE, standard error.
a-c Means with different letters are significantly differ ($P < 0.05$).

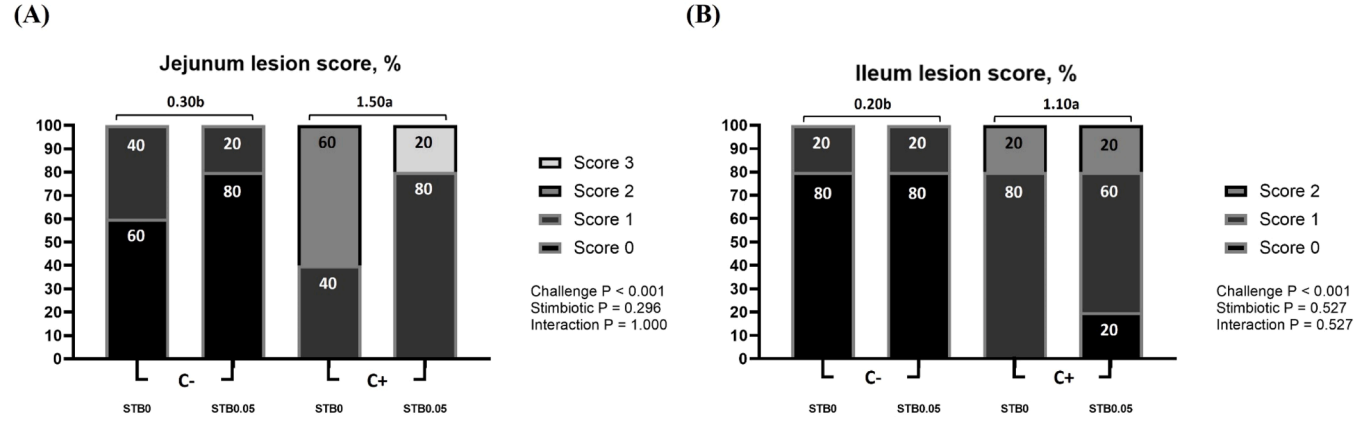

Fig. 1. Effects of supplementation with 0.05 % stimbiotic (STB) on the intestinal lesion score of broilers challenged with necrotic enteritis (NE) on day 21. -C, non-challenge; +*C*, NE challenge; 0, basal diet with non-supplementation of STB; 0.05, basal diet with supplementation of 0.05 % STB. $n = 10$ broilers/treatment. (A) $x^2 = 42.794$, $P < 0.001$. (B) $x^2 = 21.333$, $P < 0.05$. Numbers inside the bar indicate percentage of score out of total (100 %). a, b Means with different letters are significantly differ ($P < 0.05$).

compared to the non-supplementation group. For ileum, the supplementation of 0.05 % STB significantly upregulated ($P < 0.05$) the *ZO-1* and downregulated ($P < 0.05$) the *MUC2* compared to the non-supplementation group. On day 28, the NE challenge group significantly downregulated ($P < 0.05$) the *ZO-1* and upregulated ($P < 0.05$) the *MUC2* of jejunum compared to the non-challenge group. For ileum, the NE challenge group significantly downregulated ($P < 0.05$) the *ZO-1* and *CLDN-1* and upregulated ($P < 0.05$) the *MUC2* and *TLR4* compared to the non-challenge group. The supplementation of 0.05 % STB significantly downregulated ($P < 0.05$) the *MUC2* and *TLR4* of jejunum and ileum compared to the non-supplementation group. There were no interactions between the NE challenge and the supplementation of STB in gene expression on days 21 and 28.

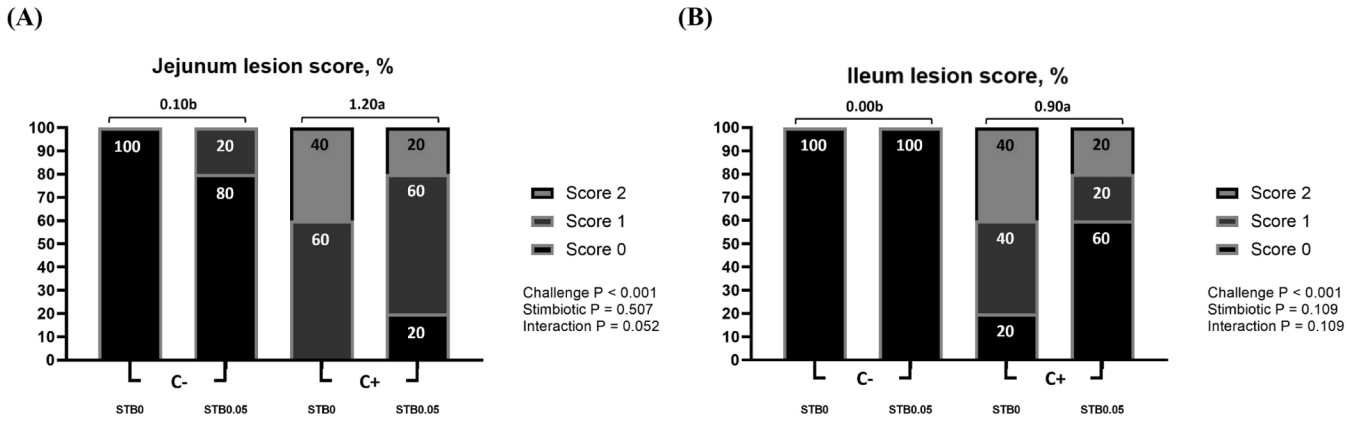

Fig. 2. Effects of supplementation with 0.05 % stimbiotic (STB) on the intestinal lesion score of broilers challenged with necrotic enteritis (NE) on day 28. -C, non-challenge; +*C*, NE challenge; 0, basal diet with non-supplementation of STB; 0.05, basal diet with supplementation of 0.05 % STB. $n = 10$ broilers/treatment. (A) $x^2 = 28.648$, $P < 0.001$. (B) $x^2 = 20.952$, $P < 0.05$. Numbers inside the bar indicate percentage of score out of total (100 %). a, b Means with different letters are significantly differ ($P < 0.05$).

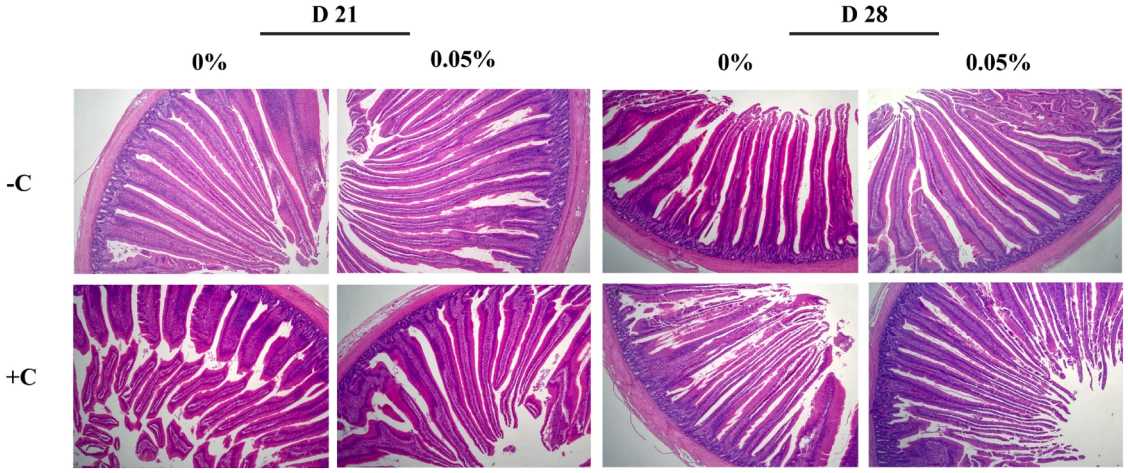

Fig. 3. Effects of supplementation with 0.05 % stimbiotic (STB) on the jejunum microscopic morphology (H&E staining) of broilers challenged with necrotic enteritis (NE) on day 21 and 28. -C, non-challenge; +*C*, NE challenge; 0, basal diet with non-supplementation of STB; 0.05, basal diet with supplementation of 0.05 % STB. The scale bar is 500 μm.

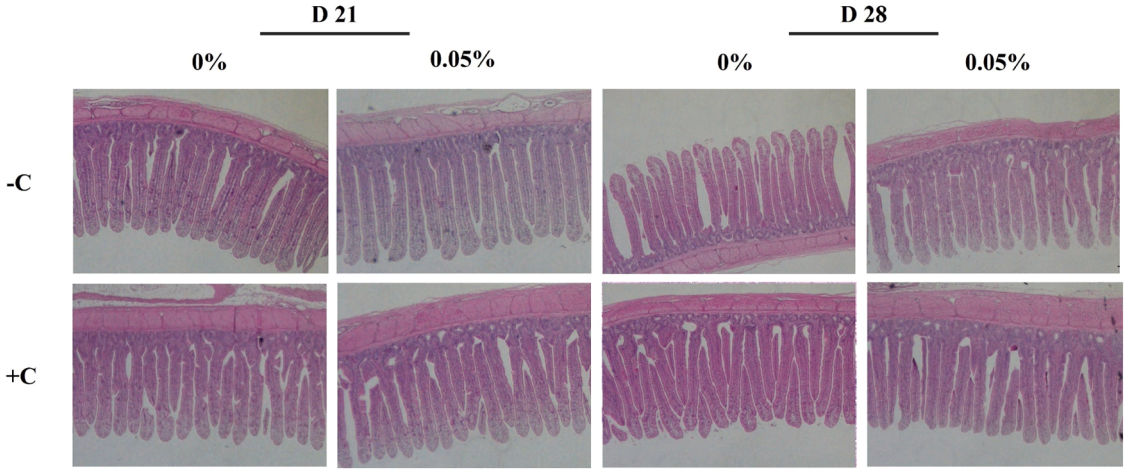

Fig. 4. Effects of supplementation with 0.05 % stimbiotic (STB) on the ileum microscopic morphology (H&E staining) of broilers challenged with necrotic enteritis (NE) on day 21 and 28. -C, non-challenge; +*C*, NE challenge; 0, basal diet with non-supplementation of STB; 0.05, basal diet with supplementation of 0.05 % STB. The scale bar is 500 μm.

Table 6
Effects of supplementation with 0.05 % stimbiotic (STB) on the intestinal morphology of broilers challenged with necrotic enteritis (NE).

Items	-C		+C		SE	C		STB		*P*-value		
	0	0.05	0	0.05		-	+	0	0.05	C	STB	C×STB
d 21												
Jejunum												
VH, μm	1186.20[a]	1187.12[a]	1012.95[b]	1193.07[a]	40.688	1186.66	1103.01	1099.58	1190.09	0.047	0.033	0.034
CD, μm	132.49	139.06	159.88	141.53	9.896	135.77	150.71	146.18	140.29	0.140	0.555	0.216
VH:CD	9.01	9.11	6.82	8.62	0.625	9.06	7.72	7.91	8.86	0.038	0.138	0.181
Ileum												
VH, μm	619.89	616.46	599.54	615.46	20.947	618.18	607.50	609.72	615.96	0.613	0.767	0.647
CD, μm	135.63	115.33	166.09	153.98	11.962	125.48	160.04	150.86	134.66	0.007	0.184	0.734
VH:CD	4.76	5.39	3.70	4.62	0.361	5.07	4.16	4.23	5.00	0.016	0.040	0.688
d 28												
Jejunum												
VH, μm	1278.20	1309.12	1194.95	1295.07	33.375	1293.66	1245.01	1236.58	1302.09	0.154	0.057	0.307
CD, μm	142.49	151.06	171.88	149.53	10.200	146.77	160.71	157.18	150.29	0.180	0.504	0.138
VH:CD	9.05	9.15	7.37	8.85	0.556	9.10	8.11	8.21	9.00	0.084	0.167	0.223
Ileum												
VH, μm	730.89	747.46	710.54	726.46	20.311	739.18	718.50	720.72	736.96	0.315	0.429	0.987
CD, μm	127.33	125.63	178.09	145.98	7.637	126.48	162.04	152.71	135.81	<0.001	0.033	0.054
VH:CD	5.78	5.99	4.08	5.27	0.285	5.88	4.67	4.93	5.63	<0.001	0.019	0.094

-C, non-challenge; +*C*, NE challenge; 0, basal diet with non-supplementation of STB; 0.05, basal diet with supplementation of 0.05 % STB; VH, villus height; CD, crypt depth; VH:CD, villus height:crypt depth ratio; SE, standard error.
a,b Means with different letters are significantly differ ($P < 0.05$).

Table 7
Effects of supplementation with 0.05 % stimbiotic (STB) on the gene expression of tight junction proteins, mucin, and toll-like receptor of broilers challenged with necrotic enteritis (NE).

Items	-C		+C		SE	C		STB		*P*-value		
	0	0.05	0	0.05		-	+	0	0.05	C	STB	C×STB
d 21												
Jejunum												
ZO-1	1.00	1.05	0.52	0.84	0.114	1.03	0.68	0.76	0.95	0.005	0.105	0.238
CLDN-1	1.00	1.04	0.64	0.83	0.157	1.02	0.73	0.82	0.93	0.077	0.476	0.639
MUC2	1.00	0.41	1.64	1.43	0.330	0.71	1.54	1.32	0.92	0.017	0.235	0.571
TLR4	1.00	0.41	1.15	0.73	0.106	0.71	0.94	1.07	0.57	0.035	<0.001	0.422
Ileum												
ZO-1	1.00	1.18	0.52	0.87	0.093	1.09	0.69	0.76	1.02	<0.001	0.008	0.366
CLDN-1	1.00	1.08	0.40	0.74	0.215	1.04	0.57	0.70	0.91	0.034	0.331	0.551
MUC2	1.00	0.54	1.47	1.23	0.133	0.77	1.35	1.23	0.88	<0.001	0.013	0.403
TLR4	1.00	0.66	1.41	0.76	0.200	0.83	1.08	1.20	0.71	0.213	0.019	0.449
d 28												
Jejunum												
ZO-1	1.00	1.37	0.77	1.12	0.096	1.19	0.94	0.88	1.25	0.017	<0.001	0.943
CLDN-1	1.00	1.44	0.93	1.00	0.156	1.22	0.97	0.97	1.22	0.113	0.110	0.243
MUC2	1.00	0.62	1.44	1.14	0.144	0.81	1.29	1.22	0.88	0.002	0.026	0.760
TLR4	1.00	0.39	1.20	0.73	0.142	0.69	0.97	1.10	0.56	0.062	<0.001	0.603
Ileum												
ZO-1	1.00	1.10	0.36	0.51	0.064	1.05	0.44	0.68	0.81	<0.001	0.062	0.741
CLDN-1	1.00	1.64	0.43	0.80	0.146	1.32	0.62	0.72	1.22	<0.001	0.001	0.350
MUC2	1.00	0.28	1.76	1.21	0.157	0.64	1.49	1.38	0.74	<0.001	<0.001	0.605
TLR4	1.00	0.40	1.25	0.68	0.105	0.70	0.96	1.12	0.54	0.016	<0.001	0.865

-C, non-challenge; +*C*, NE challenge; 0, basal diet with non-supplementation of STB; 0.05, basal diet with supplementation of 0.05 % STB; *ZO-1*, zonula occludens-1; *CLDN-1*, claudin-1; *MUC2*, mucin-2; *TLR4*, toll-like receptor 4; SE, standard error.

Diversity of the cecal microbiome

On day 21, the NE challenge group was significantly higher ($P < 0.05$) alpha diversity parameters (Simpson and Shannon) than the non-challenge group (Table 8). The supplementation of 0.05 % STB was significantly lower ($P < 0.05$) Shannon indices than the non-supplementation group. There were no interactions between the NE challenge and the supplementation of STB in alpha diversity.

Table 8
Effects of supplementation with 0.05 % stimbiotic (STB) on the cecal alpha diversity of broilers challenged with necrotic enteritis (NE).

Items	-C		+C		SE	C		STB		*P*-value		
	0	0.05	0	0.05		-	+	0	0.05	C	STB	C×STB
Simpson	0.96	0.95	0.97	0.96	0.006	0.95	0.97	0.96	0.96	0.036	0.314	0.786
Shannon	5.75	5.54	5.98	5.77	0.100	5.65	5.88	5.87	5.65	0.026	0.039	0.976
Chao1	350.98	334.18	389.48	311.04	15.972	342.58	350.26	370.23	322.61	0.753	0.064	0.217

-C, non-challenge; +*C*, NE challenge; 0, basal diet with non-supplementation of STB; 0.05, basal diet with supplementation of 0.05 % STB; SE, standard error.

The supplementation of 0.05 % STB showed a difference ($P < 0.05$) from a non-supplementation group in unweighted unifrac distance (Figs. 3 and 4). There were no significant differences in weighted unifrac distance among the treatment groups (Figs. 5 and 6).

Relative abundance

At the family level, the NE challenge group significantly decreased ($P < 0.05$) the abundance of *Eubacterium coprostanoligenes* group, *Erysipelatoclostridiaceae*, and *Anaerovoracaceae* compared to the non-challenge group (Table 9). The supplementation of 0.05 % STB significantly decreased ($P < 0.05$) the abundance of *Muribaculaceae* and increased ($P < 0.05$) the abundance of *Monoglobaceae* compared to the non-supplementation group. There was an interaction ($P < 0.05$) between the NE challenge and the supplementation of STB in the abundance of *Oscillospiraceae* and *Muribaculaceae*. The supplementation of 0.05 % STB with the NE challenge group significantly increased ($P < 0.05$) the abundance of *Oscillospiraceae* and decreased ($P < 0.05$) the abundance of *Muribaculaceae* compared to the non-supplementation with the NE challenge group.

At the genus level, the NE challenge group significantly decreased ($P < 0.05$) the abundance of *Eubacterium coprostanoligenes* group and *Erysipelatoclostridium* and increased ($P < 0.05$) the abundance of *Harryflintia, Monoglobus, Gordonibacter*, and *Eggerthella* compared to the non-challenge group (Table 10). The supplementation of 0.05 % STB significantly decreased ($P < 0.05$) the abundance of *Muribaculaceae* and increased ($P < 0.05$) the abundance of *Paludicola, Intestinimonas*, and *Caproiciproducens* compared to the non-supplementation group. There was an interaction ($P < 0.05$) between the NE challenge and the supplementation of STB in the abundance of *Muribaculaceae, Caproiciproducens,* and *Colidextribacter*. The supplementation of 0.05 % STB with the NE challenge group significantly decreased ($P < 0.05$) the abundance of *Muribaculaceae* compared to the non-supplementation with the NE challenge group.

Discussion

The NE is a common multifactorial disease in 2 to 5 weeks old broilers. Its acute form causes a high mortality and its subclinical form leads to reduced growth performance (Wu et al., 2010; Guo et al., 2023). In this study, no mortality occurred due to NE challenge, although decreases of feed intake and body weight were observed (data not shown), suggesting that a subclinical form of infection occurred. Subclinical forms of NE can damage the intestinal epithelium and impair nutrient absorption (Kulkarni et al., 2022). In this study, NE challenge decreased expression of tight junction proteins such as *ZO-1* and *CLDN-1*, increased expression of *TLR4* in the jejunum and intestinal lesion score, and negatively affected the intestinal barrier function. The VH:CD was also decreased in NE challenge group, leading to a decrease in nutrient digestibility. In addition, IFN-γ and heterophil in the blood were increased while lymphocytes and IL-10 were decreased in NE challenge group, indicating a decrease in immunity. These results were consistent with previous study results (Cao et al., 2012; Wang et al., 2017), suggesting that NE was successfully induced.

This study was conducted to investigate effects of STB supplementation on nutrient digestibility and intestinal health in broilers with NE. In this study, the addition of STB improved nutrient digestibility. In broilers challenged with NE, the digestibility of GE increased compared to that in the group without STB supplementation. This might be due to improvement of intestinal morphology through STB supplementation consisting of enzymes and prebiotics. In this study, STB supplementation also increased VH in the jejunum and VH:CD in the ileum. An increase in VH indicates an increase in surface area for nutrient absorption (Silva et al., 2009). Additionally, XYL breaks down complex carbohydrates such as xylan into low-molecular-weight oligosaccharides, making them easier to digest and absorb in the small intestine (Kiarie et al., 2013). The XOS in STB is a prebiotic that can improve tissue morphology by creating an environment favorable for the growth of intestinal microflora (Xu et al., 2003). The STB used in this study was designed to deliver XOS to the fibrinolytic microbiota of the colon by adding small amounts

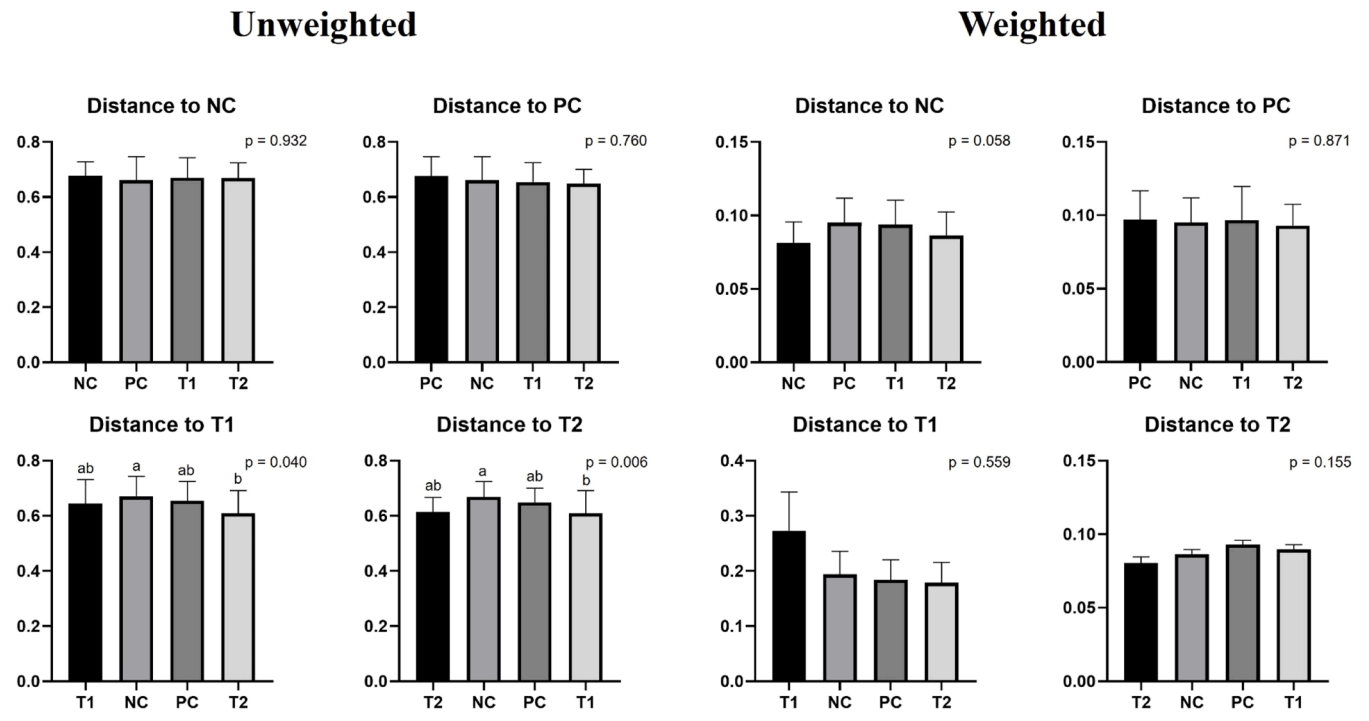

Fig. 5. Unweighted and weighted Unifrac measurement in positive control (PC), basal diet; negative control (NC), basal diet with NE challenge; T1, PC + 0.05 % STB; T2, NC + 0.05 % STB. Each treatment group was placed as the control group, and treatment groups were compared by using one-way PROC MIXED with Dunnett's post-hoc test.

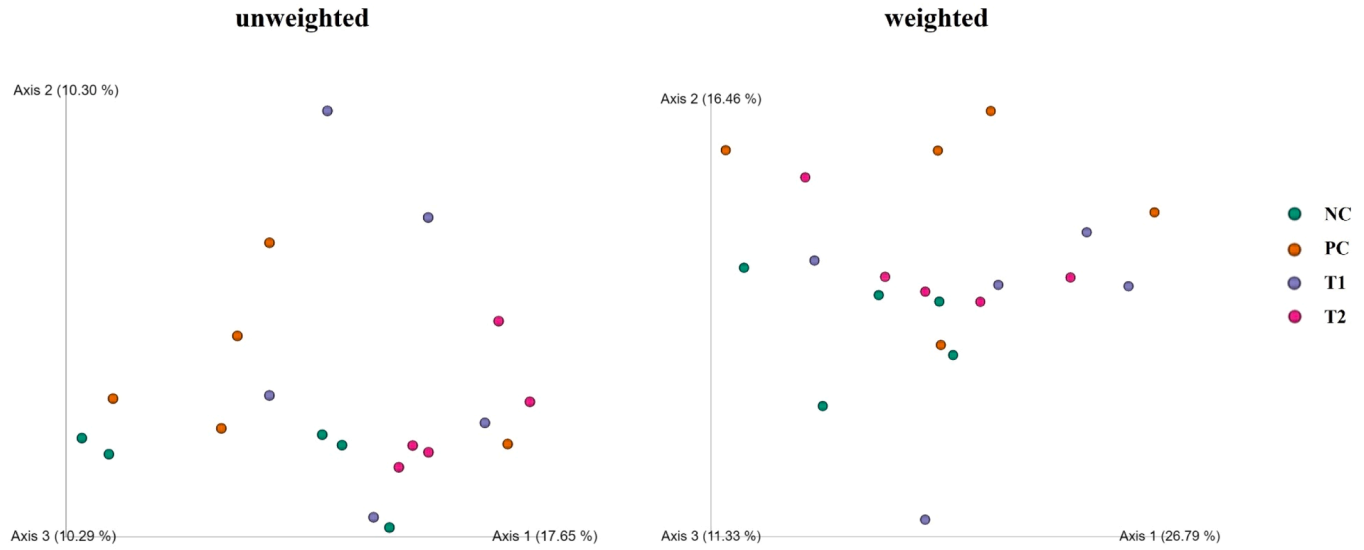

Fig. 6. Visualized beta-diversity indices including unweighted and weighted emperor in positive control (PC), basal diet; negative control (NC), basal diet with NE challenge; T1, PC + 0.05 % STB; T2, NC + 0.05 % STB.

Table 9
Effects of supplementation with 0.05 % stimbiotic (STB) on the relative abundance of cecal microbiota at the family level of broilers challenged with necrotic enteritis (NE).

Items, %	-C		+C		SE	C		STB		*P*-value		
	0	0.05	0	0.05		-	+	0	0.05	C	STB	C×STB
Lactobacillaceae	2.88	3.45	4.07	4.34	1.415	3.16	4.20	3.47	3.89	0.475	0.770	0.916
Lachnospiraceae	36.63	39.11	36.40	33.35	3.260	37.87	34.88	36.52	36.23	0.371	0.932	0.409
Oscillospiraceae	20.42^{ab}	16.70^{b}	16.56^{b}	23.41^{a}	2.028	18.56	19.99	18.49	20.06	0.493	0.451	0.019
Ruminococcaceae	14.35	22.90	20.15	20.36	2.172	18.63	20.26	17.25	21.63	0.464	0.061	0.073
Muribaculaceae	0.05^{ab}	0.06^{a}	0.07^{a}	0.03^{b}	0.006	0.06	0.05	0.06	0.04	0.318	0.013	0.001
Eubacterium coprostanoligenes group	2.07	2.65	1.03	1.27	0.530	2.36	1.15	1.55	1.96	0.037	0.448	0.757
Bacteroidaceae	0.03	0.03	0.04	0.02	0.008	0.03	0.03	0.04	0.02	0.836	0.153	0.091
Erysipelatoclostridiaceae	5.97	3.93	2.55	3.17	0.920	4.95	2.86	4.26	3.55	0.037	0.451	0.167
Butyricicoccaceae	0.31	0.31	0.19	0.44	0.096	0.31	0.31	0.25	0.38	0.954	0.198	0.224
Enterobacteriaceae	1.10	0.35	1.66	0.81	0.523	0.73	1.23	1.38	0.58	0.346	0.145	0.919
Streptococcaceae	0.37	0.33	0.39	0.21	0.110	0.35	0.30	0.38	0.27	0.631	0.327	0.507
Christensenellaceae	0.35	0.52	0.27	0.26	0.193	0.44	0.27	0.31	0.39	0.386	0.674	0.649
Monoglobaceae	0.23	0.28	0.16	0.51	0.087	0.26	0.34	0.20	0.39	0.375	0.037	0.103
Bifidobacteriaceae	0.18	0.16	0.21	0.10	0.056	0.17	0.15	0.20	0.13	0.734	0.250	0.421
Anaerovoracaceae	0.10	0.15	0.06	0.09	0.021	0.13	0.07	0.08	0.12	0.024	0.066	0.498
Rest	14.96	9.04	16.19	11.64	2.610	12.00	13.91	15.57	10.34	0.474	0.062	0.797

-C, non-challenge; +*C*, NE challenge; 0, basal diet with non-supplementation of STB; 0.05, basal diet with supplementation of 0.05 % STB; SE, standard error.
a, b Means with different letters are significantly differ ($P < 0.05$).

of short-chain XOS produced in vitro together with β−1,4-endo xylanase, which hydrolyzes the arabinoxylan moiety of the feed to XOS (Lee et al., 2022). The STB may maintain intestinal mucosal health by altering microbial fermentation processes to increase SCFA production and promote nutrient transporter expression to improve overall nutrient utilization (You et al., 2022). Also, STB can have a positive effect on the nutrient transport mechanism by increasing the efficiency of nutrient decomposition and absorption through the interaction of enzymes and microorganisms and improving the intestinal morphology (Davies et al., 2024; Veluri et al., 2024). Excessive proliferation of *C. perfringens*, the main cause of NE, can stimulate cell proliferation and increase CD (Alizadeh et al., 2022). When NE is infected, supplementation of STB can reduce the viscosity of the digesta associated with proliferation of pathogenic bacteria in the intestine and promote the growth of beneficial bacteria (Choct et al., 2010; Nguyen et al., 2022). This suggests that the reduction in pathogenic bacteria can decrease the need for cell proliferation, leading to a reduction in CD and an improvement in the VH:CD ratio.

In this study, OPG counts increased with NE challenge, while STB supplementation reduced these counts. The increase of OPG counts during NE infection suggests that NE can deteriorate the intestinal environment, creating favorable conditions for coccidial proliferation (Kaldhusdal et al., 2021). The reduction in OPG counts with STB supplementation appears to be due to STB's antimicrobial and anti-inflammatory effects, which can inhibit coccidial proliferation (Chang et al., 2024; Li et al., 2024). The STB can stimulate the growth of short-chain fatty acid (SCFA) producers, increasing the content of SCFAs such as butyric acid in the intestinal microbiota (Ren et al., 2023). Butyric acid, the primary energy source for intestinal epithelial cells, has been reported to be able to enhance intestinal barrier function and modulate immune responses (Kaczmarek et al., 2016). Through these effects of STB, the increase of OPG counts caused by NE infection can be reduced.

In this study, STB supplementation increased lymphocyte and IL-10 levels while decreasing heterophil and IFN-γ levels. The heterophil-to-lymphocyte ratio can be used as an indicator of acute or chronic stress (Weimer et al., 2018). An increase in heterophils is considered an indicator of infection severity, while a decrease in lymphocytes indicates

Table 10
Effects of supplementation with 0.05 % stimbiotic (STB) on the relative abundance of cecal microbiota at the genus level of broilers challenged with necrotic enteritis (NE).

Items	-C		+C		SE	C		STB		*P*-value		
	0	0.05	0	0.05		-	+	0	0.05	C	STB	C×STB
Lactobacillus	2.88	3.45	4.07	4.34	1.415	3.16	4.20	3.47	3.89	0.475	0.770	0.916
Lachnoclostridium	9.98	13.15	7.46	11.72	2.846	11.57	9.59	8.72	12.43	0.497	0.210	0.851
Eubacterium coprostanoligenes group	2.07	2.65	1.03	1.27	0.530	2.36	1.15	1.55	1.96	0.037	0.448	0.757
Anaerostignum	0.32	0.08	0.53	0.36	0.168	0.20	0.44	0.43	0.22	0.171	0.245	0.828
Ruminococcus torques group	8.25	10.28	13.09	6.82	2.597	9.27	9.96	10.67	8.55	0.794	0.426	0.129
Muribaculaceae	0.05^{ab}	0.06^{a}	0.07^{a}	0.03^{b}	0.006	0.05	0.05	0.06	0.04	0.500	0.026	0.002
Bacteroides	0.03	0.03	0.04	0.02	0.008	0.03	0.03	0.04	0.02	0.836	0.153	0.091
Pseudoflavonifractor	2.00	0.83	0.59	1.12	0.591	1.42	0.85	1.30	0.97	0.355	0.590	0.170
Erysipelatoclostridium	5.97	3.92	2.55	3.14	0.917	4.94	2.85	4.26	3.53	0.036	0.440	0.168
Paludicola	2.64	4.34	4.23	5.11	0.586	3.49	4.67	3.44	4.73	0.060	0.043	0.493
Intestinimonas	1.90	3.25	2.51	3.63	0.475	2.58	3.07	2.20	3.44	0.317	0.019	0.811
Butyricicoccus	0.24	0.28	0.16	0.33	0.090	0.26	0.25	0.20	0.31	0.895	0.274	0.462
Anaerotruncus	0.82	1.12	2.73	1.00	0.876	0.97	1.86	1.77	1.06	0.323	0.428	0.266
Harryflintia	0.24	0.33	0.76	0.91	0.208	0.29	0.83	0.50	0.62	0.019	0.577	0.897
Escherichia-Shigella	1.09	0.35	1.66	0.80	0.523	0.72	1.23	1.38	0.58	0.345	0.145	0.914
Caproiciproducens	6.69^{b}	15.45^{a}	8.52^{b}	8.29^{b}	1.659	11.07	8.40	7.60	11.87	0.128	0.020	0.016
Oscillibacter	2.67	2.57	2.84	4.66	0.564	2.62	3.75	2.76	3.61	0.063	0.148	0.109
Flavonifractor	2.87	3.87	3.20	4.12	0.761	3.37	3.66	3.03	4.00	0.708	0.225	0.959
Colidextribacter	9.81^{a}	4.58^{b}	4.51^{b}	8.80^{ab}	1.691	7.20	6.66	7.16	6.69	0.755	0.784	0.013
Lactococcus	0.36	0.33	0.39	0.20	0.110	0.34	0.30	0.38	0.26	0.659	0.330	0.507
Tyzzerella	0.15	0.14	0.24	0.11	0.042	0.14	0.17	0.19	0.13	0.478	0.145	0.166
Ruminococcus	2.20	0.80	1.76	3.46	1.134	1.50	2.61	1.98	2.13	0.341	0.896	0.192
Monoglobus	0.23	0.28	0.16	0.51	0.087	0.26	0.34	0.20	0.39	0.375	0.037	0.103
Candidatus_Soleaferrea	0.02	0.03	0.03	0.02	0.009	0.02	0.03	0.02	0.03	0.687	0.668	0.189
Bifidobacterium	0.18	0.16	0.21	0.10	0.056	0.17	0.15	0.20	0.13	0.734	0.250	0.421
Vallitalea	0.92	1.58	1.77	1.88	0.908	1.25	1.82	1.34	1.73	0.535	0.674	0.767
Pseudomonas	0.02	0.02	0.01	0.01	0.006	0.02	0.01	0.01	0.01	0.200	0.799	0.669
Gordonibacter	0.02	0.02	0.01	0.04	0.006	0.02	0.02	0.01	0.03	0.812	0.016	0.078
Eggerthella	0.04	0.09	0.02	0.04	0.010	0.06	0.03	0.03	0.06	0.008	0.008	0.264
Anaerostipes	0.23	0.09	0.12	0.05	0.088	0.16	0.08	0.17	0.07	0.401	0.238	0.682
Rest	35.11	25.87	34.73	27.11	3.040	30.49	30.92	34.92	26.49	0.889	0.014	0.793

-C, non-challenge; +*C*, NE challenge; 0, basal diet with non-supplementation of STB; 0.05, basal diet with supplementation of 0.05 % STB; SE, standard error.
a, b Means with different letters are significantly differ ($P < 0.05$).

immunosuppression in poultry (Erinle et al., 2023). IL-10 is an immunoregulatory cytokine that plays a crucial inhibitory role in various inflammatory responses. It can suppress IFN-γ expression to control host immune responses, thereby limiting cellular damage during inflammation (Rajput et al., 2013). The increase of IL-10 observed with STB supplementation suggests stimulation of anti-inflammatory factors, potentially stabilizing the intestinal environment and effectively regulating inflammatory responses. Furthermore, this study showed that STB supplementation resulted in higher lymphocyte counts compared to the non-supplemented group when infected with NE. During NE infection, lymphocytes in the blood can migrate to intestinal tissues due to inflammatory responses, leading to a decrease in blood lymphocyte level (Emami et al., 2019). The observed increase in lymphocytes with STB supplementation might be attributed to reduced inflammation and stabilization of the intestinal environment, resulting in decreased lymphocyte migration to the intestines.

The *TLR4* is distributed in almost all cell lines. It is expressed in cells involved in host defense functions (Akira and Takeda, 2004). When *TLR4* receptor binds to its ligand, the signal is transmitted to the *TLR4* domain. Nuclear factor kappa B (NF-kB) and mitogen-activated protein kinase (MAPK) signaling pathways are then further activated to promote the expression and activation of various inflammatory cytokine genes (Hwang, 2001). The *TLR4* is overexpressed during NE infection. It differentially regulates the expression of intestinal immune-related genes and growth factor genes to activate intestinal immune inflammatory responses (Khalique et al., 2019; Pham et al., 2020). In this study, the expression of *TLR4* in the jejunum and ileum was suppressed when STB was added. The XOS produced by XYL can directly affect the immune system by binding to toll-like receptors (Niewold et al., 2012). Down-regulation of *TLR4* can also inhibit the NF-kB signaling pathway, which might have reduced the expression of inflammatory cytokines such as IFN-γ in this study. The intestinal barrier is regulated by tight junction proteins, which can maintain the integrity and function of the intestinal barrier and protect the intestine from pathogen invasion (Pham et al., 2020). *ZO-1* is a peripheral membrane protein and a support protein that acts as a cytoplasmic adapter to connect the actin cytoskeleton and membrane proteins within cells (Turner, 2009). The *CLDN-1* plays an important role in barrier formation and intercellular selectivity of various tissues (Suzuki, 2013). The *MUC2* secreted by goblet cells can cover the intestinal epithelial surface, protect the intestinal epithelium from infection, and maintain integrity of the intestinal mucosal barrier, immune hemostasis, and intestinal health (Kim and Ho, 2010). In this study, when STB was added, *ZO-1* in the jejunum was up-regulated, *CLDN-1* expression was increased, and *MUC2* expression was decreased in the ileum. In previous studies, adding XOS to the diet of laying hens up-regulated the expression of *CLDN-1* in the ileal mucosa and increased the expression of *CLDN-1* and *ZO-1* in broilers (Luo et al., 2021; Zhou et al., 2021). This might be because XOS can act as a prebiotic, directly binding to pathogens, reducing biological activity of pathogenic bacteria, and promoting production of antimicrobial compounds to reduce adhesion of pathogens in the mucus layer (Brink et al., 2006). The decrease in the expression of *MUC2* was thought to be due to reduced amount of mucus needed to fight against pathogens through XOS, which could lead to improved intestinal health (Kufe, 2009).

The present study suggests that NE infection can increase Simpson and Shannon indices, indicating that changes in the intestinal environment due to NE infection could promote the growth of diverse microorganisms. The STB supplementation resulted in a decrease in the Shannon index and significant differences in β-diversity. This implies that STB could altere compositions of the gut microbiota and selectively promote or inhibit the growth of specific microorganisms, thereby

reducing overall diversity. However, previous studies have reported that XOS supplementation could either increase α-diversity including Shannon or show no significant differences (Sutton et al., 2021; Rao et al., 2024). These discrepancies might be due to factors such as the presence or absence of NE challenge and the age of broilers at cecal sampling. In this study, NE infection decreased the relative abundance of generally beneficial bacteria (such as the *Eubacterium coprostanoligenes* group, *Erysipelatoclostridiaceae*, and *Anaerovoracaceae*) but increased potential pathogens such as *Harryflintia, Monoglobus, Gordonibacter*, and *Eggerthella*. However, STB supplementation induced increases of *Paludicola, Intestinimonas*, and *Caproiciproducens. Paludicola*, a relatively recently discovered microorganism, has been reported to be involved in maintaining intestinal homeostasis (Kotlyarov, 2024). *Intestinimonas*, a fiber-degrading bacterium associated with SCFA production, has been reported to be increased in broilers fed STB (Lee et al., 2017). These results suggest that STB can stimulate gut microbiota to improve intestinal function, potentially leading to higher dietary fiber utilization in the cecum (Ren et al., 2023). Fermentation and utilization of fiber by fiber-degrading bacteria can produce SCFAs, which can lead to reduced intestinal inflammation and improve intestinal barrier function (Makki et al., 2018). Although this study did not show interactions between STB and NE challenge for fiber-degrading bacteria, it suggested that STB supplementation could alter the gut microbiota and significantly impact broiler gut health.

Conclusion

The NE challenge decreased nutrient digestibility, IFN-γ, heterophil levels in blood, and VH:CD compared to the non-challenge group. Also, the NE challenge lower *ZO-1* and higher *MUC2* gene expression than the non-challenge group. Supplementation of 0.05 % STB with NE challenge increased GE digestibility and decreased the OPG counts compared to the NE-challenged group. Supplementation of 0.05 % STB increased the VH:CD in ileum compared to the non-supplementation group. Also, supplementation of 0.05 % STB lower *MUC2* and *TLR4* gene expression in ileum than the non-supplementation group. The STB increased beneficial bacteria (*Paludicola, Intestinimonas*, and *Caproiciproducens*) in maintaining intestinal homeostasis and decomposing fiber. In conclusion, supplementation of 0.05 % STB in a diet could positively regulate the cecal microflora and gene expression of tight junction protein and alleviate the decline in nutrient digestibility caused by NE.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jinho Cho reports financial support was provided by National Research Foundation of Korea. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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