Study on the interactive effect of deoxynivalenol and *Clostridium perfringens* on the jejunal health of broiler chickens

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ABSTRACT With global warming and ban on antibiotics, it occurs occasionally that deoxynivalenol (**DON**) together with *Clostridium perfringens* impairs the gut health of broiler chickens. However, the interactive effect of DON and *C. perfringens* on intestinal health is still unknown. A total of 120 one-day-old Arbor Acres broilers were randomly distributed to 4 groups. Birds were gavaged with *C. perfringens* (8×10^8 CFU/d per bird) or sterile medium and fed a DON diet (0 or 5 mg of DON per kg diet) to investigate the interactive effects. The main effect analysis showed that DON diet significantly downregulated ($P < 0.05$) the mRNA expression of mucin-2, B-cell lymphoma-2–associated X, and cysteinyl aspartate–specific proteinase-3 of jejunal mucosa; decreased ($P < 0.05$) the indexes of ACE, Chao1, Shannon, and Simpson; and also decreased the relative abundance of the phylum *Bacteroidete* and the genera *Lactococcus* in jejunal contents of broilers chickens. Meanwhile, *C. perfringens* significantly increased ($P < 0.05$) crypt depth; decreased ($P < 0.05$) the ratio of villi height to crypt depth, the activity of jejunal diamine oxidase, and the relative abundance of *Lactococcus*; and upregulated ($P < 0.05$) the relative expression of B-cell lymphoma-2 and cysteinyl aspartate–specific proteinase-8. Furthermore, the interactions between DON and *C. perfringens* were most significant ($P < 0.05$) in the mRNA expression of lipopolysaccharide-induced TNF factor (***LITAF***) and *TLR-4*, the abundance of the genera *Lactococcus* in jejunal contents, and butyric acid concentrations in cecal contents of birds. Finally, Spearman correlation analysis suggested that the most negative correlations ($P < 0.05$) with the abundance of the genera except *Lactobacillus* were observed within the mRNA expression of *LITAF*. The abundance of *Lactococcus* had a positive correlation ($P < 0.05$) with the expression of Caspase-3. Most genera except *Lactobacillus* negatively correlated ($P < 0.05$) with acetic acid, butyric acid, and total short-chain fatty acids. In conclusion, dietary deoxynivalenol and *C. perfringens* challenge had a harmful effect on the jejunal health and should be carefully monitored in broiler production.

Key words: broiler chicken, *Clostridium perfringens*, deoxynivalenol, jejunal health, microbiota

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INTRODUCTION

Deoxynivalenol (**DON**), a mycotoxin belongs to group B of trichothecene, is the secondary metabolites of *Fusarium*, especially *Fusarium graminearum* and *Fusarium culmorum* (Khaneghah et al., 2018). The detection rate of DON could be up to 70%, which is often found in wheat, barley, oat, corn, and other crops (Streit et al., 2013). It could exist from agriculture crops to human food and animal feed, hence it brings great risk to the human food safety and the animal feeding industry because of its resistance to grind, process, and heat. In addition, DON has been shown to involve in acute and chronic illnesses in both humans and farm animals (Girardet et al., 2011). DON could bind to ribosome, causing dysfunction of protein and nucleic acid biosynthesis so as to influence cell proliferation, differentiation, and apoptosis (Ueno, 1983). Therefore, the high-protein turnover tissues such as the intestine and liver are major targets of the DON. DON is reported to damage the barrier function of the intestine, leading to mucosal necrosis, diarrhea, vomiting, reduced feed conversion, and immune dysfunction of human and poultry (Chen et al., 2017; Mishra et al., 2020). As a biotic barrier that separate pathogenic agent from visceral organs, "leaky gut" indicates a serious health challenge to the animal. What's more, DON could affect the absorption of amino acid, carbohydrate, and other nutrients, and the fermentation of mass nutriment in the large intestine could

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disrupt the balance of intestinal microflora, which could be a potential mechanism of its harmful effects (Awad and Zentek, 2015). The toxicity of the DON is dependent on exposal dose and duration (Payros et al., 2016). Low-dose DON promotes the secretion of cytokines, induces inflammatory responses, and increases the risk of chronic immune disease infection, whereas acute feeding leads to immune cell apoptosis, immunosuppression, and impaired intestinal function of the animal.

Clostridium perfringens, a resident gram-positive bacteria of poultry intestinal contents, is the major etiologic agent for necrotic enteritis (**NE**). *C. perfringens* is classed into type-A to type-E by the 4 major secreted toxins (α, β, ε, and ι). The intestine of healthy birds typically contains approximately 1×10^4 CFU *C perfringens*/g of digesta, but it would overgrow reaching a critical concentration of about 1×10^7 to 1×10^9 CFU/g of digesta with various unfavorable factors such as intestinal injury, excessive animal protein content in the diet, impaired immune function, a plethora of coccidiosis, and intestinal microflora disorder (Uzal et al., 2014). Some exotoxin and harmful active substances such as hyaluronidase, acid phosphatase, protease, collagenase, sulfatase, and so on would be secreted excessively to injure the intestinal mucosa of poultry (Wu et al., 2018b). Then the bacteria would pass the mucosa layer and attach to intestinal epithelial cells, active pathogen-associated molecular patterns, and result in an inflammation response, inducing cell death. It is reported that the pathogenicity of *C. perfringens* is associated with the secreted exotoxin such as α-toxin, NE B–like toxin, and so on. α-Toxin, a toxin secreted by all type (type A, B, C, D, and E) of *C. perfringens*, is a zinc-containing metalloenzyme, which has the function same as phospholipase and sphingomyelinase, could induce cell death and destroy the epithelial cell membrane (Immerseel et al., 2009). NE B–like toxin, a novel exotoxin discovered, is produced by type-A *C. perfringens*, which could induce avian intestinal cells hemolytic and pore-forming (Zhou et al., 2017). But the pathogenesis of NE is still contradictory, and the mechanisms need to be further explored.

Owing to the change of climate with higher temperature and humidity and global ban on the use of antibiotics, mycotoxin and pathogens take great challenges to the poultry industry (Antonissen et al., 2015). And the cooccurrence of mycotoxin and pathogens happened occasionally (Antonissen et al., 2014). Studies have revealed that mycotoxin could exacerbate the pathogens' toxicity on the intestine through injuring the intestine, but few studies have concentrated on the interaction between mycotoxin and pathogens. Thus, we choose broiler chicken as an animal model to investigate the interactive effects of dietary DON and *C. perfringens* challenge on gut health, trying to provide another insight into the interaction of mycotoxin and pathogens.

MATERIALS AND METHODS

Ethics Statement

The protocol for the animal experimental procedures was approved by the Institutional Animal Care and Use Committee of Northwest A&F University (Permit Number: NWAFAC 1008).

Birds and Treatment

In a 2 × 2 factorial arrangement, a total of 120 hatched 1-day-old healthy (Arbor Acres) broilers with similar body weight were randomly assigned into 4 treatment groups: 1) Birds of group CON were fed a basal diet; 2) Birds of group DON were fed a basal diet contaminated with DON at 5 mg per kg diet; 3) Birds of group CP were challenged with *C. perfringens* CVCC2030 culture medium at 8×10^8 CFU/mL by instilling using 1-mL injector, and the dose was

Table 1. Ingredients and composition of the control experimental diets.

Ingredients	Content (%)	Nutrient levels[2]	Content (%)
Corn	60.86	Crude protein	21.00
Soybean meal	23.52	Calcium	0.90
Corn gluten meal	4.00	Total phosphorus	0.70
Cottonseed meal	3.00	Available phosphorus	0.46
Distiller's dried grain	3.00	ME/(MJ/kg)	12.13
$CaHPO_4$	1.83	Lysine	1.40
Soybean oil	1.10	Methionine	0.55
Limestone	1.00	Available lysine	1.20
L-Lys•H_2SO_4	0.80	Available methionine	0.50
Salt	0.30		
DL-Met	0.23		
L-Threonine	0.12		
Choline chloride	0.10		
Premix[1]	0.40		
Total	100.00		

[1]The premix provided the following per kg of diets: VA 8,000 IU, VB1 4 mg, VB2 3.6 mg, VB5 40 mg, VB6 4 mg, VB12 0.02 mg, VD3 3,000 IU, VE 20 IU, VK3 2 mg, biotin 0.15 mg, folic acid 1.0 mg, D-pantothenic acid 11 mg, nicotinic acid 10 mg, antioxidant 100 mg, Cu (as copper sulfate) 10 mg, Fe (as ferrous sulfate) 80 mg, Mn (as manganese sulfate) 80 mg, Zn (as zinc sulfate) 75 mg, I (as potassium iodide) 0.40 mg, Se (as sodium selenite) 0.30 mg.

[2]All the nutrient levels were calculated values.

Table 2. Primers used in real-time quantitative PCR.

Gene	Gene bank ID	Primer sequence (5′-3′)
GAPDH	L08165	F: TGGGCATCAAGGGCTACA
		R: TCGGGTTGGTTGGTGATG
Bax	XM_025145467.1	F: ATCGTCGCCTTCTTCGAGTT
		R: ATCCCATCCTCCGTTGTCCT
Bcl-2	NM_205339.2	F: TCGCGCCGCTACCAGAGGGACTTC
		R: CCGGTTGACGCTCTCGACGCACAT
Caspase-3	NM_204725.1	F: GGCTCCTGGTTTATTCAGTCTC
		R: ATTCTGCCACTCTGCGATTT
Caspase-8	NM_204592.3	F: TCACTTTGCCAGAGCCTGAG
		R: CCCTTGTTCCTCCTGTCGTC
Claudin-1	NM_001013611.2	F: ACCCACAGCCTAAGTGCTTC
		R: AGGTCTCATAAGGCCCCACT
C-myc	NM_001030952.1	F: ACAGTGGCAGGGTCCTCAAACAGA
		R: GTCACGCAGGGCAAAGAAACTCAG
IFN-γ	NM_205149.1	F: AAGCTCCCGATGAACGACT
		R: CCTCTGAGACTGGCTCCTTTT
IL-1β	NM_204524.1	F: TGGGCATCAAGGGCTACA
		R: TCGGGTTGGTTGGTGATG
LITAF	NM_204267.1	F: TGTGTATGTGCAGCAACCCGTAGT
		R: GGCATTGCAATTTGGACAGAAGT
Muc-2	NM_001318434.1	F: CTACTTCACCTTCAACCATTACAAC
		R: TCATAGTCACCACCATCTTCTTCAG
Occludin	NM_205128.1	F: TCATCGCCTCCATCGTCTAC
		R: TCTTACTGCGCGTCTTCTGG
TLR-2	NM_204278.1	F: GATTGTGGACAACATCATTGACTC
		R: AGAGCTGCTTTCAAGTTTTCCC
TLR-4	NM_001030693.1	F: AGTCTGAAATTGCTGAGCTCAAAT
		R: GCGACGTTAAGCCATGGAAG
ZO-1	XM_015278981.2	F: GGGATGTTTATTTGGGCGGC
		R: TCACCGTGTGTTGTTCCCAT

Abbreviations: Bax, Bcl2-associated X; Bcl-2, B-cell lymphoma-2; Caspase-3, cysteinyl aspartate specific proteinase-3; Caspase-8, cysteinyl aspartate specific proteinase-8; F, forward; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; IFN-γ, interferon-γ; IL-1β, interleukin-1β; LITAF, lipopolysaccharide induced TNF factor; Muc-2, mucin-2; R, reverse; TLR-2, toll-like receptor-2; TLR-4, toll-like receptor-2; ZO-1, zona occludens-1.

l mL 1 d per bird from day 17 to 20; 4) Birds of group DC were fed basal diet contaminated with 5 mg of DON per kg diet and challenged with *C. perfringens* CVCC2030 medium at 8×10^8 CFU/mL by instilling using 1-mL injector, and the dose was l mL 1 d per bird from day 17 to 20. To avoid cross-contamination, the unchallenged birds and challenged birds were reared in 2 separate rooms with 2 layers of metal cage, and the brooding temperature was maintained at 35°C for the first week and gradually decreased to 27°C in the 3rd wk. All birds were allowed ad libitum access to feed and water. The experiment lasted for 21 d.

Diets, C. perfringens *Challenge, and Sampling*

The ingredients and nutrient levels of the basic diet are shown in Table 1. The DON was produced by inoculating *Fusarium graminea* PH-1 into the rice at 25°C. The final DON concentration in fermentative rice analyzed by LC-MS/MS method was 453.9 ppm. And the DON feed was mixed with fermentative rice and basic diet by a ratio of 1:90 to reach 5 mg of DON/kg of diet approximately referred to European Commission (European Commission, 2006), which was a maximum observed level in animal feeds (Rodrigues and Naehrer, 2012). The *C. perfringens* challenged method was based on the study by Antonissen et al. (2014) with slight modifications. Briefly, *C. perfringens* (CVCC2030) was obtained from the China Veterinary Culture Collection Center (Beijing, China) which isolated them from NE-infective broilers. *C. perfringens* was cultured anaerobically on tryptose-sulfite-cycloserine for 18 h at 37°C and then aseptically transferred into a cooked meat medium (HB0259; Qingdao Hope Bio-Technology Co., Ltd.) and incubated anaerobically for 18 h at 37°C.

At day 21, 6 birds per group were randomly selected, weighed, and euthanized by cervical dislocation. The middle complete jejunal segments which were fixed in 10% buffered formalin were used for the analysis of intestinal morphology (Yang et al., 2017). The jejunal mucosa scraped from the jejunum was snap-frozen in liquid nitrogen and stored at −80°C until further mRNA analysis. The jejunal and cecal content samples were collected into 2-mL Eppendorf tubes and frozen immediately in liquid nitrogen, and the rest were stored at −80°C immediately for 16s rDNA and short-chain fatty acid (**SCFA**) contents analysis.

Quantitative RT-PCR Analysis of Gene Expression

The total RNA of mucosa samples was extracted using the Trizol Reagent kit (Invitrogen Co., Carlsbad, CA).

The quantity and quality of extracted total RNA were determined using a spectrophotometer (NanoDrop-2000; Thermo Fisher Scientific, Waltham, MA) at 260 and 280 nm. First-strand cDNA was synthesized from 2 μg of total RNA using an EasyScript First-Strand cDNA Synthesis SuperMix kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. Primer sequences of the β-actin, zona occludens-1 (**ZO-1**), occludin, claudin-1, mucin-2 (**Muc-2**), toll-like receptor-2 (**TLR-2**), lipopolysaccharide-induced TNF factor (**LITAF**), interleukin-1β (**IL-1β**), interferon-γ (**IFN-γ**), toll-like receptor-4 (**TLR-4**), B-cell lymphoma-2 (**Bcl-2**), C-myc, cysteinyl aspartate specific proteinase-3 (**Caspase-3**), cysteinyl aspartate–specific proteinase-8 (**Caspase-8**), and Bcl2-associated X (**Bax**) are listed in Table 2, and all the genes' sequences were quoted from NCBI. A total volume of 20 μL of reaction system included 10 μL of SYBR Promerx Ex Taq, 0.4 μL upstream primers (10 μM/L), 0.4 μL downstream primers (10 μM/L), 2 μL cDNA, and 7.2 μL DEPC H_2O. The reactions of real-time PCR were carried out at 95°C for 15 s, followed by 40 cycles at 95°C for 15 s, and at 60°C for 60 s. The mRNA expression was calculated as being equal to $2^{-\Delta\Delta CT}$ and normalized for *GAPDH* gene expression.

Determination of Jejunal Diamine Oxidase Activity

The frozen jejunal tissue samples were thawed on ice, and 0.1 g of each tissue was homogenized in 0.9 mL of cold phosphate buffered saline. The resulting homogenate was then centrifuged at 2,500 rpm under 4°C for 10 min, and the supernatant was collected for estimation of diamine oxidase (**DAO**) activity (A088-2-1, DAO assay kit; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The DAO activity was calculated by the decline rate of OD 340 nm.

Microbial DNA Extraction, 16S rRNA Gene Amplification of the V3 + V4 Region, Sequencing, and Bioinformatics Analysis

A total of 24 collected jejunal content samples (1 g) from CON, DON, CP, and DC groups of 21-day-old broilers were used for DNA extraction using DNeasy PowerSoil Kit (QIAGEN, Inc., Netherlands). The quantity and quality of extracted DNA were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and agarose gel electrophoresis, respectively.

PCR amplification of the bacterial 16S rRNA genes V3–V4 region was performed and purified with Agencourt AMPure Beads (Beckman Coulter, Indianapolis, IN) and quantified using the PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA). After the individual quantification step, amplicons were pooled in equal amounts, and paired-end 2 × 300-bp sequencing was performed using the Illumina MiSeq platform with MiSeq Reagent Kit v3 at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China). The Quantitative Insights into Microbial Ecology (QIIME, v1.8.0) pipeline was used to process the sequencing data. Briefly, raw sequencing reads with exact matches to the barcodes were assigned to respective samples and identified as valid sequences. The low-quality sequences were filtered through the following criteria: sequences that had a length of less than 150 bp, sequences that had average Phred scores less than 20, sequences that contained ambiguous bases, and sequences that contained mononucleotide repeats more than 8 bp. Paired-end reads were assembled using FLASH (v1.2.7, http://ccb.jhu.edu/software/FLASH/). After chimera detection, the remaining high-quality sequences were clustered into operational taxonomic units (OTUs) at 97% sequence identity by UCLUST. A representative sequence was selected from each OTU using default parameters. OTU taxonomic classification was conducted by BLAST searching the representative sequences set against the Green genes Database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) using the best hit. The taxon abundance of each sample was separated into the phylum, class, order, family, and genera levels. The MetaStat (http://metastats.cbcb.umd.edu/) was used to identify the effect of DON and *C. perfringens* on gut microbiota, and the R software 2.15.3 was used to calculate Spearman's rank correlation coefficients between the relative abundance of the gut microbiome and the concentrations of SCFAs, as well as the mRNA expression of immunity-related and apoptosis-regulatory genes, and exhibited the result by the heatmap.

Determination of Bacterial Metabolites

The cecal contents were collected and immediately placed into sterile plastic tubes, sealed, and frozen at −80°C for subsequent SCFAs analysis. The concentrations of SCFAs (acetic acid, propionic acid, isobutyric acid, n-butyric acid, isopentanoic acid, n-pentanoic acid) were measured using gas chromatography (Agilent 7820A GC). SP-2560 capillary column and hydrogen flame ion detector was used. The chromatographic conditions are as follows: The carrier gas was N_2, the split ratio was 40:1, the flow rate was 2.0 mL/min, the average linear velocity was 38 cm/s, the column pressure was 11.3 psi (1psi≈6.89kpa). The initial temperature of the capillary column was 120°C (3 min), and the temperature is increased to 180°C at 10°C/min (1 min). The temperature of the hydrogen flame detector was 250°C, the flow rate of H_2 was 40 mL/min, and the airflow rate was 450 mL/min. Column flow + makeup gas flow was 45 mL/min, the temperature of the inlet was 210°C, and the injection volume was 0.6 μL.

Statistical Analysis

The SPSS 13.0 software for Windows was used for all statistical analyses in this study. Univariate analysis was used to analyze the main effect and interactive effect. All

Table 3. The effect of DON and *C. perfringens* on jejunal morphology.

						P values		
Parameter	CON	DON	CP	DC	SEM	DON	*C. perfringens*	DON × *C. perfringens*
VH (μm)	734.28	675.33	700.53	678.11	18.33	0.311	0.689	0.669
CD (μm)	69.22	63.82	73.15	86.95	2.72	0.691	0.015	0.103
VH/CD	10.59	10.61	9.62	8.13	0.32	0.325	0.009	0.315

Abbreviations: CD, crypt depth; CON, basal diet; CP, basal diet + *C. perfringens* challenge; DC, basal diet extra deoxynivalenol + *C. perfringens* challenge; DON, basal diet extra deoxynivalenol; VH, villi height; VH/CD, the ratio of villi height to crypt depth.

results were represented in terms of mean ± SEM, and $P < 0.05$ was implied a significant difference.

RESULTS

Effect of DON and C. perfringens *on the Jejunal Morphology*

The gut is the first target of DON and *C. perfringens*, so we explored the effect of dietary DON and *C. perfringens* challenge on the morphology of jejunum. As shown in Table 3, *C. perfringens* challenge increased ($P < 0.05$) the jejunal crypt depth (**CD**) and decreased ($P < 0.05$) the ratio of villi height to crypt depth (**VH/CD**) showing that *C. perfringens* could damage the construction of jejunum. Dietary DON had no effect on the morphology of jejunum. And no interaction was seen between dietary DON and *C. perfringens* challenge.

Effect of DON and C. perfringens *on the Gut Barrier Permeability*

The intestinal mucosa acts as a selectively permeable barrier, absorbing the nutrients and defending against pathogens and toxins. We tested the mRNA expression of tight junctions, Muc-2, and activity of the related enzyme (Table 4). It was shown that dietary DON and *C. perfringens* challenge had no significant effect on the gene expression of occludin, claudin-1, and *ZO-1*, while dietary DON significantly downregulated ($P < 0.05$) the mRNA expression of *Muc-2* and *C. perfringens* reduced ($P < 0.05$) the activity of DAO of jejunum. These results indicated impaired barrier function. There was no interaction between dietary DON and *C. perfringens* challenge.

Effect of DON and C. perfringens *on the Inflammation and Apoptosis Response*

In the present study, we tried to investigate the effects of dietary DON and *C. perfringens* challenge and their interactive effects on the inflammation response in a broiler jejunum model. For this purpose, we determined the relative expression of cytokines using RT-qPCR (Table 5). As shown, dietary DON and *C. perfringens* challenge had no effect on the relative expression of cytokines. However, the significant interactions between DON and *C. perfringens* were clear ($P < 0.05$) in the expression of *TLR-4* and *LITAF*.

Inflammation is accompanied by cell death, so we test the expression of apoptosis-related genes (Table 6). Results showed dietary DON significantly downregulated ($P < 0.05$) the relative expression of *Bax* and *Caspase-3*, inversely *C. perfringens* significantly upregulated ($P < 0.05$) the relative expression of *Bcl-2* and *Caspase-8*. The trend of interactions ($P = 0.086$) between DON and *C. perfringens* was in the expression of *Caspase-8*.

Effect of DON and C. perfringens *on the Jejunal Microflora and Bacterial Metabolites*

To get the diversity and abundance of microflora in jejunal digesta, we performed 16s rDNA gene sequencing. It can be seen from Table 7 that dietary DON decreased ($P < 0.05$) the ACE, Chao1, Shannon, and Simpson index significantly. We analyzed the effect of dietary DON and *C. perfringens* challenge on the relative abundance of top 5 phylum and genera in jejunal digesta (Table 8). Results showed that dietary DON tended to increase the relative abundance of *Firmicutes* ($P = 0.079$) and decrease *Proteobacteria* ($P = 0.085$),

Table 4. The effect of DON and *C. perfringens* on the relative expression of tight junction, *Muc-2* and DAO activity of jejunum.

						P values		
Parameter	CON	DON	CP	DC	SEM	DON	*C. perfringens*	DON × *C. perfringens*
Claudin	1.14	0.49	0.57	0.52	0.12	0.121	0.224	0.178
Muc-2	1.13	0.21	1.20	0.69	0.14	0.013	0.299	0.434
Occludin	1.02	0.89	1.36	1.36	0.11	0.768	0.104	0.768
ZO-1	0.79	0.54	0.62	0.95	0.11	0.872	0.608	0.209
DAO (U/L)	1.97	2.29	1.17	1.29	0.15	0.286	<0.001	0.618

Abbreviations: CON, basal diet; CP, basal diet + *C. perfringens* challenge; DAO, diamine oxidase; DC, basal diet extra deoxynivalenol + *C. perfringens* challenge; DON, basal diet extra deoxynivalenol; Muc-2, mucin-2; ZO-1, zona occludens-1.

Table 5. The effect of DON and *C. perfringens* on the relative expression of cytokines.

						P-values		
Parameter	CON	DON	CP	DC	SEM	DON	*C. perfringens*	DON × *C. perfringens*
IFN-γ	1.42	0.80	0.61	0.75	0.19	0.508	0.244	0.309
IL-1β	1.04	1.81	1.44	2.14	0.18	0.231	0.543	0.950
LITAF	1.02^{c}	2.17^{b}	2.50^{a}	1.81^{b}	0.17	0.565	0.162	0.028
TLR-2	1.14	1.13	1.72	0.71	0.3	0.425	0.896	0.437
TLR-4	0.81^{b}	1.60^{a}	0.99^{b}	0.63^{c}	0.11	0.438	0.154	0.046

Numbers within a row with different superscripts differ statistically at $P \leq 0.05$.

Abbreviations: CON, basal diet; CP, basal diet + *C. perfringens* challenge; DC, basal diet extra deoxynivalenol + *C. perfringens* challenge; DON, basal diet extra deoxynivalenol; IFN-γ, interferon-γ; IL-1β, interleukin-1β; LITAF, lipopolysaccharide-induced TNF factor; TLR-2, toll-like receptor-2; TLR-4, toll-like receptor-2.

Actinobacteria ($P = 0.070$), and *Bacteroidetes* ($P < 0.05$) significantly. In the genus level, similarly, dietary DON increased ($P < 0.05$) the relative abundance of *Lactobacillus*, decreased ($P < 0.05$) the relative abundance of *Lactococcus*, and tended to decrease ($P = 0.074$) the relative abundance of *Sediminibacterium*. In addition, *C. perfringens* challenge decreased ($P < 0.05$) the relative abundance of *Lactococcus*. There was significant interaction ($P < 0.05$) between DON and *C. perfringens* for the abundance of *Lactococcus*.

SCFAs are produced by the microflora, and it is not only the energy source of bacteria and intestinal cells but it also plays potent roles in maintaining intestinal barrier function, regulation on immune response, and cell proliferation (Dalile et al., 2019). We examined the content of SCFAs in the cecal digesta (Table 9). Results showed that dietary DON and *C. perfringens* challenge had no main effect ($P > 0.05$) on the content of SCFAs. The interaction between DON and *C. perfringens* was most significant ($P < 0.05$) in the content of butyric acid.

The Analysis of KEGG Pathway PICRUSt

To best understand the function of microflora, we used LDA analysis on the Kyoto Encyclopedia of Genes and Genomes pathway PICRUSt in level 2 of microflora in the jejunal digesta (Figure 1). As found, compared with group DC, the most abundant KEGG pathways of group DC were amino acid metabolism, xenobiotic biodegradation and metabolism, and metabolism of cofactors, vitamins. However, the pathways predicted of group DON were membrane transport, replication, and repair, translation et al. When compared between groups CP and DC, the pathways enriched in group CP were membrane transport, cell motility, and amino acid metabolism, and those in group DC were replication and repair, translation, and nucleotide metabolism.

Correlation Analysis

Spearman correlation analysis was conducted to characterize the associations of top 50 genera bacterial abundances with SCFAs' concentrations and the relative expression of apoptosis and inflammation genes. In total, the most abundance of the genera was significantly negatively correlated with acetic acid, butyric acid, isobutyric acid, and total SCFAs' concentrations (Figure 2A). However, the abundance of *Lactobacillus* had a positive ($P < 0.05$) correlation with all the SCFA indicator except for propionic acid. The abundance of *Lactococcus* was significantly negatively ($P < 0.05$) correlated with acetic acid and total SCFAs and positively correlated ($P < 0.05$) with isovaleric acid and propionic acid contents.

To study the role of gut microbiota in DON-induced and *C. perfringens*–induced inflammation and apoptosis response of broiler chickens, we examined the relationship between the relative abundance of gut microbiota and the mRNA expression of immunity-related and apoptosis-regulatory genes (Figure 2B). In total, most negative correlations ($P < 0.05$) were observed between genera abundances and *LITAF*, a proinflammation cytokine. And surprisingly, *Lactobacillus* was positively

Table 6. The effect of DON and *C. perfringens* on the relative expression of apoptotic genes.

						P values		
Parameter	CON	DON	CP	DC	SEM	DON	*C. perfringens*	DON × *C. perfringens*
Bax	0.80	0.42	1.00	0.34	0.12	0.038	0.802	0.551
Bcl-2	0.79	0.30	1.67	1.27	0.18	0.192	0.012	0.883
Bcl-2/Bax	0.92	0.79	0.74	0.86	0.04	0.964	0.605	0.220
Caspase-3	1.03	0.42	1.67	0.45	0.20	0.018	0.355	0.402
Caspase-8	0.84	0.85	3.59	1.10	0.17	0.090	0.046	0.086
C-myc	0.92	0.72	1.34	0.45	0.20	0.213	0.879	0.407

Abbreviations: Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X; Caspase-3, cysteinyl aspartate specific proteinase-3; Caspase-8, cysteinyl aspartate specific proteinase-8; CON, basal diet; CP, basal diet + *C. perfringens* challenge; DC, basal diet + 5 mg of deoxynivalenol per kg diet + *C. perfringens* challenge; DON, basal diet + 5 mg of deoxynivalenol per kg diet.

Table 7. The effect of DON and *C. perfringens* on the abundance and diversity of microflora in jejunal digesta.

						P values		
Parameter	CON	DON	CP	DC	SEM	DON	*C. perfringens*	DON × *C. perfringens*
ACE	1,152.69	600.59	1,022.24	639.85	86.71	0.007	0.772	0.591
Chao1	1,078.02	580.43	971.15	620.66	81.97	0.011	0.826	0.628
Shannon	5.88	3.30	4.53	3.61	0.33	0.007	0.380	0.170
Simpson	0.90	0.65	0.75	0.72	0.03	0.037	0.514	0.107

Abbreviations: CON, basal diet; CP, basal diet + *C. perfringens* challenge; DC, basal diet + 5 mg of deoxynivalenol per kg diet + *C. perfringens* challenge; DON, basal diet + 5 mg of deoxynivalenol per kg diet.

correlated ($P < 0.05$) with *LITAF*. *Lactococcus* was not correlated ($P > 0.05$) with *LITAF*, but had a positive correlation ($P < 0.05$) with *Caspase-3*. Furthermore, the abundance of *Enterococcus* was positively correlatied ($P < 0.05$) with *Caspase-8*.

DISCUSSION

The digestion and absorption capacity are tight correlative with intestinal morphology, and VH, CD, and VH/CD are sensitive indicators reflecting the development and function of the gut. As the main target of the DON, the development of the gut would be impaired when DON was consumed by animals. But the toxicity of DON for chicken is still controversial. Osselaere et al. (2013) reported that jejunal VH was decreased and CD was increased when 7.54 mg of DON/kg of feed was consumed. On the contrary, dietary DON would not influence the morphology of jejunum when the dose was 10 mg/kg (Ghareeb et al., 2015; Wu et al., 2018a). In this study, it is found that dietary DON had no influence on the morphology of jejunum. The result may be related to the dose we choose according to the European Commission standards (European Commission, 2006). And the tolerant capacity of broilers for DON is strengthened with the breeding and genetic modification. *C. perfringens* is an intestinal pathogenic agent that could induce impairment on the mucosa for its secretion of exotoxin and bioactive matter. Xue et al. (2018) reported that *C. perfringens* infection could result in obstacle to the development of villi and crypt and decrease the VH/CD ratio. Partially consistent with the aforementioned report, the results of this study noted that *C. perfringens* could increase the CD and decrease the VH/CD ratio. Variation of results among different studies may be due to the different effects from different *C. perfringens* species and infection methods. Crypt, the villus factory, palyes potent role in the renewal of villus as needed in response to inflammation from pathogens or toxins (Willing and Van, 2007). In accordance with the results of morphology, *C. perfringens* significantly reduced the activity of jejunal DAO. As a sensitive indicator of intestinal barrier function, DAO is an intracellular enzyme in the intestinal cells that would be released into blood from mucosal cells when the intestine was damaged (Liu et al., 2020). In this experiment, the decreased DAO indicated impaired intestine.

Intestinal mucosa layer act as a selective barrier that prevents intestinal microbial from translocation and maintains intestinal homeostasis, among which Muc-2 is an important part of the mucosa layer secreted by global cells. Results showed that dietary DON downregulated the mRNA expression of Muc-2, and this may be the inhibition effect of dietary DON on resistin-like molecule beta through the activation of protein kinase R and mitogen-activated protein kinase p38 (Pinton et al., 2015). Intestinal immune function plays a potent role in defending against gut pathogenic agents. DON could bind to the ribosome to induce "ribotoxic stress

Table 8. The effect of DON and *C. perfringens* on the relative abundance of jejunal microbial flora.

						P values		
Parameter	CON	DON	CP	DC	SEM	DON	*C. perfringens*	DON × *C. perfringens*
Phylum level								
Firmicutes	49.35	75.86	54.50	73.53	6.18	0.079	0.909	0.763
Proteobacteria	30.22	13.84	27.83	16.62	3.81	0.085	0.980	0.737
Actinobacteria	13.13	6.76	11.63	6.78	1.48	0.070	0.803	0.797
Bacteroidetes	2.83	1.23	2.72	1.18	0.39	0.050	0.913	0.969
Cyanobacteria	2.18	1.07	1.62	0.99	0.25	0.102	0.527	0.642
Genera level								
Lactobacillus	34.95	71.72	50.02	71.24	6.62	0.032	0.567	0.542
Ochrobactrum	14.55	6.63	14.68	9.79	1.9	0.109	0.670	0.696
Amycolatopsis	9.26	4.87	7.71	5.05	1.05	0.113	0.751	0.689
Lactococcus	0.52^{a}	0.11^{b}	0.21^{b}	0.13^{b}	0.04	0.026	0.001	0.013
Sediminibacterium	1.97	0.82	1.79	0.91	0.27	0.074	0.934	0.797

Numbers within a row with different superscripts differ statistically at $P \leq 0.05$.

Abbreviations: CON, basal diet; CP, basal diet + *C. perfringens* challenge; DC, basal diet + 5 mg of deoxynivalenol per kg diet + *C. perfringens* challenge; DON, basal diet + 5 mg of deoxynivalenol per kg diet.

Table 9. The effect of DON and *C. perfringens* on the contents of short chain fatty acids of cecal digesta.

						P values		
Parameter	CON	DON	CP	DC	SEM	DON	*C. perfringens*	DON × *C. perfringens*
Acetic acid	63.16	76.11	72.13	56.97	4.01	0.891	0.532	0.095
Propionic acid	3.83	3.88	4.26	3.23	0.29	0.430	0.866	0.384
Butyric acid	9.91^{a,b}	13.41^{a}	12.56^{a}	8.46^{b}	0.89	0.862	0.508	0.039
Isobutyric acid	0.44	0.44	0.51	0.46	0.04	0.781	0.538	0.768
Valeric acid	1.05	1.07	1.22	0.98	0.04	0.342	0.728	0.237
Isovaleric acid	0.72	0.62	0.63	0.51	0.05	0.146	0.171	0.927
Total SCFAs	79.11	95.53	91.31	70.61	4.91	0.827	0.518	0.070

Numbers within a row with different superscripts differ statistically at $P \leq 0.05$.

Abbreviations: CON, basal diet; CP, basal diet + *C. perfringens* challenge; DC, basal diet + 5 mg of deoxynivalenol per kg diet + *C. perfringens* challenge; DON, basal diet + 5 mg of deoxynivalenol per kg diet.

response," activating downstream mitogen-activated protein kinase, which plays a critical role in modulating cell growth, differentiation, apoptosis, and immune response, of which p38 and ERK affect cytokine production and lead to the upregulation of *LITAF* gene expression (Ueno, 1983). Especially p38 appears to be involved in the genotoxicity of DON. Our laboratory reported that 10 mg/kg of DON in the diet had no influence on the relative expression of *IL-10*, *IL-12*, *LITAF*, and *IFN-γ* (Wu et al., 2018a). According to the present result, the extra addition of DON has no influence on the mRNA expression of *TLRs* and cytokines. To conclude, the low content of DON cannot induce inflammation response of broilers. *C. perfringens* could attach to epithelial cells, and the pathogen-associated molecular patterns, which are unique "microbial signatures" found in different pathogens, are recognized by pathogen-recognition receptors, triggering the final activation of nuclear factor kappa-light-chain-enhancer of activated B cells and inducing inflammation response (Oh and Lillehoj, 2016). Previous studies revealed that the relative expression of *IFN-γ*, *LITAF*, and *IL-1β* was upregulated in *C. perfringens*–induced NE broilers (Li et al., 2018; Wang et al., 2019). However, another study demonstrated *C. perfringens* challenge failed to influence the expression of *IFN-γ*, *LITAF*, and *IL-10* in the NE model (Li et al., 2018). In this study, we found that *C. perfringens* did not change the expression of *TLRs* and cytokines. The difference in results may be attributed to the difference in infection patterns. However, interactive analysis results showed that dietary DON and *C. perfringens* challenge had an antagonistic effect on the expression of *TLR-4* and *LITAF*. TLRs are sensitive sites that participate in the host immune response by recognizing molecular patterns associated with pathogens. They can active downstream TIR domain-containing adaptor inducing interferon-β or myeloid differentiation factor 88 to regulate the chemoresistance, cytokine, and chemokine production inducing cell apoptosis or proliferation (Lu et al., 2019). Among those,

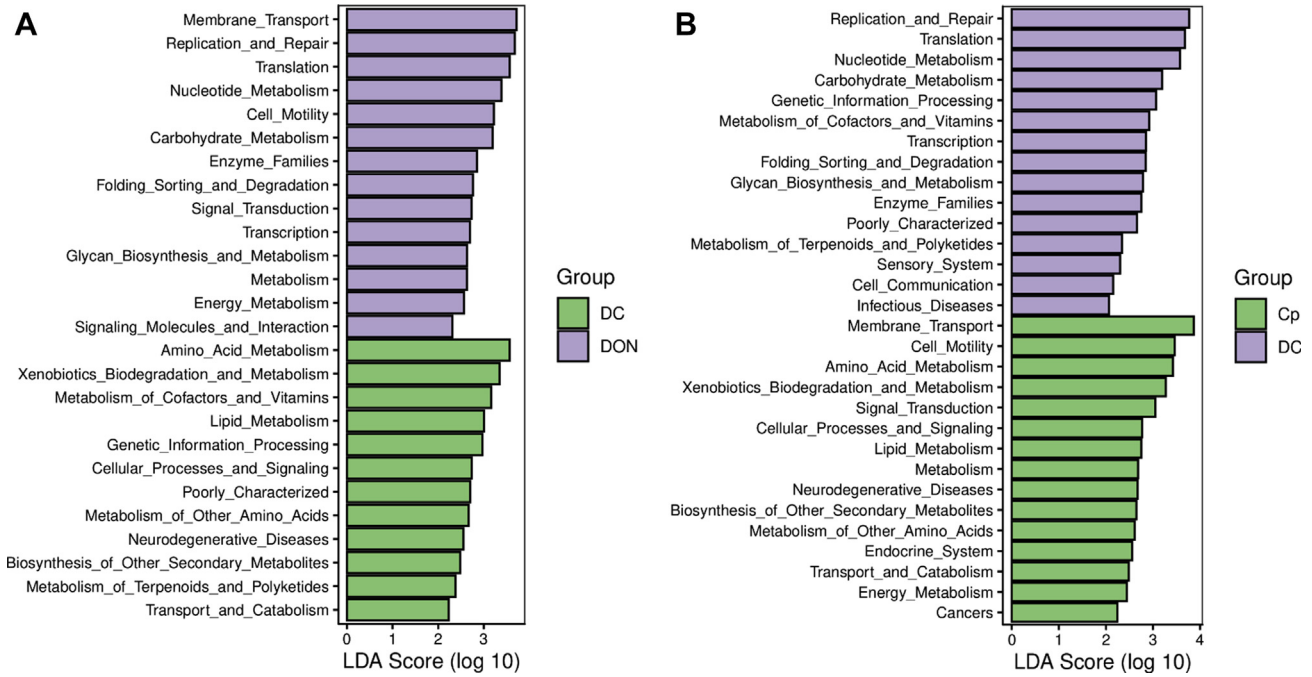

Figure 1. The linear discriminant analysis (LDA) of KEGG pathway PICRUSt of microflora in jejunal digesta of 21-day-old broilers at level 2. (A) between group DON and DC and (B) between group CP and DC, of which the LDA scores ≥2. The treatments were as follows: DON = basal diet + 5 mg of deoxynivalenol per kg diet; Abbreviations: CP, basal diet + *C. perfringens* challenge; DC, DON diet + *C. perfringens* challenge.

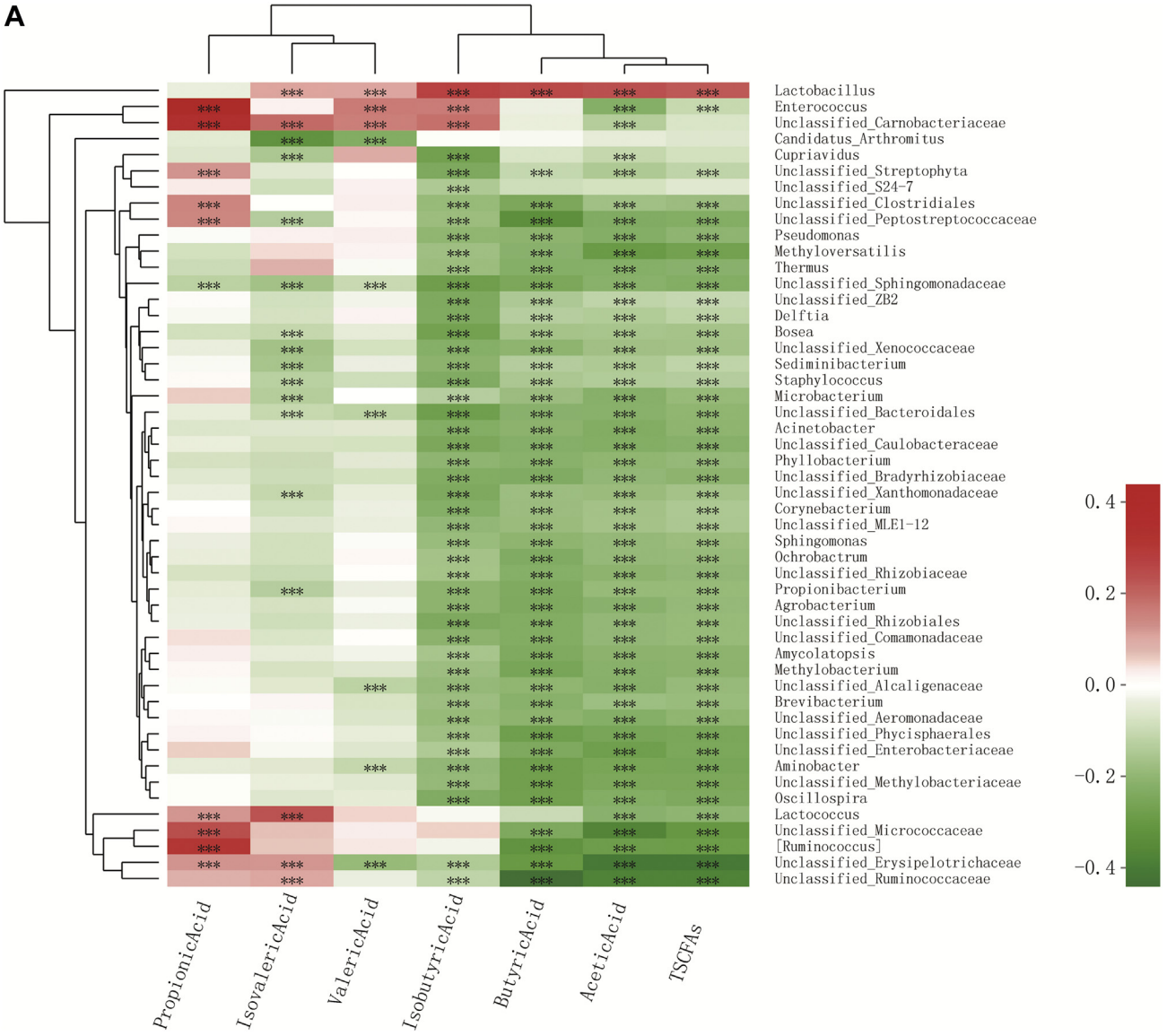

Figure 2. Correlation analysis (A) between SCFA concentrations in cecal digesta and relative abundance of the top 50 genera in jejunal digesta of 21-day-old broilers and (B) between mRNA expression of immunity-related and apoptosis-regulatory genes with the relative abundance of the top 50 genera in jejunal digesta of 21-day-old broilers. Colors refer to the degree of correlation. *$0.01 < P \leq 0.05$, **$0.001 < P \leq 0.01$, ***$P \leq 0.001$.

TLR4 is the key factor mediating LPS-induced inflammation. LITAF, a proinflammation cytokine that could promote the activation and chemotaxis of inflammatory cells, could induce acute inflammatory response (Sattler, 2017). Above all, dietary DON at 5 mg/kg and *C. perfringens* challenge (duration of day 17–day 21) had no effect on the jejunal inflammation response, but they had an interactive effect on the inflammation response.

Inflammation responce can eliminate antigens, but too acute inflammation response could result in cell death and injure the intestine. Apoptosis and proliferation are important mechanisms to update epithelial cells to maintain the normal construction and function of the intestine (Der Flier and Clevers, 2009). Meanwhile, abnormal apoptosis would lead to various diseases and dysfunction of the intestine. Apoptosis, an ATP-dependent cell death method, is a procedural death controlled by cells (Benjelloun et al., 2010). Under basal conditions, Bax is inactive and localized to cytoplasm. When various unfavorable conditions happened, Bax accumulates at the mitochondria and can be directly activated by BH3-only proteins (Letai et al., 2002). Afterward, BH3-only proteins inhibit antiapoptotic Bcl-2 proteins and activate the effector proapoptotic Bcl-2 proteins Bax and Bcl-2-antagonist/killer1, leading to mitochondrial outer membrane permeabilization which activated Caspase-3 and 7 to facilitate apoptosis (Cogliati et al., 2013). In addition, Caspase-8–mediated cleavage and activation of BH3-only protein BID also regulated the apoptosis pathway (Bock and Tait, 2020). Results showed that dietary DON significantly downregulated the expression of proapoptosis genes Bax and Caspase-3 indicating an antiapoptosis state, and this may be due to the dose- and time-dependent toxicity of DON (Payros et al., 2016). Bcl-2 can inhibit apoptosis by limiting the release of cytochrome c from

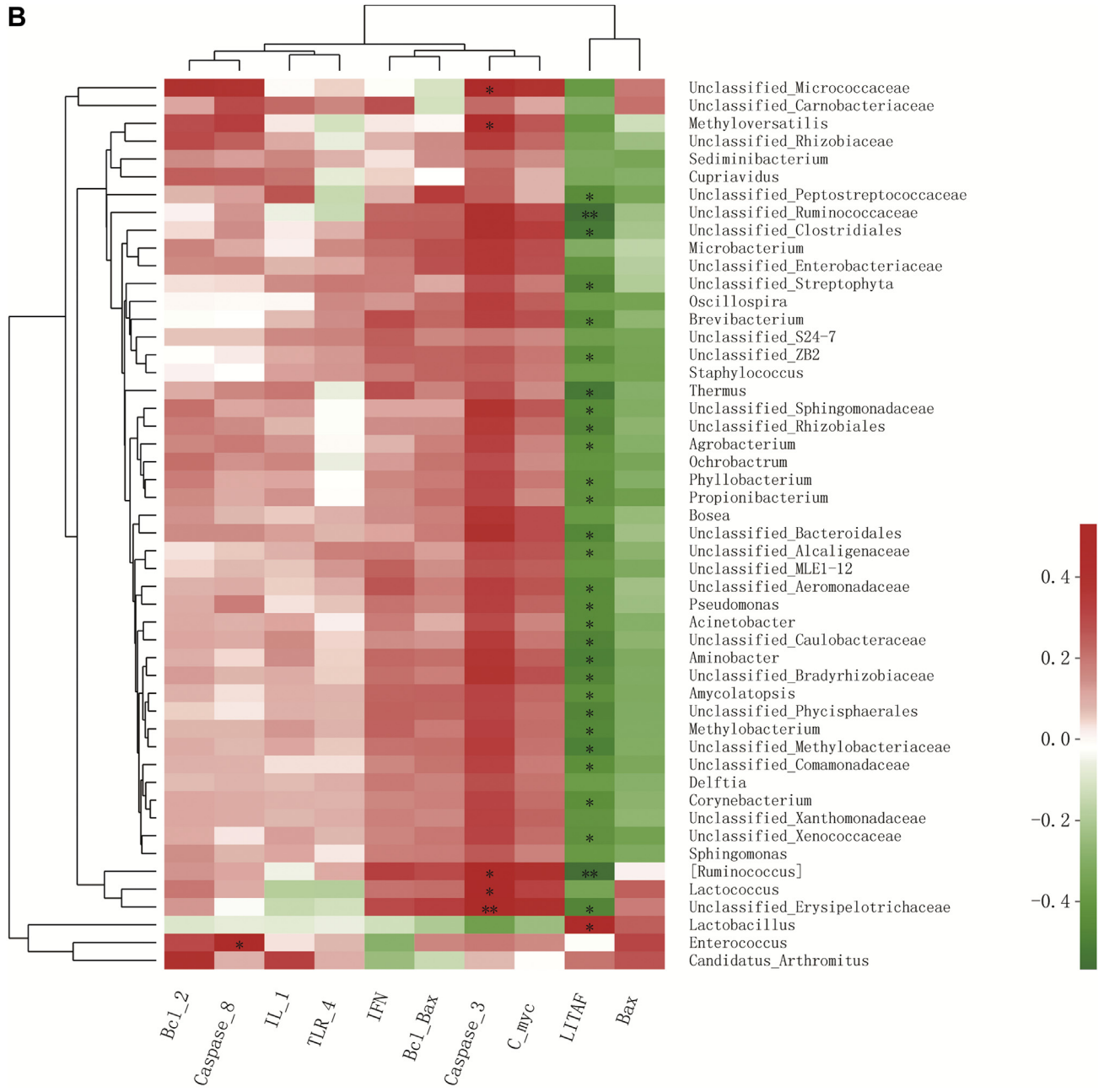

Figure 2. Continued

mitochondria, which has the ability as Caspases to activate apoptosis (Yang et al., 1997). Furthermore, Bcl-2 impedes the activation of Bax to prevent apoptosis (Scaffidi et al., 1998). Caspase-8, as a cell death regulator, could induce cell apoptosis via death receptors such as tumor necrosis factor receptor 1 (**TNFR1**). Moreover, it could depress cell necroptosis via regulating the receptor-interacting serine/threonine kinase 3 and mixed lineage kinase domain-like protein (Alvarez-Diaz et al., 2016). Effects of *C. perfringens* on the upregulated relative expression of *Bcl-2* and *Caspase-8* indicated that the broilers were in a proapoptosis status. In effect, *C. perfringens* promoted the apoptosis of jejunal epithelial cells. The death receptors pathway is accomplished by the combination of a signal molecule and death receptors located in the cell surface, such as LITAF and TNFR. When secreted, signal molecule LITAF combined with TNFR1, TNFR1-associated death domain, receptor-interacting protein kinase 1, TNF receptor–associated factor 2, and cellular inhibitor of apoptosis proteins 1/2 were recruited, and Caspase-8 was activated (Haas et al., 2009). Activated Caspase-8 cleaves and activates Caspases-3 and Caspases-7, leading to wide-scale cleavage of cellular components and rapid cell death (Bock and Tait, 2020). The interactive analysis noted that the combined effect of DON and *C. perfringens* trended to antagonize the expression of Caspase-8. Combined with their antagonistic effect on the expression of TLR-4 and LITAF, it could be conjectured that DON and *C. perfringens* may have interaction in the apoptotic death receptor pathway of epithelial cells by antagonizing the expression of LITAF in the TLR4 pathway.

There is abundant microbiota harbored in the intestine, which plays an important role in the regulation of digestion, absorption, and immunity. As known the toxicity of DON on intestine is through the impact on the microflora (Robert et al., 2017). To verify whether the interactive effect of DON and *C. perfringens* is through their impact on the microflora, the construction of microflora in the jejunum was determined using the 16s rDNA gene-sequencing approach. Alpha diversity indices including Chao1, ACE, Shannon, and Simpson indices, are commonly used to measure the abundance and diversity of the community. Chao1 and ACE indices suggest the abundance of the community, and the Shannon and Simpson indexes suggest diversity (Shannon, 1948; Chao, 1984; Chao and Yang, 1993). In this experiment, dietary DON significantly reduced Simpson, Chao1, ACE, and Shannon indices of the flora, exhibiting that dietary DON reduced the abundance and diversity of microflora, according to previous studies (Vignal et al., 2018; Awad et al., 2019). In agreement with the aforementioned results, dietary DON reduced the abundance of *Bacteroidetes* and tended to reduce the abundance of *Firmicutes*, *Proteobacteria*, and *Actinobacteria*. *C. perfringens* challenge had no effect on the abundance of microbiota on the phylum level. On the genus level, results revealed that dietary DON affected the abundance of *Lactobacillus* and *Lactococcus*. Similarly, the *C. perfringens* challenge had the same effect on *Lactococcus*, but interactive analysis revealed that they antagonized the relative abundance of *Lactococcus* among the top 5 genera. Variation of bacteria mainly related to the shift of SCFAs' contents. Dietary DON and *C. perfringens* have no effect on the content of SCFAs in the cecal contents of broilers. However, dietary DON and *C. perfringens* challenge had an interaction effect on butyric acid content. The changes of apoptosis and intestinal inflammation status are driven by the modified microbiota (Yang et al., 2020). Spearman correlation analysis was used to characterize associations of bacterial relative abundances with SCFAs' concentrations in cecal digesta and mRNA expression of inflammation and apoptosis-relative genes. In total, most negative correlations were observed within the isobutyric acid, butyric acid, acetic acid, and total acid contents. In contrast, most positive correlations were observed within the genus *Lactobacillus*. These results indicated that the composition differences of bacterial metabolites reflected the changes of microbiota. To study the role of gut microbiota in dietary DON and *C. perfringens* challenge–induced apoptosis and intestinal inflammation in broilers, the relationship between the relative abundance of the gut microbiome and the mRNA expression of immunity-related and apoptosis-regulatory genes was also examined. Results revealed most of the top 50 genera were negatively correlative with the mRNA expression of *LITAF*. Interestingly, the relative abundance of *Lactobacillus* was positively correlated with *LITAF*. We speculate that it may be the regulative role of the *Lactobacillus* under infection conditions (Sanders et al., 2019). The relative abundance of *Lactococcus* had no significant correlation with *LITAF* gene expression, but a significant positive correlation was seen with *Caspase-3* gene expression. *Lactococcus* are among the most important lactic acid bacteria involved in the dairy industry (Barbieri et al., 2019), and some species are the pathogenic agent in fish such as *Lactococcus garvieae* (Raissy et al., 2018). Above all, we speculated that the interactive effect of dietary DON and *C. perfringens* challenge on the relative abundance of *Lactococcus* to influence the death receptor apoptosis pathway may be a potential mechanism of their interactive effect on the apoptosis response of jejunal epithelial cells. The results of the KEGG pathway PICRUSt agreed with the aforementioned results, showing the metabolism was altered by the shifty microflora. Different stimuli such as DNA damage and mitotic processes accompany with apoptosis. DON is potentially genotoxic, and exposure to DON enhances DNA damage and apoptosis (Mishra et al., 2016). Pierron et al. (2016) reported that the de-epoxidation or epimerization of DON led to an absence of MAPK activation and a reduced toxicity, and it is reported that there are intestinal bacteria that belong to the *Clostridium* sp. category which had the ability to de-epoxidize or epimerize DON (Li et al., 2017). Furthermore, it is reported that the downregulated expression of genes related to the energy metabolism of *Salmonella* may be the reason of the toxicity of DON to *Salmonella* (Vandenbroucke et al., 2011). The toxicity between DON and pathogenic bacteria may be another explanation for the interactive effect of dietary DON and *C. perfringens* challenge. Although the underlying mechanism still needs further investigation, our results confirm that there are antagonistic effects between DON and *C. perfringens* on the enterotoxication of broilers, implying that the complex mode of mycotoxin and pathogenic bacteria and microflora may play a crucial role in their interaction.

CONCLUSIONS

Both DON and *C. perfringens* had a harmful effect on the jejunal health of broiler chickens. The interaction effect between the 2 factors on the mRNA expression of LITAF and TLR-4 may have evolved by manipulating the bacterial community composition and bacterial metabolites, especially *Lactococcus* and butyric acid.

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DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.psj.2020.10.061.

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