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Curcumin alleviates cecal oxidative injury in diquat-induced broilers by regulating the Nrf2/ARE pathway and microflora

Fengyang Wu [*,†,1] Man Zhao,[*,1] Zhaohong Tang,[‡,1] Fengxia Wang,[*] Shuaijuan Han,[*] Shudong Liu,[*] and Baojiang Chen [*,2]

[*]*College of Animal Science and Technology, Hebei Agricultural University, Baoding, 071000 China;* [†]*College of Food Science and Technology, Hebei Agricultural University, Baoding, 071000 China; and* [‡]*Hebei Research Institute of Microbiology Co., LTD, Baoding, 071000 China*

ABSTRACT This study evaluated the alleviative effect of curcumin (**CUR**) on the diquat (**DQ**)-induced cecal injury in broilers. A total of 320 one-day-old Cobb broilers were selected and randomly divided into 4 treatments, namely control, DQ, CUR 100, and CUR150 groups. The control and DQ groups were fed a basal diet, while the CUR 100 and CUR150 groups were fed the basal diet supplemented with 100 and 150 mg/kg CUR, respectively. Each group had 8 replicates, with 10 broilers per replicate. On day 21 of the experiment, 1 broiler was selected from each replicate and intraperitoneally injected 20 mg/kg body weight of DQ for DQ, CUR 100, and CUR 150 groups. Broilers in control group received equivalent volume of saline. Broilers were euthanized 48h post-injection for tissue sampling. The results showed that DQ injection could cause oxidative stress and inflammatory reactions in the cecum, affecting the fatty acid production and flora structure, thus leading to cecum damage. Compared with the DQ group, the activity of superoxide dismutase, the level of interleukin 10, acetic acid, and total volatile fatty, and the abundance of nuclear factor E2-related factor 2, copper and zinc superoxide dismutase and catalase mRNA in the cecal mucosa of broilers in the CUR group increased significantly ($P < 0.05$). However, the levels of malondialdehyd, reactive oxygen species, tumor necrosis factor-alpha, and the expression of cysteine-aspartic acid protease-3 and tumor necrosis factor-alpha decreased significantly ($P < 0.05$) in the CUR group. In addition, CUR treatment alleviated the damage to the cecum and restored the flora structure, and *Lactobacillus* and Lactobacillaceae promoted the alleviative effect of CUR on DQ. In summary, CUR could alleviate the cecal injury caused by DQ-induced oxidative damage and inflammatory reactions by regulating the Nrf2-ARE signaling pathway and intestinal flora, thus protecting the cecum.

Key words: curcumin, broiler, diquat, cecum, intestinal flora

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INTRODUCTION

In the poultry industry, oxidative stress commonly arises from an imbalance and excessive accumulation of free radicals in chickens (Yang et al., 2016). The primary consequence of oxidative stress in broilers is a decline in both growth performance and meat quality (Han et al., 2020; Hafez et al., 2022; Mashkoor et al., 2023). In severe cases, oxidative stress adversely impacts biological macromolecules, cell proliferation, differentiation, maturity, apoptosis, and various physiological or pathological processes, serving as a catalyst in the occurrence and development of diseases (Gorrini et al., 2013; Filomeni et al., 2015; Jaganjac et al., 2022). Broilers are particularly susceptible to oxidative stress due to their rapid growth and high metabolism as well as the access to varied environmental conditions, diets, management programs, and diseases (Hafez et al., 2022; Jimoh et al., 2022; Mashkoor et al., 2023; Sang et al., 2023). To ensure optimal growth performance and overall broiler health, antioxidant feed additives are frequently utilized to alleviate the imbalance of free radicals and counteract oxidative stress in broilers (Damiano et al., 2022).

Curcumin (**CUR**) is a diketone compound extracted from the rhizomes of *Curcuma longa* (radix curcumae, a member of Zingiberaceae family), demonstrating diverse biological activities, including antioxidant, anti-inflammatory, and antiviral effects (Ak et al., 2008; Kotha et al., 2019). Previous research has shown that CUR can safeguard the liver, serum, and gut from damage

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[1]These authors contributed equally.

[2]Corresponding author: chenbaojiang@vip.sina.com

induced by oxidative stress in broilers (Zhang et al., 2019; Liu et al., 2023; Wu et al., 2023). Additionally, the supplementation of CUR in feed has been observed to mitigate cecal lesions resulting from coccidiosis infection, attributed to its antioxidant properties (Yadav et al., 2020). Despite the recognized antioxidant effects on various broiler organs, there remains a knowledge gap concerning the modulatory pathways of CUR in the broiler gut and microbiome. The gut and microbiome function as direct interfaces for interaction and manipulation with feed (Sylvestre et al., 2023), and gut oxidative stress levels are intricately linked to various feed additives (Shi et al., 2017; Jia et al., 2023) and stress conditions (Bhattacharyya et al., 2014; Scazzocchio et al., 2020). Understanding the pathway and microbiome response of CUR to oxidative stress can yield valuable insights into the mechanism underlying CUR's antioxidant properties in broilers.

Diquat (**DQ**) is a chemical commonly utilized in the development of broiler oxidative stress models (Chen et al., 2021). Previous research has consistently demonstrated liver and intestinal damage resulting from DQ injection, indicating that DQ injection can establish a reliable oxidative stress model in broilers (Wu et al., 2023; Zha et al., 2023). Therefore, the objective of this study was to investigate the possible alleviation mechanisms involved in dietary curcumin (CUR) supplementation on intestinal signaling and gut microbiome under DQ-induced oxidative stress in broilers.

MATERIALS AND METHODS

The experimental protocols were approved by the Animal Care and Use Committee of Hebei Agriculture University (Baoding, China) (Protocol No.: 2022003). All animal experiments complied with the ARRIVE guidelines were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

Experimental Animals and Materials

One-day-old male Cobb broilers were purchased from Jiuxing Farming Co., Ltd. (Baoding, China). CUR (turmeric extract of Zingiberaceae, purity ≥ 95%) was purchased from the Chenguang Biotechnology Group Co., Ltd. (Handan, China). DQ (diquat's dibromide monohydrate) was purchased from Sigma Aldrich (St Louis, MO).

Experimental Design and Experimental Diet

A total of 320 healthy Cobb broilers with an average weight close to 44.18 ± 1.05 g were randomly divided into 4 groups, with 8 replicates for each group and 10 broilers in each replicate. The control and DQ groups were fed a basal diet, and CUR supplementation groups (CUR 100 and CUR 150) were fed with a basal diet supplemented with 100 and 150 mg/kg CUR. The experiment was lasted for 23 d. The composition and nutritional levels of the basal diet are shown in Table 1. The growth performance was previously reported (Wu et al., 2023).

Experiment Management

The experiment was conducted on the Hebei Agricultural University Animal Husbandry Teaching Center broiler house. A total of 32 flat pens were used for this study. Broilers were fed ad libitum, and don't have access to liter. The temperature in the broiler house was kept at 32℃ to 34℃ from day 1 to day 7 of the experiment, then decreased by 2℃ to 3℃ every 7 d, and kept constant at 26℃ for the rest of the experiment. Moreover, the humidity of the hen house was kept at about 70% from day 1 to day 3 of the experiment and then decreased to 60 to 65% for the rest of the experiment.

On day 21 of the experiment, one broiler was randomly selected from each replicate based on the average weight (n = 8). The broilers in DQ, CUR 100, and CUR150 groups were intraperitoneally injected with saline-diluted 20 mg/mL DQ solution at a rate of 1 mL/kg body weight (final dosage is 20 mg/kg body weight), while those in the control group were injected with equivalent amount of normal saline. The broilers were then slaughtered 48 h postinjection.

Morphology of Cecum Tissues

Cecum tissue samples were collected and rinsed in sterile saline to remove any remaining cecal content.

Table 1. Composition and nutrient levels of the basal diet (air-dry basis, %).

	Trial period	
Items	1−21 d	22−24 d
Ingredients		
Corn	48.20	51.45
Soybean meal	38.50	33.30
Vegetable oil	5.00	7.05
Premix[1]	5.00	5.00
Limestone	1.60	1.50
Calcium phosphate	1.25	1.25
Sodium chloride	0.35	0.35
Choline chloride	0.10	0.10
Total	100.00	100.00
Nutrient levels[2]		
Metabolizable energy /(MJ/kg)	12.76	13.39
Crude protein	24.14	22.91
Crude fiber	3.48	3.15
Calcium	0.83	0.81
Available phosphorus	0.42	0.37
Total phosphorus	0.60	0.58
Lysine	1.26	1.11
Methionine	0.53	0.51
Threonine	0.93	0.85

[1]The premix provided the following per kg of diets: vitamin A, 10,000 IU; vitamin D3, 4,000 IU; vitamin E, 20 IU; vitamin K3, 2 mg; vitamin B1, 2 mg; vitamin B2, 6 mg; vitamin B6, 3 mg; vitamin B12, 0.02 mg; nicotinamide, 40 mg; calcium pantothenate, 10 mg; folic acid, 1 mg; biotin, 0.12 mg; Cu, 16 mg; Fe, 80 mg; Zn, 110 mg; Mn, 120 mg; I, 1.5 mg; Se, 0.3 mg.

[2]Crude protein, calcium and total phosphorus in nutrients are measured values, while the rest are calculated values.

Subsequently, the samples were rapidly fixed in a 4% paraformaldehyde solution, and the fixed cecum tissues were trimmed, dehydrated, waxed, embedded, sliced, dyed (hematoxylin and eosin dye solution), and sealed. A microscope camera system (BA200Digital, Motic, Xiamen, China) was then employed for slice observation and image acquisition. The cecum damage conditions were described by 2 trained poultry veterinaries.

Cecal Mucosa Indexes

The broilers were euthanized by cervical dislocation, and the cecal mucosa samples were collected using sterilized surgical tools and stored at −80℃ for further analysis. Commercial kits were utilized to detect cecal mucosa indexes following the manufacturer's instructions. Enzyme activity assay kits were purchased from Jiangsu Meimian Industrial Co., Ltd. (Yancheng, China) for the determination of total antioxidant capacity (**TAOC**), superoxide dismutase (**SOD**), glutathione peroxidase (**GSH-Px**) and catalase (**CAT**). Enzyme-linked immunosorbent assay kits were purchased from Jiangsu Meimian Industrial Co., Ltd. (Yancheng, China) for the determination of malondialdehyde (**MDA**), tumor necrosis factor-α (**TNF-α**), interleukin-1β (**IL-1β**), interleukin 4 (**IL-4**), interleukin 6 (**IL-6**) and interleukin 10 (**IL-10**), while reactive oxygen species (**ROS**) kit (chemical fluorescence method) was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The protein concentration of mucosa was measured using a BCA protein assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Volatile Fatty Acids

Cecum contents (0.5 g) were diluted at the dilution power of 3 and centrifuged at 5,400 × g for 10 min at 4℃. Thereafter, 1 mL of the supernatant was mixed with 0.2 mL of 25% metaphosphate solution (containing 2- ethylbutyric acid) and incubated in an ice bath for 30 min. The mixture was centrifuged at 10,000 × g for 10 min at 4°C to remove the protein precipitate. The supernatant was then collected and used to measure the concentration of volatile fatty acids via gas chromatography (Agilent 7890A, Santa Clara, CA). The injector and detector temperatures were set at 220°C and 230°C, respectively. The column temperature was programmed to increase at a rate of 7°C/min within the range of 70°C to 150°C, facilitating optimal separation. Peak identification was conducted by comparing the results with commercial standard solutions of acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, and isovaleric acid from Sigma Chemical Co. (St Louis, MO).

Relative Expression Levels of Genes in the Cecum Mucosa

Specific primers were designed using Primer 6.0 software and synthesized by Shenggong Biotech Co., Ltd. (Shanghai, China) (Table 2). Total RNA was extracted from cecum mucosa (50−100 mg) using a Trizol kit (Invitrogen, Carlsbad), and NanoDrop Lite (Thermo-Fisher Scientific, Waltham) was employed to measure the RNA concentration. Thereafter,1 μg of the RNA sample was used to synthesize cDNA using a reverse transcription kit (Vazyme, Nanjing, China). Real-time fluorescence quantitative PCR (qRT-PCR) analysis was conducted on a CFX96 contact real-time PCR detection system (Bio-Rad, Hercules) using a qRT-PCR kit (Vazyme, Nanjing, China), with *GAPDH* as the internal reference gene. The qRT-PCR premix contained quantitative primers and the cDNA template. The experiment was conducted in triplicate, and the expression level of the target gene was calculated using the delta-delta ($2^{-\Delta\Delta Ct}$) method.

DNA Extraction and 16S rRNA Sequencing

Fresh cecal digesta samples of broilers in the 4 treatment groups (control, DQ, CUR 100, and CUR 150) were used to evaluate the cecal microflora community (n = 6). The cecal digesta microbiota genomic DNA *16S rRNA* v3-v4 region was sequenced using high-throughput sequencing method (Illumina NovaSeq 6000, 250 PE). Briefly, microbial genomic DNA was extracted from the samples using the E.Z.N.A. DNA Kit (Omega Bio-tek, Norcross, GA), according to the manufacturer's protocol. After the *16S rRNA* gene v3-v4 region amplification, the resulting PCR products were extracted and purified. The purified amplicons were then pooled in equimolar and subjected to paired-end sequencing on an Illumina MiSeq platform (Illumina, San Diego). The sequencing process and instruments were provided by Personalbio Technology CO., Ltd (Shanghai, China).

Bioinformatics Analyses of the 16S rRNA Sequencing Data

The principal coordinate analysis (**PCoA**), based on Bray-Curtis distance, and analysis of similarity (**ANOSIM**) were used to detect differences between the groups. Differential bacteria in the pairwise comparisons were determined using the linear discriminant analysis (**LDA**) effect size (**LEfSe**) method (LDA>2.5). Mantel's correlation was used to examine the correlation between the bacteria and the inflammatory and anti-antioxidant parameters. All analyses were conducted on the free online platform of Genes Cloud Platform (www.genescloud.cn).

Statistical Analysis

SPSS 26.0 software was used for statistical data analysis, and the control and DQ groups were compared using the t-test. ANOVA and Duncan's method were adopted for the multiple comparisons between the DQ and control group. The orthogonal polynomial method was employed to detect the linear and quadratic effects

Table 2. Sequence of primers for real-time PCR.

Genes	Primer sequence (5′→3′)	Accession No.
Keap1[1]	F: TGCCCCTGTGGTCAAAGTG R: GGTTCGGTTACCGTCCTGC	XM_015274015.1
Nrf2[2]	F: CACCAAAGAAAGACCCTCCT R: GAACTGCTCCTTCGACATCA	XM_015289381.3
HO-1[3]	F: CCGCTATTTGGGAGACCT R: CTCAAGGGCATTCATTCG	NM_205344.1
NQO-1[4]	F: TCTCTGACCTCTACGCCAT R: TCTCGTAGACAAAGCACTCGG	NM_001277621.1
GSH-Px[5]	F: GCTGTTCGCCTTCCTGAGAG R: GTTCCAGGAGACGTCGTTGC	NM_001277853.1
SOD1[6]	F: AGGGAGGAGTGGCAGAAGT R: GCTAAACGAGGTCCAGCAT	NM_205064.1
CAT[7]	F: GTTGGCGGTAGGAGTCTGGTCT R: GTGGTCAAGGCATCTGGCTTCTG	NM_001031215.2
Bcl-2[8]	F: GCTGCTTTACTCTTGGGGGT R: CTTCAGCACTATCTCGCGGT	NM_205339.2
Bax[9]	F: GGTGACAGGGATCGTCACAG R: TAGGCCAGGAACAGGGTGAAG	XM_422067
Caspase-3[10]	F: TGGTGGAGGTGGAGGAGC R: TGTCTGTCATCATGGCTCTTG	NM_204725.1
TNF-α[11]	F: TGTGTATGTGCAGCAACCCGTAGT R: GGCATTGCAATTTGGACAGAAGT	NM_204267.1
IL-1β[12]	F: GGTCAACATCGCCACCTACA R: CATACGAGATGCAAACCAGCAA	NM_204524.1
IL-4[13]	F: GTGCCCACGCTGTGCTTAC R: AGGAAACCTCTCCCTGGATGTC	NM_001007079.1
IL-6[14]	F: AAATCCCTCCTCGCCAATCT R: CCCTCACGGTCTTCTCCATAAA	NM_204628.1
IL-10[15]	F: ATCCAACTGCTCAGCTCTGAACTG R: GGCAGGACCTCATCTGTGTAGAAG	NM_001004414.2
GAPDH[16]	F: GGCTGCTAAGGCTGTGGG R: ATCATCATACTTGGCTGGTTTC	NM_204305.1

[1-16]*Keap1*, kelch-like ECH-associated protein 1; *Nrf2*, nuclear factor E2-related factor 2; *HO-1*, heme oxygenase-1; *NQO-1*, NAD (P) H: quinone oxidoreductase; *GSH-Px*, glutathione peroxidase; *SOD1*, copper and zinc superoxide dismutase; *CAT*, catalase; *Bcl-2*, B-cell lymphoma/leukemia; Abbreviations: *Bax*, BCL2 Associated X protein; *Caspase-3*, cysteine-aspartic acid protease-3; *TNF-α*, tumor necrosis factor-α; *IL-1β*, interleukin-1β; *IL-4*, interleukin-4; *IL-6*, interleukin-6; *IL-10*, interleukin-10; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

of CUR. The experimental results were expressed as the mean with standard error mean, and $P < 0.05$ indicated a significant difference. Differential taxa among the 4 groups were assessed using the Kruskal−Wallis test with $P < 0.05$.

RESULTS

Antioxidant Indexes of the Cecal Mucosa

Compared with the control group, the CAT activity and ROS level in the cecal mucosa of the DQ group increased significantly ($P < 0.05$) (Table 3). The TAOC activity in the cecum of broilers in the CUR group exhibited an increasing trend ($P = 0.052$), and their SOD activity increased ($P < 0.05$), but the MDA and ROS levels decreased significantly ($P < 0.05$) compared with DQ group. The activities of TAOC, CAT, and SOD presented quadratic curve changes ($P < 0.05$). The MDA and ROS levels decreased linearly ($P < 0.05$) with the increasing CUR concentration.

Inflammation Indexes of the Cecal Mucosa

As shown in Table 4, the levels of TNF-α and IL-6 in the cecal mucosa of broilers in the DQ group were

Table 3. Effect of curcumin on antioxidant indexes of cecal mucosa of diquat-challenged broilers.

Items	Control group	DQ[1]	CUR 100[2]	CUR 150[3]	SEM[4]	*P*-value[5]		
						ANOVA	Linear	Quadratic
TAOC[6] (U/mg prot)	34.86	32.49	38.43	35.31	1.03	0.052	0.221	0.032
GSH-Px[7] (U/g prot)	36.19	39.18	37.28	43.76	1.36	0.131	0.158	0.138
SOD[8] (U/g prot)	651.26	619.44[a]	721.44[b]	720.19[b]	14.88	0.001	0.001	0.027
CAT[9] (U/mg prot)	4.25*	4.77	4.46	4.79	0.08	0.124	0.909	0.045
MDA[10] (nmol/g prot)	2.14	2.34[b]	2.01[a]	1.93[a]	0.06	0.003	0.001	0.198
ROS[11] (U/mg prot)	724.40*	802.52[b]	735.04[a]	746.95[a]	10.94	0.016	0.021	0.051

*There was significant difference ($P < 0.05$) between the control and DQ groups.

[a,b,c]Within a row, means with different superscripts differ significantly ($P < 0.05$). Values are means (n = 8).

[1-11]DQ, diquat group; CUR 100, 100 mg/kg curcumin supplementation group; CUR 150, 150 mg/kg curcumin supplementation group; SEM, the standard error of the means; The linear and quadratic effects of curcumin were detected by orthogonal polynomials (DQ, CUR 100 and CUR 150 groups); TAOC, total antioxidant capacity; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase; MDA, malondialdehyde; ROS, reactive oxygen species.

Table 4. Effect of curcumin on inflammatory indexes of cecal mucosa of diquat-challenged broilers.

Items	Control group	DQ[1]	CUR 100[2]	CUR 150[3]	SEM[4]	*P*-value[5]		
						ANOVA	Linear	Quadratic
TNF-α[6], pg/mg prot	29.54*	39.07[b]	32.92[ab]	25.65[a]	1.88	0.006	0.002	0.855
IL-1β[7], pg/mg prot	23.22	26.25	19.45	21.98	1.20	0.053	0.116	0.053
IL-4[8], pg/mg prot	5.98	7.18	6.58	6.47	0.24	0.453	0.247	0.645
IL-6[9], pg/mg protт	71.99*	83.06	77.52	86.40	2.13	0.240	0.519	0.121
IL-10[10], pg/mg prot	15.31	13.75[a]	18.83[b]	17.80[b]	0.77	0.008	0.014	0.028

*There was significant difference ($P < 0.05$) between the control and DQ groups.
[a,b,c]Within a row, means with different superscripts differ significantly ($P < 0.05$). Values are means (n = 8).
[1-10]DQ, diquat group; CUR 100, 100 mg/kg curcumin supplementation group; CUR 150, 150 mg/kg curcumin supplementation group; SEM, the standard error of the means; The linear and quadratic effects of curcumin were detected by orthogonal polynomials (DQ, CUR 100 and CUR 150 groups); TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; IL-4, interleukin-4; IL-6, interleukin-6; IL-10, interleukin-10.

significantly higher than those in the control group ($P < 0.05$). Compared with the DQ group, the level of TNF-α decreased significantly ($P < 0.05$) in the CUR 150 group, while the levels of IL-10 increased significantly ($P < 0.05$) in the CUR 100 and CUR 150 groups. However, the level of TNF-α decreased linearly ($P < 0.05$), while that of IL-10 was increased quadratically ($P < 0.05$) with increasing CUR concentration.

Contents of Volatile Fatty Acid in the Cecum

Compared with the control group, the content of total volatile fatty acids in the cecum of broilers in the DQ group decreased significantly ($P < 0.05$) (Table 5). The levels of acetic acid and total volatile fatty acids increased linearly with the increasing CUR concentration, and the increment was significantly higher ($P < 0.05$) in the CUR 150 group compared with DQ group.

Structure and Morphology of the Cecum

As shown in Figure 1, the mucosal layer, submucosa, muscular layer, and outer membrane structure of cecum tissue of samples in control group were clear with obvious layers. In the control group, mucosal epithelium and lamina propria protruded, forming folds that were long, with low intestinal villi, and the lymph follicles could be observed in some areas. The submucosa mainly contained loose connective tissues, and the muscular layer was developed and thick and was closely connected with the adventitia. No obvious pathological changes were found. However, compared with the control group, the cecum of broilers in the DQ group showed local epithelial necrosis and shedding, with an expanded intestinal gland in the lamina propria containing necrotic cell fragments. A small amount of epithelial necrosis was found in the cecum of broilers in the CUR 100 group, with some areas missing. Compared with the control group, no obvious pathological changes were found in the cecum of broilers in the CUR 150 group.

Expression Level of Genes Related to the Cecum Mucosa

Compared with the control group, the expression of *SOD1* in the cecum mucosa of broilers in the DQ group decreased significantly ($P < 0.05$), while that of *CAT, BAX, Caspase-3, TNF-α*, and *IL-4* increased significantly ($P < 0.05$) (Table 6). Moreover, the expression of *GSH-Px* and *SOD1* increased significantly ($P < 0.05$), while that of *Caspase-3* decreased significantly ($P < 0.05$) in the CUR 100 group compared with the DQ group. In the CUR 150 group, the expression of *Nrf2, SOD1,* and *CAT* increased significantly ($P < 0.05$), while that of *Caspase-3* and *TNF-α* decreased significantly ($P < 0.05$). The expression of *Caspase-3* showed a quadratic curve change ($P < 0.05$) with the increasing CUR concentration. The expression of *Nrf2, SOD1, CAT*, and *IL-10* increased linearly ($P < 0.05$), while that

Table 5. Effect of curcumin on volatile fatty acids in cecum of diquat-challenged broilers.

Items	Control group	DQ[1]	CUR 100[2]	CUR 150[3]	SEM[4]	*P*-value[5]		
						ANOVA	Linear	Quadratic
Acetic acid, mmol/L	25.10	20.92[a]	23.49[ab]	29.06[b]	1.24	0.013	0.004	0.487
Propionic acid, mmol/L	5.22	4.89	5.08	4.51	0.16	0.379	0.358	0.296
Butyric acid, mmol/L	6.17	6.35	6.54	6.61	0.20	0.877	0.627	0.897
Isobutyric acid, mmol/L	0.65	0.72	0.70	0.58	0.03	0.147	0.072	0.443
Pentanoic acid, mmol/L	2.51	3.00	3.16	2.85	0.10	0.470	0.551	0.287
Isopentanoic acid, mmol/L	0.50	0.56	0.53	0.59	0.02	0.679	0.691	0.439
TVFA[6], mmol/L	43.62*	38.83[a]	42.79[ab]	47.92[b]	1.37	0.014	0.004	0.803

*There was significant difference ($P < 0.05$) between the control and DQ groups.
[a,b,c]Within a row, means with different superscripts differ significantly ($P < 0.05$). Values are means (n = 8).
[1-6]DQ, diquat group; CUR 100, 100 mg/kg curcumin supplementation group; CUR 150, 150 mg/kg curcumin supplementation group; SEM, the standard error of the means; The linear and quadratic effects of curcumin were detected by orthogonal polynomials (DQ, CUR 100 and CUR 150 groups); TVFA, total volatile fatty acids.

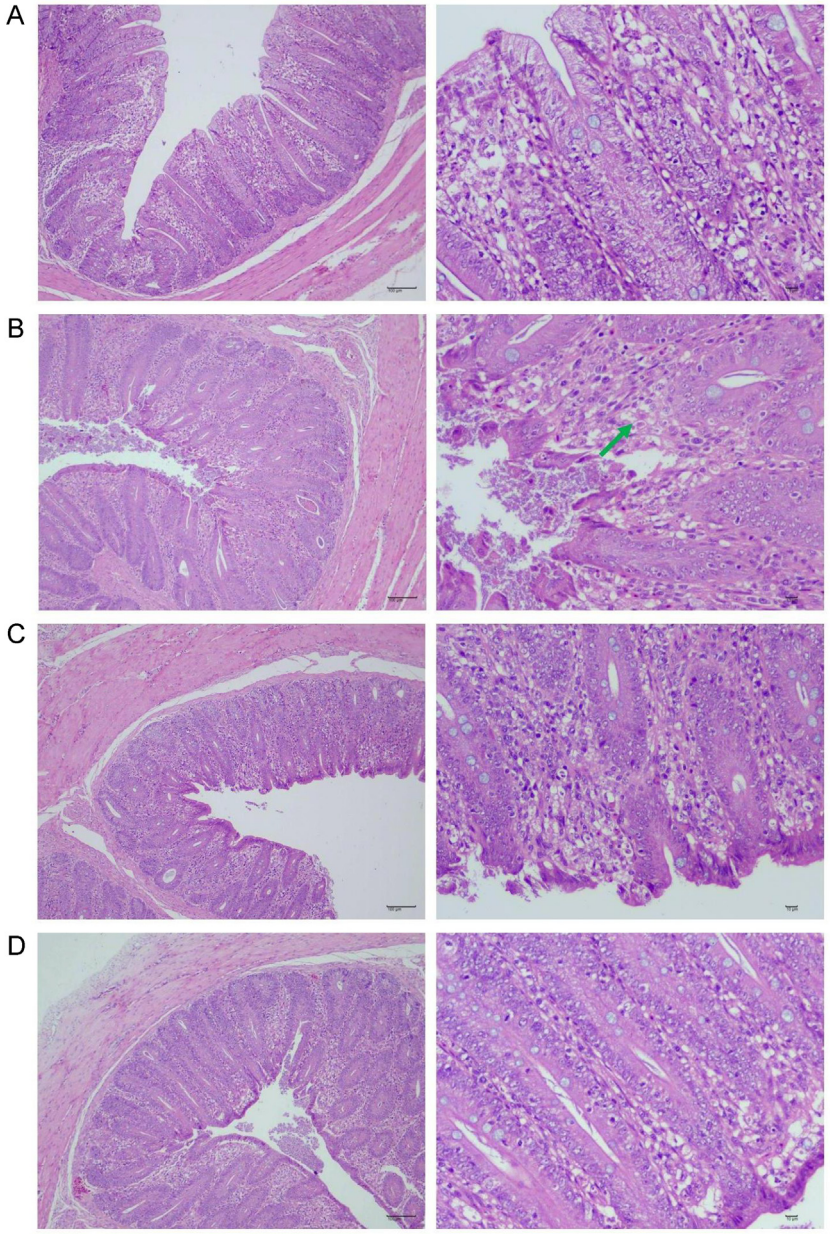

Figure 1. Effects of curcumin on the cecal structure and morphology of diquat-challenged broilers. (A) control group, (B) group treated with diquat, (C) group treated with 100 mg/kg curcumin + diquat and (D) (group treated with 150 mg/kg curcumin + diquat). Images in Figure 1, labeled as A, B, C, and D, were captured at magnifications of X100 (left) and X400 (right) (n = 8). Key: green- lamina propria degeneration and necrosis. The magnifications used were X100 and X400, with scales of 100 μm and 10 μm, respectively.

of *Bax* and *TNF-α* decreased linearly ($P < 0.05$) with the increasing CUR concentration.

Cecal Microflora Structure and Composition of Broilers

The dilution curve showed a continuous trend, indicating that the sequencing data was appropriate, and the diversity of all samples was saturated (Figure 2A). The operational taxonomic unit (OTU) rank curve showed that all samples were close to saturation, suggesting that the data had enough depth to capture the diversity information of most samples (Figure 2B). We compared the alpha diversity indexes for intestinal microbial groups in each group, and the results showed no significant differences in the Shannon and Chao1 indexes among groups (Figure 2C). The Venn diagram shows that there were 2,033 unique OTUs in the control group, 2,070 in the DQ group, 2,764 in the CUR 100 group, and 2,428 in the CUR 150 group, with 686 common OTUs in the 4 groups (Figure 2D). The PCoA analysis showed that principal component 1 (**PCA1**) could explain 52.0% of the total cecal flora, while principal component 2 (**PCA2**) could explain 16.4% of the total cecal flora, and both PCA1 and PCA2 could explain 68.4% of the total cecal flora (Figure 2E). Furthermore, ANOSIM analysis demonstrated significant differences in intestinal flora structure among the groups ($P < 0.05$) (Figure 2F).

At the phylum level, the top 10 abundant flora in the cecal contents of broilers were *Firmicutes,*

Table 6. Effects of curcumin on expression level of related genes in cecal mucosa of diquat-challenged broilers.

						P-value[5]		
Items	Control group	DQ[1]	CUR 100[2]	CUR 150[3]	SEM[4]	ANOVA	Linear	Quadratic
Keap1[6]	1.00	0.90	1.29	1.14	0.08	0.099	0.171	0.088
Nrf2[7]	1.00	0.97[a]	1.11[ab]	1.23[b]	0.04	0.032	0.010	0.891
HO-1[8]	1.00	1.07	1.16	1.13	0.03	0.519	0.455	0.390
NQO-1[9]	1.00	0.89	0.97	1.04	0.04	0.302	0.128	0.952
GSH-Px[10]	1.00	0.85[a]	1.24[b]	1.13[ab]	0.07	0.044	0.072	0.064
SOD1[11]	1.00*	0.66[a]	0.84[b]	0.88[b]	0.04	0.039	0.016	0.374
CAT[12]	1.00*	1.32[a]	1.26[a]	1.73[b]	0.08	0.032	0.031	0.098
BAX[13]	1.00*	1.42	1.32	1.10	0.06	0.071	0.026	0.602
Bcl-2[14]	1.00	0.93	1.25	1.18	0.06	0.065	0.076	0.106
Caspase-3[15]	1.00*	1.86[b]	1.22[a]	1.39[a]	0.09	0.002	0.008	0.007
TNF-α[16]	1.00*	1.30[a]	1.10[ab]	1.03[b]	0.05	0.040	0.016	0.408
IL-1β[17]	1.00	1.15	1.17	0.98	0.04	0.079	0.071	0.154
IL-4[18]	1.00*	1.27	1.15	1.31	0.05	0.484	0.781	0.248
IL-6[19]	1.00	0.86	0.94	0.87	0.04	0.687	0.871	0.403
IL-10[20]	1.00	0.92	1.07	1.21	0.05	0.059	0.019	1.000

[*]There was significant difference ($P < 0.05$) between the control and DQ groups.

[a,b,c]Within a row, means with different superscripts differ significantly ($P < 0.05$). Values are means (n = 8).

[1-20]DQ, diquat group; CUR 100, 100 mg/kg curcumin supplementation group; CUR 150, 150 mg/kg curcumin supplementation group; SEM, the standard error of the means; The linear and quadratic effects of curcumin were detected by orthogonal polynomials (DQ, CUR 100 and CUR 150 groups); *Keap1*, Kelch-like ECH-associated protein 1; *Nrf2*, nuclear factor E2-related factor 2; *HO-1*, Heme oxygenase-1; *NQO-1*, NAD (P) H: quinone oxidoreductase 1; *GSH-Px*, Glutathione peroxidase; *SOD1*, copper and zinc superoxide dismutase; *CAT*, catalase; *Bax*, BCL2 Associated X protein; *Bcl-2*, B-cell lymphoma/leukemia 2; *Caspase-3*, cysteine-aspartic acid protease-3; *TNF-α*, tumor necrosis factor-α; *IL-1β*, interleukin-1β; *IL-4*, interleukin-4; *IL-6*, catalase; *IL-10*, interleukin-10.

Bacteroidetes, Proteobacteria, Tenericutes, Cyanobacteria, Verrucomicrobia, Actinobacteria, Lentisphaerae, Fusobacteria, and *Gemmatimonadetes* in that order (Figure 3A). At the family level, the top 10 abundant flora in the cecal contents of broilers were *Ruminococcaceae, Rikenellaceae, Lachnospiraceae, Bacteroidaceae, Barnesiellaceae, Helicobacteraceae, Porphyromonadaceae, Lactobacillaceae, Erysipelotrichaceae* and *Odoribacteraceae* in that order (Figure 3B). At the genus level, the top 10 abundant flora in the cecal contents of broilers were *Bacteroides, Oscillospira, Alistipes, Helicobacter, Ruminococcus, Parabacteroides, Lactobacillus, Butyricicoccus, Faecalibacterium* and *AF12* in that order (Figure 3C). Figure 3D shows the taxonomic composition of gut bacteria. *Firmicutes* was largely represented by *g_ Oscillospira* (6%) and *g_ Ruminococcus* (4%). *Bacteroidetes* was dominated by *g_ Bacteroides* (10%) and *g_ Alistipes* (4%).

Differential Flora Among Groups

As shown in Figure 4, DQ significantly reduced the relative abundance of Lactobacillaceae at the family level, but the supplementation of 100 and 150 mg/kg CUR significantly reversed this effect ($P < 0.05$). Similarly, the relative abundance of *Lactobacillus* was increased at the genus level, and the inhibitory effect of DQ was significantly reversed by supplementing 100 mg/kg CUR ($P < 0.05$). Compared with the control group, the supplementation of 150 mg/kg CUR significantly increased the relative abundance of *Ruminococcaceae_ Clostridium* ($P < 0.05$), but DQ did not have significant effects on the strain. However, DQ significantly reduced the relative abundance of *Victivallis* and *Bacillus* ($P < 0.05$) compared with control group, but the effect was not alleviated after supplementing CUR. At the species level, DQ significantly increased the relative abundance of *Saccharopolyspora_ hirsuta* and *Staphylococcus_ succinus* compared with control group, but the relative abundance of these 2 strains was significantly decreased after supplementing 100 and 150 mg/kg CUR ($P < 0.05$). As shown in Figure 3D, *Lactobacillus* was the most abundant genus in Lactobacillaceae, with a relative abundance of 2%. In addition, LEfSe analysis was conducted to further screen CUR-related probiotics. As shown in Figure 5, the LDA values of Lactobacillaceae and *Lactobacillus* were the highest in the pairwise comparison group. Therefore, we speculated that Lactobacillaceae and *Lactobacillus* may play a key role in the alleviative effect of CUR on oxidative stress and inflammatory reactions caused by DQ.

Relationship Between the Intestinal Flora and the Antioxidant Activity and Inflammatory Indexes

Mantel analysis was employed to identify the correlation between intestinal flora and differential indexes. As shown in Figure 6A, there was a strong correlation between bacteria and SOD and ROS activities (SOD: $R = 0.760$, $P = 0.001$; ROS: $R = 0.613$, $P = 0.001$). Bacteria also showed a significant correlation with the MDA and IL-10 levels (MDA: $R = 0.237$, $P = 0.02$; IL- 10: $R = 0.204$, $P = 0.029$). *Lactobacillaceae* and *Lactobacillus* were negatively correlated with the ROS and TNF-α levels ($P < 0.05$), while *Ruminococcaceae_ Clostridium* was positively correlated with CAT activity ($P < 0.05$) (Figure 6B).

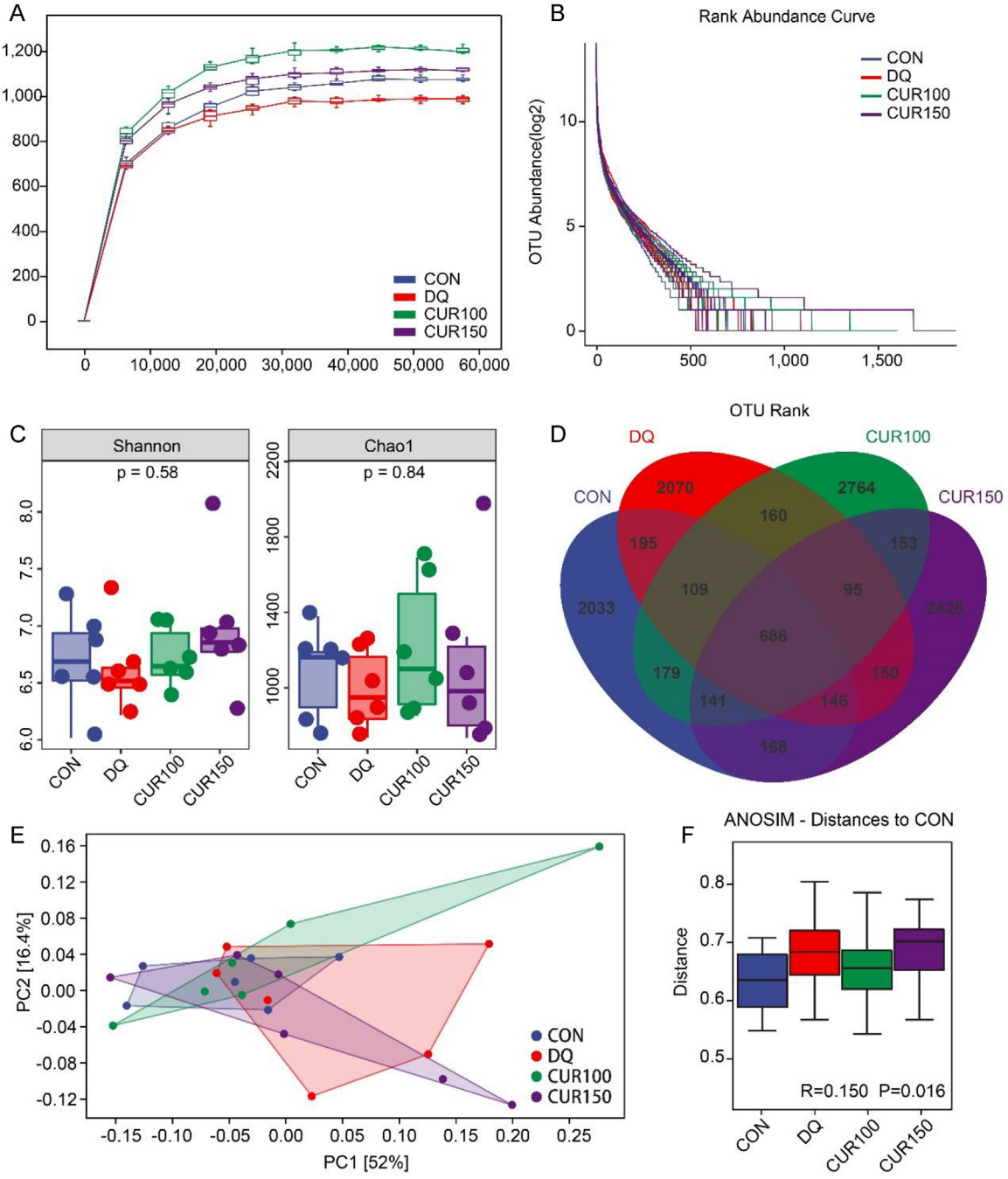

Figure 2. The composition and structure of intestinal microflora in different treatments. (A) Rarefaction curve. (B) OTU rank abundance curve. (C) Effect of curcumin on α-diversity of intestinal microbiota. (D) Venn of the OTUs in the caecum content among different dietary treatments. (E) Weighted UniFrac Principal coordinate analysis (PCoA) plots of the intestinal microbiota of different treatments. (F) Analysis of similarities (ANOSIM) based on Bray−Curtis distance (n = 6). CON, control group; DQ, group treated with diquat; CUR 100, group treated with 100 mg/kg curcumin + diquat; CUR 150, group treated with 150 mg/kg curcumin + diquat.

DISCUSSION

Diquat has been commonly used to develop oxidative stress models for various experimental animals, including broilers, mice, and pigs (Chen et al., 2020; Jones and Vale, 2000; Li et al., 2022; Qiao et al., 2022). In this experiment, the ROS level in the cecum mucosa of broilers in the DQ group increased, indicating that DQ induced excessive production of free radicals in the cecum. However, this effect was reversed by CUR dietary supplementation. SOD activity was significantly higher in the CUR 100 and CUR 150 groups as compared to the DQ group, but their MDA and ROS levels were significantly lower as compared to the DQ group. This might have been due to the antioxidant activity of CUR including inhibiting the chain reaction and reducing the level of free radicals (Kaneko and Baba, 1999). In addition, CUR can regulate the expression of genes encoding antioxidant enzymes and increase the activity of antioxidant enzymes thus enhance the antioxidant capacity of the body (Zhang et al., 2018). The Nrf2/ARE signaling pathway is a part of the antioxidant

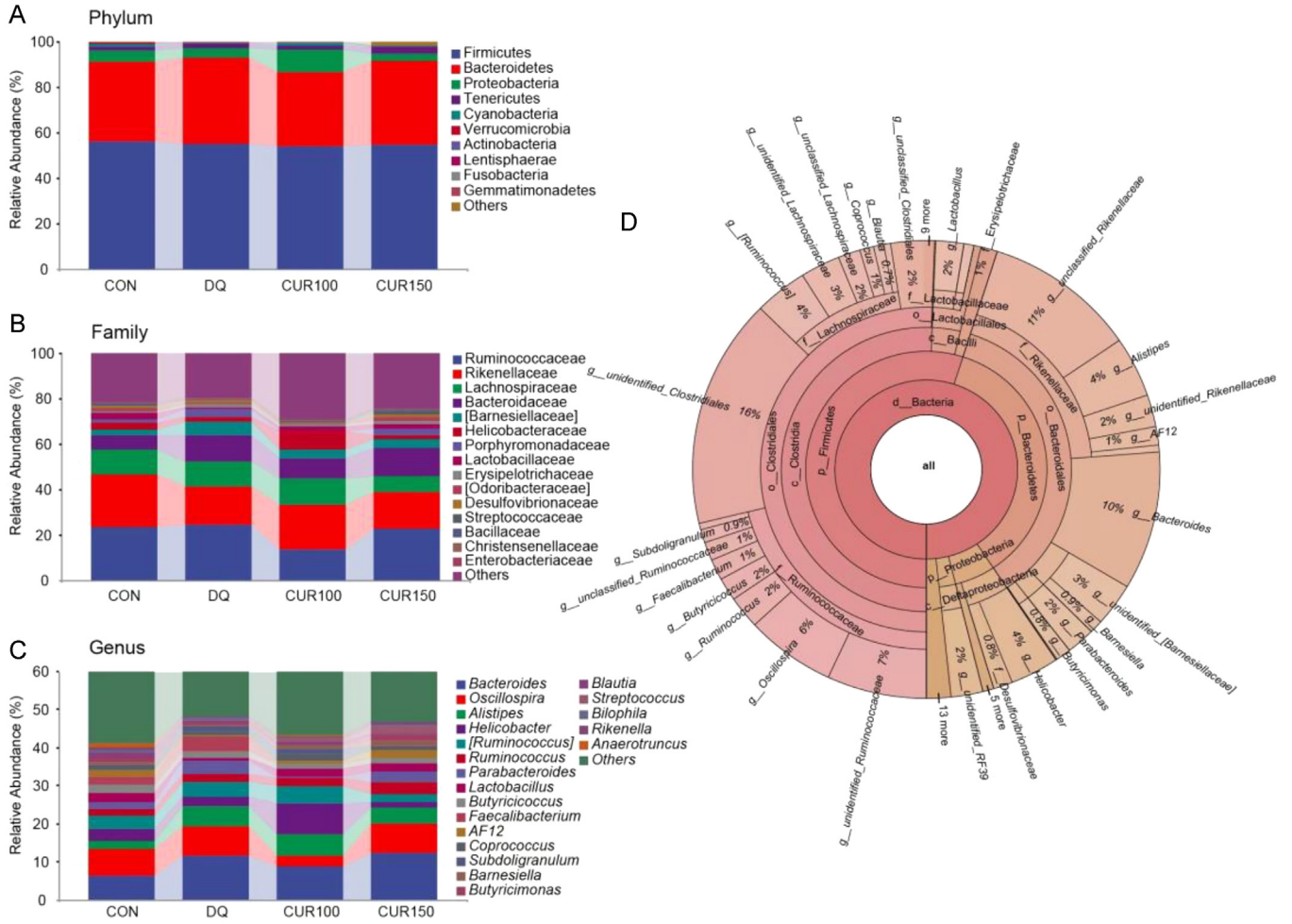

Figure 3. Taxonomic composition of intestinal microflora in different treatments. The relative abundance of gut bacteria at the (A) phylum, (B) family and (C) genus level in different treatments. (D) Taxonomic composition of gut bacteria (n = 6). CON, control group; DQ, group treated with diquat; CUR 100, group treated with 100 mg/kg curcumin + diquat; CUR 150, group treated with 150 mg/kg curcumin + diquat.

system and an important regulator of free radical homeostasis (Liu et al., 2022). In this experiment, the expression of *Nrf2, GSH-Px, SOD1*, and *CAT* in the cecal mucosa was significantly increased after CUR supplementation, suggesting that CUR enhanced the activity of downstream antioxidant enzymes through the Nrf2/ARE signaling pathway and alleviated the effect of DQ on the broiler cecum. It has been reported that CUR could also alleviate acute ileal injury caused by aflatoxin B1 in ducks through the Nrf2-ARE and NF-κB signaling pathways (Jin et al., 2021). These reports are similar to the results of this experiment.

The antioxidant system is closely associated with inflammatory reactions when maintaining the homeostasis of the immune response in the body. ROS can induce inflammation through the NF-κB or NALP-3 pathway, and the inflammatory reaction can, in turn, promote ROS production (Martinon et al., 2010). In this experiment, the expression level of *TNF-α* in the cecal mucosa of broilers in the DQ group increased significantly, indicating that an inflammatory reaction was induced. However, the CUR supplementation significantly reduced the expression level of *TNF-α* and significantly increased the level of IL-10, indicating that the inflammatory reaction was alleviated by CUR supplementation. This was due to the anti-inflammatory activity of CUR, which can inhibit the activities of key enzymes in the synthesis of inflammatory mediators such as cyclooxygenase and lipoxygenase (Rao et al., 2007). Furthermore, CUR play an anti-inflammatory role by regulating the expression of key inflammatory genes and proteins (Tian et al., 2016). A previous study reported that CUR could modulate NF-κB inflammatory pathway, thus alleviating the spleen inflammation in broilers caused by decabrominated diphenyl ethers (Wang et al., 2023). Volatile fatty acids are one of the energy sources for intestinal mucosal epithelial cells, which can promote the proliferation and maturation of intestinal cells and are important in regulating intestinal function and immune response and maintaining the stability of flora structure. In this experiment, the concentration of acetic acid and total volatile fatty acids in the cecum of broilers in the CUR 150 group was significantly higher than that in the DQ group, indicating that CUR promoted acetic acid production. This might have been due to the relative abundance of

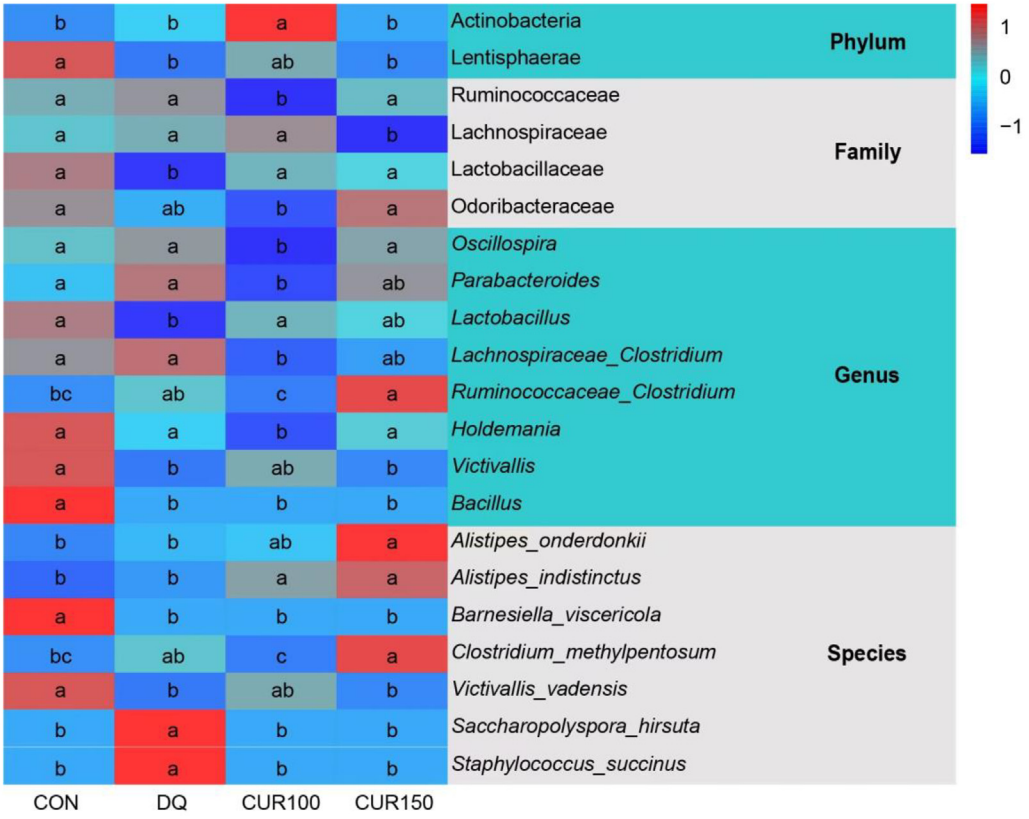

Figure 4. Difference test of the relative abundance of dominant gut bacteria in different treatments (Kruskal−Wallis test) (n = 6). CON, control group; DQ, group treated with diquat; CUR 100, group treated with 100 mg/kg curcumin + diquat; CUR 150, group treated with 150 mg/kg curcumin + diquat.

Ruminococcaceae_ Clostridium in the cecum of broilers in the CUR 150 group was significantly higher than that in the control group. It has been reported that Ruminococcaceae exhibits an important role in starch degradation and fiber fermentation and can promote the production of volatile fatty acids (Shi et al., 2023). As the main component of volatile fatty acids, acetic acid is directly correlated with the content of total volatile fatty acids. In addition, the positive effects of CUR on maintaining cecum homeostasis might have also promoted acetic acid production.

DQ treatment resulted in intestinal epithelial necrosis and shedding, intestinal gland expansion in lamina propria, intestinal cell necrosis, and other pathological injuries in the cecum of broilers, similar to the previously reported DQ-induced damage to duodenum, jejunum and ileum in broilers (Nong et al., 2023). This result indicates that the DQ injection was successfully induced a cecal injury model at the physiological level. *Bax* and *Caspase-3* genes play an important role in apoptosis regulation and thus can promote apoptosis (Ola et al., 2021). The expression of *Bax* and *Caspase-3* in the cecum of broilers in the DQ group was significantly higher than that of the control group, further confirmed the DQ-induced damage to the cecum. This might have been due to the oxidative stress and inflammatory reaction caused by DQ in the cecum. The cecum injury in broilers was alleviated in the CUR 100 group, and no obvious pathological changes were observed in the CUR 150 group. This might have been because CUR can alleviate the oxidative stress and inflammatory reaction caused by DQ through the Nrf2/ARE pathway. It was reported that CUR could alleviate the ileum injury caused by aflatoxin B1-induced oxidative stress and inflammatory reaction in ducks through the Nrf2-ARE and NF-κB signaling pathways (Jin et al., 2021). This report is similar to the results of the present study. Intestinal microorganisms in chickens play an important role in digesting and absorbing nutrients, establishing and developing immune systems, and maintaining the intestinal environment and immune homeostasis (Mindus et al., 2021; Shehata et al., 2022). Among the intestinal organs of broilers, the cecum has the largest number of microorganisms. In this experiment, there were significant differences in the cecum flora structure among the groups, indicating that DQ and CUR treatments significantly impacted the cecum flora structure. This was probably because oxidative stress is one of the causes of intestinal microecological and structural imbalance of flora, and CUR can alleviate the effect of stress on intestinal microorganisms by enhancing intestinal antioxidation and anti-inflammatory activities (Tomasello et al., 2016; Lopresti et al., 2018). Through analyzing the cecal microbial composition in the cecum of broilers, Broom et al. (2018) found that the F*irmicutes, Bacteroides* and *Proteus* were the most abundant species at the phylum

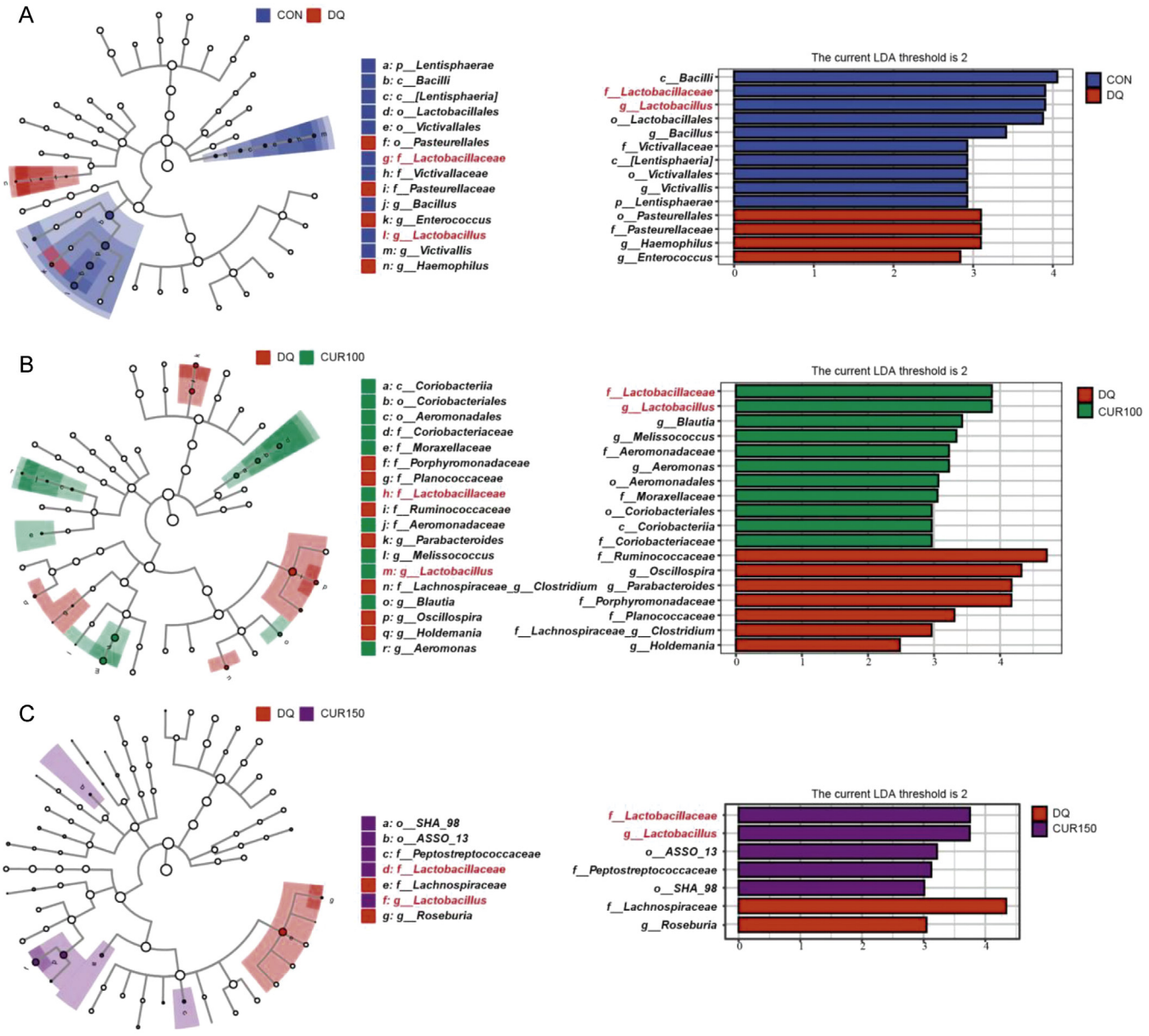

Figure 5. LEfSe analysis of significant difference species in broiler gut microbiota. (A) DQ vs. CON. (B) CUR100 vs. CON. (C) CUR150 vs. CON. (Left: cladogram diagram; Right: LDA diagram; LDA≥2.5) (n = 6). CON, control group; DQ, group treated with diquat; CUR 100, group treated with 100 mg/kg curcumin + diquat; CUR 150, group treated with 150 mg/kg curcumin + diquat.

level, consistent with the results of this experiment. Firmicutes and Bacteroides play an important role in digesting nonstarch polysaccharides, thus promoting the production of volatile fatty acids in the cecum (Clavijo et al., 2018). This experiment indicated that *Lactobacillus* and Lactobacillaceae were significantly negatively correlated to ROS and TNF-α levels in cecum mucosa and might have promoted the alleviative effect of CUR on DQ-induced oxidative stress and inflammatory reaction in the cecum. *Lactobacillus spp* can inhibit ROS production by inhibiting the activities of key enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and enhance the ROS scavenging ability of the antioxidant system by regulating the Nrf2/ARE and NF-κB signal pathways (Clavijo et al., 2018). Moreover, *Lactobacillus spp* can inhibit the expression of *TNF-α* through NF-κB pathway to alleviate the inflammatory reaction in the intestine (Kong et al., 2020). In addition, there was a significant positive correlation between the abundance of *Ruminococcaceae_ Clostridium* and the CAT activity in the cecum. CUR could alleviate the oxidative stress and inflammatory reaction caused by DQ in the cecum by regulating cecal flora, and *Lactobacillus* and Lactobacillaceae may promote the alleviative effect of CUR. Thus, based on the current dosage levels, the recommended amount of CUR supplementation in the broiler diet is 150 mg/kg.

In summary, CUR could alleviate the cecal injury caused by DQ-induced oxidative damage and inflammatory reactions through regulating the Nrf2-ARE signaling pathway and intestinal flora. The recommended dosage for broiler diets is 150 mg/kg curcumin.

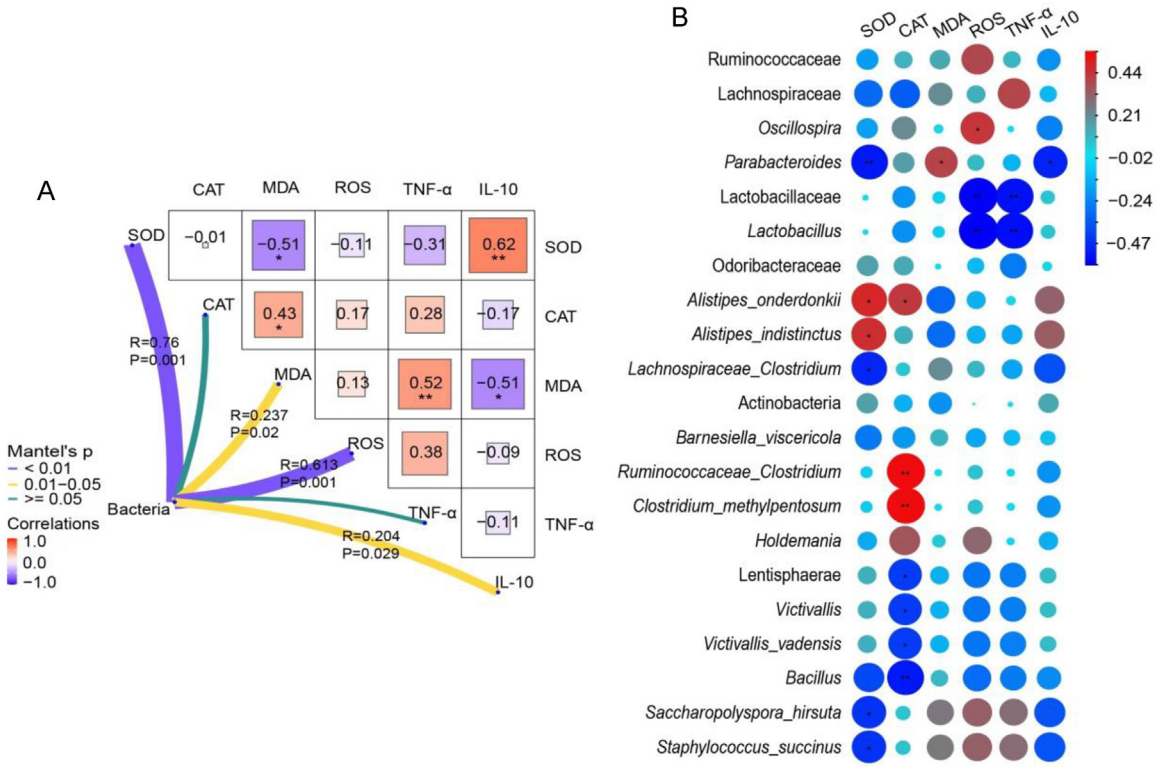

Figure 6. Correlation between bacteria and inflammatory and antiantioxidant parameters. (A) Pairwise comparisons of inflammatory and anti-antioxidant parameters, with a color-gradient denoting Spearman's correlation coefficients. The correlation between differently abundant taxa and parameters was tested by partial Mantel tests. Edge width corresponds to the Mantel's statistic for the corresponding distance correlations, and edge color denotes the statistical significance. (B) Correlation coefficients between bacteria and inflammatory and anti-antioxidant parameters. Correlation coefficients >0.5 or ≤0.5, $^{*}P < 0.05$, $^{**}P < 0.01$. Color intensity is proportional to Spearman's rank correlation values (n = 6). CON, control group; DQ, group treated with diquat; CUR 100, group treated with 100 mg/kg curcumin + diquat; CUR 150, group treated with 150 mg/kg curcumin + diquat.

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DISCLOSURES

The authors declare no conflict of interest.

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