Chlorogenic acid improves growth performance and intestinal health through autophagy-mediated nuclear factor erythroid 2-related factor 2 pathway in oxidatively stressed broilers induced by dexamethasone

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ABSTRACT The effects of chlorogenic acid (**CGA**) on growth performance, intestinal morphology, antioxidant capacity, and the autophagy-mediated nuclear factor erythroid 2-related factor 2 (**Nrf2**) pathway in oxidatively stressed broilers were investigated. A total of 400 one-day-old male Cobb broilers were divided randomly into 4 groups using a 2 × 2 factorial arrangement with 2 CGA supplemental levels (0 and 500 mg/kg) and 2 dexamethasone (**DEX**) challenge levels (0 and 3 mg/kg body weight). All the broilers were injected intraperitoneally with DEX or sterile saline beginning at the age of 15 d for 6 consecutive days. The experiment lasted for 21 d. The CGA increased average daily gain (**ADG**), villus height, villus height/crypt depth (**V/C**) value, and the protein expressions of Occludin and ZO-1 in the ileum and decreased the feed:gain (**F:G**) ratio, which were impaired by the DEX challenge. Superoxide dismutase (**SOD**), catalase (**CAT**), gutathione S-transferase (**GST**), and heme oxygenase-1 (**HO-1**) activities in the serum and ileum were increased by CGA, whereas protein carboxyl (**PCO**) level in the serum and ileum, and malondialdehyde (**MDA**) level in the ileum were decreased of the DEX challenged broilers. The DEX challenge decreased microtubule-associated protein 1 light chain 3 (***LC3***)-II, *Beclin1*, and autophagy-related gene (***ATG***) 7 mRNA expressions, and the LC3-II/LC3-I value and increased *LC3-I*, cysteinyl aspartate specific proteinase (***Caspase***)-3 and Caspase-9 mRNA expressions in the ileum, which were improved by CGA. DEX also decreased the protein expressions of Kelch-like ECH-associated protein-1 (**Keap1**), Nrf2, HO-1, NADPH quinone oxidoreductase-1 (**NQO-1**) and increased sequestosome 1 (**p62**) in the ileum, which were improved by CGA. Interactions occurred between DEX and CGA for the ADG, F:G ratio, villus height, crypt depth, V/C value, and SOD, CAT, GST, and HO-1 activities, MDA and PCO levels, LC3-II/LC3-I value, and expressions of *LC3-I, LC3-II, Beclin1, ATG7, Caspase-3, Caspase-9*, Occludin, ZO-1, Keap1, Nrf2, HO-1, NQO-1, and p62. In conclusion, CGA improved the growth performance and intestinal health of oxidatively stressed broilers by activating the autophagy-mediated Nrf2 pathway.

Key words: autophagy, broiler, chlorogenic acid, intestinal health, oxidative stress

INTRODUCTION

Oxidative stress is hazardous to poultry health because of the frequent occurrence in intensive husbandry, and the high probability of affecting the intestinal health and growth performance of broilers (Sun et al., 2020; Miao et al., 2021). Some stress factors can stimulate the hypothalamic-pituitary-adrenal axis to release plethora of glucocorticoids (Zeng et al., 2014), inducing oxidative stress (Pan et al., 2019). An oxidative stress challenge by dexamethasone (**DEX,** a synthetic glucocorticoid) can affect the physiological and biochemical processes of animals and influence intestinal metabolism and functions (Vicuña et al., 2015; Osho and Adeola, 2020). Therefore, an injection of DEX has been used widely to establish an oxidative stress model in broilers (Gao et al., 2010). Previous studies showed that the adverse effects of oxidative stress, such as growth inhibition, leaky gut, and meat quality damage can be modulated by the dietary supplementation of natural antioxidants including vitamins and plant extracts

([Barekatain et al., 2019](); [Pan et al., 2019]()). Thus, it is of great interest to identify an effective natural antioxidant that may modulate the antioxidant system to protect broilers against oxidative stress.

Chlorogenic acid (**CGA**) is a polyphenol that is widely found in Chinese herbal medicines including *Flos lonicerae* and *Eucommia ulmoides* ([Upadhyay and Mohan Rao, 2013](); [Miao and Xiang, 2020]()). The CGA has a vicinal hydroxyl group on an aromatic residue that binds directly with free radicals to stop the chain reaction of reactive oxygen species generation ([Kono et al., 1997](); [Wu, 2007]()) and protect the enzymatic activity of the antioxidant system ([Koriem and Soliman, 2014]()). Our previous study demonstrated that CGA-enriched extract could alleviate the adverse effects of stress on growth performance and improve oxidative stability in heat-stressed broilers ([Zhao et al., 2019]()) and oxidatively stressed finishing pigs ([Li et al., 2020]()). In addition, autophagy serves as a defense mechanism to clear oxidatively damaged proteins and organelles during oxidative stress ([Scherz-Shouval and Elazar, 2007]()). A recent study reported that autophagy induced by CGA was involved in decreasing oxidative stress in human chondrocyte C28/I2 cells ([Zada et al., 2021]()). However, the autophagy property and mechanisms of CGA are still unknown in broilers, especially those subjected to oxidative stress. Therefore, the present study was designed to evaluate the effects of CGA on growth performance, intestinal morphology, antioxidant capacity, and autophagy-mediated nuclear factor erythroid 2-related factor 2 (**Nrf2**) pathway in oxidatively stressed broilers.

MATERIALS AND METHODS

Materials

The CGA (with a purity of 98%) was obtained from Changsha E.K Herb Co. Ltd., (Changsha, China). The DEX was purchased from the Beian Feilong Animal Pharmaceutical Factory (Beian, China).

Animals and Experimental Design

All procedures were approved by the Animal Care and Use Committee of Qingdao Agricultural University (Qingdao, China). The DEX is a synthetic glucocorticoid widely used to establish the stress models ([Gao et al., 2010]()). A total of 400 one-day-old male Cobb broilers were purchased from a commercial hatchery (Qingdao Zhengda Co., Ltd., Qingdao, China) and weighed (initial body weight 34.8 ± 0.18 g). The broilers were randomly assigned into one of 4 treatment groups, with 10 replicates in each group and 10 broilers per replicate. The 4 groups were basal diet (Control group), basal diet and intraperitoneal injection of DEX at 3 mg/kg body weight (DEX group), basal diet containing 500 mg/kg CGA (CGA group), and basal diet containing 500 mg/kg CGA and intraperitoneal injection of DEX at 3 mg/kg body weight (DEX-CGA group), in a 2×2 factorial arrangement with 2 CGA supplemental levels

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

Item	Content
Ingredients	
Corn	60.3
Soybean meal	32.4
Fish meal	1.89
Soybean oil	2.00
Limestone	1.29
CaHPO$_4$	1.29
DL-Methionine	0.22
NaCl	0.29
Premix[1]	0.23
Choline chloride	0.09
Total	100
Nutrient levels[2]	
Metabolizable energy (MJ/kg)	12.5
Crude protein (%)	20.6
Crude fat (%)	4.50
Calcium (%)	0.960
Available phosphorus (%)	0.440
Lysine (%)	1.11
Methionine (%)	0.540
Total sulfur amino acids (%)	0.840
Tryptophan (%)	0.260
Threonine (%)	0.790

[1]The premix provided the following per kg of diets: Vitamin A (trans-retinyl acetate) 10000 IU; vitamin D$_3$ (cholecalciferol) 2700 IU; vitamin E (all-rac-a-tocopherol acetate) 20 IU; vitamin B$_1$ (thiamin) 4.18 mg; vitamin B$_2$ (riboflavin) 4.03 mg; vitamin B$_6$ (pyridoxine HCl) 4.48 mg; vitamin B$_{12}$ (cobalamin) 0.014 mg; biotin 0.10 mg; folic acid 0.89 mg; *D*-pantothenic acid 10.7 mg; nicotinic acid 9.75 mg; Cu (as copper sulfate) 10.3 mg; Fe (as ferrous sulfate) 79.8 mg; Mn (as manganese sulfate) 79.8 mg; Zn (as zinc sulfate) 74.7 mg; I (as potassium iodide) 0.7 mg; Se (as sodium selenite) 0.3 mg.

[2]The nutrient levels were calculated values.

(0 and 500 mg/kg) and 2 DEX challenge levels (0 and 3 mg/kg body weight). Broilers in the challenge groups were injected intraperitoneally once daily with DEX beginning at the age of 15 d for 6 consecutive d, while the nonchallenged broilers were injected with the same volume of 0.9% (w/v) of saline solution. The experiment lasted for 21 d. During the experiment, fresh water and feed were provided ad libitum. The temperature in the broiler room was set at 34°C, then progressively lowered by 0.5°C per day to a final temperature of around 25°C. The basal diet was formulated to meet the requirements suggested by the National Research Council (NRC, 1994; [Table 1]()). The amounts of feed provided and refused were measured daily on a replicate basis to calculate the average daily feed intake (**ADFI**). Body weight was measured at d 1, 15, and 22 to calculate average daily gain (**ADG**) and the feed:gain (**F:G**) ratio on a replicate basis.

Sample Collections

At the end of the experiment and fasting for 12 h, one broiler of each replicate was selected randomly and killed by cervical dislocation. Blood samples were collected by cardiac puncture into vacuum tubes containing coagulant and centrifuged for 10 min ($3000 \times g$) at 4°C, and serum samples were stored in 1.5 mL Eppendorf tubes at −20°C until analysis. Two-centimeter segments from the median sections of the ileum were collected and

preserved in 4% paraformaldehyde for further morphological measurements. The midregions of the ileum (approximately 1 cm) were collected, rapidly frozen in liquid N$_2$, and stored at −80°C for further analysis.

Intestinal Morphology

The fixed ileum tissue was embedded in paraffin after being treated in 4% paraformaldehyde. Transverse 5 μm sections were stained with hematoxylin-eosin. The ileal villus height and crypt depth was measure using an Olympus microscope (Olympus, Tokyo, Japan) and HMIAS-2000 image analysis system. Villus height was measured from the tip of the villus to the crypt opening and the associate crypt depth was measured from the base of the crypt to the level of the crypt opening. Then the average values of villus height and crypt depth in each section were calculated, respectively. The ratio of villus height to relative crypt depth was calculated from these measurements (Shan et al., 2019).

Antioxidant Indices in Serum and Ileum

Approximately 0.1 g of each ileum sample was weighed and collected into 1.5 mL sterile centrifuge tubes. Then the sample was diluted 10 times with sterile saline and homogenized using a high-throughput tissue homogenizer (Jingxin, Shanghai, China) and centrifuged for 10 min (1000 × g) at 4°C to collect the supernatant for further analysis. The levels of malonaldehyde (**MDA**, Cat. MM-251702) and protein carboxyls (**PCO**, Cat. MM-3327502) and the activities of superoxide dismutase (**SOD**, Cat. MM-253302), catalase (**CAT**, Cat. MM-3328402), glutathione peroxidase (**GSH-Px**, Cat. MM-3419202), glutathione S-transferase (**GST**, Cat. MM-6021301), and hemeoxygenase-1 (**HO-1**, Cat. MM-193601) were determined spectrophotometrically in the serum and ileum using commercial kits (Jiangsu Enzyme industry Co., Ltd., Yancheng, China) according to the manufacturer's instructions.

Real-Time Quantitative PCR Analysis

Total RNA from the ileum was isolated using Trizol reagent (Tiangen Biochemical Technology Co., Ltd., Beijing, China), and the RNA purity and integrity were evaluated through a spectrophotometer (Thermo Scientific, Waltham, MA) using the 260/280 nm absorbance ratio. Total RNA (1 μg) was reverse transcribed by a PrimeScript RT Master Mix Kit (TaKaRa, Osaka, Japan) according to the manufacturer's instruction. Reverse transcription was performed for 15 min at 37°C, followed by heat inactivation for 5 s at 85°C. All of the cDNA preparations were stored at −20°C. Real-time quantitative PCR was performed in duplicate reactions including nuclease-free water, the forward and reverse primers of each gene, cDNA, and TB Green Premix Ex Taq Kit (TaKaRa), as a detector on a Bio-Rad CFX96 Real-Time PCR system (Bio-Rad Laboratories, Hercules, CA). Pairs of primers were designed and checked for target identity using GenBank from the National Center for Biotechnology Information. Gene-specific primer sequences are shown in Table 2. Each sample was analyzed in triplicate under the following PCR conditions: 95°C for 30 s, 95°C for 5 s, followed by 40 cycles, 95°C for 5 s, 60°C for 30 s, and 95°C for 10 s. The specificity of the PCR products was evaluated by the analysis of the melting curve. The relative levels of mRNA expression were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), which was normalized to the reference mRNA level of *GAPDH*.

Western Blot Analysis

The protein expressions of tight junction proteins (Occludin and ZO-1), Kelch-like ECH-associated protein-

Table 2. Primers used for quantitative RT-Qpcr.

Gene[1]	Primer sequence (5'-3')	Product size (bp)	GenBank accession No.
LC3-I	Forward: GTGTAGATTAGCCCCGCTCC	474	XM_417327.7
	Reverse: CTCCCCATTCCAACCTGTCC		
LC3-II	Forward: TGAATCCCACCCAGGCTTTC	361	XM_040688401.1
	Reverse: GGAGCGGGGCTAATCTACAC		
Beclin1	Forward: GTGTGGAGAACCAGATGCGT	372	NM_001006332.1
	Reverse: AGAAAGCCACCATCGCATGA		
ATG5	Forward: TGGCAAAAGCAAATAA-TAAGGTTCA	188	NM_001006409.2
	Reverse: GGAGTTTCAGTGGGTTTAGGT		
ATG7	Forward: CTGAGGGGCATGGAGGATGTC	136	NM_001030592.2
	Reverse: CATGATTGTAGTTCATCTCGCCC		
Caspase-3	Forward: CTGCGTACCCTCAGCTCACT	284	NM_204725.2
	Reverse: ACCTGATGGATCGCCATTGTA		
Caspase-8	Forward: TGCATGGGCTGCTTAAAGAGA	305	NM_204592.4
	Reverse: GACGGGAGCTTTCTGACACA		
Caspase-9	Forward: TGACAAGAGCGACCACAGAC	280	XM_424580.7
	Reverse: ATGGGAGAGGATGACCACGA		
GAPDH	Forward: GGGCACGCCATCACTATCTT	148	NM-204305.1
	Reverse: TAACACGCTTAGCACCACCC		

[1]*LC3-I* = microtubule-associated protein 1 light chain 3-I; *LC3- II* = microtubule-associated protein 1 light chain 3-II; *ATG5* = autophagy-related gene 5; *ATG7* = autophagy-related gene 7; *Caspase-3* = cysteinyl aspartate specific proteinase-3; *Caspase-8* = cysteinyl aspartate specific proteinase-8; *Caspase-9* = cysteinyl aspartate specific proteinase-9.

1 (**Keap1**), Nrf2, HO-1, NADPH quinone oxidoreductase-1 (**NQO-1**), and sequestosome 1 (**p62**) in the ileum of broilers were determined. Total protein extracts were obtained by homogenizing the ileum in the RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) supplemented with phosphatase inhibitors. Equal amounts of proteins from each sample were subjected to 15% SDS-PAGE (Solarbio Biotechnology Co., Ltd., Beijing, China) before transferring onto polyvinylidene difluoride membrane (Merck Millipore, Darmstadt, Germany). Membranes were blocked by 5% skimmed milk (Beyotime Institute of Biotechnology, Shanghai, China) and then incubated with primary antibodies including anti-β-actin (Cat. AF5003, Beyotime), anti-Occludin (Cat. GB11149, Servicebio, Wuhan, China), anti-ZO-1 (Cat. GB111402, Servicebio), anti-Keap1 (Cat. A1820, Abclonal, Woburn, MA), anti-Nrf2 (Cat. ab31163, Abcam, Cambridge, UK), anti-HO-1 (Cat. bs-2075R, Bioss, Beijing, China), anti-NQO-1 (Cat. bs-2184R, Bioss), and anti-p62 (Cat. NBP1-48320, Novus Biologicals, Littleton, CO) overnight at 4°C. After washing with TBST 3 times, blots were incubated with goat anti-rabbit IgG-HRP (Cat. AF5006, Beyotime) for 1 h at room temperature. After incubation, these blots were washed with TBST 5 times. An enhanced chemiluminescence Kit (Beyotime) was used to capture the bands via a CanoScan LiDE 100 scanner (Canon, Tokyo, Japan), and western blots were analyzed by ImageJ software (Bethesda, MD).

Statistical Analysis

All data were analyzed by two-factorial analysis of variance to examine the main effects of CGA and DEX, and their interaction using general linear model procedure SPSS 18.0 (SPSS Inc., Chicago, IL). When a significant interaction between the main effects was observed, Tukey's multiple comparison was used to compare the differences among the 4 groups. Significant differences were defined as $P < 0.05$.

RESULTS

Growth Performance

As shown in Table 3, a significant interaction was observed between DEX challenge and CGA supplementation on ADG and the F:G ratio ($P < 0.01$) of broilers from d 14 to 21. Broilers in the Control, CGA, and DEX-CGA groups had a higher ADG ($P < 0.05$) and a lower F:G ratio ($P < 0.05$) than those in the DEX group. Broilers in the DEX-CGA group had a lower ADG ($P < 0.05$) and a higher F:G ratio ($P < 0.05$) than those in the Control group. There was no significant difference in ADG or the F:G ratio ($P > 0.05$) between the Control and CGA groups.

Intestinal Morphology in the Ileum

As shown in Figure 1 and Table 4, the interaction between the effects of DEX challenge and CGA

Table 3. Effect of chlorogenic acid (CGA) on growth performance of broilers from d 14 to 21.

Item[1]	Body weight (g, day 14)	ADG (g)	ADFI (g)	F:G
Control[2]	336	54.7[a]	74.9	1.38[c]
DEX	329	26.8[c]	67.0	2.53[a]
CGA	331	54.9[a]	77.8	1.42[c]
DEX-CGA	334	43.2[b]	73.4	1.73[b]
SEM	4.20	1.98	2.78	0.110
Main effect				
CGA				
0 mg/kg	-	40.7	70.9	1.96
500 mg/kg	-	49.1	75.6	1.58
DEX				
0 mg/kg	-	54.8	76.3	1.40
3 mg/kg	-	35.0	70.2	2.13
P-value				
CGA	0.814	<0.010	0.022	<0.010
DEX	-	<0.010	<0.010	<0.010
Interaction	-	<0.010	0.376	<0.010

[1]Abbreviations: ADG, average daily gain; ADFI, average daily feed intake; F:G, feed:gain.

[2]Control group, basal diet; dexamethasone (DEX) group, basal diet and intraperitoneal injection of DEX at 3 mg/kg body weight; CGA group, basal diet containing 500 mg/kg CGA; DEX-CGA group, basal diet containing 500 mg/kg CGA and intraperitoneal injection of DEX at 3 mg/kg body weight.

[abc]Different letters indicate significant differences for the interaction effect ($P < 0.05$).

supplementation influenced villus height ($P < 0.05$), crypt depth ($P < 0.05$), and V/C value ($P < 0.01$) in the ileum. Broilers in the DEX group had lower villus height and V/C value ($P < 0.05$) compared with the Control group, whereas dietary CGA supplementation significantly increased villus height and V/C value ($P < 0.05$) in DEX challenge broilers (DEX-CGA group), restoring control levels. The crypt depth ($P < 0.05$) in the DEX group was higher than those in the Control group, but broilers in the DEX-CGA group had a lower crypt depth ($P < 0.05$) than those in the Control group. No differences were observed between the CGA and DEX-CGA groups ($P > 0.05$).

Tight Junction Protein Expressions in the Ileum

The expressions of tight junction protein Occludin and ZO-1 are shown in Figure 2. The interaction between the effects of DEX challenge and CGA supplementation influenced Occludin and ZO-1 ($P < 0.01$) protein expressions in the ileum. The protein expressions of Occludin and ZO-1 ($P < 0.05$) in the DEX group were lower compared with the other groups, but no differences were observed among the Control, CGA, and DEX-CGA groups ($P > 0.05$).

Antioxidant Indices in the Serum and Ileum

As shown in Table 5, in the serum, there were interactive effects between DEX challenge and CGA supplementation on the activities of SOD ($P < 0.05$), CAT ($P < 0.01$), GST ($P < 0.01$), and HO-1 ($P < 0.01$) and the levels of MDA ($P < 0.01$) and PCO ($P < 0.01$).
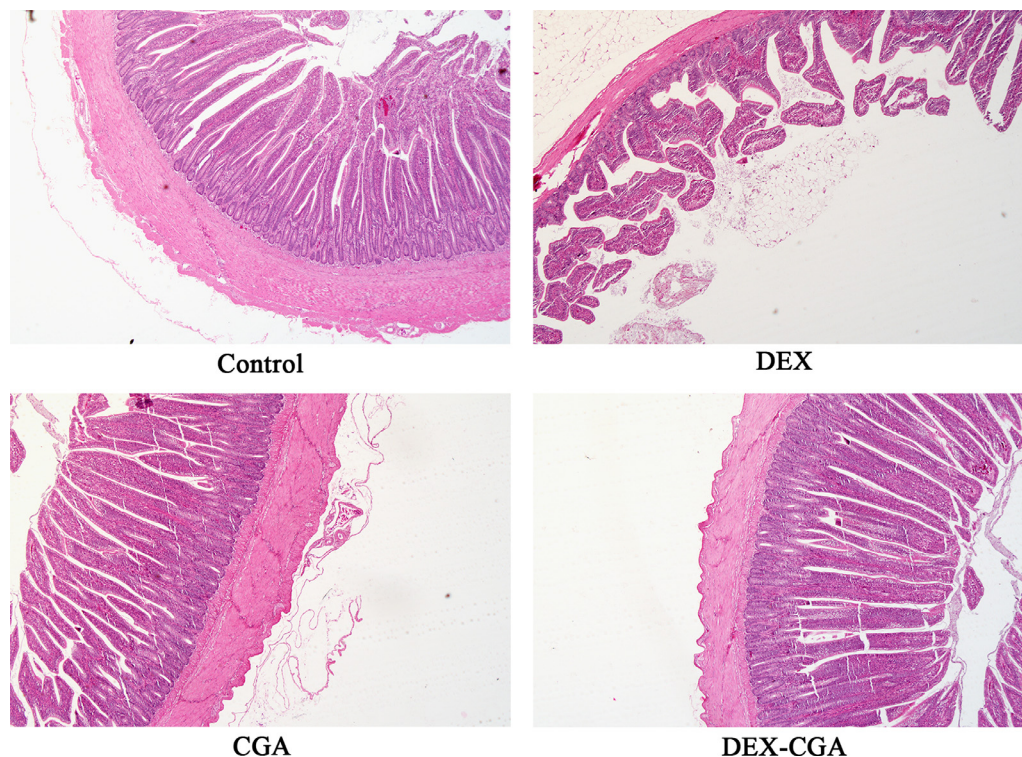
Figure 1. Effect of chlorogenic acid (CGA) on intestinal morphology in the ileum of broilers. Control group, basal diet; dexamethasone (DEX) group, basal diet and intraperitoneal injection of DEX at 3 mg/kg body weight; CGA group, basal diet containing 500 mg/kg CGA; DEX-CGA group, basal diet containing 500 mg/kg CGA and intraperitoneal injection of DEX at 3 mg/kg body weight.

Compared with the Control group, the DEX group had lower SOD, CAT, GST, and HO-1($P < 0.05$) activities, whereas dietary CGA supplementation (DEX-CGA group) significantly increased SOD, CAT, GST, and HO-1($P < 0.05$) activities, restoring control levels. The levels of MDA and PCO ($P < 0.05$) in the DEX group were higher than those of the Control. No differences were observed among the Control, CGA, and DEX-CGA groups ($P > 0.05$). The interactive effects of SOD ($P < 0.01$), CAT ($P < 0.01$), GST ($P < 0.05$), and HO-1 ($P < 0.01$) and the levels of MDA ($P < 0.01$) and PCO ($P < 0.01$) in the ileum were observed between DEX challenge and CGA supplementation (Table 6). Broilers in the DEX group showed lower activities of SOD, CAT, GST, and HO-1($P < 0.05$) and higher MDA and PCO levels ($P < 0.05$) compared with the Control group. Broilers in the CGA and DEX-CGA groups had higher SOD, CAT, GST, and HO-1 activities ($P < 0.05$) and lower MDA and PCO levels ($P < 0.05$) than those in the DEX groups.

mRNA Expressions of Autophagy and Apoptosis-Related Genes in the Ileum

The mRNA expressions of autophagy and apoptosis-related genes are shown in Figure 3. The interactive effects on the expressions of microtubule-associated protein 1 light chain 3 (***LC3***) -I ($P < 0.05$), *LC3-II* ($P < 0.05$), *Beclin1* ($P < 0.01$), and autophagy-related gene (***ATG***) *7* ($P < 0.05$) and the LC3-II/LC3-I value ($P < 0.01$) in the ileum were observed between DEX challenge and CGA supplementation (Figure 3A). Broilers in the DEX group showed lower the mRNA expressions of *LC3-II, Beclin1*, and *ATG7* and LC3-II/LC3-I value ($P < 0.05$) and a higher *LC3-I* mRNA expression ($P < 0.05$) compared with the other groups, but no differences were observed among the Control, CGA, and DEX-CGA groups ($P > 0.05$). The interaction of the effects between DEX challenge and CGA supplementation influenced cysteinyl aspartate specific proteinase (***Caspase***)-*3* and *Caspase-9* mRNA expressions ($P < 0.05$) in the ileum

Table 4. Effect of chlorogenic acid (CGA) on intestinal morphology in the ileum of broilers.

Item[1]	Villus height (μm)	Crypt depth (μm)	V/C
Control[2]	185[a]	50.74[b]	3.65[a]
DEX	130[b]	59.94[a]	2.17[b]
CGA	194[a]	44.69[bc]	4.34[a]
DEX-CGA	179[a]	39.62[c]	4.52[a]
SEM	12.7	3.62	0.351
Main effect			
CGA			
0 mg/kg	158	55.3	2.93
500 mg/kg	186	42.2	4.48
DEX			
0 mg/kg	190	47.7	4.03
3 mg/kg	154	49.8	3.38
P-value			
CGA	<0.010	<0.010	<0.010
DEX	<0.010	0.430	0.018
Interaction	0.041	0.013	<0.010

[1]Abbreviation: V/C, Villus height/Crypt depth.
[2]Control group, basal diet; dexamethasone (DEX) group, basal diet and intraperitoneal injection of DEX at 3 mg/kg body weight; CGA group, basal diet containing 500 mg/kg CGA; DEX-CGA group, basal diet containing 500 mg/kg CGA and intraperitoneal injection of DEX at 3 mg/kg body weight.
[a,b,c]Different letters indicate significant differences for the interaction effect ($P < 0.05$).

CGA: P = 0.000
DEX: P = 0.000
Interaction: P = 0.000

CGA: P = 0.000
DEX: P = 0.000
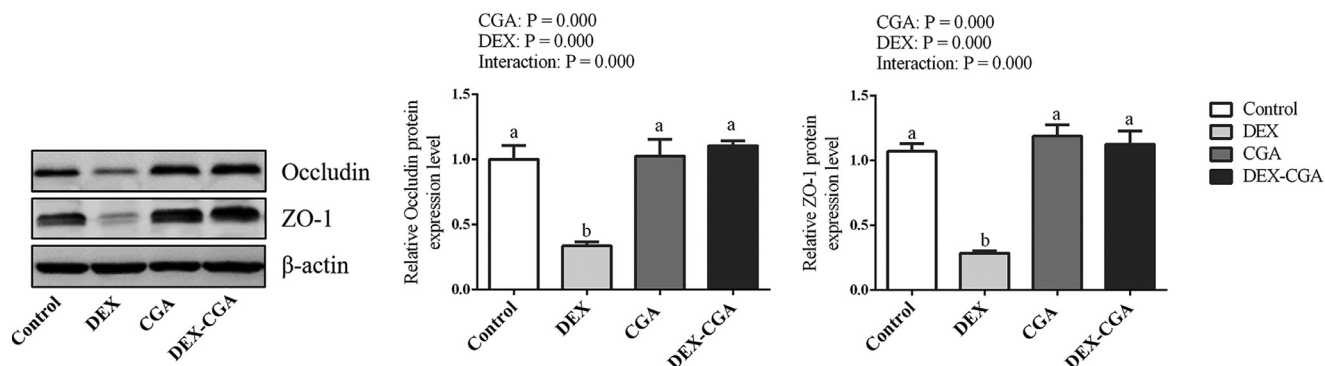Interaction: P = 0.000

Figure 2. Effect of chlorogenic acid (CGA) on the protein expressions of tight junction proteins in the ileum of broilers. Control group, basal diet; dexamethasone (DEX) group, basal diet and intraperitoneal injection of DEX at 3 mg/kg body weight; CGA group, basal diet containing 500 mg/kg CGA; DEX-CGA group, basal diet containing 500 mg/kg CGA and intraperitoneal injection of DEX at 3 mg/kg body weight. [ab] Different letters indicate significant differences for the interaction effect ($P < 0.05$).

Table 5. Effect of chlorogenic acid (CGA) on antioxidant indices in the serum of broilers.

Item[1]	SOD (U/mL)	CAT (U/mL)	GSH-Px (U/mL)	GST (U/mL)	HO-1 (U/mL)	MDA (nmol/mL)	PCO (pg/mL)
Control[2]	55.5[a]	13.9[a]	7.58	324[a]	330[a]	35.5[b]	75.8[b]
DEX	43.5[b]	9.30[b]	6.18	198[b]	260[b]	44.8[a]	176[a]
CGA	53.9[a]	14.6[a]	8.63	287[a]	315[a]	41.0[ab]	84.5[b]
DEX-CGA	55.7[a]	13.6[a]	7.71	276[a]	358[a]	39.8[ab]	75.4[b]
SEM	3.97	0.377	0.755	21.1	18.4	2.29	15.9
Main effect							
CGA							
0 mg/kg	49.5	11.6	6.88	260	295	40.1	126
500 mg/kg	54.8	14.1	8.17	282	336	40.4	80.0
DEX							
0 mg/kg	54.7	14.3	8.11	304	323	38.2	80.2
3 mg/kg	49.6	11.5	6.95	237	309	42.3	126
P-value							
CGA	0.072	<0.010	0.025	0.151	<0.010	0.871	<0.010
DEX	0.083	<0.010	0.042	<0.010	0.295	0.021	<0.010
Interaction	0.024	<0.010	0.657	<0.010	<0.010	<0.010	<0.010

[1]Abbreviations: CAT, catalase; GSH-Px, glutathione peroxidase; GST, glutathione S-transferase; HO-1, heme oxygenase-1; MDA, malonaldehyde; PCO, protein carboxyls; SOD, superoxide dismutase.
[2]Control group, basal diet; dexamethasone (DEX) group, basal diet and intraperitoneal injection of DEX at 3 mg/kg body weight; CGA group, basal diet containing 500 mg/kg CGA; DEX-CGA group, basal diet containing 500 mg/kg CGA and intraperitoneal injection of DEX at 3 mg/kg body weight.
[abc]Different letters indicate significant differences for the interaction effect ($P < 0.05$).

Table 6. Effect of chlorogenic acid (CGA) on antioxidant indices in the ileum of broilers.

Item[1]	SOD (U/mg protein)	CAT (U/mg protein)	GSH-Px (U/mg protein)	GST (U/mg protein)	HO-1 (U/mg protein)	MDA (nmol/mg protein)	PCO (pg/mg protein)
Control[2]	109[a]	72.4[a]	38.1	258[a]	384[a]	77.2[b]	185[b]
DEX	69.8[b]	64.7[b]	35.9	225[b]	247[b]	88.5[a]	258[a]
CGA	110[a]	72.7[a]	38.8	241[a]	400[a]	78.8[b]	202[b]
DEX-CGA	114[a]	72.1[a]	38.7	258[a]	384[a]	67.9[c]	212[b]
SEM	3.84	1.09	1.43	16.4	16.7	1.57	10.5
Main effect							
CGA							
0 mg/kg	89.6	68.5	37.0	342	316	82.9	221
500 mg/kg	112	72.4	38.8	250	392	73.3	207
DEX							
0 mg/kg	110	72.5	38.5	250	392	78.0	193
3 mg/kg	91.8	68.4	37.3	242	316	78.2	235
P-value							
CGA	<0.010	<0.010	0.094	0.489	<0.010	<0.010	0.065
DEX	<0.010	<0.010	0.257	0.489	<0.010	0.826	<0.010
Interaction	<0.010	<0.010	0.326	0.046	<0.010	<0.010	<0.010

[1]Abbreviations: CAT, catalase; GSH-Px, glutathione peroxidase; GST, glutathione S-transferase; HO-1, heme oxygenase-1; MDA, malonaldehyde; PCO, protein carboxyls; SOD, superoxide dismutase.
[2]Control group, basal diet; dexamethasone (DEX) group, basal diet and intraperitoneal injection of DEX at 3 mg/kg body weight; CGA group, basal diet containing 500 mg/kg CGA; DEX-CGA group, basal diet containing 500 mg/kg CGA and intraperitoneal injection of DEX at 3 mg/kg body weight.
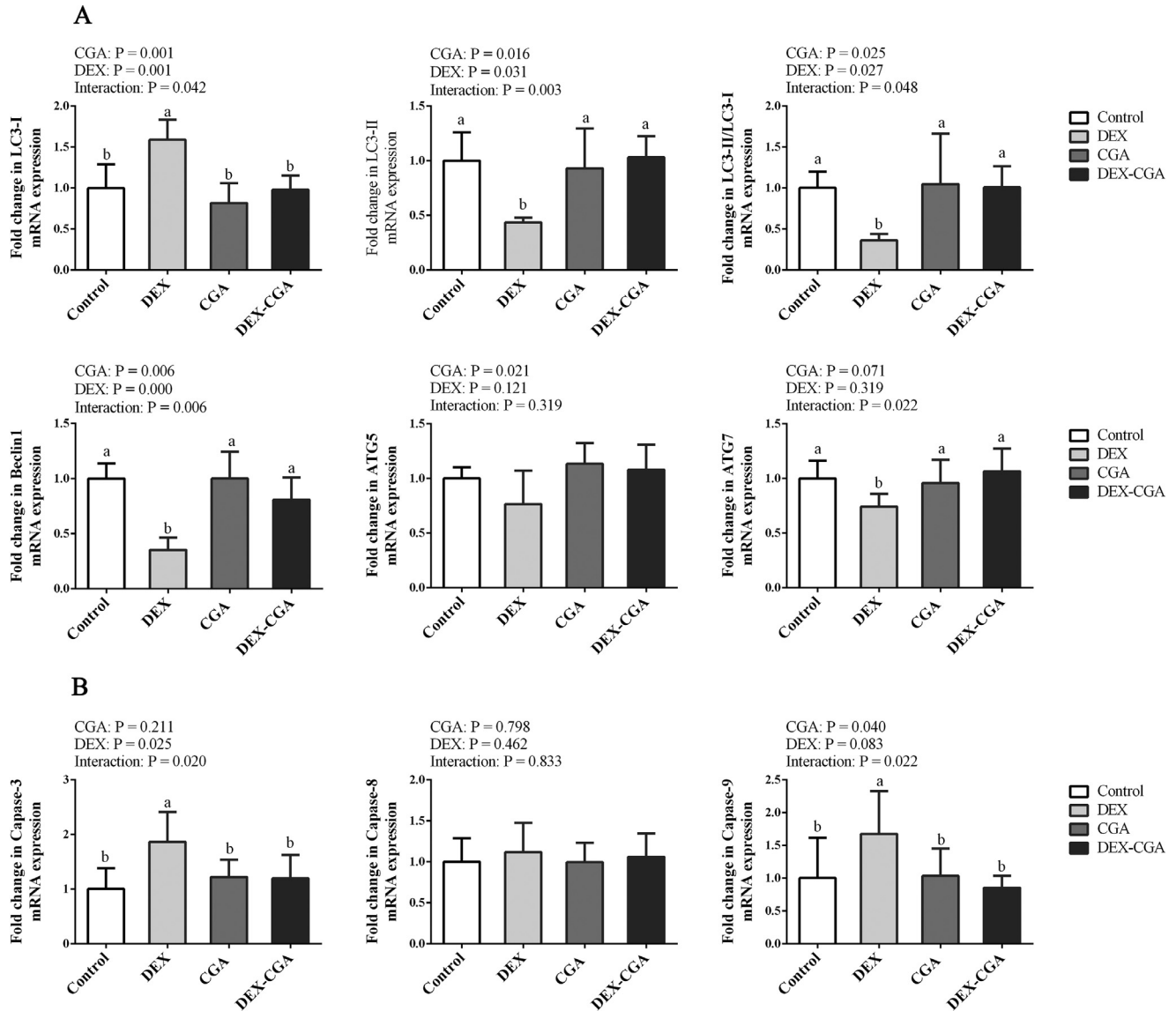[abc]Different letters indicate significant differences for the interaction effect ($P < 0.05$).

Figure 3. Effect of chlorogenic acid (CGA) on the autophagy and apoptosis-related mRNA expressions in the ileum of broilers. A, Autophagy-related mRNA expressions; B, Apoptosis-related mRNA. Control group, basal diet; dexamethasone (DEX) group, basal diet and intraperitoneal injection of DEX at 3 mg/kg body weight; CGA group, basal diet containing 500 mg/kg CGA; DEX-CGA group, basal diet containing 500 mg/kg CGA and intraperitoneal injection of DEX at 3 mg/kg body weight. [ab] Different letters indicate significant differences for the interaction effect ($P < 0.05$).

(Figure 3B). The mRNA expressions of *Caspase-3* and *Caspase-9* ($P < 0.05$) in the ileum of broilers in the DEX group were higher than that of other groups, but no differences were observed among Control, CGA, and DEX-CGA groups ($P > 0.05$).

Autophagy-Mediated Nrf2 Pathway Protein Expression in the Ileum

The protein expressions of the autophagy-mediated Nrf2 pathway are shown in Figure 4. Interactive effects on Keap1 ($P < 0.01$), Nrf2 ($P < 0.01$), HO-1 ($P < 0.01$), NQO-1 ($P < 0.05$), and p62 ($P < 0.05$) protein expressions in the ileum were observed between DEX challenge and CGA supplementation. Compared with the Control group, broilers in the DEX group had lower expressions of Keap1, Nrf2, HO-1, and NQO-1 ($P < 0.05$) and a higher protein expression of p62 ($P < 0.05$), and dietary

CGA supplementation significantly increased Keap1, Nrf2, HO-1, and NQO-1 ($P < 0.05$) protein expressions and decreased p62 ($P < 0.05$) protein expression in DEX challenge broilers (DEX-CGA group), restoring control expressions levels. No differences were observed in Nrf2, HO-1, NQO-1, and p62 protein expressions ($P > 0.05$) in the ileum between the Control and CGA groups.

DISCUSSION

In the present study, the DEX challenge impaired growth performance in broilers, which is in line with the results of Liu et al. (2021), who reported that the DEX challenge decreased ADG and increased the F:G ratio in broilers. We observed that the dietary supplementation of CGA increased the ADG and decreased the F:G ratio in the DEX challenged broilers. According to a previous study, dietary supplementation of CGA alleviated the
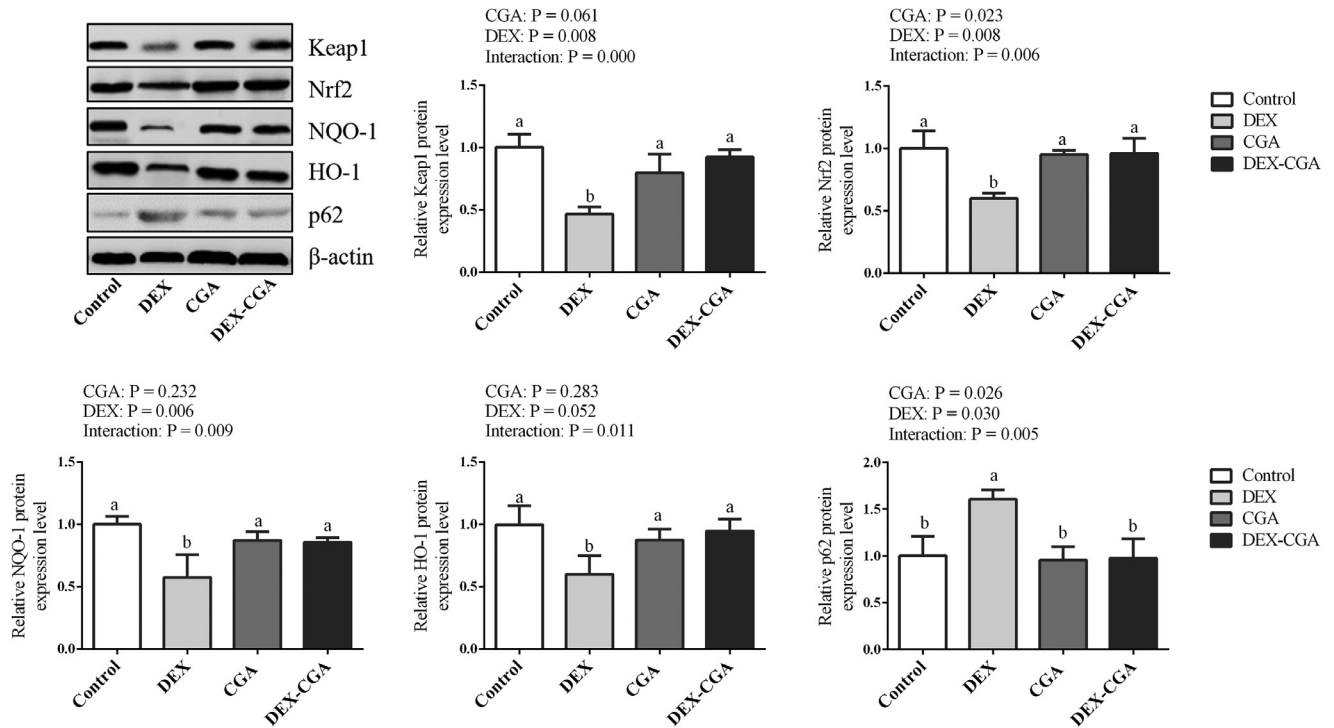
Figure 4. Effect of chlorogenic acid (CGA) on the protein expressions of autophagy-mediated Nrf2 pathway in the ileum of broilers. Control group, basal diet; dexamethasone (DEX) group, basal diet and intraperitoneal injection of DEX at 3 mg/kg body weight; CGA group, basal diet containing 500 mg/kg CGA; DEX-CGA group, basal diet containing 500 mg/kg CGA and intraperitoneal injection of DEX at 3 mg/kg body weight. [abc] Different letters indicate significant differences for the interaction effect ($P < 0.05$).

depressed growth performance of heat-stressed broilers (Zhao et al., 2019). Chen et al. (2018a) reported that CGA could improve the growth performance of weaned pigs through maintaining antioxidant capacity and intestinal digestion and absorption function.

The intestinal morphology is an important indicator of intestinal health, such as intestinal barrier integrity, nutrient digestion, and the absorption capacity of the small intestine (Sabry Abd Elraheam Elsayed et al., 2021). In the present study, our results showed that the DEX challenge significantly damaged the intestinal morphology in the ileum of broilers, which was consistent with the results reported by previous studies (De Grande et al., 2020; Berenjian et al., 2021). Moreover, we found that dietary supplementation of CGA also increased the villus height and V/C value and decreased the crypt depth of the ileum in oxidatively stressed broilers. The improved intestinal morphology observed in our study is probably one of the reasons for the improvement of growth performance. Similarly, recent studies have indicated that dietary supplementation of CGA improved intestinal morphology in necrotizing enteritis broilers (Zhang et al., 2020) and weaned piglets (Zhang et al., 2018). Tight junction proteins such as Occludin and ZO-1 have been used to consider the key components of intestinal permeability in broilers (Chen et al., 2015). Our data demonstrated that dietary supplementation of CGA significantly increased the ileal protein expressions of Occludin and ZO-1 of DEX challenged broilers. Similar results were found in a study by Chen et al. (2018b), who found that dietary supplementation of CGA up-regulated the mRNA expressions of tight junction proteins

in the small intestine of weaned pigs. These results suggested that CGA could improve intestinal barrier function partly by maintaining tight junction protein expression.

Antioxidant enzymes, such as SOD, GSH-Px, and CAT, are the most important intracellular antioxidant defense system to prevent intestinal damage induced by oxidative stress in broilers (Tang et al., 2019). In the present study, the DEX challenge significantly decreased SOD, CAT, GSH-Px, and GST activities and increased MDA and PCO levels, which was consistent with a report by Lv et al. (2018) who found that antioxidant enzyme activities and mRNA expressions were decreased and that MDA levels were increased by the DEX challenge. Previous studies have shown that CGA can ameliorate intestinal mitochondrial injury by decreasing H_2O_2-induced reactive oxygen species production (Zhou et al., 2016), and provide even higher antioxidant capacity than many other phenols (Ulluwishewa et al., 2011). Our data showed that dietary supplementation of CGA increased the activity of SOD, CAT, GST, and HO-1 and decreased PCO level in the DEX challenged broilers, indicating that CGA could alleviate oxidative stress by improving antioxidant enzymes activities in broiler. Similar results were found in the study by Zhao et al. (2019).

To further explore the molecular mechanism of CGA in attenuating intestinal oxidative damage, we investigated the effects of CGA on the activation of the Nrf2 pathway. The Nrf2 pathway acts against oxidative stress by regulating the levels of endogenous antioxidant enzymes (Ma, 2013). A previous study in oxidatively

stressed broilers showed that the activities of antioxidant enzymes could be enhanced by increasing the mRNA and protein expressions levels of Nrf2 (Li et al., 2019). Similarly, our present study showed that dietary supplementation of CGA could increase the Nrf2 protein expression level in the ileum of oxidatively stressed broilers, which is consistent with the increased activities of antioxidant enzymes. Several studies have suggested that polyphenols possess the potential of activating Nrf2 and then up-regulating the expressions of many antioxidative and cytoprotective genes in the small intestine (Scapagnini et al., 2011; Cheng et al., 2013). *HO-1* and *NQO-1* genes are the downstream targets of Nrf2 (Li et al., 2014). Previous studies have reported that CGA could relieve oxidative stress by increasing Nrf2, HO-1, and NQO-1 proteins expressions in H_2O_2 induced rat pheochromocytoma cells (Yao et al., 2019). Similarly, in this study, the dietary supplementation of CGA significantly up-regulated the proteins expressions levels of HO-1 and NQO-1 of DEX challenged broilers. Therefore, these combined findings demonstrate collectively that the enhanced activities of antioxidant enzymes mediated by the Nrf2 pathway might partially explain the reasons why CGA could improve the antioxidative capacity of oxidatively stressed broilers.

Autophagy serves as a defense mechanism to clear oxidatively damaged proteins and organelles and is activated in response to oxidative stress (Scherz-Shouval and Elazar, 2007; Tang et al., 2021). The Nrf2-p62 pathway has been reported to play an important role in autophagy by modulating the expression of multiple autophagy genes (Kageyama et al., 2018). In the present study, the DEX challenge decreased *LC3-II* and *Beclin1* mRNA expressions and LC3-II/LC3-I value, but increased the mRNA expression of *LC3-I* and p62 protein expression in the ileum, indicating that oxidative stress could disrupt autophagy. However, we noted that dietary supplementation of CGA induced autophagy in oxidatively stressed broilers, as evidenced by the increased *LC3-II, Beclin1* and *ATG7* mRNA expressions and LC3-II/LC3-I value and decreased mRNA expression of *LC3-I* and protein expression of p62, implying CGA inhibits oxidative stress through the activation of autophagy. Similar results were found in human neuroblastoma cells (Gao et al., 2021) and zebrafish (Ji et al., 2021). Moreover, with regards to apoptosis, our results showed that dietary supplementation of CGA decreased the mRNA expression of *Caspase-9* in oxidatively stressed broilers, which was consistent with a previous study (Liu et al., 2020) that found CGA inhibited the expressions of apoptosis-related proteins (caspase-3 and caspase-9) in the ischemia/reperfusion injury of rats. Overall, these outcomes demonstrated that CGA can promote autophagy and decrease apoptosis levels in oxidatively stressed broilers by activating the Nrf2-p62 pathway, thus alleviating oxidative stress.

Our study showed interactions between DEX and CGA in growth performance, intestinal morphology, tight junction proteins, antioxidant indices, autophagy-related mRNA expressions, and LC3-II/LC3-I value,

apoptosis-related mRNA expressions, and autophagy-mediated Nrf2 pathway protein expressions. This implies that dietary supplementation of CGA improved the condition of broilers more robustly under stressful conditions than under unchallenged conditions. Similar results were found in a study by Liu et al. (2018), in which CGA-enriched extract had a more robust protective effect under transport stressed conditions than under none-stressed conditions in lambs.

In conclusion, we demonstrated that CGA has an apparent protective effect on growth performance and intestinal health in the DEX challenged broilers. Dietary supplementation of CGA could increase antioxidant capacity and decrease apoptosis levels through the activation of the autophagy-mediated Nrf2 pathway in oxidatively stressed broilers. Moreover, dietary supplementation of CGA improved the condition of broilers more robustly under stressful conditions than under none-stressed conditions. Therefore, our findings provide new evidence for the mechanism of CGA as an effective natural antioxidant to protect intestinal health in broilers.

DISCLOSURES

The authors declare that there is no conflict of interest.

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