Comparison of the effects of chlorogenic acid isomers and their compounds on alleviating oxidative stress injury in broilers

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ABSTRACT The development of large-scale and intensive breeding models has led to increasingly prominent oxidative stress issues in animal husbandry production. Chlorogenic acid (**CGA**) is an important extract with a variety of biological activities. It is an effective antioxidant drug and shows different antioxidant capacities due to its different chemical structures. Therefore, it is a new research target to determine the proportion of chlorogenic acid isomers with high antioxidant activity to resist the damage caused by oxidative stress. In this experiment, the antioxidant activities of the chlorogenic acid monomer and its compounds were compared by a series of in vitro antioxidant indexes. Based on the above experiments, it was found that LB and LC have superior antioxidant abilities ($P < 0.05$). Subsequently, 300 healthy 1-day-old Arbor Acres (**AA**) male broilers with no significant difference in body weight (about 44 g) were randomly selected and randomly divided into 5 groups with 6 replicates in each group and 10 chickens in each replicate. One group was the control group, 1 group was the model group, and the remaining 3 groups were the experimental groups. At 37 d of age, animals in the control group were injected with normal saline, and animals in the other 4 groups were injected with 1 mL/kg 5% hydrogen peroxide (H_2O_2) through the chest muscle before the supplementation. The control group (control) and the model group (PC) were fed a standard diet. The remaining 3 groups included the CGA group, LB group (CIB), and LC group (CIC). In these groups, 50 g/t chlorogenic acid, LB compound, or LC compound were added to the basal diet, respectively, and the other feeding conditions remained consistent. The addition of the LB complex to the diet could significantly improve the growth performance and antioxidant performance of broilers ($P < 0.05$), upregulate the expression of Nuclear factor erythroid 2-related factor 2 (**Nrf2**) pathway-related genes in liver and jejunum ($P < 0.05$), regulate the disordered intestinal flora, and alleviate the damage caused by oxidative stress. These results suggested for the first time that the LB complex exhibited superior effects in vitro and vivo.

Key words: broiler, chlorogenic acid isomer, antioxidant activity, compound

INTRODUCTION

In recent times, people's attention to the adverse effects of using phytogenic feed additives to combat oxidative stress in poultry has gradually increased. As a plant extract, CGA is a phenolic acid compound condensed by the 1-carboxyl of caffeic acid and the 3-hydroxy of quinic acid. It is a phenylpropanoid substance synthesized by plant cells through aerobic respiration via the shikimic acid pathway (Clifford, 1999; Lallemand et al., 2012; Nabavi et al., 2017). As a

bioactive substance, CGA is widely detected in higher dicotyledonous plants and ferns. Among them, honeysuckle, Eucommia leaves, sunflower seeds, coffee beans, and other plants exhibit high CGA content (Jin et al., 2015, Zhang et al., 2018; De Oliveira Filho and Egea, 2021; Muchtaridi et al., 2021) and antibacterial, anti-inflammatory, antiviral and antioxidant physiological functions (Meng et al., 2013; Liang and Kitts, 2015; Tan et al., 2020). Because of their nontoxic and pollution-free use characteristics, these plants are widely used in food and medical treatment (Li et al., 2021; Tian et al., 2021).

Oxidative stress is a potential cause of many physiological and pathological phenomena and plays a role in many processes, including aging, autoimmune diseases, inflammation, cancer, and neurodegenerative diseases (Taeb et al., 2017). In terms of exerting antioxidant effects, CGA and its isomers can protect against lipid

oxidation and balance mitochondrial membrane potential to reduce oxidative damage, but current research on this topic remains lacking (Liang et al., 2016a; Tsai et al., 2018). The structure of natural chlorogenic acid in nature is different, and its efficacy is also different. Comparing the antioxidant activity of different chlorogenic acid isomers and determining the best compound proportions to improve antioxidant effects can not only further improve the economic value and practical value of natural plant extracts but also provide a theoretical basis for human disease resistance and antiaging.

The chemical formula of CGA is $C_{16}H_{18}O_9$. Some scholars refer to CGA as 3-CQA, but it is more appropriately named 5-o-caffeoylquinic acid (5-CQA) according to the numbering rules of IUPAC(Ltd, 1976). Neochlorogenic acid (3-CQA), cryptochlorogenic acid (4-CQA), isochlorogenic acid A (3,5-DICQA), isochlorogenic acid B (3,4-DICQA), and isochlorogenic acid C (4,5-DICQA) are common CGA isomers, and their structural formulas are presented in Figure 1 (Meinhart et al., 2017; Liang and Kitts, 2018). The binding site and number of caffeic acid and quinic acid are different. The esters formed between 1 molecule of caffeic acid and 1 molecule of quinic acid are called single caffeoylquinic acids (CQAs), and include chlorogenic acid, neochlorogenic acid, and cryptochlorogenic acid; the esters formed between 2 molecules of caffeic acid and 1 molecule of quinic acid are called double caffeoylquinic acid (DICGA), such as isochlorogenic acid A, isochlorogenic acid B, and isochlorogenic acid C (Liang et al., 2019).

The majority of current literature studies examine the application effect of chlorogenic acid monomers in broiler chickens, but the application of different proportions of chlorogenic acid isomer complexes has rarely been reported. In this experiment, Arbor Acres white feather broilers were used as experimental animals,

supplemented by previous chemical and cell experiments, to identify the best relative proportions of chlorogenic acid compound. The exploration of this topic can provide a reference for the further development and utilization of chlorogenic acid isomers of natural plant extracts.

MATERIALS AND METHODS

Experimental Design

Comparison of the Antioxidant Effects of Chlorogenic Acid and Its Isomers In Vitro To compare the antioxidant activity of different isomers and the combination of these isomers, the 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS), 1,1-Diphenyl-2-picrylhydrazyl radical 2,2-Diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl (DPPH) free radical scavenging ability and ferric ion reducing antioxidant power (FRAP) ferrous ion reduction ability were measured. Chlorogenic acid isomers (CGA, cryptochlorogenic acid, neochlorogenic acid, isochlorogenic acid A, isochlorogenic acid B and isochlorogenic acid C) were measures. The activity of the chlorogenic acid isomer combination were then measures, and the composition of these combinations are presented in Table 1. Additionally, cell experiments were conducted to extract chicken primary duodenal epithelial cells, and cells were treated with 400 μM tert-butyl hydroperoxide (tBHP) for 1 h to construct an oxidative stress model. In previous studies, it pretreatment with 200 μM chlorogenic acid for 1 h was found to alleviate oxidative stress, and pretreatment with 25 μM isochlorogenic acid A, B, and C for 1 h could also achieve the same mitigation effect. According to the previous results, chlorogenic acid monomer and compound samples were added to the cells for pretreatment. The compound proportions are presented in Table 1. The cell viability and ROS levels of different treatment groups under the oxidative stress model were detected.

Comparison of the Antioxidant Effects of Chlorogenic Acid Mixture In Vivo A total of 300 one-day-old AA broilers with no significant difference in body weight were randomly divided into 5 treatment groups with 6 replicates per treatment group and 10 chickens per replicate. This experiment began at 1 day old and ended at 42 days old. The control group (Control) and the model group (PC) were fed a basal diet. Animals in the chlorogenic acid group (CGA), chlorogenic acid compound mixture LB group (CIB), and chlorogenic acid compound mixture LC group (CIC) were treated with 50 g/t chlorogenic acid, LB, and LC. Animals in the control group were injected with 0.75% saline, and animals in the other groups were injected with 5% H_2O_2 (1 mL/kg BW).

Net rearing was used as the feeding method for this experiment. During the experiment, all conditions were maintained according to the hygienic requirements standard for broiler feeding (GB14925-1994). The temperature and humidity were adjusted with the actual growth of broiler chickens (The temperature of the house is
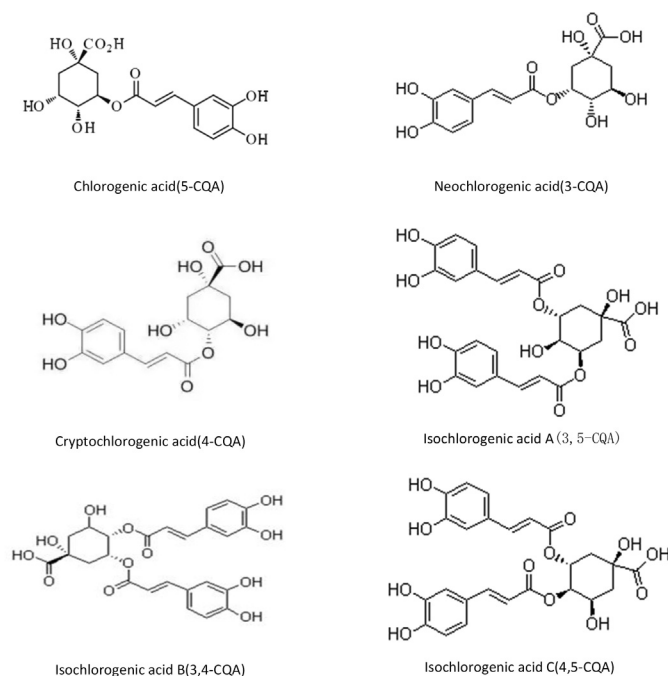

Figure 1. Chlorogenic acid isomer.

Table 1. Compound proportions of chlorogenic acid isomers.

Group	Compound composition	Composition of total isochlorogenic acid
YA	Chlorogenic acid: Total Isochlorogenic acid = 2:8	Isochlorogenic acid A: Isochlorogenic acid B: Isochlorogenic acid C = 4:2:2
YB		Isochlorogenic acid A: Isochlorogenic acid B: Isochlorogenic acid C = 2:4:2
YC		Isochlorogenic acid A: Isochlorogenic acid B: Isochlorogenic acid C = 2:2:4
PA	Chlorogenic acid: Total Isochlorogenic acid = 5:5	Isochlorogenic acid A: Isochlorogenic acid B: Isochlorogenic acid C = 4:2:2
PB		Isochlorogenic acid A: Isochlorogenic acid B: Isochlorogenic acid C = 2:4:2
PC		Isochlorogenic acid A: Isochlorogenic acid B: Isochlorogenic acid C = 2:2:4
LA	Chlorogenic acid: Total Isochlorogenic acid = 8:2	Isochlorogenic acid A: Isochlorogenic acid B: Isochlorogenic acid C = 4:2:2
LB		Isochlorogenic acid A: Isochlorogenic acid B: Isochlorogenic acid C = 2:4:2
LC		Isochlorogenic acid A: Isochlorogenic acid B: Isochlorogenic acid C = 2:2:4

controlled as follows : $33-34°C$ at $1-7$ d of age, and then decreased by $1°C$ every day until the temperature of the house is controlled at $25-26°C$. The humidity of the house is about $60-70\%$). The chickens were normally immunized. Other management measures were the same as conventional feeding management. During the feeding period, the chickens were allowed to feed and drink freely. The diet composition and nutritional levels are presented in Table 2. The diet was prepared and granulated according to the NRC (1994) and the People's Republic of China industry standard NY/T 33-2004.

Cell Culture

The air chamber at the blunt end of the eggshell of 18-day-old SPF live embryos was crushed with the blunt head of a large tweezers, and the shell membrane was torn off using small tweezers to identify the chicken

Table 2. Basic diet nutrition formula.

Composition	Ratio(%)	
	0−21 d	22−42 d
Corn	55.24	59.37
Soybean	36.92	31.90
Soybean oil	3.50	5.00
Limestone	1.12	1.23
CaHPO$_4$	2.10	1.50
Met	0.28	0.27
98% Lys	0.22	0.11
NaCl	0.30	0.30
Premix Vitamins[1]	0.03	0.03
Premix mineral[2]	0.20	0.20
70% Choline chloride	0.09	0.09
Total	100.00	100.00
Nutritional level[3]		
kcal/kg	2950	3100
CP	21.00	19.00
Ca	1.00	0.90
TP	0.67	0.56
AP	0.45	0.35
Lys	1.20	1.00
Sulfur containing amino acid	0.85	0.80
Thr	0.66	0.60
Trp	0.22	0.20

[1]Premix vitamins provided per kilogram of diet: vitamin A, 8000 IU; vitamin D3, 1000 IU; vitamin E, 20 IU; vitamin K3, 0.50 mg; vitamin B1, 2.00 mg; vitamin B2, 8.00 mg; vitamin B6, 3.50 mg; vitamin B12, 10.00 μg; nicotinic acid, 35.00 mg; calcium pantothenate hydrate, 10.00 mg; folic acid, 0.55 mg; biotin, 0.18 mg.
[2]Premix mineral provided per kilogram of diet: iron, 80.00 mg; copper, 8.00 mg; manganese, 100.00 mg; zinc, 80.00 mg; iodine, 0.70 mg; selenium, 0.30 mg.
[3]Nutritional level is calculated.

embryo wings or legs. The embryo was pinched and removed (being careful not to pinch the neck), separated from the egg yolk, and placed on a plate. Eye scissors were used to cut the abdominal cavity, and the complete duodenum was removed and placed onto a plate containing D-Hanks' solution previously preheated to $37°C$ using a water bath. The pancreas was removed with tweezers and washed twice with D-Hank's. Subsequently, the intestinal mucosa was carefully squeezed out with curved tweezers and straight tweezers, placed on another plate containing prewarmed D-Hanks, and then transferred to two 15-mL centrifuge tubes with a Pasteur pipet. The remaining precipitate was dissolved in 20 mL Hank's, 2 mL type I collagenase was added to a final concentration of 1 mg/mL, and the sample was digested in a $37°C$ constant temperature water bath with magnetic stirring for 10 min. After digestion, the sample was centrifuged at 800 rpm for 8 min, and the digestive juice was discarded. D-Hank's ($3-5$ mL) solution was used to suspend and mix the precipitate. The sample was centrifuged again, the supernatant was removed, and the wash was repeated once. Finally, complete medium (10% fetal bovine serum + 1% penicillin and streptomycin) was used to suspend and mix evenly, followed by straining through 50-mesh and 200-mesh sieves. The cells were inoculated into 96-well plates with 100 μL suspension per well at a cell density of 5×10^6 cells per well. The cells were cultured at $37°C$ and 5% CO_2 for approximately 48 h.

Cell Viability Assay

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) according to the manufacturer's protocols. The cultured cells were placed in a 96-well plate at a density of 1×10^5 cells per well. Ten microliters of CCK-8 reagent were added to each well, and the plates were placed in a cell incubator for 2 h. After reagent treatment, the optical density was measured at 450 nm with a microplate reader (TECAN, Switzerland). Relative cell viability (%) is expressed as the percentage of treated cells relative to untreated control cells. Each treatment had 6 replicates.

Assessment of Intracellular ROS

Intracellular Reactive oxygen species (**ROS**) production was analyzed using a ROS Assay Kit (Nanjing

Jiancheng Bioengineering Institute, Nanjing, China). Approximately 100 μL cell suspension was added to the wells of a 96-well cell culture plate, add PBS solution around the suspension, and a blank well was created. When the cells in the 96-well plate were confluent, they were treated. The DCFH-DA probe was added to serum-free medium; DCFH-DA was generally diluted with serum-free medium at 1:1,000 (final concentration 10 μM). After removing the treatment solution, 100 μL of diluted DCFH-DA was added to each well. A positive control well and negative control well were set up. For the positive control, the probe was applied to the cells, and a reactive oxygen species donor was added to the cells. For the negative control, media not containing the probe was added to the cells. The cells were incubated at 37°C for 30 min. The cells were washed with PBS or serum-free medium twice to remove excess DCFH-DA and reduce errors. The fluorescence intensity was read by a fluorescence microplate reader with an excitation wavelength of 488 nm and an absorption wavelength of 525 nm. Each treatment had 6 replicates.

Determination of ABTS Free Radical Scavenging Ability

The ABTS free radical solution was prepared. The ABTS solution (7 mmol/L) and potassium persulfate solution (2.45 mmol/L) were mixed and placed in the dark for 16 to 24 h. The ABTS free radical solution was diluted with ultrapure water to obtain an absorbance at 734 nm of 0.70 ± 0.20. Under light-protected conditions, 990 μL of diluted ABTS free radical solution mixed with 10 μL of chlorogenic acid buffer solution for 5 min, and the absorbance was measured at a wavelength of 734 nm. Ultrapure water was used as a blank control. The ABTS radical scavenging rate (%) was defined as (Ao-A1)/Ao * 100%, where Ao is the absorbance of the blank control and A1 is the absorbance of the sample.

Determination of DPPH Free Radical Scavenging Ability

The sample was diluted in methanol (1 mg/mL) to the desired concentration, and the DPPH solution (60 μM) was prepared with methanol. Approximately 0.5 mL sample was mixed with 2.5 mL DPPH solution. The mixture was shaken vigorously and incubated in the dark for 30 min. The absorbance was measured at 517 nm, and ultrapure water was used as a blank control. The DPPH free radical scavenging rate (%) was calculated as [(Ao-A1)/Ao] * 100%, where Ao is the absorbance of the blank control solution and A1 is the absorbance of the sample added to the DPPH solution. The DPPH free radical scavenging activity of the sample is expressed by the EC50 value, which is defined as the sample concentration when the absorbance reaches 0.5.

Determination of FRAP Ferrous Ion Reduction Capacity

The TPTZ solution (10 mM/L) was prepared with sodium acetate buffer solution (300 mM/L, pH 3.6) and hydrochloric acid (40 mM/L) as solvent. Ultrapure water was used as a solvent in the FeCl3 solution (20 mM/L). The above 3 solutions were mixed at a proportion of 10:1:1 to form a FRAP solution. Ultrapure water (3.1 mL), 1.8 mL FRAP solution, and 0.1 mL sample were mixed in test tubes and incubated for 30 min. The absorbance was measured at 593 nm. Ultrapure water was used as a blank control. The FRAP ferrous ion reducing ability was defined as the difference between the solution and the blank control. The IC50 value is the effective concentration at which a 50% FRAP ferrous ion reduction was observed.

Sample Collection

On the 21st and 42nd days of the experiment, the production performance of each group was recorded. On the 42nd day, one chicken wing vein was randomly selected from each replicate, and three 1.5-mL blood samples were collected from the wing vein of the test chicken, which was similar in average weight. The blood samples were placed in coagulation-promoting blood vessels and centrifuged at 3,000 r/min for 15 min, and the supernatant was taken and stored at −20°C. The duodenum, jejunum, ileum, cecum, and liver were separated and weighed, and the intestinal length of each intestinal segment was measured. One-centimeter-thick slices of the duodenum, jejunum, and ileum were embedded in 4% paraformaldehyde. The liver sample, 3 intestinal mucosal samples, and cecal chyme were quickly frozen in liquid nitrogen and stored at −80°C. The pectoral muscles were separated and weighed, and the meat color was measured; the muscles were then stored in a self-sealing bag. Abdominal fat, heart, thymus, liver, spleen, and bursa were separated and weighed.

Meat Quality

Meat color: An automatic colorimeter (chroma meter CR-400, KONICA MINOLTA, Tokyo) was used to measure each sample 3 times. In general, smaller brightness (L*) values indicate muscle that is less white, which reflects less water seepage from the muscle surface and better meat quality. Higher redness (a*) values and lower the yellowness (b*) values indicate better muscle color.

Water loss rate: The water loss rate was determined by the filter paper extrusion method. At 24 h after slaughter, approximately 5 g samples of breast muscle were removed, and the pre-pressure weight was recorded. The meat samples were wrapped with filter paper, and 35 kg pressure was applied for 5 min. The samples were weighed and recorded, and the water loss rate was calculated. The water loss rate (%) was defined

Table 3. Primer sequences for fluorescence-based quantitative real-time PCR.

Genes	GenBank number		Primer sequences (5′→3′)	Amplicon size (bp)
β-actin	NM_205518.2	F	ATCTTTCTTGGGTATGGAGTC	141
		R	TCAGCAATGCCAGGGTA	
Nrf2	XM_046921130.1	F	GGTGACACAGGAACAACA	223
		R	AAGTCTTATCTCCACAGGTAG	
HO-1	NM_205344.2	F	CTGAAGGAAGCCACCAAG	136
		R	CCAGAGCAGAGTAGATGAAG	
SOD	NM_205064.2	F	GCTTGTGGTGTAATTGGAAT	159
		R	AGACAGCAGAGTAGTAATGAG	
GCLC	XM_046915268.1	F	AGGCTATGTGTCCGATATTG	100
		R	GTTGTTCTTCAGTGGCTCTA	
GCLM	NM_001007953.2	F	GCTGCTAACTCACAATGACC	174
		R	TGCATGATATAGCCTTTGGAC	
NQO1	NM_001277620.2	F	CACCATCTCTGACCTCTAC	173
		R	CCGCTTCAATCTTCTTCTG	

as follows: (weight before pressure − weight after pressure)/weight before pressure × 100.

Antioxidant Indices of Serum, Jejunum and Liver

A kit (Nanjing Jiancheng Biotechnology Co, Ltd, Nanjing, China) was used to detect the changes in the antioxidant indices malondialdehyde (**MDA**), total antioxidant capacity (**T-AOC**), and glutathione peroxidase (**GSH-Px**) in the serum, jejunum and liver and superoxide dismutase (**SOD**) in the jejunum and liver.

Jejunal Tissue Morphology

On the 42nd day of the experiment, one sampling chicken was selected from each replicate group and sacrificed. The jejunum samples were collected and stored in 4% paraformaldehyde, dehydrated in graded alcohol, clarified in xylene, and embedded in paraffin blocks. The embedded samples were cut into 5 μm sections, and slides were mounted. Paraffin sections were stained with hematoxylin-eosin (**H & E**). The villus height and crypt depth of the jejunum were measured under an optical microscope (50 × magnification, DM-1000, Leica Microsystems Inc, Germany), and the villus/crypt ratio was calculated.

RNA Extraction

The total RNA of the jejunum mucosa and liver was extracted according to the steps of Yeasen's cell/tissue total RNA extraction kit (item number 19221ES50).

Reverse Transcription

The concentration and purity of total RNA were determined by an ultramicro spectrophotometer (Nano-Drop ND 2000, Thermo, Wilmington, DE) in a low-temperature environment. The sample selection should have a total RNA OD260/OD280 ratio between 1.8 and 2.0 and an OD260/OD230 ratio between 2.0 and 2.2. A reverse transcription kit from Yeasen Biotechnology Co, Ltd., Shanghai, China,

Real-Time Fluorescent Quantitative PCR

Real-time fluorescence quantitative PCR (**RT–PCR**): RT–PCR was performed according to the RT–PCR kit of Yeasen Biotechnology Co., Ltd., Shanghai, China (No. 11884ES50). The instrument was a StepOnePlus. The specific steps of the test were as follows: pre-denaturation at 95°C for 5 min followed by 40 cycles of 95°C denaturation for 10 s and 60°C annealing/extension for 30 s. The reaction system is presented in Table 5 below. The primers were synthesized by Suzhou Jinweizhi Bioengineering Company. The gene sequence is presented in Table 3. The relative expression was calculated using the 2 − ΔΔCT method.

Cecal Microbial Analysis

After genomic DNA was extracted from the samples, the conserved region of rDNA was amplified with specific primers with barcodes. The PCR amplification products were recovered and quantified by a Quanti-Fluor^{TM} fluorometer. The purified amplification products were mixed with the same amount, the sequencing adapter was connected, the sequencing library was constructed, and Illumina PE250 was sequenced on the machine. After sequencing the raw reads, we first filtered out the low-quality reads, assembled the reads, spliced the 2-terminal reads into a tag, and then filtered the tag. The resulting data are referred to as the clean tag. Next, clustering based on clean tags removed the chimera tags detected in the process of clustering, and the final data reflected the effective tags. After obtaining OTUs, OTU abundance statistics were calculated based on effective tags. According to the analysis process, species annotation, α diversity analysis, and β diversity analysis were carried out in turn. If there were effective groups, the differences between groups were compared and statistically tested.

Statistical Analysis of Data

The experimental data are expressed as the mean $\pm$ standard error. The experimental data were analyzed by one-way ANOVA in SPSS 21.0, followed by Duncan's
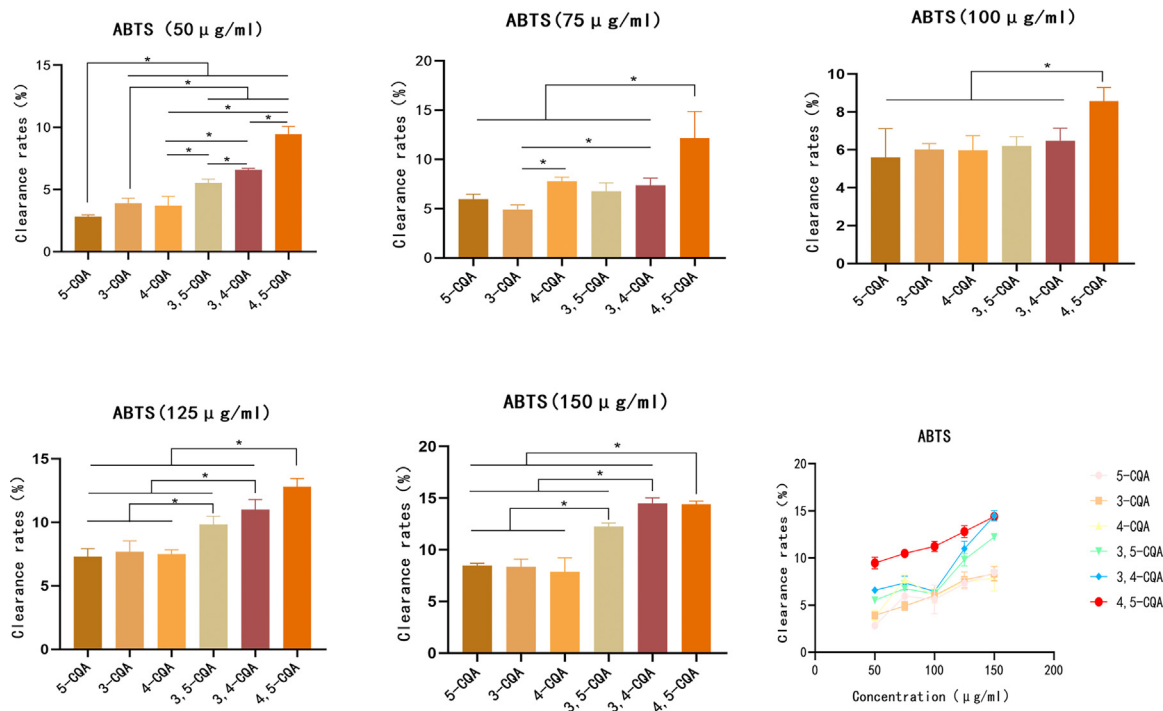
Figure 2. Comparison of ABTS free radical scavenging ability of chlorogenic acid isomers at 50 to 150 μg/mL.

multiple comparison tests. Statistical significance was set at $P < 0.05$.

RESULTS

Initial Screening of the Antioxidant Capacity of Chlorogenic Acid Isomers

The ABTS free radical scavenging ability, DPPH free radical scavenging ability, and FRAP ferrous ion reducing ability of chlorogenic acid isomers (CGA, cryptochlorogenic acid, neochlorogenic acid, isochlorogenic acid A, isochlorogenic acid B and isochlorogenic acid C) were tested. In the ABTS free radical scavenging test the ABTS free radical scavenging ability of the different chlorogenic acid isomers increased with increasing concentrations. At high concentrations, the free radical scavenging ability of DICQA (isochlorogenic acid A, isochlorogenic acid B and isochlorogenic acid C) was higher than CQA (CGA, cryptochlorogenic acid, and neochlorogenic acid), and isochlorogenic acid C performed best (Figure 2) ($P < 0.05$). In contrast to ABTS detection, although the DPPH free radical scavenging ability of different chlorogenic acid isomers increased with increasing concentration, the difference between DICQA and CQA was more significant at low concentrations, and isochlorogenic acids A and B performed best (Figure 3) ($P < 0.05$). In the FRAP ferrous ion reduction ability test, the reduction ability of chlorogenic acid isomers also increased in a dose-dependent manner, and the ferrous ion reduction ability of isochlorogenic acid B differed significantly from cryptochlorogenic acid at each concentration (Figure 4) ($P < 0.05$).

At a concentration of 25 μM, pretreatment with isochlorogenic acids A, B and C significantly improved cell viability after the addition of BHP ($P < 0.05$). Among them, the viability of the isochlorogenic acid B group was significantly higher than that of the CGA group but did not differ significantly from other isochlorogenic acid groups. at 100 μM, the cell viability of isochlorogenic acid A, B, and C pretreatment was significantly higher than that of the model and CGA groups ($P < 0.05$), At 200 μM, CGA, isochlorogenic acid A, B, and C pretreatment significantly improved cell viability after the addition of BHP ($P < 0.05$), and there was no significant difference among the isomers (Figure 5A). In addition, at 25 μM, the ROS level of the isochlorogenic acid A, B, and C pretreatment groups was significantly lower than that of the Pcon group, model group or CGA group, and there was no significant difference from DICQA ($P < 0.05$). At 100 μM, CGA and isochlorogenic acid A, B, and C pretreatment significantly reduced the ROS level after BHP addition. The ROS level in the isochlorogenic acid B group was significantly higher than in the Pcon group, model group, CGA group, or other isochlorogenic acid groups ($P < 0.05$). At 200 μM, CGA, isochlorogenic acid A, B, and C pretreatment significantly improved the cell activity after adding BHP ($P < 0.05$), and there was no significant difference among the isomers (Figure 5B).

Preliminary Determination of the Proportion of Chlorogenic Acid Isomers With the Best Antioxidant Capacity

As shown in Figure 6A, LC exhibited the best ABTS free radical scavenging ability in the in vitro antioxidant test, which was significantly different from other
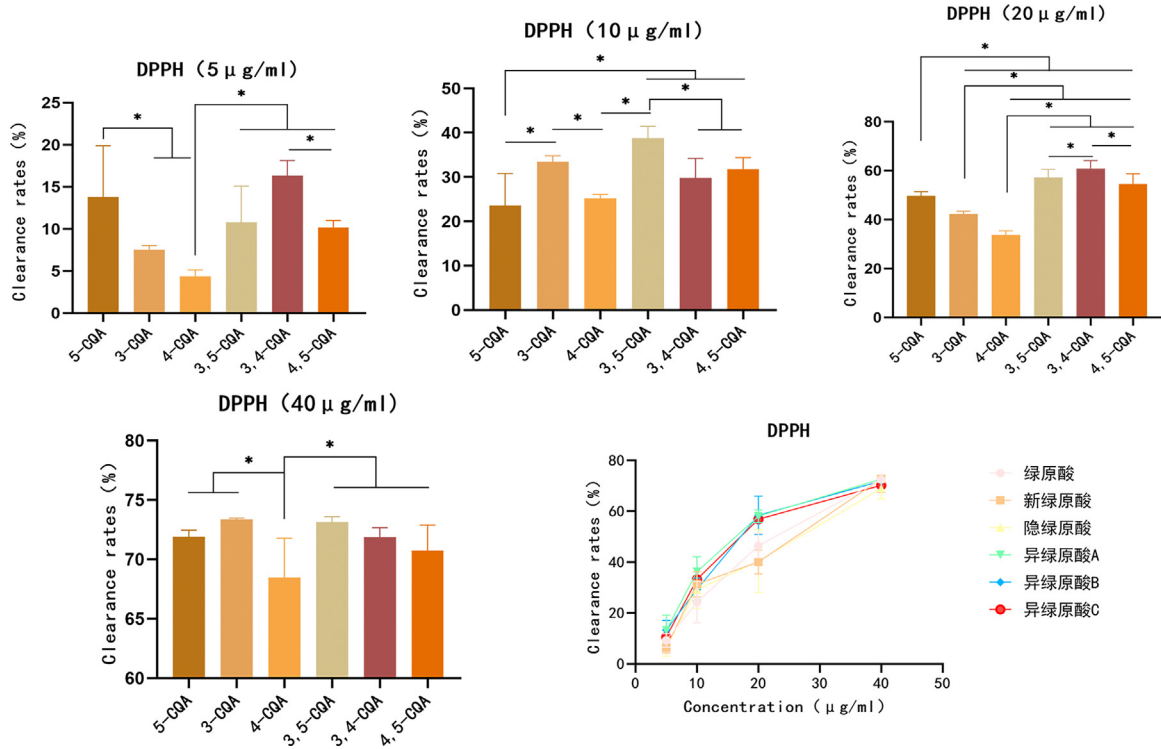
Figure 3. Comparison of DPPH free radical scavenging ability of chlorogenic acid isomers at 5 to 40 μg/mL.

compound types and single products ($P < 0.05$). The DPPH free radical scavenging ability of LB and LC was the best, which was significantly different from other compound types and single products ($P < 0.05$), but there was no significant difference between them (Figure 6B). The FRAP ferrous ion reducing ability of LB was the best, which differed significantly from most of the compounding types and single products

(Figure 6C) ($P < 0.05$). In the cell experiment, we first assessed the antioxidant capacity of the compound mixture.

When the oxidation model was constructed, the cell viability decreased to approximately 60%, and the ROS level increased significantly ($P < 0.05$). However, after pretreatment with LB and LC, the cell viability increased to 80 to 90%, and the ROS level of the YB,
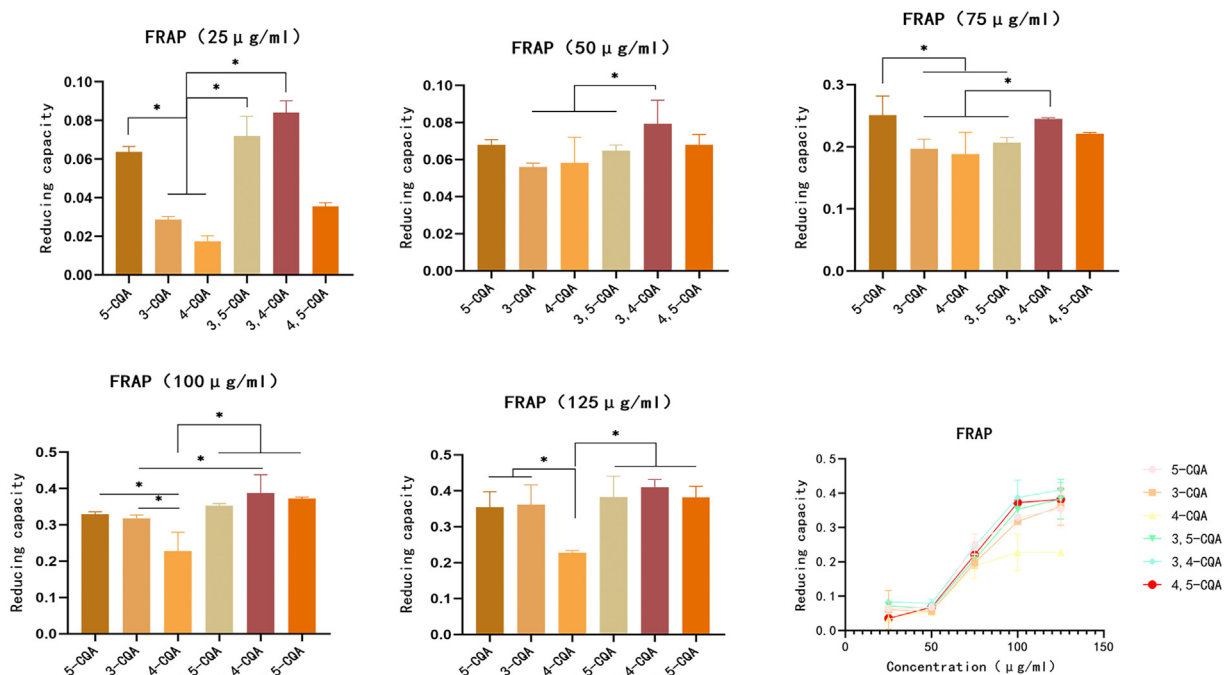

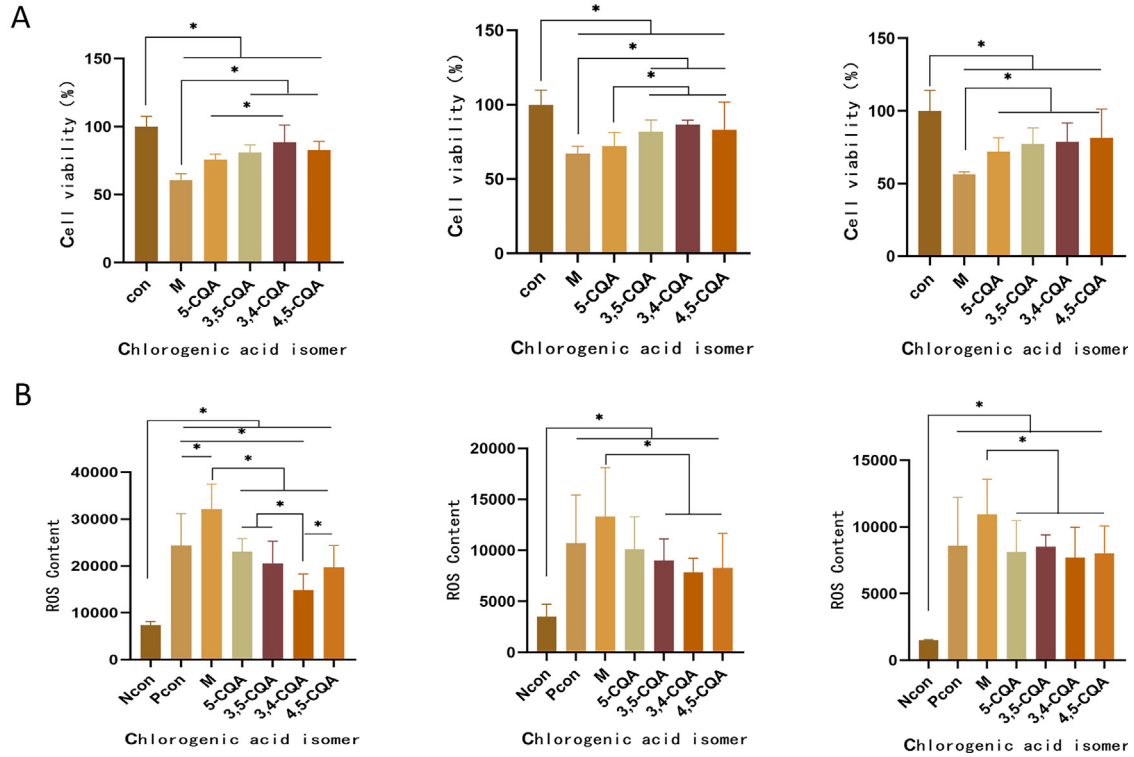**Figure 4.** Comparison of FRAP ferrous ion reduction ability of chlorogenic acid isomers at 25 to 125 μg/mL.

Figure 5. Chlorogenic acid isomers can alleviate oxidative stress by reducing ROS content. The 3 plots represent 25 μM, 100 μM, and 200 μM chlorogenic acid isomers from left to right. This dose was explored in previous experiments. (A) Changes in intestinal epithelial cell viability after pretreatment with different chlorogenic acid isomers in the oxidative stress model. (B) Changes in ROS content in intestinal epithelial cells after pretreatment with different chlorogenic acid isomers in the oxidative stress model.

YC, LB, and LC treatment groups decreased significantly (Figure 7A) ($P < 0.05$). Subsequently, combined with the previous test results, YA, YB, LA, and LB were compared with chlorogenic acid isomers. Under the same oxidative stress model, the cell activity increased to 70 to 80% after YC and LC pretreatment, and the ROS level decreased significantly (Figure 7B) ($P < 0.05$).
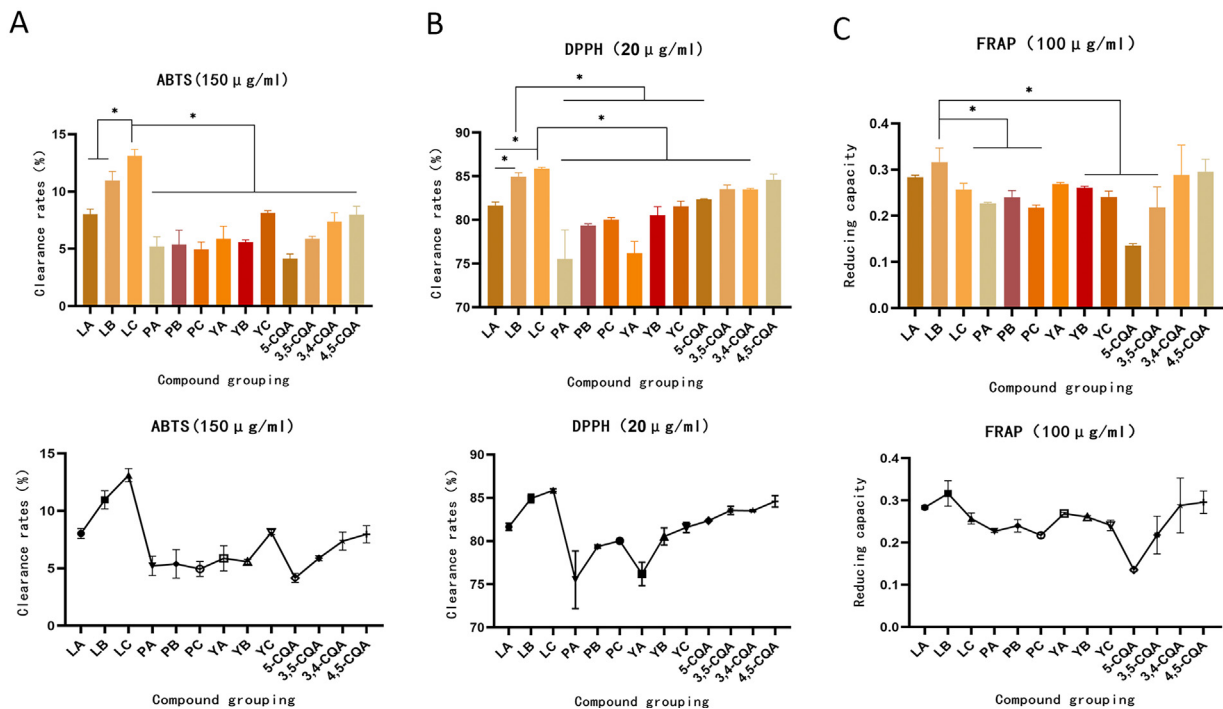

Figure 6. (A) Comparison of the ABTS free radical scavenging ability of chlorogenic acid isomer compound products at 150 μg/mL. (B) Comparison of the DPPH free radical scavenging ability of chlorogenic acid isomer compound products at 40 μg/mL. (C) Comparison of FRAP ferrous ion reducing ability of chlorogenic acid isomer compounds at 100 μg/ma.
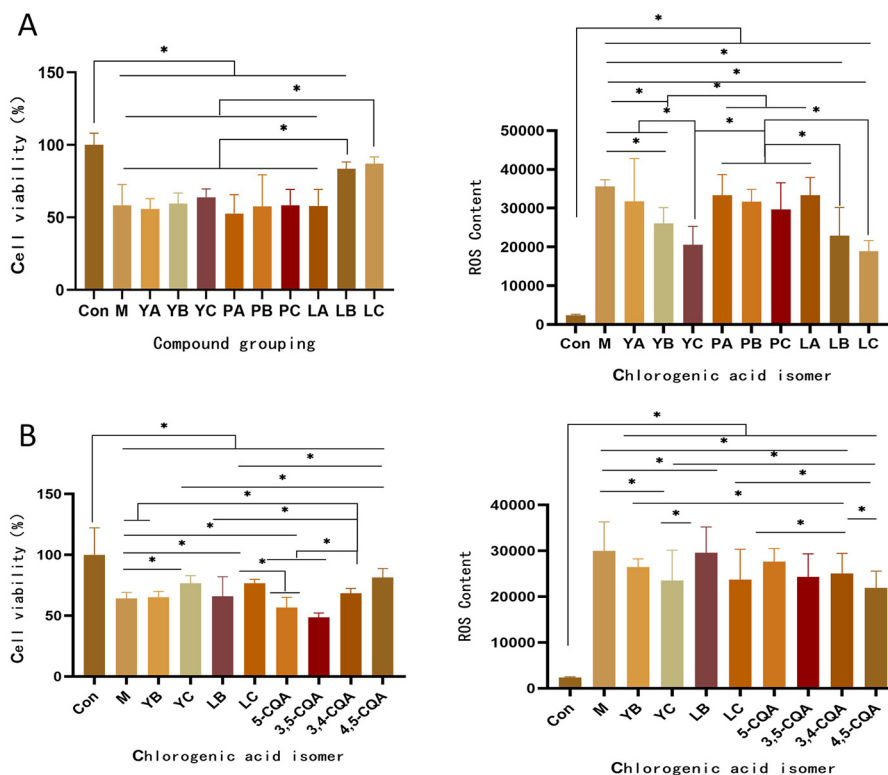
Figure 7. The concentration of the mixture of chlorogenic acid isomers was 200 μM. (A) Changes in intestinal epithelial cell viability after pretreatment with different chlorogenic acid isomer mixtures under an oxidative stress model. (B) Changes in ROS content in intestinal epithelial cells after pretreatment with different chlorogenic acid isomers in an oxidative stress model.

The Chlorogenic Acid Compound LB Can Alleviate the Damage of Oxidative Stress on the Production Performance of Broilers

As shown in Table 4, oxidative stress significantly reduced 42 d BW, 22 to 42 d ADG, and 1 to 42 d ADG and significantly increased 22 to 42 d FCR and 1 to 42 d FCR ($P < 0.05$). Compared with the model group, the CIB Group 1 to 21 d ADFI, CIC Group 42 d BW, 22 to 42 d ADG, 1 to 42 d ADG increased significantly and the CIB Group 22 to 42 d FCR decreased significantly ($P < 0.05$). Compared with the chlorogenic acid group,

the ADFI of the CIB group was significantly increased from 1 to 21 d, and the FCR was significantly decreased from 22 to 42 d ($P < 0.05$).

As shown in Table 5, oxidative stress significantly reduced the water holding capacity of broilers and significantly increased their yellowness (b*) value ($P < 0.05$); compared with the model group, the water holding capacity of the CIB group was significantly increased, and the yellowness (b*) value of the CIB group was significantly decreased ($P < 0.05$). In addition, the brightness (L*) value of the CIB group was significantly lower than that of the chlorogenic acid group ($P < 0.05$).

Table 4. Effects of chlorogenic acid isomers and their compounds on the production performance of broilers under oxidative stress.

Items	Control /	PC /	CGA CGA (50g/t)	CIB LB (50g/t)	CIC LC (50g/t)	SEM	P Value
1d BW/g	44.20	44.20	44.46	44.48	44.18	0.659	0.381
21d BW/kg	1.24[ab]	1.22[abc]	1.19[c]	1.21[bc]	1.26[a]	0.007	0.019
1−21d ADG/g	56.99[ab]	56.19[abc]	54.44[c]	55.70[bc]	58.08[a]	0.360	0.019
1−21d ADFI/g	65.29[b]	65.56[b]	65.36[b]	67.90[a]	68.24[a]	0.355	0.023
1−21d FCR	1.15[b]	1.17[ab]	1.23[a]	1.22[a]	1.17[ab]	0.009	0.031
42d BW/kg	3.77[a]	3.51[b]	3.61[ab]	3.65[ab]	3.79[a]	0.033	0.024
22−42d ADG/g	120.23[a]	108.70[b]	115.80[ab]	116.02[ab]	121.04[a]	1.445	0.043
22−42d ADFI/g	193.53	213.13	207.16	201.57	194.08	2.951	0.147
22−42d FCR	1.61[b]	1.97[a]	1.76[ab]	1.71[b]	1.61[b]	0.039	0.010
1−42d ADG/g	88.61[a]	82.45[b]	84.84[ab]	85.86[ab]	89.26[a]	0.780	0.023
1−42d ADFI/g	129.41	139.34	136.72	134.55	131.16	1.545	0.230
1−42d FCR	1.46[b]	1.69[a]	1.58[ab]	1.59[ab]	1.47[b]	0.026	0.016

In the same rank, values with different letter superscripts indicate significant difference ($P < 0.05$), while the same letter or no letter indicates no significant difference, similar to below.

Table 5. Effects of chlorogenic acid isomers and their compounds on the meat quality of broilers under oxidative stress.

Items	Control /	PC /	CGA CGA (50 g/t)	CIB LB (50 g/t)	CIC LC (50 g/t)	SEM	P value
Breast muscle weight ratio	120.37	112.77	115.83	118.11	120.32	1.188	0.206
L*	53.64	53.21	56.62	52.75	54.43	0.663	0.251
b*	6.85^{bc}	8.59^{a}	7.73^{ab}	6.92^{bc}	5.88^{c}	0.276	0.016
Water binding capacity	51.32	46.00	49.57	50.48	51.02	0.788	0.196

Table 6. Effects of chlorogenic acid isomers and their compounds on the antioxidant properties of broilers under oxidative stress.

Items	Control /	PC /	CGA CGA (50 g/t)	CIB LB (50 g/t)	CIC LC (50 g/t)	SEM	P value
Jejunum MDA (nmol/mL)	2.05^{ab}	2.24^{a}	1.52^{ab}	1.18^{b}	2.07^{ab}	0.105	0.041
Jejunum GSH-Px	24.11^{b}	22.51^{b}	24.55^{b}	44.52^{a}	25.38^{b}	2.237	0.004
Jejunum T-AOC (mmol/g)	0.075	0.042	0.055	0.077	0.046	0.006	0.181
Liver SOD (U/mg)	49.81^{a}	35.96^{ab}	31.58^{b}	51.60^{a}	29.51^{b}	2.903	0.025
Liver MDA (nmol/mL)	2.40^{b}	3.31^{a}	2.23^{b}	1.67^{b}	1.56^{b}	0.171	0.002
Liver GSH-Px	15.67^{a}	11.77^{b}	10.13^{b}	10.28^{b}	9.21^{b}	0.702	0.022
Liver T-AOC (mmol/g)	0.070	0.040	0.053	0.046	0.053	0.004	0.099
Serum T-AOC (mmol/g)	0.81	0.68	0.57	0.69	0.56	0.033	0.109
Serum GSH-Px	2274.88^{b}	$1,746.66^{c}$	2845.97^{a}	2514.74^{b}	2435.67^{b}	81.553	0.000

The Chlorogenic Acid Compound LB Can Affect Oxidative Stress in Broilers

As shown in Table 6, oxidative stress significantly reduced GSH-Px activity, T-AOC in the liver, GSH-Px activity in the serum and T-AOC in the jejunum of broilers and significantly increased MDA content in the liver ($P < 0.05$). Compared with the model group, the MDA content in the liver decreased significantly in other groups ($P < 0.05$). The addition of CIB significantly reduced the MDA content in the jejunum of broilers and increased the GSH-Px activity in the jejunum and serum and T-AOC in the jejunum ($P < 0.05$). Furthermore, the activity of GSH-Px in the jejunum of the CIB group was significantly higher than that of the other groups, the liver SOD activity was significantly higher than that of the chlorogenic acid and CIC groups, and T-AOC in the jejunum was significantly higher than that in the CIC group ($P < 0.05$).

Figure 8 demonstrates that oxidative stress significantly downregulated the expression of the Nrf2 and
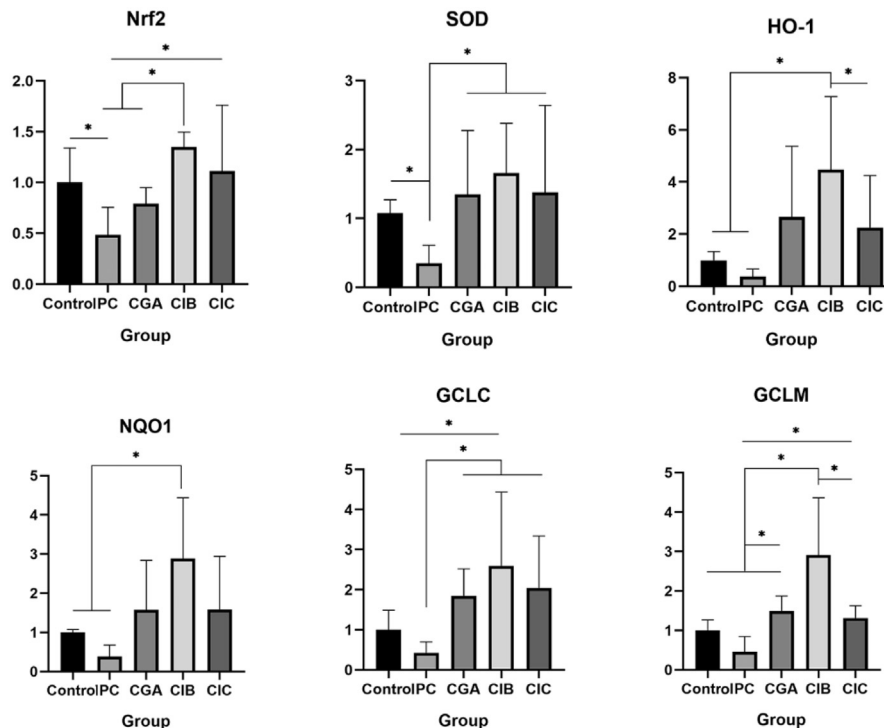
Figure 8. Effects of chlorogenic acid isomers and their compounds on Nrf2 pathway gene expression in the liver of broilers under oxidative stress.
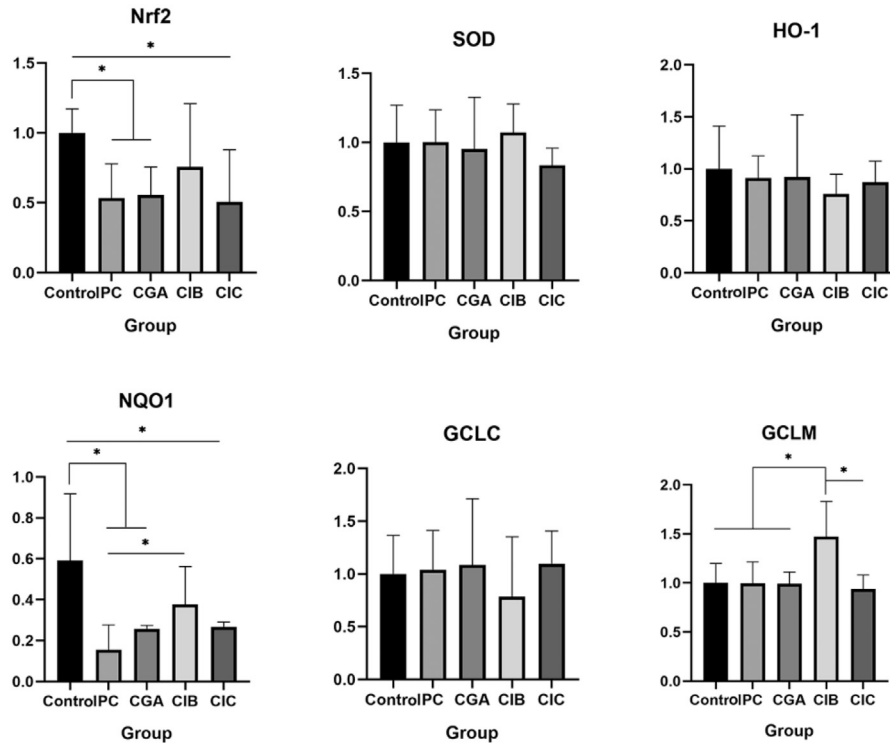
Figure 9. Effects of chlorogenic acid isomers and their compounds on Nrf2 pathway gene expression in the jejunum of broilers under oxidative stress.

SOD genes in the liver of broilers ($P < 0.05$). Compared with the model group, the addition of CIB significantly upregulated the expression of the Nrf2, SOD, Heme oxygenase-1 (**HO-1**), NADPH: Quinone oxidoreductase 1 (**NQO1**), Recombinant glutamate cysteine ligase, catalytic (**GCLC**) and Recombinant glutamate cysteine ligase, modifier subunit (**GCLM**) genes in the liver of broilers ($P < 0.05$). In addition, the expression of Nrf2 in the liver of the CIB group was significantly higher than that of the chlorogenic acid group, the expression of HO-1 was significantly higher than that of the control group and CIC group, the expression of NQO1 and GCLC was significantly higher than that of the control group, and the expression of GCLM was significantly higher than that of the other groups ($P < 0.05$).

As shown in Figure 9, oxidative stress significantly downregulated the expression of the Nrf2 and NQO1 genes in the jejunum of broilers ($P < 0.05$). Compared with the model group, the addition of CIB significantly upregulated the expression of the NQO1 and GCLM genes in the jejunum of broilers ($P < 0.05$).

The Chlorogenic Acid Compound LB Can Regulate Disordered Intestinal Flora in Broilers

Figure 10(A) demonstrates that 10 phyla were detected in all cecal chyme samples at the phylum level (phylum). The relative abundance of the bacteria detected in the cecal samples was calculated, and *Firmicutes, Bacteroidetes*, and *Proteobacteria* were identified as the dominant bacteria (content greater than 2%). Oxidative stress significantly increased the relative abundance of *Proteobacteria*, decrease the relative abundance of Firmicutes at the phylum level, and increase the relative abundance of *Desulfobacterota* at the phylum level ($P < 0.05$). Compared with the model group, the CIB group exhibited a trend toward reductions in the relative abundance of *Desulfobacterota* and *Proteobacteria* at the phylum level ($P < 0.05$). As shown in Figure 10(B), the dominant flora (content > 2%) at the top 10 family level (Family) in each group included *Bacteroidaceae, Ruminococcaceae, Lachnospiraceae, Oscillospiraceae,* and *Enterobacteriaceae*. Oxidative stress significantly reduced the abundance of *Lachnospiraceae* at the family level and had a tendency to reduce the relative abundance of *Ruminococcaceae* ($P < 0.05$). Compared with the model group, the CIB group exhibited a significant increase in the relative abundance of *Ruminococcaceae* and had a significant reduction in the relative abundance of *Sphingomonadaceae* ($P < 0.05$). As shown in Figure 10(C), the dominant bacteria at the top 50 genus level (genus) in each group included *Bacteroides, Faecalibacterium, Escherichia-Shigella*, and *Ruminococcus torques group* (content greater than 2%). Oxidative stress tended to increase the relative abundance of *DTU089* at the genus level and tended to reduce the relative abundance of *Shuttleworthia* at the genus level ($P < 0.05$). Compared with the model group, the CIB group exhibited a reduction trend in the relative abundance of *Negativibacillus* at the genus level, an increasing trend in the relative abundance of *Subdoligranulum* at the genus level, a significant increase in the
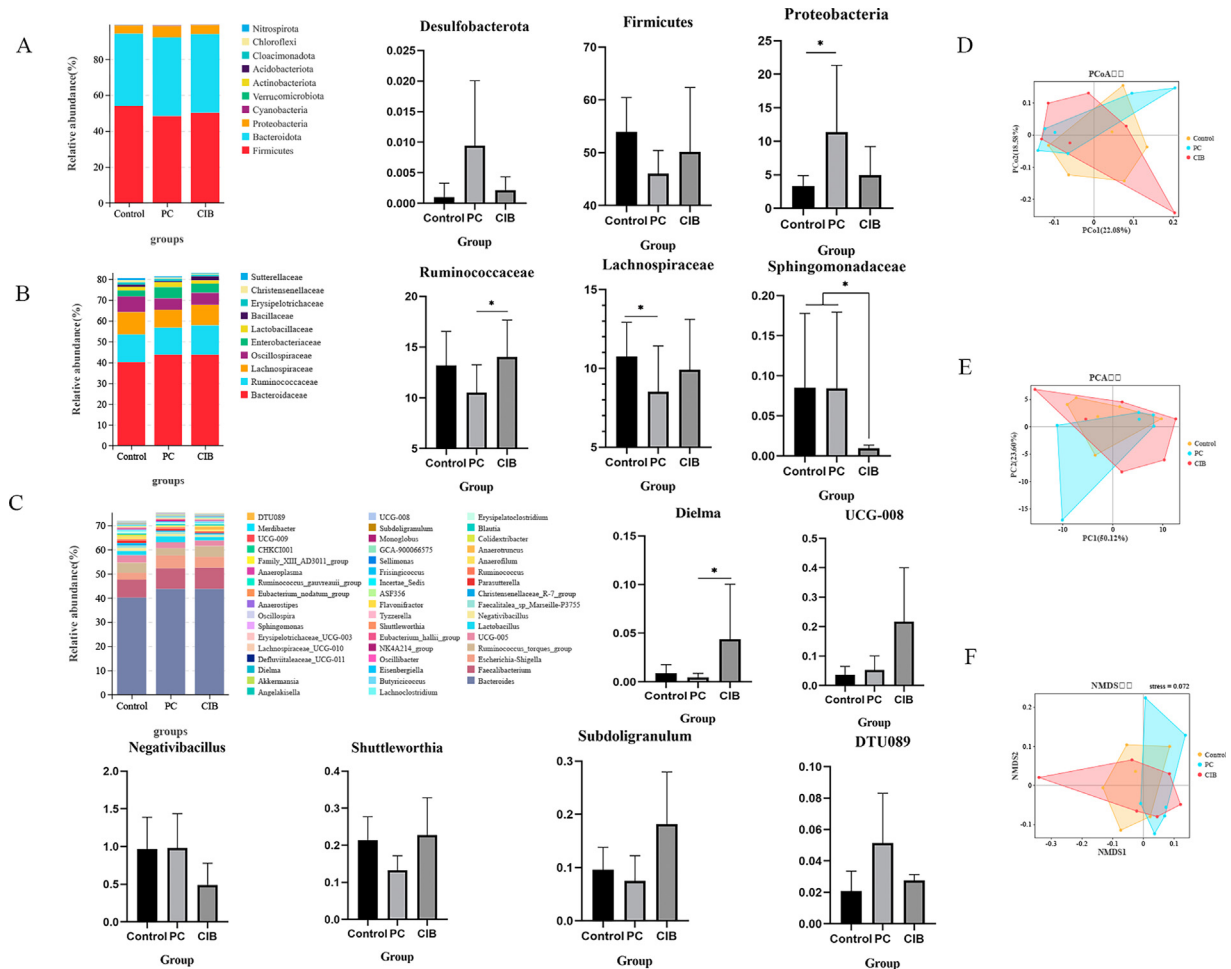
Figure 10. (A−C) Effects of chlorogenic acid isomers and their compounds on the composition of the cecal microbial phylum, family, and genus levels in broilers under oxidative stress. (D−F) Effects of chlorogenic acid isomers and their mixtures on PCoA, PCA, and NMDS clustering of the cecal microorganisms of broilers under oxidative stress.

relative abundance of *Dielma* at the genus level, and an increasing trend in the relative abundance of *Shuttleworthia* and *UCG-008* at the genus level ($P < 0.05$).

As shown in Figures 10 (D−F), compared with other groups, the overlapping area between the CIB group and the control group was larger, indicating that the PCoA, PCA, and NMDS clustering similarity between the control group and the CIB group was higher than that between the control group and other groups.

DISCUSSION

In this experiment, we observed that the antioxidant activity of DICGA was higher than that of caffeoylquinic acids, and the difference between caffeoylquinic acids was not obvious. In previous studies, the relative effects of 6 chlorogenic acid isomers in coffee and other plant foods on the antioxidant status of inflammatory Caco-2 intestinal cells were measured. CGA isomers improved oxidative stress, scavenged intracellular ROS, increased GSH, and activated Nrf2 signaling. Furthermore, DICQA isomers exhibited superior reduction of IL-8, which was consistent with the results of this

experiment (Liang and Kitts, 2018, Liang et al., 2019). Previously, some scholars measured the antioxidant activity of chlorogenic acid isomers of coffee bean extract by ORAC, ABTS, and DPPH assays (Liang et al., 2016b). However, the unique feature of this experiment is that the combination of a free radical scavenging assay and a cell assay increases the credibility of the in vitro comparison of the antioxidant capacity of chlorogenic acid isomers. Overall, this study provides clear experimental evidence to confirm the superiority of DICQA antioxidant capacity.

However, most of the natural chlorogenic acid isomers in plants do not exist as monomers but are widely present in compound forms. In honeysuckle, in addition to CGA, high-purity isochlorogenic acids A, B, and C can be obtained (Rosliuk et al., 2020). Abundant chlorogenic acid isomers can be detected in various functional plants, such as coffee (Craig et al., 2016), stevia (Craig et al., 2016), and *Eucommia ulmoides* (Wang and Clifford, 2008). Therefore, after determining the test results of chlorogenic acid isomer monomers, this experiment studied chlorogenic acid compounds, which have been less well studied by previous reports. CGA (5-CQA) is the most abundant caffeoylquinic acid isomer in plants

and the most commonly used caffeoylquinic acid isomer in daily life. Therefore, CGA and DICQA were selected for further comparison in cells.

In the subsequent experiment, the antioxidant capacity of LB, LC, YB, and YC was demonstrated to be superior to that of other compound mixtures and some monomers. Particular with respect to the free radical scavenging test, the groups with high CGA proportions generally exhibited higher antioxidant capacity. In previous studies, it was found that when the ethanol extract of *Centella* asiatica was combined with α-tocopherol, the higher the content of ethanol extract of *Centella* asiatica with low antioxidant activity, the higher the synergistic antioxidant effect (Zhao et al., 2022). CQA is equivalent to the ethanol extract of *Centella* asiatica, DICQA is equivalent to α-tocopherol, and the synergistic antioxidant effect of the system is increased at high doses of CQA. The article suggests that the synergistic effect is due to the low antioxidant activity of the ethanol extract of *Centella asiatica*. The regeneration of α-tocopherol with higher antioxidant activity may also be related to the high antioxidant capacity of LB and LC in this experiment (Thoo et al., 2013). In addition, some scholars have confirmed that in the binary complex system of CQA and DICQA chlorogenic acid, as the CQA contents gradual increases, the degree of synergism in the ABTS free radical scavenging ability of the binary complex system increases linearly, and the phenomenon from antagonism to additive to synergistic effect was observed. In the detection of β-carotene bleaching inhibition, a concave trend was observed with increasing compound ratios, and the degree of synergism was lowest when the ratio of the 2 tended to be equal; the degree of synergism was highest when the ratio of the 2 differed significantly (Yang, 2016), which was consistent with the higher antioxidant capacity of LB, LC, YB, and YC in cell experiments. We speculate that this may be related to differential effects of the mixture in different reaction environments, although the specific reasons for this phenomenon remain to be further elucidated.

The growth performance of broiler chickens is the most direct indicator to measure growth and development status. Improvements in growth performance are positively correlated with increasing economic benefits. Growth performance is affected by many factors, such as heredity, environment, and nutrition. Growth performance is often measured by daily gain, daily feed intake, body weight, and feed conversion ratio. In broiler breeding, oxidative stress often causes loss of appetite, resulting in decreased body weight and daily feed intake, slowed growth rate, and significant economic losses in animal husbandry. In their study, Zha et al. (2023) reported that adding 500 mg/kg pure chlorogenic acid to the diet can effectively increase the daily weight gain and daily feed intake of broilers and alleviate the decline in production performance caused by oxidative stress. In this experiment, after H_2O_2 injection, the daily gain and daily feed intake of the model group decreased significantly, and the feed-to-meat ratio increased significantly. This finding is consistent with the previous experimental results and indicates that when the oxidative stress model was successfully constructed, the production performance would be affected. The CIB and CIC groups supplemented with LB and LC compounds reversed the decline in production performance; however, the effect in the chlorogenic acid group was not significant, which is inconsistent with the previous test results. This inconsistency may be due to differences in the volume of additives. In addition, at the same dose, the CIB and CIC groups were more effective than the chlorogenic acid group in increasing daily weight gain, daily feed intake and body weight and in reducing the ratio of feed to gain, indicating that the CIB and CIC better alleviate damage than the chlorogenic acid monomer even at low doses.

Meat quality is the most direct and important index to measure the meat quality of livestock and poultry and include measurements of shear force, weight ratio, pH, water loss rate, and meat color (Yu et al., 2020). In the detection of meat quality, the water holding capacity of muscle generally refers to the water retention capacity of muscle, which is negatively correlated with drip loss and cooking loss. Poor water holding capacity is an important factor leading to a decrease in meat quality and tenderness. Meat color is an important factor in judging the appearance of chicken, which can affect the sales of chicken. Generally, lower brightness (L*) values reflect less white muscle, less water exuded from the muscle surface, and better meat quality. Higher redness (a*) values reflect lower yellowness (b*) values and indicate a superior muscle color (Warriss and Brown, 1987). Previous studies have found that oxidative stress induced by H_2O_2 significantly increased meat drip loss (Yan et al., 2022). In our study, oxidative stress significantly decreased breast muscle weight and water retention capacity, while the yellowness (b*) value increased significantly. These findings are consistent with the results of previous studies, indicating that oxidative stress significantly reduces the quality of chicken meat. Under stress, the addition of 1000 mg/kg chlorogenic acid could significantly increase the pH_{24} value and a * value of chicken; reduce the drip loss, cooking loss and L * value; and reduce the meat quality damage caused by oxidative stress (Zhao et al., 2019). Through this experiment, the CIB and CIC groups exhibited improvements in water retention capacity and reduced yellowness (b*) values. In addition, the brightness (L*) value of the CIB group was significantly lower than that of the chlorogenic acid group. Previous experiments demonstrated that chlorogenic acid can reverse the effect of meat quality damage at high doses. However, the lower dose used in this experiment also produces an antioxidant effect that tenderizes the meat tender and produces a more healthy and beautiful meat color. These findings demonstrate that the chlorogenic acid isomer compound, especially CIB, exhibited a more prominent meat quality improvement ability.

Due to the short breeding cycle of broilers and the sealed breeding environment, oxidative stress occurs during broiler production. Broilers have evolved a

variety of antioxidant defense systems to fight stress and protect cells from attack by reactive oxygen species (Halliwell, 1999). Chi et al. (2020) found that H_2O_2-induced oxidative stress can significantly decrease the activity of T-AOC, GSH-Px, and SOD in lymphocytes and increase cellular MDA content. In this experiment, oxidative stress caused a significant decrease in GSH-Px activity, T-AOC, serum GSH-Px activity, and jejunum T-AOC in broiler livers and increased MDA content in the jejunum and liver, which is consistent with previous results. These findings demonstrate that the activity of antioxidant enzymes is inhibited during oxidative stress, resulting in lipid peroxidation and excessive accumulation of MDA. This stress damage is more prominent in the liver, which may be related to the fact that the liver is the primarily site of ROS attack (Sharma et al., 2012). The addition of chlorogenic acid or its compound to the diet can alleviate the damage caused by oxidative stress (Zha et al., 2023). The CIB group significantly reduced MDA content in the liver and jejunum and increased GSH-Px activity in the jejunum and serum. The activity of GSH-Px in the jejunum and the activity of SOD in the liver were significantly higher than the respective activities in the chlorogenic acid and CIC groups, and the T-AOC in the jejunum was also significantly higher than that in the CIC group. Together, these data demonstrate that the CIB group with the LB compound exhibited stronger antioxidant capacity, which could reverse the stress-induced oxidase inhibition and protect the body from oxidative loss.

As a transcription factor, Nrf2 regulates cell defense against toxic and oxidative through downstream expression of genes involved in the oxidative stress response and drug detoxification. More than 90% of antioxidant genes are regulated by the Nrf2 pathway (Xu et al., 2018; He et al., 2020; Ghanim et al., 2021). As an important antioxidant factor, Nrf2 typically forms a complex with Keap1 in the cytoplasm. In the presence of oxidative stress, the complex dissociates, and Nrf2 binds to ARE in the nucleus to promote the transcriptional activation of downstream antioxidant factors, including NQO1, HO-1, SOD and GSH-Px, as well as GCLC and GCLM (Yi et al., 2020; Yang et al., 2021). HO-1 is considered to be an important promoter of the antioxidant effect of Nrf2 (Ryter, 2021). Liu et al. found that oxidative stress downregulated the expression of the Nrf2 and HO-1 genes in the ovaries of aging laying hens (Liu et al., 2018). In addition, bromoacetic acid-induced oxidative stress also significantly downregulated the expression of the Nrf2, HO-1, NQO1, GCLM, and GCLC genes in chicken kidneys (Wu et al., 2022). Consistent with the results of previous studies, the expression of Nrf2 and SOD in the liver and Nrf2 and NQO1 in the jejunum of broilers in the model group was significantly downregulated, indicating that when broilers were injected with H_2O_2, the rapidly accumulated ROS in vivo inhibited the antioxidant capacity of the Nrf2 pathway (including Nrf2 and its downstream genes).

The intestine is the largest digestive organ in the human body (Depoortere, 2014). To protect the body from potential pathogens and external stimuli, the gut has evolved regional immune characteristics. The intestinal microenvironment created by the intestinal flora and its products significantly affects immune function. In turn, stress and disease regulate and affect the composition of the intestinal flora (Zhou et al., 2020). In recent years, human and animal experiments have revealed that the intestinal flora possesses metabolic, nutritional, and immunomodulatory functions (Sebastián Domingo et al., 2018). Changes in its composition and function (intestinal flora imbalance) are closely related to the development of various diseases, including inflammatory bowel disease, irritable bowel syndrome, and abdominal diseases (Jin et al., 2019, Li et al., 2020). Many studies have reported that excessive ROS produced by oxidative stress can destroy the intestinal structure and lead to disordered intestinal flora (Assimakopoulos et al., 2004). Furthermore, oxidative stress can promote the proliferation of harmful bacteria such as *Escherichia coli* and inhibit the proliferation of probiotics such as Lactobacillus (O'Mahony et al., 2017), leading to inflammation and resulting in decreased production performance and even the death of broilers. Chlorogenic acid can regulate the imbalance of intestinal flora and inhibit the proliferation of harmful bacteria by enhancing the body's antioxidant capacity and destroying bacterial cell membranes (Fattouch et al., 2007). In this experiment, the statistical results of alpha diversity demonstrated that oxidative stress did not change the richness and diversity structure of the cecal microbial community. The results of beta diversity clustering demonstrated that the cross area of PCoA clustering, PCA clustering and NMDS clustering between the CIB group and the control group was larger than that of other groups, indicating that the addition of LB compound to the diet played a more prominent role in improving the species similarity between cecal microorganisms under oxidative stress. As demonstrated through the significant difference analysis of the intestinal flora of broilers, the relative abundance of *Proteobacteria* at the phylum level in the model group was significantly increased. *Firmicutes* tended to decrease, whereas the relative abundance of *Proteobacteria* was restored after adding LB compound. *Proteobacteria*, a major bacterial phylum, contains many pathogens, such as *Escherichia coli* and *Salmonella*. Some scholars have noted that the increase in *Proteobacteria* is a potential diagnostic marker of dysbacteriosis and disease risk (Shin et al., 2015). Studies have demonstrated that *Firmicutes* are beneficial to the absorption and digestion of nutrients in the intestine (Hou et al., 2020). At the family level, the relative abundance of *Lachnospiraceae* in the model group decreased significantly, and the relative abundance of *Ruminococcaceae* tended to decrease. The relative abundance of *Ruminococcaceae* in the CIB group increased significantly. *Lachnospiraceae* can regulate immune and inflammatory responses by producing large amounts of short-chain fatty acids (Smith et al., 2013; Reichardt et al., 2014; Chen et al., 2017). *Ruminococcaceae* is the primary microorganism that converts primary bile acids

into secondary bile acids, and decreases in its abundance decreases may lead to intestinal inflammation (Sinha et al., 2020). At the genus level, the relative abundance of *Dielma* in the CIB group was significantly higher than that in the model group. *Dielma* is a new genus found in human feces that is positively correlated with fat metabolism in broilers. In summary, oxidative stress can cause intestinal flora imbalance in broilers, affect intestinal function, and cause an inflammatory response, and the LB compound can reverse this imbalance, regulate fat metabolism, alleviate damage caused by inflammation, and maintain body health.

In summary, the LB compound exhibited outstanding antioxidant effects in vivo and in vitro compared with the other groups. One proposed mechanism is regulation of the Nrf2 signaling pathway, thereby increasing the activity of antioxidant enzymes in vivo, promoting the secretion of antioxidant factors, enhancing the antioxidant capacity of the body, reducing oxidative damage to the liver and intestinal morphology, maintaining the normal function of the liver and intestine, regulating the intestinal flora and alleviating oxidative stress. The results of this experiment can provide some directions and ideas for the screening and research of antioxidative stress additives.

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DISCLOSURES

The authors declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service, and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

REFERENCES

Assimakopoulos, S. F., C. E. Vagianos, N. Patsoukis, C. Georgiou, V. Nikolopoulou, and C. D. Scopa. 2004. Evidence for intestinal oxidative stress in obstructive jaundice-induced gut barrier dysfunction in rats. Acta Physiol. Scand. 180:177–185.

Chen, L., J. E. Wilson, M. J. Koenigsknecht, W. C. Chou, S. A. Montgomery, A. D. Truax, W. J. Brickey, C. D. Packey, N. Maharshak, G. K. Matsushima, S. E. Plevy, V. B. Young, R. B. Sartor, and J. P. Ting. 2017. NLRP12 attenuates colon inflammation by maintaining colonic microbial diversity and promoting protective commensal bacterial growth. Nat. Immunol. 18:541–551.

Chi, X., X. Ma, Z. Li, Y. Zhang, Y. Wang, L. Yuan, Y. Wu, W. Xu, and S. Hu. 2020. Protective effect of epigallocatechin-3-gallate in hydrogen peroxide-induced oxidative damage in chicken lymphocytes. Oxid. Med. Cell Longev. 2020:7386239.

Clifford, M. N. 1999. Chlorogenic acids and other cinnamates-nature, occurrence and dietary burden. J. Sci. Food Agric 79:362–372.

Craig, A. P., C. Fields, N. Liang, D. Kitts, and A. Erickson. 2016. Performance review of a fast HPLC-UV method for the quantification of chlorogenic acids in green coffee bean extracts. Talanta 154:481–485.

De Oliveira Filho, J. G., and M. B. Egea. 2021. Sunflower seed byproduct and its fractions for food application, an attempt to improve the sustainability of the oil process. J. Food Sci. 86:1497–1510.

Depoortere, I. 2014. Taste receptors of the gut, emerging roles in health and disease. Gut 63:179–190.

Fattouch, S., P. Caboni, V. Coroneo, C. I. Tuberoso, A. Angioni, S. Dessi, N. Marzouki, and P. Cabras. 2007. Antimicrobial activity of *Tunisian quince* (Cydonia oblonga Miller) pulp and peel polyphenolic extracts. J. Agric. Food Chem. 55:963–969.

Ghanim, B. Y., M. I. Ahmad, Q. M. Abdallah, L. A. Qatouseh, and N. A. Qinna. 2021. Modulation of NRF2/ARE pathway- and cell death-related genes during drug-induced liver injury. Hum. Exp. Toxicol. 40:2223–2236.

Halliwell, B. 1999. Antioxidant defence mechanisms, from the beginning to the end (of the beginning). Free Radic. Res. 31:261–272.

He, F., X. Ru, and T. Wen. 2020. NRF2, a transcription factor for stress response and beyond. Int. J. Mol. Sci. 21:4777.

Hou, D., Q. Zhao, L. Yousaf, B. Chen, Y. Xue, and Q. Shen. 2020. A comparison between whole mung bean and decorticated mung bean, beneficial effects on the regulation of serum glucose and lipid disorders and the gut microbiota in high-fat diet and streptozotocin-induced prediabetic mice. Food Funct. 11:5525–5537.

Jin, M., Z. Qian, J. Yin, W. Xu, and X. Zhou. 2019. The role of intestinal microbiota in cardiovascular disease. J. Cell Mol. Med. 23:2343–2350.

Jin, S., C. Chang, L. Zhang, Y. Liu, X. Huang, and Z. Chen. 2015. Chlorogenic acid improves late diabetes through adiponectin receptor signaling pathways in db/db mice. PLoS. One 10: e0120842.

Lallemand, L. A., C. Zubieta, S. G. Lee, Y. Wang, S. Acajjaoui, J. Timmins, S. Mcsweeney, J. M. Jez, J. G. Mccarthy, and A. A. Mccarthy. 2012. A structural basis for the biosynthesis of the major chlorogenic acids found in coffee. Plant Physiol. 160:249–260.

Li, X., S. Zhu, P. Yin, S. Zhang, J. Xu, Q. Zhang, S. Shi, and T. Zhang. 2021. Combination immunotherapy of chlorogenic acid liposomes modified with sialic acid and PD-1 blockers effectively enhances the anti-tumor immune response and therapeutic effects. Drug Deliv. 28:1849–1860.

Li, X. Y., C. He, Y. Zhu, and N. H. Lu. 2020. Role of gut microbiota on intestinal barrier function in acute pancreatitis. World J. Gastroenterol. 26:2187–2193.

Liang, N., J. H. Dupuis, R. Y. Yada, and D. D. Kitts. 2019. Chlorogenic acid isomers directly interact with Keap 1-Nrf2 signaling in Caco-2 cells. Mol. Cell Biochem. 457:105–118.

Liang, N., and D. D. Kitts. 2018. Amelioration of oxidative stress in Caco-2 cells treated with pro-inflammatory proteins by chlorogenic acid isomers via activation of the Nrf2-Keap1-ARE-signaling pathway. J. Agric. Food Chem. 66:11008–11017.

Liang, N., and D. D. Kitts. 2015. Role of chlorogenic acids in controlling oxidative and inflammatory stress conditions. Nutrients. 8:16.

Liang, N., X. Lu, Y. Hu, and D. D. Kitts. 2016a. Application of attenuated total reflectance-fourier transformed infrared (ATR-FTIR) spectroscopy to determine the chlorogenic acid isomer profile and antioxidant capacity of coffee beans. J. Agric. Food Chem. 64:681–689.

Liang, N., W. Xue, P. Kennepohl, and D. D. Kitts. 2016b. Interactions between major chlorogenic acid isomers and chemical changes in coffee brew that affect antioxidant activities. Food Chem. 213:251–259.

Liu, X., X. Lin, S. Zhang, C. Guo, J. Li, Y. Mi, and C. Zhang. 2018. Lycopene ameliorates oxidative stress in the aging chicken ovary via activation of Nrf2/HO-1 pathway. Aging (Albany. NY) 10:2016–2036.

Ltd, P. P. 1976. IUPAC Commission on the Nomenclature of Organic Chemistry (CNOC) and IUPAC-IUB Commission on Biochemical Nomenclature (CBN). Nomenclature of cyclitols. Recommendations, 1973. Biochem. J. 153:23–31.

Meinhart, A. D., F. M. Damin, L. Caldeirão, T. F. F. Da Silveira, J. T. Filho, and H. T. Godoy. 2017. Chlorogenic acid isomer

contents in 100 plants commercialized in Brazil. Food Res. Int. 99:522–530.

Meng, S., J. Cao, Q. Feng, J. Peng, and Y. Hu. 2013. Roles of chlorogenic Acid on regulating glucose and lipids metabolism, a review. Evid. Based. Complement. Alternat. Med. 2013:801457.

Muchtaridi, M., D. Lestari, N. K. Khairul Ikram, A. M. Gazzali, M. Hariono, and H. A. Wahab. 2021. Decaffeination and neuraminidase inhibitory activity of arabica green coffee (Coffea arabica) beans, chlorogenic acid as a potential bioactive compound. Molecules. 26:3402.

Nabavi, S. F., S. Tejada, W. N. Setzer, O. Gortzi, A. Sureda, N. Braidy, M. Daglia, A. Manayi, and S. M. Nabavi. 2017. Chlorogenic acid and mental diseases, from chemistry to medicine. Curr. Neuropharmacol. 15:471–479.

O'mahony, S. M., G. Clarke, T. G. Dinan, and J. F. Cryan. 2017. Early-life adversity and brain development, Is the microbiome a missing piece of the puzzle? Neuroscience 342:37–54.

Reichardt, N., S. H. Duncan, P. Young, A. Belenguer, C. Mcwilliam Leitch, K. P. Scott, H. J. Flint, and P. Louis. 2014. Phylogenetic distribution of three pathways for propionate production within the human gut microbiota. ISMe J. 8:1323–1335.

Rosliuk, D., R. Rutkaite, L. Ivanauskas, and V. Jakstas. 2020. Interaction between cross-linked cationic starch microgranules and chlorogenic acid isomers in artichoke and green coffee bean aqueous extracts. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 1160:122385.

Ryter, S. W. 2021. Heme oxgenase-1, a cardinal modulator of regulated cell death and inflammation. Cells 10:515.

Sharma, V., P. Singh, A. K. Pandey, and A. Dhawan. 2012. Induction of oxidative stress, DNA damage and apoptosis in mouse liver after sub-acute oral exposure to zinc oxide nanoparticles. Mutat. Res. 745:84–91.

Shin, N. R., T. W. Whon, and J. W. Bae. 2015. Proteobacteria, microbial signature of dysbiosis in gut microbiota. Trends Biotechnol. 33:496–503.

Sinha, S. R., Y. Haileselassie, L. P. Nguyen, C. Tropini, M. Wang, L. S. Becker, D. Sim, K. Jarr, E. T. Spear, G. Singh, H. Namkoong, K. Bittinger, M. A. Fischbach, J. L. Sonnenburg, and A. Habtezion. 2020. Dysbiosis-induced secondary bile acid deficiency promotes intestinal inflammation. Cell Host. Microbe 27:659–670.e655.

Smith, P. M., M. R. Howitt, N. Panikov, M. Michaud, C. A. Gallini, Y. M. Bohlooly, J. N. Glickman, and W. S. Garrett. 2013. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science 341:569–573.

Taeb, M., S. S. Mortazavi-Jahromi, A. Jafarzadeh, M. R. Mirzaei, and A. Mirshafiey. 2017. An in vitro evaluation of anti-aging effect of guluronic acid (G2013) based on enzymatic oxidative stress gene expression using healthy individuals PBMCs. Biomed. Pharmacother 90:262–267.

Tan, S., J. Gao, Q. Li, T. Guo, X. Dong, X. Bai, J. Yang, S. Hao, and F. He. 2020. Synergistic effect of chlorogenic acid and levofloxacin against *Klebsiella pneumonia* infection in vitro and in vivo. Sci. Rep. 10:20013.

Thoo, Y. Y., F. Abas, O. M. Lai, C. W. Ho, J. Yin, R. V. Hedegaard, L. H. Skibsted, and C. P. Tan. 2013. Antioxidant synergism between ethanolic Centella asiatica extracts and α-tocopherol in model systems. Food Chem 138:1215–1219.

Tian, L., M. Wu, W. Guo, H. Li, Z. Gai, and G. Gong. 2021. Evaluation of the membrane damage mechanism of chlorogenic acid against *Yersinia enterocolitica* and *Enterobacter sakazakii* and its

application in the preservation of raw pork and skim milk. Molecules. 26:6748.

Tsai, K. L., C. H. Hung, S. H. Chan, P. L. Hsieh, H. C. Ou, Y. H. Cheng, and P. M. Chu. 2018. Chlorogenic acid protects against oxLDL-induced oxidative damage and mitochondrial dysfunction by modulating SIRT1 in endothelial cells. Mol. Nutr. Food Res. 62:e1700928.

Wang, Z., and M. N. Clifford. 2008. Comparison of the profiles of chlorogenic acids and their derivatives from three Chinese traditional herbs by LC-MSn. Yao Xue Xue Bao 43:185–190.

Warriss, P. D., and S. N. Brown. 1987. The relationships between initial pH, reflectance and exudation in pig muscle. Meat. Sci. 20:65–74.

Wu, Y., J. Liu, S. Liu, W. Fan, C. Ding, Z. Gao, Z. Tang, Y. Luo, X. Shi, L. Tan, and S. Song. 2022. Bromoacetic acid causes oxidative stress and uric acid metabolism dysfunction via disturbing mitochondrial function and Nrf2 pathway in chicken kidney. Environ. Toxicol. 37:2910–2923.

Xu, D., M. Xu, S. Jeong, Y. Qian, H. Wu, Q. Xia, and X. Kong. 2018. The role of Nrf2 in liver disease, novel molecular mechanisms and therapeutic approaches. Front Pharmacol. 9:1428.

Yan, Y., X. Chen, J. Huang, C. Huan, and C. Li. 2022. H(2)O(2)-induced oxidative stress impairs meat quality by inducing apoptosis and autophagy via ROS/NF-κB signaling pathway in broiler thigh muscle. Poult. Sci. 101:101759.

Yang, K. Z. 2016. Antioxidant activity of chlorogenic acids and homologous chlorogenic acids for the search for the synergistic antioxidant mechanism. CAU.

Yang, C., P. Luo, S. J. Chen, Z. C. Deng, X. L. Fu, D. N. Xu, Y. B. Tian, Y. M. Huang, and W. J. Liu. 2021. Resveratrol sustains intestinal barrier integrity, improves antioxidant capacity, and alleviates inflammation in the jejunum of ducks exposed to acute heat stress. Poult. Sci. 100:101459.

Yi, G., J. U. Din, F. Zhao, and X. Liu. 2020. Effect of soybean peptides against hydrogen peroxide induced oxidative stress in HepG2 cells via Nrf2 signaling. Food Funct. 11:2725–2737.

Yu, M., Z. Li, T. Rong, G. Wang, Z. Liu, W. Chen, J. Li, J. Li, and X. Ma. 2020. Different dietary starch sources alter the carcass traits, meat quality, and the profile of muscle amino acid and fatty acid in finishing pigs. J. Anim. Sci. Biotechnol. 11:78.

Zha, P., L. Wei, W. Liu, Y. Chen, and Y. Zhou. 2023. Effects of dietary supplementation with chlorogenic acid on growth performance, antioxidant capacity, and hepatic inflammation in broiler chickens subjected to diquat-induced oxidative stress. Poult. Sci. 102:102479.

Zhang, B., T. G. Nan, Z. L. Zhan, L. P. Kang, J. Yang, C. J. Lai, Y. Yuan, B. M. Wang, and L. Q. Huang. 2018. A monoclonal antibody-based enzyme-linked immunosorbent assay for the determination of chlorogenic acid in honeysuckle. J. Pharm. Biomed. Anal. 148:1–5.

Zhao, J. S., W. Deng, and H. W. Liu. 2019. Effects of chlorogenic acid-enriched extract from *Eucommia ulmoides* leaf on performance, meat quality, oxidative stability, and fatty acid profile of meat in heat-stressed broilers. Poult. Sci. 98:3040–3049.

Zhao, J. Y., C. X. Nie, and S. S. Nan. 2022. Effects of dietary lipoic acid on growth performance, slaughter performance, fat metabolism, and intestinal microflora of broilers. J Anim Nutr 34:5730–5740.

Zhou, B., Y. Yuan, S. Zhang, C. Guo, X. Li, G. Li, W. Xiong, and Z. Zeng. 2020. Intestinal flora and disease mutually shape the regional immune system in the intestinal tract. Front. Immunol. 11:575.