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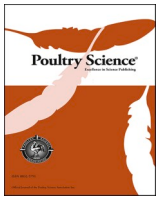

Protective effects of carnosic acid on growth performance, intestinal barrier, and cecal microbiota in yellow-feathered broilers under lipopolysaccharide challenge

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ABSTRACT

This research was performed to investigate protective effects of carnosic acid on growth performance, intestinal barrier, and cecal microbiota of lipopolysaccharide-challenged broilers. Three hundred 1-day-old yellow-feathered broilers (male) were allocated randomly into 5 treatments, with 6 replicates per treatment, and 10 birds per replicate cage. Birds in both the control group (CON) and the lipopolysaccharide-challenged group were provided with a basal diet, while others were fed a basal diet supplemented with 20, 40, and 60 mg/kg carnosic acid (CA20, CA40, CA60), respectively. At 17, 19, and 21 days of age, birds were injected intraperitoneally with lipopolysaccharide (500 μg/kg body weight), except those in CON, which were injected with saline. Compared with challenged birds, the CA20, CA40, and CA60 increased ($P < 0.05$) the final body weight, average daily gain, and average daily feed intake, and the CA40 and CA60 also decreased diarrhea rate. Compared with challenged birds, carnosic acid reduced ($P < 0.05$) plasmal levels of *D*-lactic acid and endotoxin, increased ($P < 0.05$) the villus height to crypt depth ratio, and the number of goblet cells in duodenum. The CA40 and CA60 elevated ($P < 0.05$) relative expression of cell junction proteins (*Claudin-1/-2* and *ZO-1/-2/-3*) and *MUC-2* in duodenum, while decreased ($P < 0.05$) relative expression of *TLR2, TLR4*, and the concentrations of IL-6, IL-10, TNF-α, TGF-β1 in duodenum. CA40 also increased ($P < 0.05$) the α-diversity of the cecal microbiota and boosted ($P < 0.05$) the relative abundance of beneficial phyla and genera, particularly Firmicutes, *Anaerofilum*, and *Papilibacter*. In conclusion, dietary supplementation with carnosic acid showed protective effects on the growth performance and intestinal health in challenged broilers by down-regulating the expression of *TLRs* (*TLR2/4*) and inhibiting the production of inflammatory cytokines, strengthening the tight junction in intestinal epithelial cells, and enhancing the diversity of microbiota and the relative abundance of beneficial bacteria. When supplemented to diet of broilers, 40 mg/kg carnosic acid was recommended.

Introduction

Intestinal health is maintained by the synergistic work of the intestinal barrier and immune system (de Souza et al., 2021; Alam et al., 2023), which protect animals from harmful substances and pathogenic microorganisms to avoid inflammation and disease (Stolfi et al., 2022). In poultry production, a series of factors such as feed mold, inflammation, and bacterial infection causes intestinal stress, increasing intestinal permeability and damaging intestinal barrier (Bhattacharyya et al., 2014). Lipopolysaccharide, a complex macromolecular compound found in the outer membrane of bacterial cells (Mao et al., 2020), induces the production of pro-inflammatory cytokines that activate the immune system to fight against pathogens (Yang et al., 2019). When the immune system is over-activated, the production of inflammatory cytokines (such as TNF-α, IFN-γ, IL-6, and IL-1β) is elevated, disrupting the balance of the gut microbiota, further increasing the intestinal

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permeability, finally leading to diseases such as enteritis (Yang et al., 2019).

Because of the growing health problems associated with bacterial resistance, more and more countries have banned the use of growth-promoting antibiotics in animal production. Therefore, finding effective alternatives to antibiotics has become a great challenge. Currently, many phenolic plant extracts have been used in livestock production due to their green, safe, and efficient properties (de Oliveira et al., 2018). Carnosic acid (phenolic compound) is widely found in plants such as sage and rosemary (*Rosmarinus officinalis* L.), and shows biological properties such as antioxidant, antibacterial (de Oliveira et al., 2018), and anti-inflammatory activities (Vlavcheski et al., 2024). So far, carnosic acid has been less used in livestock farming, and its protective effect on the intestinal barrier and the specific mechanisms still need to be thoroughly investigated. In our previous research, dietary supplementation with 10 to 40 mg/kg carnosic acid showed beneficial effects on growth performance of broilers, while 80 mg/kg significantly reduced the survival rate of the birds (unpublished data) Therefore, in the current research, 20/40/60 mg/kg carnosic acid was used. In addition, before the formal test, the inflammation-related variables (such as the levels of IL-6, IL-10 and TNF-α) in the duodenum, jejunum and ileum were determined, and it was found that in the current research, the results in duodenum was more significant, so the duodenum was chosen for the further research.

Therefore, this study investigated the protective effects of carnosic acid on growth performance, intestinal barrier, and cecal microbiota composition of lipopolysaccharide-challenged broilers, to explore the effective ways to improve intestinal health in broilers using bioactive additives, thus providing theoretical support for the scientific application of carnosic acid in poultry production.

Materials and methods

Experimental animals, diet, and design

The experimental protocol was approved by the Animal Care Committee of the Institute of Animal Science, Guangdong Academy of Agriculture Science, Guangzhou, P. R. China, with the approval number GAASISA-2022-017. In this experiment, 300 one-day-old fast-growing Lingnan yellow-feathered broilers (male, 40.0 ± 0.6 g) were allocated into five treatments based on the principle of weight consistency, with 6 replicates per treatment and 10 birds per replicate cage.

The basal diet was formulated according to recommended nutrition of broilers and nutritive values of feedstuffs in Nutrient Requirements of Yellow Chickens (NY/T3645-2020, Ministry of Agriculture PRC, 2020). The composition and nutrient content of diets are shown in Table 1.

Birds in the control group (**CON**) and lipopolysaccharide-challenged group (**LPS**) were fed a basal diet, while others were fed basal diets supplemented with 20, 40, or 60 mg/kg of carnosic acid (90.34%, Shanghai Yuanye Biotechnology Co., Ltd, Shanghai, China), respectively (**CA20, CA40**, and **CA60**). At 17, 19, and 21 days of age, birds in LPS, CA20, CA40, and CA60 were injected intraperitoneally with lipopolysaccharide (500 μg/kg body weight, from E. coli O55:B55, Sigma Aldrich Trading Co., Ltd, Shanghai, China), and birds in CON were injected with the equivalent normal saline. The trial lasted for 56 days. The experimental design was shown in Fig. 1.

Assessment of growth performance

Mortality was monitored daily, with dead birds recorded and weighed to adjust estimates of gain, intake, and feed conversion ratio as needed. Birds were weighed per cage on days 1, 21, and 56, and the average daily gain (**ADG**), average daily feed intake (**ADFI**), and feed-to-gain ratio (**F/G**) were calculated for each phase (1~21, 22~56, and 1~56 days of age) (Wang et al., 2021).

Table 1
Composition and nutrient content of diets (air dry-basis).

Items	1 to 21 days of age	22 to 42 days of age	43 to 56 days of age
Ingredient, %			
Corn	52.50	50.70	51.00
Wheat	3.50	10.00	10.70
Soybean meal	33.30	26.30	25.80
Double-low rapeseed meal	2.50	5.00	5.00
Soybean oil	3.20	3.40	3.50
L-Lys HCl (78%)	0.25	0.25	0.10
DL-Met	0.25	0.25	0.15
L-Thr	0.10	0.10	0.10
Zeolite powder	0.90	1.00	1.00
Dicalcium phosphate	2.25	1.70	1.50
NaCl	0.25	0.30	0.15
Premix[1]	1.00	1.00	1.00
Total	100.00	100.00	100.00
Nutrient levels[2]			
Nitrogen-corrected metabolizable energy, kcal/kg	2850.00	2960.00	2990.00
Crude protein, %	21.27	19.91	19.20
Calium, %	0.97	0.86	0.83
Total phosphorus, %	0.49	0.55	0.48
Non-phytate phosphorus, %	0.47	0.41	0.38
Lys, %	1.30	1.17	1.04
Met, %	0.57	0.54	0.43
Met+Cys, %	0.92	0.86	0.76
Thr, %	0.89	0.82	0.81
Trp, %	0.24	0.22	0.22
Ile, %	1.31	1.13	1.12

[1] Referring to previous studies (Wang et al., 2021) premixes were provided for day 1 to 21, 22 to 42 and 43 to 56.
[2] Values of crude protein, calcium and total phosphorus were determined by analysis, and others were calculated according to Nutrient Requirements of Yellow Chickens (Ministry of Agriculture PRC, 2020).

Observation of diarrhea

At d 21, 4 h after lipopolysaccharide challenge, the condition and the motion range of birds was observed, and the number of birds with diarrhea in each group was recorded, and the severity of diarrhea was assessed and quantified on a scale of 0 to 3. Birds scoring above 1 were classified as having diarrhea. The scoring rules are shown in Table 2 (Marchetti et al., 2023).

$$Diarrhea\ rate = number\ of\ diarrhea\ birds/number\ of\ birds \times 100\%$$

$$Diarrhea\ index = sum\ of\ diarrhea\ scores/number\ of\ birds$$

Sample collection

At d 21, after the observation of diarrhea, two birds with average body weight were chosen from each replicate for sample collection. Blood (5 mL) were collected from the wing veins with vacutainers containing anticoagulant (BD™ Vacutainer, Franklin Lakes, NJ, USA), and then centrifuged at 3500 × g for 10 min at 4 °C to obtain plasma. Then the birds were euthanized. The intestine was immediately removed. Segments of mid-duodenum were fixed in 4% solution of paraformaldehyde (~ 1 cm long) and 2.5% solution of glutaraldehyde (1 × 1 × 2 mm^3), respectively. In addition, samples of mid-duodenum and cecal contents were collected.

Enzyme-linked immunosorbent assay

Duodenal samples (~ 0.1 g) were homogenized in ice-cold saline, centrifuged (3000 × g, 15 min, 4°C), and processed into a 10% homogenate. Levels of *D*-lactic acid (***D*-LA**), endotoxin (**ET**), interleukin 6/10 (**IL-6/10**), interferon γ (**IFN-γ**), transforming growth factor β1 (**TGF-β1**), and tumor necrosis factor α (**TNF-α**) in plasma and duodenum were

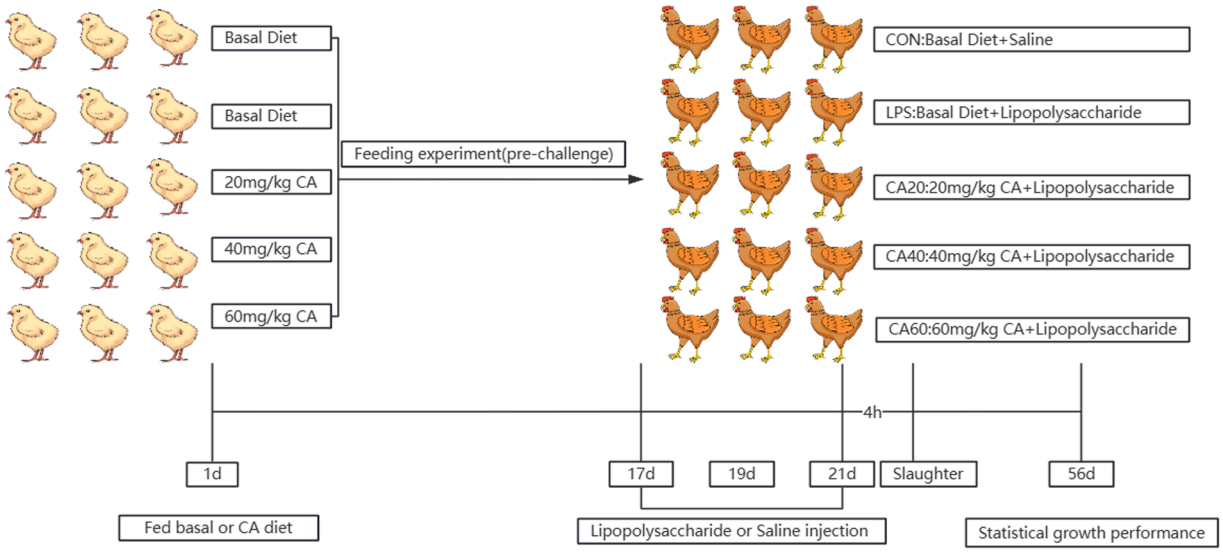

Fig. 1. Schematic illustration of the feeding experiment and lipopolysaccharide challenge.

Table 2
Diarrhea score.

Score	Degree	Appearances
0	Normally shaped feces	Molded and dry feces
1	Mild diarrhea	Shapeless (loose) feces
2	Moderate diarrhea	Thick, liquid (soft) feces
3	Severe diarrhea	Thin, liquid feces and watery diarrhea

(Marchetti et al., 2023)

measured using ELISA kits (Jiangsu Meimian Industrial Co., Ltd., Jiangsu, China) and a Microplate Reader (Bio-Rad, Hercules, CA, USA). Results of duodenum were normalized to protein concentration, quantified by the BCA Protein Assay Kit (Thermo Fisher Scientific, USA).

Intestinal morphological analysis

The duodenum, fixed in paraformaldehyde, was dehydrated and embedded in paraffin at a thickness of 5 μm. The duodenal morphology was observed after hematoxylin-eosin staining or Alicin blue-periodate acid schiff staining. Morphological images were scanned with a white light scanner (Hamamatsu Photonics Co., Ltd., Japan), then the field of view was intercepted with NDP.view2.9.22 software, and the villus height, crypt depth, and the number of goblet cells were measured with Image J software (Media Cybernetics, Bethesda, MD, USA).

Duodenal tissues were fixed in glutaraldehyde, rinsed in 0.1 M PBS (pH 7.4), and then fixed in 1% osmium acid in 0.1 M PBS at room temperature, shielded from light for 2 h. The tissues underwent rapid dehydration through increasing ethanol concentrations (30%, 50%, 70%, 95%, and 100%), followed by transfer to a 1:1 mixture of propylene oxide and epoxy aldehyde resins. After polymerization, 70 nm ultrathin sections were cut using an ultramicrotome (Leica Microsystems, IL) and stained with 2% uranyl acetate in alcohol and lead citrate solution. Cell junctions and microvilli were examined using transmission electron microscopy (Hitachi, Tokyo, Japan).

Real-time PCR analysis

The relative expressions of *IL-6, TGF-β1, TNF-α, INF-γ*, Toll-like receptor 2/3/4 (***TLR2/3/4***), *Occludin, Claudin-1, Claudin-2*, Zonula occluden-1/-2/-3 (***ZO-1/-2/-3***) and Mucin-2 (***MUC-2***) were detected by RT-PCR. As previously described (Wang et al., 2021), total RNA was extracted from duodenal tissue using TRIzol reagent (TaKaRa, Tokyo, Japan), and reverse-transcribed with the Prime Script II 1st Strand cDNA Synthesis Kit (TaKaRa, Tokyo, Japan). Real-time PCR was conducted on the CFX 96 RT-PCR detection system (Bio-Rad, CA, USA). The primer sequences are listed in Table 3. Relative mRNA expression levels were determined using the $2^{-\Delta\Delta Ct}$ method, with each target transcript normalized to *β-actin* mRNA levels.

Bacterial DNA extraction and 16S rRNA gene sequencing

Genomic DNA from the cecal microbial community was extracted using the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA), and its concentration and purity were measured with a NanoDrop2000 (Thermo Scientific, USA). The recovered products were purified with a DNA gel recovery and purification kit (PCR Clean-Up Kit, Passover, China) and quantified using a Qubit 4.0 (Thermo Fisher Scientific, USA). The V3-V4 hypervariable region of the 16S rRNA gene was PCR-amplified using the upstream primer 338F (5′-ACTCCTACGGGGAGG-CAGCAG-3′) and the downstream primer 806R (5′-GGAC-TACHVGGGGTWTCTAAT-3′).

The operational classification unit ASV (Amplicon Sequence Variant) clustering of the QC spliced sequences was performed and chimeras were removed based on 97% similarity using UPARSE v7.1 software. Data analysis was carried out on the Meiji Biological Cloud platform.

Statistical analysis

The experimental data of growth performance, diarrhea, *D*-LA and ET levels, inflammatory cytokines levels, intestinal morphological measurements, and genes levels were analyzed by one-way analysis of variance (ANOVA) in SPSS software (version 27.0), and when $P < 0.05$, multiple comparisons between treatments were performed using Duncan's multiple range tests. The results were presented as the mean ± standard error (SEM), and differences were considered to be statistically significant at $P < 0.05$.

Alpha diversity (Chao 1, Shannon index, etc.) were calculated using Mothur software, and the Kruskal-Walli's sum-rank test was used for the analysis of between-group differences in α diversity, and combined with the PERMANOVA non-parametric test to analyse whether the differences in microbiota structure between sample groups were significant. LEfSe analysis (linear discriminant analysis effect size) (LDA > 2, $P <$ 0.05) was used to identify bacterial taxa with significant differences in abundance from phylum to genus level between different groups. The data obtained were analysed using R software version 3.3.1 for statistics

Table 3
RT-qPCR primer sequence.

Gene	Forward primers (5′-3′)	Reverse primers (5′-3′)	Genbank ID
β-actin	GAGAAATTGTGCGTGACATCA	CCTGAACCTCTCATTGCCA	NM_001101.5
Claudin-1	TCATCCTGCTCTGCCTCATCT	CATCCGCCACGTTCTTCAC	NM_001013611.2
Claudin-2	ATAGCACGGGCATCACTCAG	CCACCACGGCTATAAGGCAA	NM_001277622.1
IL-6	CAGAAATCCCTCCTCGCCAA	CAAATAGCGAACGGCCCTCA	NM_204628.1
INF-γ	GCCGCACATCAAACACATATCT	TGAGACTGGCTCCTTTTCCTT	NM_205149.1
MUC-2	CATTCAACGAGGAGAGCTGC	TTCCTTGCAGCAGGAACAAC	NM_001318434.1
Occludin	TCATCCTGCTCTGCCTCATCT	CATCCGCCACGTTCTTCAC	NM_205128.1
TGF-β1	CTCGACACCGACTACTGCTT	TTCCACTGCAGATCCTTGCG	XM_046937641.1
TNF-α	AATTTGCAGGCTGTTTCTGC	TATGAAGGTGGTGCAGATGG	MF_000729.1
TLR2	CTGCTGCAGTCCAACCAAAT	CAGGTCGCTGTAGGAATTGC	XM_046915414.1
TLR3	TGACAAACTTCACCTCTCTGGA	TCTTCCTGCTCCTTCTTATGC	NM_001011691
TLR4	AGTCTGAAATTGCTGAGCTCAAAT	GCGACGTTAAGCCATGGAAG	NM_001030693.2
ZO-1	CCAAAGACAGCAGGAGGAGA	TGGCTAGTTTCTCTCGTGCA	XM_015278981.1
ZO-2	GCAAGGTGAAGCAGTTTGGG	ATGCACCTCCCTCTCCATCT	XM_046934796.1
ZO-3	ATCCCCTAAGACCTCCCCAC	GCAGCATTCCTGCTTGATCG	XM_040692502.2

IL-6: Interleukin-6, *INF-γ*: Interferon gamma, *MUC-2*: Mucin 2, *TGF-β1*: Transforming growth factor-β1, *TNF-α*: Tumor necrosis factor-alpha, *TLR2/3/4*: Toll-like receptor 2/3/4, *ZO-1*: Zonula occluden-1.

and mapping.

Results

Effects of carnosic acid on growth performance and diarrhea of lipopolysaccharide-challenged broilers

As shown in Table 4, from 1 to 21 days, compared with birds in CON, lipopolysaccharide challenge decreased ($P < 0.05$) the final body weight, ADG, and ADFI of birds. Compared with birds in LPS, the CA20 and CA40 increased the final body weight, ADG, and ADFI ($P < 0.05$), and CA40 was more effective compared to CA20 ($P < 0.05$).

Compared with birds in CON, lipopolysaccharide challenge reduced ($P < 0.05$) the final body weight and ADG from 1 to 56 days, and increased the F/G from 22 to 56 days in birds. Conversely, when compared to birds in LPS, the CA20, CA40, and CA60 showed improvements ($P < 0.05$) in final body weight and ADG from 1 to 56 days, also reduction ($P < 0.05$) in the F/G from 22 to 56 days.

As shown in Fig. 2 (A and B), birds in LPS were curled up in a corner with reduced activity, glazed eyes, and diarrhea, and in CA20, CA40, and CA60, the diarrhea was alleviated, and birds showed greater vitality. As shown in Fig. 2 (C and D), lipopolysaccharide challenge increased ($P < 0.05$) the diarrhea rate and diarrhea index of birds, which were offset by supplementation with carnosic acid (CA20 and CA60).

Effects of carnosic acid on concentrations of plasmal D-LA and ET of lipopolysaccharide-challenged broilers

As illustrated in Fig. 3, lipopolysaccharide challenge increased ($P < 0.05$) concentrations of plasmal *D*-LA and ET compared to birds in CON. In contrast, supplementation with 20, 40, and 60 mg/kg carnosic acid resulted in a significant decline ($P < 0.05$) in *D*-LA and ET levels when compared to birds in LPS. Additionally, no significant differences were found among treatments with different levels of carnosic acid ($P > 0.05$).

Effects of carnosic acid on concentrations of inflammatory cytokines of lipopolysaccharide-challenged broilers

The concentrations of plasmal and duodenal cytokines are shown in Table 5. The lipopolysaccharide challenge enhanced ($P < 0.05$) plasmal concentrations of IL-6 and TGF-β1, and duodenal concentrations of IL-6, IL-10, IFN-γ, TGF-β1, and TNF-α, compared to birds in CON. When contrasted with birds in LPS, carnosic acid decreased ($P < 0.05$) the levels of IFN-γ (CA20), IL-6, and TGF-β1 (CA40) in plasma. Additionally, 40 or 60 mg/kg carnosic acid decreased ($P < 0.05$) levels of those cytokines (IL-6, IL-10, TGF-β1, and TNF-α) in duodenum.

Table 4
Effects of carnosic acid on growth performance of lipopolysaccharide-challenged broilers.

Item	CON	LPS	CA20	CA40	CA60	SEM	*P*-value
1 to 21days of age							
Initial body weight, g	40.42	40.53	40.48	40.42	40.48	0.02	0.192
Final body weight, g	471.83^{b}	452.11^{c}	476.50^{b}	494.50^{a}	465.83^{bc}	3.53	< 0.001
ADG, g	18.75^{b}	17.89^{c}	18.95^{b}	19.74^{a}	18.49^{bc}	0.19	< 0.001
ADFI, g	31.44^{b}	30.22^{c}	31.43^{b}	32.39^{a}	30.77^{bc}	0.15	< 0.001
F/G	1.67	1.68	1.65	1.62	1.67	0.01	0.060
Survival rate, %	100.00	98.75	100.00	100.00	100.00	0.25	0.421
22 to 56 days of age							
ADG, g	44.41	43.59	44.89	45.64	45.65	0.44	0.546
ADFI, g	121.52	121.45	120.69	120.47	121.48	0.36	0.850
F/G	2.64^{b}	2.76^{a}	2.60^{b}	2.53^{b}	2.62^{b}	0.02	0.013
Survival rate, %	100.00	100	100.00	100.00	100.00	0.00	1.000
1 to 56 days of age							
Final body weight, g	1960.00^{a}	1870.00^{b}	1970.00^{a}	2020.00^{a}	1990.00^{a}	0.02	0.021
ADG, g	34.30^{a}	32.63^{b}	34.49^{a}	35.39^{a}	34.77^{a}	0.29	0.021
ADFI, g	82.54	81.64	81.87	81.75	81.20	0.22	0.059
F/G	2.44	2.47	2.37	2.32	2.35	0.02	0.433
Survival rate, %	100.00	98.75	100.00	100.00	100.00	0.25	0.421

^{a,b}Within a row, means with the same superscripts under a main effect are not significantly different ($P < 0.05$). Values are means of 12 replicate samples (two birds per cage). SEM indicates standard error of mean. CON: Saline-challenged group, LPS: Lipopolysaccharide-challenged group, CA20/40/60: 20/40/60 mg/kg carnosic acid + Lipopolysaccharide-challenged group.

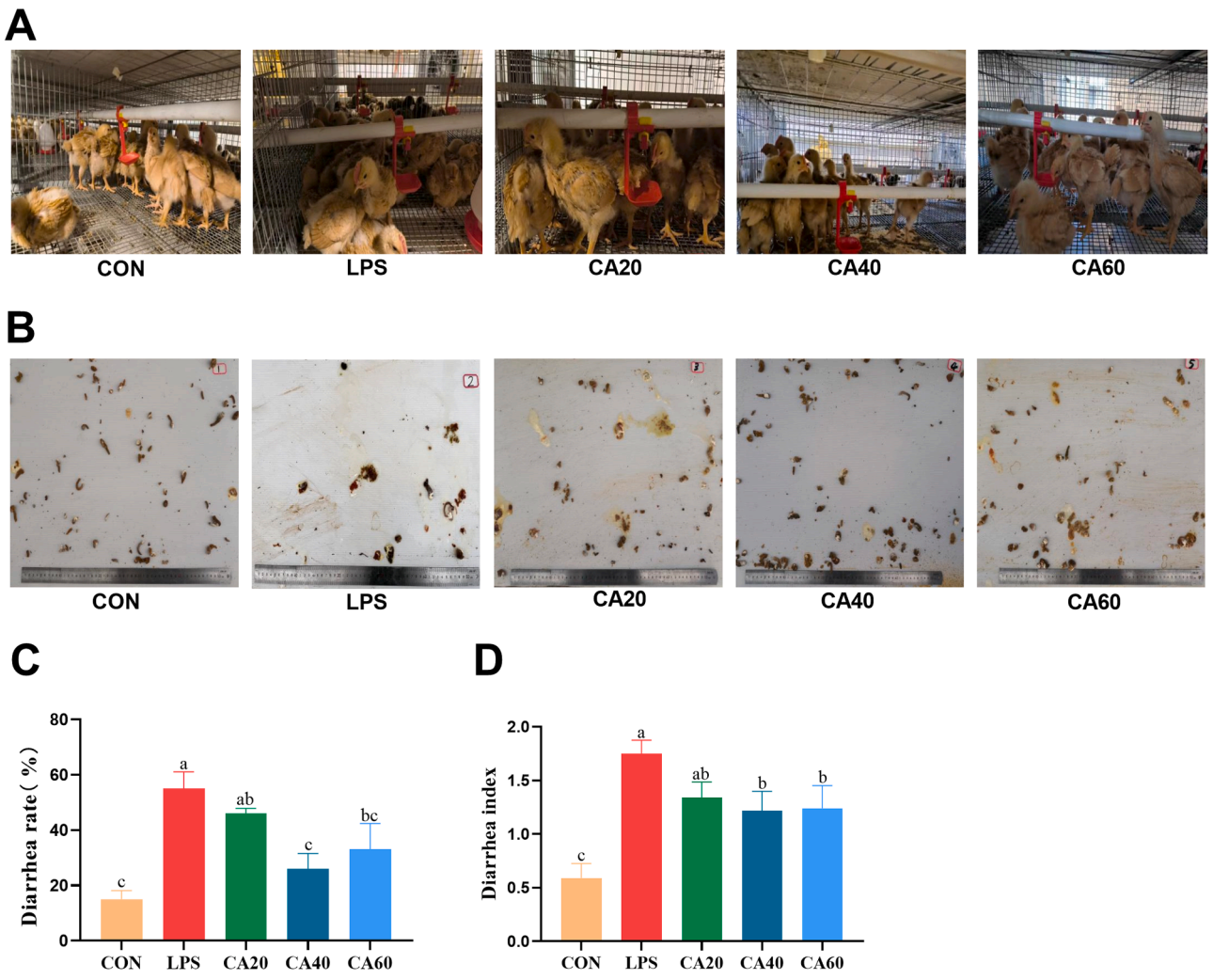

Fig. 2. Effect of carnosic acid on diarrhea of lipopolysaccharide-challenged broilers. [a,b]Means with the same superscripts under a main effect are not significantly different ($P < 0.05$). Values are means of 12 replicate samples (two birds per cage). Photograph of (A) bird and (B) feces, (C) Diarrhea rate, (D) Diarrhea index. CON: Saline-challenged group, LPS: Lipopolysaccharide-challenged group, CA20/40/60: 20/40/60 mg/kg carnosic acid + Lipopolysaccharide-challenged group.

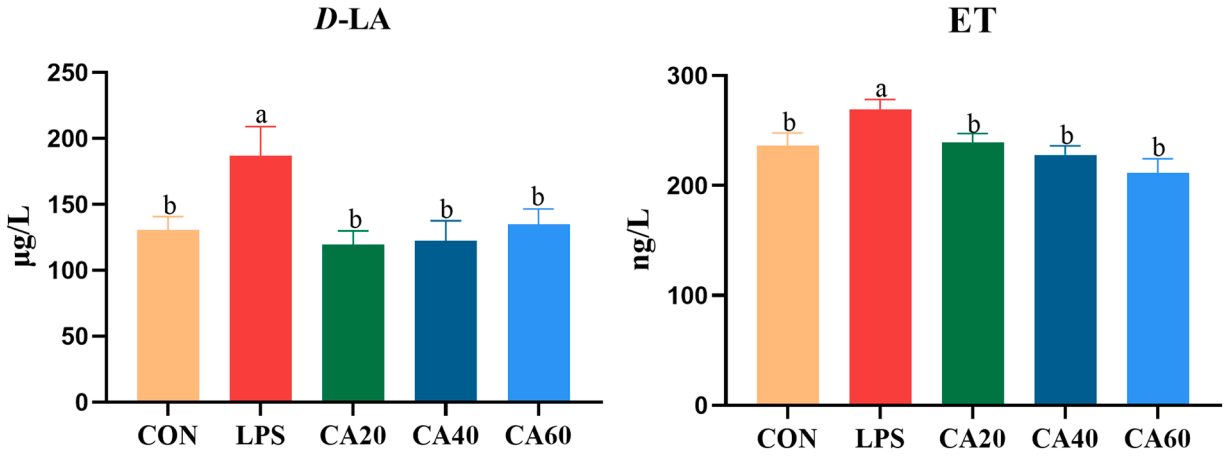

Fig. 3. Effects of carnosic acid on plasmal concentrations of *D*-LA and ET of lipopolysaccharide-challenged broilers. [a,b] Means with the same superscripts under a main effect are not significantly different ($P < 0.05$). Values are means of 12 replicate samples (two birds per cage). CON: Saline-challenged group, LPS: Lipopolysaccharide-challenged group, CA20/40/60: 20/40/60 mg/kg carnosic acid + Lipopolysaccharide-challenged group.

Table 5
Effect of carnosic acid on inflammatory cytokines concentrations of lipopolysaccharide-challenged broilers.

Items	CON	LPS	CA20	CA40	CA60	SEM	*P* value
In plasma, pg/mL							
IL-6	36.42^{b}	42.82^{a}	35.87^{b}	34.54^{b}	37.66^{ab}	0.93	0.041
IL-10	53.27^{b}	53.30^{b}	58.02^{b}	68.23^{a}	60.18^{ab}	1.64	0.020
IFN-γ	65.44^{a}	69.25^{a}	55.84^{b}	59.95^{ab}	62.69^{ab}	1.47	0.040
TNF-α	69.33	77.33	70.58	70.04	67.04	1.99	0.580
TGF-β1	128.88^{b}	176.51^{a}	145.91^{ab}	138.58^{b}	147.84^{ab}	5.11	0.035
In duodem, pg/mg prot							
IL-6	6.51^{b}	8.41^{a}	8.47^{a}	6.39^{b}	6.38^{b}	0.24	< 0.001
IL-10	9.06^{b}	11.68^{a}	9.72^{ab}	8.28^{b}	7.80^{b}	0.36	0.003
IFN-γ	9.36^{b}	13.60^{a}	10.84^{ab}	11.35^{ab}	9.98^{ab}	0.55	0.134
TNF-α	10.05^{b}	12.57^{a}	10.05^{b}	9.95^{b}	9.86^{b}	0.36	0.074
TGF-β1	34.62^{b}	40.84^{a}	35.58^{ab}	32.32^{b}	31.96^{b}	1.24	0.154

^{a,b}Within a column, means with the same superscripts under a main effect are not significantly different ($P < 0.05$). Values are means of 12 replicate samples (two birds per cage). CON: Saline-challenged group, LPS: Lipopolysaccharide-challenged group, CA20/40/60: 20/40/60 mg/kg carnosic acid + Lipopolysaccharide-challenged group.

Effects of carnosic acid on intestinal morphology of lipopolysaccharide-challenged broilers

In Fig. 4A and B, after challenge, the duodenal villus was broken, the lamina propria was extensively infiltrated by inflammatory cells (eosinophils, plasma cells, lymphocytes, neutrophils, and monocytes), and numbers of goblet cells were decreased. The above injury was alleviated in birds from CA20, CA40, and CA60.

As shown in Fig. 4C, duodenal microvilli, intermediate junctions, and desmosomes were intact and dense in birds from CON. The duodenal villus was broken and dispersed into the lumen of the intestine in birds from LPS, with no intact intermediate junctions and desmosomes. Moreover, the duodenal villus was tightly arranged in birds from CA40 and CA60, and the birds in CA40 also showed intact and dense intermediate junctions and desmosomes.

As shown in Fig. 4D and E, compared to birds in CON, lipopolysaccharide challenge significantly declined the villus height to crypt depth ratio (**V/C**) and the number of goblet cells, and increased crypt depth of duodenum ($P < 0.05$). Compared to birds in LPS, supplementation with carnosic acid (CA20, CA40, and CA60) offset the change in crypt depth, V/C, and the CA40 and CA60 also increased villus height and the number of goblet cells.

Effects of carnosic acid on relative expression of tight junction and mucin genes of lipopolysaccharide-challenged broilers

Fig. 5 demonstrated that the lipopolysaccharide injection significantly reduced the relative expression of *Claudin-2, ZO-1*, and *ZO-2* in duodenum compared to birds in CON. Compared to birds in LPS, 40 and 60 mg/kg carnosic acid increased ($P < 0.05$) the relative expression of *ZO-2* and *ZO-3* in duodenum, and supplementation with carnosic acid also enhanced ($P < 0.05$) the relative expression of *ZO-1* (CA20 and CA40) and *MUC-2* (CA40).

Effects of carnosic acid on relative expression of cytokines and TLRs of lipopolysaccharide-challenged broilers

As illustrated in Fig. 6, compared with birds in CON, lipopolysaccharide challenge increased ($P < 0.05$) the relative expression of *IL-6, TLR2*, and *TLR4*, while decreased *TLR3* expression ($P < 0.05$) in duodenum. When compared with birds in LPS, a significant reduction in the relative expression of *TLR2* and *TLR4* was observed in birds supplemented with carnosic acid ($P < 0.05$), and the CA20 and CA40 also down-regulated the *IL-6* expression ($P < 0.05$).

Effects of carnosic acid on intestinal microbiota of lipopolysaccharide-challenged broilers

Based on the results on growth performance and intestinal barrier shown above, birds with 40 mg/kg carnosic acid were used for analysis of the intestinal microbiota.

Alpha diversity

As shown in Fig. 7A-D, compared with birds in CON, lipopolysaccharide injection decreased ($P < 0.05$) α diversity (ace, chao, and sobs indices) of cecal microbiota. Compared with birds in LPS, α diversity (ace, chao, sobs, and shannon indices) was significantly increased in CA40.

Microbial composition

In Fig. 7E and 7F, at the phyla level, there were 9 common phyla (60.00%) among the three groups, and 1, 2 and 0 phyla (6.67%, 13.33% and 0.00%) were unique in birds from CON, LPS, and CA40, respectively.

At the genus level, there were 132 common genera among the three groups, and 19, 20, and 49 genera were unique in birds from CON, LPS, and CA40, respectively.

As shown in Fig. 7G and H, the main phyla of the cecal microbiota in birds were Firmicutes, Bacteroidota, Campilobacterota, and Proteobacteria, and the main genera were *Alistipes, Bacteroides, Barnesiella*, and *Faecalibacterium.*

Differential microorganisms

Fig. 8A and C indicated that at the phylum level, compared to birds in CON, lipopolysaccharide injection significantly reduced the relative abundance of Verrucomicrobiota. Compared to birds in LPS, supplementation with 40 mg/kg carnosic acid increased the relative abundance of Firmicutes ($P < 0.05$).

The differential genera among treatments were shown in Fig. 8B and D. In Table 6, the change of 7 genera caused by lipopolysaccharide were offset by supplementation with carnosic acid. In detail, compared with birds in CON, lipopolysaccharide challenge significantly decreased the relative abundance of *Clostridia_UCG-014, Eubacterium_coprostanoligenes_group, Anaerofilum, Defluviitaleaceae_UCG-011*, and *Papillibacter*, and increased ($P < 0.05$) the relative abundance of *Pelomonas* and *Erysipelotrichales* ($P < 0.05$) in the cecum, the above change caused by lipopolysaccharide injection were alleviated by supplementation with carnosic acid.

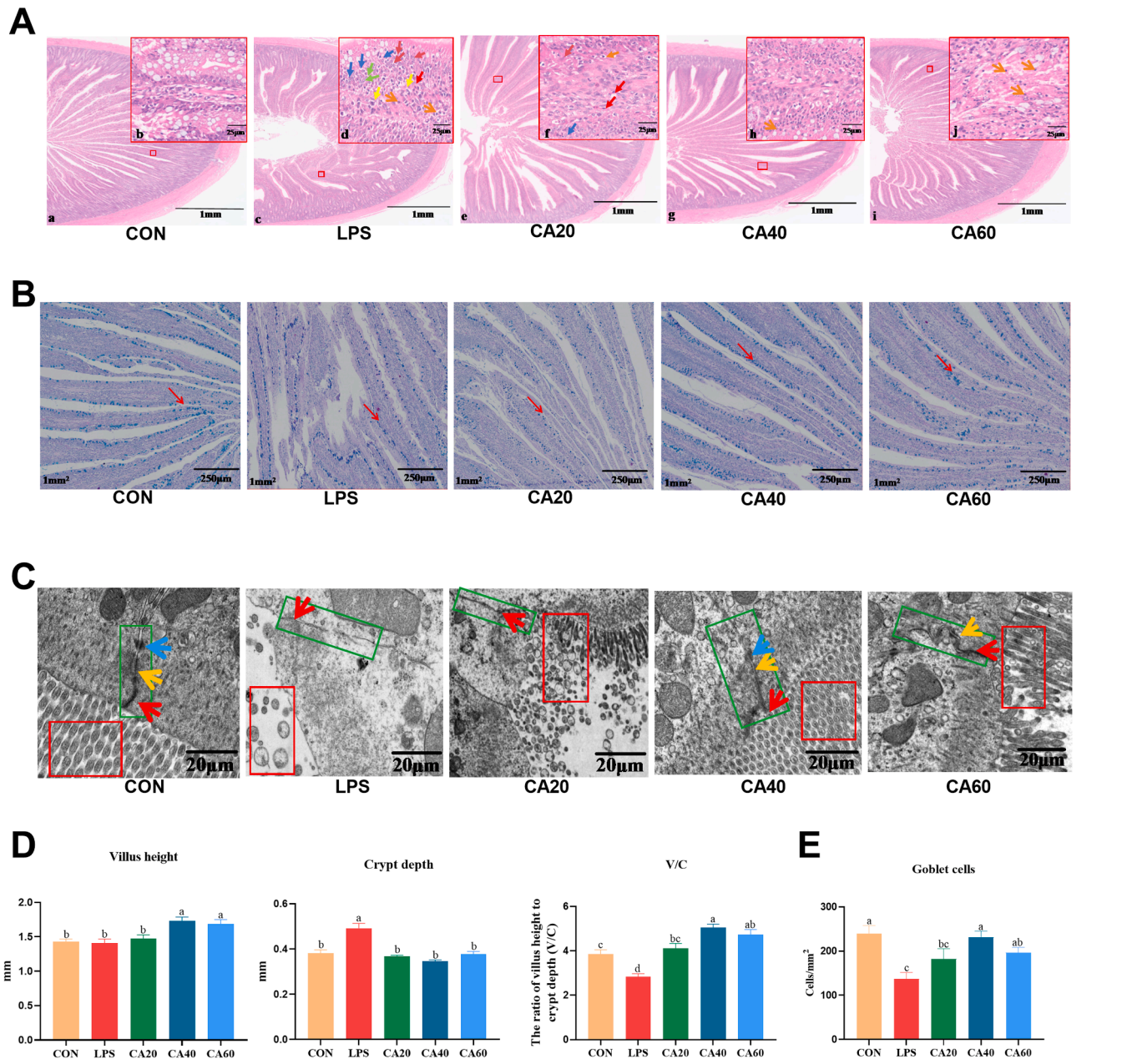

Fig. 4. Effect of carnosic acid on duodenal morphology of lipopolysaccharide-challenged broilers. [a,b]Means with the same superscripts under a main effect are not significantly different ($P < 0.05$). Values are means of 12 replicate samples (two birds per cage). A: Representative images of e morphological structure of duodenal villi (hematoxylin-eosin staining), at 250 × or 8000 × magnification, respectively. Red arrows: eosinophils, Blue arrows: plasma cells, Orange arrows: lymphocytes, Green arrows: neutrophils, Yellow arrows: monocytes. B, Representative images of duodenal goblet cells (Alicin blue-periodate acid schiff staining). at 1000 × magnification. C, Representative images of microvilli morphology and cell junctions under transmission electron microscopy (TEM), resolution: 1.00 μm, magnification: 15 K. The red box: microvilli, The green box: cell junction, Red arrow: tight junction, Yellow arrow: intermediate junction, Blue arrow: desmosome. D, Villus height, crypt depth, and the ratio of villus height to crypt depth (V/C). E, Number of goblet cells in the 1 mm^2 field of view. CON: Saline-challenged group, LPS: Lipopolysaccharide-challenged group, CA20/40/60: 20/40/60 mg/kg carnosic acid + Lipopolysaccharide-challenged group.

Discussion

Effects of carnosic acid on growth performance and diarrhea of lipopolysaccharide-challenged broilers

Lipopolysaccharide, a metabolite of gram-negative bacteria, has been shown to trigger strong systemic inflammation and is an important cause of inhibited growth performance in poultry (Wang et al., 2024). Studies have shown that lipopolysaccharide increased F/G in broilers from 1 to 42 days of age (Wang et al., 2024) and decreased ADG and ADFI in Cobb broilers from 1 to 21 days of age (Hong et al., 2023). Comparable results were observed in this experiment, suggesting that lipopolysaccharide has a negative effect on the growth performance of yellow-feathered broilers. In the current research, supplementation with different doses of carnosic acid effectively alleviated the decline of growth performance of broilers (the final body weight, ADG, and ADFI) caused by lipopolysaccharide, and the optimal level was 40 mg/kg. Wan et al. (2024) suggested that dietary supplementation with 20, 40, and 80 mg/kg of carnosic acid linearly increased the body weight in lipopolysaccharide-challenged broilers, but the effect was not significant, which was possibly attributed to the short feeding cycle of carnosic acid (1 week). The above studies suggested that carnosic acid had a

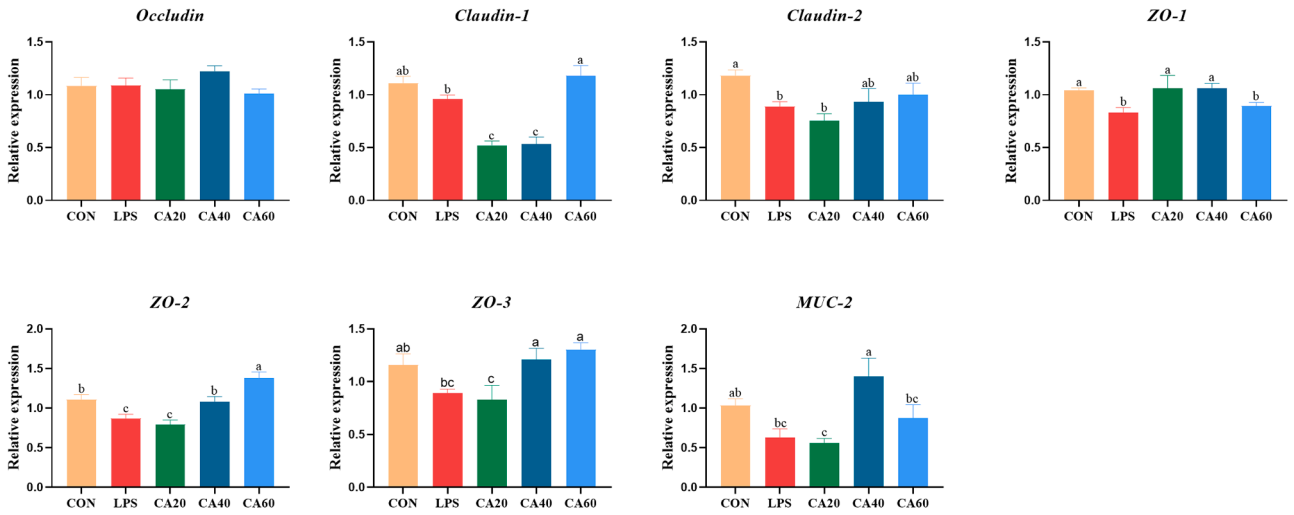

Fig. 5. Effect of carnosic acid on expression of tight junction and mucin genes of lipopolysaccharide-challenged broilers. [a,b]Means with the same superscripts under a main effect are not significantly different ($P < 0.05$). Values are means of 12 replicate samples (two birds per cage). CON: Saline-challenged group, LPS: Lipopolysaccharide-challenged group, CA20/40/60: 20/40/60 mg/kg carnosic acid + Lipopolysaccharide-challenged group.

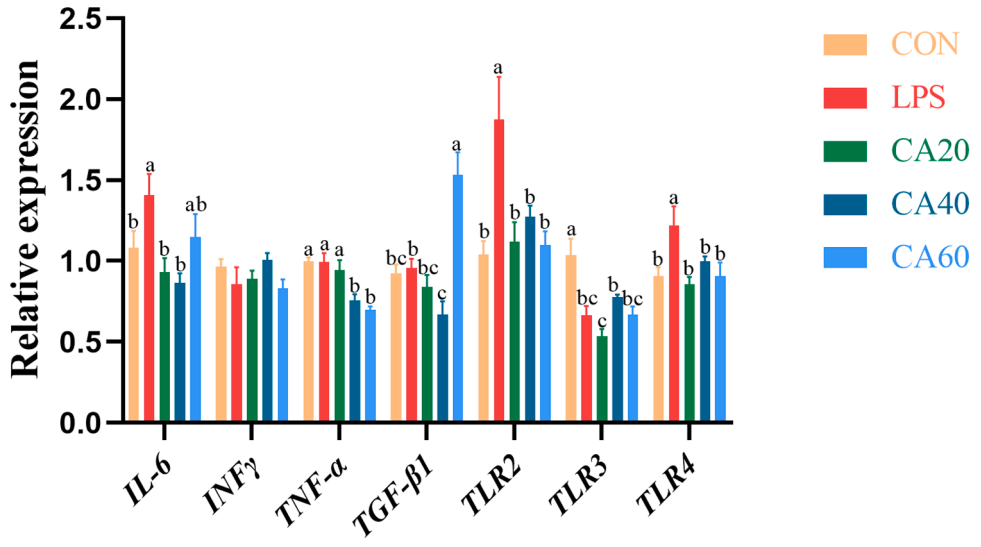

Fig. 6. Effect of carnosic acid on relative expression of cytokines and TLRs of lipopolysaccharide-challenged broilers. [a,b]Means with the same superscripts under a main effect are not significantly different ($P < 0.05$). Values are means of 12 replicate samples (two birds per cage). CON: Saline-challenged group, LPS: Lipopolysaccharide-challenged group, CA20/40/60: 20/40/60 mg/kg carnosic acid + Lipopolysaccharide-challenged group.

protective effect on the growth performance of lipopolysaccharide-challenged broilers.

For poultry, after intraperitoneal lipopolysaccharide challenge, cellular damage and inflammatory responses were exacerbated (Wang et al., 2024), resulting in intestinal vasodilatation, congestion, and mucosal damage, which in turn led to intestinal dysfunction and diarrhea (Sun et al., 2020). In the study, lipopolysaccharide injection increased the diarrhea rate and index in birds, and 40 and 60 mg/kg carnosic acid alleviated diarrhea in challenged birds. In DSS-challenged mice, carnosic acid alleviated the induced diarrhea and fecal bleeding (Xu et al., 2022). Also, other phenolic compounds alleviated the lipopolysaccharide-induced diarrhea in piglets (Lu et al., 2022), through anti-inflammatory and antioxidant properties (Luo et al., 2022). These suggested that phenolics such as carnosic acid might alleviate diarrhea and growth performance through anti-inflammatory and antioxidant effects in lipopolysaccharide-challenged birds.

Effects of carnosic acid on inflammation of lipopolysaccharide-challenged broilers

Lipopolysaccharide injection is a key factor of inflammation, which activates the production of cytokines (Zheng et al., 2016). Cytokines (IL-6, IL-10, TNF-α and IFN-γ) produced by immune cells play a key role in the immune system and are highly correlated with the severity of inflammation (Zhang et al., 2022). Similar to the current research, lipopolysaccharide, activated the expression of *IL-6*, and *TNF-α*, increased the levels of IL-6, IFN-γ, and TNF-α, thus induced inflammation in liver and jejunum of broilers (Xiao et al., 2024). In the current research, supplementation with carnosic acid reduced inflammatory cytokines increased by lipopolysaccharide, especially 40 mg/kg carnosic acid, which reduced the relative expression of *IL-6, TGF-β1, TNF-α* and decreased levels of IL-6, IL-10, TGF-β1, and TNF-α in duodenum. Various studies, both in vitro and in vivo, have shown that carnosic acid has anti-inflammatory activity. Xu et al. (2022) found that carnosic acid reduced the relative expression of *TNF-α, IL-6*, and *IFN-γ* in DSS-injured

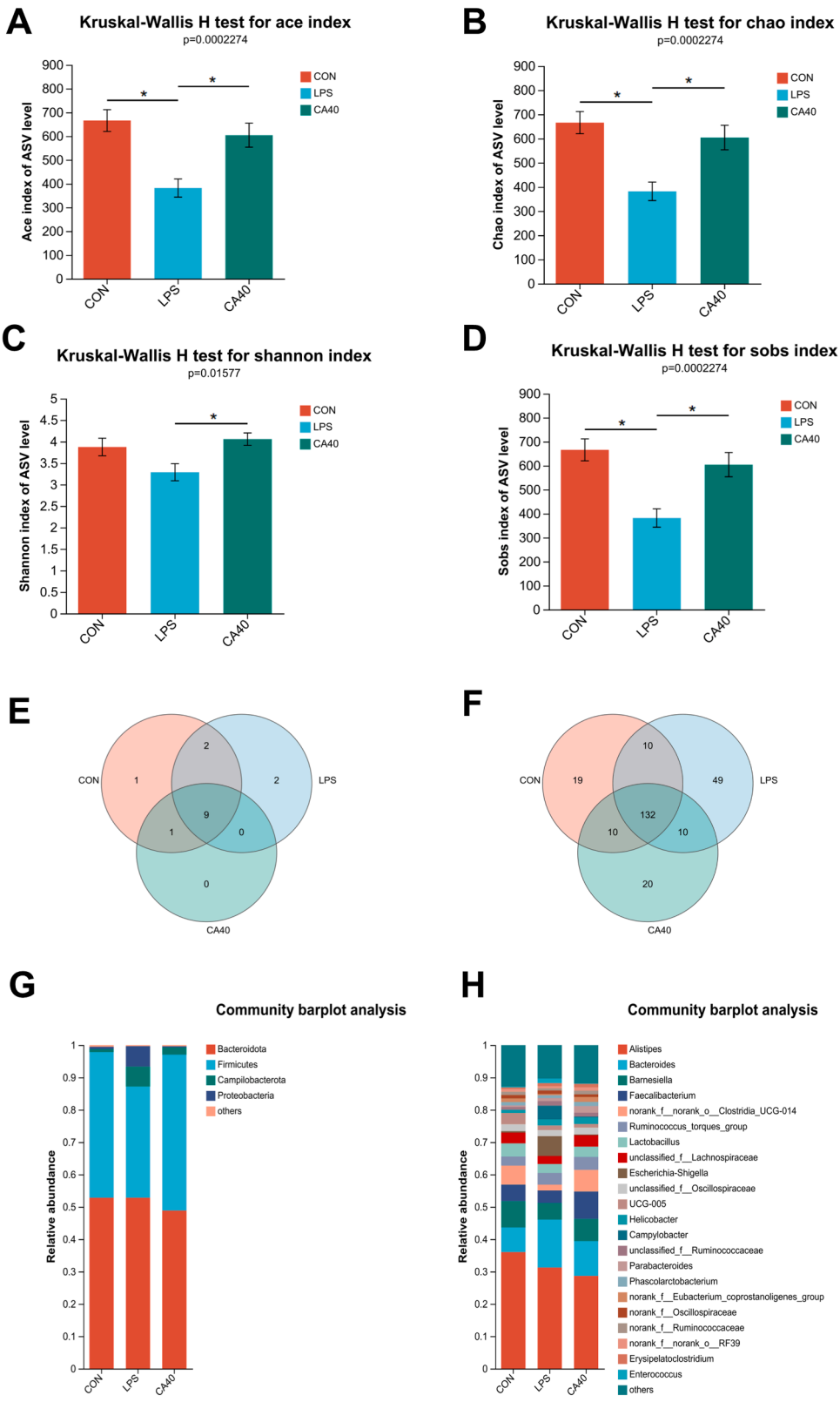

Fig. 7. Effect of carnosic acid on α diversity and microbial composition of the cecum. CON: Saline-challenged group, LPS: Lipopolysaccharide-challenged group, CA40: 40 mg/kg carnosic acid + Lipopolysaccharide-challenged group. A–D Alpha-diversity (ace, chao, shannon, sobs), number of species at the phylum (E) and genus (F) level, The microbial composition in cecal contents at phylum (G) and genus (H) levels. Date were shown as mean ± SEM (n = 6), * $P < 0.05$.

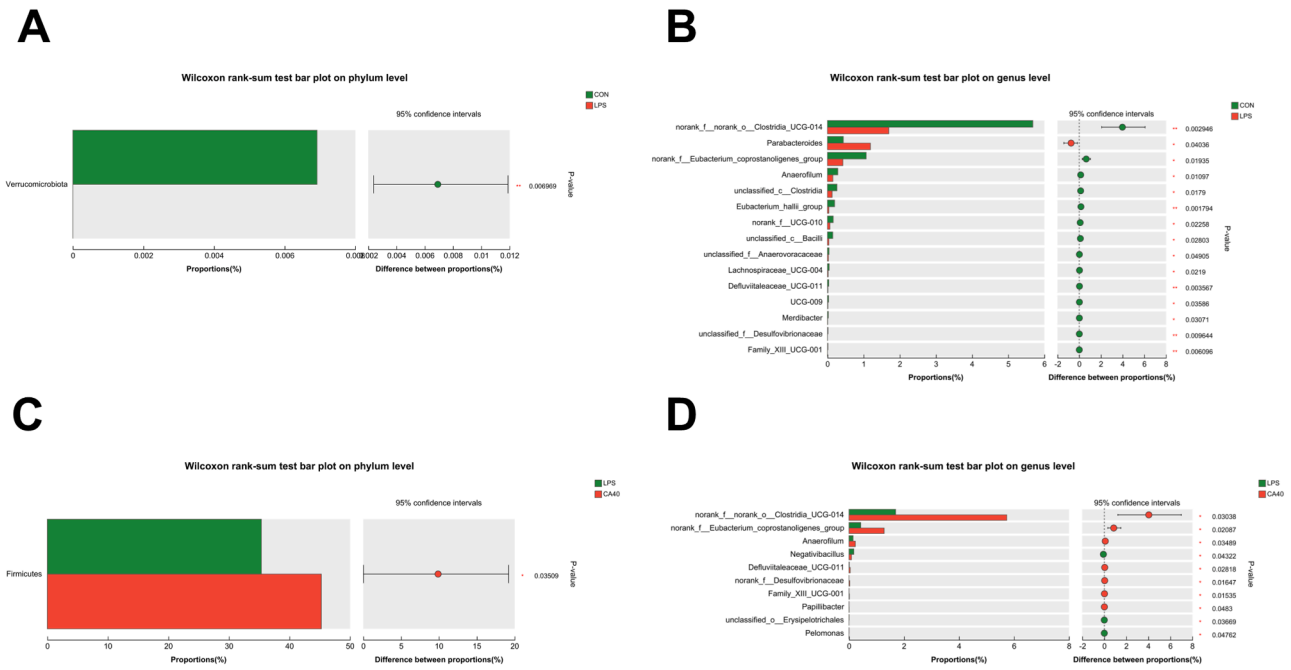

Fig. 8. Effect of carnosic acid on differential microorganisms of the cecum. CON: Saline-challenged group, LPS: Lipopolysaccharide-challenged group, CA40: 40 mg/kg carnosic acid + Lipopolysaccharide-challenged group. Differences microbes in cecal between CON and LPS at the phylum (A) and genus (B) levels. Differences microbes in cecal between LPS and CA40 at the phylum (C) and genus (D) levels. Date were shown as mean ± SEM (n = 6), *$P < 0.05$.

Table 6
Effect of carnosic acid on microbial differences at the genus level.

Genus	CON VS LPS	LPS VS CA40
Clostridia_UCG-014	down	up
Eubacterium_coprostanoligenes_group	down	up
Anaerofilum	down	up
Defluviitaleaceae_UCG-011	down	up
Papillibacter	down	up
Pelomonas	up	down
Erysipelotrichales	up	down

CON: Saline-challenged group, LPS: Lipopolysaccharide-challenged group, CA40: 40 mg/kg carnosic acid + Lipopolysaccharide-challenged group. 'CON vs LPS' refers to the changes in LPS when compared to the CON. 'LPS vs CA40' refers to the changes in CA40 when compared to the LPS, 'down' means that the relative abundance of genera was significantly decreased, 'up' means that the relative abundance of genera was significantly increased.

mouse colon. In addition, co-culture of 20 μg/mL carnosic acid with HaCaT cells reduced LPS-induced skin inflammation by decreasing the levels of inflammatory cytokines (IL-6, IL-8, IL10, and TNF-α) (Oh et al., 2012). Toll-like receptor family members TLR2, TLR3, and TLR4 interact in pathogen recognition and innate immune activation (Kimand Karin, 2011), and cause inflammation through stimulating and transfering the production of chemokines and cytokines (Matveichuk et al., 2024). In this experiment, lipopolysaccharide also increased the relative expression of *TLR2* and *TLR4*, supplementation with carnosic acid mitigated this effect of lipopolysaccharide. In summary, carnosic acid alleviated the intestinal inflammation in broilers caused by lipopolysaccharide, through the inhibited expression of *TLRs* (*TLR2/4*) and reduced levels of cytokines.

Effects of carnosic acid on intestinal barrier function of lipopolysaccharide-challenged broilers

The intestinal barrier defenses against foreign bacteria or viruses (Zhang et al., 2017), and lipopolysaccharide damages the intestinal barrier and increases permeability (Li et al., 2015). Plasmal levels of *D*-LA and ET were generally indicators of increased intestinal permeability (Zhang and Wu, 2020). The level of *D*-LA is associated with bacterial metabolic processes and is produced during the metabolism of certain intestinal bacteria. Normally, most LA exists in the form of *L*-LA, whereas in pathological conditions, LA crosses the intestinal epithelium and enters the blood circulation in the form of *D*-LA (Zha et al., 2024; Shen et al., 2010). A class of toxic substances known as ET is present in the bacterial cell wall and interacts with specific receptors in animals (Shen et al., 2010), causing the inflammatory response (Ramery et al., 2014). Previous studies showed that lipopolysaccharide enhanced abnormal plasmal levels of *D*-LA and ET (Wu et al., 2013; Zhang et al., 2023), which indicated severe impairment of the intestinal barrier in broilers (Su et al., 2022; Yang et al., 2022). Dietary supplementation with carnosic acid reduced the elevated levels of *D*-LA and ET in lipopolysaccharide-challenged birds, suggesting that carnosic acid mitigated the increasing intestinal permeability and maintained intestinal health under inflammation.

Villus height, crypt depth, and V/C are key variables used to assess intestinal health and nutrient absorption capacity (Ducatelle et al., 2018). There is a positive correlation between the number of intestinal epithelial cells (as goblet cells) and villus height, with the contact surface area of the intestinal villi being increased and facilitating nutrient absorption (Grego-Bessa et al., 2016). The main function of goblet cells is the secretion of mucus, with this mucus secreting the mucin family MUC-2, which protects the intestinal mucosa (Shan et al., 2013). Consistent with a previous study (Zhang et al., 2021), the depth of intestinal crypts in lipopolysaccharide-challenged broilers increased V/C and goblet cells decreased in the current research. This was also consistent with the previous results for growth performance. Damage to intestinal villi by lipopolysaccharide reduces intestinal absorptive area and decreases the capacity to digest and absorb nutrients, thus affecting the growth performance of the broiler (Csernus et al., 2020). Consistent with the results of this experiment, supplementation with carnosic acid increased the duodenal V/C and alleviated the villus damage in lipopolysaccharide-challenged broilers (Wan et al., 2024). Moreover, in present research, supplementation with carnosic acid also increased both the number of duodenal goblet cells and the relative expression of

MUC-2 in lipopolysaccharide-challenged broilers. A previous study showed that 50 mg/kg/day of carnosic acid increased the number of intestinal goblet cells and the relative expression of *MUC-2* in colitis mice (Xu et al., 2022). Carnosic acid improved the infiltration of immune cells, crypt structure, and goblet cell damage in DSS-induced intense colitis mice (Yang et al., 2017).

Tight junction in intestinal epithelia including Occludin, Claudin-1, Claudin-2 (Lee et al., 2017), ZO-1, ZO-2, and ZO-3 played an important role in regulating intestinal permeability (Gadde et al., 2017). Lipopolysaccharide challenge decreased the expression of *MUC-2, Claudin-1, Occludin*, and *ZO-1* in the intestinal barrier (Bavananthasivam et al., 2019; Tan et al., 2023), and disrupted tight junction in intestinal epithelia, which leading to increased intestinal permeability (Kong et al., 2022, 2023). Lipopolysaccharide challenge decreased the expression of duodenal *Claudin-2, ZO-1*, and *ZO-2* in the current study, which resulted in widened gaps between intestinal tight junction and the absence of intact intermediate junctions, desmosomes, and tight junctions. Supplementation with carnosic acid alleviated the above harmful change caused by lipopolysaccharide. 50 mg/kg/day of carnosic acid protected intestinal barrier of DSS-induced mouse by the increased the expression of *Claudin-1, ZO-1*, and *Occludin* (Xu et al., 2022). In conclusion, carnosic acid protected the intestinal mucosa and maintained the integrity in lipopolysaccharide-challenged broilers through the increased expression of intestinal tight junction proteins and *MUC-2*. These studies suggested that carnosic acid may have potential in the prevention or treatment of intestinal disorders caused by lipopolysaccharide injection.

Effects of carnosic acid on intestinal microbiota of lipopolysaccharide-challenged broilers

The intestinal microbiota is a biological barrier that resists colonization by pathogenic bacteria, the change in composition and structure of which are crucial in disease pathogenesis (Zhong et al., 2019). Lipopolysaccharide injection destroyed the ecological balance of the intestinal microbiota, boosted intestinal inflammation, and reduced growth performance of animals (Metzler-Zebeli et al., 2020). The cecum is crucial for microbial organisms as the site of the highest microbial number and richness. Analysis of microbial α-diversity in the cecum of broilers revealed that lipopolysaccharide injection reduced ace, chao, and sobs indices, and disorganized intestinal microbiota in AA broilers (Keerqin et al., 2021), which was consistent with the current research. Supplementation with 40 mg/kg of carnosic acid increased the α-diversity of the cecal microbiota and alleviated microbiota dysbiosis in challenged broilers. It has been shown that dietary supplementation with carnosic acid moderately increased microbial α-diversity in the mouse colon (Fu et al., 2024). The main phyla of cecal microbiota were Bacteroidota and Firmicutes in broiler, which is consistent with previous studies (Zhang et al., 2021; Lobionda et al., 2019). In the research, carnosic acid supplementation increased the relative abundance of Firmicutes and decreased the relative abundance of Verrucomicrobiota in cecal microbiota of challenged broilers. The decomposition of polysaccharides such as cellulose can be promoted by Firmicutes (Ge et al., 2024), resulting in beneficial metabolites such as short-chain fatty acids (Lin et al., 2018), which are crucial for maintaining gut health and nutrient absorption (Sun et al., 2016). Verrucomicrobiota regulated intestinal metabolism (Liu et al., 2022), and maintained intestinal barrier homeostasis (Tu et al., 2023). Therefore, lipopolysaccharide injection reduced the relative abundance of Verrucomicrobiota in the current experiment, which might contribute to disrupting the intestinal barrier and increasing inflammation response.

At genus level, the relative abundance of *Clostridia_UCG-014, Anaerofilum*, and *Papilibacter* was decreased by lipopolysaccharide challenge. It was shown that the production of short-chain fatty acids, like propionic acid and butyric acid, which belong to the Firmicutes, were associated with *Clostridia_UCG-014* and *Anaerofilum* (Zhong et al., 2019). *Papilibacter* was known as a butyrate-producing bacterium (Ge et al., 2024). The relative abundance of these above genera in the cecum was increased by carnosic acid, suggesting that the harm caused to the intestinal microbial system by lipopolysaccharide was alleviated by carnosic acid. To be sum, the adverse effects of lipopolysaccharide challenge on the cecal microbiota could be alleviated by dietary supplementation with carnosic acid, through increasing the α-diversity, and promoting the beneficial flora, thus maintaining the balance of the intestinal flora.

Conclusion

In summary, dietary supplementation with carnosic acid improved growth performance and intestinal health in lipopolysaccharide-challenged broilers by decreasing intestinal barrier permeability, inhibiting the expression of *TLRs* (*TLR2/4*), decreasing inflammatory cytokine production, and enhancing cecal microbiota diversity and beneficial bacteria abundance. Under the conditions of this research, the optimal dosage of carnosic acid was 40 mg/kg.

Ethics statement

Experimental procedures were approved by the Institutional Animal Care and Use Committee, Guangdong Academy of Agricultural Sciences in China (GAASISA-2022-017).

Disclosures

No conflict of interest.

CRediT authorship contribution statement

Qin Wang: Investigation, Data curation, Writing – original draft. **Jiawei Li:** Investigation, Data curation. **Guanhuo Li:** Investigation, Data curation. **Yingan Zang:** Investigation, Data curation. **Qiuli Fan:** Investigation, Data curation. **Jingling Ye:** Investigation. **Yibing Wang:** Investigation, Funding acquisition, Resources, Writing – review & editing. **Shouqun Jiang:** Investigation, Funding acquisition, Resources, Writing – review & editing.

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Data availability

All data generated or analyzed during this study are available from the corresponding author on request.

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