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Effect of coated organic acids on growth performance, *Clostridium perfringens* colonization, gut integrity and immune response in broilers challenged with subclinical necrotic enteritis

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ABSTRACT

Necrotic enteritis (**NE**) is considered a major enteric disease that compromises intestinal health and causes significant economic losses in broiler production. This study aimed to evaluate the effect of dietary coated organic acids (**COA**) supplementation on growth, *Clostridium perfringens* colonization, gut integrity, and immune response in broilers challenged with NE. A total of 420 one-day-old broilers were allocated to 30 cages for 28 days. Five treatments included a non-challenged control and four NE-challenged groups with different dietary COA supplementation levels (0, 0.1, 0.2, and 0.4 %). Birds in NE groups were challenged with *Eimeria maxima* on day 14 and *C. perfringens* on days 18 and 19. Major measurements included daily feed intake (**FI**); weekly body weight gain (**BWG**) and feed conversion ratio (**FCR**); intestinal *C. perfringens* load on days 20, 21, and 28; NE lesion score and gut permeability on days 20 and 21; and gene expression of tight junction proteins and cytokines in the jejunum on day 21. Results showed that increasing dietary COA dose-dependently improved FCR from day 0 to 14, with the lowest FCR observed at 0.1 % COA from day 7 to 14 ($P < 0.05$). Under NE, dietary COA linearly improved BWG and cumulative FI from day 14 to 28 and FCR from day 14 to 21 ($P < 0.05$). On day 21, dietary COA decreased intestinal *C. perfringens* loads, NE lesions, and gut permeability in a dose-dependent manner ($P < 0.05$). Additionally, dietary COA dose-dependently increased the expression of claudin-2 and zonula occludin-2 but decreased the expression of claudin-1, interleukin-1β, interferon-γ, and interleukin-10 ($P < 0.05$). These results indicate that dietary COA may improve early feed efficiency at a lower dose (0.1 %) and mitigate growth loss in NE-challenged broilers by reducing intestinal *C. perfringens* colonization and modulating gut integrity and immune responses in a dose-dependent manner. In conclusion, this study suggests that dietary COA could be a potential intervention to alleviate the adverse effects of NE and improve broiler productivity.

Introduction

In poultry production, necrotic enteritis (**NE**) is recognized as a significant enteric disease that adversely affects growth performance, health, and welfare, leading to substantial economic losses (Lee and Lillehoj, 2021). Overgrowth of *Clostridium perfringens* strains producing NE B-like (**NetB**) toxin, one of the key virulence factors, in response to intestinal disturbances triggered by predisposing factors, has been implicated in the pathogenesis of NE (Lee and Lillehoj, 2021; Abd El-Hack et al., 2022). Major predisposing factors contributing to the development of NE include *Eimeria* infection, rearing condition, physiological stress, immunosuppression, and dietary characteristics (Dahiya et al., 2006; Shojadoost et al., 2012; Goo et al., 2024, 2025). They collectively impair intestinal integrity, disrupt the microbial balance, and compromise immune defenses, thereby increasing susceptibility to *C. perfringens* colonization and NE development (Abd El-Hack et al., 2022; Goo et al., 2023a, 2025).

Necrotic enteritis in the broiler production could be classified into clinical and subclinical, depending on its severity. Clinical NE is characterized by significant mortality during the acute phase, whereas subclinical NE leads to chronic intestinal damage, impaired nutrient absorption, and reduced growth performance (Timbermont et al., 2011).

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Subclinical NE is particularly challenging to diagnose and has a more significant economic impact due to its effects on feed efficiency and increased risk of secondary health issues (Timbermont et al., 2011; Shojadoost et al., 2012). Furthermore, as the use of antibiotics becomes increasingly restricted in poultry production, the burden of subclinical NE has intensified (Abd El-Hack et al., 2022). According to previous reports, NE incidents in the antibiotic-free rearing system were higher than in the conventional system in poultry production sectors (Gaucher et al., 2015). Therefore, the development of alternative strategies to mitigate NE without relying on antibiotics has become a critical priority in poultry production.

Organic acids have been widely studied as potential antibiotic alternatives in the poultry industry due to their antimicrobial properties, gut health benefits, and ability to improve feed efficiency (Dittoe et al., 2018; Adhikari et al., 2020; Abd El-Ghany, 2024; Du et al., 2024). The antimicrobial effects of organic acids are attributed to their ability to lower intestinal pH, disrupt bacterial metabolism, and regulate microbial composition, ultimately inhibiting pathogens such as *C. perfringens* (Gómez-García et al., 2019; Melaku et al., 2021). However, the efficacy of organic acids varies across studies and may not always produce the expected antimicrobial effects, depending on factors such as rapid dissociation in the upper gastrointestinal tract (**GIT**), the type and dosage of the acid used, target pathogens, and animal species. (Kasprowicz-Potocka et al., 2009; Du et al., 2024). In general, undissociated organic acids are considered the most effective form of showing antimicrobial effects. However, organic acids tend to dissociate rapidly in the upper GIT due to the pH-dependent dissociation and may not reach the lower small intestine, where most pathogenic bacteria reside (Du et al., 2024). This limitation raises concerns about whether organic acids can sufficiently inhibit enteric pathogens in the small intestine and may explain the inconsistent findings observed in broiler studies applying dietary organic acids.

Matrix coating or encapsulation technologies have been developed to enhance the retention time of nutrients or active compounds in food and pharmaceuticals (Anton et al., 2012; Calderón-Oliver and Ponce-Alquicira, 2022). These technologies have been applied to prevent the early dissociation of organic acids in the upper GIT and deliver undissociated form to the lower small intestine in animal production (Tugnoli et al., 2020; Nouri, 2023). Several studies have demonstrated that dietary encapsulated or matrix-coated organic acids improved gut health, humoral immune response, and growth performance in broilers under normal conditions (Nguyen et al., 2018; Nguyen and Kim, 2020; Manvatkar et al., 2022). Additionally, the blend of encapsulated or matrix-coated organic acids and bioactive compounds has been shown to alleviate NE and coccidiosis in broilers (Stefanello et al., 2020; Pham et al., 2020; Swaggerty et al., 2022). The alleviation effects of these various applications of organic acids were primarily associated with gut microbiota balance, intestinal integrity improvement, and immune modulation during intestinal diseases in poultry (Pham et al., 2020; Swaggerty et al., 2022). However, the effectiveness of organic acids may vary depending on types, supplementation levels, combination of organic acids, and interactions with other feed supplements in poultry (Abd El-Ghany, 2024; Du et al., 2024). The variable efficacy necessitates further research into coated organic acids, which may help develop a nutritional strategy to mitigate the adverse effects of NE in broilers.

Considering the functional role of organic acids, the efficacy of coated organic acids may vary with dietary inclusion levels, as different doses can influence their release dynamics in the GTI and consequently alter their antimicrobial and immunomodulatory activity. Therefore, the present study hypothesized that different supplementation levels of coated organic acids (**COA**) would influence broilers: (1) during start phase, COA supplementation would affect growth performance; and (2) during grower phase with NE challenge, COA levels would influence NE-induced responses, including changes in growth, *C. perfringens* colonization, gut health parameters, and immune responses. Accordingly, this study aimed to evaluate the effects of different supplementation levels of dietary COA on broiler performance throughout the production period, as well as on NE-associated outcomes, including *C. perfringens* colonization, gut integrity, and immune response following NE challenge during the grower phase.

Materials and methods

Experimental design, birds, management, experimental diets and supplement

The current study was approved by the Institutional Animal Care and Use Committee (A2021 12-012) and was performed at the Poultry Research Center at the University of Georgia. A total of 420 broilers (one-day-old, male, Cobb 500) were obtained from the commercial hatchery (Cobb-Vantress Inc., Cleveland, GA). Birds were randomly allocated to 30 battery cages (14 birds per cage) in a completely randomized design, which comprised five treatments with six replicate cages for 28 d: starter, d 0 to 14; grower with NE challenge, d 15 to 28. The five treatments included a non-challenged control (**NC**) group receiving basal diets, and four NE-challenged groups receiving 0 %, 0.1 %, 0.2 %, or 0.4 % dietary COA, designated as challenge control (**CC**), T1, T2, and T3, respectively. During the starter phase, both NC and CC groups were considered a unified control group as one experimental group in this study, as no NE challenge had yet been introduced, and both groups received the same basal diet under identical management conditions. However, during the grower phase, the NC and CC groups were treated as separate experimental groups, as only the CC group was subjected to the NE challenge.

This study was conducted in the biosafety level 2 facility, and each cage was equipped with a trough feeder and nipple drinkers. Feed and water were provided *ad libitum*. General management of the environments, such as temperature and light and dark cycles in the trial room, was controlled by the automatic housing system according to the Cobb management guide (Cobb-Vantress, 2021) during the whole experimental period. The experimental diets for starter (d 0 to 14) and grower (d 14 to 28) were corn-soybean meal-based mash diets formulated to meet or exceed the nutrient requirement following the manufactural nutrient specification (Cobb-Vantress, 2022). The nutrient composition of basal diets is presented in Table 1. Basal diets without filler (sand) were produced as one batch, and COA was supplemented into separate mixing batches following the supplementation levels of COA (0, 0.1, 0.2, and 0.4 %) by substituting the filler. The COA used in the experiment was provided by a commercial company (Morningbio Co., Ltd., Cheonan, South Korea). This product contained approximately 40 % organic acids, including fumaric acid, citric acid, and malic acid, and was coated within a lipid-based matrix.

Growth performance

Feed intake (**FI**) was recorded daily at a consistent time, with adjustments made for any mortality that occurred prior to measurement. Average body weight (**BW**), body weight gain (**BWG**), cumulative FI, and feed conversion ratio (**FCR**) were calculated weekly. Mortality was recorded daily throughout the experimental period.

Necrotic enteritis challenge

The NE challenge in the experiment was conducted based on the previously established NE models in the broiler with slight modification (Goo et al., 2023a,b, 2024). All individual birds in the NE-challenged groups were orally challenged with 1 mL of a suspension containing 10,000 sporulated oocysts of *E. maxima* on d 14 and with 1 mL of bacterial suspension including 1×10^9 CFU/mL *C. perfringens* (stain Del-1; NetB-positive) on d 18 and 19. Birds in the NC group were sham-challenged with 1 mL of sterile phosphate-buffered saline (**PBS**) at the corresponding time points.

Table 1
Experimental diets.

	Starter (d 0 to 14)	Grower (d 14 to 28)
Ingredients, %		
Corn	61.70	66.44
Soybean meal	31.38	28.22
Soybean oil	0.30	0.20
Limestone	0.97	1.12
Dicalcium phosphate	2.33	1.35
Choline Cl 60 %	0.09	0.09
Common salt	0.23	0.23
Sodium bicarbonate	0.20	0.17
Vitamin premix[1]	0.10	0.10
Mineral premix[2]	0.08	0.08
L-Arg	0.23	0.20
L-Ile	0.06	0.05
L-Val	0.16	0.13
L-Lys HCl	0.40	0.38
DL-Met	0.48	0.44
L-Thr	0.39	0.33
Sand[3]	0.91	0.47
Total	100.00	100.00
Calculated values		
ME, kcal/kg	2,900	2,970
Crude protein, %	21.09	19.85
Ether extract, %	2.77	2.81
Ca, %	0.97	0.81
Available P, %	0.58	0.40
Digestible Arg, %	1.36	1.25
Digestible His, %	0.44	0.42
Digestible Ile, %	0.81	0.75
Digestible Leu, %	1.45	1.38
Digestible Lys, %	1.26	1.16
Digestible Met, %	0.72	0.67
Digestible TSAA, %	0.94	0.88
Digestible Phe, %	0.86	0.81
Digestible Thr, %	0.86	0.78
Digestible Trp, %	0.21	0.19
Digestible Val, %	0.96	0.88

[1] Supplemented per kg of diet: vitamin A, 3,527 IU; vitamin D3, 1,400 ICU; vitamin E, 19.4 IU; vitamin B12, 0.01 mg; menadione, 1.10 mg; riboflavin, 3.53 mg; d-pantothenic acid, 5.47 mg; thiamine, 0.97 mg; niacin, 20.28 mg; vitamin B6, 1.46 mg; folic acid, 0.57 mg; biotin, 0.08 mg.
[2] Supplemented in per kg of diet: Ca, 24 mg; Mn, 100.5 mg; Zn, 80.25 mg; Mg, 20.1 mg; Fe, 19.73 mg; Cu, 3 mg; I, 0.75 mg; Se, 0.3 mg.
[3] The inclusion of coated organic acids was substituted with sand by following the supplementation levels (0, 0.1, 0.2, and 0.4 %).

Gut permeability, lesion scoring, excreta moisture, and oocyst shedding

On d 20 and 21, gut permeability was evaluated using fluorescein isothiocyanate-dextran (FITC-d) assay. In brief, one bird was randomly selected from each cage (a total of 30 birds; 6 per treatment) was orally gavaged with 1 mL of FITC-d solution (2.2 mg/mL; Sigma-Aldrich Co., St. Louis, MO) and detained. Blood samples were collected after 2 hours post-gavage and protected from light during the assay (dos Santos et al., 2020). The serum was separated by centrifugation at 1,500 × g for 13 minutes. Then, 100 μL of serum samples and FITC-d standards were transferred to a black 96-well microplate (Ref. 655077; Greiner Bio-One, Monroe, NC), and serum FITC-d concentration was measured using a spectrophotometer (Spectramax M5, Molecular Devices, San Jose, CA) at excitation and emission wavelengths of 485 nm and 528 nm, respectively.

Necrotic enteritis lesion scoring was performed on d 20 and 21 following the previous studies (Goo et al., 2023a,b, 2024). In brief, two birds from each cage (excluding the bird used for FITC-d assay) were euthanized by cervical dislocation, and jejunum to the upper ileum (around 15 cm lower from the Meckel's diverticulum) was collected and evaluated by two independent experienced evaluators. Lesion scoring was based on the 4 scales (0 to 3) system following the previous studies (Goo et al., 2023a,b, 2024). The average lesion score of the two birds was used as the representative value per cage for statistical analysis.

Before measuring excreta moisture and oocyst shedding, all excreta were removed from the excreta trays on d 19. Approximately 300 g of fresh excreta from each replicate cage was collected from d 19 to 20 and d 20 to 21 and homogenized (Lee et al., 2025). Approximately 100 g of homogenized fresh excreta was dried in a drying oven (Thelco, Winchester, VA) at 120 ℃ for 48 h. Differences in the weight of excreta samples before and after drying were used to calculate excreta moisture content. In addition, homogenized feces were also subjected to oocyst shedding. In brief, 5 g of gently homogenized fresh excreta was mixed with 35 mL of tap water in a 50 mL conical tube. Then, 1 mL of the mixed fecal solution was diluted with tap water to obtain a measuring range. One mL of diluted sample was transferred to the new tubes and mixed with 9 mL of saturated salt solution. Approximately 600 μL of the mixed solution was collected right after inversely shaking a few times and then added to a McMaster counting chamber (Vetlab Supply, Palmetto Bay, FL) to count the *E. maxima* oocysts in 6 columns. The number of *E. maxima* oocyst in the excreta was transformed into $\log_{10}$ before statistical analysis.

Enumeration of Clostridium perfringens

Clostridium perfringens loads in digesta and cecal contents were measured on d 20, 21 and 28 according to the previous studies with slight modifications (Goo et al., 2023a,b, 2024). On d 20 and 21, samples were collected from the same birds used for lesion scoring, whereas on d 28, two additional birds per cage were euthanized by cervical dislocation for *C. perfringens* enumeration. In brief, digesta from Meckel's diverticulum area (from mid-low jejunum to upper ileum), and cecal contents were collected aseptically into sterile filter bags (Whirl-Pak, Nasco, Fort Atkinson, WI). The collected samples were diluted approximately three-fold (w/v) with sterile PBS and homogenized for 90 sec using a homogenizer (Masticator Silver Panoramic, Neutec Group Inc., Farmingdale, NY). The homogenized contents were filtered and transferred into a 10 mL sterile test tube, followed by a 10-fold serial dilution using sterile PBS. One hundred μL of the diluted content from selected dilution tubes were transferred onto Tryptose Sulfite Cycloserine and Shahidi Ferguson Perfringens (TSC/SFP; Oxoid Ltd., Hampshire, UK) agar plates and spread evenly. The plates were anaerobically incubated in the anaerobic camber system (AnaeroPack, Thermo Scientific, MA) at 37°C for 48 to 72 h. After incubation, *C. perfringens* colonies were counted and recorded. The average value from two individual birds per cage was calculated and then transformed into $\log_{10}$ before statistical analysis.

Quantitative real-time reverse transcript PCR for Gene expression

The lower part of the jejunal tissue from one of the two lesion-scored birds (d 21) per cage was gently rinsed with PBS, snap-frozen in liquid nitrogen, and stored at –81 °C until RNA extraction. Approximately 100 mg of jejunal tissues were placed in the bead tubes with QIAzol lysis reagent (Qiagen, Valencia, CA) and homogenized using a bead beater (Biospec Products, Bartlesville, OK), and total RNA was extracted following the manufacturer's procedure. The quantity and quality of collected RNA samples were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) (Tejeda and Kim, 2021). The RNA was reverse transcribed for cDNA using high-capacity cDNA synthesis kits (Applied Biosystems, Foster City, CA). Quantitative real-time reverse transcript PCR (**qRT-PCR**) was performed by Step One Plus real-time PCR system (Thermo Fisher Scientific, Waltham, MA) with iTaq Universal SYBR Green Supermix (**SYBR**; Bio-Rad Laboratories Inc., Waltham, MA) for gene expression. The total volume of the qRT-PCR mixture was 10 μL (5.0 μL of SYBR Green mix, 2.0 μL of cDNA, 0.5 μL of forward primer, 0.5 μL of reverse primer, and 1.0 μL of nuclease-free water). Primers were selected based on previous studies (Teng et al., 2021; Liu et al., 2023; Shi et al., 2023; Sharma et al., 2024; Goo et al., 2024). The primer sets and brief descriptions of the

corresponding gene functions are presented in Table 2. All gene samples were duplicated, *β-actin* was used as the housekeeping gene, and the target genes were expressed using the $2^{-\text{delta delta CT}}$ method (Livak and Schmittgen, 2001).

Statistical analysis

Statistical analysis was conducted using SAS software (SAS version 9.3, SAS Ins., Cary, NC), and figures were created using GraphPad Prism software (GraphPad Prism version 10.2.3, GraphPad Software Inc., San Diego, CA). Individual cages were considered as one experimental unit in this study. Continuous data were presented as means with standard error of means, whereas ordinal data were expressed as medians with interquartile range. The normality and homogeneity of continuous data were evaluated using the Shapiro–Wilk and Levene's tests. Continuous data were analyzed using one-way ANOVA, followed by Scheffé's post hoc test for data from d 0 to 14 and Tukey's post hoc test for data from d 14 to 28 to compare all treatment groups. Orthogonal polynomial contrast was performed to evaluate the linear and quadratic effects of dietary COA supplementation levels. For this analysis, data collected before the NE challenge included only cages assigned to the NE-challenged groups (CC, T1, T2, and T3), whereas data after the NE challenge used the same NE-challenged groups. Ordinal data were analyzed using the Kruskal-Wallis test with Bonferroni-adjusted pairwise comparisons among all treatment groups. The Jonckheere-Terpstra trend test was conducted to assess the dose-dependent effects of dietary COA within the NE-challenged groups. Statistical significance was set at $P < 0.05$, and the trend or numerical change was defined as $0.05 < P \leq 0.10$ in this study (Choi et al., 2021).

Results

Growth performance

The growth performance results during the starter phase are presented in Table 3. Dietary COA linearly increased BW on d 14 and BWG from d 7 to 14 and d 0 to 14 (Linear, $P = 0.013$, $P = 0.001$, and 0.020, respectively). In addition, the highest BWG was found in the T3 group from d 7 to 14 ($P = 0.012$). The quadratic effects of dietary COA were observed in FCR during d 7 to 14 and d 0 to 14 (Quadratic, $P = 0.020$ and 0.007, respectively). The FCR of the T1 was lower than the control group from d 7 to 14 ($P = 0.017$).

The growth performance results after the NE challenge are exhibited in Table 4. BW in the NE-challenged groups was lower than the NC, and BW in the T2 and T3 was significantly higher than the CC on d 21 and 28 ($P < 0.001$). Dietary COA linearly increased BW among the NE-challenged groups at these time points. BWG in the CC from d 14 to 21, d 21 to 28, and d 14 to 28 was lower than the NC ($P < 0.05$), and dietary COA supplementation level increased BWG among the NE-challenged groups from d 14 to 21 and d 14 to 28 (Linear, $P = 0.001$ and $P = 0.023$, respectively). Cumulative FI in the NE-challenged groups was lower than the NC from d 14 to 21, d 21 to 28, and d 14 to 28 ($P < 0.001$), but linear effects were found among the NE-challenged groups from d 21 to 28 and d 14 to 28 (Linear, $P = 0.035$ and $P = 0.027$, respectively). The CC group exhibited a significantly higher FCR from d 14 to 21 and d 14 to 28 than the NC ($P < 0.001$ and $P = 0.012$, respectively). However, dietary COA supplementation linearly decreased FCR among the NE-challenged groups from d 14 to 21 (Linear, $P = 0.001$).

Daily feed intake

The daily FI results after the NE challenge are presented in Fig. 1. Daily FI in the NE-challenged groups was significantly lower compared to the NC during d 19 to 22 ($P < 0.001$). On d 23, the T1 and T3 groups showed lower daily FI than the NC ($P = 0.003$). The lowest daily FI was found in the CC among the treatments on d 24, 25, and 26 ($P < 0.05$), and linear effects of dietary COA were also observed within the NE-challenged groups (Linear, $P < 0.05$).

Table 2
Primer sequences and general functions of the target genes.

Gene symbol[1]	Accession No.	Primer sequence, 5′–3′	Amplicon size, bp	Annealing Temp.,°C	General function
CLDN1	NM_001013611.2	F: TGGAGGATGACCAGGTGAAGA R: CGAGCCACTCTGTTGCCATA	115	60	Sealing tight junction protein that enhances paracellular barrier (Krause et al., 2008).
CLDN2	NM_001277622.1	F: TTGGAGGCTTCATCCTCTGT R: ACTCACTCTTGGGCTTCTGC	126	59	Channel-forming tight junction protein involved in paracellular transport of Na^+ and water (Venugopal et al., 2019).
OCLN	NM_205128.1	F: ACGGCAGCACCTACCTCAA R: GGCGAAGAAGCAGATGAG	122	59	Maintains tight junction barrier integrity (Cummins, 2012).
ZO1	XM_046925213.1	F: AGGTGAAGTGTTTCGGGTTG R: CCTCCTGCTGTCTTTGGAAG	171	58	Scaffold protein linking tight junction components to the cytoskeleton (Hartsock and Nelson, 2008).
ZO2	NM_001396728.1	F: GGCAAATCATTGAGCAGGA R: ATTGATGGTGGCTGTAAAGAG	239	56	Tight junction-associated scaffold protein (Hartsock and Nelson, 2008).
JAM2	NM_001397141.1	F: AAGGATTCTGGGACCTACCG R: GTTCCCGTCATTGCAGAGTT	143	59	Cell adhesion molecule involved in barrier function (Paradis et al., 2021).
TNFα	MF000729.1	F: CGTGGTTCGAGTCGCTGTAT R: CCGTGCAGGTCGAGGTACT	100	60	Pro-inflammatory cytokine involved in immune response (Navegantes et al., 2017).
IFNγ	NM_205149.2	F: CACATATCTGAGGAGCTCTATAC R: GTTCATTCGCGGCTTTG	130	55	Pro-inflammatory cytokine; Th1 cytokine that promotes cellular immunity (Fries-Craft and Bobeck, 2024).
IL1β	NM_204524.2	F: TGCCTGCAGAAGAAGCCTCG R: GACGGGCTCAAAAACCTCCT	204	60	Mediator of inflammation and immune activation (Navegantes et al., 2017; Kan et al., 2021).
IL6	NM_204628.2	F: GCTACAGCACAAAGCACCTG R: GACTTCAGATTGGCGAGGAG	112	59	Proinflammatory cytokine involved in inflammation and immune activation (Navegantes et al., 2017).
IL10	NM_001004414.4	F: AGCAGATCAAGGAGACGTTC R: ATCAGCAGGTACTCCTCGAT	103	58	Anti-inflammatory cytokine that regulates immune responses (Arendt et al., 2019).
β-actin	NM_205518.2	F: CAACACAGTGCTGTCTGGTGGTA R: ATCGTACTCCTGCTTGCTGATCC	205	62	Housekeeping gene for normalization (Livak and Schmittgen, 2001).

[1] CLDN, claudin; OCLN, Occludin; ZO, zonula occludin; JAM, junctional adhesion molecule; TNF, tumor necrosis factor; IFN, interferon; IL, interleukin.

Table 3
Effect of dietary coated organic acids supplementation levels on growth performance from days 0 to 14.

Treatments[1]	Control[2]	T1	T2	T3	SEM	*P*-value[3]	Contrast[4]	
Dietary COA, %	0	0.1	0.2	0.4			Linear	Quadratic
Bodyweight, g/bird								
d 7	156	151	156	156	1.318	0.560	0.589	0.819
d 14	435	442	449	455	2.986	0.054	0.013	0.511
Bodyweight gain, g/bird								
d 0 to 7	114	109	114	114	1.318	0.560	0.589	0.819
d 7 to 14	279[b]	291[ab]	293[ab]	299[a]	2.552	0.012	0.001	0.391
d 0 to 14	393	400	407	413	2.986	0.054	0.020	0.538
Feed intake, g/bird								
d 0 to 7	134	131	134	137	1.217	0.546	0.378	0.459
d 7 to 14	349	337	347	364	3.843	0.164	0.045	0.124
d 0 to 14	483	468	482	501	4.888	0.207	0.160	0.218
Feed conversion ratio								
d 0 to 7	1.18	1.21	1.18	1.21	0.010	0.714	0.876	0.577
d 7 to 14	1.25[a]	1.16[b]	1.19[ab]	1.22[ab]	0.012	0.017	0.609	0.020
d 0 to 14	1.23	1.17	1.18	1.21	0.008	0.051	0.691	0.007

[1] Birds fed diets containing different levels of COA: 0 % (Control; $n = 12$), 0.1 % (T1; $n = 6$), 0.2 % (T2; $n = 6$), and 0.4 % (T3; $n = 6$).
[2] Control group included NC and CC before challenge; control ($n = 12$) used for ANOVA; only CC subset ($n = 6$) used for the contrast.
[3] All data were analyzed using one-way ANOVA followed by Scheffé's post hoc test ($P < 0.05$).
[4] Orthogonal polynomial contrast was performed to evaluate the linear and quadratic effects of dietary COA levels ($P < 0.05$).
[a,b] Means in the same row with different superscripts are statistically different ($P < 0.05$).
COA, coated organic acids; SEM, standard error of the means; NC, non-challenged control; CC, NE-challenged control.

Table 4
Effect of dietary coated organic acids supplementation levels on growth performance in broilers challenged with necrotic enteritic.

Treatments[1]	NC	CC	T1	T2	T3	SEM ($n = 6$)	*P*-value[3]	Contrast[4]	
Dietary COA, %	0	0	0.1	0.2	0.4				
NE challenge[2]	-	+	+	+	+			Linear	Quadratic
Bodyweight, g/bird									
d 21	800[a]	634[c]	662[bc]	684[b]	709[b]	11.591	<0.001	<0.001	0.900
d 28	1,413[a]	1,137[c]	1,221[bc]	1,252[b]	1,247[b]	19.756	<0.001	0.007	0.116
Bodyweight gain, g/bird									
d 14 to 21	365[a]	200[c]	220[bc]	235[bc]	254[b]	11.362	<0.001	0.001	0.954
d 21 to 28	612[a]	503[b]	559[ac]	568[ab]	538[ab]	10.677	0.012	0.246	0.056
d 14 to 28	977[a]	703[b]	779[b]	803[b]	791[b]	19.638	<0.001	0.023	0.113
Feed intake, g/bird									
d 14 to 21	595[a]	473[b]	485[b]	479[b]	496[b]	9.014	<0.001	0.092	0.770
d 21 to 28	998[a]	865[b]	901[b]	919[b]	921[b]	10.732	<0.001	0.035	0.364
d 14 to 28	1,593[a]	1,337[b]	1,386[b]	1,397[b]	1,417[b]	18.583	<0.001	0.027	0.537
Feed conversion ratio									
d 14 to 21	1.63[c]	2.38[a]	2.21[ab]	2.07[b]	1.96[b]	0.055	<0.001	0.001	0.726
d 21 to 28	1.64	1.73	1.63	1.63	1.72	0.027	0.575	0.874	0.145
d 14 to 28	1.63[b]	1.91[a]	1.79[ab]	1.75[ab]	1.79[ab]	0.027	0.012	0.112	0.142
Mortality, %									
d 14 to 21	0.00	2.38	0.00	1.19	0.00	0.398	0.222	*NA*	*NA*
d 21 to 28	1.19	1.19	0.00	0.00	0.00	0.331	0.567	*NA*	*NA*
d 14 to 28	1.19	3.57	0.00	1.19	0.00	0.494	0.129	*NA*	*NA*

[1] NC, non-challenged control group with a basal diet; CC, NE-challenged control group with a basal diet; T1, NE-challenged group with diet containing 0.1 % COA; T2, NE-challenged group with diet containing 0.2 % COA; T3, NE-challenged group with diet containing 0.4 % COA.
[2] All birds in the NE-challenged groups (CC, T1, T2, and T3) were challenged with 10,000 sporulated oocysts of *E. maxima* on d 14 and 1×10^9 CFU per mL of *C. perfringens* on d 18 and 19.
[3] All data were analyzed using one-way ANOVA followed by Tukey's post hoc test ($P < 0.05$).
[4] Orthogonal polynomial contrast was applied among the NE-challenged groups (CC, T1, T2, and T3) to evaluate the linear and quadratic effects of dietary COA levels ($P < 0.05$).
[a,b,c] Means in the same row with different superscripts are statistically different ($P < 0.05$).
NE, necrotic enteritis; COA, coated organic acids; SEM, standard error of the means; NA, not applicable for statistical analysis.

Clostridium perfringens colonization

Clostridium perfringens loads in digesta and cecal contents on d 20, 21, and 28 are summarized in Table 5. The NE challenge tended to increase *C. perfringens* loads in digesta on d 20 ($P = 0.057$). Digesta *C. perfringens* loads increased in the CC and T1 compared to the NC on d 21, whereas the T2 and T3 had significantly lower *C. perfringens* loads than the CC. Furthermore, digesta *C. perfringens* loads were linearly reduced as dietary COA supplementation increased in the NE-challenged groups ($P = 0.001$; Linear, $P = 0.006$). On d 28, digesta *C. perfringens* loads in the CC were higher than those in the NC ($P = 0.025$).

The cecal *C. perfringens* loads in the CC on d 20 were higher than the dietary COA supplementation groups, and the NC showed the lowest cecal *C. perfringens* loads ($P < 0.001$). In addition, linear and quadratic effects of dietary COA supplementation levels were observed in cecal *C. perfringens* loads on d 20 (Linear, $P < 0.001$; Quadratic, $P = 0.012$). On d 21, cecal *C. perfringens* loads were higher in the CC and T1 than those in the NC, whereas there were no significant differences between the NC, T2, and T3. In addition, a linear effect on cecal *C. perfringens* loads among the NE-challenged groups were observed ($P = 0.002$;

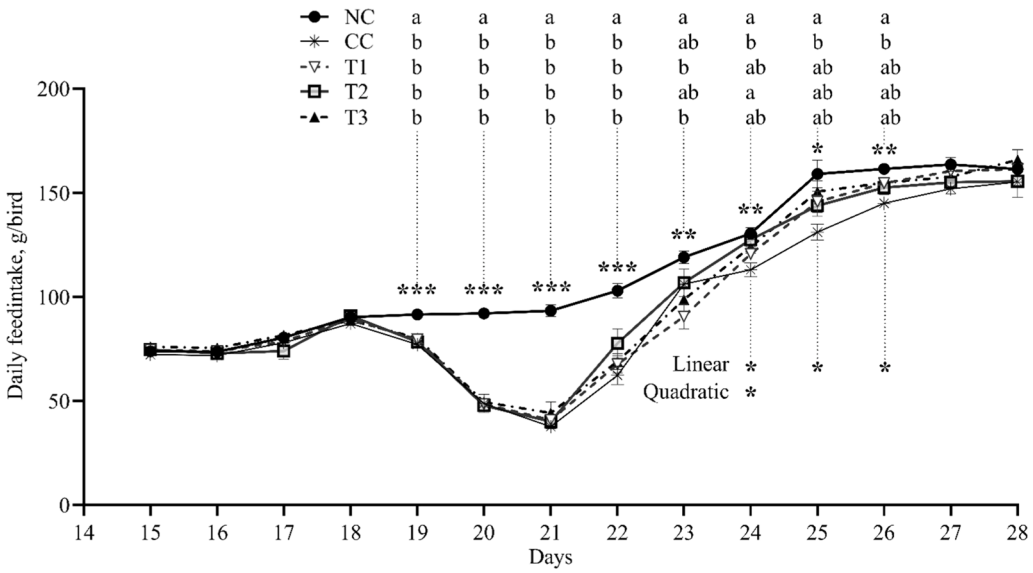

Fig. 1. Effect of dietary coated organic acids supplementation levels on daily feed intake in broilers challenged with necrotic enteritis. Data are presented as a line graph with standard error of the mean ($n = 6$). NC, non-challenged control group with a basal diet; CC, NE-challenged control group with a basal diet; T1, NE-challenged group with diet containing 0.1 % COA; T2, NE-challenged group with diet containing 0.2 % COA; T3, NE-challenged group with diet containing 0.4 % COA. All birds in the NE-challenged groups (CC, T1, T2, and T3) were challenged with 10,000 sporulated oocysts of *E. maxima* on d 14 and 1×10^9 CFU per mL of *C. perfringens* on d 18 and 19. All data were analyzed using one-way ANOVA followed by Tukey's post hoc test ($P < 0.05$). Orthogonal polynomial contrast was applied among the NE-challenged groups (CC, T1, T2, and T3) to evaluate the linear and quadratic effects of dietary COA levels. A star (*) above the error bar indicates significant differences from ANOVA, and a star below the error bar indicates significant effects from polynomial contrast (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Different letters (a, b) next to the legend indicate statistical differences ($P < 0.05$). NE, necrotic enteritis; COA, coated organic acids.

Table 5
Effect of dietary coated organic acids supplementation levels on intestinal *Clostridium perfringens* colonization in broilers on days 20, 21, and 28.

Treatments[1]	NC	CC	T1	T2	T3	SEM ($n = 6$)	*P*-value[3]	Contrast[4]	
Dietary COA, %	0	0	0.1	0.2	0.4				
NE challenge[2]	-	+	+	+	+			Linear	Quadratic
Digesta, Log_{10}CFU/g									
d 20	5.12	7.35	6.76	6.68	6.06	0.257	0.057	0.133	0.977
d 21	5.44[c]	8.48[a]	7.21[ab]	6.46[bc]	6.68[bc]	0.255	0.001	0.006	0.111
d 28	5.59[b]	7.57[a]	6.53[ab]	6.58[ab]	6.64[ab]	0.197	0.025	0.138	0.180
Cecal content, Log_{10}CFU/g									
d 20	6.17[c]	9.36[a]	7.93[b]	7.62[b]	7.61[b]	0.216	<0.001	<0.001	0.012
d 21	6.89[b]	9.06[a]	8.69[a]	8.11[ab]	7.69[ab]	0.205	0.002	0.007	0.945
d 28	7.26	8.84	8.63	8.33	7.65	0.307	0.460	0.249	0.749

[1] NC, non-challenged control group with a basal diet; CC, NE-challenged control group with a basal diet; T1, NE-challenged group with diet containing 0.1 % COA; T2, NE-challenged group with diet containing 0.2 % COA; T3, NE-challenged group with diet containing 0.4 % COA.

[2] All birds in the NE-challenged group (CC, T1, T2, and T3) were challenged with 10,000 sporulated oocysts of *E. maxima* on d 14 and 1×10^9 CFU per mL of *C. perfringens* on d 18 and 19.

[3] All data were analyzed using one-way ANOVA followed by Tukey's post hoc test ($P < 0.05$).

[4] Orthogonal polynomial contrast was applied among the NE-challenged groups (CC, T1, T2, and T3) to evaluate the linear and quadratic effects of dietary COA levels ($P < 0.05$).

[a,b,c] Means in the same row with different superscripts are statistically different ($P < 0.05$).

NE, necrotic enteritis; COA, coated organic acids; SEM, standard error of the means.

Linear, $P = 0.007$).

Necrotic enteritis lesion and gut permeability

The results of NE lesion scoring are presented in Table 6. The CC had significantly higher NE lesion scores compared to the NC on d 20 and 21 ($P = 0.022$ and $P = 0.004$). In addition, on d 21, the NE lesion decreased dose-dependently with an increase in dietary COA supplementation level among the NE-challenged groups ($P = 0.002$).

In the present study, gut permeability was assessed based on serum FITC-d concentration (Table 7). The NE challenge increased gut permeability compared to the NC on d 20 ($P < 0.001$). Linear effects of dietary COA supplementation levels were observed on serum FITC-d concentration among the NE-challenged groups on d 20 and 21 (Linear, $P = 0.044$ and 0.048, respectively).

Excreta moisture and oocyst shedding

Excreta moisture content and oocyst shedding results are summarized in Table 8. Oocysts were only found in the NE-challenged groups, not in the NC; however, no statistical differences were observed. On d 21, excreta moisture increased in the CC compared to the NC and linearly decreased among the NE-challenged groups as dietary COA was increased ($P = 0.05$; Linear, $P = 0.019$).

Table 6
Effect of dietary coated organic acids supplementation levels on necrotic enteritis lesion on days 20 and 21.

Treatments[1]	NC	CC	T1	T2	T3	*P*-values	
Dietary COA, %	0	0	0.1	0.2	0.4		
NE challenge[2]	-	+	+	+	+	KW[3]	JT[4]
NE lesion score, median (interquartile range)							
d 20	0.50[b] (0.38)	1.50[a] (0.63)	1.00[ab] (0.75)	1.25[ab] (0.63)	1.25[ab] (1.13)	0.022	0.206
d 21	0.25[b] (1.00)	2.00[a] (1.00)	1.50[ab] (0.38)	1.00[ab] (1.63)	0.50[ab] (0.75)	0.004	0.002

[1] NC, non-challenged control group with a basal diet; CC, NE-challenged control group with a basal diet; T1, NE-challenged group with diet containing 0.1 % COA; T2, NE-challenged group with diet containing 0.2 % COA; T3, NE-challenged group with diet containing 0.4 % COA.
[2] All birds in the NE-challenged group (CC, T1, T2, and T3) were challenged with 10,000 sporulated oocysts of *E. maxima* on d 14 and 1×10^9 CFU per mL of *C. perfringens* on d 18 and 19.
[3] All treatments were compared using the Kruskal–Wallis test followed by Bonferroni-adjusted pairwise comparisons ($P < 0.05$).
[4] Jonckheere–Terpstra trend test was applied among the NE-challenged groups (CC, T1, T2, and T3) to assess the dose-dependent effect of dietary COA levels ($P < 0.05$).
[a,b] Medians in the same row with different superscripts are statistically different ($P < 0.05$).
NE, necrotic enteritis; COA, coated organic acids; KW, Kruskal–Wallis test; JT, Jonckheere–Terpstra trend test.

Table 7
Effect of dietary coated organic acids supplementation levels on gut permeability on days 20 and 21.

Treatments[1]	NC	CC	T1	T2	T3	SEM ($n = 6$)	*P*-value[3]	Contrast[4]	
Dietary COA, %	0	0	0.1	0.2	0.4				
NE challenge[2]	-	+	+	+	+			Linear	Quadratic
Serum FITC-d concentration, ng/mL									
d 20	32.24[b]	315.10[a]	199.09[a]	194.11[a]	190.38[a]	22.302	<0.001	0.044	0.170
d 21	27.92	43.28	36.26	32.55	30.17	2.370	0.279	0.048	0.617

[1] NC, non-challenged control group with a basal diet; CC, NE-challenged control group with a basal diet; T1, NE-challenged group with diet containing 0.1 % COA; T2, NE-challenged group with diet containing 0.2 % COA; T3, NE-challenged group with diet containing 0.4 % COA.
[2] All birds in the NE challenged group (CC, T1, T2, and T3) were challenged with 10,000 sporulated oocysts of *E. maxima* on d 14 and 1×10^9 CFU per mL of *C. perfringens* on d 18 and 19.
[3] All data were analyzed using one-way ANOVA followed by Tukey's post hoc test ($P < 0.05$).
[4] Orthogonal polynomial contrast was applied among the NE-challenged groups (CC, T1, T2, and T3) to evaluate the linear and quadratic effects of dietary COA levels ($P < 0.05$).
[a,b] Means in the same row with different superscripts are statistically different ($P < 0.05$).
NE, necrotic enteritis; COA, coated organic acids; SEM, standard error of the means; FITC-d, fluorescein isothiocyanate-dextran.

Table 8
Effect of dietary coated organic acids supplementation levels on oocyst shedding and excreta moisture on days 20 and 21.

Treatments[1]	NC	CC	T1	T2	T3	SEM ($n = 6$)	*P*-value[3]	Contrast[4]	
Dietary COA, %	0	0	0.1	0.2	0.4				
NE challenge[2]	-	+	+	+	+			Linear	Quadratic
Oocysts count in excreta, Log_{10}Oocysts/g									
d 20	ND	4.90	4.81	4.86	4.81	0.031	0.679	0.438	0.743
d 21	ND	5.32	5.18	5.19	5.17	0.038	0.470	0.196	0.470
Excreta moisture, %									
d 20	74.71	81.31	78.99	76.23	76.92	0.979	0.242	0.137	0.519
d 21	74.98[b]	82.50[a]	78.48[ab]	76.53[ab]	75.98[ab]	0.893	0.050	0.019	0.369

[1] NC, non-challenged control group with a basal diet; CC, NE-challenged control group with a basal diet; T1, NE-challenged group with diet containing 0.1 % COA; T2, NE-challenged group with diet containing 0.2 % COA; T3, NE-challenged group with diet containing 0.4 % COA.
[2] All birds in the NE challenged group (CC, T1, T2, and T3) were challenged with 10,000 sporulated oocysts of *E. maxima* on d 14 and 1×10^9 CFU per mL of *C. perfringens* on d 18 and 19.
[3] All data were analyzed using one-way ANOVA followed by Tukey's post hoc test ($P < 0.05$); Oocyst data from the NC group were excluded from statistical analysis because no oocysts were detected.
[4] Orthogonal polynomial contrast was applied among the NE-challenged groups (CC, T1, T2, and T3) to evaluate the linear and quadratic effects of dietary COA levels ($P < 0.05$).
[a,b] Means in the same row with different superscripts are statistically different ($P < 0.05$).
NE, necrotic enteritis; COA, coated organic acids; SEM, standard error of the means; ND, not detected.

Gene expression

Relative mRNA expression of tight junction proteins in jejunal tissue is presented in Fig. 2. The relative expression of claudin-1 (***CLDN1***) tended to be upregulated in the CC but linearly downregulated among the NE-challenged groups ($P = 0.056$; Linear, $P = 0.023$). However, the relative expression of *CLDN2* in the CC was downregulated compared to the NC and T2, and linear and quadratic effects were also observed among the NE-challenged groups ($P = 0.01$; Linear, $P = 0.015$; Quadratic, $P = 0.018$). The relative expression of occludin (***OCLN***), zonula occludens-1 (***ZO1***), and *ZO2* were downregulated in the NE-challenged groups compared to the NC ($P < 0.001$). In addition, dietary COA supplementation levels linearly upregulated *ZO2* expression among the NE-challenged groups (Linear, $P = 0.007$).

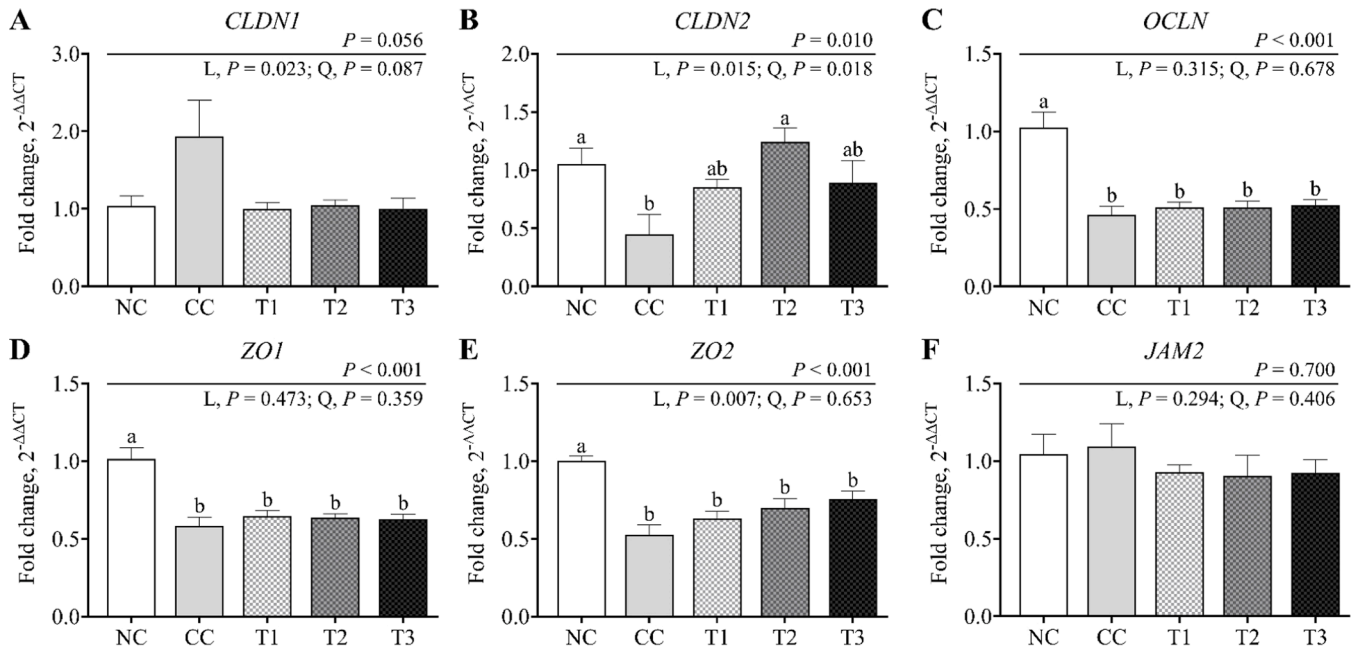

Fig. 2. Effect of dietary coated organic acids supplementation levels on the expression of tight junction-related genes in the jejunal tissue on day 21. (A) The expression of *CLDN1*. (B) The expression of *CLDN2*. (C) The expression of *OCLN*. (D) The expression of *ZO1*. (E) The expression of *ZO2*. (F) The expression of *JAM2*. Data are presented as a bar graph with standard error of the mean ($n = 6$). NC, non-challenged control group with a basal diet; CC, NE-challenged control group with a basal diet; T1, NE-challenged group with diet containing 0.1 % COA; T2, NE-challenged group with diet containing 0.2 % COA; T3, NE-challenged group with diet containing 0.4 % COA. All birds in the NE-challenged groups (CC, T1, T2, and T3) were challenged with 10,000 sporulated oocysts of *E. maxima* on d 14, 1×10^9 CFU per mL of *C. perfringens* on d 18 and 19. All data were analyzed using one-way ANOVA followed by Tukey's post hoc test ($P < 0.05$). Orthogonal polynomial contrast was applied among the NE-challenged groups (CC, T1, T2, and T3) to evaluate the linear (L) and quadratic (Q) effects of dietary COA levels ($P < 0.05$). Different letters (a, b) above the error bar indicate significant ($P < 0.05$). NE, necrotic enteritis; COA, coated organic acids; CLDN, claudin; OCLN, Occludin; ZO, zonula occludin; JAM, junctional adhesion molecule.

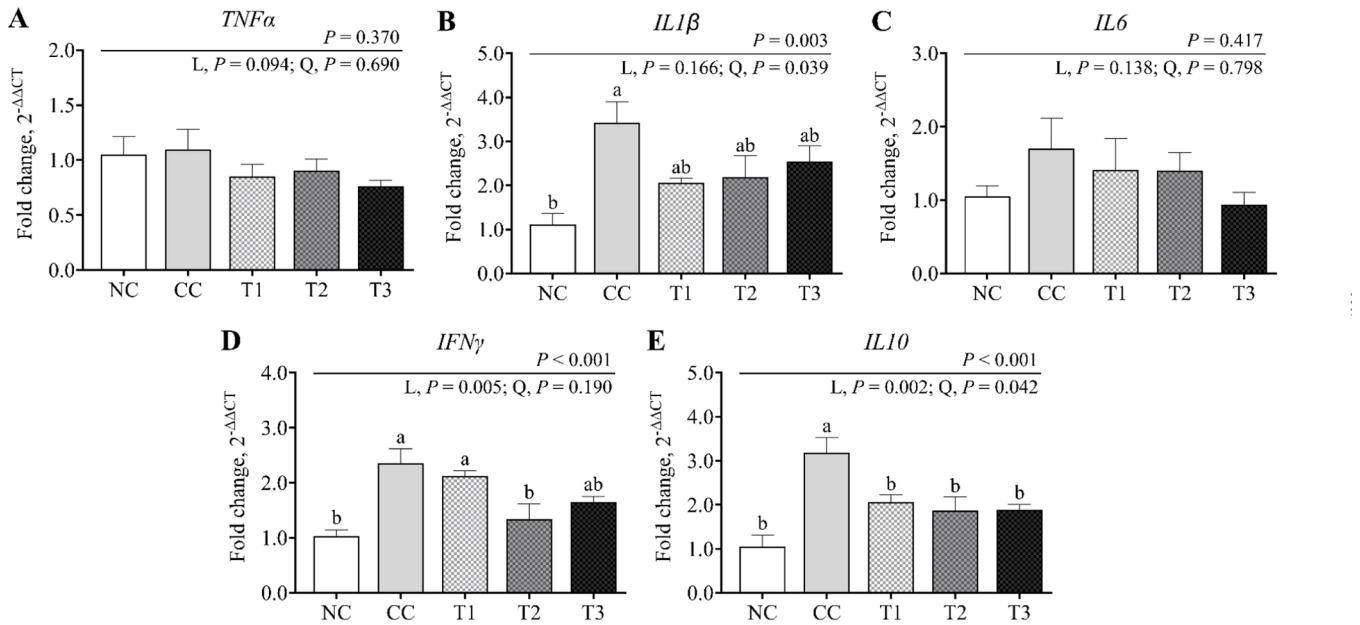

Fig. 3. Effect of dietary coated organic acids supplementation levels on the expression of immune-related genes in the jejunal tissue on day 21. (A) The expression of *TNFα*. (B) The expression of *IL1β*. (C) The expression of *IL6*. (D) The expression of *IFNγ*. (E) The expression of *IL10*. Data are presented as a bar graph with standard error of the mean ($n = 6$). NC, non-challenged control group with a basal diet; CC, NE-challenged control group with a basal diet; T1, NE-challenged group with diet containing 0.1 % COA; T2, NE-challenged group with diet containing 0.2 % COA; T3, NE-challenged group with diet containing 0.4 % COA. All birds in the NE-challenged groups (CC, T1, T2, and T3) were challenged with 10,000 sporulated oocysts of *E. maxima* on d 14, 1×10^9 CFU per mL of *C. perfringens* on d 18 and 19. All data were analyzed using one-way ANOVA followed by Tukey's post hoc test ($P < 0.05$). Orthogonal polynomial contrast was applied among the NE-challenged groups (CC, T1, T2, and T3) to evaluate the linear (L) and quadratic (Q) effects of dietary COA levels ($P < 0.05$). Different letters (a, b) above the error bar indicate significant ($P < 0.05$). NE, necrotic enteritis; COA, coated organic acids; TNF, tumor necrosis factor; IL, interleukin; IFN, interferon.

The expression of inflammatory cytokines in the jejunal tissue is summarized in Fig. 3. Interleukin-1β (***IL1β***) expression was upregulated in the CC than the NC, and a quadratic effect of dietary COA supplementation levels was found among the NE-challenged groups ($P = 0.003$; Quadratic, $P = 0.039$). The interferon-γ (***IFNγ***) expression was upregulated in the CC and T1 compared to the NC and T2, and a linear effect of dietary COA supplementation levels was found among the NE-challenged groups ($P < 0.001$; Linear, $P = 0.005$); the *IFNγ* expression was reduced when dietary COA levels increased. Among all treatments, the CC exhibited the highest expression of *IL10*, whereas dietary COA supplementation exhibited linear and quadratic effects on *IL10* expression among the NE-challenged groups ($P < 0.001$; Linear, $P = 0.002$; Quadratic, $P = 0.042$).

Discussion

Necrotic enteritis has threatened efficient poultry production, posing challenges to food security and economic sustainability (Lee and Lillehoj, 2021). Thus, finding effective strategies to mitigate its impact is essential. The present study investigated the effects of dietary COA supplementation on growth performance, NE lesions, *C. perfringens* colonization, gut integrity, and immune responses in broilers under NE conditions.

Before the NE challenge, the current findings suggest that broiler performance responses to different supplementation levels of COA may vary with age during the starter period. Nguyen et al. (2018) reported that dietary supplementation with matrix-coated organic acids combined with medium-chain fatty acids at different levels did not affect broiler performance from d 0 to 7, which shows a similar pattern to the present findings. The minimal effect of COA during the early phase (d 0 to 7) may be associated with the immaturity of the digestive system in young broilers. Although broilers exhibit rapid growth immediately after hatching, their digestive functions are not fully developed (Lilburn and Loeffler, 2015; Ravindran and Abdollahi, 2021). Specifically, bile acid secretion and lipase activity are limited in early-age broilers (Noy and Sklan, 1998). Because COA was coated within a lipid-matrix polymer, the lower emulsification capacity with lipase activity in young broilers may have restricted the breakdown of COA, indicating that the effectiveness of organic acids released from COA may be limited.

However, improved BWG and FCR from d 7 to 14 were found as COA supplementation increased, exhibiting the most effective improvement at 0.1 % of COA in the current study. As birds age, broilers are capable of producing enough bile acids and pancreatic lipase activity, which facilitates the effective release and utilization of organic acids from COA (Ravindran and Abdollahi, 2021). However, excessive organic acids in the lumen may lower intestinal pH, inhibiting the activity of certain digestive enzymes or disrupting the homeostasis of intestinal epithelial cells, thereby impairing nutrient absorption (Nourmohammadi and Khosravinia, 2015; Montoro-Huguet et al., 2021). Previous studies have also reported that high concentrations of organic acids tend to reduce growth performance in young broilers (Biggs and Parsons, 2008; Nourmohammadi and Khosravinia, 2015). This evidence suggests that COA 0.1 % supplementation may balance the beneficial effects of organic acids on growth and the potential risk of excessive acidification in the gut. Furthermore, improving growth performance in the COA supplementation groups from d 7 to 14 in the current study may have contributed to the overall enhancement pattern in BWG and FCR observed from d 0 to 14. Therefore, these findings suggest that COA supplementation positively impacts broiler performance from d 0 to 14 under normal conditions in a dose-dependent manner. These results also raise the hypothesis that the efficacy of COA may vary depending on intestinal development, proposing further investigation into its relationship with the lipid matrix coating system in young broilers.

In the current study, the broiler was challenged with *E. maxima* as a predisposing factor on d 14 and *C. perfringens* on d 18 and 19 to induce NE, which led to increased oocyst shedding and *C. perfringens* proliferation in the NE-challenged groups, verifying the induction of NE. Following *E.* maxima challenge, alterations in the intestinal environment provide a favorable condition for *C. perfringens* proliferation, leading to NE development (Timbermont et al., 2009; Yang et al., 2019; Deslauriers et al., 2024). The toxin NetB, produced by *C. perfringens*, increases intestinal epithelial permeability, induces cell apoptosis, and accelerates mucosal damage (Goo et al., 2023a,b). As NE progresses, intestinal mucosal damage and severe inflammatory response ultimately lead to a decline in growth performance (Goo et al., 2023a,b, 2024). Similarly, in the present study, the CC group exhibited 28 and 16 % reductions in BWG and FI, respectively, compared to the NC, and FCR also increased by 0.28 points under the NE condition. Thus, the compromised growth in the NE-challenged groups may be attributed to pathophysiological changes, including increased intestinal lesions, inflammatory cytokines, and gut permeability during NE. However, no notable mortality was observed, indicating that even subclinical NE can substantially impair broiler performance.

Daily feed intake patterns in the present study reflected the birds' physiological response to NE, capturing both the acute impact and the recovery trajectory. In the NE-challenged groups, daily FI decreased sharply from d 19 to 21 but gradually recovered subsequently. Previous studies have reported similar daily feed intake patterns in *Eimeria spp.* and NE challenge models in poultry (Sharma et al., 2022; Goo et al., 2023a,b; Liu et al., 2023). These changes in daily FI align with the period when NE severity is at its peak, with similar NE model studies confirming that NE severity peaks from 6 to 7 days post inoculation (Goo et al., 2023a,b, 2024). Consequently, the NE-challenged groups exhibited significant physiological changes during the acute phase of NE. In addition, the results showed a dip followed by a rise in daily FI after d 21, and there was no difference in FCR among the groups from d 21 to 28. These results suggest that broilers entered a recovery phase from acute NE. Nevertheless, FI and BWG remained lower in the CC compared to the NC during this period. The lack of FCR difference between NC and CC groups may reflect proportionate reductions in both feed intake and growth, rather than full performance restoration. Thus, the sustained reduction in FI and BWG may be attributed to changes in metabolic prioritization for supporting immune responses and tissue repair (Eming et al., 2021; Lee and Rochell, 2022). Collectively, these findings support the idea that incomplete restoration of intestinal integrity and metabolic functions after acute NE may result in prolonged impairment of growth performance.

Dietary COA supplementation under NE conditions appeared to alleviate the performance deterioration caused by NE in the present study. This finding aligns with previous studies reporting that the supplementation of encapsulated organic acids with other bioactive compounds positively influenced broiler performance under NE conditions (Abdelli et al., 2020; Swaggerty et al., 2022; Pham et al., 2020). During the acute phase of NE in the current study, dietary COA supplementation showed a linear trend in improving BWG and FCR, with a significant improvement observed at the 0.4 % inclusion level. The alleviation effect of dietary COA supplementation could be linked to changes in immune response, intestinal lesions, and intestinal integrity parameters observed in the current study, which might be driven by reduced *C. perfringens* loads. These results suggest that dietary COA may play a role in mitigating NE severity during the acute phase, and a relatively higher COA level (0.4 %) might be required for effective mitigation. Furthermore, a linear improvement in daily FI was observed from d 23 to 26, depending on the dietary COA supplementation level, indicating that dietary COA may accelerate the recovery from NE in a dose-dependent manner. Overall, the NE challenge led to a significant deterioration in growth performance, but BWG and FI showed a linear improvement in the dietary COA supplementation groups. Notably, FCR in the COA supplementation groups did not show a remarkable difference compared to the NC, suggesting a potential mitigation effect of dietary COA at 0.4 % or higher under NE conditions.

The current study demonstrated that dietary COA supplementation

led to a reduction in *C. perfringens* loads in both the jejunal and cecal contents. A similar study reported that dietary encapsulated organic acids blend with essential oils leads to a reduction in *C. perfringens* colonization in broilers challenged with NE (Pham et al., 2020). The reduction in *C. perfringens* loads may be related to the antimicrobial effects of organic acids. In general, the antimicrobial mechanism of organic acids operates by lowering the external pH, disrupting bacterial cellular homeostasis, and inducing metabolic stress in bacterial cells (Treesuwan et al., 2023). Components of COA, such as fumarate, citrate, and malate, have been documented to inhibit *C. perfringens* and other pathogenic bacteria in vitro studies (Skřivanová et al., 2006; Giovagnoni et al., 2019). However, the antimicrobial efficacy of organic acids may vary depending on their form, as most dietary organic acids tend to dissociate before reaching the small intestine, thereby limiting their direct antibacterial activity (Dibner and Buttin, 2002; Du et al., 2024). Despite this limitation, the matrix coating may enable the release of undissociated organic acids in the small intestine, where they can directly interact with bacteria (Tugnoli et al., 2020). Previous studies have also demonstrated that encapsulated organic acid blends in poultry diets contributed to a reduction in pathogenic bacteria (Rodjan et al., 2018; Nguyen and Kim, 2020). These findings support the hypothesis that the release of organic acids from COA in the jejunum could have contributed to *C. perfringens* suppression.

Furthermore, dietary organic acids may alter the intestinal environment by lowering gut pH, thereby inhibiting pathogenic bacteria that typically thrive in neutral to alkaline conditions (Giannenas et al., 2014; Dittoe et al., 2018; Xu et al., 2020). Previous studies evaluating dietary organic acids in poultry have reported that these compounds suppressed the proliferation of pathogenic bacteria selectively but supported beneficial bacteria and contributed to microbial balance in the gut (Giannenas et al., 2014; Abdelli et al., 2020). *Clostridium perfringens* is an opportunistic pathogen that could be suppressed by competition with other commensal bacteria in the gut (La Ragione et al., 2004; Antonissen et al., 2016). These findings support that the inhibition effects of organic acids against *C. perfringens* may not be due only to the antimicrobial properties of organic acids but are also associated with microbial balance in the gut. However, further studies will be needed to investigate the relationship between gut microbiota shift by organic acids and *C. perfringens*.

Organic acids potentially improve gut health, supporting gut barrier function and intestinal recovery (Vieira et al., 2022). These properties may be expected to alleviate the effects of the NE. In the current study, intestinal barrier damage caused by NE exhibits an increase in intestinal lesions and intestinal permeability observed in NE-challenged broilers, consistent with previous reports demonstrating that NE can impair gut barrier integrity (Goo et al., 2023a,b, 2024). However, COA supplementation dose-dependently reduced intestinal lesion scores on d 21 and decreased gut permeability on d 20 and 21 in the current study. Similar studies have reported that dietary organic acids reduce intestinal lesions and improve intestinal permeability under NE conditions (Song et al., 2017; Pham et al., 2020; Kumar et al., 2021). Furthermore, the reduction of pathogenic bacteria in the gut may be associated with decreased intestinal lesions (Macdonald et al., 2017). Thus, the dose-dependent reduction in the NE lesion with increasing COA levels among the NE-challenged groups potentially resulted from the decreased *C. perfringens* loads, a key factor in NE pathogenesis.

The tight junction complex acts as a key regulator of paracellular permeability, adjusting its opening and sealing in response to environmental changes to maintain gut barrier integrity (Paradis et al., 2021; Choi et al., 2022). In the CC group, the expression of *OCLN, ZO1*, and *ZO2* was significantly reduced, which is consistent with previous NE studies demonstrating NE disturbs gut integrity (Goo et al., 2023a). Occludin is known to maintain tight junction structural stability, involving cell-to-cell adhesion, whereas *ZO2* functions as scaffolding proteins that link tight junction components to the cytoskeleton and maintain epithelial integrity (Cummins, 2012; Hartsock and Nelson, 2008). The downregulation of these proteins can weaken tight junction complexes, increase gut permeability, and facilitate pathogen translocation and inflammation (Chen et al., 2015; Paradis et al., 2021). However, dietary COA supplementation linearly increased *ZO2* expression among the NE-challenged groups in the current study, indicating its potential role in tight junction restoration and gut barrier integrity. Additionally, *CLDN2* expression was reduced in the CC group but tended to recover with increasing dietary COA supplementation level. As a channel-forming tight junction protein, *CLDN2* regulates sodium and water flux to maintain osmotic balance in the gut (Luettig et al., 2015; Venugopal et al., 2019). Interestingly, a previous study observed the upregulation of *CLDN2* in jejunal tissue following the NE challenge (Goo et al., 2023). This inconsistency may be related to the severity of NE, which potentially exhibits different inflammation responses. Under inflammatory conditions, proinflammatory signaling can induce *CLDN2* overexpression, exacerbating gut permeability and intestinal inflammation (Raju et al., 2020). Because *CLDN2* can either protect or aggravate gut barrier function depending on its expression level, the regulation and function of *CLDN2* under COA supplementation requires further investigation. In the present study, *CLDN1* expression tended to increase in the CC but decreased with COA supplementation. Similarly, the increase in *CLDN1* was also observed in the previous studies on *Eimeria* challenge model in the broiler, suggesting that upregulation of *CLDN1* under infection could represent a compensatory response for gut barrier protection or adaptive response to inflammation (Poritz et al., 2011; Paradis et al., 2021; Teng et al., 2021). Thus, the current results suggest that dietary COA supplementation may mitigate the damaged gut integrity by supporting mucosal recovery under the NE condition.

Under the NE challenge, both innate and adaptive immune responses are activated simultaneously, involving acute inflammatory reactions and immune regulation (Oh and Lillehoj, 2016; Lee et al., 2022; Goo et al., 2023a,b). Peptidoglycans in the cell wall of *C. perfringens* act as typical pathogen-associated molecular patterns (**PAMP**), which are recognized by host pattern recognition receptors (Wolf and Underhill, 2018). This recognition is primarily mediated by macrophages and dendritic cells in the intestinal mucosa, leading to the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (**NF-κB**) pathway and subsequent *IL1β* production (Kan et al., 2021). The resulting *IL1β* enhances inflammatory responses and plays a crucial role in early immune defense (Navegantes et al., 2017). In addition, the NE promotes Th1 cell activation, leading to the release of *IFNγ*, which enhances macrophage-mediated pathogen clearance (Fries-Craft and Bobeck, 2024). Consistent with this, the observed upregulation of *IL1β* and *IFNγ* in the present study is indicative of a heightened pro-inflammatory state driven by NE challenge, reflecting host immune responses aimed at controlling infection. However, sustained secretion of these pro-inflammatory cytokines can exacerbate intestinal mucosal damage (Arendt et al., 2019). Anti-inflammatory cytokine *IL10* is mainly secreted by regulatory T cells and functions to suppress excessive immune responses, protecting tissues from damage (Arendt et al., 2019; Fries-Craft and Bobeck, 2024). Accordingly, in the present study, the elevated IL10 expression in the CC group may suggest a compensatory response to NE-induced inflammation. Therefore, the increased expression of *IL1β, IFNγ*, and *IL10* in the CC group indicates that the NE induced both an acute inflammatory response and an immune regulatory process in the present study.

In contrast, dietary COA supplementation exhibited downregulated expression of *IL1β, IFNγ*, and *IL10* under the NE challenge. This immunomodulatory effect may be associated with the reduction in *C. perfringens* loads. The decreased *C. perfringens* burden may have led to reduced PAMP stimulation, subsequently attenuating NF-κB activation and decreasing pro-inflammatory cytokine production. These results align with previous studies showing that dietary organic acids reduced *C. perfringens* colonization and modulated inflammatory gene expression, including NF–κB–related pathways and cytokines such as *IL10* and *IFNγ* in broilers challenged with NE (Pham et al., 2020; Swaggerty et al.,

2022). Moreover, some studies have shown that dietary organic acids influence intestinal bacterial populations and enhance humoral immune responses in poultry, suggesting that microbiota-related mechanisms may also be involved (Nguyen and Kim, 2020; Reda et al., 2021; Xue et al., 2023). Therefore, the present findings indicate that COA may help mitigate NE-induced inflammation, potentially through the suppression of *C. perfringens* and associated microbial changes. However, further studies are needed to clarify the precise immunoregulatory mechanisms of COA, particularly its interactions with gut microbiota under the NE conditions.

In summary, dietary COA supplementation during the starter showed beneficial effects on growth after d 7, with the most significant improvement in FCR at 0.1 % supplementation. Following the NE challenge, the effects of COA varied between the acute phase and the recovery phase, with linear improvement observed in major performance indicators depending on the COA inclusion level. Notably, during the acute phase of NE, COA supplementation exhibited a linear trend in improving BWG and FCR, with significant benefits at the 0.4 % inclusion level. These effects were associated with reduced NE severity, as shown by dose-dependent reductions in intestinal *C. perfringens* loads and lesion scores, improved gut integrity, and modulated immune responses. Additionally, dietary COA supplementation enhanced daily FI recovery during the NE recovery phase, suggesting a potential role in shortening the duration of recovery. In conclusion, the present study indicates that dietary COA supplementation at 0.1 % may enhance feed efficiency during starter phase, whereas 0.4 % supplementation could serve as a potential dietary intervention to mitigate the adverse effects of NE in broilers.

Disclosures

There is no conflict of interest.

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