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Phytochemicals act holistically to enhance host defenses during poultry coccidiosis

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ABSTRACT

This study was conducted to investigate the effects of a phytochemical mixture containing full spectrum cinnamon, clove, and oregano essential oils (**CCO**) on the growth performance, intestinal immunity, and intestinal integrity of broilers infected with coccidiosis. In chicken macrophage cells (**CMCs**), inflammation was induced with 1.0 μg/mL LPS, followed by stimulating with CCO at three concentrations (1.0, 10.0, and 100 μg/mL) and measuring the gene expression levels of IL-1β and IL-8. In chicken intestinal epithelial cells (**IECs**), CCO was added and cultured, and the gene expression levels of occludin, ZO-1, and MUC2 were measured. In the *in vivo* experiment, one hundred and twenty male broiler chickens (0-day-old) were allocated into three treatment groups: (1) basal diet without infection (**NC**), (2) basal diet with *E. maxima* infection (**PC**), and (3) CCO at 4.5 mg/kg feed with *E. maxima* infection (**CCO**). Body weight (**BW**) was measured on days 0, 7, 14, 20, and 22. PC and CCO groups were orally infected with *E. maxima* on day 14. Jejunal samples were collected on day 22 to conduct gene expression analysis of cytokines, TJ proteins, and antioxidant enzymes. CCO significantly decreased IL-1β and IL-8 in CMCs and increased ZO-1 and MUC2 in IECs in a dose-dependent manner. In the *E. maxima*-infected groups, dietary CCO tended to mitigate BW loss due to infection. Upon infection, proinflammatory cytokines were suppressed in the CCO group compared to the PC group. Dietary CCO also increased the expression of occludin and JAM-2 in the jejunum. However, CCO did not reduce the oocyst number in coccidiosis-infected chickens. These results suggest that dietary CCO supplementation may improve intestinal immunity and permeability, helping to reduce productivity losses in *E. maxima*-infected broilers through gut physiological responses, rather than direct antimicrobial effects. These results show the advantage of using *in vitro* screening based on host-mediated responses, and not on direct pathogen killing, when exploring new phytochemicals to mitigate disease response to reduce economic losses due to coccidiosis.

Introduction

As we search for phytochemicals that can be used as alternatives to antibiotics, one of the challenges is that we have approached the search by using *in vitro* screening based on direct pathogen killing. However, the doses used in such screenings are not relevant to animal feeding due to safety and/or economic concerns (Lillehoj et al., 2018; Al AlSheikh et al., 2020; Khare et al., 2021). While the ability to directly kill pathogens is an important function of phytochemicals, the ideal phytochemical is one that can favorably modulate intestinal health at relatively low concentrations through physiological mechanisms in the host, such as regulating the intestinal inflammatory response, enhancing the immune response, providing antioxidative effects, and improving intestinal permeability. This regulation helps mitigate the damage caused by pathogens or mask certain disease-causing aspects, thereby preserving growth performance during infections (Lillehoj et al., 2018; Hotea et al., 2022). While the use of a single phytochemical that focuses on one mechanism of action offers many benefits, to effectively achieve this, it might be more beneficial to use a combination of various phytochemicals that provide synergistic effects through diverse mechanisms of action (Seidavi et al., 2022; Al-Mnaser et al., 2022).

Our laboratory has already conducted *in vitro* tests on 25

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phytochemicals, evaluating their effects on intestinal epithelial cells, macrophage cells, and muscle cells, focusing on cytotoxicity, pro-inflammatory responses, tight junction proteins (**TJPs**), and muscle growth. Among the tested phytochemicals, clove essential oil, oregano essential oil, and cinnamon essential oil showed beneficial effects on cellular inflammation and intestinal integrity (Park et al., 2023, 2024a). Therefore, we hypothesized that a combination of these three phytochemicals could synergistically improve intestinal health in broilers infected with *Eimeria maxima* (***E. maxima***), alleviating the growth depression effects associated with coccidiosis. *Eimeria* species target different parts of the intestine, and *E. maxima* specifically target the jejunum, where most nutrient absorption occurs. It is also known to cause necrotic enteritis in conjunction with *Clostridium perfringens*. To test this hypothesis, we investigated the characteristics of the phytochemical mixture (**CCO**) through *in vitro* tests on intestinal epithelial cells, macrophage cells, and muscle cells, focusing on cytokine expression levels, TJPs, and muscle growth. Additionally, the effects of dietary CCO supplementation were evaluated on growth performance, intestinal inflammatory responses, TJPs, and antioxidant enzymes in young broilers infected with *E. maxima*. Thus, this study provides a foundation for using combinations of phytochemicals, effectively screened through *in vitro* tests representing host-mediated responses, to alleviate coccidiosis in broilers and improve growth performance.

Materials and methods

For the *in vitro* study, CCO provided by AVT Natural Products, composed of natural, full-spectrum essential oils, was used. CCO was a combination of cinnamon essential oil, clove essential oil, and oregano essential oil, mixed in equal parts (1:1:1) to form a solution with a concentration of 100 mg/mL in a tween 80 solvent. The *in vitro* characteristics of individual ingredients were described by Park et al. (2023 and 2024a). The CCO solution was initially diluted to a concentration of 10 mg/mL using a culture medium mixed with 10 % dimethyl sulfoxide (**DMSO,** Sigma-Aldrich, St. Louis, MO, USA). Subsequently, it was further diluted as needed for each experiment using a culture medium without DMSO. Therefore, as the dilution ratio increased, the concentrations of both Tween 80 and DMSO decreased. The concentrations of tween 80 and DMSO in the vehicle control (**VC**), as shown in the proinflammatory cytokines (Fig. 5), were maintained at the same level as the highest concentration of CCO (100 μg/mL) used in the experiment. The CCO concentrations mentioned in this study refer to the final concentrations post-CCO administration.

For the *in vivo* study, the CCO, composed of natural, full-spectrum essential oils, was produced by AVT in a granulated form. This granulated CCO was then mixed into a mash type of broiler feed manufactured by the USDA-ARS feed mill for 10 min. The final concentration of the CCO in the feed was 4.5 mg of CCO/kg of feed. All animal care procedures were approved by the Beltsville Agricultural Research Center Institutional Animal Care and Use Committee (# 20-014).

Experiment 1: in vitro study

Culture of chicken intestinal epithelial cells and chicken macrophage cells

Chicken macrophage cells (**CMCs**) and chicken intestinal epithelial cells (**IECs**) were maintained as described previously (Park et al., 2022). In brief, IECs (2×10^5/mL, 8E11 cell line) and CMCs (2×10^5/mL, HD11 cell line) were seeded into 24-well plates. These cells were then grown in Dulbecco's Modified Eagle Medium (**DMEM**)/F-12 (HyClone, Logan, UT, USA), supplemented with 10 % heat-inactivated Fetal Bovine Serum (**FBS**; HyClone) and 1 % Penicillin (10,000 unit/mL, Gibco, Grand Island, NY, USA)/Streptomycin (10 mg/mL, Gibco). The two types of cells were incubated at 41°C in a humidified atmosphere with 5 % CO_2 for 24 h to allow for cell adhesion. After 24 h, lipopolysaccharide (**LPS**; Sigma-Aldrich) was administered at 1.0 μg/mL to all the treatment groups, except for the control group. Immediately after this, CCO was administered at concentrations of 0.0, 1.0, 10.0, and 100 μg/mL. After 18 h, all cells were lysed using lysis buffer (Qiagen, Valencia, CA, USA) and 1 % 2-mercaptoethanol (Sigma-Aldrich). RNA was isolated from the IECs and the CMCs using the RNeasy Isolation Kit (Qiagen) in a QIAcube (Qiagen) for performing quantitative real-time PCR (**qRT-PCR**) analysis. All experiments were independently replicated six times.

Quail muscle cell culture

Quail muscle cells (**QMCs**) were seeded in 24-well plates at a concentration of 2×10^5 cells/mL as described previously (Park et al., 2021). The QMCs were then grown in Medium 199 (Hyclone) supplemented with 10 % FBS and 1 % penicillin/streptomycin. This process continued until the cells reached an approximate confluence of 70 %. To stimulate cell differentiation, the culture medium in 12 wells was substituted with Medium 199, which included 0.5 % FBS and 1 % penicillin/streptomycin. On the other hand, the remaining 12 wells were freshly sustained in the basic Medium 199 supplemented with 10 % FBS to maintain cell proliferation. Each well was treated with varying concentrations (0.0, 0.5, 1.0, and 10.0 μg/mL) of CCO. After an 18 h incubation at 41°C in a humidified atmosphere with 5 % CO_2, all cells were lysed in lysis buffer and 2-mercaptoethanol. RNA was extracted from the QMCs using the RNeasy Isolation Kit within the QIAcube, followed by a qRT-PCR analysis. All experiments were independently repeated six times.

Chicken embryonic muscle cell culture

Chicken embryonic muscle cells (**EMCs**) were cultured using fertilized eggs sourced from Moyer's Hatchery (Quakertown, PA, USA). The procedure for establishing the EMC culture was conducted as described previously (Park et al., 2024b). The eggs were incubated in a GQF 1500 professional automated incubator (Savannah, GA) set at 41°C and a relative humidity of 70 %. After 13 days of incubation, the pectoralis major area of the embryos was carefully removed, finely chopped, and then digested at 37°C for 20 min using 0.05 % trypsin-EDTA (Sigma-Aldrich). The EMCs were subsequently rinsed 3 times with HBSS (Sigma-Aldrich) and seeded at a concentration of 2×10^5 cells/mL in 24-well plates. The EMCs were then cultured in DMEM (Hyclone) supplemented with 10 % FBS and 1 % penicillin/streptomycin until they reached approximately 70 % confluence. To induce cell differentiation, the culture media in 12 wells were switched with DMEM containing 2 % FBS and 1 % penicillin/streptomycin. In contrast, the other 12 wells were freshly maintained in basic DMEM supplemented with 10 % FBS to encourage cell proliferation. Each well was treated with different concentrations (0.0, 0.5, 1.0, and 10.0 μg/mL) of the CCO. After an 18-h incubation at 41°C in a humidified atmosphere with 5 % CO_2, all cells were lysed in lysis buffer and 2-mercaptoethanol. Total RNA was extracted from the EMCs using the RNeasy Isolation Kit in the QIAcube, followed by qRT-PCR analysis. The RNA was eluted in 30 μL of RNase-free water. All experiments were independently replicated six times.

Complementary DNA synthesis

RNA quantification was carried out using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA), with the absorbance measured at a 260 nm wavelength. The RNA's purity was determined by an OD260/OD280 ratio ranging from 1.8 to 2.0. Following this, 1 μg of the total RNA was converted into complementary DNA (**cDNA**) using the QuantiTect reverse transcription kit (Qiagen). The cDNA samples obtained were then aliquoted and stored at −20°C in cryotubes for subsequent use.

Analysis of cytokines, tight junction proteins, and markers of muscle cell growth by qRT-PCR

In CMCs, RNA samples were used to measure the levels of proinflammatory cytokines (IL-1β and IL-8). For the evaluation of TJPs (occludin, ZO-1, and MUC-2), RNA samples from IECs were subjected to

a quantitative real-time polymerase chain reaction (**qRT-PCR**). The expression of muscle cell proliferation and differentiation markers, Pax7 and MyoG, were assessed using RNA samples derived from QMCs and EMCs. The qRT-PCR was performed with the Applied Biosystems QuantStudio 3 Real-Time PCR Systems (Life Technologies, Carlsbad, CA, USA) and SYBR Green qPCR Master Mix (PowerTrack, Applied Biosystems, Foster City, CA, USA). The oligonucleotide primer sequences and GenBank Accession Numbers utilized for qRT-PCR are listed in Table 1. A melting curve was generated at the end of each run to confirm the presence of a single amplification product and the absence of primer dimers. Standard curves were established using serial, 5-fold dilutions of cDNA. The fold changes in each transcript were normalized to glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**) and are relative to the transcript expression in the unstimulated control group (normalized to 1) using the comparative $2^{-\Delta\Delta Ct}$ method as outlined by Kim et al. (2014).

Statistical analysis for in vitro test

In vitro responses were evaluated using the GLM procedure in SAS software version 9.4 (SAS Inc., Cary, NC, USA). For linearity assessment, orthogonal polynomial contrasts were utilized, which were generated via the PROC IML procedure in SAS for doses that were not uniformly distributed. In the event of a significant linear value in the treatment groups, the mean values across treatments were compared pairwise using the PDIFF option in SAS. The outcomes are presented as least squares mean values ± standard error of the mean. A probability value less than 0.05 was considered to be a significant difference.

Experiment 2: in vivo study

Chickens and experimental design

A total of one hundred twenty male broiler chickens (Ross 708) at zero-day old (Longenecker's hatchery, Elizabethtown, PA, USA) were weighed and allocated to three dietary treatments in a randomized complete block design with initial body weight (**BW**; heavy and light) as a block. Each treatment group had eight cages with five chickens per cage (0.65 × 0.75 m^2). The dietary treatments (Table 2) included basal diet for non-infected chickens (**NC**), basal diet for *E. maxima*-infected chickens (**PC**), PC supplemented with CCO at 4.5 mg/kg feed (CCO). All diets were prepared in a mash form. During the experimental period, the chickens were provided with ad libitum access to experimental feed and water. The experimental design used for the study is illustrated in Fig. 1.

Table 2
Ingredient composition of basal diet (as-fed basis, %, unless otherwise indicated).

Ingredients (%)	Basal diet
Corn	55.78
Soybean meal	37.03
Soybean oil	2.97
Dicalcium phosphate	1.80
Calcium carbonate	1.51
Salt	0.38
Poultry Vit Mix[1]	0.22
Poultry Mineral Mix[2]	0.15
DL-Methionine	0.10
Choline-chloride, 60 %	0.06
Total	100
Calculated values (%)	
CP, %	24.00
Ca, %	1.20
AP, %	0.51
Lys, %	1.40
Met, %	0.49
Cys + Met, %	0.80
ME, Mcal/kg	3.5

[1] Vitamin mixture provided the following nutrients per kg of diet: vitamin A, 2,000 IU; vitamin D3, 22 IU; vitamin E, 16 mg; vitamin K, 0.1 mg; vitamin B_1, 3.4 mg; vitamin B_2, 1.8 mg; vitamin B_6, 6.4 mg; vitamin B_{12}, 0.013 mg; biotin, 0.17 mg; pantothenic acid, 8.7 mg; folic acid, 0.8 mg; niacin, 23.8 mg.
[2] Mineral mixture provided the following nutrients per kg of diet: Fe, 400 mg; Zn, 220 mg; Mn, 180 mg; Co, 1.3 mg; Cu, 21 mg; Se, 0.2 mg. CP = crude protein, AP = available phosphorus.

Determination of body weight

The BWs of chickens were recorded at intervals on days 0, 7, 14, 20, and 22 to compute the average daily gain (**ADG**). Dead chickens were removed and weighed to ensure accurate adjustments of the growth data.

Table 1
Oligonucleotide primer sequences for qRT-PCR.

Type	Target gene	Primer sequence (5′–3′)	GenBank accession Nomber
Reference	GAPDH	F: GGTGGTGCTAAGCGTGTTAT R: ACCTCTGCCATCTCTCCACA	K01458
Proinflammatory	IL-1β	F: TGGGCATCAAGGGCTACA R: TCGGGTTGGTTGGTGATG	NM_204524.1
	IL-8	F: GGCTTGCTAGGGGAAATGA R: AGCTGACTCTGACTAGGAAACTGT	AJ009800
TJ proteins	Occludin	F: GAGCCCAGACTACCAAAGCAA R: GCTTGATGTGGAAGAGCTTGTTG	NM205,128.1
	ZO-1	F: CCGCAGTCGTTCACGATCT R: GGAGAATGTCTGGAATGGTCTGA	XM01,527,8981.1
	MUC2	F: GCCTGCCCAGGAAATCAAG R: CGACAAGTTTGCTGGCACAT	NM0,013,18434.1
Muscle cell	MyoG	F: GGCTTTGGAGGAGAAGGACT R: CAGAGTGCTGCGTTTCAGAG	D90157.1
	Pax7	F: AGGCTGACTTCTCCATCTCTCCT R: TGTAACTGGTGGTGCTGTAGGTG	NM_205065.1
Antioxidant enzymes	CAT	F: ACTGCAAGGCGAAAGTGTTT R: GGCTATGGATGAAGGATGGA	NM001031215.1
	HMOX-1	F: CTGGAGAAGGGTTGGCTTTCT R: GAAGCTCTGCCTTTGGCTGTA	NM205344
	SOD-1	F: ATTACCGGCTTGTCTGATGG R: CCTCCCTTTGCAGTCACATT	NM205064.1

IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; TJ, tight junction; JAM, junctional adhesion molecule; ZO, zonula occludens. CAT, catalase; HMOX, heme oxygenase;.
SOD, superoxide dismutase.

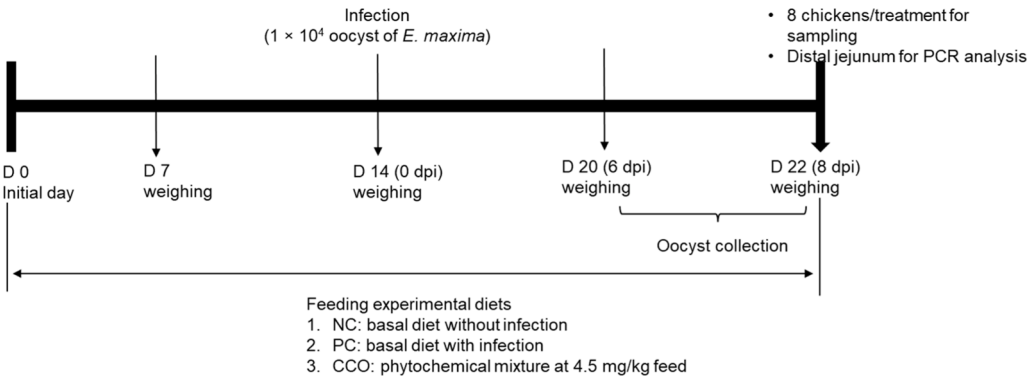

Figure 1. Schematic outline of the experimental design in experiment 2.

Oral administration of E. maxima

All chickens, with the exception of those in the NC group, received an oral dose of *E. maxima* (10,000 sporulated oocysts/chicken, using the Beltsville strain 41A) on day 14 following the previously described methods (Park et al., 2022). To identify the genetic consistency of the *E. maxima* strain used, a DNA genotyping assay was conducted (Haug et al., 2007).

Collection of jejunal samples

On day 22, a representative chicken from each cage, chosen for its BW approximating the group's average, was euthanized via cervical dislocation. A 2 cm segment of the distal jejunum, devoid of contents, was excised and preserved in RNAlater® (Invitrogen, Carlsbad, CA, USA), and subsequently stored at −20°C for further analysis.

Fecal oocyst shedding

From days 20 to 22 (6 to 8 days post-infection: dpi), the whole fecal samples per cage were collected for analysis, and the quantification of oocysts was counted in accordance with protocols described previously (Lee et al., 2022) using the McMaster chamber according to the formula below:

Total oocysts/chicken = [counted oocyst × dilution factor × (fecal sample volume/counting chamber volume)]/number of chickens per cage.

Isolation of RNA and reverse transcription from jejunal samples

Total RNA isolation was performed on the jejunal samples that were stored in the RNAlater® (Invitrogen). Total RNA was extracted using TRIzol reagent (Invitrogen) followed by DNase digestion as described (Park et al., 2024b). Briefly, 100 mg of jejunum samples were dissected and placed into a 1.5 mL tube. The samples were then homogenized in 1.0 mL of Trizol reagent (Qiagen TissueRuptor®, Qiagen) on ice at 30, 000 rpm for 1 min. The homogenate was centrifuged at 4°C at 3,000 rpm × 15 min, and the supernatant was carefully transferred to a new 1.5 mL tube. To this, 200 µL of chloroform (Sigma-Aldrich) was added, and the tube was gently inverted five times to mix. The mixture was then centrifuged at 4°C at 14,000 rpm × 15 min, and 500 µL of the supernatant was cautiously transferred to a new 1.5 mL tube. Subsequently, 500 µL of isopropanol (Sigma-Aldrich) was added to the same tube, and the tube was slowly inverted five times to mix. After another centrifugation at 4°C at 14,000 rpm × 15 min, the supernatant was discarded, leaving only the pellet. The pellet was then washed by adding 500 µL of 75 % ethanol solution and centrifuging at 4°C at 14,000 rpm × 15 min. The supernatant was drained off, and the pellet was left to dry completely at room temperature. Once dry, the pellet was resuspended in 200 µL of nuclease-free water (Invitrogen), and the RNA concentration was measured using a Nanodrop spectrophotometer. The assessment of RNA quantity, RNA purity, and cDNA production followed the methods mentioned for *in vitro* testing.

Gene expression analysis by qRT-PCR from the cDNA

The oligonucleotide primer sequences used for qRT-PCR analysis are shown in Table 1. The expression of various cytokines (IL-1β, IL-8, IFN-γ, and TNFSF15), TJ proteins (claudin, JAM-2, occludin, and ZO-1), antioxidant enzymes (CAT, HMOX, and SOD-1) was evaluated with the cDNA manufactured from the jejunal samples. The synthesized cDNA was diluted 1:10 in RNase-free water (Invitrogen), and 5 µL of cDNA was amplified with SYBR Green qPCR Master Mix (PowerTrack, Applied Biosystems, Vilnius, Lithuania) using a qRT-PCR (QuantStudio 3, Carlsbad, CA, USA). Standard curves were generated using log10 diluted RNA standards, and the transcript levels were normalized to those of GAPDH using the Q-gene program (Park et al., 2024c).

Statistical analysis

Growth data from the animal trial were analyzed using a mixed model (PROC MIXED) in SAS. The cage was considered the experimental unit. If a significant value in treatment groups, the mean values between treatments were compared through the Tukey-Kramer test in SAS. The results are reported as least squares mean values ± standard error of the mean. Probability values less than 0.05 were considered significantly different. For the oocyst number, the Shapiro-Wilk test was conducted using SAS, and the W-value and p-value were 0.960 and 0.658, respectively, confirming that the data follows a normal distribution.

Results

Experiment 1: in vitro study

Gene expression levels of IL-1β and IL-8 in phytochemical mixture-induced CMCs

Stimulation of CMCs with 1.0 µg/mL of LPS resulted in an increase in gene expression levels of IL-1β by 7.5-fold (Fig. 2a, $P < 0.0001$) and IL-8 by 18-fold (Fig. 2b, $P < 0.0001$), indicating the induction of an inflammatory response. Upon administration of three different concentrations of CCO (1.0, 10.0, and 100 µg/mL), a linear decrease ($P < 0.0001$) in IL-1β levels to 5.5, 4.4, and 3.5-fold, respectively, was observed. Similarly, IL-8 levels also decreased ($P < 0.0001$) linearly to 15.5, 13.3, and 10.4-fold, respectively. These results indicate that CCO mitigates the inflammatory response.

Gene expression levels of occludin, ZO-1 and MUC2

Administration of CCO at 1.0 and 10 µg/mL did not affect ($P > 0.05$) the gene expression level of occludin (Fig. 3a). However, administration of CCO at 100 µg/mL resulted ($P < 0.001$) in a 2-fold increase in occludin expression compared to other treatments, although no significant dose-dependent change was observed ($P = 0.184$). For ZO-1 (Fig. 3b) and MUC2 (Fig. 3c), there was ($P > 0.05$) no change at 1.0 µg/mL of CCO, but at 10.0 µg/mL and 100 µg/mL, ZO-1 levels increased

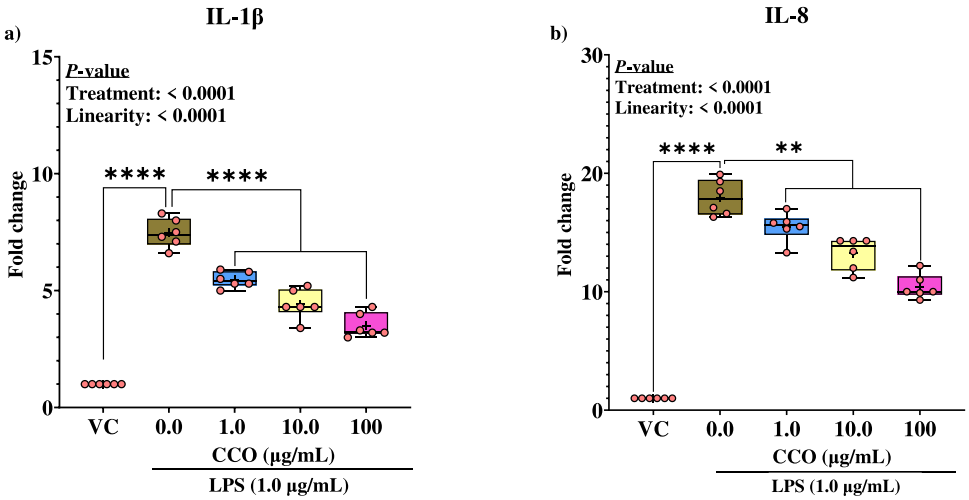

Figure 2. Gene expression of IL-1β and IL-8 in phytochemical mixture (CCO)-treated chicken macrophage cells. Cells were treated with three different concentrations (1.0, 10.0, and 100 μg/mL) of CCO for 18 h in the presence of lipopolysaccharide (LPS) at 1.0 μg/mL. The data represents an average of six independent experiments. $P < 0.01$ (**) and $P < 0.0001$ (****) were considered statistically significant compared to the mean of each treatment with the mean of every other treatment. The fold changes in each transcript were normalized to glyceraldehyde-3-phosphate dehydrogenase and are relative to the transcript expression in unstimulated control group (normalized to 1) using the comparative $^{\Delta\Delta}$Ct method. VC = vehicle control. Box and whisker plot represents (from top to bottom) the maximum, upper quartile, median, lower quartile, and minimum value. Average measured by six independent experiments was shown as "+".

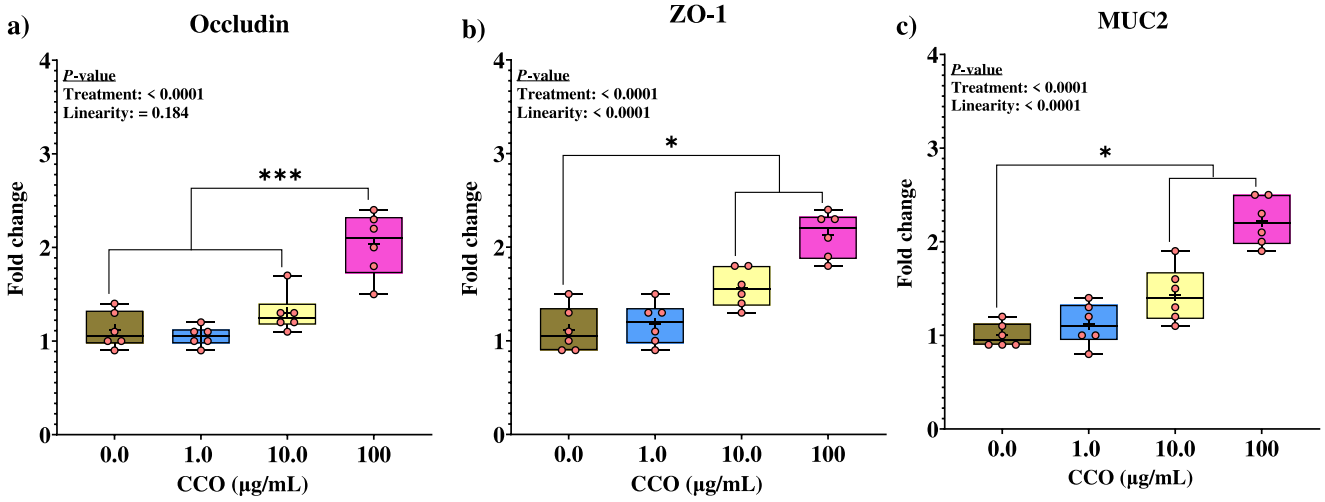

Figure 3. Gene expression of tight junction and mucin proteins in phytochemical mixture (CCO)-treated chicken intestinal epithelial cells. Cells were treated with three different concentrations (1.0, 10.0, and 100 μg/mL) of CCO for 18 h. The data represents an average of six independent experiments. The fold changes in each transcript were normalized to glyceraldehyde-3-phosphate dehydrogenase and are relative to the transcript expression in untreated control group (normalized to 1) using the comparative $^{\Delta\Delta}$Ct method. Box and whisker plot represents (from top to bottom) the maximum, upper quartile, median, lower quartile, and minimum value. Average measured by six independent experiments was shown as "+".

($P < 0.05$) by 1.6-fold and 2.1-fold, respectively, and MUC2 levels increased ($P < 0.05$) by 1.4-fold and 2.2-fold, respectively. Thus, both ZO-1 and MUC2 levels showed ($P < 0.0001$) a significant dose-dependent response. These results suggest that administering CCO may enhance intestinal permeability in chickens.

Gene expression levels of MyoG and Pax7 on muscle cells growth

In the egg, on the 13th day of growth, breast muscle was harvested from the embryo and cultured EMCs were brought to 90 % confluency (Fig. 4a,b). When the concentration of FBS in the culture media was changed from 10 % to 2 %, the MyoG level significantly increased ($P < 0.01$) by 1.8-fold (Fig. 4a), while the Pax7 level did not show ($P > 0.05$) a significant change (Fig. 4b). This finding suggests that EMCs are in the cell differentiation stage and CCO did not affect the cell differentiation stage at any concentration (Fig. 4a). 1.0 μg/mL of CCO significantly increased ($P < 0.05$) the Pax7 level by 1.4-fold compared to 10 % FBS (Fig. 4b), indicating that it supports cell proliferation even at the differentiation stage in EMCs. In QMCs (Fig. 4c,d), when the FBS was changed from 10 % to 0.5 %, both MyoG and Pax7 levels significantly increased by 5.1-fold (Fig. 4c) and 2.1-fold (Fig. 4d), respectively. However, no concentration of CCO altered the MyoG (Fig. 4c) or Pax7 (Fig. 4d) levels in QMCs. These results suggest that QMCs appear to undergo both cell differentiation and proliferation simultaneously at 0.5 % FBS, and that the cell growth stage may be different between EMCs and QMCs.

Experiment 2: in vivo study

Body weight, average daily gain, and oocyst shedding

Dietary CCO supplementation did not affect the BW or ADG of

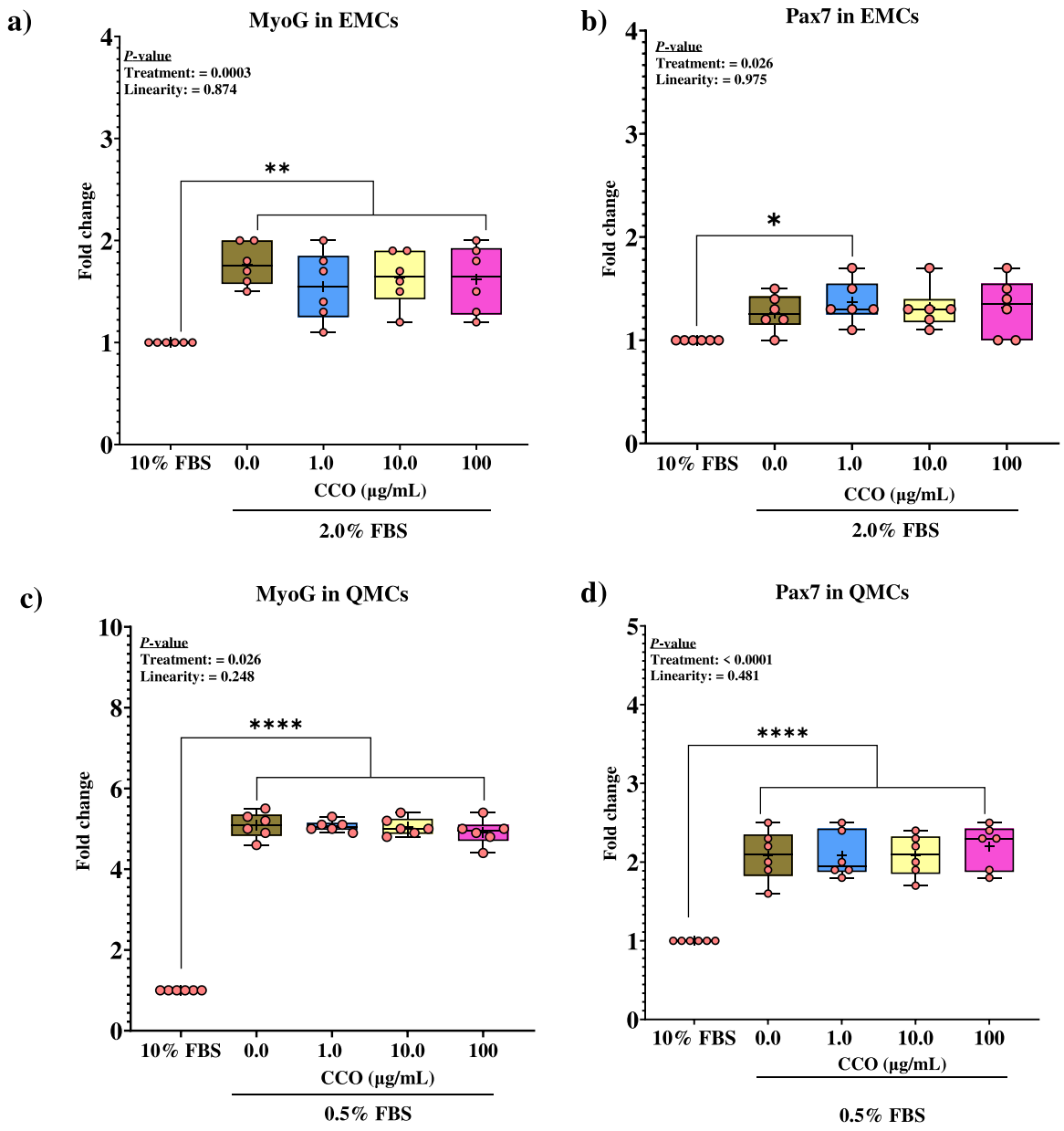

Figure 4. Gene expression of Myogenin (MyoG) and Paired box protein 7 (Pax7) in phytochemical mixture (CCO)-treated quail muscle cells (QMCs) and embryonic chick breast muscle cells (EMCs). To induce the transition from the proliferation stage to the differentiation stage in muscle cells, the concentration of 10 % fetal bovine serum (FBS) was changed to 0.5 % in QMCs and 2.0 % in EMCs. Subsequently, the cells were treated with three different concentrations (1.0, 10.0, and 100 μg/mL) of CCO for 18 h. The data represents an average of six independent experiments. $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.0001$ (****) were considered statistically significant compared to the mean of each treatment with the mean of every other treatment. The fold changes in each transcript were normalized to glyceraldehyde-3-phosphate dehydrogenase and are relative to the transcript expression in untreated control group with 10 % of FBS (normalized to 1) using the comparative $^{\Delta\Delta}$Ct method. Box and whisker plot represents (from top to bottom) the maximum, upper quartile, median, lower quartile, and minimum value. Average measured by six independent experiments was shown as "+".

chickens prior to *E. maxima* infection (Table 3). After *E. maxima* infection, the average BW of the infected group (PC) significantly decreased from 819 g to 669 g at 6 dpi (d 20), a reduction of approximately 18 %. The CCO group also showed a significant decrease ($P < 0.05$) from 819 g to 719 g, approximately 12 %. At 8 dpi (d 22), the BW of the PC group decreased ($P < 0.05$) by about 22 %, from 968 g to 750 g. In the CCO group, the BW decreased ($P < 0.05$) by approximately 13 %, reaching 840 g compared to the NC group. Among the infected treatment groups, the CCO group significantly increased ($P < 0.05$) from 750 g to 840 g, by about 12 %, compared to the PC group. *E. maxima* infection (NC) decreased ($P < 0.05$) ADG at 6 dpi (60.5 to 37.7 g) and 8 dpi (64.2 to 38.3 g) compared to the NC. Dietary CCO supplementation increased the ADG at 6 dpi (37.7 to 44.4 g) and 8 dpi (38.3 to 48.5 g) in *E. maxima*-infected chickens compared to those of the PC. According to the growth results, the *E. maxima* infection clearly reduced BW and ADG, and dietary CCO supplementation mitigated growth loss effects caused by the infection. Dietary CCO supplementation did not affect ($P = 0.824$) the oocyst number of fecal samples collected from 6 to 8 dpi. (Fig. 5). Mortality was not observed during the experimental period.

Proinflammatory cytokines, antioxidant enzymes, and tight junction proteins in jejunum at 8 days post-infection

The levels of pro-inflammatory cytokines IL-1β (Fig. 6a, $P < 0.05$,

Table 3
Growth performance of broiler chickens fed on feed with blended phytochemicals (CCO).

	NC	PC	CCO	SEM	*P*-value
BW, g					
Initial	44.4	44.4	44.5	0.6	0.990
d 7	172	168	171	4.8	0.762
d 14	456	443	454	11.5	0.698
d 20 (6 dpi)	819[a]	669[b]	719[b]	18.8	< 0.0001
d 22 (8 dpi)	968[a]	750[c]	840[b]	23.4	< 0.0001
ADG, g					
d 0 to 7[1]	18.2	17.6	18.1	0.7	0.796
d 7 to 14[1]	40.7	39.3	40.4	1.2	0.641
d 14 to 20[2]	60.5[a]	37.7[c]	44.4[b]	1.8	< 0.0001
d 14 to 22[2]	64.2[a]	38.3[c]	48.5[b]	2.9	< 0.0001

NC = basal diet, PC = basal diet for *E. maxima*-infected chickens, CCO = phytochemical mixture with clove essential oil, oregano essential oil, and cinnamon essential oil, SEM = standard error of the mean.

[1] before infection.

[2] after infection, BW = body weight, ADG = average daily gain, d = day, dpi = days post-infection, all chickens in PC and CCO groups were infected by oral gavage at day 14 with 1.0×10^4 oocysts/chicken of *E. maxima*.

[a,b] Means in the same row with different superscripts differ ($P \leq 0.05$) and the difference was revaluated by Tukey-Kramer test in SAS when *P*-value between treatments was less than 0.05.

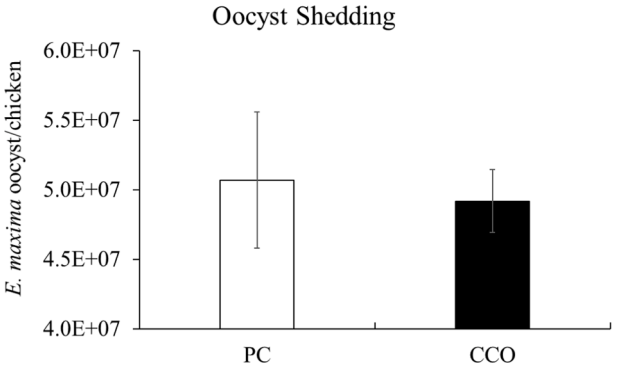

Figure 5. Oocyst shedding of chickens fed diet supplemented with phytochemical mixture (CCO) at 100 mg/kg feed during infection with *E. maxima* in experiment 2. PC = basal diet for infected chickens, CCO, phytochemical mixture at 4.5 mg/kg feed, all chickens were infected by oral gavage at day 14 with 1.0×10^4 oocysts/chicken of *E. maxima*. Each bar represents the mean ± SEM ($n = 8$). The fecal sample was collected from 6 to 8 dpi to calculate the oocyst shedding.

0.001 to 0.002), TNF-α (Fig. 6d, $P < 0.05$, 0.004 to 0.006), and IFN-γ (Fig. 6e, $P < 0.01$, 0.0001 to 0.0004) were all found to increase following *E. maxima* infection, indicating that an inflammatory response was activated post-infection. Dietary CCO supplementation decreased ($P < 0.05$) the levels of these cytokines (IL-1β: 0.002 to 0.001, TNF-α: 0.006 to 0.004 and IFN-γ: 0.0004 to 0.0002), which had increased due to the infection. However, no changes were observed ($P > 0.05$) in IL-6 (Fig. 6b) and IL-8 (Fig. 6c) levels following *E. maxima* infection or dietary CCO supplementation. *E. maxima* infection reduced the gene expression levels of occludin (Fig. 7b, $P < 0.05$ 0.063 to 0.045) and JAM-2 (Fig. 7c, $P < 0.001$, 0.045 to 0.025) but did not affect ($P > 0.05$) the levels of claudin-1 (Fig. 7a) and ZO-1 (Fig. 7d). In this condition, dietary CCO supplementation was observed to increase ($P < 0.05$, 0.025 to 0.036) the level of JAM-2. In this study, no change in antioxidant enzymes HMOX (Fig. 8a), CAT (Fig. 8b), and SOD-1 (Fig. 8c) ($P > 0.05$) due to either the infection or dietary CCO supplementation.

Discussion

Previously, we demonstrated the ability of clove essential oil (Park et al., 2024a), oregano essential oil (Park et al., 2024a), and cinnamon essential oil (Park et al., 2023) to individually regulate intestinal inflammatory responses and permeability. Therefore, in this study, we explored the potential synergistic effects of CCO, a mixture of these three components in a 1:1:1 ratio, by evaluating its anti-inflammatory capabilities in CMCs, its ability to regulate intestinal permeability in IECs, and its potential to promote muscle growth in QMCs and EMCs *in vitro*. Additionally, we investigated the impact of dietary CCO supplementation on the growth performance and intestinal health of broilers infected with *E. maxima*.

In *in vitro* studies, among the pro-inflammatory cytokines that play a key role in innate immunity, IL-1β and IL-8, which have consistently shown responses in previous studies, were measured as a priority. CCO inhibited the production of these cytokines induced by inflammatory responses in CMCs. Consistent with previous reports (Owolabi et al., 2018; Saleh et al., 2021; Pantalos et al., 2024), the administration of CCO significantly suppressed the LPS-induced increase in IL-1β by 10-fold and IL-8 by 20-fold in CMCs in a dose-dependent manner, demonstrating its potential to mitigate inflammatory responses. Although the exact mechanisms by which CCO regulates inflammatory responses and mitigates cytokine release need to be better understood, there is increasing evidence that CCO interferes with the processing and maturation of microRNA (miRNA) induced by inflammation-activated NF-κB, thereby regulating miRNA expression (Shao et al., 2018; Slezak-Prochazka et al., 2010; Srivastava et al., 2015). Therefore, further studies are necessary to better understand the molecular mechanisms involved in CCO-induced cytokine regulation. In our study, we analyzed the expression of occludin, ZO-1, and MUC2 genes in IECs using PCR. Occludin and ZO-1 are crucial components of TJs, playing a significant role in maintaining the integrity and function of the epithelial barrier (Otani and Furuse, 2020). On the other hand, MUC2 is a major mucin protein that forms the protective mucous layer in the gastrointestinal tract (Pelaseyed et al., 2014). Understanding the expression levels of these genes provides insights into the mechanisms regulating epithelial barrier function. Treatment of IECs with 100 μg of CCO resulted in more than a two-fold increase in the gene expression levels of occludin, ZO-1, and MUC2 compared to the control, suggesting an enhancement of intestinal permeability in chickens. These findings are supported by similar results from other studies using various phytochemicals (Leonardo et al., 2020; Felix et al., 2021; Park et al., 2023). Based on reports suggesting that pro-inflammatory cytokines (Fahey and Doyle, 2019) or tight junction proteins (Citi, 2019) can appropriately regulate inflammatory responses to promote tissue and vascular regeneration, thereby indirectly supporting the regeneration and growth of muscle cells, the current *in vitro* experiment evaluated whether CCO could directly affect muscle cells. Contrary to our expectations, CCO did not directly affect muscle growth in QMCs and EMCs. However, other studies conducted on polyphenols, flavonoid glycosides, curcumin, and Curcuma longa (Zhang et al., 2017; Oh et al., 2021; Kim et al., 2021; German et al., 2024) have reported positive effects of these phytochemicals on muscle cell growth. Therefore, future research should investigate the effects of the compounds that constitute CCO on muscle cells. Additionally, the PI3K/Akt/mTOR pathway and Smad pathway, utilized in previous studies, are also worth considering in subsequent experiments.

This *in vitro* study demonstrated that CCO alleviated the increased cytokine release during inflammatory responses and enhanced TJPs and Mucin. These results suggest the potential to improve intestinal immunity and permeability in chickens with coccidiosis. For this reason, we conducted a dietary CCO supplementation experiment with broilers infected with *E. maxima* to evaluate whether dietary CCO supplementation could assist the chickens during coccidiosis.

Before *E. maxima* infection, dietary CCO supplementation did not

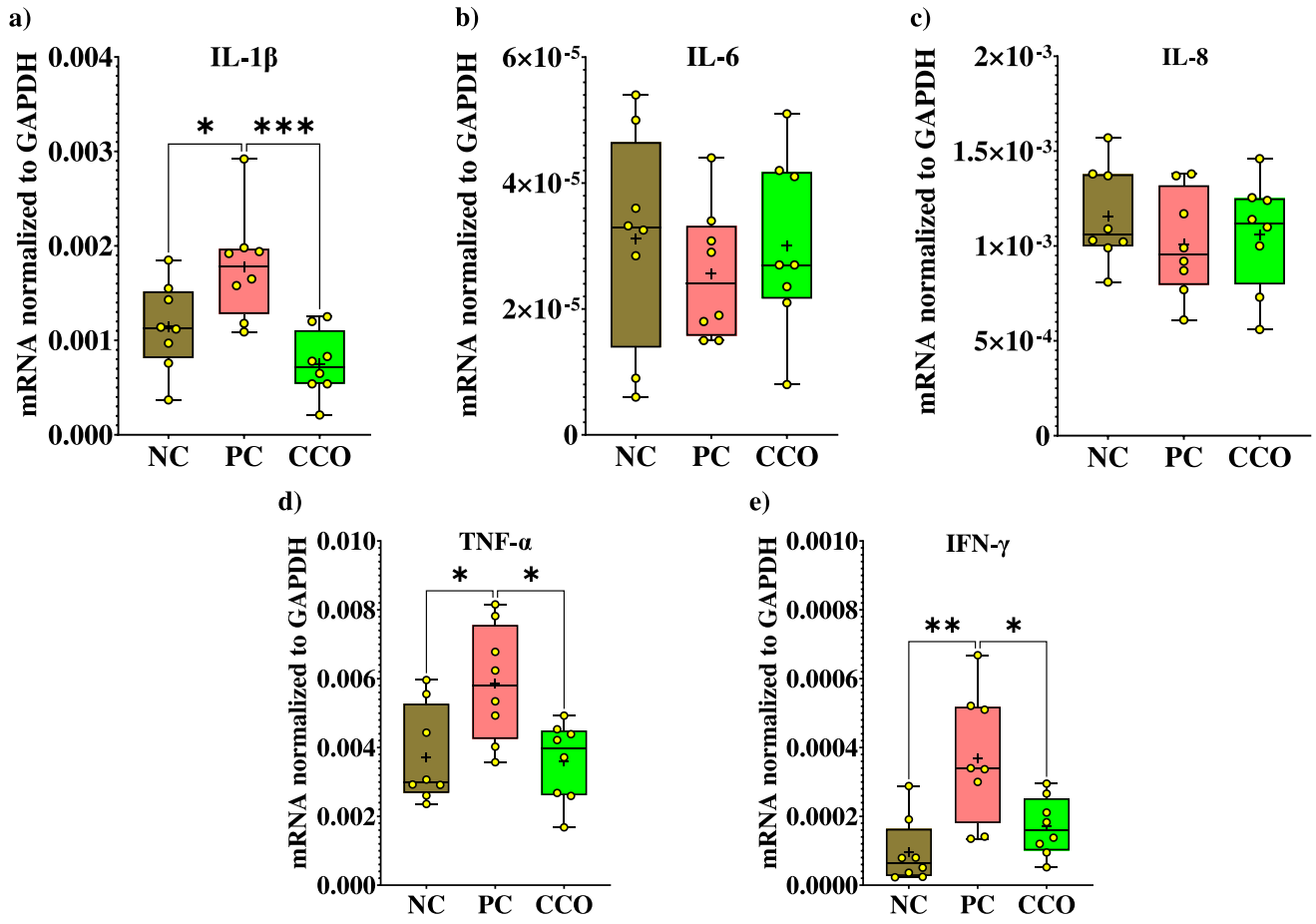

Figure 6. Proinflammatory cytokine transcripts in jejunum of chickens fed diet supplemented with phytochemical mixture (CCO) at 4.5 mg/kg feed during infection with *E. maxima* in experiment 2. NC, basal diet; PC, basal diet for infected chickens; CCO, phytochemical mixture at 4.5 mg/kg feed; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon. All chickens, except for NC, were infected by oral gavage on day 14 with 1.0×10^4 oocysts/chicken of *E. maxima*. $P < 0.05$ (*) and $P < 0.001$ (***) were considered statistically significant. The data were collected from jejunal tissues of 8 chickens per treatment on d 22 (8 days post-infection). Transcript levels of the cytokines were measured using quantitative RT-PCR and normalized to GAPDH transcript levels. Box and whisker plot represents (from top to bottom) the maximum, upper quartile, median, lower quartile, and minimum value. Average measured by six independent experiments was shown as "+".

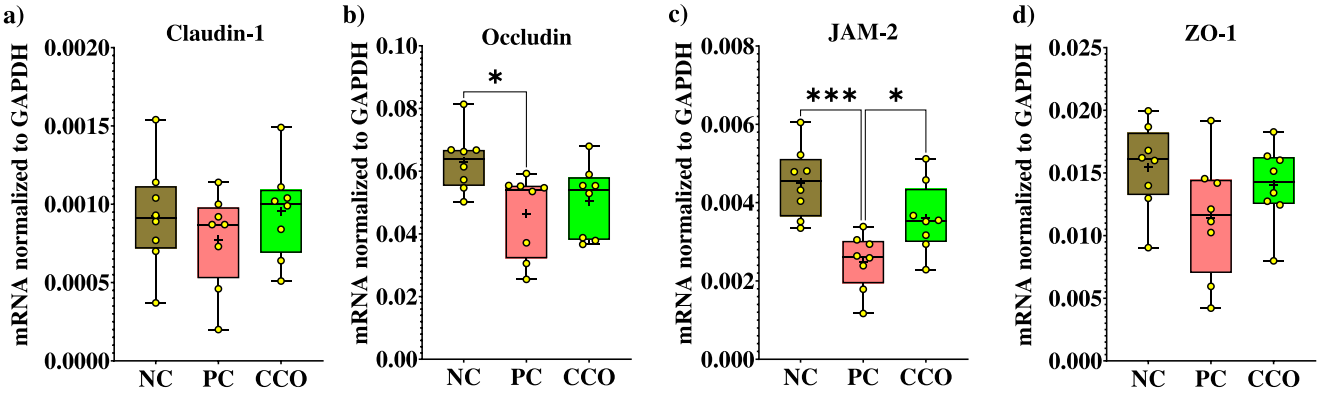

Figure 7. Tight junction protein transcripts in jejunum of chickens fed diet supplemented with phytochemical mixture (CCO) at 4.5 mg/kg feed during infection with *E. maxima* in experiment 2. NC, basal diet; PC, basal diet for infected chickens; CCO, phytochemical mixture at 4.5 mg/kg feed; ZAM, junctional adhesion molecule; ZO, zonula occludins. All chickens, except for NC, were infected by oral gavage on day 14 with 1.0×10^4 oocysts/chicken of *E. maxima*. $P < 0.05$ (*) and $P < 0.001$ (***) were considered statistically significant. The data were collected from jejunal tissues of 8 chickens per treatment on d 22 (8 days post-infection). Transcript levels of the cytokines were measured using quantitative RT-PCR and normalized to GAPDH transcript levels. Box and whisker plot represents (from top to bottom) the maximum, upper quartile, median, lower quartile, and minimum value. Average measured by six independent experiments was shown as "+".

affect BW or ADG compared to the basal diet (NC group), indicating that this supplementation level is not harmful. However, in the presence of *E. maxima* infection (PC group), BW was reduced by 18.4 % on 6 dpi and by 22.5 % on 8 dpi compared to the non-infected NC group, indicating that the chickens continued to suffer from the infection. The reduction in ADG was even more pronounced, reaching 37.7 % on day 6 dpi and 40.3

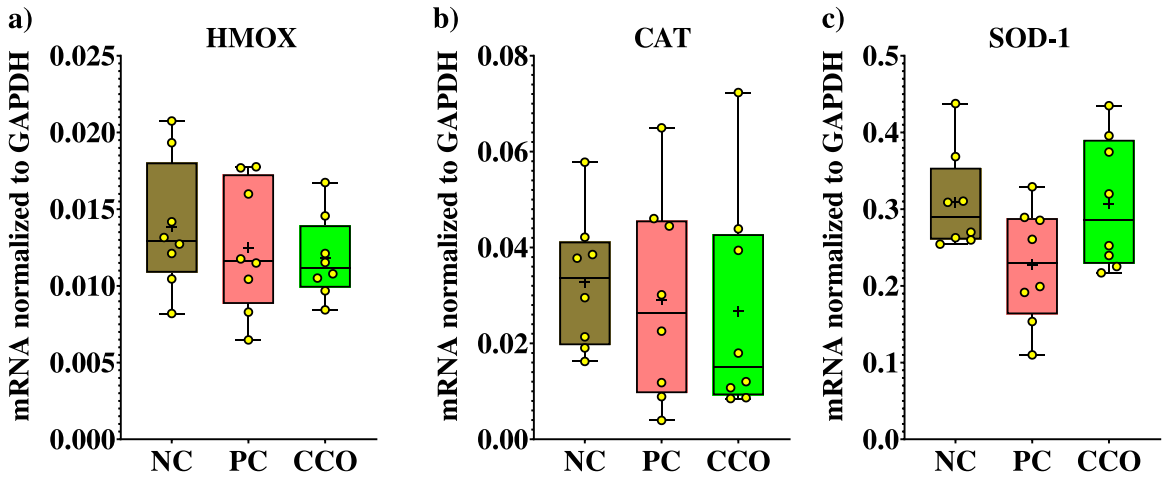

Figure 8. Antioxidant enzyme transcripts in jejunum of chickens fed diet supplemented with phytochemical mixture (CCO) at 4.5 mg/kg feed during infection with *E. maxima* in experiment 2. NC, basal diet; PC, basal diet for infected chickens; CCO, phytochemical mixture at 4.5 mg/kg feed; CAT, catalase; HMOX, heme oxygenase; SOD, superoxide dismutase. All chickens, except for NC, were infected by oral gavage on day 14 with 1.0×10^4 oocysts/chicken of *E. maxima*. The data were collected from jejunal tissues of 8 chickens per treatment on d 22 (8 days post-infection). Transcript levels of the cytokines were measured using quantitative RT-PCR and normalized to GAPDH transcript levels. Box and whisker plot represents (from top to bottom) the maximum, upper quartile, median, lower quartile, and minimum value. Average measured by six independent experiments was shown as "+".

% on day 8 dpi. Under these infection conditions, dietary CCO supplementation also showed a significant difference in BW at 6 dpi and 8 dpi compared to the uninfected group, however, compared to the PC group, the CCO group showed significantly higher BW (12 %) at 8 dpi, thereby demonstrating that dietary CCO supplementation can reduce the growth-inhibitory effects of *E. maxima* infection after 8 dpi. Regarding ADG, dietary CCO supplementation led to an increase of 17.8 % on 6 dpi and 26.6 % on 8 dpi, and these ADG results provided evidence that dietary CCO supplementation was able to mitigate the reduction in BW caused by *E. maxima* infection. Reports indicating that phytochemical mixtures alleviate weight loss in chickens infected with various pathogens support our findings (Kim et al., 2013; Bravo et al., 2014; Lee et al., 2017; Park et al., 2023). However, to definitively demonstrate the efficacy of CCO in mitigating the growth reduction effects of *E. maxima* infection in broilers, it is important to measure feed intake and feed conversion ratio in future studies with a larger number of chickens. Additionally, a longer experimental period, including a phase where the 22 % difference in infection severity between NC and PC further decreases, would be valuable. Interestingly, dietary CCO supplementation did not affect the fecal oocyst number in broilers infected with *E. maxima*. The reason why CCO did not alter the number of *E. maxima* oocysts cannot be definitively explained through this experiment. However, these results suggest that CCO at 4.5 mg/kg feed does not seem to directly affect the oocysts of *E. maxima*. Additionally, we speculated that the mitigation of growth reduction caused by the infection might be attributed to the effects of CCO on host-mediated responses, such as reducing intestinal inflammation and improving permeability, rather than its direct removal of oocysts. Consequently, this experiment suggests that a concentration of 4.5 mg of CCO per kg of feed might be an effective lower dosage for controlling coccidiosis in chickens infected with *E. maxima*.

Excessively released cytokines during the inflammatory response can divert nutrients that should be used for growth towards the production of various immune proteins to counteract and alleviate inflammation, resulting in a waste of nutrients that would otherwise support growth (Klasing, 2007; Broom and Kogut, 2018). This negative outcome was also related to TJPs, which play a crucial role in intestinal permeability. In general, a decrease in the gene expression of TJPs is observed during *E. maxima* infection (Goo et al., 2023; Park et al., 2024c). Numerous studies have shown that the increase in pro-inflammatory cytokines activates myosin light chain kinase (MLCK), which induces the endocytosis of TJPs (Jin and Blikslager, 2020; Yokoyama et al., 2021). This leads to changes in the intestinal barrier, specifically a reduction in TJPs, ultimately weakening TJ integrity and increasing paracellular permeability (He et al., 2020). However, in the current study, MLCK was not investigated, indicating the need for future experiments to measure MLCK to confirm these results.

Dietary CCO supplementation demonstrated an anti-inflammatory effect by suppressing the expression of pro-inflammatory cytokines, IL-1β, TNF-α, and IFN-γ, which were elevated due to *E. maxima* infection. However, while these results were described as anti-inflammatory responses, a reduction in cytokines may not always be positive for immune responses. For example, IFN-γ plays an important role in limiting parasite replication in *E. maxima*. Therefore, diminishing its expression may not necessarily be advantageous for host responses (Chen et al., 2024). The study by Chen et al. (2024) suggests that specific molecules derived from *E. maxima* inhibit IL-12 secretion by increasing phosphorylated protein expression through the ERK-MAPK pathway, thereby enabling *E. maxima* to evade host immune responses. This provides a new approach to controlling coccidiosis in chickens. Thus, when interpreting cytokine-related results, a balanced understanding tailored to the experimental conditions is required. Additionally, the current experiment on inflammatory responses was focused on the jejunum, where most digestion occurs and which is targeted by *E. maxima*. Since positive effects of CCO were observed in the jejunum, it is considered worthwhile to examine systemic responses in future experiments. CCO supplementation also increased the gene expression level of JAM-2 compared to the infected group, thereby enhancing intestinal permeability. The positive effects of dietary CCO supplementation on inflammatory responses and intestinal permeability may have contributed to partially mitigating the growth reduction effects of BW and ADG caused by *E. maxima*. In this regard, dietary CCO supplementation showed potential as in-feed coccidiostats and feed additive without directly killing the *E. maxima*.

In conclusion, CCO composed of three phytochemicals inhibited cytokine production induced by inflammatory responses in CMCs and increased the expression of TJPs in IECs. These beneficial effects were also observed in young broilers infected with *E. maxima*, where 4.5 ppm of CCO alleviated inflammatory responses and improved intestinal permeability, partially mitigating the growth reduction caused by *E. maxima* infection. Therefore, Using the phytochemical mixture selected through *in vitro* screening as a dietary supplement can

effectively mitigate intestinal damage in broilers infected with *E. maxima*, maintaining growth, and demonstrating its potential as in-feed coccidiostats. The findings of this work support the value of screening approaches based on host-mediated responses, and not direct pathogen killing, when searching for phytochemicals to be used as feed additives in poultry.

Author contributions

IP and HL designed the research. IP and NH conducted the in vitro study. IP carried out the animal study. IP analyzed data. IP wrote an original draft. IP, SR, EHW, and HL edited the original draft. IP, HN, SR, EHW, and HSL had responsibility for the content.

CRediT authorship contribution statement

Inkyung Park: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft. **Hyoyoun Nam:** Software, Visualization, Formal analysis, Writing – review & editing. **Sripathy Ravichandran:** Resources, Writing – review & editing. **Emma H. Wall:** Investigation, Resources, Writing – review & editing. **Hyun S. Lillehoj:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Disclosures

Author SR and EHW were employed by AVT Natural and Nutreco Exploration, respectively. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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