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Article Title (within 20 words without abbreviations)	Effects of gallic acid and essential oil supplementation on performance, health and cecal bacterial count in broilers challenged with coccidiosis
Running Title (within 10 words)	Effect of phytogenic feed additives against coccidiosis
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Abstract

This study was conducted to investigate the effects of different phytogenic feed additives (PFA) on growth performance, immune organ weight, lesion score, footpad dermatitis, oocyst shedding counts, intestinal morphology, blood profile and cecal bacterial counts in broiler chickens challenged with coccidiosis. 200 one-day-old Arbor Acres broiler chickens (BW 37.65 ± 0.82 g) were allocated in a randomized complete block design to 4 treatments: (1) NC: no-challenge / no-supplementation; (2) PC; Coccidiosis-challenge / no-supplementation; (3) GA: Coccidiosis-challenge / gallic acid (GA) at 100 mg/kg; (4) PFA: Coccidiosis-challenge / mixture of PFA including carvacrol, thymol and GA at 100 mg/kg. Broilers were fed for 32 days. Coccidiosis challenge decreased ($p < 0.05$) body weight (BW), body weight gain (BWG), feed intake (FI), villus height to crypt depth (VH:CD) and *Lactobacillus* count, while increased ($p < 0.05$) feed conversion ratio (FCR), FCR at 1.5 kg, incidence of high lesion score and average lesion score of upper, middle, ceca and footpad, oocyst shedding count, heterophil count in serum and *Escherichia coli* count. On the other hand, anticoccidial feed additives (GA and PFA) mitigated ($p < 0.05$) impaired growth performance and intestinal morphology by coccidiosis challenge. Moreover, they alleviated ($p < 0.05$) high lesion score, oocyst shedding count and harmful bacteria count induced by coccidiosis challenge. Therefore, GA and mixture of PFA including carvacrol, thymol and GA can be considered as an effective alternative for anticoccidial treatment in broilers.

Keywords: Coccidiosis, intestinal health, gallic acid, essential oil, phytogenic feed additive

INTRODUCTION

Coccidiosis in poultry is a parasitic disease caused by several species of the genus *Eimeria*. It impairs intestinal barrier function and causes diarrhea [1]. Coccidiosis is an expensive disease, estimated to cost the global poultry industry more than $3 billion USD annually due to reduced growth performance, prophylaxis, and treatment costs [2]. Previous studies have shown that coccidiosis outbreaks cause intestinal damage, thereby reducing body weight (BW), feed intake (FI), feed conversion ratio (FCR) and nutrient digestibility, and increased intestinal lesions, and diarrhea [3-5]. Antibiotics have been used for decades to control poultry diseases, including coccidiosis. However, antibiotics are gradually being banned as antimicrobial growth promoters (AGPs) due to rising public concerns about drug residues in poultry meat and the development of antimicrobial resistance [6,7]. Therefore, many researchers have been studying alternative strategies to control enteric diseases, including coccidiosis.

Phytogenic feed additives (PFAs) are classified as sensory and flavoring compounds. They primarily consist of plant extracts, such as essential oils (EOs), gallic acid (GA), oleoresin, and flavonoids [8]. PFAs mainly control potential pathogens by modulating the intestinal microbiota, making them a suitable alternative to AGPs [9].

GA, known as 3,4,5-trihydroxybenzoic acid, has been used in various industries, including the poultry industry, due to its positive benefits, such as antioxidant, anti-inflammatory, and antibacterial effects, and may serve as an alternative to AGPs [10-13]. Dietary GA supplementation was reported to decrease crypt depth and plasma malondialdehyde, improving growth performance and meat yields [13]. Furthermore, providing supplemental GA to broilers alleviated the negative effects of an *Aspergillus flavus* challenge on the histology of organs, such as lungs and liver [14]. The combination of GA and other plant extracts (i.e., eugenol and oregano) alleviated mortality and improved growth performance, and decreased lesion scores and oocyst counts of *Eimeria* spp. in broilers challenged with necrotic enteritis and coccidiosis [15,16], while single GA addition did not positively influence broilers challenged with coccidiosis [16].

EOs have been widely used as an alternative to AGPs to improve the growth performance and health of broilers [17,18]. Moreover, previous studies reported that EOs had positive effects, including antioxidant, antibacterial, anti-inflammatory, and antiparasitic properties [19-21]. Due to the properties mentioned above, the supplementation of poultry diets with EO alleviated impaired intestinal morphology and gut microbiota by controlling pathogenic bacteria, such as *Salmonella*, *Escherichia coli*, *Clostridium perfringens*, and *Eimeria* spp., thereby reducing economic losses [22-25].

However, although many studies have verified the efficacy of these additives, studies focusing on coccidia challenge and comparative studies of various additives are lacking.

Therefore, we hypothesized that different natural anticoccidials might enhance growth performance by protecting intestinal health and enhancing the immunity of birds under conditions of coccidia challenge. This experiment was conducted to investigate the efficacy of different natural feed additives, including GA and PFAs, by evaluating growth, organ weight, oocyst shedding, intestinal morphology, lesion scores, and immunity cecal bacteria count in broilers under coccidia challenge and comparing their potential to mitigate the impact of coccidiosis on intestinal health.

MATERIALS AND METHODS

Ethics approval and consent to participate

The experimental protocols describing the management and care of animals were reviewed and approved by the Animal Care and Use Committee of Chungbuk National University (CBNUA-1742-22-02).

Experimental design, birds and treatment

A total of 200 one-day-old male Arbor Acres broiler chickens (initial body weight of 37.65 ± 0.82 g) were obtained from a local hatchery (Cherrybro Co., Eumseong, South Korea) and used in this experiment for 32 days. All broilers were randomly allocated into four experimental groups in a randomized complete block design, and each group had twenty-five replicate pens with two broilers per pen. The dietary treatments were as follows: 1) negative control group, No coccidiosis challenge and no PFAs supplementation, (NC); 2) Coccidiosis infected group, Coccidiosis challenge and no PFAs supplementation, (PC); 3) Supplemented group 1, Coccidiosis challenge and GA supplementation at 100 mg/kg; VantiPEARL™, Kemin industries, Asia Pacific, Senoko Drive, Singapore, (GA); 4) Supplemented group 2, Coccidiosis challenge and phytogenic feed additives including carvacrol, thymol and gallic acid (PFA) supplementation at 100 mg/kg; ORSENTIAL™ extend, Kemin industries, Asia Pacific, Senoko Drive, Singapore, (PFA). Each cage was 100 cm in width, 40 cm in depth, and 45 cm in height. The experiment initiation temperature was 33 ± 1℃, and thereafter, the temperature was gradually lowered to maintain 25 ± 1℃. All diets were formulated to meet or exceed National Research Council (NRC, 1994) [26] for the starter (1-7 d), grower (8-21 d), and finisher (22-32 d) periods. The experimental diets were provided in mash form to avoid potential loss of bioactive compounds in the phytogenic additives during the heat-treatment process of pelleting. Table 1 presents the basal feed composition and calculated nutrient value. All broilers were given ad libitum access to feed and water throughout the experiments.

Establishment of Coccidiosis Model

On d 14, all broilers except NC group broilers were orally challenged by overdosing with coccidia and Infectious bursal disease (IBD) vaccines (20 times of recommended dose, respectively). The above-mentioned vaccines were the Hipra Evalon® (Hipra Evalon®, Laboratorios Hipra, Girona, Spain) and IBD vaccine (IBD blen®, Boehringer Ingelheim Animal Health USA Inc., Georgia, USA). The freeze-dried live intermediate strain of infectious bursitis virus contained in the IBD vaccine is IBD Winterfield 2512. Hipra Evalon® vaccine

contained *Eimeria acervuline* (Strain 003, 332–450 oocysts per dose), *Eimeria brunetti* (Strain 034, 213–288 oocysts per dose), *Eimeria maxima* (Strain 013, 196–265 oocysts per dose), *Eimeria necatrix* (Strain 033, 340–460 oocysts per dose) and *Eimeria tenella* (Strain 004, 276–374 oocysts per dose) strains. NC group broilers received the same dosage of sterile phosphate-buffered saline (PBS) via oral gavage.

Growth performance

BW and FI were weighed on d 0, d 14, d 21, and d 32 for estimating body weight gain (BWG), FI, and FCR. Mortality was recorded as it occurred. Adjusted FCR at 1.5 kg BW was calculated as follows: FCR at 1.5 kg BW = FCR – (average BW-1.5) × 0.3. Production index was calculated depending on the following formula: Production index = {(Average body weight (kg) × livability) / (Duration of the period (day) × FCR)} × 100.

Relative immune organs weight

On d 21 and d 32, twenty-five birds per treatment were euthanized by cervical dislocation. After euthanasia, the liver, spleen, and Bursa of Fabricius were excised and weighed. The weights of immune organs were recorded, and the relative organ weights were calculated using the following formula: Relative organ weight (g/kg) = organ weight (g)/live BW (kg).

Intestine and foot-pad dermatitis lesion scores

On d 21 and d 32, twenty-five broilers per treatment were euthanized, and intestinal lesion score was measured according to the method of Johnson and Reid [27]. Intestinal lesion score from upper intestine (from the pyloric junction to the most distal point of insertion of the duodenal mesentery), middle intestine (10 cm posterior from the Meckel's diverticulum junction; upper ileum), and caeca were scored as follows: 0 (non), 1 (contain some bloody spot and normal digesta), 2 (serosal surface has red petechiae and contains some bloody spots) 3 (contain large quantities of blood, caecal mould and mucus) 4 (increased its thickness and contain bloody spots which have specific color and smell).

On d 32, Foot pad lesion was measured twenty-five broilers per treatment according to the method of Eichner et al. [28]. Foot pad lesion score was scored as follows: 0 (no lesion), 1 (lesions cover less than 25% of the footpad), 2 (lesions in wide areas, covering between 25% and 50% of the footpad), 3 (more

than 50% lesion on the footpads). Assessments were performed on both paws. The average score was used for statistical analysis.

Oocyst shedding

On d 21 through d 25 and d 32, clean plastic was placed under each pen and fresh feces were collected. From each pen, approximately 200 g of representative excreta were collected in a sample bag and stored at 4℃ until further processing following the modified procedure by Teng et al. [29]. Five g of fecal samples were diluted with 45 mL water. After proper mixing, 1 mL mixture was taken and diluted with 9 mL saturated salt solution. Homogenized mixtures were incubated for 30 s for letting oocysts float. Using a water dropper pipette (Thermo Fisher Scientific, Waltham, MA), the final samples were loaded in McMaster chambers (Jorgensen Laboratories, Loveland, CO), and the total oocysts were counted. The number of oocysts per gram of feces (OPG) was calculated using the following formula: The number of OPG = (Number of oocysts counted / 0.15) × Dilution. Where 0.15 is the volume of the McMaster counting chamber.

Intestinal morphology

Twenty-five broilers per treatment were euthanized by cervical dislocation on d 21 and d 32 to collect intestinal tissue samples. Segments of the duodenum (the middle 4 cm), ileum (10 cm from close to the ileocecal junction), and caeca (the middle 4 cm) were excised for morphological evaluation. Each segment was freed of mesenteric attachments and rinsed clean with 10% neutral buffered formalin. The intestinal segment was submerged in approximately 20 mL of 10% neutral buffered formalin for 24 hours. Slides of intestinal cross-sections (5 μm thick) were processed in low-melt paraffin and stained with hematoxylin and eosin. The slides were examined using an Olympus IX51 inverted phase-contrast microscope (Olympus Optical Co., Ltd., Tokyo, Japan). The villus height (VH) was measured from the tip of the villus to the crypt orifice. Crypt depth (CD) was measured from the junction of the villus to the crypt base. And then, the villus height to crypt depth ratio (VH:CD) was calculated.

Blood profile

Blood samples were collected from the brachial wing vein into a sterile syringe on d 21 and d 32. At the time of collection, blood samples were collected into vacuum tubes containing tripotassium ethylene

diamine tetra-acetic acid (K_3EDTA; Becton, Dickinson and Co., Franklin Lakes, NJ, USA) for complete blood count analysis. The white blood cell (WBC), red blood cell (RBC), heterophil, and lymphocyte levels in the whole blood were measured using an automatic blood analyzer (ADVIA 120, Bayer, NY, USA).

Caeca bacterial enumeration

On d 21 and d 32, the cecal digesta of twenty-five broilers per treatment was aseptically collected into individual sterile culture tubes and then placed on ice for transportation to the laboratory, where analysis was immediately carried out. One gram of cecal sample was blended with 9 mL of 1 × PBS buffer and vortexed for 1 min. Samples were used for measuring the number of viable cells in the cecal by serial dilution from 10^{-1} to 10^{-7}. To measure the number of colonies of *Escherichia coli* (*E. coli*) and *Lactobacillus*, MacConkey agar (KisanBio, Seoul, Korea) was used for *E. coli*, and de Man, Rogosa and Sharpe (MRS) agar (KisanBio, Seoul, Korea) for *Lactobacillus*. The MacConkey agar plates were cultured at 37℃ for 24 hours. The MRS agar plates were cultured at 37℃ for 48 hours. After the incubation periods, colonies of the respective bacteria were counted and expressed as the logarithm of colony-forming units per gram (log_{10} CFU/g).

Statistical analysis

Data of growth performance, relative immune organ weight, average intestinal lesion score, oocyst counts, blood profile, and caeca bacterial count were statistically analyzed as a randomized complete block design using general linear model procedure of SAS (Statistical Analysis System 9.1, SAS Institute, Cary, NC, USA). Total mortality and footpad dermatitis and intestine lesion scoring were compared with a chi-squared test, using the FREQ procedures of SAS. Differences between treatment means were determined using Tukey's multiple range test. A probability level of $p < 0.05$ was indicated to be statistically significant, and a level of $0.05 < p < 0.10$ was considered statistically tendency.

RESULTS

Growth performance

The effects of supplementing GA and EO on growth performance in coccidiosis challenged broilers are presented in table 2. During the period before the coccidiosis challenge (d 0 to 14), there was no significant difference ($p > 0.05$) on BW, BWG, FI, and FCR among the treatments. After the coccidiosis challenge, BW and BWG were significantly decreased ($p < 0.05$) compared to the NC group until the end of the experiment (d 14 to 21 and d 21 to 32). On d 14 to 21, coccidiosis challenge significantly decreased ($p < 0.05$) in BWG and FI, while increased ($p < 0.05$) FCR and FCR at 1.5 kg compared to the NC group. Anticoccidial feed additives (GA and PFA) improved ($p < 0.05$) impaired BWG, FCR at 1.5 kg and production index by coccidiosis challenge. Moreover, GA groups showed ($p > 0.05$) similar growth performance with NC groups. There was no significant difference ($p > 0.05$) in mortality among the treatments on d 14 to 21 and during the entire period.

Immune organ weight

The effects of supplementing GA and EO on relative immune organ weight in coccidiosis challenged broilers are presented in table 3. There was no significant difference ($p > 0.05$) among the treatments in relative weight of liver and spleen on d 21. On d 21, the PC and PFA groups showed a decreased tendency ($p = 0.073$) in the relative weight of the Bursa of Fabricius compared with the NC group. The PC and PFA groups showed an increased tendency ($p = 0.065$) in the relative weight of the liver and spleen while significantly decreasing ($p < 0.05$) the relative weight of the Bursa of Fabricius compared with the NC group. There was no significant difference ($p > 0.05$) among the treatments in the relative weight of the spleen on d 32.

Intestine and foot-pad dermatitis lesion scores

The effects of supplementing GA and EO on upper and middle intestinal lesion score on d 21 and d 32 in coccidiosis challenged broilers are presented in tables 4 and 5. The lesion score of the upper and middle intestine on d 21 and d 32 were significantly higher ($p < 0.05$) in the coccidiosis challenged groups than the NC group. GA groups showed ($p < 0.05$) lower lesion score in the upper and middle intestine on d 21 compared to PC group. The effects of supplementing GA and EO on caeca lesion score in coccidiosis challenged broiler are presented in table 6. The lesion score of the caeca on d 21 and d 32 (d 18 PI) was significantly higher ($p < 0.05$) in the coccidiosis challenged groups than the NC group. However, anticoccidial feed additive (GA and PFA) did not affect ($p > 0.05$) the lesion score of the caeca. The effects of supplementing GA and EO on foot-pad

dermatitis lesion score in coccidiosis challenged broilers are presented in table 7. There was no significant difference ($p > 0.05$) among the treatments in foot-pad dermatitis lesion score.

Oocyst shedding

The effects of supplementing GA and EO on OPG count in NE-challenged broilers are presented in table 8. There were no oocysts were detected in the feces obtained from the NC group. The pattern of oocyst shedding showed a decrement during d 22 to d 25. Also, the pattern of oocyst shedding showed a decrement between d 25 and d 32. The shedding pattern was common for all coccidiosis challenged groups. On d 22 to d 25 and d 32, the GA group showed the significantly lowest ($p < 0.05$) OPG count among the coccidiosis challenged groups. On d 23 and d 25, the PFA group significantly decreased ($p < 0.05$) the OPG count compared to the PC group.

Intestinal morphology

The effects of supplementing GA and EO on intestinal morphology in coccidiosis challenged broilers are presented in table 9. coccidiosis challenge decreased ($p < 0.05$) villus height and VH:CD, while increased ($p < 0.05$) crypt depth compared to those of NC groups on d 21 and 32. The villus height in the duodenum was significantly increased ($p < 0.05$) in the GA group compared to the PC group. The GA and PFA groups significantly decreased ($p < 0.05$) the crypt depth in the ileum compared to the PC group. The GA group significantly increased ($p < 0.05$) the villus height and crypt depth of the ileum compared to the PC group. On d 32, the crypt depth in the duodenum was significantly decreased ($p < 0.05$) in the GA group compared to the PC and PFA groups. The GA group significantly improved ($p < 0.05$) the villus height and crypt depth ratio of the duodenum compared to the PC and PFA groups. There was no significant difference ($p > 0.05$) among the treatments in the crypt depth in the caeca on d 21 and d 32.

Blood profile

The effect of supplementing GA and EO on blood profile in coccidiosis challenged broilers is presented in table 10. WBCs levels in blood were significantly higher ($p < 0.05$) in coccidiosis challenged groups than those in the NC group. Heterophils percentages were also significantly increased ($p < 0.05$) in the coccidiosis challenged groups compared with the PC group on d 21. However, increased heterophils percentage by coccidiosis challenge was lower ($p < 0.05$) in supplementing GA and EO in diets. On d 32, WBCs levels in

blood were significantly decreased ($p < 0.05$) in the GA group compared to the PC group. RBC, heterophil, and lymphocyte on 18 PI showed no significant difference ($p > 0.05$) among the treatments.

Caeca bacterial enumeration

The effects of supplementing GA and EO on caeca bacterial counts in coccidiosis challenged broilers are presented in table 11. On d 21, the *E. coli* counts were significantly higher ($p < 0.05$) in coccidiosis challenged groups than the NC group. The GA group was significantly lower ($p < 0.05$) *E. coli* counts than the PFA group on d 21. For *Lactobacillus* counts, the NE-challenged groups were significantly lower ($p < 0.05$) than the NC group on d 21. However, on d 32, the GA and PFA groups significantly recovered ($p < 0.05$) the *Lactobacillus* counts compared to the PC group and showed a similar value to the NC group.

DISCUSSION

In this study, we investigated the effects of PFAs on growth performance, relative organ weight, *Eimeria* oocyst excretion, intestinal and footpad lesions, intestinal morphology, blood profiles, and cecal bacterial counts in broilers challenged with coccidiosis infection. We found that coccidiosis challenge induced poor growth performance, as evidenced by reduced BWG and FI, and increased FCR compared to groups without coccidiosis challenge. These findings are consistent with previous studies reporting that broilers challenged by coccidiosis showed a significant reduction in BWG, which can result in immense economic losses [2,30]. Sharma et al. [31] reported that FI started to decrease from d 4 post-injection (PI) with coccidiosis and was lowest on d 5 to 6 PI. Also, FI in the high-dosage coccidiosis group did not return to normal until d 12 PI. In our results, coccidiosis challenge significantly decreased FI from d 0 to d 7 PI, confirming that the method of inducing coccidiosis used in this study was very effective. Moreover, previous studies have shown that FI reduction is the main factor contributing to BWG reduction [29,32]; thus, reductions in BWG in this study were due to decreased FI. GA supplementation alleviated BWG and FI affected by coccidiosis challenge. Moreover, supplementation with GA restored growth performance measurements, such as BWG, FI, FCR, and FCR at 1.5 kg body weight, with production index values similar to those in the group not challenged with coccidiosis in this study. These results agree with those of previous studies, in which GA improved the performance of birds challenged with coccidiosis [33-36]. Gallic is known to have many activities such as anti-inflammatory, antimicrobial and antioxidant effects, and exerts especially effective therapeutic activity against coccidiosis [37-39]. In this study, we observed a remarkable reduction in *Eimeria* oocyst excretion from 8 to 12 PI in the GA groups compared to the PFA groups. Nawarathne et al. [40] reported that GA significantly reduced the number of oocysts shed in feces by up to 10%. Reduced oocyst shedding suggests that GA may suppress parasite replication or development within the intestinal tract. Similarly, it is considered that improvements in growth performance by adding GA to diets are due to the positive effects of GA on coccidiosis. In contrast, supplementation with PFAs had little effect on growth performance or *Eimeria* oocyst shedding compared to the PC groups. Many researchers confirmed the beneficial effect of PFAs on performance, as well as oocyst excretion, in broiler chickens challenged with coccidiosis [41-43]. These inconsistent results may be related to the different forms and dosages of PFAs used in the studies.

We estimated the relative organ weight of the liver, spleen, and the bursa of Fabricius because the weight of lymphoid organs is associated with the ability to deliver lymphoid cells during an immune response [44]. In this study, coccidiosis challenge numerically increased the relative weight of the liver and spleen on d 21 and 32,

and significantly decreased the relative weight of the bursa of Fabricius compared to non-*Eimeria* spp.-challenged birds. Our results agree with previous studies reporting that coccidiosis challenge caused lower relative bursa of Fabricius weights and higher relative spleen weights [45,46]. The reduction in bursa weight may reflect lymphoid depletion following immune activation induced by Eimeria infection. Supplementation with GA significantly alleviated decreases in the relative weight of the bursa of Fabricius, similar to un-coccidiosis-challenged groups. However, no significant difference was seen in relative organ weights between the PC groups and the PFA groups. The effects of PFAs on relative organ weights are controversial. Some studies reported that various PFAs altered the relative weight of immune organs, including the thymus, spleen, and bursa of Fabricius, by improving humoral immune responses under normal and abnormal conditions [47-49]. However, other studies showed that PFAs, including GA, did not affect the relative weight of immune organs [50,51]. According to previous research, changes in relative organ weight may be attributed to organ atrophy and/or enlargement caused by lymphocyte depletion and/or activation to meet the requirements of humoral and cellular immunity [52,53]. Thus, changes in relative organ weight in this study may be due to changes in immunity in the broilers.

A primary target of *Eimeria* spp. is the intestinal tract, and it causes coccidiosis in poultry [54]. The *Eimeria* strains used in this study consisted of *Eimeria acervuline, Eimeria brunetti, Eimeria maxima, Eimeria necatrix*, and *Eimeria tenella*. Generally, each *Eimeria* spp. affects a particular part of the gastrointestinal tract. *E. acervulina* infects the upper intestine, *E. maxima* and *E. necatrix* infect the middle intestine, and *E. tenella* and *E. brunetti* infect the hind intestine, including the ceca and rectum [55]. Previous studies reported that *Eimeria* spp. infection significantly increased gastrointestinal leakage, lesion scores, and oocyst excretion [29, 56]. Consistent with these long-known features, coccidiosis challenge increased lesion scores in the respective intestinal parts in this study, as well as oocyst excretion. *Eimeria* spp. causes intestinal lesions by replicating within the intestinal walls of poultry [57]. More specifically, sporozoites spread into the gastrointestinal tract and then become trophozoites in 12 to 48 h. Trophozoites become schizonts through asexual reproduction, and then, mature schizonts change to merozoites and spread within the gastrointestinal tract [58]. The released merozoites penetrate epithelial cells and induce severe damage to the intestine [29,31,59]. The intestinal lesions and increased oocyst shedding in this study were attributed to this process.

Furthermore, intestinal morphology, used as a marker to identify coccidiosis, is related to lesion scores and oocyst shedding [29]. Intestinal VH, CD, and VH:CD are important markers for estimating gut health, recovery, and function. Many researchers observed that *Eimeria* spp. Infections caused poor intestinal morphology, along with high lesion scores and oocyst shedding [60-63]. Our previous studies showed that *Eimeria* spp. challenge decreased VH and VH:CD in broilers [64]. Consistent with these studies, our findings indicated that coccidiosis challenge decreased VH and VH:CD in the upper and middle intestine, and increased CD in the ceca compared to the non-NE-challenged group. As mentioned above, *Eimeria* spp. challenge damages the epithelium, allowing the increased penetration and replication of merozoites. These processes cause rapid damage to gastrointestinal tract epithelium, resulting in impaired intestinal morphology, such as low VH and high CD [29]. However, supplementation with GA and PFAs mitigated severe intestinal lesion scores, damaged intestinal morphology, and high oocyst excretion caused by coccidiosis challenge in this study. These findings agree with previous studies in which PFAs, including GA and EO, reduced lesion scores and fecal oocyst shedding counts, and improved intestinal morphology [40, 65, 66, 67]. Fatemi et al. [68] reported that natural compounds could alter the formation of oocyst walls, causing sporozoite destruction. These compounds can affect parasites directly, as well as up-regulate positive functions, such as antioxidative and anti-inflammatory properties, against coccidiosis [69, 70]. In this study, GA and PFAs reduced oocyst excretion, suggesting that these compounds may affect oocyst formation, as mentioned above. These effects may also contribute to preventing *Eimeria*'s penetration into the intestinal epithelium, thereby alleviating high intestinal lesion counts and poor intestinal morphology caused by coccidiosis. In contrast, other studies reported that PFA supplementation did not affect intestinal morphology under challenged and unchallenged conditions [71, 72]. These inconsistent results may be due to differences in feed additive types, dosages, and *Eimeria* spp dosages.

The role of heterophils in avian species is similar to that of neutrophils in mammals and, therefore, they are involved in innate immunity (first-line defense) against bacteria, fungi, protozoa, and some viruses [73]. The number of heterophils is significantly increased in acute *Eimeria tenella* infections [74, 75]. Also, the main role of lymphocytes is in the immunological response, humoral antibody production, and cell-mediated immunity. An initial increase in lymphocytes may be induced as an immune response defense mechanism against *Eimeria* infection [76]. However, Matthew et al. reported that the lymphocyte count increased on d 4 PI and then decreased on d 7 PI [77]. Consistent with these studies, our findings indicated that coccidiosis challenge induced high heterophil and low lymphocyte counts compared to the non-coccidiosis-challenged group. These changes suggest that the *Eimeria* infection in this study stimulated host innate immune responses. Supplementation with

GA and PFAs alleviated changes in heterophil and lymphocyte counts caused by coccidiosis challenge. Many previous studies have reported that PFAs boost host defenses against various infections [78, 79]. Natural compounds, including mustard, safflower, thistle, and turmeric, were reported to stimulate innate immunity in poultry [80, 81]. Therefore, GA and PFAs may enhance immunity in broilers challenged with coccidiosis, altering blood parameters.

Eimeria spp. infection causes severe intestinal damage, impairs digestion, and causes nutrient and plasma protein leakage, unbalancing the gut microbial community [82-85]. Furthermore, the infection disrupts thereby causing the proliferation of other pathogens, such as *Clostridium perfringens* [86-88]. Consistent with these studies, our findings indicated that coccidiosis challenge increased *E. coli* and decreased *Lactobacillus* populations in the ceca. This shift in microbial populations is likely associated with epithelial damage and altered gut environment following infection. However, supplementation with GA and PFAs decreased pathogenic bacteria counts, including *E. coli*, and increased *Lactobacillus* in broilers challenged with coccidiosis. These findings are consistent with previous studies reporting that supplementation with PFAs, including thymol, carvacrol, and piperine, increased *Clostridiales* and *Lactobacillales* [23, 89, 90]. Other studies also reported that natural compounds derived from green tea and pomegranate up-regulated beneficial bacteria in the intestine [91, 92]. This positive change may be attributed to the promotion of intestinal integrity by PFAs against coccidiosis.

CONCLUSION

This study showed that coccidiosis challenge remarkably caused decreased growth performance, poor gut integrity including high lesion and oocyst excretion, and reduced immunity and cecal bacteria balance compared to non-coccidiosis challenge. However, GA and EO alleviated negative effects such as poor growth performance, damaged epithelial cell in intestine, lower number of oocyst excretion and imbalance of cecal bacteria by coccidiosis. It may be concluded that GA and EO used in this study can be considered as an effective alternative for anticoccidial treatment in broiler. Therefore, it is suggested that GA and EO can effectively, and naturally, help farms suffer from economic loss by coccidiosis.

CONFLICT OF INTEREST

The authors declare no conflict of interest

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Tables and figures

Table 1. Ingredient composition of basal diets

Items, %	Starter, 1-7 d	Grower, 8-21 d	Finisher, 22-32 d
Corn	40.645	47.357	53.292
Soybean meal (CP 45%)	33.263	26.750	23.675
Wheat	10.000	10.000	10.000
DDGS 28%	4.000	5.000	3.000
Tankage ML-60	3.000	3.000	1.900
MBM 50%	1.800	1.700	1.700
Wheat flour	2.000	2.000	2.000
Poultry oil	2.633	1.700	2.108
L-lysine-SO_4	0.501	0.510	0.392
DL-Methionine	0.418	0.367	0.411
L-Threonine	0.141	0.141	0.105
L-Tryptophan	0.010	0.010	0.100
Salt	0.224	0.250	0.234
Limestone	0.445	0.450	0.438
Mono-dicalcium phosphate	0.435	0.300	0.200
Mineral premix	0.220	0.220	0.220
Vitamin premix	0.150	0.130	0.130
Choline	0.100	0.100	0.080
Phytase1000 (HiPhos 10000 GT)	0.010	0.010	0.010
Xylanase (WX 2000)	0.005	0.005	0.005
Total	100.00	100.00	100.00
Calculated value (%)			
Metabolic energy (ME), kcal/kg	3000	3020	3100
Crude protein (CP)	22.000	20.500	19.000
Available Lysine	1.310	1.170	1.010
Available Methionine	0.570	0.540	0.560
Available Threonine	0.850	0.770	0.680
Available Tryptophan	0.210	0.190	0.160
Available SAA	0.990	0.900	0.900
Arginine	1.510	1.350	1.160
Iso-Leucine	0.970	0.880	0.780
Valine	1.120	1.010	0.890
Calcium (Ca)	0.850	0.800	0.700
Available P	0.480	0.450	0.400
Na	0.150	0.150	0.150

Provided per kg of diet: 37.5 mg Zn (as $ZnSO_4$), 37.5 mg of Mn (MnO_2), 37.5 mg of Fe (as $FeSO_4 \cdot 7H_2O$), 3.75 mg of Cu (as $CuSO_4 \cdot 5H_2O$), 0.83 mg of I (as KI), and 0.23 mg of Se (as $Na_2SeO_3 \cdot 5H_2O$). [2]Provided per kg of diet: 15,000 IU of vitamin A, 3,750 IU of vitamin D_3, 37.5 mg of vitamin E, 2.55 mg of vitamin K_3, 3 mg of thiamin, 7.5 mg of riboflavin, 4.5 mg of vitamin B_6, 24 μg of vitamin B_{12}, 51 mg of niacin, 1.5 mg of folic acid, 0.2 mg of biotin and 13.5 mg of pantothenic acid

Table 2. Effect of supplementing gallic acid and essential oils on growth performance in broilers challenged with coccidiosis

Items	NC	PC	GA	PFA	SEM	*p*-value
BW, g						
d 0	37.60	37.80	37.60	37.60	0.13	0.615
d 14	407.40	403.00	402.40	401.80	7.43	0.950
d 21	974.80^{a}	865.60^{c}	931.60^{b}	901.60^{b}	12.77	<0.0001
d 32	1826.40^{a}	1640.80^{c}	1770.60^{ab}	1707.00^{bc}	27.65	<0.0001
d 0 to 14						
BWG, g	369.80	365.20	364.80	364.20	7.32	0.946
FI, g	481.42	475.25	472.54	476.60	4.04	0.470
FCR	1.30	1.30	1.30	1.31	0.03	0.911
Mortality, %	0.00	0.00	0.00	0.00	-	-
d 14 to 21						
BWG, g	570.00^{a}	460.00^{d}	528.80^{b}	499.80^{c}	6.18	<0.0001
FI, g	855.18^{a}	819.14^{bc}	826.53^{b}	806.80^{c}	4.82	<0.0001
FCR	1.50^{c}	1.78^{a}	1.56^{b}	1.61^{b}	0.02	<0.0001
Mortality, %	2.00	6.00	2.00	0.00	-	0.273
d 21 to 32						
BWG, g	863.60^{a}	772.40^{c}	839.60^{ab}	803.60^{bc}	15.68	0.001
FI, g	1536.60	1503.59	1550.68	1528.30	14.79	0.155
FCR	1.78^{b}	1.95^{a}	1.85^{bc}	1.90^{ab}	0.04	0.002
Mortality, %	0.00	0.00	0.00	0.00	-	-
d 0 to 32						
BWG, g	1788.76^{a}	1603.00^{c}	1732.95^{ab}	1669.37^{bc}	27.51	<0.0001
FI, g	2873.20^{a}	2797.98^{b}	2849.75^{ab}	2811.69^{b}	18.57	0.020
FCR	1.61^{c}	1.75^{a}	1.64^{bc}	1.68^{b}	0.02	0.0003
FCR at 1.5kg	1.51^{c}	1.71^{a}	1.56^{bc}	1.62^{b}	0.03	0.0002
Mortality, %	2.00	6.00	2.00	0.00	-	0.273
Production index	348.10^{a}	275.29^{c}	330.53^{ab}	318.08^{b}	8.99	<0.0001

Abbreviation: NC, basal diet without NE challenge (negative control); PC, basal diet with NE challenge (positive control); GA, PC + 100 mg/kg VantiPEARL™; PFA, PC + 100 mg/kg ORSENTIAL™ extend; BW, body weight; BWG, body weight gain; FI, feed intake; FCR, feed conversion ratio; SEM, standard error of mean.

Each value is the mean value of 25 replicates.

[a-c] Means in the same row with different superscripts differ ($p < 0.05$).

Table 3. Effect of supplementing gallic acid and essential oils on relative immune organ weight in broilers challenged with coccidiosis

Items, g/kg of live BW %	NC	PC	GA	PFA	SEM	*p* -value
d 21						
Liver	3.38	3.59	3.47	3.60	0.09	0.226
Spleen	0.10	0.12	0.11	0.12	0.01	0.111
Bursa of Fabricius	0.16	0.13	0.15	0.14	0.01	0.073
d 32						
Liver	2.90	3.13	3.00	3.13	0.07	0.065
Spleen	0.09	0.10	0.09	0.10	0.01	0.353
Bursa of Fabricius	0.14[a]	0.12[b]	0.13[ab]	0.11[b]	0.01	0.041

Abbreviation: NC, basal diet without NE challenge (negative control); PC, basal diet with NE challenge (positive control); GA, PC + 100 mg/kg VantiPEARL™; PFA, PC + 100 mg/kg ORSENTIAL™ extend; BW, body weight; SEM, standard error of mean.
Each value is the mean value of 25 replicates.
[a,b] Means in the same row with different superscripts differ ($p < 0.05$).

Table 4. Effect of supplementing gallic acid and essential oils on intestinal lesion score at d 21 in broilers challenged with coccidiosis

Items	NC	PC	GA	PFA	SEM	*p*-value
Frequency of intestinal lesion score, %						
Upper-intestine						
Score 0	100.00	0.00	20.83	16.00		
Score 1	0.00	40.91	50.00	56.00		
Score 2	0.00	40.91	29.17	28.00	-	<0.0001
Score 3	0.00	18.18	0.00	0.00		
Score 4	0.00	0.00	0.00	0.00		
Average Score	0.00[c]	1.77[a]	1.08[b]	1.12[b]	0.13	
Middle-intestine						
Score 0	100.00	0.00	16.67	16.00		
Score 1	0.00	4.55	37.50	32.00		
Score 2	0.00	45.45	29.17	20.00	-	<0.0001
Score 3	0.00	50.00	16.67	32.00		
Score 4	0.00	0.00	0.00	0.00		
Average Score	0.00[c]	2.45[a]	1.46[b]	1.68[b]	0.17	

Abbreviation: NC, basal diet without NE challenge (negative control); PC, basal diet with NE challenge (positive control); GA, PC + 100 mg/kg VantiPEARL™; PFA, PC + 100 mg/kg ORSENTIAL™ extend; SEM, standard error of mean.

Lesion score: Lesion score was determined as follows: 0, normal; 1, contain some bloody spot and normal digesta; 2, serosal surface has red petechiae and contains some bloody spots; 3, contain large quantities of blood, caecal mould and mucus; 4, increased its thickness and contain bloody spots which have specific color and smell.

Average score is the mean value of 25 broiler per treatment.

[a-c] Means in the same row with different superscripts differ ($p < 0.05$).

Table 5. Effect of supplementing gallic acid and essential oils on intestinal lesion score at d 32 in broilers challenged with coccidiosis

Items	NC	PC	GA	PFA	SEM	*p* -value
Frequency of intestinal lesion score, %						
Upper-intestine						
Score 0	100.00	8.00	16.00	8.00		
Score 1	0.00	56.00	48.00	60.00		
Score 2	0.00	36.00	36.00	32.00	-	<0.0001
Score 3	0.00	0.00	0.00	0.00		
Score 4	0.00	0.00	0.00	0.00		
Average Score	0.00[b]	1.28[a]	1.20[a]	1.24[a]	0.11	
Middle-intestine						
Score 0	100.00	0.00	0.00	0.00		
Score 1	0.00	12.00	40.00	28.00		
Score 2	0.00	44.00	28.00	24.00	-	<0.0001
Score 3	0.00	44.00	32.00	48.00		
Score 4	0.00	0.00	0.00	0.00		
Average Score	0.00[b]	2.32[a]	1.92[a]	2.20[a]	0.14	

Abbreviation: NC, basal diet without NE challenge (negative control); PC, basal diet with NE challenge (positive control); GA, PC + 100 mg/kg VantiPEARL™; PFA, PC + 100 mg/kg ORSENTIAL™ extend; SEM, standard error of mean.

Lesion score: Lesion score was determined as follows: 0, normal; 1, contain some bloody spot and normal digesta; 2, serosal surface has red petechiae and contains some bloody spots; 3, contain large quantities of blood, caecal mould and mucus; 4, increased its thickness and contain bloody spots which have specific color and smell.

Average score is the mean value of 25 broiler per treatment.

[a,b] Means in the same row with different superscripts differ ($p < 0.05$).

Table 6. Effect of supplementing gallic acid and essential oils on caeca lesion score in broilers challenged with coccidiosis

Items	NC	PC	GA	PFA	SEM	*p* -value
Frequency of caeca lesion score, %						
d 21						
Score 0	100.00	63.64	75.00	72.00		
Score 1	0.00	36.36	25.00	28.00		
Score 2	0.00	0.00	0.00	0.00	-	0.018
Score 3	0.00	0.00	0.00	0.00		
Score 4	0.00	0.00	0.00	0.00		
Average Score	0.00[b]	0.36[a]	0.25[a]	0.28[a]	0.08	0.017
d 32						
Score 0	100.00	64.00	72.00	68.00		
Score 1	0.00	36.00	28.00	32.00		
Score 2	0.00	0.00	0.00	0.00	-	0.012
Score 3	0.00	0.00	0.00	0.00		
Score 4	0.00	0.00	0.00	0.00		
Average Score	0.00[b]	0.36[a]	0.28[a]	0.32[a]	0.08	0.011

Abbreviation: NC, basal diet without NE challenge (negative control); PC, basal diet with NE challenge (positive control); GA, PC + 100 mg/kg VantiPEARL™; PFA, PC + 100 mg/kg ORSENTIAL™ extend; SEM, standard error of mean.

Lesion score: Lesion score was determined as follows: 0, normal; 1, contain some bloody spot and normal digesta; 2, serosal surface has red petechiae and contains some bloody spots; 3, contain large quantities of blood, caecal mould and mucus; 4, increased its thickness and contain bloody spots which have specific color and smell.

Average score is the mean value of 25 broiler per treatment.

[a,b] Means in the same row with different superscripts differ ($p < 0.05$).

Table 7. Effect of supplementing gallic acid and essential oils on food-pad dermatitis lesion score in broilers challenged with coccidiosis

Items	NC	PC	GA	PFA	SEM	*p* -value
Frequency of foot-pad dermatitis lesion score, %						
Score 0	60.00	48.00	52.00	44.00	-	0.755
Score 1	16.00	20.00	28.00	36.00		
Score 2	24.00	24.00	16.00	16.00		
Score 3	0.00	8.00	4.00	4.00		
Average Score	0.64	0.92	0.72	0.80	0.18	0.736

Abbreviation: NC, basal diet without NE challenge (negative control); PC, basal diet with NE challenge (positive control); GA, PC + 100 mg/kg VantiPEARL™; PFA, PC + 100 mg/kg ORSENTIAL™ extend; SEM, standard error of mean.
Lesion score: Lesion score was determined as follows: 0, no lesion; 1, lesions cover less than 25% of the footpad; 2, lesions in wide areas, covering between 25% and 50% of the footpad; 3, more than 50% lesion on the footpads.
Average score is the mean value of 25 broiler per treatment.

Table 8. Effect of supplementing galic acid and essential oils on oocysts per gram in feces (OPG) in broilers challenged with coccidiosis

Items, 10^3	NC	PC	GA	PFA	SEM	*p* -value
OPG count						
d 22 (8 PI)	0^{c}	431.84^{a}	256.93^{b}	428.72^{a}	8.64	<0.0001
d 23 (9 PI)	0^{d}	354.19^{a}	224.08^{c}	306.11^{b}	5.82	<0.0001
d 24 (10 PI)	0^{c}	261.95^{a}	167.79^{b}	248.37^{a}	5.67	<0.0001
d 25 (11 PI)	0^{d}	198.77^{a}	125.17^{c}	180.53^{b}	4.19	<0.0001
d 26 (12 PI)	0^{c}	129.49^{a}	87.31^{b}	121.95^{a}	3.85	<0.0001

Abbreviation: NC, basal diet without NE challenge (negative control); PC, basal diet with NE challenge (positive control); GA, PC + 100 mg/kg VantiPEARL™; PFA, PC + 100 mg/kg ORSENTIAL™ extend; PI, post inoculation; SEM, standard error of mean.

Each value is the mean value of 25 broiler per treatment.

$^{a-d}$ Means in the same row with different superscripts differ ($p < 0.05$).

Table 9. Effect of supplementing gallic acid and essential oils on intestinal morphology in broilers challenged with coccidiosis

Items	NC	PC	GA	PFA	SEM	*p* -value
d 21						
Duodenum						
Villus height	1703.32[a]	1496.68[c]	1623.90[ab]	1546.84[bc]	38.46	0.002
Crypt depth	130.16	144.24	139.07	148.26	6.72	0.262
VH:CD	13.82[a]	10.83b	12.23[ab]	11.16[b]	0.64	0.006
Ileum						
Villus height	890.36[a]	816.57[b]	817.26[b]	810.86[b]	13.89	0.0002
Crypt depth	104.41[b]	140.83[a]	105.38[b]	116.23[b]	6.18	0.0001
VH:CD	9.35[a]	6.13[c]	8.35[ab]	7.47[bc]	0.48	<0.0001
Caeca						
Crypt depth	146.60	167.07	162.77	165.01	9.25	0.388
d 32						
Duodenum						
Villus height	2031.17[a]	1749.17[b]	1843.07[b]	1712.68[b]	51.01	0.0001
Crypt depth	148.01[b]	184.06[a]	150.67[b]	180.69[a]	9.53	0.009
VH:CD	15.48[a]	10.01[b]	13.27[a]	10.27[b]	0.88	<0.0001
Ileum						
Villus height	896.63[a]	805.66[b]	877.55[ab]	825.89[ab]	24.55	0.033
Crypt depth	101.34	122.42	108.70	110.42	6.66	0.168
VH:CD	9.53[a]	7.15[b]	8.39[ab]	8.29[ab]	0.50	0.012
Caeca						
Crypt depth	141.44	161.11	168.02	177.12	10.55	0.110

Abbreviation: NC, basal diet without NE challenge (negative control); PC, basal diet with NE challenge (positive control); GA, PC + 100 mg/kg VantiPEARL™; PFA, PC + 100 mg/kg ORSENTIAL™ extend; SEM, standard error of mean.

Each value is the mean value of 25 broiler per treatment.

[a-c] Means in the same row with different superscripts differ ($p < 0.05$).

Table 10. Effect of supplementing gallic acid and essential oils on blood profile in broilers challenged with coccidiosis.

Items	NC	PC	GA	PFA	SEM	*p* -value
d 21						
RBC, $10^6/\mu\ell$	2.50	2.22	2.46	2.30	0.08	0.060
WBC, $10^3/\mu\ell$	20.91^{b}	25.14^{a}	23.76^{a}	24.56^{a}	0.65	<0.0001
Heterophil, %	21.16^{c}	25.97^{a}	23.37^{b}	23.14^{b}	0.61	<0.0001
Lymphocyte, %	73.73	70.85	72.38	72.83	0.74	0.0626
d 32						
RBC, $10^6/\mu\ell$	2.00	1.90	1.97	1.92	0.08	0.816
WBC, $10^3/\mu\ell$	19.84^{c}	23.80^{a}	21.85^{b}	22.20^{ab}	0.65	0.001
Heterophil, %	24.33	25.32	24.59	24.03	0.47	0.266
Lymphocyte, %	70.82	71.61	70.23	71.42	0.84	0.645

Abbreviation: NC, basal diet without NE challenge (negative control); PC, basal diet with NE challenge (positive control); GA, PC + 100 mg/kg VantiPEARL™; PFA, PC + 100 mg/kg ORSENTIAL™ extend; RBC, red blood cell; WBC, white blood cell; SEM, standard error of mean.
Each value is the mean value of 25 broiler per treatment.
$^{a-c}$ Means in the same row with different superscripts differ ($p < 0.05$).

Table 11. Effect of supplementing gallic acid and essential oils on cecal bacterial counts in broilers challenged with coccidiosis

Items, log10 CFU/g	NC	PC	GA	PFA	SEM	*p* -value
d 21						
E. coli	6.76[c]	7.25[b]	7.18[b]	7.46[a]	0.05	<0.0001
Lactobacillus	8.65[a]	8.50[b]	8.45[b]	8.43[b]	0.05	0.005
d 32						
E. coli	6.70	6.84	6.75	6.79	0.07	0.516
Lactobacillus	9.28[a]	9.04[b]	9.36[a]	9.29[a]	0.03	<0.0001

Abbreviation: NC, basal diet without NE challenge (negative control); PC, basal diet with NE challenge (positive control); GA, PC + 100 mg/kg VantiPEARL™; PFA, PC + 100 mg/kg ORSENTIAL™ extend; SEM, standard error of mean.

Each value is the mean value of 25 broiler per treatment.

[a-c] Means in the same row with different superscripts differ ($p < 0.05$).