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Original Article

Evaluation of diclazuril and amprolium against *Eimeria* spp. in Vietnamese broilers: Implications for intestinal integrity and claudin gene expression

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Abstract

This study aimed to assess the resistance status of *Eimeria* spp. in Vietnam to diclazuril and amprolium and to evaluate their effects on intestinal recovery following infection. One hundred 12-day-old chicks were randomly allotted to four groups: a non-infected negative control (NC), an infected untreated as positive control (PC), an infected group treated with diclazuril (DI), and an infected group treated with amprolium (AM). At day 14, chicks from all groups except NC were orally challenged with 2×10^4 sporulated *Eimeria* oocysts (*E. tenella*, *E. maxima*, *E. acervulina*, and *E. mitis*). On day 7 post-infection, chickens were euthanized to assess gross and microscopic intestinal lesions and to analyze the expression of tight-junction genes. Anticoccidial resistance was determined using four indicators: anticoccidial index, percentage of optimum anticoccidial activity, reduction of lesion score, and reduction of oocyst production. The results showed that Eimeria isolate exhibited complete resistance to diclazuril across all four indices, while remaining fully sensitive to amprolium. Chicks in the AM group demonstrated markedly reduced oocyst shedding, lower gross and microscopic lesion scores, and enhanced expression of Claudin family genes, particularly Claudin-3, which plays a key role in reinforcing epithelial barrier integrity during recovery. These findings identify amprolium as an effective therapeutic option for controlling coccidiosis under the current field conditions in Vietnam and highlight the necessity of ongoing monitoring of anticoccidial drug resistance to inform sustainable poultry health management strategies.

Keywords: amprolium, anticoccidial resistance, diclazuril, *Eimeria* spp., gut health, tight junction genes, Vietnam

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Introduction

Coccidiosis, induced by protozoan parasites of the *Eimeria* genus, remains one of the most widespread intestinal diseases and a primary reason for substantial economic losses within the worldwide poultry production system. The disease is characterized by intestinal inflammation, hemorrhage, and necrosis of the mucosa, leading to impaired digestion and nutrient absorption, diarrhea, growth retardation, and high mortality in severe cases. The invasion and destruction of intestinal epithelial cells by *Eimeria* spp. increase intestinal permeability, resulting in the loss of nutrients and proteins that negatively affect digestive efficiency and production performance in poultry (Nabian *et al.*, 2018; Madlala *et al.*, 2021).

Because *Eimeria* oocysts are highly resistant to conventional disinfectants, controlling the disease through environmental management is difficult. Consequently, prevention and treatment in practical poultry production rely mainly on anticoccidial drugs. Among these, amprolium – a synthetic thiamine analogue – is widely used for both prophylaxis and therapy. Amprolium acts by competitively inhibiting thiamine transport into the parasite, leading to intracellular thiamine deficiency that interferes with carbohydrate metabolism and energy production during the schizogony stage, thereby suppressing parasite replication (Chapman *et al.*, 2022). Another commonly used compound, diclazuril, is a benzeneacetonitrile derivative of the triazinone family that disrupts both asexual (schizogony) and sexual (gametogony) developmental stages of *Eimeria* spp., causing degeneration of schizonts and gametocytes, and inhibiting oocyst shedding (Maes *et al.*, 1988).

For many decades, diclazuril and amprolium have been widely employed worldwide as key chemoprophylactic and therapeutic agents against *Eimeria* spp. infections in poultry (Flores *et al.*, 2022). However, growing evidence from recent field and laboratory studies indicates a concerning rise in anticoccidial resistance. For example, a sensitivity test of field isolate in Iran showed partial to complete resistance to diclazuril and amprolium + ethopabate. Similarly, studies conducted in Brazil revealed that several *Eimeria* isolate from commercial broiler farms were resistant or partially resistant to diclazuril. In a large-scale epidemiological investigation in Korean chicken farms, *Eimeria* field samples showed severe resistance to diclazuril and other commonly used anticoccidial drugs. In Vietnam, coccidiosis is widespread in both backyard and commercial production systems, with the highest prevalence observed in young chickens. *Eimeria* infection was detected in 50.92% of backyard-raised native chickens (Huynh *et al.*, 2016), while the infection rate in commercial broiler farms was approximately 40%, despite regular use of anticoccidial drugs for prevention and treatment (Pham *et al.*, 2023). These findings indicate that the long-term and widespread use of these anticoccidials has significantly reduced their effectiveness.

Eimeria infection can impair intestinal barrier integrity through disruption of the tight junction complex between adjacent epithelial cells. Alterations in tight junction-associated proteins, particularly claudins and zonula occludens-1 (ZO-1), may increase paracellular permeability, thereby facilitating the passage of luminal microorganisms, toxins, and other harmful substances across the intestinal epithelium and contributing to inflammatory responses (Collier *et al.*, 2008; Lee *et al.*, 2020). Claudins constitute a major family of transmembrane proteins that regulate selective paracellular transport, whereas ZO-1 serves as a scaffolding protein linking tight junction components to the actin cytoskeleton (Fanning *et al.*, 1998). Preservation of intestinal barrier function is therefore critical for maintaining gut health, nutrient utilization, and productive performance in poultry. Although the importance of tight junction proteins in epithelial barrier maintenance is well recognized, limited information is available regarding the effects of anticoccidial treatments on the expression of these molecular markers during *Eimeria* infection. In particular, the influence of commonly used anticoccidial drugs on tight junction-related gene expression in infected chickens has not been adequately investigated.

Previous studies investigating anticoccidial drug resistance have primarily focused on macroscopic indicators, including oocyst shedding, body weight gain, mortality, and gross lesion scores (Freitas *et al.*, 2023). Although these parameters are useful for evaluating infection severity and treatment efficacy, they provide limited information regarding microscopic intestinal damage and epithelial barrier integrity. In particular, the effects of diclazuril and amprolium on intestinal histopathology and tight junction-associated gene expression remain poorly characterized. A better understanding of these responses is important because alterations in epithelial barrier integrity may compromise intestinal function and overall poultry performance. Therefore, this study was undertaken to assess drug resistance and evaluate the effects of diclazuril and amprolium on intestinal health in broiler chickens experimentally infected with *Eimeria* spp., using indicators including body weight gain, oocyst shedding, gross and histopathological intestinal lesions, and the expression of genes related to tight junction integrity.

Materials and Methods

Ethical approval: All animal procedures were reviewed and approved by the Animal Ethics Committee of Hue University (Approval No. HUVN0040).

Animals: Broiler chicks at one day of age (MD02 strain) were obtained from Minh Du Co., Ltd., Vietnam. The chickens were housed in pens that had been treated at high temperature to ensure the absence of *Eimeria* oocyst contamination. To confirm the absence of natural coccidial infection, fecal collections were carried out each day from chickens aged 7 to 14 days and examined using the flotation technique. The chickens were fed a diet consisting of corn, soybean meal, fish meal, and rice bran, supplemented with

vitamins and minerals to meet their nutritional requirements.

Experimental design: At 12 days of age, 100 broiler chickens of comparable body weights were randomly assigned to four treatment groups: negative control (NC), positive control (PC), amprolium-treated group (AM), and diclazuril treated group (DI). Each treatment included 25 chickens, with 5 replicates per treatment (Table 1). Chickens in each treatment were housed in separate wire cages with individual fecal collection trays cleaned daily to minimize cross-contamination and reinfection. All chickens were weighed to record initial body weight. Following group allocation until the end experiment (21 days of age), chickens in the AM and DI groups received anticoccidial treatment at therapeutic doses according to the manufacturer's recommendations. Specifically, Amprolium 20% was administered therapeutically at the manufacturer's recommended dose of 10 g per 10 L of drinking water. Diclazuril 3% was administered therapeutically at a dose of 1 mL per 5 L of drinking water. The drugs were administered in drinking water after a 1–2 h water withdrawal period. Control groups (NC and PC) did not receive any treatment throughout the experimental period.

At 14 days of age, chickens in the AM and DI groups, as well as those in the PC group, were orally challenged with 2×10^4 *Eimeria* spp. oocysts per chicken. The isolate was a field strain collected from broiler farms in Hue City, Central Vietnam. Field isolates of *Eimeria* were identified by sequencing and confirmed to include four species: *E. tenella*, *E. acervulina*, *E. mitis*, and *E. maxima*. Seven days post-infection (dpi), all chickens were weighed to record final body weight and subsequently euthanized for necropsy to assess macroscopic and microscopic intestinal lesions, as well as the expression of tight junction–related genes in the intestinal epithelium.

Oocyst shedding determination: Oocyst shedding was quantified daily between 4 and 10 dpi using a sucrose flotation protocol. For each cage, fecal material was homogenized, and triplicate samples (2 g each) were prepared. Two grams of feces were weighed and placed into a container, and 28 mL of saturated sucrose solution was added. The mixture was thoroughly homogenized using a spatula, then filtered through a fine mesh sieve into a second container. The filtrate was mixed again with a Pasteur pipette, and while continuously stirring, a sub-sample was withdrawn. The suspension was stirred once more before filling the first compartment of the McMaster counting chamber with the sub-sample; the process was repeated to fill the second compartment. The chamber was allowed to stand for 5 minutes to permit the oocysts to float to the surface while debris settled at the bottom. The number of oocysts per gram (OPG) was then calculated by counting the oocysts within the grid area of both chambers (excluding those outside the squares), summing the total count, and multiplying the result by 50 to obtain the number of eggs per gram of feces.

Evaluation of gross intestinal lesions: Macroscopic lesions were evaluated in three intestinal segments (duodenum, ileum, and cecum) according to the method described by Johnson and Reid (Johnson and Reid, 1970; Ho *et al.*, 2021). Lesions were scored on a scale from 0 to 4 as follows: a score of 0 was assigned when no visible lesions were observed; a score of 1 indicated the presence of a few hemorrhagic spots on the intestinal mucosal surface; a score of 2 represented more numerous hemorrhagic spots and orange intestinal contents, with lesions affecting up to 25% of the observed area; a score of 3 indicated thickened, distended intestinal walls with or without blood clots, affecting approximately 25.1–50% of the affected intestinal area; and a score of 4 was given when the intestinal walls were severely thickened and distended with blood present in the lumen, involving more than 50% of the intestinal area or frequency observed, or when death occurred.

Evaluation of histopathological lesions: Histopathological lesions in the duodenum, ileum, and cecum were observed on tissue specimens stained with Hematoxylin and Eosin (H&E). For each sample, six sections spaced 200 µm apart were observed under a light microscope at 100× to assess the degree of microscopic damage to the intestinal epithelium. The severity of lesions was scored on a scale from 0 to +4 according to Balestrin *et al.* (2022). All H&E-stained slides were independently assessed by two pathologists to ensure objectivity, and a blinded evaluation procedure was applied to prevent any knowledge of treatment groups during analysis.

Evaluation of Eimeria drug resistance: The drug resistance of *Eimeria* spp. was evaluated based on four indices: Anticoccidial Index (ACI), Percent of Optimum Anticoccidial Activity (POAA), Reduction of Lesion Scores (RLS), and Relative Oocyst Production (ROP). When 3 or 4 of these indices indicated resistance, the *Eimeria* isolate was categorized as showing strong resistance. When 2 indices indicated resistance, it was classified as moderately resistant, and when 1 or 0 of the indices exhibited resistance, the isolate was considered sensitive to the tested drug.

The ACI was determined according to Zou *et al.* (2019) using the following formula:

$$ACI = (RBWG + SR) - (LSI + OI)$$

where relative body weight gain rate (RBWG, %) was calculated as:

$$RBWG = \frac{Average\ BW\ at\ day\ 21 - Average\ BW\ at\ day\ 12}{BW\ gain\ in\ the\ NC\ group} \times 100$$

where survival rate (SR, %) was calculated as:

$$SR = \frac{Total\ surviving\ chickens\ at\ day\ 21}{Total\ number\ of\ chickens\ at\ day\ 12} \times 100$$

where lesion score index (LSI) was obtained by multiplying the mean macroscopic lesion score of each group by 10. Lesion scores were evaluated on a scale from 0 to 4 according to Johnson and Reid (1970).

and, where oocyst index (OI, %) was calculated as:

$$OI = \frac{OPG\ of\ the\ treated - infected\ group}{OPG\ of\ the\ PC\ group} \times 100$$

The degree of drug resistance was determined based on the ACI value following Lan *et al.* (2017): *Eimeria* isolate with an ACI value ≥160 were considered sensitive, whereas those with ACI < 160 were considered resistant.

The POAA was determined according to Arabkhazaeli *et al.* (2013) using the following formula:

$$POAA = \frac{(GSR\ of\ medicated\ group\ -\ GSR\ of\ PC)}{(GSR\ of\ NC\ -\ GSR\ of\ PC)} \times 100$$

where the Growth and Survival Ratio (GSR) was calculated as follows:

$$GSR = \frac{Final\ body\ weight\ (day\ 21)}{Initial\ body\ weight\ (day\ 12)} \times 100$$

The drug resistance of *Eimeria* spp. was classified based on the POAA value following Arabkhazaeli *et al.* (2013): isolate with POAA > 50% were considered sensitive, whereas those with POAA ≤ 50% were considered resistant.

The RLS was calculated according to the lesion scoring system of Johnson and Reid (1970) as follows:

$$RLS = \frac{(Average\ lesion\ score\ of\ PC - Average\ lesion\ score\ of\ medicated\ group)}{Average\ lesion\ score\ of\ PC} \times \times 100$$

The interpretation of RLS followed McDougald *et al.* (1987): isolate with RLS ≥ 50% were considered sensitive, while those with RLS < 50% were considered resistant.

The ROP was determined following Lan *et al.* (2017), based on the OPG count, using the formula:

$$ROP = \frac{OPG\ of\ medicated\ group}{OPG\ of\ PC} \times 100$$

Drug resistance was interpreted according to Lan *et al.* (2017): isolate with ROP ≥ 15% were classified as resistant, while those with ROP < 15% were considered sensitive.

Determination of tight junction gene expression: On day 7 post-infection (21 days of age), a 5 cm segment from the middle portion of the small intestine was collected from 20 chickens across the four treatment groups (five chickens randomly selected from five independent cages per group) and preserved in 1.5 mL microcentrifuge tubes. Samples were immediately snap-frozen at -80°C for later assessment of gene expression. Total RNA was extracted from intestinal tissue using Freezol Reagent (Nanjing Vazyme Biotech Co., Ltd., China) following the manufacturer's protocol. RNA concentration and purity were measured using a NanoDrop Lite Spectrophotometer (Thermo Scientific, USA), yielding concentrations ranging from 600–1000 ng/µL and A260/280 ratios between 1.8 and 2.2. The extracted RNA was preserved at −80 °C until subsequent use. For each sample, 1 µg of total RNA was reverse-transcribed into cDNA by the FIREScript RT cDNA Synthesis MIX with oligo(dT) and random primers (Solid Biodyne, Estonia), following the manufacturer's protocol. All cDNA samples were preserved at -20°C until later analysis. RT-qPCR was performed on a QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific, USA) using the PCR Supermix Kit (Solid Biodyne, Estonia). Relative target gene expression was normalized to RPS17 as the housekeeping gene for each sample. Primer sequences used for gene analysis (E-cadherin, Occludin, Zonula occludens (ZO)-1, Claudin-1, Claudin-2, Claudin-3) are shown in Table 2.

PCR amplification was undertaken with the thermal cycling parameters described below: an initial denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min (Pham *et al.*, 2021). Each treatment group included five independent biological samples, and each reaction was conducted in triplicate on the same plate. The 2^−ΔΔCt approach was applied to calculate relative mRNA expression levels (Livak and Schmittgen, 2001).

Statistical analysis: Statistical analyses were performed using SPSS software. The cage was considered the experimental unit for average daily gain and fecal oocyst shedding, whereas one bird per cage was used as the experimental unit for lesion score and gene expression analyses. Lesion score and gene expression data were analyzed by one-way ANOVA followed by Tukey's test. Fecal oocyst shedding was analyzed using a repeated-measures generalized linear model. Differences were considered significant at $P < 0.05$.

Table 1 Experimental design and treatment groups in broilers challenged with *Eimeria* spp.

Treatments	NC	PC	AM	DI
Challenged with *Eimeria*	No	Yes	Yes	Yes
Drug administration	No	No	Amprolium	Diclazuril

Notes: NC = non-infected negative control; PC = infected untreated as positive control; AM = infected group treated with amprolium; DI = infected group treated with diclazuril.

Table 2 Primer sequences used for tight junction genes analysis by RT-qPCR.

Gene Name		Primer sequences (5' to 3')	GenBank Accession No.
Housekeeping genes			
RPS17	Forward	AAGCTGCAGGAGGAGGAGAGG	NM_204217.1
	Reverse	GGTTGGACAGGCTGCCGAAGT	
Junctional molecular genes			
E-Cadherin	Forward	TCACGGGCAGATTTCTAT	NM_001039258.2
	Reverse	CACGGAGTTCGGAGTTTA	
Occludin	Forward	ACGGCAAAGCCAACATCTAC	NM_205128.1
	Reverse	ATCCGCCACGTTCTTCAC	
ZO-1	Forward	AAGTGGGAAGAATGCCAAAA	XM_015278981.2
	Reverse	GGTCCTTGGATCCCGTATCT	
Claudin-1	Forward	AAGGTGTACGACTCGCTGCT	NM_001013611.2
	Reverse	CAGCAACAAACACACCAACC	
Claudin-2	Forward	CCTGCTCACCCTCATTGGAG	NM_001277622.1
	Reverse	GCTGAACTCACTCTTGGGCT	
Claudin-3	Forward	GCCAAGATCACCATCGTCTC	NM_204202.1
	Reverse	CACCAGCGGGTTGTAGAAAT	

Results

Evaluation of the effects of anticoccidial drugs on body weight gain: Body weight gain results in the experimental chickens are shown in Table 3. The positive control group showed the lowest body weight gain of 52.36 ± 4.17 g/chicken, which differed significantly from the negative control group (77.64 ± 4.34 g/chicken) ($P < 0.05$). The DI group achieved a mean gain of 60.67 ± 4.27 g/chicken, which was higher than that of the PC group but not significantly different ($P > 0.05$). Weight gain in the DI group was significantly lower than that in the NC ($P < 0.05$). In contrast, the amprolium group exhibited a markedly higher gain of 89.22 ± 4.43 g/chicken, showing a significant difference ($P < 0.05$) compared with both the PC and DI groups.

Oocyst shedding: The oocyst shedding patterns in all chicken groups are presented in Table 4. The NC group remained free of oocysts throughout the study, confirming the absence of natural *Eimeria* infection and supporting the validity of the experimental model. In contrast, oocyst shedding in the PC began on day 4 post-infection, peaked on day 5 (6.83 ± 0.29), and gradually declined thereafter until day 7.

Oocyst counts in the AM group remained consistently lower than those in both the PC and DI groups throughout the observation period. Moreover, chickens treated with amprolium exhibited a significantly lower total oocyst output (19.65 ± 0.15) compared with the PC group ($P < 0.05$). Meanwhile, the total oocyst output in the DI group (21.70 ± 0.14) was lower than that observed in the PC group (23.21 ± 0.18).

Table 3 Effects of amprolium and diclazuril on the body weight gain of experimental chickens.

Treatment groups	Initial body weight ($\bar{x}$ ± SD, g)	Final body weight ($\bar{x}$ ± SD, g)	Weight gain ($\bar{x}$ ± SD, g)
NC	110.52^{a} ± 13.25	188.16^{a} ± 30.92	77.64^{a} ± 4.34
PC	117.23^{a} ± 13.08	169.59^{b} ± 25.58	52.36^{b} ± 4.17
AM	111.77^{a} ± 13.19	200.89^{a} ± 35.76	89.22^{a} ± 4.43
DI	111.59^{a} ± 11.98	171.82^{b} ± 33.11	60.67^{b} ± 4.27

Notes: NC = non-infected negative control; PC = infected untreated as positive control; AM = infected group treated with amprolium; DI = infected group treated with diclazuril. Means within the same column followed by different superscript letters differ significantly ($P < 0.05$).

Table 4 Oocyst counts (Ln(OPG+1)) in fecal samples of chickens from 3-7 dpi.

Treatment groups	3 dpi	4 dpi	5 dpi	6 dpi	7 dpi	Total
NC	0	0	0	0	0	0
PC	0	5.07 ± 0.11	6.83 ± 0.29	5.85 ± 0.14	5.46 ± 0.18	23.21^{a} ± 0.18
AM	0	3.54 ± 0.08	5.53 ± 0.11	5.15 ± 0.25	5.43 ± 0.17	19.65^{b} ± 0.15
DI	0	4.56 ± 0.07	5.78 ± 0.11	5.77 ± 0.26	5.59 ± 0.10	21.70^{a} ± 0.14

Note: dpi = days post-infection; NC = non-infected negative control; PC = infected untreated as positive control; AM = infected group treated with amprolium; DI = infected group treated with diclazuril. Values are expressed as mean ± standard deviation (SD). OPG values were transformed to natural logarithm scale [Ln(OPG+1)]. Values within the same column bearing different superscript letters (a, b) differ significantly ($P < 0.05$).

Assessment of gross lesion severity: The results of gross lesion evaluation in the duodenum, ileum, and cecum are presented in Figure 1. In the duodenum, chickens in NC group showed very low lesion scores (0.8 ± 0.37), which were significantly lower ($P < 0.05$) than those observed in the PC group (3.0 ± 0.00), where severe lesions were recorded. Chickens treated with diclazuril (2.80 ± 0.44) and amprolium (2.80 ± 0.44) exhibited lower lesion scores than the PC group; however, these differences were not statistically significant ($P > 0.05$). In the ileum, the lesion scores were generally low, ranging from 0.00 in the NC group to 1.33 ± 0.70 in the PC group, with no statistically significant differences observed among the treatment groups ($P > 0.05$). In the cecum, the PC group showed the highest lesion score (3.00 ± 0.10), which was significantly higher ($P < 0.05$) than that in the NC group (0.06 ± 0.06) and the amprolium-treated group (0.80 ± 0.34). The diclazuril-treated group (2.93 ± 0.28) had a slightly lower lesion score than the PC group, with no statistically significant difference ($P > 0.05$).

Assessment of microscopic lesion severity: The microscopic lesion scores in the duodenum, ileum, and cecum after *Eimeria* spp. infection are presented in Figures 2 and 3. In the duodenum, the PC group exhibited the highest lesion severity (3.58 ± 0.20), which was significantly different from the NC group (0.63 ± 0.20; $P < 0.05$). The microscopic lesions in the AM group were significantly lower than those in the PC group ($P < 0.05$). The DI group (2.72 ± 0.40) showed a slight reduction in lesion severity compared to the PC group but remained significantly higher than the AM group (1.74 ± 0.20; $P < 0.05$). In the ileum, lesions were mainly observed in the PC (1.58 ± 0.12) and DI (1.60 ± 0.32) groups, both significantly higher than those observed in the NC group (0.06 ± 0.06). Meanwhile, the AM group (0.50 ± 0.04) showed low lesion severity, with no significant difference from the NC group. In the cecum, the PC group showed the highest lesion score (2.38 ± 0.34), significantly different from the NC group (0.12 ± 0.08). The DI group (1.24 ± 0.54) showed reduced lesion severity but did not differ significantly from the PC group. In contrast, the AM group (0.24 ± 0.09) maintained low lesion severity, similar to the NC group and significantly lower than the PC group ($P < 0.05$).

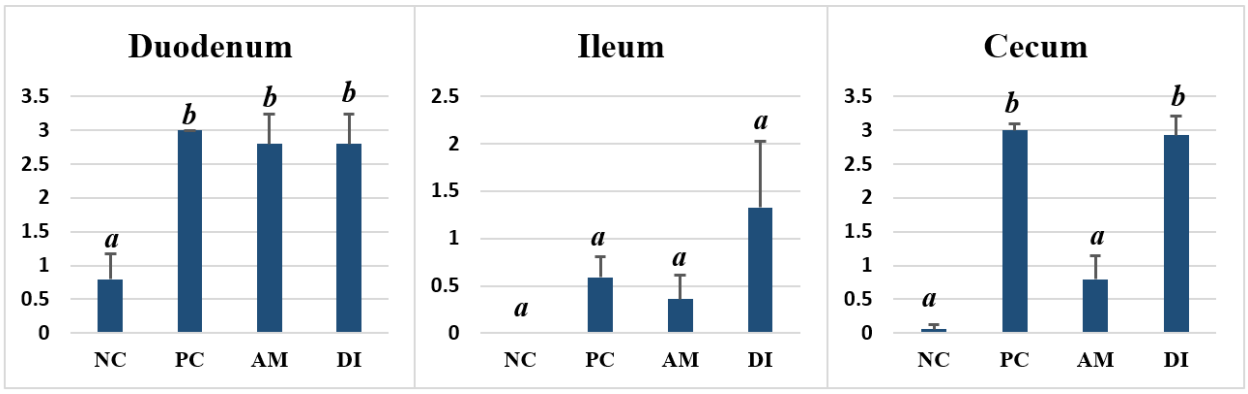

Figure 1 Gross lesion score. The results are shown as mean ± SE. NC = non-infected negative control; PC = infected untreated as positive control; AM = infected group treated with amprolium; DI = infected group treated with diclazuril. The letters "a" and "b" denote statistically significant differences between treatments ($P < 0.05$).

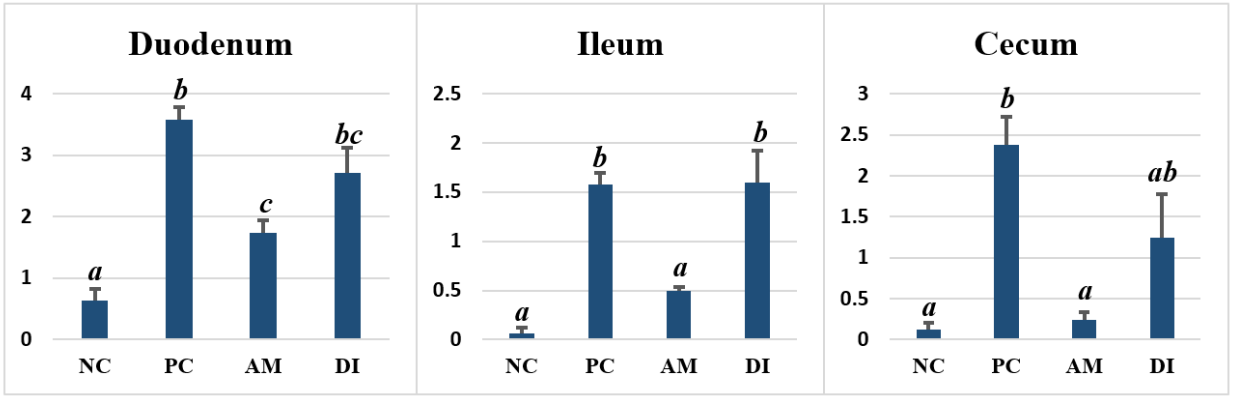

Figure 2 Microscopic Lesion score. The results are shown as mean ± SE. NC = non-infected negative control; PC = infected untreated as positive control; AM = infected group treated with amprolium; DI = infected group treated with diclazuril. The letters "a" and "b" denote statistically significant differences between treatments ($P < 0.05$).

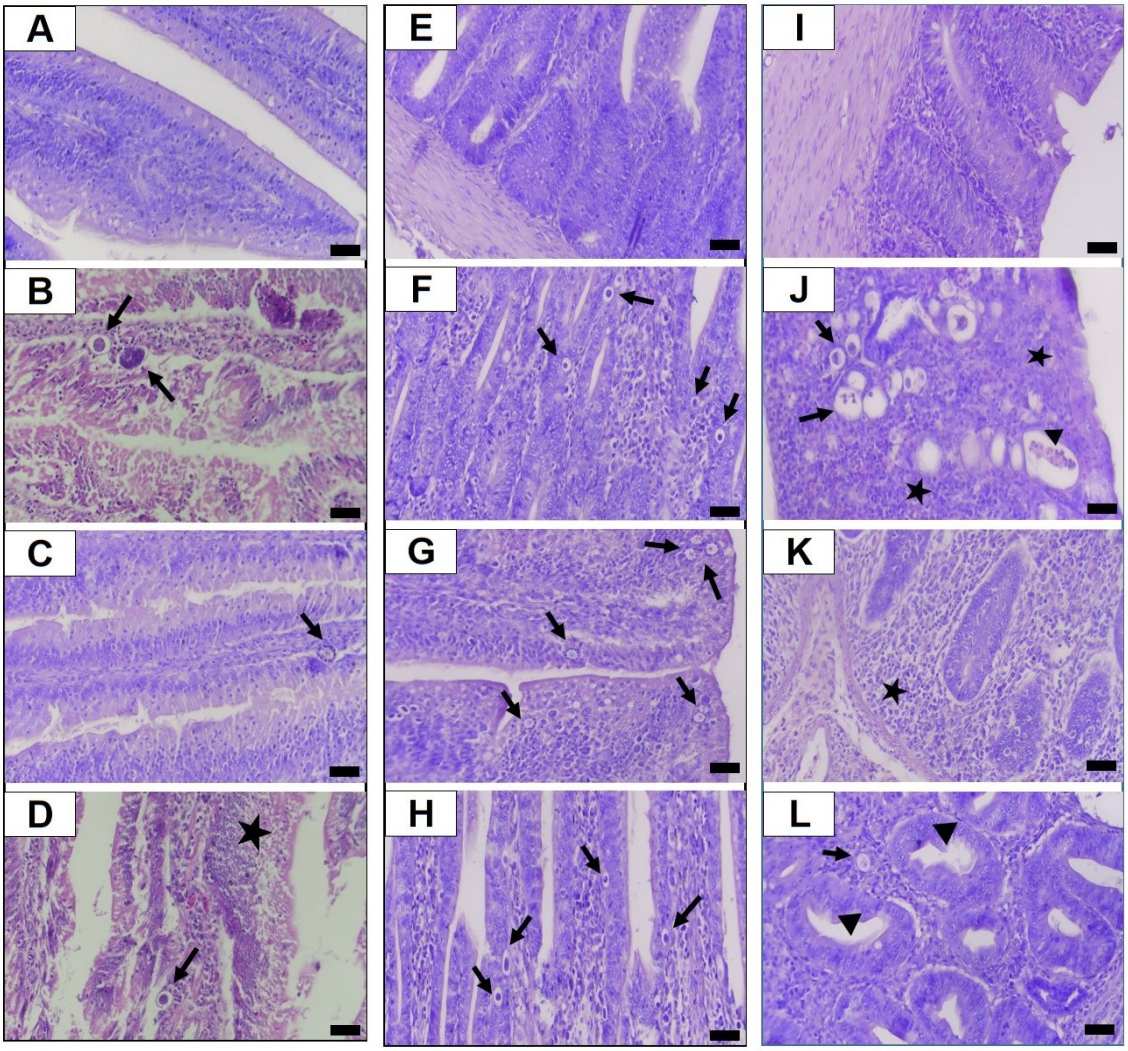

Figure 3 Histological images of the chicken intestine. The duodenum of chickens in the non-infected negative control group (NC; A), infected untreated positive control group (PC; B), infected group treated with amprolium (AM; C), and infected group treated with diclazuril (DI; D) at 40× magnification. The ileum of chickens in the NC (E), PC (F), AM (G), and DI (H) groups at 40× magnification. The cecum of chickens in the NC (I), PC (J), AM (K), and DI (L) groups at 40× magnification. The arrows indicate schizonts and oocysts of coccidia invading and disrupting intestinal epithelial cells, while arrowheads indicate destruction of intestinal crypts and stars indicate areas of inflammatory cell infiltration in the intestinal tissue. Scale bars = 50 μm.

Evaluation of anticoccidial drug resistance: Drug susceptibility of *Eimeria* spp. was examined using four combined indices: ACI, POAA, RLS, and ROP, as presented in Supplementary Tables S1 – S3 and Table 5.

The overall resistance evaluation of *Eimeria* spp. isolate indicates that the isolate remained sensitive to amprolium but showed evidence of resistance to diclazuril. Notably, the diclazuril-treated group exhibited resistance across all four indicators (4/4), confirming a high level of resistance.

Expression levels of tight junction associated genes: The expression levels of tight junction–associated genes (Claudin-1, Claudin-2, Claudin-3, ZO-1, Occludin, and E-cadherin) in the experimental groups are presented in Figure 4. No significant differences were observed among the experimental groups in the expression of ZO-1, Occludin, or E-cadherin. Notably, chickens infected with *Eimeria* and treated with amprolium exhibited a tendency toward increased expression of Claudin family genes, including Claudin-1, Claudin-2, and Claudin-3. In particular, the amprolium-treated group showed a statistically significant upregulation of Claudin-3 expression compared with the other three groups.

Discussion

Minor gross and microscopic lesion scores were occasionally observed in the negative control group. However, no oocyst shedding was detected in these birds throughout the study, indicating the absence of *Eimeria* infection or cross-contamination. Therefore, these minimal lesions are more likely attributable to normal biological variation, physiological mucosal turnover, minor mechanical irritation, or other non-specific factors rather than coccidial infection. The assessment of anticoccidial drug resistance in *Eimeria* spp. using combined indicators such as the ACI,

POAA, RLS, and ROP has been widely reported in prior investigations. In the present study, we also applied these four parameters to assess the resistance status of diclazuril and amprolium against *Eimeria* species isolated in Vietnam. The results demonstrated that the isolates were resistant to diclazuril, whereas they remained sensitive to amprolium.

Studies have demonstrated that resistance to diclazuril in *Eimeria* spp. is a practical concern, particularly in species such as *E. acervulina* and *E. tenella* (Arabkhazaeli *et al.*, 2013; Flores *et al.*, 2022). Continuous or improper use of diclazuril may contribute to the emergence of drug-resistant variants (Kawazoe and Fabio, 1994; Lan *et al.*, 2017). Conversely, several field studies have demonstrated that amprolium remains effective against many *Eimeria* isolate (Ahmed *et al.*, 2023; Chalalai *et al.*, 2025, Karmakar *et al.*, 2025). Under the conditions of this experiment, amprolium exhibited an advantage over diclazuril in controlling lesions, especially in the cecum. These findings align with prior reports on the superior efficacy of amprolium in controlling *E. tenella* (Chalalai *et al.*, 2025). Diclazuril only slightly reduced the number of oocysts, but the reduction was not statistically significant compared to the positive control group. This finding suggests that the field isolate collected in Hue City, Vietnam exhibited resistance to diclazuril under the conditions of the present study.

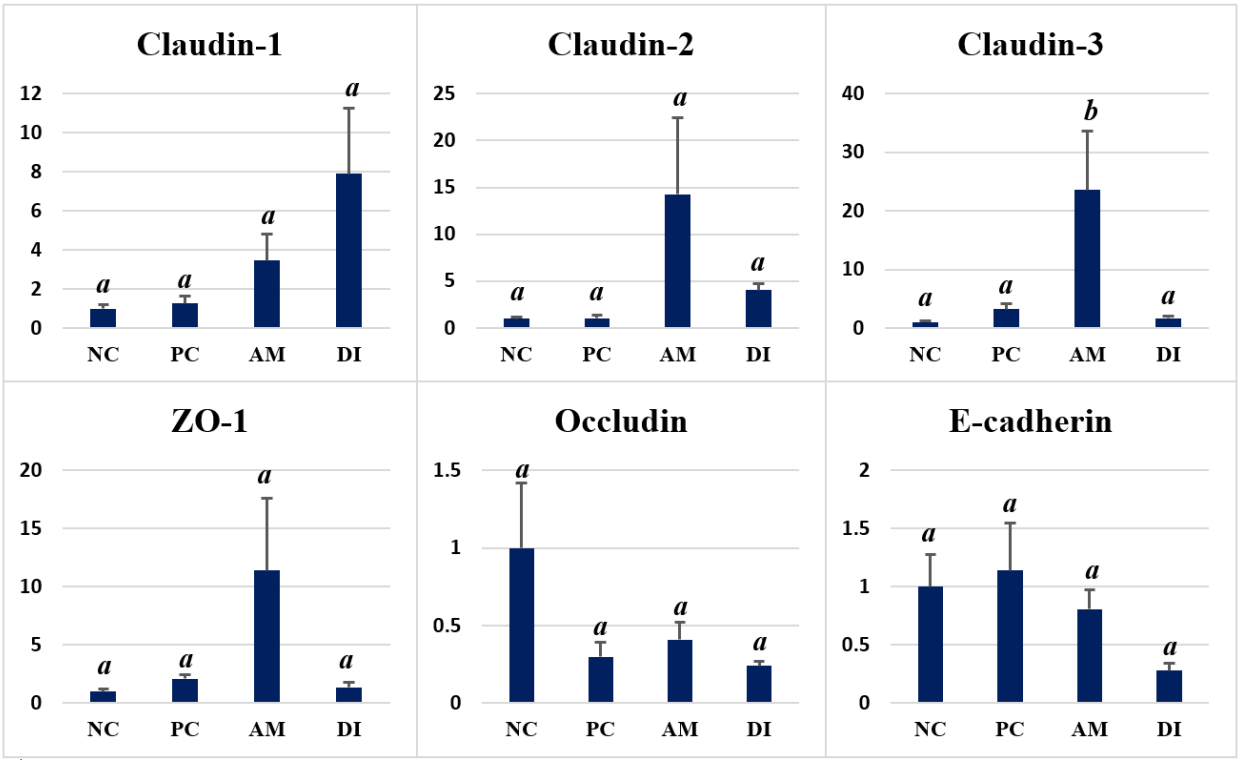

Figure 4 mRNA expression levels of tight junction genes. The results are shown as mean ± SE. NC = non-infected negative control; PC = infected untreated as positive control; AM = infected group treated with amprolium; DI = infected group treated with diclazuril. Different letters (a, b) indicate statistically significant differences among treatments ($P < 0.05$).

Table 5 Summary evaluation of anticoccidial drug resistance.

Treatment groups	ACI	POAA	RLS	ROP	Overall assessment
Diclazuril	+	+	+	+	+
Amprolium	-	-	-	-	-

Note: ACI – Anticoccidial Index; POAA – Percent of Optimum Anticoccidial Activity; RLS – Reduction of Lesion Scores; ROP – Relative Oocyst Production. + indicates that *Eimeria* spp. have developed resistance to the drug; - indicates that *Eimeria* spp. remain sensitive to the drug.

Amprolium demonstrated beneficial effects not only on macroscopic indicators, such as reducing oocyst shedding and lowering gross lesion scores in the intestine, but also produced favorable outcomes at the microscopic level, including decreased histopathological damage (duodenum, ileum, cecum) and enhanced expression of tight-junction–associated genes in the small intestine. These findings indicate that the use of amprolium for prevention and treatment may inhibit the replication of coccidia within intestinal epithelial cells, thereby reducing epithelial cell rupture during the parasite's life cycle and consequently lowering gross lesion scores as well as microscopic structural alterations. Tight-junction proteins of the Claudin family play a critical role in preserving intestinal barrier integrity through the regulation of paracellular permeability and preventing the translocation of pathogens and toxins across the

mucosal surface (Lu *et al.*, 2013; Garcia-Hernandez *et al.*, 2017, Suzuki, 2021). Among them, Claudin-3 is particularly important due to its strong sealing properties, contributing significantly to the restoration of epithelial tightness during and after enteric infections (Milatz *et al.*, 2010). The upregulation of Claudin-3 observed in this study is therefore consistent with a protective response, indicating that amprolium treatment not only mitigates epithelial damage but may also promote barrier reinforcement at the molecular level (McDougald *et al.*, 1987). The observed reduction in histopathological lesion scores in the amprolium-treated group further suggests improved recovery of the intestinal epithelium in chickens following infection.

A limitation of the present study is that the expression of tight junction-related genes was evaluated only at the mRNA level. Neither tight junction protein abundance nor functional intestinal permeability was assessed. Therefore, the observed transcriptional changes should be interpreted with caution, as they may not necessarily reflect corresponding alterations in protein expression or barrier function. Future studies incorporating protein-level analyses and direct measurements of intestinal permeability are warranted to provide a more comprehensive understanding of intestinal epithelial integrity during coccidial infection and recovery.

In conclusion, the findings of this study indicate that the *Eimeria* population evaluated in Hue City, Vietnam exhibited resistance to diclazuril under the present experimental conditions, while remaining susceptible to amprolium. The use of amprolium for both prevention and treatment resulted in a marked reduction in oocyst shedding, decreased gross and microscopic intestinal lesions, and was associated with increased expression of tight junction-related genes during recovery. These results highlight amprolium as a viable therapeutic option for managing coccidiosis under current field conditions and underscore the need for continued monitoring of anticoccidial drug resistance in poultry production systems.

Conflicts of interest: The authors have no conflicts of interest to declare.

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