Inhibitory effect of morin on aldolase 2 from Eimeria tenella

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ABSTRACT

Eimeria tenella (E. tenella) is a protozoal parasite that can cause severe cecal lesions and death in chickens, seriously harming the chicken industry. Conventional control strategies mainly rely on anticoccidial drugs. However, the emerging problems of anticoccidial resistance and drug residues necessitate exploring potential drug targets for developing new anticoccidial drugs. Fructose-1,6-bisphosphate aldolase (ALD) is an essential enzyme for parasite energy metabolism that has been considered a potential drug target. In this study, we analyzed the molecular and biochemical properties of E. tenella ALD2 (ETALD2). ETALD2 mRNA expression was highest in second-generation merozoites, whereas the protein level was highest in unsporulated oocysts. Indirect immunofluorescence showed that ETALD2 was mainly distributed in sporozoite cytoplasm. The natural product inhibitor (morin) was screened by computer-aided drug screening. Enzyme kinetic and inhibition kinetic assays showed that morin had a good inhibitory effect on ETALD2 activity (IC50 = 10.37 μM, Ki = 48.97 μM). In vitro inhibition assay demonstrated that morin had an inhibitory effect on E. tenella development, with an IC50 value of 3.98 μM and drug selection index of 177.49. In vivo, morin significantly improved cecal lesions (p < 0.05) and reduced oocyst excretion (p < 0.05) in E. tenella-infected chickens compared with the untreated group. The anticoccidial index of the group receiving 450 mg morin per kg feed was 162, showing a good anticoccidial effect. These findings suggest that ETALD2 could be a novel drug target for E. tenella treatment, and morin should be further evaluated as a therapeutic candidate for chicken coccidiosis.

1. Introduction

Coccidiosis is one of the most critical diseases in chickens (Blake et al., 2020; El-Shall et al., 2022; Lee et al., 2022) caused by the coccidian parasites Eimeria species infecting the intestinal epithelial cells of chickens. Eimeria species have a worldwide distribution and seriously harm the chicken industry, resulting in substantial economic losses (El-Liu et al., 2020). The annual cost of coccidiosis prevention and treatment exceeds USD 14.5 billion worldwide (Blake et al., 2020). Among the seven known species (Zhang et al., 2013), Eimeria tenella (E. tenella) is the most common and clinically pathogenic species that can cause severe cecal lesions and death in chickens (Zhang et al., 2022). Although coccidia control mainly depends on anticoccidial drugs, there are growing concerns about anticoccidial resistance and drug residues. Thus, more efforts are required to identify potential drug targets and develop new anticoccidial therapeutics for controlling coccidiosis in chickens.

During the endophytic developmental stage, the parasitic protozoa mainly live in hypoxic or anaerobic environments and thus use glycolysis as one of the primary ways to obtain energy. Fructose-1,6-bisphosphate aldolase (EC:4.1.2.13) is an essential enzyme for glycolysis. This enzyme catalyzes the reversible cleavage of fructose-1,6-
biphosphate (FBP) to dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP) (Yang et al., 2019). Aldolase plays a central role in glycolysis, gluconeogenesis, and fructose metabolism (Bu et al., 2018; Pirovich et al., 2021). Previous studies have reported that aldolase (ALD) is essential for energy metabolism in some protozoan parasites (Dux et al., 2006; Cáceres et al., 2010; Pirovich et al., 2021). Therefore, ALD has been considered a potential antiprotozoal drug target. However, the molecular and biochemical properties of E. tenella aldolase 2 (EtALD2) have been poorly studied.

Morin is a vital flavonoid compound in mulberry, with anti-inflammatory (Wei et al., 2015), antibacterial (Li et al., 2020), and anti-tumor effects (Gao et al., 2021). While morin has been extensively researched, there are few reports on its antiparasitic effects. Preliminary virtual screening and molecular docking suggested that morin may have high inhibitory activity on EtALD2.

In this study, we investigated the molecular and biochemical properties of EtALD2 in E. tenella for the first time. Additionally, we analyzed the inhibitory effects of the natural product morin on EtALD2 enzymatic activity. Our results suggest EtALD2 as a potential drug target against E. tenella, and morin may be a promising anticoccidial drug candidate for chicken coccidiosis.

2. Materials and methods

2.1. Animals and parasites

Chickens used in this study were bred by the Institute of Animal Science, Guangdong Academy of Agricultural Sciences (GAAS) and then transported to the experimental animal house of the Institute of Animal Health, GAAS. The Guangdong strain of E. tenella was isolated and preserved by the Laboratory of Parasitic Biology, Institute of Animal Health, GAAS, with propagation every four months. Unsporulated oocysts (UO) were isolated from the cecal content of 2-week-old chickens (Vazyme, Nanjing, China). The oocysts were incubated overnight at 4 °C with shaking for 1 h at 37 °C. For Western blot analysis, blocked membranes were incubated with the corresponding secondary antibody. After washing in PBS, membranes were incubated with the corresponding secondary antibody. After washing in PBS, membranes were incubated with the corresponding secondary antibody. After washing in PBS, membranes were incubated with the corresponding secondary antibody. After washing in PBS, membranes were incubated with the corresponding secondary antibody.
antibodies, goat anti-mouse IgG-HRP (Horseradish Peroxidase-conjugated) antibodies (1:2000, CWBIO, Beijing, China) and rabbit anti-chicken IgG-HRP antibodies (1:2000, Cell Signaling Technology, Shanghai, China) for 1 h at 37 °C. After washing with PBST, immunoreactive bands were visualized using the diaminobenzidine (DAB) Western blotting detection system (Beyotime, Shanghai, China) for enhanced chemiluminescence (ECL).

2.6. Transcriptional level analysis of EtALD2

Quantitative reverse transcription PCR (qRT-PCR) was used to analyze the differential transcriptional level of EtALD2 using cDNA isolated from E. tenella at the four developmental stages as a template. Et18srRNA was selected as the reference gene and EtALD2 gene as the target gene. Primers for qRT-PCR were designed to analyze the differences in EtALD2 mRNA expression at the four developmental stages of E. tenella. The primer sequences for the target gene were EtALD2-F: 5'-TCGATGGGGTGGAAGAACAC-3' and EtALD2-R: 5'-TATCCCCGGAATTGGATCCCTCC-3' and 18srRNA-R: 5'-CCTGCTGCTCTTCTTAGATG-3'. qPCR reactions (10 μL) contained cDNA (1 μL), forward and reverse primers (10 μmol/L, 0.8 μL of each), TB Green Premix Ex TaqII (5 μL, TaKaRa, Guangzhou, China), and ddH₂O (2 μL). qPCR cycling program was 95 °C for 30 s; 35 cycles of 95 °C for 15 s, 60 °C for 30 s; 95 °C for 10 s; and melt curve stage at 65–95 °C, increasing 0.5 °C every 5 s. Each sample was examined in triplicates. The relative expression levels of EtALD2 in the different developmental stages were calculated by the 2⁻ΔΔCt method.

2.7. Translation level analysis of EtALD2

The EtALD2 protein levels were analyzed by Western blot. Samples (0.2 g) of E. tenella four developmental stages (UO, SO, Spz, and Mrz) were placed into 1.5 mL centrifuge tubes. After adding 300 μL radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) and 3 μL protease inhibitor (Sigma-Aldrich, Guangzhou, China), centrifuge tubes were oscillated at 60 HZ for 40 min to extract total proteins. The total proteins were separately extracted from the four developmental stages, and the protein concentration was determined using the Bradford Protein Concentration Assay Kit. Based on the measured protein concentrations, the amount of protein loaded in SDS-PAGE from the four stages was adjusted to keep final concentrations consistent. Western blot was performed using mouse anti-EACTIN monoclonal antibodies and mouse polyclonal antibodies against EtALD2 (prepared by GenScript Company, Nanjing, China) as primary antibodies. EtACTIN was used as a reference. The secondary antibody was goat anti-mouse IgG-HRP (1:2000, CWBIO, Beijing, China). The results were analyzed using ImageJ software.

2.8. Indirect immunofluorescence of EtALD2 localization in sporozoites

We determined the localization of EtALD2 protein in sporozoites by indirect immunofluorescence assay (IFA). The sporozoites were fixed on slides with 4% paraformaldehyde for 30 min, permeated with 1% Triton® X-100 for 25 min, and blocked with 10% bovine serum albumin (BSA, Sangon Biotech, Shanghai, China) for 1 h. The slides were then incubated with mouse anti-Δ2ΔALD2 polyclonal antibodies (1:300) and mouse pre-immune serum as a negative control (1:100) overnight at 4 °C and with AlexaFlour™ 488 rabbit anti-mouse-IgG-R (1:500, Cell Signaling Technology, Shanghai, China) for 1 h at 37 °C in the dark. Nuclei were stained with 10 μg/mL of 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) for 10 min at room temperature. After three washes with phosphate buffer saline (PBS), the sporozoites were examined for fluorescent staining under a Zeiss LSM710 confocal microscope (Zeiss Microscopy, White Plains, NY, USA).

2.9. Analysis of EtALD2 enzyme kinetics and inhibition

We evaluated the catalytic activity of EtALD2 on fructose 1,6-bisphosphate (FBP or F16BP) cleavage by measuring the NADH consumption rate using the change in relative fluorescence units (ΔRFU), measured by Varioskan™ LUX with an excitation wavelength of 340 nm and an emission wavelength of 460 nm. Reactions were conducted for 30 min at 27.5 °C in 50 mM Tris-HCl buffer (pH 7.5, 100 μL total volume). The reaction system contained 75 μg EtALD2, 0.3 mM NADH (Sigma-Aldrich), 0.2 mg/mL BSA, 0.2 U/mL triosephosphate isomerase (TPi, Sigma-Aldrich), 2.25 U/mL α-glycerophosphate dehydrogenase (DHG, Sigma-Alrdich), and 0.1 mM FBP (Sangon Biotech). EtALD2 enzyme kinetic Michaelis-Menten curves were determined using different substrate concentrations, including NADH (3.125, 6.25, 12.5, 25, 50, and 100 μM) and FBP (25, 50, 75, 100, 200, and 300 μM). One unit (U) of EtALD2 activity represented the amount of enzyme that catalyzes the cleavage of fructose-1,6-bisphosphate to α-glycerol phosphate (glycerol-3-phosphate) and produces 1.0 mol/L of NADH per minute at 27.5 °C. Dimethyl sulfoxide (DMSO, Sigma-Aldrich) was used to dissolve morin (Yuanye, Shanghai, China) at a storage concentration of 12.5 mM. The inhibition constant (Ki) of morin on EtALD2 was determined. The reaction system (100 μL) contained 75 ng EtALD2, 0.2 mg/mL BSA, 0.2 U/mL TP1, 2.25 U/mL GDH, 1 mM FBP/0.5 mM NADH, different concentrations of NADH/FBP, and 25 μM morin. All experiments were performed using 75 ng of EtALD2 and repeated three times. Kinetic data were plotted using GraphPad Prism software. The initial rate (RFU/min) was determined based on Michaelis-Menten enzyme kinetics, and Km, Vmax, Kcat, Ki, and IC50 values of the substrate were calculated.

2.10. MTT cytotoxicity assay

Referring to Jin et al. (2019), Madin-Darby bovine kidney cells (MDBK, Jennio, Guangzhou, China) grown to 80% confluency were digested with 0.25% trypsin when they reached 80% confluency. Then, 2 mL cells of each group were set, with continuing incubation at 5% CO₂/37 °C. After 48 h incubation, 10 μL MTT (2,5-diphenyl-2H-tetrazolium bromide) reagent (5 mg/mL, Roche, Beijing, China) was added to each well and then cultured in the cell culture incubator for 4 h. A 100 μL MTT solution was added to dissolve Metzan crystal in each well, followed by shaking on a rotating shaker for 10 min. A multifunctional microplate reader was used to measure the absorbance at 550 nm. The OD550 values of the experimental and the blank control groups were determined to judge the cell viability and the toxicity of the inhibitors on the cells. Data were processed using GraphPad Prism7 software, and CC50 values (half cytotoxicity concentration) were calculated. All cytotoxicity tests were repeated three times.

Relative cell viability (%) = OD550 value of drug group/ OD550 value of blank control group × 100%

2.11. Inhibitory effect of morin on E. tenella in vitro

In vitro inhibition of E. tenella by morin was studied according to Marugan-Hernandez et al. (2020), Thabet et al. (2017), and Sun et al. (2016). MDBK cells were digested with 0.25% trypsin when they reached 80% confluency. Then, 2 × 10⁶ cells were seeded in 24-well plates with Minimum Essential Medium (Gibco™, Thermo Fisher Scientific, Guangzhou, China) with 10% fetal bovine serum (FBS) and incubated at 37 °C, 5% CO₂ for 24 h. When the cells reached 80%
confluency, $5 \times 10^5$ E. tenella sporozoites were inoculated per well, and the medium was discarded after 4 h. The MDBK/sporozoites suspensions were washed three times with PBS, followed by centrifugation to discard the supernatant, and MEM with 5% FBS was added to each well. Morin was added at final concentrations of 0.01, 0.1, 1, and 10 μM, with three replicates per experimental condition. Meanwhile, a blank control (MDBK cells without sporozoites and inhibitors) and positive control (MDBK cells with sporozoites and without inhibitors) were prepared. Then, 10 μM sulfachlorpyrazine sodium (SS) was used as a positive drug control with MDBK cells and sporozoites. The prepared plate was incubated in 5% CO$_2$ at 37 °C for 48 h before discarding the medium. Cells in each well were washed three times with PBS before being used to extract total RNA by the MicroElute Total RNA Kit (Omega Bio-tek). qRT-PCR was used to detect the effect of morin on the development of E. tenella using the One-Step TB Green® PrimeScript™ RT-PCR Kit II (TaKaRa). Primers used were EGGAPDH-F: 5'-TGGAGTCATTACGGAAC AAGGA-3', EGGAPDH-R: 5'-ACCCCATCAAAACATCCGAAGTA-3', BGAPDH H-F: 5'-GAAGGTGGAGTGAACGGAT-3', and BGAPDH-R: 5'-GAAGGT CCGAGTGAAAGGAT-3'. EGGAPDH and BGAPDH primers were used to assess the mRNA expression of E. tenella GAPDH (target gene) and MDBK cells GAPDH (internal reference gene), respectively. qRT-PCR reactions (20 μL) contained RNA (1 μL), forward and reverse primers (1 μmol/L), 2 × One-Step TB Green RT-PCR Buffer (10 μL), PrimeScript 1 Step Enzyme Mix (1 μL), and RNase Free dH$_2$O (6 μL). qRT-PCR conditions were 42 °C for 5 min; 95 °C for 30 s; 95 °C for 5 s, 60 °C for 20 s, 40 cycles; 95 °C for 10 s; and Melting curve at 65–95 °C, increasing 0.5 °C every 5 s. The following formula was used to calculate the inhibitory effect of morin on E. tenella.

$$\text{Inhibition} (\%) = 100 \times \left(1 - \frac{\Delta\Delta CT}{\Delta\Delta CT_{\text{control}}} \right)$$

According to Jin et al. (2019), safety interval (SI) was calculated by measuring the ratio of C$_{C50}$ to I$_{C90}$ (half effective concentration), that is, SI = C$_{C50}$/I$_{C90}$. Theoretically, the index >1 indicates that the drug is effective and safe, and the higher the SI value is, the safer the drug will be.

### 2.12 Evaluation of anticoccidial effect of morin in vivo

Forty 12-day-old healthy Lingnan yellow chickens of similar size and body shape were randomly selected and divided into four groups. Each group was weighed (denoted as the initial weight before infection), and the weight was adjusted appropriately to keep the weight of each group consistent. Chickens receiving morin at 150 and 450 mg/kg feed doses were classified as Group1 (G1 group) and Group2 (G2 group), respectively. The infected group was designated as the positive control group (PC group), while the non-infected group was designated as the blank control group (NC group). The G1 and G2 groups were given feed containing the corresponding concentration of morin two days before the coccidial challenge. PC and NC groups were given a basal diet. After two days, chickens in all groups, except the NC group, were fed on $1 \times 10^5$ E. tenella sporulated oocysts. The anticoccidial efficacy of morin was evaluated in vivo according to the anticoccidial index (ACI) by referring to Qaid et al. (2021). The ACI $\geq$ 180 was considered excellent, 160 $\leq$ ACI $< 180$ was judged good, 120 $\leq$ ACI $< 160$ was medium, and ACI $< 120$ was invalid.

### 2.13 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 26. One-way analysis of variance with Duncan’s multiple range test was used to compare groups, and $p$-values of less than 0.05 were considered statistically significant.

### 3. Results

#### 3.1 Analysis of EtALD2 sequence

Analysis of the conserved structure of EtALD2 revealed that the EtALD2 protein was composed of 368 amino acids and contained a PTZ00019 conserved domain, which belongs to the TIM superfamily (Fig. 1A). Analyzing EtALD2 homologs from different parasite species showed that the sequences of the EtALD2 domain shared a high degree of similarity with the ALD domain from apicomplexan parasites. Furthermore, the EtALD2 gene contained a class I aldolase motif (Fig. 1B). In the phylogenetic tree, EtALD2 was closely related to ALDs from other apicomplexan species, Cyclospora cayetanensis (OEH7 8062.1), Toxoplasma gondii (AD2J_A, BN1205_061240), and Neospora caninum (NC11V_050370). EtALD2 clustered in one branch with ALD from C. cayetanensis (OEH7 8062.1). However, ALD from Toxoplasma gondii and Neospora caninum clustered together (Fig. 1C).

#### 3.2 Purification and immunogenicity analysis of recombinant EtALD2 protein

SDS-PAGE analysis of the bacterial culture media before and after induction, using supernatants and precipitates after bacterial lysis, demonstrated that the target protein was partially expressed in the supernatant with visible bands at approximately 40 kDa (Fig. 2A). We successfully obtained pCold I-EtALD2 soluble protein using the Beyotime His-tag Protein Purification Kit (Fig. 2B). Western blot results showed that the protein could be specifically recognized by anti-EtALD2 polyclonal antibodies and E. tenella-infected chickens’ positive serum. However, no reaction band was found with uninfected chicken serum (Fig. 2C).

#### 3.3 Subcellular EtALD2 localization in sporozoites

Laser scanning was performed with a Zeiss LSM710 confocal microscope at an excitation light wavelength of 493 nm with an emission wavelength of 519 nm (EtALD2) and an excitation light wavelength of 405 nm with an emission wavelength of 498 nm (DAPI). In Fig. 3, blue fluorescence represents the DAPI-labeled nucleus, and red fluorescence shows the EtALD2 signal. EtALD2 proteins were evenly distributed in the cytoplasm of sporozoites.

#### 3.4 Analysis of EtALD2 transcription and translation levels

EtALD2 mRNA expression levels significantly differed ($p < 0.05$) between the four developmental stages of E. tenella. Second-generation merozoites expressed the highest level of EtALD2 mRNA, followed by sporulated oocysts, sporozoites, and unsporulated oocysts (Fig. 4A). EtALD2 protein expression was also measured in the different developmental stages of E. tenella, using EtACTIN as an internal reference. As shown in Fig. 4B and C, EtALD2 protein expression varied significantly among the four stages. The highest protein level was detected in unsporulated oocysts, followed by sporulated oocysts, second-generation merozoites, and sporozoites ($p < 0.05$).

#### 3.5 EtALD2 activity and inhibition by morin

EtALD2 had catalytic activity on fructose 1, 6-diphosphate (Fig. 5A). According to response surface methodology (RSM), the optimal reaction conditions for EtALD2 enzyme activity were 27.5 °C and pH 7.5 (Fig. 5B). We assessed EtALD2 enzyme activity by enzyme kinetic experiment. The nonlinear Michaelis-Menten equation was fitted by Enzyme Kinetics Module GraphPad Prism 7 to obtain the kinetic parameters $K_m$, $V_{max}$, and $Kcat$ (Fig. 5C and D and Table 1). For FBP, $K_m = 18.06 \pm 1.40$ μM and $V_{max} = 0.64 \pm 0.01$ mmol/min/mg; and for NADH, $K_m = 43.84 \pm 6.59$ μM and $V_{max} = 2.04 \pm 0.17$ mmol/min/mg.
In the EtALD2 catalytic activity inhibition experiment, morin could inhibit the catalytic activity of EtALD2. Fig. 5E and F shows the enzyme kinetic inhibition curve of morin. The $K_i$ value and $IC_{50}$ value of morin were 48.97 μM and 10.37 μM, respectively.

3.6. Cytotoxicity of morin on MDBK cells

We used the MTT method to evaluate the cytotoxicity of morin on MDBK cells. Results revealed that the $CC_{50}$ value of morin on MDBK cells was 706.4 μM, and MDBK cells had no toxicity at 250 μM morin and below (Fig. 6A). The negative control well supplemented with 0.5% DMSO showed no toxicity effect on MDBK cells.

3.7. Inhibition of E. tenella by morin in vitro

The effect of morin on E. tenella was tested in vitro. As a positive control, 10 μM sulfachlorpyrazine sodium (SS) inhibited parasite growth by 74.32% at the endogenous developmental stage (sporozoites) (Fig. 6B). Compared with the positive control, morin showed anti-E. tenella activity on the endogenous developmental stage, with a drug concentration in the micromolar range. A significant difference ($p < 0.05$) was identified in the inhibition rate between the morin-added and the positive control groups. The $IC_{50}$ value for E. tenella inhibition at the endogenous stage was 3.98 μM (Fig. 6B).

3.8. Cecal lesion scores

On the seventh day after the coccidial attack, chickens in each group...
were sacrificed, and the cecum was dissected. The cecum lesion scores were evaluated by the morphology of the intestinal contents, the thickness of the intestinal wall, and the degree of bleeding. The results showed that the cecum lesions in the G1 and G2 groups were lighter than those in the PC group (Fig. 7A), and the average cecum lesion score was 1.7, 1.4, and 2.7, respectively (Fig. 7B). Compared with the PC group, the reduction rate of cecal lesions in the G1 and G2 groups was 37.04% and 48.15%, respectively (Fig. 7C), with a significant difference (p < 0.05). A comprehensive analysis of cecal lesion score and cecal lesion reduction rate revealed that the G2 group (morin treatment) could significantly improve cecal lesions compared with the PC group (p < 0.05). Morin’s improving effect on cecal lesions in *E. tenella*-infected chickens was generally dose-dependent.

### 3.9. Oocyst discharge

Feces of each group were collected 96–168 h after the coccidial attack and sampled after homogenization, and oocyst per gram feces (OPG) was counted with a McMaster slide. Morin could significantly reduce the oocyst production of *E. tenella*. Compared with the PC group, the oocyst discharge in morin-treated groups was significantly decreased (p < 0.05). The oocyst reduction rate in the G1 and G2 groups was 35.15% and 32.88%, respectively, with no significant difference between the two groups (Fig. 7D).

### 3.10. Morin anticoccidial index

Morin anticoccidial effect was dose dependent. The G1 group
achieved a moderate anticoxidial effect, while the G2 group achieved a good anticoxidial effect. The anticoxidial indexes of the G1 and G2 morin administration groups were 149 and 162, respectively (Table 2), while the PC group had the lowest ACI (113).

4. Discussion

Aldolase is an attractive drug target for treating a variety of parasitic infections because of its enzymatic and non-enzymatic functions (Pir-ovich et al., 2021). In this study, we investigated the enzymatic function of EtALD2. When we used NCBI to analyze the conserved domain of the EtALD2 gene sequence, we found that EtALD2 gene encodes a protein containing a conserved PTZ00019 domain. Further analysis showed that the gene belonged to the TIM superfamily.

To further study EtALD2 protein function, we retrieved the homologous sequences of this gene in other parasite species from NCBI (https://www.ncbi.nlm.nih.gov/) and constructed the phylogenetic tree to explore the genetic relationship between ALD genes in different protozoan species. The results showed that the EtALD2 gene was most
closely related to ALD genes from *Cyclospora cayetanensis*, *Toxoplasma gondii*, and *Neospora caninum*. These protozoa are all apicomplexan species, suggesting that this gene is relatively conserved among apicomplexans. To verify this hypothesis, the five species (*Babesia bovis*, *Cyclospora cayetanensis*, *Neospora caninum*, *Plasmodium falciparum*, and *Toxoplasma gondii*) with the highest sequence homology to the *Et*ALD2 gene were compared. The *Et*ALD2 gene had high homology with the ALD domain of these other apicomplexan parasites, and the sequence was highly conserved. These data suggest that the function of *Et*ALD2 could be relatively conserved in different species, which may help *Et*ALD2 functional studies.

*Eimeria tenella* has a complex life cycle, including different developmental stages (El-Shall et al., 2022). Although *Et*ALD2 was transcribed and expressed in all four developmental stages, the *Et*ALD2 mRNA level in the second-generation merozoites was higher than that in sporozoites, sporulated oocysts, and unsporulated oocysts. However, many transcripts do not necessarily mean a corresponding number of translated proteins, which may be related to gene function at different stages of the parasite (Liang et al., 2021). This hypothesis may explain the higher protein level detected in unsporulated oocysts than in the second-generation merozoites. Generally, *Et*ALD2 protein was most highly expressed in the second-generation merozoites and unsporulated oocysts. These two developmental stages are the late endogenous stages of *E. tenella*, developing in the chicken intestinal tract and requiring high metabolic rates to obtain nutrients from host cells and escape the host immune response (Fetterer et al., 2007). The high *Et*ALD2 expression during development in host cells may be related to this protein’s role in the glycolytic pathway, especially in second-generation merozoites. This finding suggests that *Et*ALD2 may be involved in the late endogenous development of the parasite, which may be an interesting topic for further study.

*Et*ALD2 was localized in the cytoplasm of *E. tenella* sporozoites, consistent with the localization in other apicomplexans (Shen and Sibley, 2014; Nemetski et al., 2015). Glycolysis occurs in the cytoplasm, and previous reports on aldolase localization also showed that aldolase was present in the cytoplasm, where it plays a conserved role in energy metabolism.
metabolism. However, aldoses of some species, such as Trichuris trichiura, were not only localized in the cytoplasm but also on the cell membrane, where they perform many non-enzymatic functions (Pirovich et al., 2021).

We used the Michaelis-Menten model to fit the kinetic curve of the morin-mediated inhibition of EtALD2. Morin was a non-competitive inhibitor of EtALD2 and could reversibly bind to the enzyme-substrate complex and the enzyme itself. Non-competitive inhibition is more favorable than competitive inhibition in drug development because high substrate concentrations cannot reverse inhibition (Hartuti et al., 2018).

In vitro inhibition experiments demonstrated that morin could inhibit the development of E. tenella with an IC₅₀ value of 3.98 μM. Studies have shown that plant-derived phenolic compounds (including flavonoids and phenolic acids) have an inhibitory effect on coccidia, and phenolic hydroxyl groups may greatly contribute to this anticoccidial activity (El-Saadony et al., 2021; El-Shall et al., 2022). In vivo experiment results revealed that the addition of morin at a 450 mg/kg feed dose could achieve a good anticoccidial effect, and the SI value was 177.49, with a high safety factor. Additionally, morin feed supplement significantly decreased oocyst discharge and improved cecal lesions of E. tenella-infected chickens. Recently, scholars have devoted themselves to seeking “green” anticoccidial drugs from natural product extracts (Quiroz-Castañeda and Dantín-González, 2015). As a common natural compound in Chinese herbal medicine and dietary supplements, the safety of morin has been thoroughly studied and confirmed, achieving satisfactory results (Jiang et al., 2020; Khamchai et al., 2020). A recent study has shown that morin, as a feed additive, had the potential to protect poultry liver and kidneys, prevent harmful substances in feed, and improve poultry production performance (Gao et al., 2021). Together, these results indicate that morin has a good application prospect for coccidiosis control.

In the structure of morin, phenolic hydroxyl groups are present at the C3 position, A ring 5, 7 position and B ring 2, 4 position of the flavonoid skeleton. Similarly, there are phenolic hydroxyl groups in quercetin at the C3 position, A ring 5, 7 position and B ring 3, 4 position of the flavonoid skeleton; in kaempferol at the C3 position, A ring 5, 7 position and B ring 4 position of the flavonoid skeleton; and in fisetin at the C3 position, A ring 7 position and B ring 3, 4 position of the flavonoid skeleton. All three flavonoids, quercetin, kaempferol, and fisetin, showed strong antiparasitic activity (Panda and Luyten, 2018). Furthermore, Quintanilla-Licea et al. (2020) analyzed the antiparasitic activity of different flavonoids with similar structures. They displayed that adding phenolic hydroxyl at position 7 of the A ring significantly improved antiparasitic activity. However, after adding a phenolic hydroxyl group to position 4 of the B ring, there was little difference in antiprotozoal activity. Notably, phenolic hydroxyl groups in glabridin showed strong antiparasitic activity only at positions 2 and 4 of the B ring of the flavonoid skeleton (Cheema et al., 2014). These results suggest that the phenolic hydroxyl groups at position 7 of the A ring and position 2 of the B ring in the flavonoid skeleton may significantly contribute to the antiparasitic activity.

5. Conclusion

EtALD2 was localized in the cytoplasm of E. tenella sporozoites and plays a conserved role in energy metabolism. EtALD2 mRNA and protein were highly expressed in the late stage of infection, suggesting that EtALD2 may have been involved in the late stages of parasite-endogenous development. Morin significantly improved cecal lesions of E. tenella infected chickens and reduced oocyst excretion. The anticoccidial index of the morin-treated group (450 mg/kg dose) was 162, indicating a good anticoccidial effect. Morin showed strong anticoccidial activity, which may be due to the interaction of the phenolic hydroxyl group at position 7 of the A ring and position 2 of the B ring of its flavonoid skeleton with EtALD2 active site.

Declaration of competing interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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