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Assessment of the Interaction between Lactoferrin and Diclazuril On *Eimeria Stiedae* in Rabbits

Dalia, M. Azab*; Mai, O. Nada*; Rasha, A. EL- Maghnawy
and Sameh, A. El- Alfy*****

*Biochemistry Department (Pharmacology) Animal Health Research Institute, Benha-Branch. Agriculture Research Center (ARC), Egypt.
**Parasitology Department. Animal Health Research Institute, Shebin El Kom- Branch, Agriculture Research Center (ARC), Egypt.
***Parasitology Department. Animal Health Research Institute, Benha- Branch, Agriculture Research Center (ARC), Egypt.

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Abstract

Hepatic coccidiosis is one of the most common and severe protozoal infections in rabbits, caused by *Eimeria stiedae.* This disease poses a significant threat to rabbit health, resulting in high mortality rates, reduced productivity, and stunted growth. Conventional antiprotozoal drugs often fail to effectively reach the parasite's intracellular stages, leading to prolonged high-dose treatments that increase the risks of toxicity, drug residues, and resistance. Although prebiotics show some promise, their effectiveness is often limited by inconsistent ingredients and dosing. This study evaluated the combined use of lactoferrin and diclazuril to reduce the infectivity of sporulated oocysts of *Eimeria stiedae*. For this purpose, twenty-five rabbits, each 44 days old, were divided into five groups of five. Group 1 served as the negative control. Group 2 was infected with *E. stiedae* and left untreated. Group 3 received lactoferrin (0.2 mg/kg) daily for 7 days before infection and was then treated with diclazuril (1 mg/kg) daily for 7 days after the onset of symptoms. Group 4 received diclazuril (1 mg/kg) daily for 7 days before infection. Group 5 was pre-treated with both lactoferrin and diclazuril at the same dosages as Group 3. All rabbits were infected by administering 10^5 sporulated oocysts of *E. stiedae.* The study monitored several factors, including clinical signs, mortality rates, and drug concentrations, which were measured using High-Performance Liquid Chromatography (HPLC). Additionally, pharmacokinetic parameters of diclazuril were calculated using PK Solver, taking into consideration feed intake, feed conversion ratio, body weight, and weight gain. Fecal oocysts were counted (measured as oocysts per gram) along with biochemical and antioxidant markers.

Corresponding author: Dalia, M. Azab, Biochemistry Department (Pharmacology) Animal Health Research Institute (AHRI), Benha-Branch., Agriculture Research Center (ARC), Nadi El-Seid Street, Dokki P.O., Giza 12618, Egypt.
Email address: drdaliamahfouz@yahoo.com

The results showed that the combination of lactoferrin and diclazuril significantly reduced oocyst shedding and prevented mortality related to coccidiosis ($P < 0.05$). Additionally, this treatment eliminated clinical signs of the disease and greatly improved feed intake, body weight, weight gain, and feed conversion efficiency. Improvements in biochemical and antioxidant indicators, including a notable increase in serum glutathione (GSH) levels and positive effects on liver health. Pharmacokinetic analysis indicated that co-administration of diclazuril (1 mg/kg) and lactoferrin (0.2 mg/kg) resulted in a peak plasma concentration of 8.41 µg/mL, occurring 4.63 hours after dosing. The absorption half-life ($t_{1/2k_a}$) was 2.15 hours, the area under the concentration-time curve ($AUC_{0-\infty}$) was 88.56 µg/mL/h, and the mean residence time (MRT) was 9.28 hours. These findings suggest that lactoferrin may modify the pharmacokinetics of diclazuril in a potentially beneficial way. However, further research is needed to assess its clinical effectiveness, evaluate tissue residues, and establish appropriate withdrawal periods for rabbits undergoing treatment for coccidiosis.

Introduction

Coccidiosis is a common and costly protozoan infectious disease that affects rabbits, leading to significant losses in the rabbit meat industry **(Hegazi *et al.,* 2023)**. Among the most harmful species is *E. stiedae*, which infects the epithelial cells of the bile ducts. This infection can result in severe hepatic coccidiosis and considerable economic losses **(Maziz *et al.,* 2018)**. Infected rabbit populations often show symptoms such as decreased appetite, poor growth rates, diarrhea, jaundice, abdominal distension, and, in severe cases, high rates of illness and mortality **(Eladl *et al.,* 2020)**. Coccidiosis primarily spreads through the oral ingestion of contaminated water and feed. Therefore, effective hygiene practices and proper management are crucial for controlling the disease **(Mohsin *et al.,* 2021; Hussain *et al.,* 2022)**. The prophylactic use of anticoccidial drugs often supports these control measures. However, the use of coccidiostats carries risks, including the development of drug resistance and potential toxicity **(Bawm and Htun, 2021)**.

The prolonged use of anticoccidial drugs has led to the emergence of drug-resistant strains of parasites, raising significant concerns about the effectiveness of treatments and public health. These issues are important due to the potential presence of drug residues in animal-derived products. Consequently, there has been a growing focus on researching safer and more sustainable alternatives to conventional anticoccidial agents **(El Banna *et al.,* 2016)**. One commonly used anticoccidial drug is diclazuril, which disrupts both the sexual and asexual stages of the coccidian life cycle and inhibits the excretion of oocysts **(Elokil *et al.,* 2020)**. However, there are increasing reports of resistance to diclazuril and similar drugs, which presents a growing concern. Therefore, the search for new, effective anticoccidial agents remains a priority in strategies for controlling coccidiosis. Natural products appear to be a promising alternative for coccidiosis management, especially since resistance to these compounds has not yet emerged **(Abbas *et al.,* 2012)**. Recent studies have highlighted the potential of natural products and plant-based compounds as effective alternatives for managing coccidiosis **(Abd-ELrahman *et al.,* 2022; Elmahallawy *et al.,* 2022)**.

Recent research emphasizes the critical role of prebiotics in preventing and managing infectious diseases, including coccidiosis **(Cai *et al.,* 2023)**. Among these prebiotics, Lactoferrin is an exceptional iron-binding glycoprotein that significantly enhances the host's innate immune defenses. Lactoferrin is found in various biological sources, such as maternal milk, mucous secretions, tears, the secondary granules of neutrophils, and circulating blood **(Kell *et al.,* 2020)**. In mammals, lactoferrin quickly enters the bloodstream, reaching peak concentrations about 12 hours after oral ingestion. It travels through the portal vein and lymphatic system to various tissues, eventually being excreted into the gastrointestinal tract via bile **(Matsuzaki *et al.,* 2019)**. The essential functions of lactoferrin include enhancing iron absorption, neutralizing free radicals, supporting immune activity, and regulating gastrointesti-

nal function **(Kowalczyk *et al.,* 2022)**. Extensive studies demonstrate that lactoferrin functions through complex mechanisms. It influences the production of cytokines and chemokines, regulates levels of reactive oxygen species (ROS), and promotes the recruitment of immune cells. In addition to its antioxidant properties, lactoferrin acts as a natural iron-binding agent, modulating critical signaling pathways and regulating inflammatory responses by targeting the negative feedback mechanisms that control inflammation **(Legrand, 2012)**.

Lactoferrin can interact with parasites and free-living protozoa found in mucosal surfaces and the bloodstream, functioning as either a microbiostatic or microbicidal agent **(Zhao *et al.,* 2023)**. Recently, lactoferrin has gained attention as a promising natural agent for health-promoting therapies. While extensive studies have focused on its antimicrobial and antifungal properties, its potential as an antiparasitic, especially against coccidian parasites, has not been thoroughly explored and requires further investigation.

This study aimed to evaluate the effect of lactoferrin on the pharmacokinetics of diclazuril after a single oral administration. Additionally, it sought to investigate the combined effects of lactoferrin on growth performance, biochemical parameters, and oocyst output in rabbits experimentally infected with *E. stiedae*. The toxicity assessment was conducted by analyzing the impact on the antioxidant defense system.

Material and Methods

Collection of samples:

A total of one hundred samples, including gallbladders, blood, and feces, were collected from rabbits naturally infected with coccidiosis and exhibiting clinical signs of the disease. These samples were obtained from farms within Qalyoubia Governorate that had a documented history of *E. stiedae* infection. All samples were placed in sterile tubes and transported promptly to the Animal Health Research Institute (AHRI) laboratory in Benha City for analysis.

Sporulation of *E. stiedae* Oocysts

E. stiedae oocysts were collected and identified morphologically according to **Levine (2018)**. The oocysts were preserved in a 2.5% potassium dichromate solution to induce sporulation. To propagate the oocysts, five rabbits confirmed to be free of Eimeria infection were orally administered the sporulated oocysts. After 20 days, the rabbits were euthanized, and their gallbladders were collected. The contents were washed with a saline solution and concentrated using the flotation technique **(Solusby, 1968)**. The oocysts were verified and stored at 4°C until further use. The number of sporulated oocysts utilized for the infection was determined using the McMaster counting technique **(Metwally *et al.,* 2022)**.

Experimental protocol

Twenty-five New Zealand rabbits, each 44 days old and with an average body weight of 900 grams, were purchased from a farm in the Qalyoubia governorate. The rabbits were divided into five groups, each containing five rabbits. During the experimental period, each rabbit was housed individually in a metal cage. To ensure that the rabbits were free from coccidian infections, daily direct fecal analyses using the flotation technique were conducted for two consecutive weeks before the experiments. The rabbits were fed a commercial diet that contained no therapeutic additives, and both feed and water were provided ad libitum throughout the experiment.

Challenged inoculum

The isolated *E. stiedae* oocysts were used. Rabbits in all groups except the negative control group were challenged with 1ml of 10^5sporulated oocysts of *E. stiedae* via the oral route using a stomach tube on day 7 after prophylactic treatment **(Hassan *et al.,* 2016).**

Prebiotic Lactoferrin®

One sachet containing 200 mg bovine lactoferrin was purchased from Hygienic Pharmaceutical Company, Alex.

Diclosol 1%®

A milliliter containing 10 mg of diclazuril was purchased from Pharma Swede Company, located in 10th of Ramadan City, B3, Egypt.

Batch No: L250450.

Experimental design

The rabbits were randomly assigned to five groups, each containing five rabbits. Group 1 (G1) served as the negative control (non-infected, non-treated), while Group 2 (G2) was the positive control (infected, non-treated). In the prophylactic approach, Group 3 (G3) received lactoferrin daily in drinking water at a dosage of 0.2 mg/kg body weight starting 7 days before infection and continuing throughout the study. Additionally, they received diclazuril post-infection at 1 mg/kg body weight daily for 7 days, beginning after symptoms appeared. Group 4 (G4) was administered diclazuril at a dosage of 1 mg/kg body weight in the drinking water daily for 7 days, before infection **(Saleh *et al.* 2023)**. Group 5 (G5) received a combination of lactoferrin and diclazuril at the same respective doses, starting one week before infection. On the seventh day of beginning the experiment, all rabbits in groups G2, G3, G4, and G5 were orally infected with 10^5 sporulated oocysts of *E. stiedae*. The rabbits in all groups were examined daily to monitor symptoms of coccidiosis and track rabbit mortalities. Moreover, body weights and feed consumption were recorded weekly in each group. These values were then used to calculate weight gain and the feed conversion ratio (FCR) for each group, as described by (**Wanger *et al.* 1983)**, using the following formula: FCR = Average feed intake (g) rabbit per week/ Average body weight gain (g) rabbit per week.

Blood sampling

To assess the pharmacokinetic parameters, blood samples were collected at various time intervals: 0.25, 0.5, 1, 2, 4, 8, 12, and 24 hours after the rabbits received oral doses of lactoferrin and diclazuril. The concentrations of these substances were determined using high-performance liquid chromatography (HPLC). At the end of the experiment on the 30th day post-infection (dpi), blood samples were taken from the ear vein of three rabbits from each group. These samples were then centrifuged at 3,000 rpm for 10 minutes to separate the serum, which was stored at −20°C for later analysis of antioxidant and biochemical parameters.

Parasitological analysis

On the day after coccidian infection and continuing for 30 days post-infection, fresh voided fecal samples were collected individually from all groups to monitor the onset of oocyst shedding. Oocysts were counted using the McMaster technique, with results reported as oocysts per gram (OPG) of feces compared to the infected untreated control group **(Metwally *et al.,* 2022)**. The percentage of oocyst reduction was calculated using the following formulas:

Reduction percentage = X-Y / X × 100

where X represents the mean number of oocysts in the positive control group, and Y represents the mean number of oocysts in the treated group **(Lan *et al.* 2016)**.

Biochemical parameters analysis:

Biochemical parameters, including ALT and AST enzyme activities, total protein, albumin, as well as levels of uric acid and creatinine, were measured in serum samples using Bioanalytica colorimetric test kits (Spinreact S.A./ S.A.U., Ctra. Santa Coloma 7, E-17176 Sant Esteve De Bas, Girona, Spain). Serum globulin (G) was calculated by subtracting albumin from total protein.

Determination of antioxidant indices:

Levels of glutathione (GSH), malondialdehyde (MDA) and nitric oxide synthase (NOS) were measured using ELISA kits supplied by Nanjing Jiancheng Bioengineering Institute (Nanjing, China), following the protocol described by (**Dalton *et al.* 2000)**.

HPLC Analysis of Diclazuril in Serum Samples

- Standards and serum specimen preparation

A standard solution of diclazuril was prepared at a concentration of 1 mg/mL in dimethylformamide (DMF). This solution was then diluted with blank rabbit serum to create calibration standards ranging from 0.025 to 10 µg/mL. The preparation of serum samples followed a method outlined by **(Dirikolu *et al.* 1999)**, utilizing solid-phase extraction (SPE) with Bond

Elut C18 columns. The columns were preconditioned with methanol and phosphate buffer at pH 6.0. After this, the samples were loaded onto the columns, which were sequentially washed with phosphate buffer, acetic acid, and hexane, allowing for drying between each step. Elution of the samples was performed using a methanol-hydrochloric acid (HCl) solution in a 95:5 ratio. The collected eluent was then evaporated at 40°C under nitrogen gas. The resulting dried residue was reconstituted first in DMF and then in water, with both mixing processes involving vortex and sonication. Finally, 20 µL of the prepared sample was injected into the high-performance liquid chromatography (HPLC) system for analysis.

Diclazuril levels in serum were measured using high-performance liquid chromatography (HPLC). The HPLC system comprised an Agilent 1200 series setup, which included a quaternary gradient pump, an autosampler, a UV-VIS detector set to 280 nm, and 2D ChemStation software. Separation of the compound was achieved on a Phenomenex C18 column (5 µm, 150 × 4.6 mm). The mobile phase was composed of two solvents: Solvent A, which included 80% buffer (0.5% ammonium acetate and 0.01 M tetrabutylammonium hydrogen sulfate in water) and 20% acetonitrile; and Solvent B, which consisted of 80% methanol and 20% acetonitrile. The solvent ratio was maintained at 46:54 (v/v). The flow rate was set at 1 mL/min, with diclazuril exhibiting a retention time of 13.7 minutes. The method was validated by assessing recovery, sensitivity, precision, and linearity. Linearity was confirmed over a concentration range of 0.025–10 µg/mL, with a correlation coefficient (R^2) greater than 0.99. The method's limit of detection (LOD) was 0.008 µg/mL, while the limit of quantification (LOQ) was 0.025 µg/mL.

Pharmacokinetic analysis

Mean serum concentrations of diclazuril were determined at each sampling time point in rabbits. A non-compartmental analysis was performed using the PK-solver program (**Dirikolu *et al.*, 2022).** Key pharmacokinetic parameters were calculated, including: AUC_0–last: Area under the concentration-time curve, determined using the linear up/log down trapezoidal method. $T_{1/2k_a}$: Absorption half-life. MRT: Mean residence time. V/F: Apparent volume of distribution. Cl/F: Apparent clearance divided by bioavailability. Additionally, Cmax (maximum plasma concentration) and Tmax (time to reach Cmax) were obtained directly from the plasma concentration-time data.

Statistical analysis

Group differences were analyzed using one-way ANOVA, followed by Duncan's multiple comparison Post Hoc tests **(Duncan, 1955).** Statistical analyses were conducted using the Statistical Package for the Social Sciences (SPSS), version 20.0 (SPSS Inc., Chicago, IL, USA). Significance between mean values was set at a statistically significant $P<0.05$. The kinetic parameters were investigated by PK-solver, an add-in program for Microsoft Excel, version 2, and other parameters were calculated according to (**Zhang *et al.* 2010).**

Ethical approval

The experiment was done at the Faculty of Veterinary Medicine, Benha University. All procedures used in this experiment were followed by the guidelines of the National Institute of Health and approved by the Institutional Animal Ethics Committee of the Faculty of Veterinary Medicine, Benha University, Egypt **(Ethical No. ARCAHRI 54-25).**

Results

The clinical observations showed that the rabbits in the negative control group (G1) exhibited no signs of illness. In contrast, the groups of rabbits treated with lactoferrin and diclazuril (G3 and G5) displayed only mild clinical signs, with no clinical abnormalities related to hepatic coccidiosis. These rabbits maintained good health and a normal appetite throughout the experiment, especially when compared to the diclazuril-only group (G4). Conversely, rabbits in the positive control group (G2) presented clinical signs including lethargy, rough fur, decreased food intake, weight loss, and abdominal swelling. No deaths were recorded in groups G3, G4, and G5; however, one mortality occurred in group G2 on the 20th day after infection.

At the start of the experiment (day 0, weeks 1 and 2), the body weights of the rabbits were similar across all groups. However, by the third week, significant differences became evident. Rabbits in the lactoferrin-protected groups (G3 and G5) exhibited a considerable increase in body weight ($P<0.05$), with the highest gains observed from the third week to the fifth week, compared to the group treated only with diclazuril (G4) and both control groups. Notably, by the fifth week, both the negative control group (G1) and the diclazuril-treated group (G4) showed significant weight increases, with averages of 1529.3±7.55 and 1426.5±7.37, respectively. In contrast, the untreated infected group (G2) had a lower average weight of 1305.4±3.32, as shown in Table 1.

Table (1). Effectiveness of lactoferrin and diclazuril on growth performance indicators in rabbits experimentally infected with *E. stiedae*.

Growth Performance Parameters	Age	Groups				
		G1	G2	G3	G4	G5
Body Weight	0 day	920.84± 1.50^{ab}	918.67± 0.87^{ab}	921.80± 1.44^{a}	921.37± 0.62^{ab}	918.07± 0.64^{ab}
	1st week	978.67± 2.47^{a}	980.09± 1.16^{a}	986.04± 3.08^{a}	983.37± 1.42^{a}	979.33± 2.55^{a}
	2nd week	1194.4± 3.21^{a}	1191.1± 3.86^{a}	1198.3± 1.05^{a}	1190.8± 2.70^{a}	1196.1± 3.03^{a}
	3rd week	1299.6± 1.11^{c}	1289.4± 0.75^{d}	1344.4± 2.66^{b}	1298.5± 0.96^{c}	1350.8± 0.88^{a}
	4th week	1411.0± 6.47^{b}	1087.2± 4.01^{d}	1477.0± 6.52^{a}	1325.9± 2.74^{c}	1470.8± 6.56^{a}
	5th week	1529.3± 7.55^{c}	1305.4± 3.32^{e}	1684.0± 3.71^{a}	1426.5± 7.37^{d}	1617.4± 8.77^{b}
cBWG (gm)		609.0	387.0	763.0	505.0	699.0
cBWG (%)		39.8%	29.7%	45.3%	35.4%	43.2%
cFCR (%)		4.14%	6.51%	3.31%	4.99%	3.60%

a, b, c, d, e Mean values within the same row with different superscript letter are statistically different at ($p \leq 0.05$). Values are given as the mean (n=3) ±SE. G1; Control negative, G2; control positive, G3; Protected by lactoferrin daily from 7 days before infection and continuing throughout the experiment and received diclazuril for 7 days only after symptoms appeared., G4; Protected by diclazuril daily for 7 days only before infection and G5; Protected by both lactoferrin and diclazuril daily for 7 days only before infection.. cFCR: Mean group cumulative feed conversion ratio.

According to the data presented in Figure 1 and Table 2, oocyst shedding in feces was first detected 15 days after infection (the prepatent period) in rabbits from groups G3, G4, and G5. All treated groups (G3, G4, and G5) demonstrated a significant reduction in oocyst counts from day 15 to day 30 post-infection when compared to the positive control group (G2). Among all the groups, rabbits in group G2 displayed the highest oocyst counts per gram of feces. In contrast, oocyst shedding was delayed in group G5, which received both lactoferrin and diclazuril. This group exhibited the lowest oocyst counts throughout the experimental period, up to day 20 post-infection. Meanwhile, groups G3 and G4 maintained low oocyst counts until day 22 post-infection, significantly lower than those observed in the positive control group (G2).

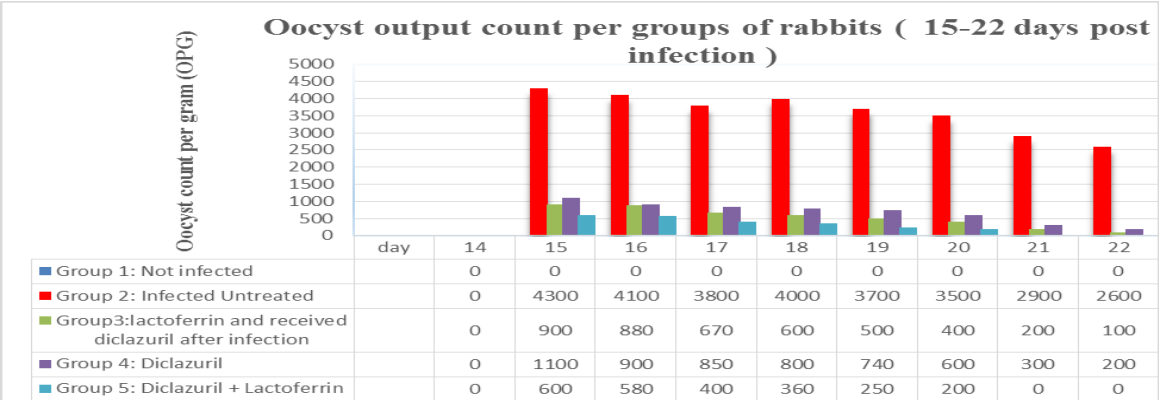


Figure (1). The number of oocyst shedding in groups of rabbits on different days post-infection

Table (2). Effectiveness of lactoferrin and diclazuril on oocysts count and reduction rate % between rabbits experimentally infected with *E. stiedae* at different days post-infection.

Days post-infection	Group1	Group2	Group3	Group4	Group5
1-14 days	0	0	0	0	0
15	0	4300	900	1100	600
16	0	4100	880	900	580
17	0	3800	670	850	400
18	0	4000	600	800	360
19	0	3700	500	740	250
20	0	3500	400	600	200
21	0	2900	200	300	0
22	0	2600	100	200	0
23	0	2400	0	0	0
24	0	2300	0	0	0
25	0	2100	0	0	0
26	0	1960	0	0	0
27	0	1900	0	0	0
28	0	1800	0	0	0
29	0	1600	0	0	0
30	0	900	0	0	0
Overall Mean	0	2622.5	265.62	343.125	149.375
R (%)	100%	0	89.87%	86.91%	94.30%

G1; Control negative, G2; control positive, G3; Protected by lactoferrin daily from 7 days before infection and continuing throughout the experiment, and received diclazuril for 7 days only after symptoms appeared., G4; Protected by diclazuril daily for 7 days only before infection, and G5; Protected by both lactoferrin and diclazuril daily for 7 days only before infection. R% = Reduction percentage = (A−B)/A × 100, where A is the mean number of oocysts in the infected non-treated group and B is the mean number of oocysts in the treated group.

The effects of combined lactoferrin and diclazuril treatment on antioxidant enzyme activities were evaluated in rabbits experimentally infected with *E. stiedae*, 30 days after infection as showed in Table (3). The groups treated with lactoferrin, specifically groups G3 and G5, exhibited significantly higher serum levels of glutathione (GSH) ($P < 0.05$) than all other groups. Notably, group G3 demonstrated a significant increase in GSH levels compared to the diclazuril-treated group, the infected but untreated group, and the negative control group. The group that received only diclazuril exhibited elevated GSH levels compared to the infected group; however, these levels were significantly lower ($P < 0.05$) than the levels in G3 and G5. Additionally, pretreatment with lactoferrin resulted in a significant reduction in serum malondialdehyde (MDA) and nitric oxide synthase (NOS) levels compared to the other groups. The lowest MDA activity was found in groups G3 and G5. Furthermore, NOS levels were significantly decreased ($P < 0.05$) in group G3 compared to all other groups. Overall, the groups that were pretreated with lactoferrin displayed the lowest concentrations of malondialdehyde and nitric oxide

Table (3). Evaluation of the combined effect of lactoferrin and diclazuril on antioxidant parameters in rabbits experimentally infected with *E. stiedae* at day 30th post-infection. (Mean±SE).

Groups		Antioxidant parameters		
		GSH (ng/ml)	MDA (nmol/ml)	NOS (nmol/ml)
		At day 30th post infection		
Group 1	**Negative control (non-infected, non-treated)**	3.753± 0.186^{c}	83.19± 3.96^{c}	6.57± 0.313^{ab}
Group 2	**Positive control (infected, non-treated).**	1.290± 0.19^{d}	138.52± 2.033^{a}	11.64± 0.563^{a}
Group 3	**Protected by lactoferrin daily from 7 days before infection and continuing throughout the experiment and received diclazuril for 7 days only after symptoms appeared.**	9.167± 0.651^{a}	65.74± 5.76^{d}	5.53± 0.639^{c}
Group 4	**Protected by diclazuril daily for 7 days only before infection.**	2.857± 0.199^{c}	102.14± 5.44^{b}	7.34± 0.65^{b}
Group 5	**Protected by both lactoferrin and diclazuril daily for 7 days only before infection.**	7.75± 0.417^{b}	66.48± 5.67^{d}	6.57± 0.417^{ab}

a, b, c, d, mean values with different superscripts in a column are statistically different at ($p \leq 0.05$) ; *mean of 3 rabbits.

The study assessed the effects of lactoferrin and diclazuril on liver and kidney function markers in rabbits infected with *E. stiedae*, as presented in Table 4. There were no significant changes in ALT and AST levels between the rabbits in diclazuril-treated (G4), protected (G3, G5), and negative control groups on day 30th post-infection. However, a significant increase in these enzyme levels was observed in the infected non-treated group (G2). Additionally, AST levels were significantly decreased ($P < 0.05$) in *E. stiedae* experimentally infected rabbits pretreated with lactoferrin (the protected groups G3 and G5) compared to the diclazuril-treated group (G4). A significant increase in serum levels of uric acid and creatinine was observed in group 2 (infected, non-treated) compared to the other groups. No significant differences in uric acid levels were found between the diclazuril-treated group (G4) and the protected groups (G3, G5). However, creatinine levels were significantly lower ($P < 0.05$) in the protected groups (G3, G5) compared to both the diclazuril-treated group (G4) and the

infected, non-treated group (G2).
When comparing the negative control group to the treated rabbits, those in the diclazuril-treated group (G4) and the protected groups (G3 and G5) did not show any significant changes in total protein, albumin, or globulin levels. However, group G3 did demonstrate a decrease in total protein levels. In contrast, groups G1, G3, G4, and G5 exhibited a significant reduction ($P < 0.05$) in total protein and albumin levels when compared to the positive control group (G2).

Table (4). Evaluation of the combined effect of lactoferrin and diclazuril on blood biochemical parameters in rabbits experimentally infected with *Eimeria stiedae* at day 30th post-infection (Mean*±SE).

Groups		Blood biochemical parameters at day 30th post infection						
		ALT (U/L)	AST (U/L)	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	Uric acid (mg/dl)	Creatinine (mg/dl)
G1	Negative control (non-infected, non-treated)	13.57± 0.923^{b}	18.99± 0.591^{c}	6.51± 0.014^{b}	3.423± 0.014^{b}	3.09± 0.012^{ab}	5.147± 0.103^{b}	1.186± 0.021^{c}
G2	Positive control (infected, non-treated).	27.09± 3.80^{a}	37.28± 1.52^{a}	7.31± 0.218^{a}	3.88± 0.081^{a}	3.43± 0.284^{a}	7.150± 0.131^{a}	1.733± 0.023^{a}
G3	Protected by lactoferrin daily from 7 days before infection and continuing throughout the experiment and received diclazuril for 7 days only after symptoms appeared.	13.27± 0.799^{b}	18.65± 0.731^{c}	5.64± 0.19^{c}	3.343± 0.113^{b}	2.296± 0.179^{b}	5.120± 0.262^{b}	1.180± 0.026^{c}
G4	Protected by diclazuril daily for 7 days only before infection.	19.29± 2.56^{b}	25.45± 2.53^{b}	6.50± 0.093^{b}	3.50± 0.029^{b}	3.00± 0.091^{ab}	5.706± 0.192^{b}	1.330± 0.042^{b}
G5	Protected by both lactoferrin and diclazuril daily for 7 days only before infection.	14.70± 0.46^{b}	19.90± 0.224^{c}	6.45± 0.32^{b}	3.406± 0.131^{b}	3.04± 0.433^{ab}	5.160± 0.201^{b}	1.193± 0.025^{c}

a, b, and c, mean values with different superscripts in a column are statistically different at ($p \leq 0.05$) ; *mean of 3 rabbits.

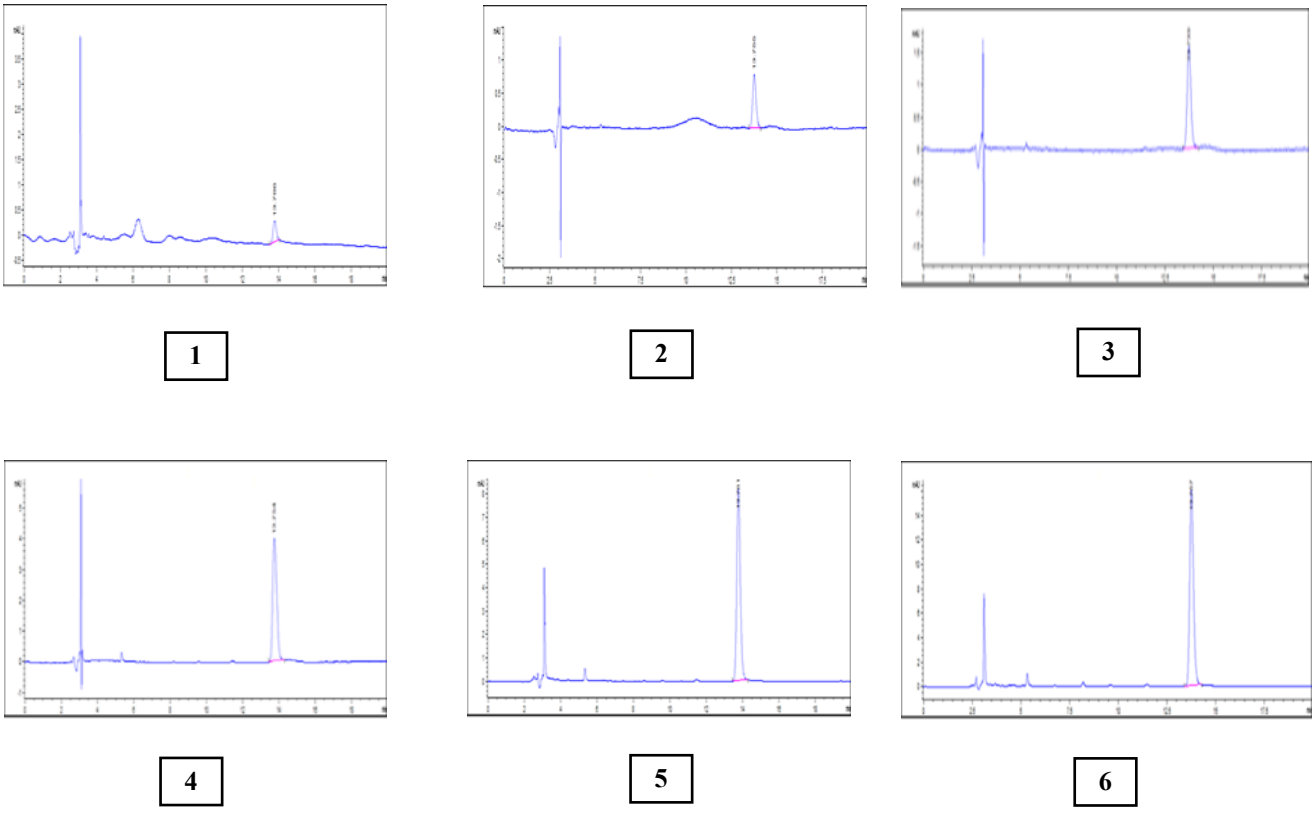
In blank serum samples, there was no detectable chromatographic interference at the retention times corresponding to either diclazuril or the internal standard. This strongly supports the method's specificity and selectivity. The calibration curves showed excellent linearity across the concentration range of 0.025–10 µg/mL, with a representative equation of $y = 0.2142x + 0.0991$ and a correlation coefficient (R^2) of 0.9988. The limit of quantification (LOQ) was successfully validated at 0.025 µg/m. Assessments of both intra-day and inter-day precision yielded relative standard deviation (RSD) below 6%, demonstrating reproducible accuracy. The average extraction recovery of diclazuril was 102.29%, indicating a remarkably consistent and concentration-independent extraction efficiency. The results of the assay are summarized in Table 5 and Figures 2 and 3.

Table (5). Validation parameters of the HPLC method for measuring diclazuril in rabbit serum after a single 1 mg/kg oral dose.

Matrix	Average recovery (%)	Intra-day RSD (%)	Inter-day RSD (%)	LOD (µg/mL)	LOQ (µg/mL)
Rabbit serum	102.29±5.96	4.62%	3.36%	0.008	0.025

Data for recovery are elucidated as mean ± Standard deviation, LOQ: Limit of quantification, LOD: Limit of detection, RSD: Relative standard deviation. Intra-day RSD and Inter-day RSD % (n = 6, 0.025µg/mL). Average recovery % (utilizing spiked concentrations in the range of 0.025–10µg/mL).

Figure (2). HPLC chromatogram of diclazuril standards (1) 25 ng/ml, (2) 50 ng/ml, (3) 100 ng/ml, (4) 250 ng/ml, (5) 500 ng/ml, (6) 1000 ng/ml.



Standard curve of diclazuril in rabbit serum

Diclazuril standards at a range of 25- 10000 ng/ml were prepared in blank rabbit serum with a correlation coefficient = 0.9988, as shown in Figure 3.

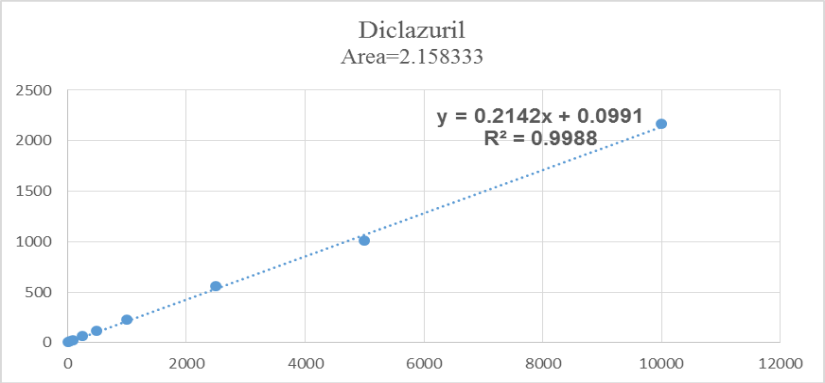


Figure (3). Standard curve of diclazuril in rabbit serum

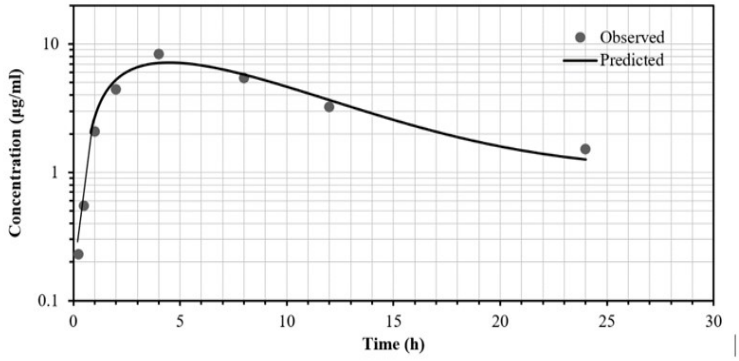


Figure (4). Mean serum concentrations of diclazuril in rabbits after a single oral administration at 1 mg/kg, with the line representing a two-compartment model prediction. Values are shown as mean ± SD (n=5).

Pharmacokinetic Oral Administration: The pharmacokinetic parameters of diclazuril and lactoferrin were analyzed using the PK Solver program (Table 6).

Table (6). Pharmacokinetic parameters of diclazuril and lactoferrin following its single oral administration in rabbits. The analytes fit into the two-compartment model.

Parameters	Diclazuril only	Diclazuril and lactoferrin
C max µg/mL	6.65	8.41
Tmax (h)	4.21	4.63
t1/2ka (h)	2.49	2.15
AUC 0-t µg/ml*h	76.79	84.96
AUC 0-inf µg/ml*h	78.85	88.56
AUMC µg/ml*h	788.44	821.42
Ka 1/h	0.278	0.321
k10 1/h	2.16	1.82
K 12 1/h	0.25	0.23
K 21 1/h	0.012	0.013
V/F µg/ml	0.058	0.069
Cl/F µg/ml	1.26	1.06
MRT (h)	9.23	9.28

C max: Maximum concentration in serum; T max: Time to achieve maximum concentration; AUC0- inf: Area under the serum concentration-time curve from 0 to last time; AUMC: area under the curve from the initial moment; V/_F: Volume of distribution scaled by bioavailability; Cl/_F: Clearance divided by bioavailability; MRT: Mean residence time; $t_{1/2k_a}$ is the absorption half time; ka is the apparent first-order absorption rate constant; k12 is the apparent first-order transfer rate constant from the central compartment to the peripheral compartment; k10 is the apparent first-order elimination rate constant from the central compartment; k21 is the apparent first-order transfer rate constant from the peripheral compartment to the central compartment.

Discussion

Hepatic coccidiosis is one of the most common and severe protozoal infections in rabbits, often resulting in fatal outcomes **(Eladl *et al.,* 2020)**. Over time, the Eimeria species have developed resistance, which has diminished the effectiveness of traditional treatments **(Abbas *et al.,* 2017)**. In recent years, natural compounds have shown promising efficacy in treating protozoan diseases in livestock **(Wink, 2012)**. However, research in this area remains limited and inconclusive. Consequently, there has been growing interest in exploring new anticoccidial agents, particularly natural additives, as potentially effective alternatives for managing coccidiosis. Compared to synthetic drugs, these natural substances are generally more cost-effective and do not leave harmful residues **(Abbas *et al.,* 2017)**. Moreover, lactoferrin is recognized as a potent immunomodulatory glycoprotein that regulates immune responses and influences key cell signaling pathways **(Demir *et al.,* 2025).**

In this study, all lactoferrin-protected groups (G3–G5) showed a significant increase in body weight and weight gain, highlighting the strong anticoccidial potential of lactoferrin. These findings are consistent with **(Abd El Monsef *et al.,* 2024),** who demonstrated that lactoferrin treatment inhibits the growth of invading gastrointestinal pathogens. This inhibition may indirectly improve the animals' health and growth performance by enhancing nutrient digestibility and absorption. In contrast, the infected control group (G2) showed a significant decline in body weight and weight gain, primarily due to reduced feed consumption and intestinal mucosal damage. This impairment likely compromised the efficiency of nutrient digestion and absorption. Also, the use of lactoferrin in poultry improves weight gain performance and modulates the adverse effects of arsenic toxicity **(Hassan *et al.,* 2024)**. These findings are consistent with those of **(Siqueiros-Cendón *et al.,* 2014)** and (**Choi *et al.,* 2021),** who reported that Eimeria parasites adversely affect intestinal function by invading and disrupting the gut lining.

The clinical symptoms observed in the infected non treated rabbits were similar to those reported by (**Allam *et al.,* 2020)**. In contrast, rabbits that were treated with diclazuril or protected with it displayed only mild symptoms. This highlights the effectiveness of diclazuril, demonstrating not only its anticoccidial activity but also its ability to control the infection.

A significant reduction in oocyst count was observed in the lactoferrin-protected groups (G3 and G5), likely due to the multifunctional nature of lactoferrin. As a natural protein, lactoferrin inhibits parasite growth through iron chelation and the ability of its peptides (Lactoferricins) to pass through the protozoal cell membrane and nuclear envelope. These findings are consistent with the study by **(Reyes-López *et al.,* 2022),** which reported that lactoferrin-treated sporozoites exhibited reduced infectivity and a lower capacity to penetrate host cells compared to untreated sporozoites.

In the present study, *E. stiedae* oocysts were first detected in the feces of the infected control group at 15 days post-infection (dpi), with the highest oocyst counts occurring between 17 and 19 dpi. Rabbits infected with oocysts treated with lactoferrin (G5) showed a significant reduction in oocyst shedding by 20 dpi, and no oocysts were detected in their feces from 21 dpi until the end of the experiment. This result is likely due to lactoferrin's ability to reduce the infective potential of sporulated oocysts. Additionally, groups G3 and G4 also exhibited a significant reduction in oocyst output by 22 dpi, with fecal oocysts disappearing by 23 dpi. In contrast, **(Hegazi *et al.,* 2023)** reported that *E. stiedae* oocyst shedding in infected controls began at 19 dpi, with peak oocyst counts occurring between 26 and 29 dpi. Similarly, **(Saleh *et al.,* 2023) and (Allam *et al.,* 2020)** observed initial oocyst detection at 17 and 18 dpi, respectively. These variations may be attributed to differences in the inoculation dose, as earlier peaks in oocyst output have been linked to higher parasite loads. Furthermore, the pathogenicity of coccidiosis depends on a combination of host and parasite factors, including the inherent virulence of different *Eimeria* species **(Choi *et al.,* 2021)**. Notably, lactoferrin is a safe protein that can be used alongside low doses of other anticoccidial drugs. In this study, the combination treatment of lac-

toferrin and diclazuril significantly enhanced anticoccidial efficacy, resulting in a marked reduction in oocyst counts compared to the untreated infected group (G2).

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are key intracellular enzymes commonly used as biomarkers for diagnosing liver injury. Their serum concentrations may increase following liver damage due to compromised structural integrity and impaired hepatic function **(Coad *et al.,* 2014).** In the current study results, no significant differences in ALT and AST levels were observed among the diclazuril-treated (G4), protected (G3 and G5), and negative control groups when compared to the positive control group (G2), which supports the hepatotoxic potential of coccidial infection. These findings indicate that lactoferrin may contribute to preserving liver function, as evidenced by the nearly unchanged ALT and AST levels in groups (G3 and G5).

A significant increase in serum liver enzymes, specifically ALT and AST, was observed in the untreated group of rabbits infected with *E. stiedae*. This suggests that the infection harms liver function. The elevated enzymatic activity is likely a result of liver cell damage caused by the parasitic invasion, which disrupts cellular integrity and allows enzymes to leak into the bloodstream. These findings are consistent with previous studies that also reported elevated liver enzymes in rabbits experimentally infected with *E. stiedae* **(Allam *et al.,* 2020)**. Additionally, the increased levels of ALT and AST may indicate injury to the epithelial lining of the bile ducts, likely caused by the accumulation of parasite oocysts and an associated inflammatory response that leads to degenerative and necrotic changes **(Hanada *et al.,* 2003)**. Interestingly, AST levels were significantly lower in the groups pretreated with lactoferrin (G3 and G5) compared to the group treated with diclazuril alone (G4). This suggests a potentially greater hepatoprotective effect of lactoferrin. These results align with the observations of **(Abd El Monsef *et al.,* 2024)**, who demonstrated that co-administration of diclazuril and lactoferrin significantly reduced ALT and AST levels, which can be attributed to improved bioavailability and synergistic therapeutic action.

Furthermore, in this study, a notable increase in serum total protein and albumin levels was detected in Group 2 at 30 days post-infection. This elevation may result from the leakage of proteins due to hepatocellular damage. On the other hand, albumin levels can also decline as a consequence of impaired liver function, since hepatic degeneration associated with the infection may hinder albumin synthesis—the liver being the central organ responsible for protein production **(Cam *et al.,* 2008).** Additionally, albumin is recognized as a negative acute-phase protein, and its levels typically decrease during inflammatory conditions **(Hashemnia *et al.,* 2014).** rabbits in groups G1, G3, G4, and G5 showed a significant decrease in total protein and albumin levels compared to the positive control group (G2) and These observations are in line with findings by **(El-Sherbeny and El-Shenawy 2023)**, who demonstrated that lactoferrin plays a protective role in improving liver and kidney functions by significantly minimizing protein loss, enzyme leakage and tissue injury.

There were no significant differences in uric acid levels between the diclazuril-treated group (G4) and the protected groups (G3 and G5). In contrast, the infected, untreated group (G2) showed a significant increase in uric acid by the end of the study, likely due to impaired liver urea cycle function **(Yaplito-Lee *et al.,* 2013).** These observations are consistent with findings from (**Allam *et al.,* 2020).** Moreover, the groups receiving lactoferrin as a preventive treatment (G3 and G5) demonstrated significantly lower creatinine levels ($P < 0.05$) 30 days post-infection compared to the therapeutic diclazuril group (G4). These findings agreed with previous research by **(Saleh *et al.,* 2023).**

In our study on the antioxidant parameters tested in the sera of rabbits, we found that groups treated with lactoferrin and experimentally infected with *E. stiedae* showed a significant reduction in serum malondialdehyde and nitric oxide levels compared to other groups. In contrast, the positive control group (G2) exhibited a notable increase in both malondialdehyde and nitric oxide levels compared to the other groups. These findings are consistent with previous studies **(Hegazi *et al.,* 2023; Abd El**

Monsef *et al.,* 2024), which reported elevated levels of malondialdehyde and a marked decrease in glutathione activity during *E. stiedae* infection, indicating oxidative stress caused by the infection and a disruption in the antioxidant -prooxidant balance. Additionally, **(Cam *et al.,* 2008)** noted that rabbits infected with *E. stiedae* had elevated plasma malondialdehyde levels, suggesting that the parasite induces lipid peroxidation through damage to the liver parenchyma and bile ducts. Furthermore, **(Alsulami and El-Saadony 2023)** indicated that oxidative stress associated with *E. stiedae* infection leads to a decrease in glutathione levels, which is an essential antioxidant enzyme that plays a critical role in neutralizing free radicals and maintaining cellular redox balance. Moreover, (**Dominguez *et al.,* 2015)** mentioned that the elevated nitric oxide levels in the positive control group were attributed to the inflammatory response triggered by the pathogenic sporozoite stage invading cecal cells. All groups treated with lactoferrin showed a significant increase in glutathione (GSH) levels compared to the untreated rabbits infected with *E. stiedae*, with the most notable improvement observed in group G3. These results align with those of **(Mohamed *et al.,* 2025) and (Abd El Monsef *et al.,* 2024)**, who demonstrated that the elevation in glutathione levels reflects the antioxidant potential of lactoferrin, likely due to its iron-binding capacity, which enables it to sequester free iron and mitigate oxidative damage.

Lactoferrin is effective against both parasites and free-living protozoa present in mucosal tissues and blood. It acts as an agent that inhibits or kills microbes **(Zhao *et al.,* 2023)**. Its antiparasitic properties are attributed to its ability to attach to the membranes of protozoa, specifically interacting with phospholipids and proteins. This binding disrupts the stability of the membrane, leading to the destruction of the parasite **(Reyes-López *et al.,* 2022).**

To investigate the effect of lactoferrin on the pharmacokinetics of diclazuril in rabbits, we analyzed the main absorption parameters. Absorption has a crucial role in pharmacokinetic studies, as it reflects how drug concentrations change over time in the bloodstream **(Zhang *et al.,* 2010)**. Two important metrics in this context are the absorption rate constant (K_a) and the absorption half-life ($t_{1/2k_a}$), which together provide insights into how effectively a drug enters the central compartment after administration. Following oral administration, diclazuril was quickly absorbed. Rabbits that received lactoferrin alongside diclazuril (Group G5) showed significantly higher K_a values and notably shorter $t_{1/2k_a}$ values compared to those that received diclazuril alone (Group G4). These findings suggest that lactoferrin enhances the absorption rate of diclazuril when both substances are administered together.

The observed increase in the volume of distribution (V/F) for diclazuril in rabbits co-administered with lactoferrin (G5) suggests an enhanced tissue distribution in the presence of lactoferrin. **El Mahdy and Zinab (2022)** emphasize that pharmacokinetic parameters such as maximum serum concentration (C_{max}), area under the concentration–time curve (AUC_{0-t}), and mean residence time (MRT) are essential indicators for evaluating drug exposure. In our study, the combination of diclazuril with lactoferrin (G5) resulted in a higher C_{max} of 8.41 µg/mL at 4.63 hours compared to diclazuril alone, which had a C_{max} of 6.65 µg/mL at 4.21 hours. Additionally, the AUC_{0-t} for the combination group was larger at 84.96 µg/ml*h, while the MRT remained similar between the two groups. Notably, serum levels of diclazuril persisted at 3.15 µg/mL 12 hours post-dosing, suggesting enhanced absorption. These findings collectively indicate a synergistic interaction when diclazuril (1 mg/kg) is combined with lactoferrin (0.2 mg/kg). Furthermore, comparable pharmacokinetic behavior has been documented in equine models by (**Hunyadi *et al.,* 2015),** who reported that administration of diclazuril at both a low dose (0.5 mg/kg) and the labeled dose (1 mg/kg) resulted in similar steady-state plasma and cerebrospinal fluid (CSF) concentrations. This supports the efficacy of lower dosing regimens for inhibiting second-generation merozoites.

Pre-treatment with lactoferrin significantly alters the pharmacokinetics profile of diclazuril in rabbits, markedly enhancing its absorption and tissue distribution relative to diclazuril administered alone. This interaction increases serum concentrations, suggesting that lactofer-

rin improves the bioavailability and therapeutic effectiveness of diclazuril. These findings highlight the potential of combining lactoferrin with diclazuril to enhance its pharmacokinetic properties and therapeutic outcomes, presenting a promising strategy for better managing coccidiosis in rabbits.

Conclusion

Lactoferrin supplementation can help reduce the harmful effects of *E. stiedae* infection when used as a preventive measure. Notably, combining lactoferrin with diclazuril significantly enhances growth performance, suggesting a synergistic interaction between the two. This study also found that lactoferrin effectively alleviates the negative impacts of coccidiosis in rabbits by improving growth metrics, decreasing oocyst shedding, suppressing inflammatory responses, and reducing oxidative stress. These positive outcomes were supported by significant reductions in serum levels of malondialdehyde (MDA) and nitric oxide (NO), along with a marked increase in serum glutathione (GSH) levels. There is an urgent need to develop effective safety strategies to protect rabbit colonies from hepatic coccidiosis. Additionally, inactivating oocysts presents a more effective method for preventing infection.

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Conflict of interest statement

The authors declare no conflict of interest.

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