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Anticoccidial Efficacy of Lactoferrin and Diclazuril against Hepatic Coccidiosis in Rabbits: Molecular Docking Insights and Experimental Validation

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Abstract

HEPATIC coccidiosis, caused by *Eimeria stiedae*, poses a significant challenge to the rabbit production industry, leading to rabbit fatalities and considerable economic losses. This study compared the effectiveness of diclazuril and bovine lactoferrin, a multifunctional glycoprotein, in preventing hepatic coccidiosis in New Zealand White rabbits. Therefore, an in-silico study was conducted to examine the molecular docking interactions of lactoferrin with rabbit tumor necrosis factor-alpha (TNF-α) as well as the surface antigen (SAG) 4 and 5 proteins of *E. stiedae*, serving as a preliminary computational assessment before experimental validation. Subsequently, an in vivo study was performed using thirty-five one-month old New Zealand White rabbits were randomly allocated into five groups: negative control group, lactoferrin-treated uninfected group, infected untreated group, infected lactoferrin-treated group, and infected diclazuril-treated group. The assessed parameters included growth performance, parasitological infection evaluation, haematological measures, and liver pathology at 28 days post-infection. Molecular docking results exhibited a strong affinity between lactoferrin and diclazuril for rabbit TNF-*α*, which heightened the inflammatory response and their binding capability to SAG4 and SAG5, thereby regulating *E. stiedae* pathogenicity. The invivo study demonstrated that lactoferrin significantly improved body weight gain and feed conversion ratio compared to the diclazuril and the untreated infected groups. Furthermore, treatment with lactoferrin delayed and reduced oocyst shedding, minimized liver injury, reduced TNF-*α* expression in immunohistochemical laboratory analysis, and decreased the inflammatory markers, suggesting its potential as an anticoccidial agent. These results underscore the efficacy of lactoferrin in alleviating *E. stiedae* infection.

Keywords: Hepatic coccidiosis, *Eimeria stiedae*, diclazuril, molecular docking, anticoccidial efficacy, lactoferrin.

Introduction

Egypt was the world's third-largest producer of rabbit meat in 2018, after China and North Korea. Egypt produced an estimated 62,143 tons of rabbit meat during this period. Consequently, Egypt's rabbit meat industry has significantly affected the country's economy [1]**.** The prevalence of rabbit diseases has increased threating the intensive production procedures [2]. Hepatic coccidiosis is a prevalent protozoal disease in rabbits that is highly harmful and frequently lethal [3]. Coccidian protozoan known

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as *Eimeria stiedae*, which is commonly observed in domestic rabbits [3, 4], induces the disease**.** Hepatic coccidiosis poses a significant challenge, resulting in a decline in the commercial value of rabbits and substantial economic losses in rabbit farms, particularly in breeding and rearing facilities characterized by inadequate sanitation practices [5]**.** Juvenile rabbits demonstrated greater susceptibility to the disease. It is crucial to remember that infected adult rabbits may act as carriers and spread the infection to others [6]**.** The parasite transmitted through the ingestion of sporulated oocysts, which subsequently attack epithelial cells in the bile ducts of rabbits and cause considerable liver damage [7]. Mortality rates in infected rabbits may reach 30%, and they display a range of symptoms including anorexia, lethargy, diarrhea, jaundice, a distended abdomen, and hepatomegaly [8, 9]**.** In rabbits with hepatic coccidiosis, postmortem examination typically reveals yellowish-white nodules (1 mm – 2 cm) filled with creamy white material on the liver and parenchyma, gallbladder enlargement, hepatomegaly, and the presence of straw-colored peritoneal fluid [3, 10]**.**

Enhancing rabbit farm productivity necessitates effective control of hepatic coccidiosis [11]**.** Despite their high cost, the preventive and therapeutic use of anti-coccidial medications has constituted the cornerstone of conventional disease management strategies [12]**.** The emergence of drug-resistant *Eimeria* strains is a direct result of the overuse and abuse of anticoccidial medications. This has led to concerns regarding drug residues in animal products and how they can affect food safety and public health [2]. Therefore, the investigation of alternative safe and effective anti-*Eimeria* drugs is becoming increasingly urgent. To diminish chemotherapy-related side effects, natural products have been investigated as possible anti-coccidial medications. Parasites can be sensitive to natural treatments without becoming resistant to them [13]**.** Lactoferrin (LF) is a multifunctional cationic glycoprotein belonging to the transferrin family. It has a molecular weight of approximately 78 kDa and is composed of amino acids arranged in a single polypeptide chain [14]**.** After being discovered in 1939 in whey, it took another 20 years to refine and extract it from both human and bovine milk [15]**.** Significant amounts of LF are present in milk and colostrum [13]. It is also found in many bodily fluids, especially those from the genital, respiratory, and gastrointestinal systems [16]**.** Furthermore, at infection sites, neutrophil secondary granules produce LF [17]**.** According to [18]**,** LF can be found in three different forms: partially iron-saturated (mono), iron-depleted (apo), and iron-saturated (holo). There is a high degree of conservation of homology along this chain across many various species of mammals [19]. As previously reported [20]**,** LF is rapidly absorbed into the bloodstream of mammals, reaching a peak concentration 12 hours after oral administration. Subsequently, the portal vein and lymphatic pathways carry LF to different tissues [21]**.** It is then excreted in the gastrointestinal tract via bile secretion [21]**.** LF has been associated with a variety of biological features, including the promotion of iron absorption, scavenging of free radicals, augmentation of the immune system, and modulation of gastrointestinal functions [22]**.** Moreover, it exhibits anti-inflammatory, antibacterial, antiviral, antifungal, antiparasitic, and anticarcinogenic properties [23, 24]**.**

The parasiticidal actions against various parasites have been ascribed to both apo-LF and holo-LF [13]**.** The LF can interact with parasites and free-living protozoa in mucosae and blood, functioning as a microbiostatic and/or microbiocidal agent either in experimental studies using parasite culture media or animal models [13, 15]**.** The effect of LF against protozoa is attributed to its capacity to engage with cellular components of protozoan membranes (phospholipids and proteins). This interaction subsequently disrupts the stability of the membrane, ultimately resulting in the breakdown of the parasite [13]**.** Studies have documented the biological interactions between LF and various parasites such as *Trypanosoma spp*., *Leishmania spp*., *Plasmodium spp*., *Babesia spp*., *Toxoplasma gondii*, and *Trichomonas vaginalis*. These studies have examined the impact of LF on the action modes or pathways of these parasites [13, 25–28]**.** In 2001, a study was carried out to evaluate the efficacy of bovine lactoferrin against *Eimeria stiedai*, both in vivo using rabbits and mice, and in vitro using rabbit hepatobiliary cells and mouse embryonic cells. The findings of this study indicated that LF can decrease the infectivity and intracellular penetration of *E. stiedai* sporozoite *in vivo* and *in vitro* [29]**.**

Considering the aforementioned therapeutic potential of lactoferrin in combating parasites and protozoa in both animals and humans, as well as the existing need for alternative treatments for coccidiosis and the limited knowledge regarding the anti-coccidial properties of lactoferrin in previous research, this current study aimed to examine the protective impact of lactoferrin as a preventive and therapeutic agent against *E. stiedae* infection in rabbits, in comparison to the effects of commercial chemotherapy.

Material and Methods

Drugs

Lactoferrin was acquired from Medizen Pharmaceutical Industries, Borg El Arab, Egypt; diclazuril oral solution was obtained from Pharma Swede, 10th of Ramadan, Egypt.

Molecular docking

The three-dimensional structures of bovine lactoferrin (Q6LEB5), rabbit TNF-α (P04924), *E. stiedae* surface antigen 4 (SAG4; A0A6H0C284), and SAG5 (A0A6H0C5Z9) were retrieved from the UniProt database (https://www.uniprot.org/). Molecular docking interactions of lactoferrin with TNF-*α*, SAG4, and SAG5 were performed using the HDOCK server [30].

The three-dimensional structure of diclazuril was sourced from the PubChem (https://pubchem.ncbi.nlm.nih.gov/) database. The molecular docking interactions of diclazuril against TNF-α, SAG4, and SAG5 were done using AutoDock Vina and Chimera 1.18 [31]. These interactions were visualized using the BIOVIA Discovery Studio 2016 (https://www.3ds.com/products/biovia/discovery-studio) software.

Isolation, propagation, and preparation of Eimeria stiedae oocysts

E. stiedae oocysts were collected from the gallbladders of naturally infected rabbits that were collected from local rabbit farms, which tested positive for *Eimeria* via fecal examinations. The obtained oocysts were morphologically confirmed and kept in a 2.5% potassium dichromate solution until sporulation.

For propagation of *E. stiedae* oocysts, five rabbits free from *Eimeria* infection were orally administered with sporulated oocysts. After 20 days, these rabbits were slaughtered according to institutional ethical guidelines, and the gall bladders were removed, crushed, sieved, rinsed with saline solution, and concentrated using the flotation method. The oocysts obtained were examined under a microscope and maintained at 4°C till required. The sporulated oocysts were counted for infection of rabbits using the McMaster technique.

Experimental animals and ethical approval

Thirty-five healthy New Zealand white weaned rabbits (*Oryctolagus cuniculus*) of both sexes free from coccidian infection were obtained from a governmental farm in Sharqia, Egypt, and kept under observation until the challenge time. The rabbits were housed individually in clean wire-floored batteries with the same nutritional, environmental, and hygienic conditions throughout the experimental period. The rabbits were fed a commercial diet free from any therapeutic additives. Food and water were supplied *ad libitum* throughout the experiment. Fecal examination of the rabbits was performed daily for three successive days before infection to verify that the rabbits were *Eimeria* species-free before the experiment. All experimental designs were conducted according to the ethical standards of the Faculty of Veterinary Medicine, Benha University, Egypt, and approved by the Institutional Animal Care and Use Committee of Benha University (ethical number BUFVTM 10-02-22).

Study Design

The study was conducted on 35 New Zealand White rabbits, randomly divided into 5 groups (n=7 per group). G1, non-infected and non-treated (control negative); G2, non-infected and lactoferrin treated (LCT), G3, *E. stiedae* infested, non-treated (E.S), G4, *E. stiedae* infected and lactoferrin treated (E.S+LCT), and G5, *E. stiedae* infected and diclazuril treated (E.S+DLZ). In G 3, 4 & 5, hepatic coccidiosis was induced by oral administration of *Eimeria stiedae* oocysts at a dose of 2×10^4 per rabbit. Treatments with lactoferrin and diclazuril were administered daily orally each morning, starting two days before infection at doses of 0.2 mg/kg BW for lactoferrin and 0.5 ppm for diclazuril, and continued until the end of the trial (28 days post-infection "dpi"). Clinical signs, body weight, liver function markers, and histopathological changes were monitored throughout the study. Rabbits were euthanized on day 28d dpi, and blood, liver, and spleen samples were collected for analysis.

The clinical signs (loss of appetite, diarrhea, depression, and ruffled hair) and mortality were recorded. Freshly voided fecal samples were collected separately from each group from coccidian infestation till 28 dpi. Blood samples were from the ear vein at the end of the experiment on the 28th dpi with and without an anticoagulant. Blood with anticoagulant was used for haematological analyses, whereas blood without anticoagulant was separated into serum and kept at -20°C for biochemical examinations. On the 28th dpi, all rabbits from each group were humanely slaughtered, and macroscopic examination and lesion scoring of the livers were performed as previously described [32]**.** Livers were weighed and fixed in neutralized 10% formaldehyde for histopathological examination. Spleen samples were preserved at -80°C for RNA isolation and gene expression analysis.

Evaluation criteria for the effectiveness of lactoferrin and diclazuril

Growth performance parameters

The live body weight (BW) and feed intake (FI) were recorded from the onset and 28 DPI to calculate body weight gain (BWG) and feed conversion ratio

(FCR), as well as to estimate relative FCR and BWG compared to the control groups.

Parasitological analysis

Freshly voided fecal samples were collected separately from various groups simultaneously on the same day following coccidian infection to facilitate the detection of the onset of oocyst shedding and the quantification of oocysts by the McMaster counting technique. The oocysts per gram of feces (OPG) and the relative OPG compared to the infected untreated group were calculated.

Haematological examinations

The haematological examination utilized an automated cell counter to estimate the following parameters: red blood cell (RBC) count, packed cell volume (PCV), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), white blood cell (WBC) count, and blood platelet count.

Histopathological examination

The fixed liver tissues were dehydrated in a graded ethanol series. The tissues were then cleared using xylene and embedded in paraffin wax for sectioning. Thin sections of 5-7 μm were prepared employing a microtome and then stained using hematoxylin and eosin (H&E), a standard histological staining technique [33]**.** These sections were subsequently examined for histopathological alterations using a computerized light microscope (Leica DM 3000 LED). Histopathological lesions scoring was performed according to a previously described method [32]**.** The scoring criteria were defined based on the nature and prevalence of lesions within randomly selected fields as follows: marked (involving 41-100% of the tissue), moderate (21-40% of the tissue involved), mild (11-20% of the tissue involved), and minimal (0-10% of the tissue involved).

Immunohistochemical analysis of TNF-α in liver tissues

The liver tissues were fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemical analysis. The staining protocol was adapted from [34]. Briefly, paraffin-embedded sections were deparaffinized, rehydrated, and subjected to antigen retrieval. Immunohistochemical staining was performed using an avidin-biotin-peroxidase complex technique with primary antibodies against Tumor Necrosis Factor-alpha (TNF-α) (Ab clonal). The sections were counterstained with Meyer's hematoxylin.

To account for non-specific background staining, a negative control was included by replacing the primary antibody step with normal serum while keeping all other staining steps consistent. For quantitative assessment, TNF-*α* immunoreactivity was analyzed using an image analysis system (Leica Q500 DMLB) at 400× magnification. Five non-overlapping fields from five sections per group were selected for evaluation. Digital images of the immune-stained sections were processed using ImageJ software (version 1.54f, National Institutes of Health, Bethesda, MD, USA). Each image was calibrated for scale and converted to grayscale for enhanced contrast. ImageJ's 'Threshold' function was employed to differentiate positive staining (brown areas) from the background. The percentage of positively stained areas (relative to the total tissue area) was calculated. This procedure was repeated consistently across ten fields for each sample to ensure reliability and accuracy.

Statistical analysis

Data was analyzed using the statistical software package SPSS for Windows (version 20.0; SPSS Inc., Chicago, IL, USA). One-way Analysis of Variance (ANOVA) was used to compare means among groups, followed by Duncan's multiple range test to determine significant differences between individual groups. A p-value < 0.05 was considered statistically significant. Results are expressed as mean ± standard error (SE).

Results

Molecular docking assessment

Bovine lactoferrin interacted with rabbit TNF-*α* and *E. stiedae* SAG4 and SAG5 with binding free energies of -324.37 (Fig. 1A), -227.11 (Fig. 1B), and -228.62 (Fig. 1C) kcal/mol, respectively.

Table 1 and Fig. 2 show the molecular docking interactions of diclazuril with rabbit TNF-*α*,*E. stiedae* SAG4 and SAG5. Diclazuril interacted with ASP124 (hydrogen bond), ASN125 (hydrogen bond), VAL214 (hydrogen bond), and PRO217 (hydrophobic interaction) residues in the binding site of TNF-*α* by binding free energy of -6.60 kcal/mol (Fig. 2A). Fig. 2B shows the interaction of diclazuril with TRP60 (hydrogen bond and hydrophobic interaction), SER61 (hydrophobic interaction), SER63 (halogen), VAL64 (halogen), VAL66 (hydrophobic interaction), LEU134 (hydrophobic interaction), GLY137 (hydrogen bond), PHE138 (hydrogen bond), and ALA141 (hydrophobic interaction) residues of SAG4 by binding free energy of -7.30 kcal/mol. In the binding site of SAG5, diclazuril bound with PRO55 (hydrophobic interaction), HIS115 (hydrogen bond and hydrophobic interaction), LYS118 (hydrophobic interaction), ILE119 (hydrophobic interaction), GLY121 (hydrophobic interaction), ALA122

(hydrogen bond and hydrophobic interaction), ASN125 (hydrogen bond), ALA134 (hydrophobic interaction), and PHE135 (hydrophobic interaction) residues by binding free energy of -7.30 kcal/mol (Fig. 2C).

Efficacy of lactoferrin and diclazuril on growth performance against hepatic coccidiosis in rabbits

At the experiment onset (0 dpi), the body weights of the tested rabbits were similar across all groups. As shown in Fig. 3, significant differences were observed at 28 dpi. The average body weight and weight gain were significantly increased in the lactoferrin groups, even with E.S. infestation, compared with all groups, including the negative control group. Significant reduction in feed intake and FCR supports the positive impact of lactoferrin on weight. E.S. infestation rabbits and the diclazuril-treated and infected rabbits (E.S+DLZ) showed the lowest body weight gain and the highest FCR.

Efficacy of lactoferrin and diclazuril on fecal oocyst shedding

Oocyst output was first detected in feces at 18 dpi in rabbits of the E.S-infected and E.S+DLZ groups. The OPG values in E.S-infected rabbits attained their highest value compared to the other groups in the study. In contrast, oocysts were delayed shedding in the fecal samples of lactoferrin-treated rabbits (E.S+LCT) until the 20^{th} dpi, with the lowest recorded OPG values through the experimental period (Table 2).

Haematological parameters

The experimental groups showed a non-significant ($p>0.05$) increase in RBCs, HB, PCV, lymphocytes, monocytes, and basophils values, indicating that neither lactoferrin nor diclazuril adversely affected these parameters. The infected and non-treated rabbits of E.S-infected had notably ($P < 0.05$) higher WBC and eosinophil counts than the other groups. The lactoferrin-treated rabbits (E.S+LCT) showed a drastic ($p<0.05$) decline in WBC and eosinophil counts when compared to E.S-infected rabbits (Tables 3 and 4).

Clinical and pathological findings

According to daily clinical observations, the infected group treated with diclazuril (E.S+DLZ) exhibited matted perineal areas, rough hair coats, and an enlarged abdomen, similar to all the rabbits in the positive control group (E.S). Additionally, they were dehydrated, depressed, and weakened. The icteric mucous membrane was visible in the five positive control rabbits (Fig. 4A). Two animals from the positive control E.S. infected group and one from the diclazuril-treated group were recorded as dead. control, LCT, and E.S+LCT groups showed no mortality or obvious clinical symptoms.

At necropsy on the 28^{th} dpi, the rabbits in the E.S infected group (infected& not treated) displayed a significant ($p < 0.05$) increase in liver weight Fig. 5A, the relative weight of the liver Fig. 5B, as well as its lesion score Fig. 5C. These lesions included thick, firm, yellowish gallbladder contents, discolored and enlarged livers with dilated bile ducts, and elevated yellowish-white nodules on the liver surface. Additionally, these livers were firm in consistency (Fig. 4C-D). As compared to the livers of the rabbits in the positive control group (E.S), the livers of the diclazuril-treated rabbits (E.S+DLZ) showed a significant ($p < 0.05$) lower in weight, liver relative weight, and macroscopic lesions score. Distinctly, a small number of pale nodules and a minor distention in the gallbladder exhibiting a greenish yellow, slightly viscous substance were noted in the E.S+DLZ rabbits (Fig. 4B and F). Regarding pathological findings resulting from the *E. stied*ae infection, the livers of the rabbits treated with lactoferrin (E.S+LCT) did not exhibit any appreciable differences from the livers of the control negative group.

Histopathological findings

Microscopic examination of the liver showed normal, well-defined hepatocytes and sinusoidal spaces, indicating the normal liver structure in the negative control group (Fig. 6A). Hepatic tissue with intact structural integrity was observed in the LCT group. The absence of notable pathological alterations was indicated by the hepatocytes, blood sinusoids, and central vein appearing morphologically normal. Fig. 6B, shows that liver histopathology was normal in both the lactoferrin-treated and negative control group. In contrast, E.S infected animals showed significant pathological alterations and extensive liver damage as a result of Coccidian infection. This demonstrates how seriously the infection affected the integrity of the liver tissue (Fig. 6C-D). Conversely, the lactoferrin-treated group showed minimal to nonexistent severity in lesions and liver architecture, with minimal desquamation of the duct epithelium and no Eimeria, suggesting an effective reduction in infection-induced liver damage because of lactoferrin treatment (Fig. 6E). In the majority of parameters, G5 displayed moderate severity. However, moderate severity in desquamated duct epithelium indicates that the diclazuril treatment may somewhat mitigate the infection's severity.

Immunohistochemical findings

As shown in Fig. 7, tumor necrosis factor-alpha in the hepatocyte was mainly distributed within the

cytoplasm and appeared yellow to brown, with granular staining. TNF-α protein expression levels were significantly higher in *E. stiedae*-infected rabbits than in the control rabbits. Control-negative rabbits showed non-significant expression of TNF-α in the cytoplasm of hepatic cells, indicating a lack of apoptosis in normal liver tissue. The LCT group appeared similar to control-negative rabbits, showing negligible expression of TNF-*α* in the hepatic cells. Conversely, the infected untreated rabbits exhibited high expression levels of TNF-α in the cytoplasm of hepatic cells, which was consistent with severe hepatic lesions, hepatocyte atrophy, and necrosis. The hepatic cells of the infected lactoferrin-treated rabbits exhibited limited TNF-*α* production, indicating a reduced level of apoptosis compared to infected untreated rabbits. The infected diclazuril-treated rabbits depict moderate expression of TNF-*α* in hepatic cells.

Discussion

Coccidiosis is the most prevalent parasitic infection in domestic rabbits. The limitations of using some chemical medications and growth enhancers have induced digestive issues and mortalities among growing rabbits [3]. This study aims to evaluate the effectiveness of lactoferrin and diclazuril as preventive and therapeutic agents for hepatic coccidiosis in rabbits. In an *in-silico* study of the molecular docking interactions of lactoferrin and diclazuril, as a reference drug, with rabbits' TNF-*α* and *E. stiedae*'s SAG4 and SAG5 was conducted as a preliminary computational tool before the experimental validation. Results showed high affinity for lactoferrin and diclazuril for rabbits' TNF-*α,* which elevated the inflammatory process, besides their binding ability for SAG4 and SAG5 that controlled host cell invasion, immune evasion, and pathogenicity of *E. stiedae*.

The notable enhancement in body weight and FCR in infected and/or lactoferrin-treated groups relative to the infected untreated group and diclazuril-treated group highlights the potential of lactoferrin as a growth promoter. This corresponds with prior research demonstrating that lactoferrin improves food absorption and facilitates growth in multiple animal models [14]. Lactoferrin's capacity to enhance feed efficiency may be attributed to its function in regulating gut flora and promoting intestinal health [13, 36]. Moreover, lactoferrin's capacity to bind iron enhances iron absorption, essential for growth and development [22]. The present study demonstrated that the parasitological analysis of feces from experimentally infected rabbits indicated a prepatent period of 18 days for *E. stiedae* infection. Comparable outcomes were noted by [37]. The delayed and reduced fecal oocyst shedding in lactoferrin-treated rabbits till 20 days post-infection, compared to the infected untreated group and diclazuril-treated group, indicates a potent anticoccidial effect. This finding is aligned with [29], who reported that lactoferrin reduced the infectivity of *Eimeria stiedae* sporozoites. The mechanism behind this effect may involve: (1) preventing the parasites from making more iron; (2) competing with them for the binding site in the host cell; (3) attaching to the parasites' internal membranes, cytoplasmic membrane, and cytoskeleton, which destabilizes these structures and causes the parasites to die; (4) influencing the induction of the immature cyst stage; and (5) by activating macrophages to phagocytize and eliminating the parasites [13]. Moreover, lactoferrin possesses antimicrobial properties, including antibacterial, antiviral, and antifungal actions, which enhance its efficacy in diminishing oocyst shedding [16]. The non-significant changes in RBC, HB, and PCV across all groups suggest that neither lactoferrin nor diclazuril adversely affected these haematological parameters. However, the significant reduction in WBC counts and eosinophil levels in lactoferrin-treated groups compared to the infected untreated group indicates an anti-inflammatory effect.

Clinical observations and pathological findings indicated that lactoferrin exceeded diclazuril in alleviating the negative drawbacks of hepatic coccidiosis on the health condition and hepatic tissues of infected animals. The lack of notable clinical symptoms and mortality in lactoferrin-treated rabbits aligns with the protective effects documented in other research [17, 38]. The marked decrease in liver weight and lesion severity in lactoferrin-treated rabbits indicates that lactoferrin could effectively mitigate hepatic damage induced by Eimeria stiedae infection. This protective effect may be attributed to lactoferrin's capacity to regulate the immune response and diminish oxidative stress, thus averting significant liver damage [39]. Lactoferrin releases micro peptides with antimicrobial properties under acidic gastric conditions; these peptides have several antimicrobial and anti-inflammatory effects. Additionally, it promotes the growth of villi in the intestines. The incorporation of lactoferrin into feed enhanced intestinal function and accelerated healing [36].

Hepatic coccidiosis appears as milky spots on the liver's surface and inside the parenchyma, along with a larger gallbladder than usual. Between days 21 and 24 after infection, sick rabbits can show hepatomegaly and ascites at the same time [40]. Histopathological analysis revealed minimal hepatic damage in lactoferrin-treated rabbits, characterized by well-preserved liver architecture and diminished inflammatory cell infiltration. Also, immunohistochemical analysis demonstrated diminished TNF-*α* expression in lactoferrin-treated rabbits, indicating decreased hepatic inflammation and apoptosis. This supports the concept that lactoferrin regulates the immune response and protects against hepatic injury [41]. The reduction in

TNF-*α* expression is notably significant as this cytokine is essential in the inflammatory response, and its overexpression correlates with severe liver pathology [18]. The study limitations include the relatively small sample size and the assessment of single doses of lactoferrin. Future research should involve larger sample sizes, multi-dose, and mechanistic investigations to clarify the exact pathways of lactoferrin's anticoccidial effect.

Conclusion

In conclusion, this study demonstrated that lactoferrin is an exceptionally effective therapeutic agent against hepatic coccidiosis in rabbits. It enhances growth performance, significantly reduces fecal oocyst shedding, and protects against liver damage. Lactoferrin's diverse biological activities are considered as a promising alternative to conventional anticoccidial drugs like diclazurilwhich may have drawbacks such as resistance and side effects. Future research should focus on uncovering the specific mechanisms underlying the anticoccidial effects of lactoferrin and exploring its applications in other species and parasitic infections. Long-term and large-scale studies with large samples size are also recommended to evaluate the safety, efficacy, and optimal dosages of lactoferrin, paving the way for its broader use in veterinary medicine.

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Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Author contributions

All authors have made substantial contributions to this work. Sawsan S. Elbasuni, Marwa I. Abdel Haleem, and Mohamed A. Abaza conceived and designed the study, supervised experimental procedures, and coordinated the preparation and revision of the manuscript as corresponding authors. Hala El Daous contributed to animal husbandry, infection management, and monitoring of growth parameters. Nagwa M. Elhawary provided expertise in parasitological assessments and the biology of **Eimeria stiedae**. Samar H. Baloza contributed to the analysis and interpretation of the molecular docking and genetic data. Maha Mamdouh conducted haematological and physiological evaluations, while Yasmeen Magdy performed histopathological examinations and assessed liver pathology. Ali ElFar was responsible for the execution and validation of molecular docking studies. All the authors participated in data interpretation, critically revised the manuscript, and approved the final version for submission.

Ethical of approval

All experimental designs were conducted according to the ethical standards of the Faculty of Veterinary Medicine, Benha University, Egypt, and approved by the Institutional Animal Care and Use Committee of Benha University (Ethical number BUFVTM 10-02-22).

TABLE 1. Molecular interactions of diclazuril with rabbits' TNF-*α* and *Eimeria stiedae*'s surface antigen 4 (SAG4) and surface antigen 5 (SAG5)

Targets	Residues	Hydrogen bond	Charge	Hydrophobic interaction	Halogen
TNF-*α*	ASP124	1	0	0	0
	ASN125	1	0	0	0
	VAL214	1	0	0	0
	PRO217	0	0	1	0
SAG4	TRP60	1	0	1	0
	SER61	0	0	1	0
	SER63	0	0	0	1
	VAL64	0	0	0	1
	VAL66	0	0	1	0
	LEU134	0	0	1	0
	GLY137	1	0	0	0
	PHE138	1	0	0	0
	ALA141	0	0	1	0
SAG5	PRO55	0	0	1	0
	HIS115	1	0	1	0
	LYS118	0	0	1	0
	ILE119	0	0	1	0
	GLY121	0	0	1	0
	ALA122	1	0	1	0
	ASN125	1	0	0	0
	ALA134	0	0	1	0
	PHE135	0	0	1	0

TABLE 2. Efficacy of the lactoferrin and diclazuril on fecal oocyst shedding of *Eimeria stiedae* infected rabbits.

Group	Fecal oocyst shedding					
	18 dpi	**20 dpi**	**22 dpi**	**24 dpi**	**26 dpi**	**28 dpi**
control	0.0 ± 0.0^{b}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
LCT	0.0 ± 0.0^{b}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
E.S	$2.66\pm0.31\times10^{6a}$	$5.39\pm0.52\times10^{6a}$	$9.40\pm0.35\times10^{6a}$	$2.43\pm0.41\times10^{7a}$	$1.66\pm0.28\times10^{7a}$	$2.43\pm0.72\times10^{7a}$
E.S+LCT	0.0 ± 0.0^{b}	$1.55\pm0.12\times10^{6c}$	$1.43\pm0.62\times10^{6c}$	$1.39\times10^{6}\pm0.22^{b}$	$2.05\pm0.52\times10^{5b}$	$3.43\pm0.82\times10^{4b}$
E.S+DLZ	$4.19\pm1.02\times10^{4b}$	$3.59\pm0.87\times10^{6b}$	$6.61\pm0.67\times10^{6b}$	$4.05\pm0.16\times10^{6b}$	$4.60\pm0.64\times10^{5b}$	$3.16\pm0.71\times10^{5b}$

[a, b, c,,,] Means with the different indices between groups are significantly different ($p<0.05$). Values are given as the mean ± SE ($n=3$)**.* dpi:** days post infection. **control;** non-infected and non-treated (control negative), **LCT;** non-infected and lactoferrin-treated animals, **E.S;** infested, non-treated (control positive), **E.S+LCT;** infected and lactoferrin treated, and **E.S+DLZ;** infected and diclazuril treated.

TABLE 3. Effect of the lactoferrin and diclazuril on haematological parameters of *Eimeria stiedae* infected rabbits.

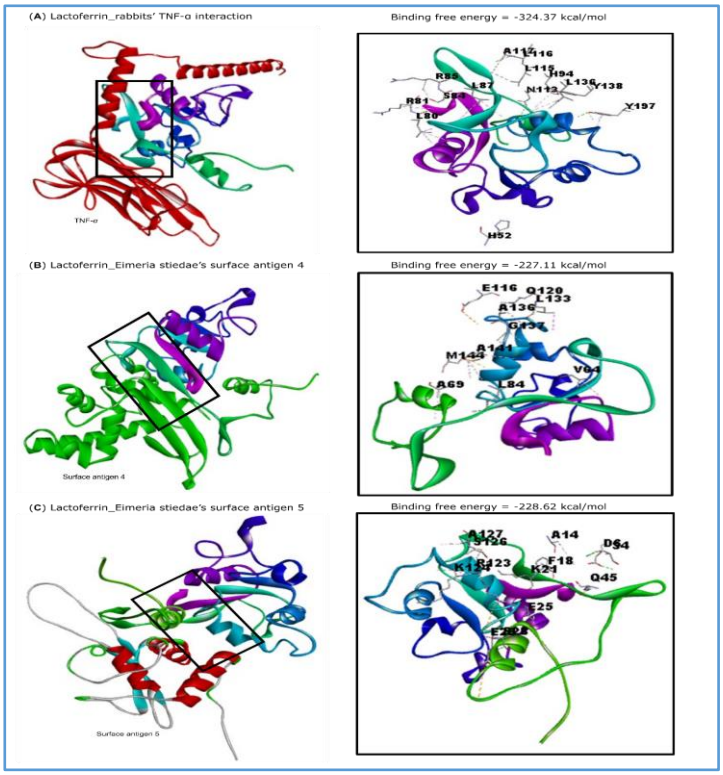
Groups	$RBC_S((10^6/\mu L))$	Hb (g/dl)	PCV (%)
control	$3.06\pm0.073*10^{6a}$	8.67 ± 0.21^{a}	25 ± 0.6^{a}
LCT	$3.00\pm0.078*10^{6a}$	8.66 ± 0.23^{a}	25.27 ± 0.7^{a}
E.S	$3.08\pm0.092*10^{6a}$	8.53 ± 0.26^{a}	25.27 ± 0.6^{a}
E.S+LCT	$2.08\pm0.041*10^{6a}$	8.53 ± 0.41^{a}	25.37 ± 0.5^{a}
E.S+DLZ	$3.08\pm0.032*10^{6a}$	9.00 ± 0.19^{a}	25.77 ± 0.6^{a}

[a, b, c,,,] Means with the different indices between groups are significantly different ($p<0.05$). Values are given as the mean ± SE ($n=3$)**. RBCs:** red blood cells**; Hb**: haemoglobin**; PCV% %:** packed cell volume. **control;** non-infected and non-treated (control negative), **LCT;** non-infected and lactoferrin-treated animals, **E.S;** infested, non-treated (control positive), **E.S+LCT;** infected and lactoferrin treated, and **E.S+DLZ;** infected and diclazuril treated.

TABLE 4. Effect of the lactoferrin and diclazuril on haematological parameters of *Eimeria stiedae* infected rabbits (Con.).

Groups	WBCs $(10^3/\mu L)$	Neutrophils$(10^3/\mu L)$	Lymphocytes $(10^3/\mu L)$	Monocytes $(10^3/\mu L)$	Eosinophils $(10^3/\mu L)$	Basophils $(10^3/\mu L)$
control	$9.4\pm0.9*10^{3b}$	1.31 ± 0.19^{b}	$7.3\pm0.\ 78^{a}$	0.68 ± 0.01^{a}	0.09 ± 0.01^{d}	0.8 ± 0.0^{a}
LCT	$9.8\pm1.1*10^{3b}$	1.05 ± 0.24^{b}	7.9 ± 0.64^{a}	0.61 ± 0.09^{a}	0.16 ± 0.03^{d}	0.08 ± 0.03^{a}
E.S	$16.7\pm3.01*10^{3a}$	2.51 ± 0.39^{a}	10.2 ± 1.4^{a}	0.66 ± 0.01^{a}	3.2 ± 0.04^{a}	0.07 ± 0.03^{a}
E.S+LCT	$10.1\pm0.08*10^{3b}$	1.33 ± 0.42^{b}	7.3 ± 2.4^{a}	0.64 ± 0.02^{a}	1.44 ± 0.08^{b}	0.07 ± 0.05^{a}
E.S+DLZ	$13\pm1.79*10^{3ab}$	1.81 ± 0.34^{ab}	9.4 ± 0.67^{a}	0.6 ± 0.09^{a}	1.12 ± 0.07^{c}	0.07 ± 0.03^{a}

[a, b, c,,,] Means that the different indices between groups are significantly different for $p<0.05$. Values are given as the mean ± SE ($n=3$)**. WBCs:** white blood cells. **control;** non-infected and non-treated (control negative), **LCT;** non-infected and lactoferrin-treated animals, **E.S;** infested, non-treated (control positive), **E.S+LCT;** infected and lactoferrin treated, and **E.S+DLZ;** infected and diclazuril treated.



Fig, 1. Molecular interactions of lactoferrin with rabbits' TNF-α and *Eimeria stiedae*'s surface antigen 4 (SAG4) and surface antigen 5 (SAG5). A: Lactoferrin rabbit TNF-α interaction B: Lactoferrin E. stiedae surface antigen 4 interaction. C: Lactoferrin E. stiedae surface antigen 5 interaction. A score of less than -200 refers to a poor score. Scores between -200 and -275 are considered moderate. Scores between -275 and -400 or more are considered promising.

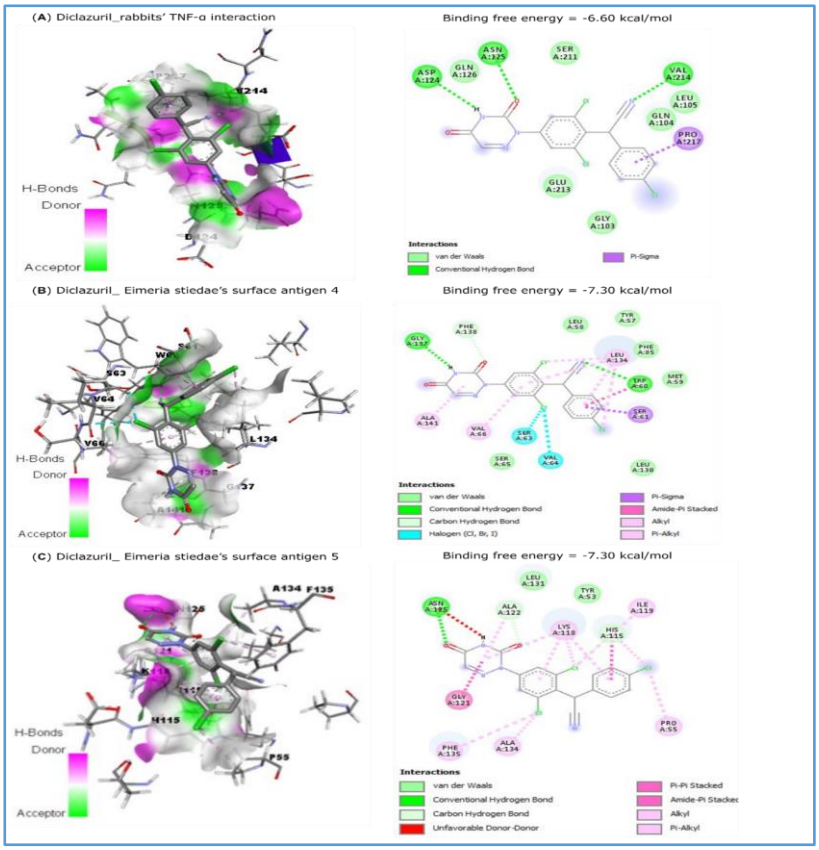


Fig. 2. Molecular interactions of diclazuril with rabbits' TNF-α and *Eimeria stiedae*'s surface antigen 4 (SAG4) and surface antigen 5 (SAG5). A: Diclazuril rabbit TNF-α interaction B: Diclazuril E. stiedae surface antigen 4 interaction. C: Diclazuril E. stiedae surface antigen 5 interaction A score of less than -200 refers to a poor score. Scores between -200 and -275 are considered moderate. Scores between -275 and -400 or more are considered promising.

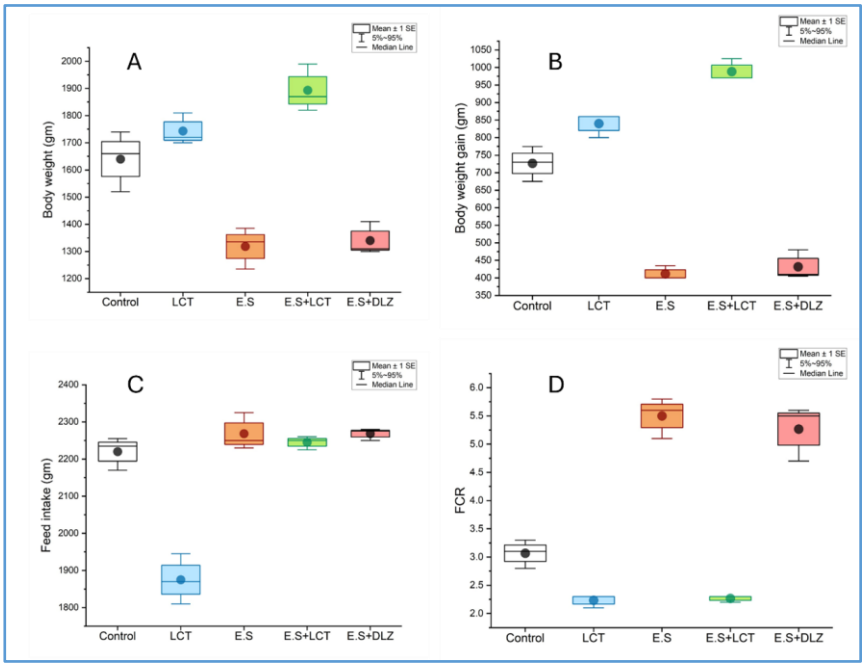


Fig. 3. Growth performance parameters in experimental groups. (A) Live body weight (g). (B) Body weight gain (g). (C) Feed intake (g). (D) Feed conversion ratio (FCR). groups: Control: Non-infected, non-treated. LCT: Non-infected, lactoferrin-treated. E.S: *E. stiedae*-infected, non-treated. E.S+LCT: *E. stiedae*-infected and lactoferrin-treated. E.S+DLZ: *E. stiedae*-infected and diclazuril-treated.

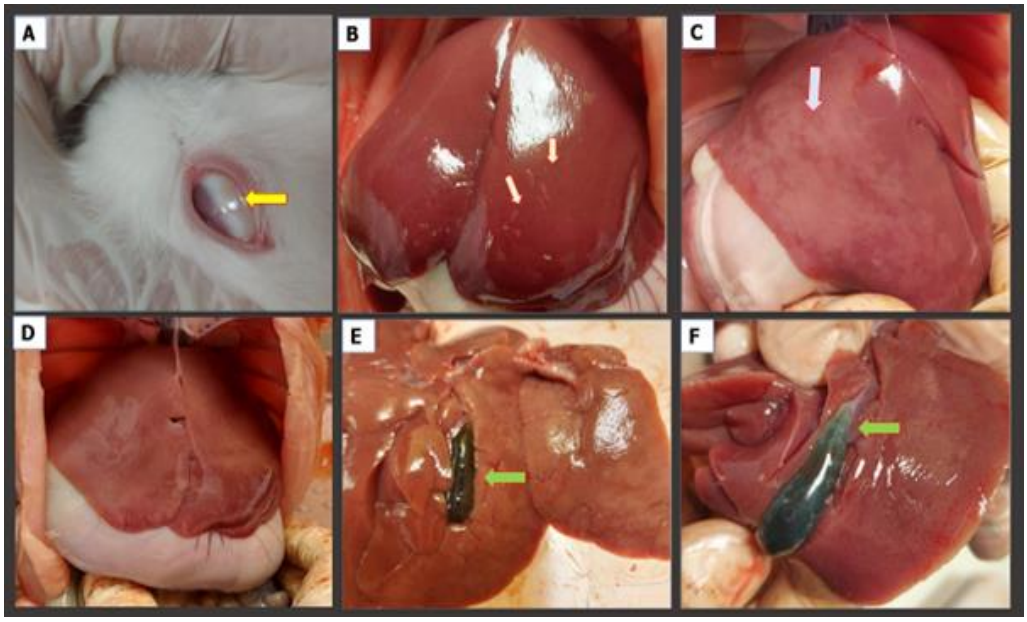


Fig. 4. Effects of the lactoferrin and diclazuril on the clinical and pathological findings of hepatic coccidiosis in rabbits. A) Icterus mucous membrane (yellow arrow pointed); B) White nodules (white arrows pointed); C-D) Enlarged pale discolored liver with diffuse white patches; E) Enlarged pale fibrous liver and distended gallbladder with a yellowish discolored semisolid content (green arrow pointed); F) Mahogany colored liver and distended gallbladder with a slightly greenish yellow slightly viscous content (green arrow pointed).

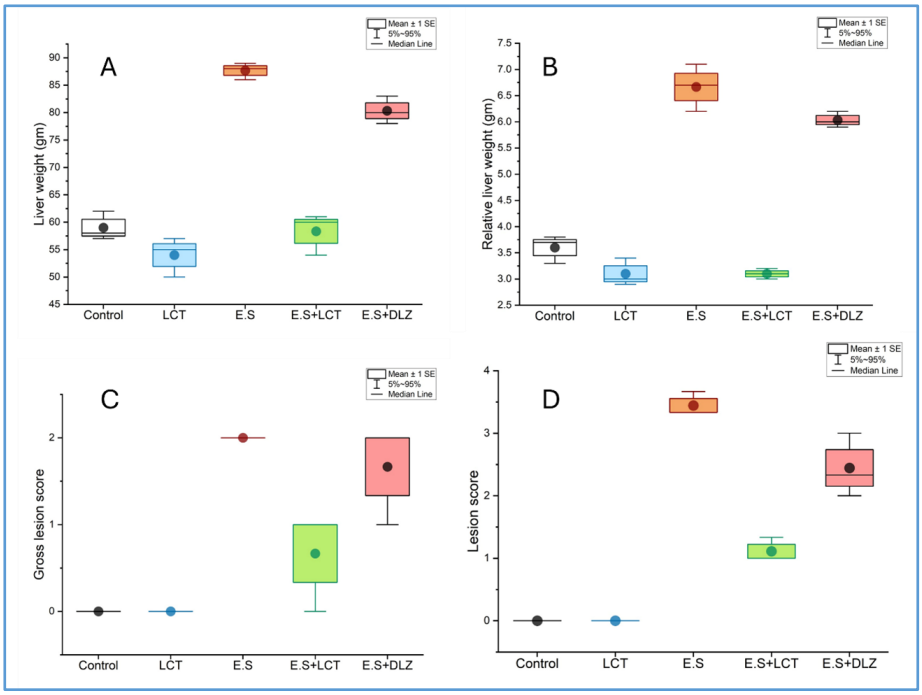


Fig. 5. Hepatic pathological assessment and lesion scoring. (A) Liver weight (g). (B) Relative liver weight (g). (C) Gross lesion score. (D) Histopathological lesion score. groups: Control: Non-infected, non-treated. LCT: Non-infected, lactoferrin-treated. E.S: *E. stiedae*-infected, non-treated. E.S+LCT: *E. stiedae*-infected and lactoferrin-treated. E.S+DLZ: *E. stiedae*-infected and diclazuril-treated

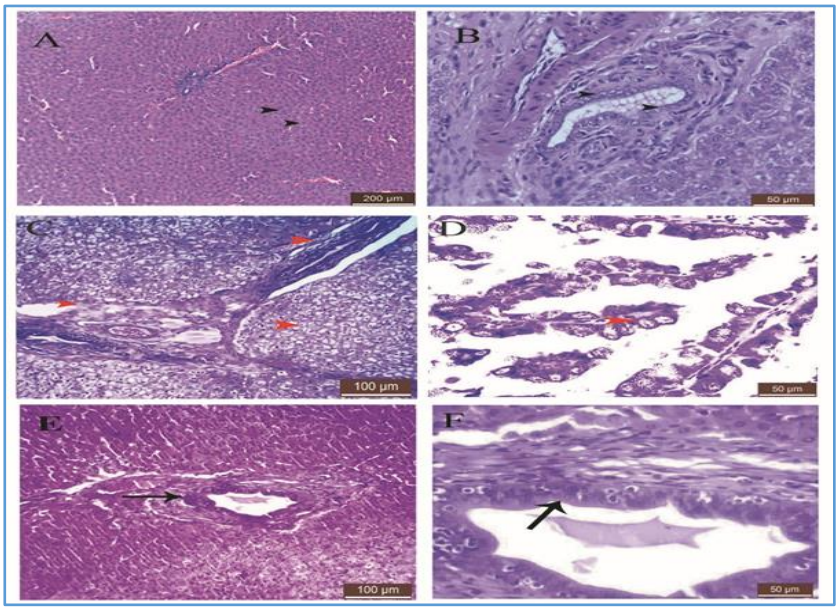


Fig. 6. Lactoferrin and diclazuril's efficacy against hepatic coccidiosis: hepatic histopathology. Panels A and B illustrate the normal liver structure in the control groups (G1 and LCT), marked by well-defined hepatocytes arranged in cords (black arrowheads), the bile duct epithelium, and sinusoidal spaces. H&E stain, bar indicates magnification. In contrast, Panel C from the infected group (G3) reveals liver congestion, infiltration by inflammatory cells, and proliferation of fibrous connective tissue in the portal and periportal areas, leading to the severe atrophy and degeneration of the neighboring hepatic parenchyma (red arrowheads). H&E stain, bar indicates magnification. Panel D highlights extensive papillary hyperplasia and various developmental stages of the parasite, as indicated by (red arrowheads), alongside desquamated epithelial folds within the lumen and developmental forms of the parasite in the epithelium in the infected group (G3). H&E stain, bar indicates magnification. Panel E shows a significant improvement in histopathological parameters in the treated group (G4), as indicated by (black arrow), as compared to the infected untreated group, evidenced by moderately distended portal areas, a noticeable reduction in the size and number of coccidial bile duct granulomas, decreased collagen deposition (fibrosis), and reduced mononuclear cell infiltration. H&E stain, bar indicates magnification. Panel F demonstrates a reduction in the hyperplasia of the biliary epithelium, with few or no developmental stages of the parasite (black arrow), indicating the therapeutic efficacy of the treatment. H&E stain, bar indicates magnification. G1; non-infected and non-treated (control negative), G2; non-infected and lactoferrin-treated animals, G3; infested, non-treated (control positive), G4; infected and lactoferrin-treated, and G5; infected and diclazuril-treated.

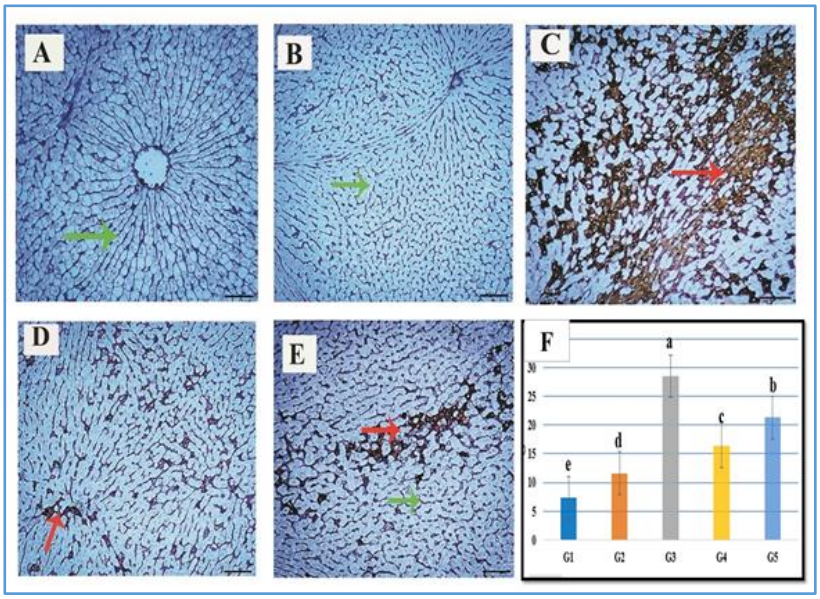


Fig. 7. Efficacy of the lactoferrin and Diclazuril on Immunohistochemically stained hepatic sections for TNF-α against Hepatic Coccidiosis. A and B: minimal TNF-α expression was observed in the uninfected rabbits (G 1 and 2). C: pronounced TNF-α expression in the infected and untreated rabbits (G3). D and E: moderate TNF-α expression in the infected and treated rabbits (G 4 and 5) was demonstrated. TNF-α expression was estimated as a percentage of positively stained cells (red arrows point to the brown positively stained cells and green arrows indicate negative expression. The bar suggests magnification. F: Graph presenting mean TNF-α expression percentages, visually comparing therapeutic efficacies across groups. [a, b, c]" Means with the different indices between groups are significantly different ($p<0.05$). Values are presented as the mean ± SE (n= 10). G1; non-infected and non-treated (control negative), G2; non-infected and lactoferrin-treated animals, G3; infested, non-treated (control positive), G4; infected and lactoferrin-treated, and G5; infected and diclazuril-treated.

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فعالية اللاكتوفيرين والديكلازوريل في مكافحة مرض الكوكسيديا الكبدي فى الأرانب: دراسة تفاعلات الترابط الجزيئي والتحقق التجريبي

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الملخص

يمثل مرض الكوكسيديا الكبدي، الذي يسببه طفيل إيميريا ستيداي، تحديًا كبيرًا لقطاع إنتاج الأرانب، مما يؤدي إلى نفوق الأرانب وخسائر اقتصادية فادحة. قارنت هذه الدراسة فعالية الديكلازوريل واللاكتوفيرين البقري، وهو بروتين متعدد الوظائف، في الوقاية من مرض الكوكسيديا الكبدي لدى أرانب نيوزيلندا البيضاء. لذلك، أجريت دراسة حاسوبية لدراسة تفاعلات الترابط الجزيئي بين اللاكتوفيرين وعامل نخر الورم ألفا (TNF-α) لدى الأرانب، بالإضافة إلى بروتيني المستضد السطحي 4 (SAG) و5 لـ E. stiedae ، وذلك كتقييم أولي قبل التحقق التجريبي. بعد ذلك، أجريت دراسة تجريبية على حيوانات حية باستخدام 35 أرنبًا من سلالة نيوزيلندا البيضاء، تبلغ من العمر شهر واحد، حيث تم توزيعها عشوائيًا إلى خمس مجموعات: مجموعة الضابط السلبية، ومجموعة غير مصابة عولجت باللاكتوفيرين، ومجموعة مصابة لم تُعالج، ومجموعة مصابة عولجت باللاكتوفيرين، ومجموعة مصابة عولجت بالديكلازوريل. شملت المعايير التي تم تقييمها: أداء النمو، وتقييم الإصابة بالديدان، والتحاليل الدموية، وأمراض الكبد بعد 28 يومًا من الإصابة. أظهرت نتائج التحليل الجزيئي ارتباطًا قويًا بين اللاكتوفيرين والديكلازوريل بروتين TNF-α لدى الأرانب، مما عزز استجابة الجسم الالتهابية وقدرة هذين المركبين على الارتباط بروتيني SAG4 وSAG5، وبالتالي تنظيم قدرة طفيل E. stiedae على إحداث المرض. أظهرت الدراسة أن اللاكتوفيرين حسّن بشكل ملحوظ زيادة وزن الجسم ونسبة تحويل الأعلاف مقارنةً بمجموعتي الديكلازوريل والمجموعة المصابة التي لم تُعالج. كما أدى استخدام اللاكتوفيرين إلى تأخير وتقليل إفراز أكياس البيوض، وتقليل تلف الكبد، وخفض مستوى بروتين TNF-α في التحليل المناعي، بالإضافة إلى خفض مؤشرات الالتهاب، مما يشير إلى فعاليته كمضاد للطفيل .E. stiedae تسلط هذه النتائج الضوء على فعالية اللاكتوفيرين في علاج العدوى الناجمة عن طفيل .E. stiedae

الكلمات الدالة: التهاب الكبد بالديدان، طفيلEimeria stiedae ، ديكلازوريل، التحليل الجزيئي، فعالية مضاد الطفيليات، اللاكتوفيرين.