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Original Article

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Probiotics lessens pathological changes in rabbits infected with hepatic coccidiosis

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ABSTRACT

Objective: This study aimed to evaluate the efficacy of probiotics in the treatment of hepatic coccidiosis in rabbits.

Methods: A total of 30 White New Zealand rabbits of both sexes, aged 4-6 weeks and weighing 600-700 g, were divided into three groups. The first group of rabbits received probiotics (100 mg/L) in drinking water for 12 days, while the second group of rabbits served as a positive control. Both groups were challenged with 1×10^5 *Eimeria stiedae* oocysts. The third group of uninfected rabbits served as the negative control. The effects of probiotic treatment on fecal oocysts output, body growth gain, liver enzymes, red blood cell count, hemoglobin level, packed cell volume, lymphocytes count and histopathological lesions were observed.

Results: Compared to the positive control, rabbits treated with probiotics exhibited no diarrhea or mortality, and there was a significant reduction in fecal oocysts output. Additionally, body growth gain was significantly higher in probiotic-treated rabbits compared to the positive control. Furthermore, liver enzymes and white blood cell counts were decreased in treated rabbits, while red blood cells, hemoglobin, packed cell volume and lymphocyte count were elevated. Severe histopathological lesions were observed in the bile ducts and hepatic parenchyma of positive control rabbits compared to probiotic-treated and negative control groups. The study findings were statistically significant, with *P* values ranging from less than 0.001 to less than 0.05.

Conclusions: Probiotics significantly improve the health performance of rabbits with hepatic coccidiosis by reducing pathological changes and stabilizing hematological parameters. Additionally, this product maintains substantial normal liver enzymes levels in infected rabbits, making it a safe and effective treatment for hepatic coccidiosis in rabbits.

KEYWORDS: Probiotics; Hepatic coccidiosis; Pathological changes; Rabbits

1. Introduction

Rabbits are recognized as an essential and healthy source of animal protein all over the world[1]. However, they are vulnerable to serious viral, bacterial, and parasitic infections that greatly affect their productivity[2]. *Eimeria* (*E.*) *stiedae* is one of the most virulent species belonging to the *Eimeriidae* family that causes liver coccidiosis in both wild and domestic rabbits[3]. Rabbit coccidiosis is one of the most prevalent protozoan parasites and causes signifiant economic losses in rabbit farms[4,5]. Young rabbits are most susceptible to disease spread than adults[6]. Coccidiosis is characterized by the rapid spread of infection, due to the shortness and direct life cycle of the parasite[7].

Significance

The study underscores the potential of probiotics as an effective alternative to the treatments for hepatic coccidiosis in rabbits, which is a significant economic concern in rabbit farming. The findings indicate that probiotics reduce symptoms and pathological changes caused by infection while enhancing immunity, making them a safe and effective option for managing this parasitic disease.

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Cirrhosis and cholestasis are considered the most important pathological changes in the livers and bile ducts, particularly in young rabbits[8,9]. Animals with severe infections show symptoms such as anorexia, brown watery diarrhea, dehydration, reduced feed efficacy, weight loss, unkempt hair, abdominal distension, and even death[10]. To prevent and control these infection, hygiene practices along with various anticoccidial medications are employed[6,11,12]. Besides protecting the gut from pathogens, the addition of dietary supplements can stimulate a robust immune response and improve physical performance[13]. One commercial probiotic, containing two beneficial bacteria, *Enterococcus* (*E.*) *faecium* and *Bacillus* (*B.*) *subtilis*, is used to treat and enhance the growth performance of birds and small animals[14,15]. *E. faecium*, which belongs to the lactic acid bacteria group, has antimicrobial effects and can improve the intestinal flora of rabbits[16,17]. *B. subtilis* can promote the immune response to infections and weight gain in rabbits[18,19]. Various products are available on the market, depending on the required microbial purity and effective dosage[16]. Therefore, this study aimed to explore the effect of the probiotics as a therapeutic agent against coccidiosis in rabbits. To evaluate this group of products on rabbits' performance, we estimated the frequency of oocysts shed after infection and analyzed hematological, histological and biochemical values in experimentally infected rabbits compared with controls.

2. Materials and methods

2.1. Experimental protocol

This study used 30 New Zealand White rabbits aged 4-6 weeks, weighing 600-700 g. The rabbits were raised in wire cages (40 cm high, 50 cm wide, and 60 cm long) in the biological animals' house under the environmental conditions of approximately 75% relative humidity and temperature of 15-25 °C during the study period. These rabbits were divided into 3 groups, which each consisting of 10 rabbits. The first group of rabbits received probiotics in drinking water at a dose of 100 mg/L for 12 days before infection date. The second group served as a positive control (infected-untreated). The third group was used as a negative control (uninfected). After a 12-day period of experimentation, the first and second groups of rabbits were orally infected with 1×10^5 sporulated oocysts of *E. stiedae* per rabbit. Rabbits in all groups received water and food without medication. Absence of *Eimeria* oocysts was confirmed by daily fecal examination for 7 days prior to the start of the experiment.

2.2. Isolation of E. stiedae samples

E. stiedae oocysts were collected from gallbladder and liver lesions from natural infected rabbits from a local market in Baghdad city. The collected oocysts were washed with normal saline to remove the biliary contents, then transferred to a clean, sterile Petri dish containing 2.5% potassium dichromate solution (K2Cr2O7) at a depth of 3-5 mm and incubated at room temperature (26 °C) and relative humidity 76%-80% for 72 h for the sporulation. The sporulation of the oocysts was examined daily under the microscope until sporulation of all oocysts was complete. The sporulated ooctys were collected and resuspended in 2.5% potassium dichromate solution then stored in a refrigerator at 4°C until used for challenge infection[20].

2.3. Preparing of probiotic product

This study used the MiaClost Probiotic product (MIAVIT, GmbH, Germany), which contains 5×10^{11} colony forming units (CFU) of *E. faecium* and 5×10^{11} CFU of *B. subtilis*. This product was diluted in a dose of 1×10^9 CFU/mL in drinking water[21].

2.4. Data collection

2.4.1. Clinical signs and mortality rate

All rabbits were monitored daily for clinical signs and mortality. Signs of hepatic coccidiosis were recorded during the experimental period of 7 days post-infection. The mortality rate for each group was recorded and calculated as a percentage.

2.4.2. Counting of oocyst of Eimeria per gram of feces

Feces were collected to calculate oocyst per gram from 7 to 28 days after infection. For oocysts count, fresh fecal samples were collected from each group's cage. Fecal samples were stored in labeled plastic containers and transported to the laboratory for counting the number of oocyst per gram in each group. McMaster slide was used for counting using the flotation method[20,22].

2.4.3. Body weight and weekly weight gain

Rabbits were weighed daily from the beginning to the end of the experiment (28 days) to calculate their body weight. Their body weight gain were record weekly using the difference between initial and final weight[23]. Moreover, the relative liver weights were also calculated by dividing the liver weight by the total body weight of each rabbit[24,25].

2.4.4. Hematological parameters

At the end of the experiment, 1 mL of blood samples were collected from all animals into sterile tubes containing ethylene diamine tetraacetic acid to prevent blood clotting for hematological analysis. Hemoglobin (Hb) was measured with a hematology analyzer (HumaCount 30TS). A 25 µL whole blood sample was evaluated for precise Hb concentration at a wave length of 540 nm. The red blood cell count (RBC), packed cell volume (PCV), mean corpuscular volume (MCV), white blood cell count (WBC) and lymphocyte (LY) percentage from an aliquot were determined using hematology analyzer[26].

2.4.4. Biochemical blood parameters

Blood was collected in anticoagulants free tubes (2 mL) and centrifuged at speed of up to 3500 rpm for 5 min. Then, the serum was aspirated by a pipette and transferred into small clean tubes for biochemical measurements. The separated serum samples were used to identify liver enzyme. Alanine amino transferase (ALT) and aspartate amino transferase (AST) were measured. These enzymes were colormetrically determined usingALT, AST, and direct bilirubin assay kits(Biosystems, Spain) according to the manufacturer's recommendations[27].

2.4.5. Gross lesion scoring and gross pathological count

Rabbits were necropsied at the end of the experiment and the liver of each animal was collected, weighed and examined for gross lesions. Gross hepatic examination included the assessment of liver lesions in white-spotted rabbits, which were classified as no lesion (0), mild (1), moderate (2,3), and severe (4,5) damage[21,28]. The size of the lesions measured from 1-8 mm was plotted.

2.4.6. Histopathological changes

At the end of the experiment, rabbits livers and bile ducts were trimmed and preserved in 10% formalin for 24 h for histopathological examination. Tissues were cut to a thickness of 3-5 µm and processed in an automated tissue processor in different chambers with different alcohol concentrations (70%, 80%, 95% and 100%). Processed tissues were cleared in xylene and embedded in paraffin. Blocks were microtomed to a size of 5 µm. They were then deparaffinized and tissues section was stained with haematoxylin and eosin staining[30]. A diystyrol-xylene softener was applied to the slides and allowed to dry before examination by light microscope.

2.4.7. Statistical analysis

Fisher's exact test was used to analyze the mortality rate data. The Kruskal-Wallis test was utilized for analyzing variables with non-normal distributions, while analysis of variance (ANOVA) was used for normally distributed variables, empolying Graph Pad Prism software version 5 (2015). A value of $P<0.05$ was considered statistically significant.

2.4.8. Ethical approval

This study was approved by the Iraqi Ethics Committee of the College of Veterinary Medicine, Baghdad University (Protocol No. D.A. 671).

3. Results

3.1. The probiotic efficacy on clinical signs and mortality rate

The rabbits treated with probiotics, which received 10^9 CFU/mL of probiotics, experinced no mortality and showed no clinical pathological symptoms after infection. Similar results were observed in the negative control throughout the experimental period, as they were not infected. In comparion, rabbits in the positive control exhibited symptoms such as anorexia, polydipsia, brown to bloody diarrhea, dehydration, rough hair and abdominal distension 10 days after infection. Compared with the probiotics, the mortality rate in positive control increased by 10% and 30% at three and four weeks after infection, respectively (0% *vs*. 10%; 0% *vs*. 30%; $P<0.05$).

3.2. Oocyst shedding

In comparison, there was a significant decrease in fecal oocyst shedding and a shorter excretion duration in probiotics while a high number of *E. steidae* oocysts were shed by positive control with a peak shedding occurring 10-16 days post-challenge, significant at $P<0.001$. No oocysts were detected in fecal samples from negative control (Figure 1).

3.3. Body weight and weight gain

In comparison, the positive control rabbits showed equivalent body weight with the negative control rabbits from day 6 to day 20 after post-infections. The rabbits treated with probiotics had a significantly higher body weight started early at day 11 post-infection as shown in Figure 2A ($P<0.001$). The weekly body weight gain of rabbits that received probiotics showed a significant improvement over the three weeks of the experiment, even more than those of negative control ($P<0.01$) (Figure 2B).

3.4. Blood hematological

At the end of the experiment, all rabbits were sacrified to assess blood hematological parameters. The results of the rabbits treated with probiotics showed a significantly increased of red blood cells (RBC), hemoglobin (Hb), packed cells volume (PCV), lymphocyte (LY) counts compared to the positive control rabbits, while the numbers of white blood cells (WBC) in probiotic-treated rabbits were significantly lower than in infected untreated rabbits (P<0.001). Similar observations were recorded in negative control rabbits, with blood parameters within the normal range as shown in Figure 3.

3.5. Blood biochemical findings (liver enzymes)

Liver function was determined by analyzing liver enzyme biomarker levels in all rabbits at the end of the experiment. The levels of enzymes alanine aminotransferase (ALT) were significantly lower in the plasma of the rabbits treated with probiotics and negative control than that in the positive infected rabbits (P<0.01). In addition, Bilirubin levels were higher in the positive infected group compared to the treated and negative rabbits, as shown in Figure 4 (P<0.01).

3.6. Gross pathological lesions

During the gross examination, the score, size and number of the liver lesions were envaluated. The positive control exhibited significantly more nodules compared to the treated and negative control. The rabbits of positive control had lesion scores ranging from 4 to 5, indicating severe damage, with the number of lesions exceeding 50. Moreover, the liver weight (176-180 g) and size (≥10 cm), as well as the gallbladder size in the positive group were significantly larger than those in the treated rabbits (P<0.001). Negative control rabbits showed no lesions in the livers and gall bladders, as illustrated in Figure 5.

3.7. Histopathological changes

The rabbits treated with probiotics showed mild hyperplasia, ectasia of bile duct with multifocal areas of periportal fibrosis. The histopathological changes of the positive control rabbits showed massive bile duct ectasia, epithelial hyperplasia with papillary projections that supported by fibrovascular stroma. Large numbers of Eimeria gametocytes (macrogametes & microgametes) and oocysts were diagnostic in the intraepithelial area of the bile duct. There was a predominant infiltration of inflammatory cells. Meanwhile, the rabbits in the negative control showed no histopathological changes (Figure 6).

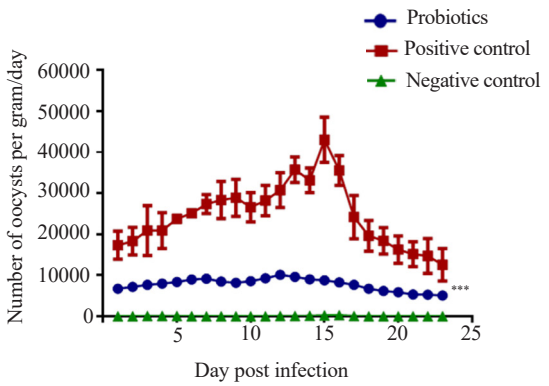


Figure 1. Comparison of fecal oocyst shedding among the three groups. $^{***}P$<0.001 *vs.* positive control.

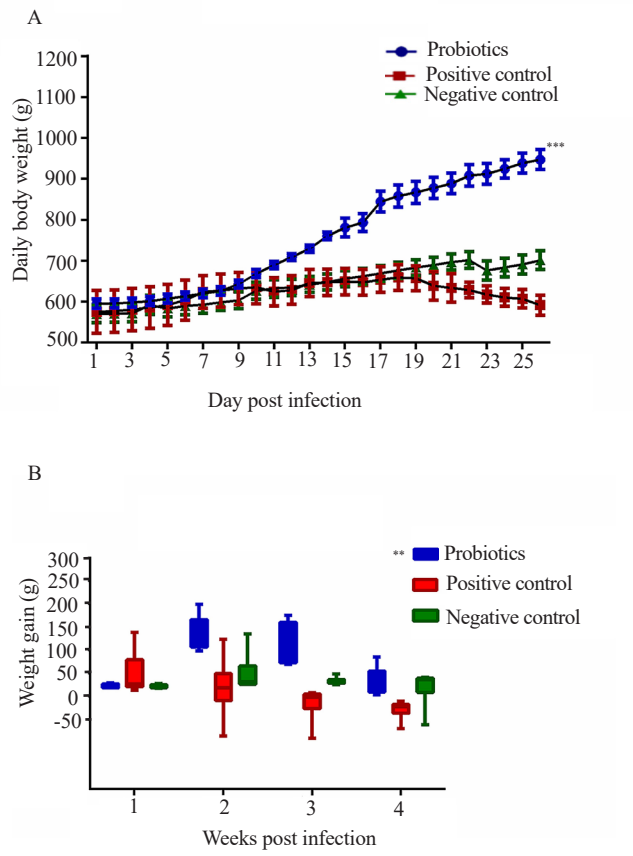


Figure 2. Comparision of daily body weight and weekly weight gain among the three groups. $^{***}P$<0.001 *vs.* positive control; $^{**}P$<0.01 *vs.* positive control.

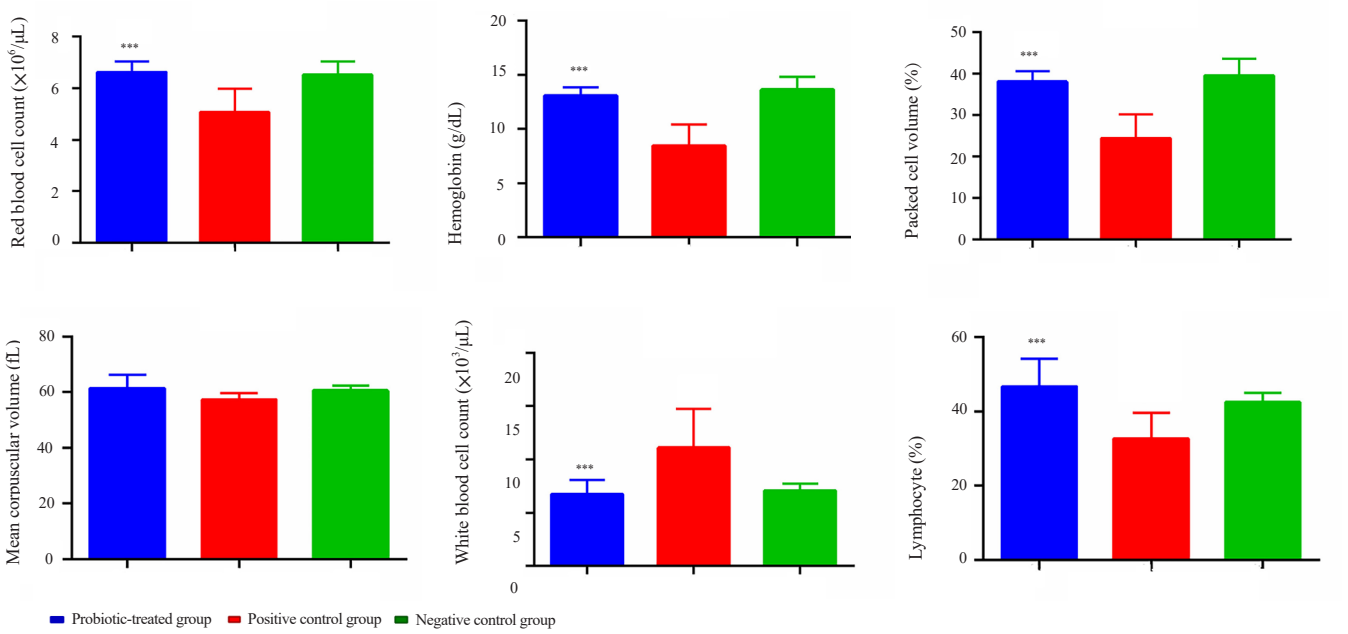


Figure 3. Comparision of hematological parameters among the three groups. ***P<0.001 *vs.* positive control.

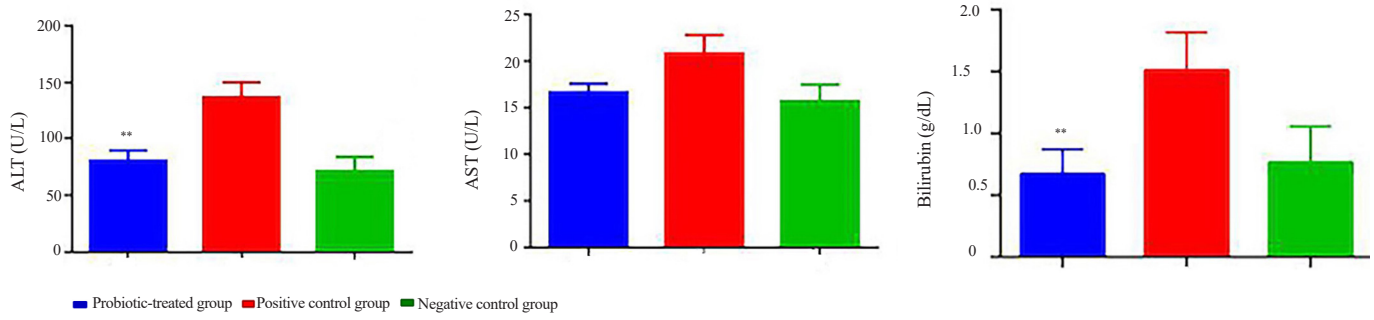


Figure 4. Comparison of biochemical parameters among the three groups. ALT: alanine aminotransferase; AST: aspartate aminotransferase; **P< 0.01 *vs.* positive control.

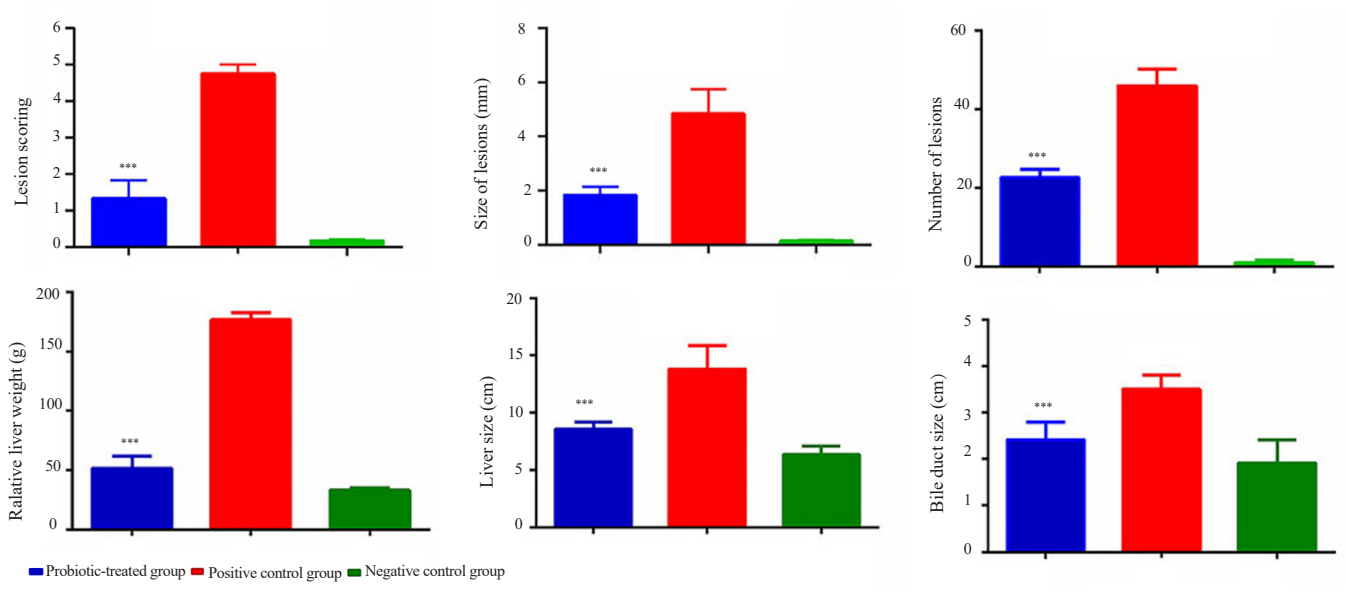


Figure 5. Comparions of gross pathological parameters among the three groups. ***P< 0.001*vs.* positive control.

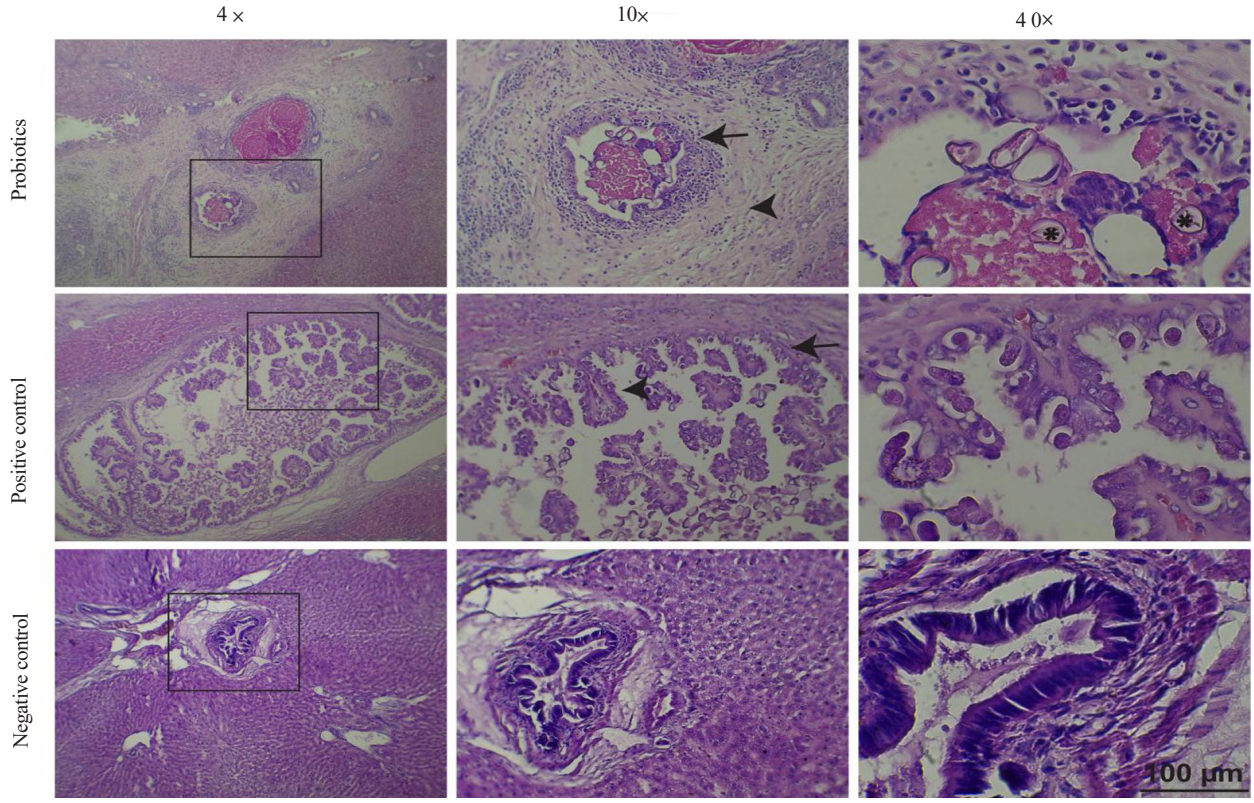


Figure 6. Comparison of histopathological images among the three groups.
Upper panel: The representative image of bile duct of the treated rabbits with probiotics shows mild bile duct hyperplasia (black arrow); ectasia of bile duct with degenerated oocyst (asterisk) and periportal fibrosis (arrow head), with inflammatory cells infiltration.
Middle panel: The representative image of the bile duct of the positive control rabbits shows massive dilatation of bile duct with hyperplasia of epithelial lining (arrow head). At 40× magnification, Eimeria macrogametes, microgametes and oocysts were found in the intraepithelial area of the bile duct. The dilated bile dust was surrounded by thick fibrous connective tissue and inflammatory infiltration. Macrogametes (20-50 µm) and microgametes (15-25 µm) are spherical shape within intracytoplasmic granules. The thin walls non-sporulated oocysts are ovoid and 20-40 µm in diameter.
Bottom panel: The representative image of the bile duct of negative control rabbits shows no pathological lesions.

4. Discussion

Beneficial effects of commercial probiotics improve gut health in domestic animals as an alternative to antibiotic growth promoters[31]. The high death rates in rabbit farms are caused by coccidiosis in many countries, which is still a problematic issue[32–34]. This study evaluated the efficacy of probiotics containing *E. faecium* and *B. subtilis* for the control of hepatic coccidiosis caused by *E. stiedae* infection in rabbits. Probiotics can regulate the intestinal microflora that improved the immunity of the animals and life performance[35]. No mortality was observed in the treated and negative group rabbits. Probiotics, which may reduce the mortality, have low level of lipid peroxides formation and higher anti-oxidant activities, which are considered crucial for anti-inflammatory and anti-oxidant effects[36,37]. In contrast, the positive control have a high mortality rate to 30% in the fourth week after infection. This could be due to the severity of the infection and the liver damage caused by hepatic coccidiosis in infected rabbits[38]. It is evident that the shedding of oocyst has a very large impact on the pathogenicity of the *E. stiedae*. A significant reduction in oocyst shedding was achieved in rabbits treated with probiotic on all days post-infection compared to positive control. The effectiveness of probiotics in preventing intestinal pathogenic microorganisms from adhering to the epithelial cells can stimulate the microbial balance in the gastrointestinal tract of the host[39]. Probiotics also benefit from reducing penetration of proinflammatory agents[40]. The innate immune mechanism enhances the ability to engulf and kill harmful bacteria and clearance is primarily carried out in the liver and by macrophages and neutrophils[41]. All of these factors have a positive impact on the reduction of oocysts shedding in probiotic treated rabbits compared to the significant output of the positive control. Similar findings have been observed when using Nigella sativa seeds oil as a treatment for hepatic coccidosis[42].

Fortunately, the impact of using probiotics on treated rabbits for 12 days before infection showed a significant increase in body weight

gain over all days of the experiment, even after infection, which was higher than that of the negative control. Meanwhile, positive control showed rapid weight loss, diarrhea and pathological signs after the first week of infection. Probiotics improve health status and enhance the nutritional value of the diet, positively convert it into fatting body mass[43]. Probiotics which used in this study contains *B. subtilis* that improved gut health and integrity by increasing the height villus to crypt depth ratio and controlled induced necrotic enteritis[21,44]. Probiotics target the small intestine site, where most enzymes work to increase the metabolic rate and improve the mucus layer of treated rabbits that lining the gut. Probiotics also play a role in the adhesion of enteric pathogens by providing antibacterial barrier in the gut[45,46]. The current study also established that the rabbits in the positive control had a significant decline of packed cells volume, Hb and RBC values with low percentage of LY compared to the treated rabbits with probiotics(P<0.001). These results were in agreement with previous studies[47]. The low PCV may indicate anemia and that attributed to the severity of infection, malnutrition, and anorexia in infected rabbits with *E. stiedae*[28]. There was no significant changes in MCV in treated rabbits with probiotics and this agreed with result of a previous study conducted by Allam T *et al*.[48]. These results indicate that probiotics supplementation caused significantly increased to the PCV, RBC, Hb, LY. These results are consistent with those observed by Rolfe *et al*. and Aattouri *et al*. that probiotics have extremely immunostimulatory effects on the immune system of rabbits[49,50]. The values of hematological parameters in the negative control rabbits were within the normal ranges, indicating that the animals were physiologically healthy. The probiotics showed serum biochemically lower levels of ALT and bilirubin than infected untreated rabbits. This product has shown improvements in liver function and enhanced immunity[39]. Conversely, the biochemical analysis of liver enzymes showed significant increases in ALT and bilirubin parameters in the positive control. These results indicate inflammation and necrosis in the liver and gallbladder of infected, untreated rabbits. Likewise, increases in serum enzymes have been attributed to hepatocellular damage, rupture of epithelial lining cells of the bile duct and an increased number of developmental stages of the parasite. In infected rabbits, hyperbilirubinemia can be accompained by biliary obstruction and cholestasis which is the most common cause of hepatic coccidiosis[51,52]. Strong histopathological changes were observed in positive control rabbits compared to probiotic-treated rabbits due to sever infiltration of inflammatory cells that increase necrotic damages of bile ducts and liver parenchyma. Probiotics have been proposed as a prevention treatment for chronic liver damage and reduce inflammation due to promoting immune response of the host[47]. In this regard, adhering of probiotics in the intestinal epithelium of treated rabbits promotes the immune response by regulating the mechanism of phagocytosis in the liver after infection[53,54].

5. Conclusions

A practical application of using probiotics to combat *E. stiedae* infection in rabbits is to add the probiotics to drinking water for 12 days. This treatment normalizes the gut flora and adjusts safer fattening management. The composition of the probiotics has been shown to significantly improve immunity in rabbits by inhibiting *Eimeria* invasion of the epithelial barrier. This was demonstrated by reduced oocyst shedding and mortality in probiotic-treated rabbits compared to positively infected animals. Moreover, increased weight gain was accompanied by maintenance of hematological and biochemical parameters in treated rabbits in contrast to infected untreated rabbits. Besides, histopothological changes was clearly reduced to mild favored by treatment with probiotics.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding author upon request.

Authors' contributions

Kalef DA designed the experiment, analyzed the data statisticlly, and prepared the manuscript. Jameel MS conducted the clinical study and performed the literature search. Both authors approved the final draft.

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