

The effect of betaine on broilers infected experimentally with *Clostridium perfringens***Ghada A. E. Mohamed^{1*}, Naglaa M. Ali², Dena E. Torra³ and Omar Amen⁴**¹Biochemistry Unit, Animal Health Research Institute, Agriculture Research Center, Assiut, Egypt.²Poultry Diseases, Animal Health Research Institute, Agriculture Research Center, Assiut, Egypt.³Pathology, and Clinical Pathology, Animal Health Research Institute, Agriculture Research Center, Assiut, Egypt. ⁴Avian and Rabbit Diseases, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt.**Abstract**

Clostridium perfringens is one of the main pathogens causing necrotic enteritis which is a highly contagious illness of poultry farm. The disease causes severe economic losses in poultry industry due to bird losses and the expensive of treatment and preventative measures. This study was investigated the effect of betaine on Hemato-biochemical parameters, histopathological picture and intestinal integrity in broiler chickens challenged with *C. perfringens*. Chickens of various ages (22-40 -day of age) they were collected from different commercial broiler farms at Assiut city. 110 intestinal contents from newly dead and diseased broilers with signs of necrotic enteritis and brownish diarrhea, the collected samples were subjected to bacteriological examination where the total percent of the isolated *C. perfringens* was 43.6%. Applied experimental study on total of 120, one- day-old broiler chicks (Rose 308) were randomly allotted for three group: 1) negative control (NC), 2) infected group (IG) and 3) treated group (TG), which were dosed with 1.0 ml betaine/L drinking water, from day one until the end of experiment. At 14 days of age, both the infected and treated groups were orally inoculated with 1×10^4 sporulated oocyte of mixed *Eimeria* species. Five days later, the same groups were orally challenged with a toxigenic strain of *C. perfringens* by inoculating each bird with 2 ml of broth culture (1×10^8 CFU) per bird. Betaine decreased the severity of necrotic enteritis in (TG) by decreasing the severity of brown diarrhea resulting in a significant ($P < 0.05$) reduction in the total count of *C. perfringens* (CFU/g) in the cecum in comparison to the infected and control groups. Betaine supplementation improves blood parameters. Additionally, betaine treatment diminishes pathological lesions in comparison to the infected group.

Keywords: Betaine, Antioxidant, Broiler, *C. perfringens*, Histopathology

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Introduction

Infection with *C. perfringens* disrupts protein metabolism, osmoregulatory homeostasis, liver enzyme activity and kidney functions (Abd El-Hamid et al., 2017; Abadeen et al., 2021). Coccidiosis and necrotic enteritis (NE) are two common diseases that can occur simultaneously in chickens. They are associated with epithelial cell damage, diarrhea with a brownish hue, intestinal osmotic stress, and susceptibility to secondary Infection with *C. perfringens* (Kettunen et al., 2001).

Antimicrobials have been utilized for decades in the animal industry. They are intended to prevent the growth of both Gram-negative and Gram-positive microbiota in the intestines and can be used as broiler growth promoters (Lozupone and Knight 2005). Due to the potential resistance of human pathogens to certain antibiotics, the European Union has currently prohibited the use of antibiotics in poultry herds (Hofacre et al., 2003). Therefore, there is a growing demand for a non-microbial alternative to improve intestinal integrity in broiler chicks in the chicken industry (Guo et al., 2004). Betaine is a methyl donor that regulates osmotic pressure in the body, and plays a significant role in several physiological processes (Wang et al., 2021).

Betaine regulates major immune functions, increases the absorption rate of nutrients, electrolytes and water, maintains homeostasis, the integrity and function of the intestinal barrier, sustains inflammation and provides protection against invading pathogens (Zhao et al., 2020; Wang et al., 2021). The administration of dietary betaine enhances serum albumin concentration (Park and Kim, 2019). Betaine is a highly effective antioxidant

with anti-inflammatory and anticarcinogenic properties (Tanaka et al., 2008). According to diffusion and MIC techniques, betaine has antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Proteus* (Pavlović et al., 2013).

Betaine has been shown to reduce the negative effects of coccidiosis by partially inhibiting coccidiosis formation and improving intestinal structure and function (Augustine et al., 1997). It can be used to preserve methionine and improve intestinal function in animals with osmotic stress such as diarrhea or coccidiosis (Amerah and Ravindran, 2015).

Nofal et al., (2015) demonstrated that dietary betaine supplementation significantly decreased the percentage of heterophils, but significantly increased the percentage of lymphocytes, and significantly decreased the H / L ratio.

Furthermore, dietary betaine may also immunomodulate the gastrointestinal tract of broilers (Hamidi et al., 2009). There is limited information on the role of betaine in treatment and prevention of *Clostridium perfringens* infections in broiler chicks. So, this study aimed to determine the effects of betaine administration on the intestinal histomorphology and some biochemical blood components of broiler chickens challenged with *C. perfringens*.

Materials and Methods

Ethical approval:

This research was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Assiut, Egypt, University. Under the No. aun/vet/3/0004. In addition to approval of the Animal Health Research Institute to research plan with ethical guidelines.

Samples:

Chickens of various ages (22-40 -day of age) were collected from different commercial broiler farms at Assiut city. One hundred and ten intestinal contents from newly dead and diseased broilers with signs of dullness, ruffled feather, brownish diarrhea, with bloody intestinal content and necrotic intestinal mucosa at examination.

Isolation and identify *Clostridium perfringens*:

Using the anaerobe Gen atmosphere generation system samples were cultured on freshly prepared cooked meat medium and incubated anaerobically at 37°C/48hr. The pure colony was streaked onto duplicate plates of reinforced clostridium agar (LAB) and incubated at 37°C/48hr in an anaerobic condition to identify the *C. perfringens*. A single colony of isolated bacteria was subjected to several biochemical tests: include lecithinase, motility, and skim milk coagulation

(stormy response) as outlined in the study by (Quinn et al., 2002).

PCR for toxin detection

PCR reactions were carried out using QIAamp DNA Mini Kit, Catalogue no.51304 .The QIAamp DNA Mini Kit provides silica-membrane-based nucleic acid purification from different types of samples.

Next, oligonucleotide primer sequences were constructed using the alpha toxin sequence according to (Yoo et al., 1997). The nucleotide sequence of each primer pair and the size of the PCR fragment are shown in (Table 1).

The PCR thermal condition included: Initial denaturation (94°C-5min.). 35 cycles (denaturation at 94 °C-30 sec.;annealing at 55 °C-40 sec.; extension at72 °C-45 sec). Final extension at72°C for 10 min. Then 10µl of the amplified product was electrophoresed in 1.5% agarose gel and stained with ethidium bromide.

Table 1. List of primers used for the PCR assay

Toxin	Primer	Sequence	Amplified product	Reference
Alpha toxin	F	GTTGATAGCGCAGGACATGTTAAG	402 bp	YOO <i>et al.</i> , 1997
	R	CATGTAGTCATCTGTTCCAGCATC		

Antimicrobial sensitivity testing via agar diffusion:

The susceptibility of 10 *C. perfringens* isolates from broilers to a panel of commonly used antibiotics and 1% betaine was determined using the Kirby–Bauer method on Mueller–Hinton plates. Agar plates (Merck, Germany) according to the criteria and breakpoints of the Clinical and Laboratory Standard Institute (CLSI, 2005). All antimicrobial discs used were purchased from Oxoid (UK), Ampicillin

(P), Amoxycillin (AML), Neomycin (N), Colistin (CT), Flurophencol (F), Streptomycin (S), Tetracycline (T), Enrofloxacin (ENR), Lincomycin (LN) and Vancomycin (VA). The susceptibility of the strains was determined by measuring the size of the inhibition zone. The isolate was categorized as a multidrug-resistant strain when *C. perfringens* was found to be resistant to at least one antibacterial agent from three or more distinct groups.

Experimental study design:

One-day-old broiler chicks (Rose 308) n=120 were placed into three groups; each group was replicated 10 times with four broilers per replicate and feed on basal commercial diet (Table 2). Consequently, random chosen represented chicks from each replicate. The experimental groups were categorized as 1) healthy NC (negative controls), 2) infected group (infected only and not treated and 3) treated group (TG) supplemented with 1.0 ml betaine/L drinking water (Natural betaine supplement) from day one until the end of the experiment. At 14 days of age,

each group was orally inoculated with 1×10^4 sporulated oocysts of mixed *Eimeria* species (Hofacre et al., 1998). After 5 days, the same groups were orally challenged with *C. perfringens* isolate by inoculation of 2 ml broth culture (1×10^8 CFU) according to (Atta et al., 2014).

The experimental period lasted 4 weeks. No antibiotic growth promoters were included in the base diet. All clinical manifestations, postmortem lesions, mortalities and cecal content associated with *C. perfringens* count were investigated.

Table 2. Feed composition

Ingredients (%)	Starter (0 to 21 days)	Grower (22 to 30 days)
Corn grain	50.30	61.20
Soybean meal (45% protein)	36.00	28.00
Corn gluten meal	3.40	3.00
Soybean oil	6.00	4.50
Limestone powder	2.14	1.85
Sodium phosphate monobasic	1.45	0.85
Common salt	0.30	0.30
Methionine	0.11	0.00
Premix*	0.30	0.30
Total	100	100
Calculated analysis (%)		
Crude protein (%)	23.02	20.21
Lysine (%)	1.31	1.01
Methionine (%)	0.50	0.38
Calcium (%)	1.00	0.90
Non-phytate phosphorus (%)	0.45	0.30
Total zinc (mg/kg)	74.21	75.74
Crude fibre (%)	3.46	3.27
ME (kcal/kg)	3193	3220

Provided per 2.5 kg: Vit. A, 1200000 IU; Vit. D3, 300,000 IU; Vit. E, 700mg; Vit.K3, 500 mg; Vit. B1, 500 mg; Vit. B2, 200 mg; Vit. B6, 600 mg; Vit. B12, 3 mg; Vit. C, 450 mg; Niacin, 3000 mg; Methionine, 3000 mg; Pantothenic acid, 670 mg; Folic acid 300 mg; Biotin, 6 mg; Choline chloride, 10,000 mg; Magnesium sulfate, 3000 mg; Copper sulfate, 3000 mg; Iron sulfate, 10,000 mg; Zinc, 400 mg; Cobalt sulfate, 300 mg.

Blood sample

At the end of the experiment blood samples were collected from wing vein puncture under aseptic precautions from chicks in each group. The first sample was 1 ml of whole blood collected on EDTA for hematological examination, the second blood sample was 3 ml of blood taken without anti-coagulant in a clean dry centrifuge tube, left to clot at room temperature and centrifuged at 3000 rpm for 10 min for serum collection. The serum was stored at -20°C until biochemical analysis

Hematological parameters

Estimation of Red blood cells (RBCs), White blood cells count (WBCs), differential leucocyte count and Hemoglobin (Hb) concentration were done according to the method of (Feldman et al., 2000). Heterophils / Lymphocytes ratio (Gross and Siegel, 1983). In addition to phagocytic activity (Kawahara et al., 1991).

Serum biochemical analysis:

The serum analysis included estimation of serum total protein (TP), albumin (Alb), and measurement of globulins (Glob) by subtracting albumin values from STP. The values of creatinine (Creat), blood urea nitrogen, Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), and also serum total antioxidant capacity (TAC) were determined using a semiautomatic biochemical analyzer and commercially available test kits (Biodiagnostic CO, Egypt) according to the manufactured structure.

Serum electrolyte measurements were conducted using ion selective electrodes for measuring Na⁺, K⁺ concentrations. The shaft and membrane of an electrode are made of glass. The

selectivity for Na⁺ ions or K⁺ is determined by the composition of the glass membrane. Cations are removed from the outer hydrated layer in an aqueous solution and replaced with Na⁺, K⁺ from the sample. This generates a potential at the boundary layer between the sample solution and membrane that dependent on the activity of ions (Na⁺, K⁺) and ions subsequently, the serum Cl⁻ concentration was determined using biodiagnostic colorimetric test kits and a manufactured structure.

Caecal microorganism counts of *C. perfringens*:

The caecum was extracted to determine caecal microb counts. 10 ml of phosphate-buffered saline with a pH of 7.0 used to homogenize the caeca (1.0 ml). The homogenate is then diluted in steps ranging from 10² to 10⁸. The selective media employed by *C. perfringens* was fortified with clostridium agar (LAB) and cooked beef broth (Oxoid). In order to determine bacterial counts, plates were incubated at 37°C for 48 h. The log of bacterial counts consistent with a gram of clean caecal content is then calculated (log 10 colony-forming unit/gram of caecal content).

Histopathological study:

Samples of chicken liver, intestine, spleen, Bursa of fabricius and thymus were fixed in 10% neutral buffered formalin. The paraffin-embedded sections were microtomed to a thickness of four microns and stained with hematoxylin and eosin. The histological preparations were analyzed with a light microscope (Bancroft et al., 1996).

Statistical analysis:

Analysis of variance (ANOVA) was utilized for statistical analysis, and nonparametric methods were used to compare groups (Mean±SEM). Kruskal–Wallis nonparametric ANOVA via the use

of GraphPad-prism software (San-Diego, co., USA, V.8).

Results

Bacteriological results:

In the current study, 48 *C. perfringens* strains were recovered out of the 110

samples analyzed. The PCR protocol was used to amplify and detect the alpha toxin gene in the *C. perfringens* strains that were isolated and contained the alpha toxin gene with a specific amplicon size of 402 bp. (Fig. 1).

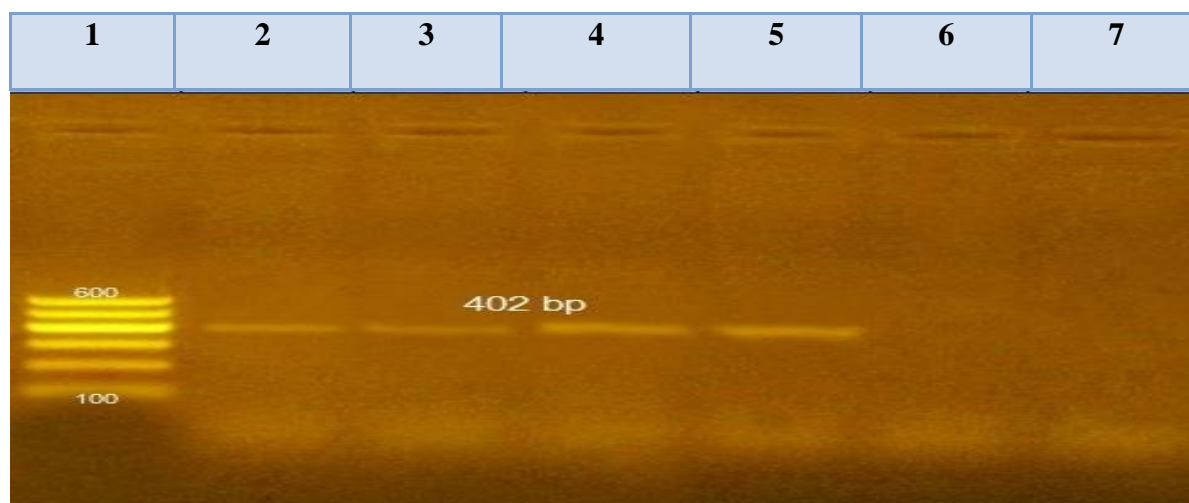


Fig.1. Electrophoretic pattern of Alpha toxin PCR assay:

Lane (1):100 bp DNA ladder

Lane (2): showed control positive

Lanes (3-5): showed positive isolated *C. perfringens* strains (402 bp).

Lane (6): showed negative isolate of *C. perfringens*.

Lane (7): showed control negative

Antimicrobial activity against *C. perfringens* strains was quantified in vitro as the zone of inhibition diameter (IZD), measured in millimeters via disc diffusion assay. The average MAR index for *C. perfringens* isolates is about 0.5. MDR *C.*

perfringens exhibited high resistance to betaine, whereas betaine exhibited poor antibacterial activity against *C. perfringens* and a negative inhibition zone on agar plates (Table 3).

Table 3. Antibiotic Sensitivity pattern of 10 *C. perfringens* strains

<i>C. perfringens</i> strains	Antibiotics resistance	Antibiotics Sensitive	MDR Index
1	P,T,N,S,CT,F,AML,ENR,LN	VA	0.9
2	P,T,N,S,CT,F,AML,ENR	VA, LN	0.8
3	P,T,N,S,CT,F,	VA, LN, ENR,AML	0.6
4	P,T,N,S,CT,F	VA, LN, ENR,AML	0.6
5	P,T,N,S,CT,	VA,LN,ENR,AML,F	0.5
6	P,T,N,S,ENR	VA,LN, AML,F, CT	0.5

7	P,T,N,S,ENR	VA, LN, AML, F, CT	0.5
8	P,T,N,S	VA, LN, AML, F, CT, ENR	0.4
9	P,T	VA, LN, AML, F, CT, ENR, N, S	0.2
10	P	VA, LN, AML, F, CT, ENR, N, S, T	0.1
Average MDR		0.51	

Clinical signs, morbidity and mortality:

Clinical symptoms such as dullness, dehydration, ruffled feathers and brown diarrhea were reported frequently in infected groups. In the betaine-treated group, the clinical manifestations and severity of brown diarrhea were less severe than in the IG.

The lesions of NE in the small intestine consisted of severe congestion, the jejunum and ileum appear dilated, with a thin friable wall and the two cecai filled with gas or containing fluid with a greenish or reddish tint, these lesions were only observed in the IG, which had a 20% mortality rate.

Lesions can also be detected in other organs, such as liver and kidney congestion. In contrast, the betaine-TG

exhibited a normal-appearing intestinal tract with minimal enteric lesions and was completely protected from death.

Total counts of *C. perfringens* in the cecum of experimental chicks:

Regarding bacterial count, the total number of *C. perfringens* count was significantly ($P < 0.05$) decrease in the betaine treated group (TG) than in infected group (IG) (Table 4).

Hematological study:

Compare to the *C. Perfringens*-IG, the betaine-TG had significantly ($P < 0.05$) higher levels of hemoglobin, (RBCs) count, hematocrit value, lymphocyte percentage and phagocytic activity. Also total (WBCs) and monocyte counts increased, but not significantly differ ($P > 0.05$) (Table 4).

Table 4. Effects of Betaine supplementation on *C. perfringens* cecal count (CFU/g of cecal content) and Hematological parameters

Groups Parameters	Control	Infected	Treated
CFU/g cecal content	3.44E+04 ± 2.07E+04 ^a	1.07E+10 ± 8.85E+09 ^b	5.00E+06 ± 2.90E+06 ^a
(RBCs) ×10⁶/μL	2.9 ± 0.3 ^a	1.5 ± 0.3 ^b	3.1 ± 0.2 ^a
Haemoglobin (Hb) (g/dl)	8.5 ± 0.6 ^a	4.1 ± 0.5 ^b	8.1 ± 0.5 ^a
Haematocrit value	26.6 ± 1.9 ^a	13.3 ± 1.5 ^b	25.5 ± 1.4 ^a
(WBCs) ×10³/μL	9.9 ± 0.9 ^a	17.6 ± 1.5 ^b	18.8 ± 2.1 ^b
Monocytes %	8.5 ± 2.11 ^a	12.3 ± 2.1 ^a	15.7 ± 1.9 ^a
Heterophils %	59.57 ± 2.7 ^a	69.71 ± 3.5 ^a	61.2 ± 1.96 ^a
Lymphocytes %	26.43 ± 5.41 ^a	23.86 ± 4.5 ^a	39.5 ± 4.8 ^b
Stress Index (H/L)	2.25 ± 0.47 ^a	2.9 ± 0.77 ^a	1.54 ± 0.41 ^b
Phagocytic activity %	56 ± 8.71 ^a	21.8 ± 5.5 ^b	43.5 ± 8.2 ^a

Mean ± SEM in the same row carry different superscripts are significantly differed at ($P < 0.05$)

Biochemical study

Compared to the control group, The IG serum biochemistry (Table 5) revealed lower values ($P < 0.05$) of TP, albumin and globulins, as well as a greater A/G ratio.

These values were restored to their original concentrations in TG and did not differ significantly ($P > 0.05$) from those of the control group.

Table 5. Proteinogram in blood serum of control, *C. perfringens* infected and *C. perfringens* treated with betaine groups.

Parameters	Control	Infected	Treated
TP (g/L)	40.9 ± 1.9 ^a	25.6 ± 2.4 ^b	39.8 ± 1.5 ^a
Albumin(g/L)	12.9 ± 0.7 ^a	8.21 ± 1.4 ^b	12.7 ± 1.3 ^a
Globulin(g/L)	28.7 ± 1.2 ^a	17.5 ± 2.2 ^b	27.5 ± 3.1 ^a
A/G ratio	0.46 ± 0.04 ^a	0.51 ± 0.06 ^b	0.46 ± 0.03 ^a

Mean±SEM in the same row carry different superscripts are significantly differed at ($P < 0.05$)

In the IG, liver function test (Table 6) revealed elevated AST and ALT activity ($P < 0.05$) compared to the control group. ALT returned to its original value after supplementation with betaine, but AST remained higher than the value of the control group. Kidney function tests (Table 6) revealed an elevated serum creatinine

concentration ($P < 0.05$) in the IG when compared to the control group, whereas the values returned to nearly control levels in the TG. On the other hand, neither infection ($P > 0.05$) nor betaine supplementation affected the serum urea concentration.

Table 6. Liver and kidney functions in blood serum of control, *C. perfringens* infected and *C. perfringens* treated with betaine groups

Parameters	Control	Infected	Treated
AST (U/L)	166.9 ± 12.2 ^a	234.6 ± 10.8 ^b	207.6 ± 11.7 ^b
ALT(U/L)	23.3 ± 1.5 ^a	30.9 ± 1.7 ^b	24.2 ± 1.8 ^a
Urea (mg/dL)	15.5 ± 0.68 ^a	18.6 ± 1.47 ^a	16.9 ± 1.41 ^a
Creatinine (mg/dL)	0.24 ± 0.02 ^a	0.35 ± 0.02 ^b	0.25 ± 0.04 ^a

Mean±SEM in the same row carry different superscripts are significantly differed at level ($P < 0.05$)

Compared to the control group, the IG had lower concentrations ($P < 0.05$) of Na⁺ and Cl⁻ ions and a higher concentration of K⁺ ions in the blood serum (Table 7). The betaine-TG had higher concentrations of Na⁺ and Cl⁻ ions than the IG ($P < 0.05$), but the K⁺ ion concentration was still greater

than the value of the control group. *C. perfringens* inhibited significantly ($P < 0.05$) the antioxidant potential biomarker TAC in serum infected group (Table 7). By supplementation with betaine, TAC was activated, and its value was restored to its initial concentration nearly control.

Table 7. Electrolytes and total antioxidant capacity (TAC) in blood serum of control, *C. perfringens* infected and *C. perfringens* treated with betaine groups

Parameters	Control	Infected	Treated
Na ⁺ (mmol/L)	149.5 ± 3.3 ^a	128.7 ± 3.96 ^b	151.1 ± 2.29 ^a
K ⁺ (mmol/L)	4.98 ± 0.33 ^a	7.28 ± 0.35 ^b	6.04 ± 0.42 ^b
Cl ⁻ (mmol/L)	71.9 ± 1.14 ^a	62.04 ± 1.90 ^b	69.61 ± 1.1 ^a
TAC (mM/L)	2.77 ± 0.18 ^a	0.83 ± 0.09 ^b	2.45 ± 0.29 ^a

Mean±SEM in the same row carry different superscripts are significantly differed at level (P < 0.05)

Histopathological study:

The spleen, thymus, Bursa of fabricius, liver and intestine all showed histopathological abnormalities. The liver of the IG showed variable histopathological changes. Hepatocytes lost their normal pattern around a congested central vein, with a massive area of hepatocyte degeneration and necrosis [Fig. 2 a]. [Fig. 2 b] There were dilated,

congested sinusoids with an area of hemorrhage. [Fig. 2 c] The findings also revealed bile duct hyperplasia with lymphocytic cell infiltration. The portal region was infiltrated with lymphocytes, and the portal vein was clogged with gram-positive, rod-shaped *C. perfringens* [Fig. 2 d]. Conversely, betaine improved the pathological picture of liver tissues compared to the IG [Fig. 2 e, f].

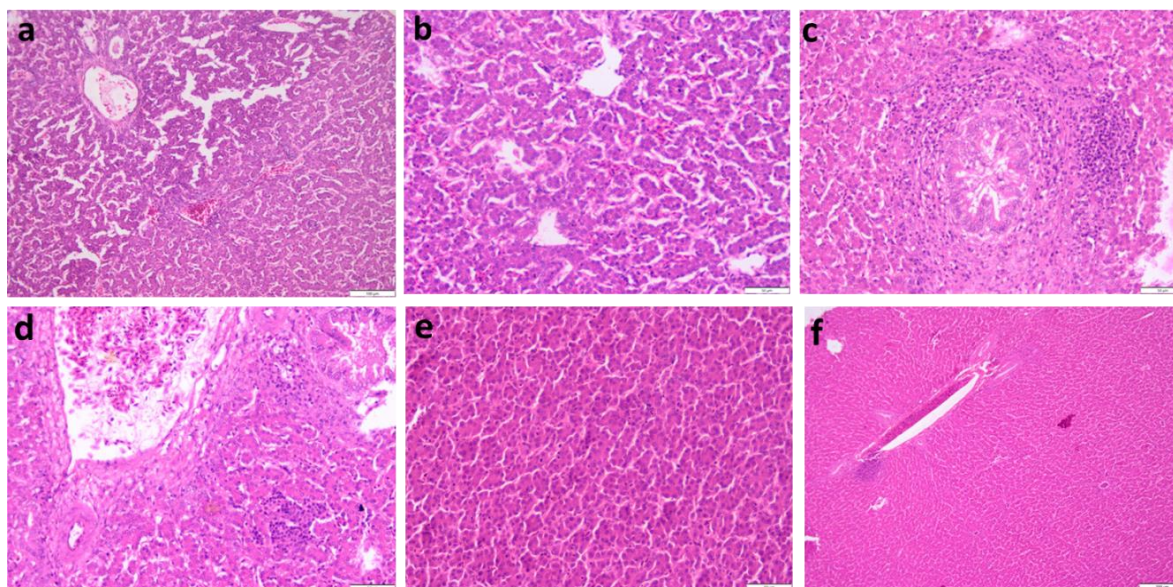


Fig.2. (a) Liver from infected group showed necrosis of hepatocytes with loss of normal pattern around the central vein (X200). (b) Liver from infected group showed dilated congested sinusoids with area of hemorrhage (X400). (c) Liver from infected group showed bile duct hyperplasia with lymphocytic cells infiltration (X400). (d) Liver from infected group showed the portal area infiltrated by lymphocytes and the portal vein is congested with gram-positive rod-shaped bacteria (X400). (e) Liver from treated group showed more or less normal hepatocytes (X400). (f) Liver from treated group showed focal lymphocytic infiltration with aggregate formation in the portal tract (X100).

The IG's spleen exhibited mild lymphocyte depletion in the white pulp [Fig. 3 a] and endothelial and medial hypertrophy in the blood vessel wall [Fig.

3 b]. We observed thickening of the arteriole wall and constriction of the lumen in conjunction with hemorrhage of red pulp [Fig. 3 c]. [Fig. 3 d] Spleen sections

revealed a significant number of pyknotic cells. In comparison to the IG, betaine

treatment reduces the pathological lesions [Fig. 3 e, f].

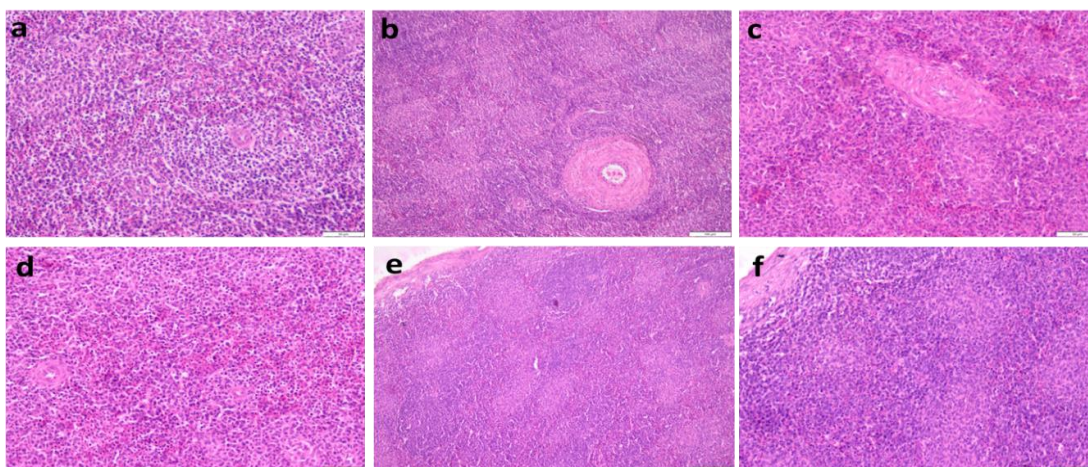


Fig.3. (a) Spleen from infected group showed mild exhaustion of lymphocytes (X400). (b) Spleen from infected group showed endothelial and medial hypertrophy in the blood vessel wall (X200). (c) Spleen from infected group showed thickness of the wall of arteriole and narrowing of the lumen with hemorrhage (X400). (d) Spleen from infected group showed large number of pyknotic cells (X400). (e) Spleen from treated group showed normal architecture consisted of red and white pulp (X200). (f) Spleen from treated group showed normal lymphocytic population (X400).

Bursa from the IG [Fig. 4 a] exhibited hyperplasia of the follicular epithelium and a thickening of the subepithelial connective tissue stroma. Interfollicular connective tissue proliferation was observed [Fig. 4 b]. [Fig. 4 c] It was observed that the lymphocyte count in the

bursal follicles was severely diminished. [Fig. 4 d] also revealed follicular atrophy with less prominent connective tissue septae. In contrast, the bursal follicles of the betaine-TG exhibited no or minimal changes [Fig. 4 e, f].

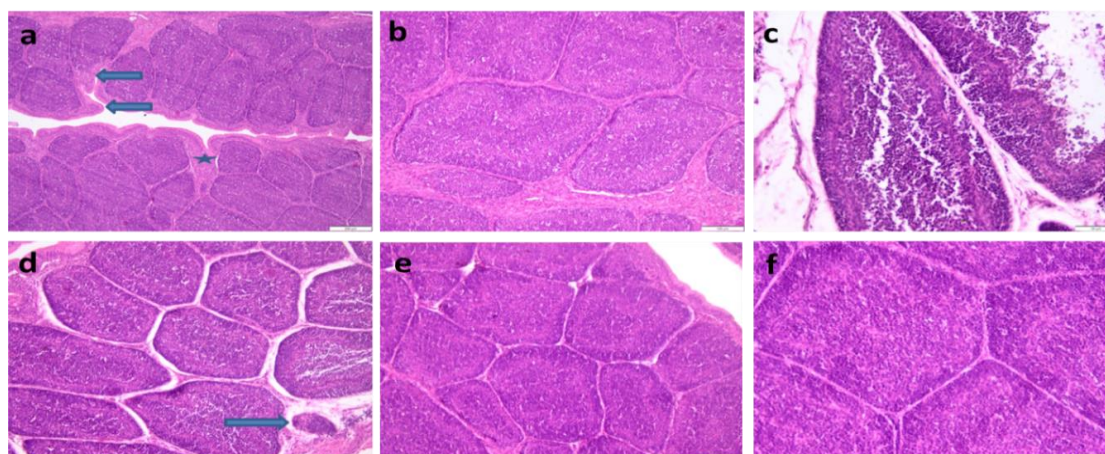


Fig.4. (a) Bursa from infected group showed hyperplasia of follicular epithelium (arrow) and thickening of subepithelial connective tissue stroma (star) (X100). (b) Bursa from infected group showed proliferation of interfollicular connective tissue (X200). (c) Bursa from infected group showed severe depletion of lymphocytes in the bursal follicles (X400). (d) Bursa from infected group showed follicular atrophy (arrow) with less prominent connective tissue septae (X200). (e) Bursa from treated group showed no or little changes in the bursal follicles (X200). (f) Bursa from treated group showed bursal follicles containing normal lymphocytic population (X400).

[Fig. 5 a] The IG's thymus displayed medullary necrosis and mild lymphocyte depletion. Mild congestion was observed in the medulla [Fig. 5 b]. In addition,

interlobular fat accumulation was documented [Fig. 5 c]. The thymus of the betaine-TG displayed normal lobulation, outer cortex, and inner medulla [Fig. 5 d].

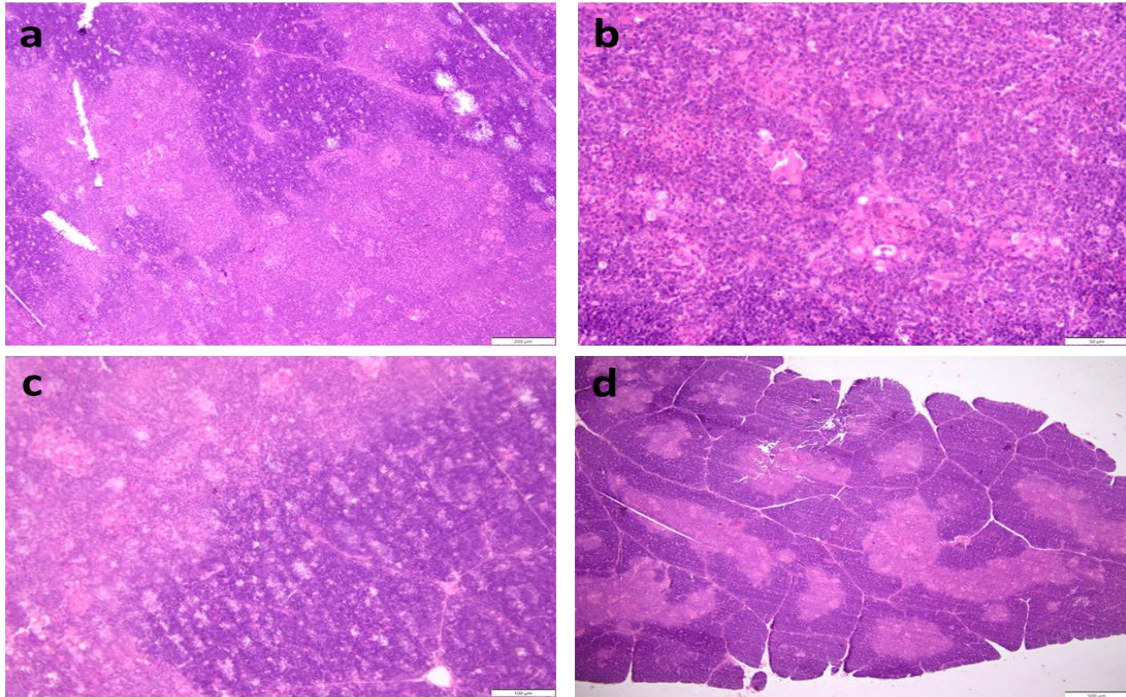


Fig.5. (a) Thymus from infected group showed medullary necrosis beside mild depletion of lymphocytes (X100). (b) Thymus from infected group showed necrosis and mild congestion in the medulla (X400). (c) Thymus from infected group showed area of fat deposition (X200). (d) Thymus from treated group showed normal lobulation, outer cortex and inner medulla (X250).

The IG's intestines exhibited NE, inflammatory cell infiltration, and the presence of *C. perfringens* as rod-shaped, gram-positive bacteria [Fig. 6 a]. [Fig. 6 b] reveals the presence of degenerated and ruptured villi and an increase in inflammatory cells in the lamina propria. The epithelium of intestinal villi and the cell lining of intestinal glands exhibited

hyperplasia [Fig. 6 c]. In addition, the detachment of the muscle layer with hemorrhage and the cell lining of the intestinal glands were predominantly replaced by various developmental stages of the coccidian parasite [Fig. 6 d]. In the betaine group, the intestine exhibited a relatively normal villi structure with few pathological changes [Fig. 6 e, f].

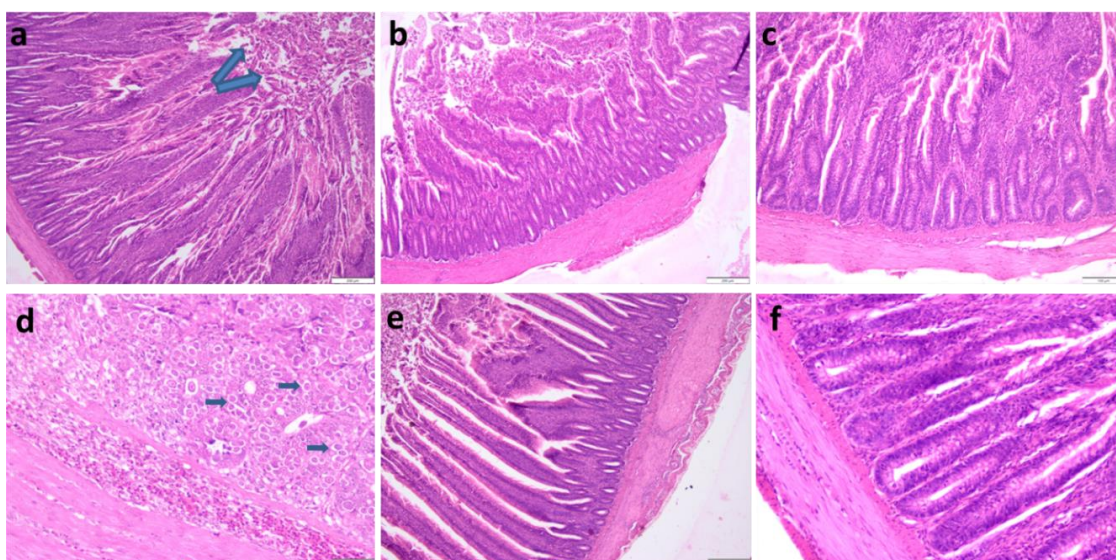


Fig.6. (a) Intestine from infected group showed necrotic enteritis with inflammatory cells infiltration and the presence of *C. perfringens* (arrow) (X100). (b) Intestine from infected group showed degenerated and bursted villi and increasing of inflammatory cells in the lamina propria (X100). (c) Intestine from infected group showed hyperplasia of the cell lining of intestinal glands and detachment of muscle layer in muscularis mucosa (X200). (d) Intestine from infected group showed different developmental stages of coccidian parasite (arrow) in intestinal glands (X400). (e) Intestine from treated group showed little remarkable pathological changes (X100). (f) Intestine from treated group showed more or less normal villi structure (X400).

Discussion

The prevalence rate of *C. perfringens* was 43.6% in 110 affected broiler chicks. Our result matched those of Abd-El All and Maysa (2014), who reported a prevalence rate of NE of 38.7% in intestinal broiler chicks. However, previous studies by (Mostafa et al., 2016) reported a lower prevalence rate of NE of 16% in intestinal broiler chicks. While a higher prevalence rate (100%) recorded by (Osman et al., 2012).

Regarding the antibiotic sensitivity test, The MAR index for bacterial isolates ranged from 0.1 to 0.90. Except for a single strain, the MAR index values for all bacterial isolates were greater than 0.2. As a result of the improper and excessive use of antibiotics in broiler farms, there is an antibiotic resistance problem (Katakweba et al., 2012).

According to in vitro studies, betaine exhibited poor antibacterial activity against MDR *C. perfringens* and a negative inhibition zone on agar plates. In contrast, Blagodatskikh et al. (2018) reported that betaine possesses antibacterial activity against both gram-positive and gram-negative bacteria.

In an in vivo study, betaine treatment significantly decreased the number of *C. perfringens*. Nevertheless, even if betaine has low antibacterial activity in vitro, the effect of betaine on the number of *C. perfringens* in the intestine may be associated with an improvement in cellular immunity by induced monocytosis and leukocytosis insignificantly and significantly increased phagocytic activity and lymphocytosis, thereby enhancing the immune response of the body. These findings support the findings of (Hamidi et

al., 2009). This may explain the decrease in *C. perfringens* in the caecum and, consequently, the decrease in intestinal damage.

In addition, the beneficial effect of betaine on gut bacteria may be associated with a significant ($P < 0.05$) improvement in antioxidant capacity, which protects against oxidative stress that can damage gut cells, increase permeability, and induce inflammation. These results correspond to those of (Wang et al., 2021).

TAC accurately reflects the antioxidant status of an organism (Erel, 2004). *C. perfringens* infection can result in inflammation, oxidative stress, and TAC inhibition (Zhao et al., 2020). In the present study, inhibited TAC activity by *C. perfringens* infection, but it can be restored with betaine supplementations. Similar results were obtained by Abudabos et al. (2018) and Chen et al. (2022). It was shown that betaine increases the TAC by triggering the activities of glutathione peroxidase, total superoxide dismutase, glutathione (GSH) level and activity of scavenging hydroxyl radicals (Yang et al. 2021). Importantly, the protective effects of betaine were associated with suppression of nuclear factor-kappa B and extracellular signal-related kinases pathway (Wang et al. 2021).

In our study, the IG had a disturbed electrolyte balance, which was restored by betaine in TG. Due to its zwitterionic structure, betaine possesses osmoprotective and osmoregulatory properties that protect intestinal cell proteins and electrolytes from various stresses. These results indicate that betaine can alleviate osmotic imbalance by maintaining constant blood electrolyte levels improve blood cation–anion balance consequently, by stabilizing osmotic pressure (Maddahian et al., 2021).

Decrease in the total number of *C. perfringens*, as well as the osmoregulatory protective effect of betaine restoring the original values of Na^+ and Cl^- ions.

In addition, the osmolytic properties of betaine can aid in the maintenance of the gut mucus membrane during digestive problems (Metzler et al., 2009), prevent intestinal inflammation as well (Wu et al., 2020). In addition to several contributing factors such as betaine-stimulated macrophage phagocytosis of coccidia and nitric oxide (Yun et al., 2000).

Although betaine has a weak direct antibacterial effect, these properties may mitigate intestinal membrane damage, dehydration, and diarrhea. In addition improving gut health and consequently, the animals' ability to withstand clostridium infections.

In the betaine-TG, the percentage of heterophils and the stress index (H/L) ratio decreased, while the percentage of lymphocytes increased significantly ($P < 0.05$). Our outcomes are consistent with the findings of Nofal et al. (2015). Based on these findings, betaine can modulate physiological stress and immunity when *C. perfringens* is present.

The group infected with *C. perfringens* exhibited both heterophilia and lymphopenia. The reduction and depletion of lymphocytes induce a hyperinflammatory response and cytokine storm, which may be linked to tissue damage (Mashaly et al., 2004). Heterophil to lymphocyte ratio (H/L) has been acknowledged for a long time as a reliable indicator of stress (Maxwell, 1993).

In our study, blood serum TP, albumin, and globulins decreased, whereas the A/G ratio increased in *C. perfringens* IG. These results were nearly achieved by (El-Sheikh et al., 2018; and Abadeen et al.,

2021). It is well known that *C. perfringens* causes a potent inflammatory reaction and can facilitate the excretion of substantial amounts of dietary protein in the feces (Abudabos et al., 2018). In this study, the addition of betaine increased serum protein levels. These results are consistent with the findings of (Abudabos et al., 2018). In addition, betaine's ability to reduce intestinal lesions and preserve intestinal integrity may help to minimize protein metabolism losses (Park and Kim, 2019). In addition to the ratio of albumin to globulin reflecting the state of the immune system in the body, a decrease in the ratio indicates an increase in globulin synthesis and an improvement in immune function in the body by betaine add (Zhang et al., 2020), this explains why the ratio decreased in the betaine-TG compared to the IG.

AST and ALT levels in serum are specific markers of liver damage and dysfunction. The elevated activity of these enzymes in serum suggests that *C. perfringens* has a negative effect on liver functions, which is consistent with the work of Abd El-Hamid et al. (2017). In the current study, betaine supplementation ameliorates hepatic disorders, and ALT enzyme functions return to their original levels. In acute and chronic animal models of hepatic injury, betaine treatment safeguards the liver by regulating mitochondrial function and reducing oxidative stress (Heidari et al., 2018). In fact, betaine has beneficial preventive and therapeutic effects on a variety of liver disorders (Wang et al., 2021).

A rise in serum creatinine as a result of *C. perfringens* infection in this study is consistent with the worsening kidney damage that may result from systemic issues. These results are consistent with

those of (Abd El-Hamid et al., 2017 and Abadeen et al., 2021). In the current study, betaine restored kidney function and returned the metabolite to normal levels. According to Ghartavol et al. (2019), betaine reduces inflammation and kidney damage by inhibiting inflammatory mediators and oxidative stress.

Our findings showed that infected chickens with *C. perfringens* revealed hypochromic anemia due to decreased RBCs and hemoglobin levels. Toxins from *C. perfringens* destroys RBCs, resulting in hemolytic anemia caused by the breakdown of phospholipids in erythrocytic membranes (El-Boraay, 1991). Clostridial infection induced monocytosis and significant leukocytosis, as leukocytosis is considered a characteristic feature of bacterial infection (Fraser et al., 1991). Moreover, *C. perfringens* caused a significant reduction in hematocrit and phagocytic activity. In our study betaine significantly enhanced phagocytic activity, antioxidant capacity, thereby decreasing inflammation and intestinal damage, these results are agreement with the findings of Nasr et al. (2019).

Leukocytosis and monocytosis were caused by bacterial infection and inflammation, and the reduction in phagocytic activity was due to the infection's detrimental effect on the immune system (Nasr et al., 2019). Our treatment with betaine enhanced certain hematological parameters. Similarly, it was reported that betaine significantly increased the number of erythrocytes and the hematocrit in ovo-injection (Park and Kim, 2017). In addition, betaine is involved in maintaining the hematocrit and increasing the erythrocyte count, which

improves hematological parameters (Gudev et al., 2011).

In the betaine group, the villi structures of the intestine were relatively normal, with few pathological changes. Betaine improved the histopathological picture of liver tissues. In addition, the pathological lesions in the spleen diminished as the normal architecture composed of red and white pulp developed. Also, the bursal follicles of the Bursa of Fabricius exhibited no or minimal change. Compared to the IG, the betaine-TG's thymus had normal lobulation, outer cortex, and inner medulla. These findings may explain why betaine has immunomodulatory properties. As demonstrated by (Hamidi et al., 2009 and Youssef et al., 2016).

It is widely believed that intestinal integrity, antioxidant capacity, and immunity are the most significant indicators of intestinal health (Song et al., 2021). Betaine improves intestinal function and structural integrity (Metzler et al., 2009). Betaine caused a partial inhibition of coccidial invasion and development, as well as a reduction in morbidity and large intestinal lesions brought on by an *Eimeria* infection (Hess et al., 1998). In addition, it has been demonstrated that betaine stabilizes cell membranes by interacting with membrane phospholipids (Klasing et al., 2002).

Conclusions

The findings of the present study suggest that betaine can effectively improve hematological, biochemical, and antioxidant values. These properties may mitigate the pathological alterations caused by *C. perfringens*, improve gut health, and consequently increase the chicks' resistance to clostridia infection.

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