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Efficacy of *Bacillus licheniformis* on *Clostridium perfringens*-inducing necrotic enteritis in broilers

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Immunohistochemistry (IHC)

Abstract

Clostridium perfringens is a major bacterial threat in poultry, responsible for necrotic enteritis (NE), which negatively affects bird health and production. Due to restrictions on antibiotics, probiotics are being investigated as alternatives probiotics *(Bacillus species),* in particular *B. licheniformis*, are non-pathogenic microorganisms that reduced intestinal inflammation and preserved the intestinal morphology in broilers challenged with *C. perfringens*. A total of 150 samples from the liver and intestine of broiler chickens from different farms in Sharkia Governorate; 70 of these isolates came from intestinal samples, 80 from liver tissue samples yielded 60 *C. perfringens* isolates. Using Real Time PCR, 60 strains of *C. perfringens* from healthy and sick broilers with gastrointestinal problems were shown to have the alpha toxin gene (*cpa*). Type A is the most common type found in *C. perfringens* samples from infected hens, while both *cpa and cpb* genes were detected in only one out of five affected birds. A sensitivity test was performed on 14 antibiotics against *C. perfringens*. For the antibiotics that showed the highest sensitivity, the minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) were measured. Bacitracin Zn was identified as the recommended treatment because the MBC values, which show the concentration needed to kill the bacterium, typically surpass the MIC values. In this study, a total of 120 Commercial Cobb broiler chicks were randomly divided into six equal groups: **Gp. (1)**: normal control group (basal diet only), **Gp. (2)**: infected control group (at day 14, infected with *C. perfringens* type A (1.5×10^8 CFU/mL), **Gp. (3)** :(fed GalliproTect® (8×10^6 CFU/g) from day 1 throughout the experiment, **Gp. (4)**: fed GalliproTect® from day 1 and

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challenged with *C. perfringens* at day 14, **Gp. (5):** infected and treated with Bacitracin Zinc at 48 hours post-infection, and **Gp. (6)**: challenged at day 14 and treated with GalliproTect® from day 1 in combination with Bacitracin Zinc after infection. The findings showed that early supplementation with GalliproTect® improved growth performance, enhanced blood parameters, and offered protection against *C. perfringens* infection. This study evaluated the effects of GalliproTect®, a probiotic formulated from *B. licheniformis*, as a dietary supplement that promotes bird growth and may serve as a potential alternative to the in-feed antibiotic Bacitracin Zn. The evaluated criteria included immunological response, growth performance, blood chemistry, serum antioxidant levels, and the probiotic's efficacy in reducing NE.

Introduction

Necrotic enteritis is a common and economically damaging disease of broilers caused by *C. perfringens,* a bacterium found in litter and intestinal contents. **(Van *et al*., 2004; Cooper and Songer, 2009 and: Osman Elhariri, 2013and Alizadeh *et al*., 2024).** Alpha-toxin is the main factor in disease severity, leading to intestinal damage and toxemia. **(Timbermont *et al*., 2009, M'Sadeq *et al*., 2015 and Villagrán-de la Mora *et al*., 2020).** The poultry feed sector is actively seeking sustainable non-antibiotic alternatives to manage necrotic enteritis (NE) during its initial phases by reducing the populations of *C. perfringens*. **(Lyras *et al*., 2009 and Elleithy *et al*., 2023).** The extensive application of antimicrobial agents for promoting growth and preventing diseases in Canada is raising concerns about the dissemination of antimicrobial resistance in the enteric flora, especially regarding the resistance observed in *C. perfringens* colonies. **(ESVAC, 2017).** Antimicrobial susceptibility assays provide an in vitro assessment of how sensitive or resistant specific microorganisms are to a variety of medications. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of many potential agents against *C. perfringens* were determined using the agar dilution technique, as advised by the **(NCCLS, 2004).** In 1945, *Bacillus licheniformis* bacteria produced the antibiotic known as Bacitracin Zn **(Hofacre *et al*., 1998),** which is a well-known metallopeptide antibiotic with potent bactericidal properties primarily targeting Gram-positive bacteria **(Russell and Strobel, 1988).** It works by inhibiting cell wall synthesis, impacting bacterial membranes, leading to a 10.8% increase in chicken body weight, reducing food conversion rate by 31%, and promoting an increase in abdominal fat in chickens **(Butaye *et al*., 2003).** Probiotics are considered potential substitutes for antibiotics and growth enhancers when utilized as a preventive measure against necrotic enteritis triggered by *C. perfringens*. **(Cheng *et al*., 2020; Hussein *et al*., 2020)**. Natural feed additives are used to maintain a healthy gut microbiota, as they contain beneficial live microorganisms that can be included in broiler diets to enhance intestinal health, promote growth, boost immunity, and improve the antioxidant status of the birds while preventing harmful microorganisms from attaching, particularly during their early growth stages. **(FAO/WHO, 2006 ; Abd Al-Fatah, 2020 and Abd El-Hack *et al*., 2022).** Bacillus bacteria, such as *Bacillus subtilis, Bacillus coagulans, Bacillus licheniformis, Bacillus cereus, and Bacillus polymyxa,* are among the top microorganisms used for probiotics and direct-fed growth enhancers. **(Chang and Yu, 2022).** Based on earlier research, *Bacillus licheniformis (BL)* ranks among the most widely used probiotics in the livestock industry due to its resistance to stress and elevated temperatures. Research has indicated that *BL* can generate various beneficial compounds, including bacteriocin, antimicrobial peptides, and digestive enzymes, which enhance animal performance and support immune system development. **(Yang *et al*., 2021; Additives *et al*., 2023)** and stop *Clostridium* from colonizing the intestines as a growth booster to increase bird growth by producing the enzymes that help break down and absorb nutrients from food **(Han *et al*., 2023).**

Immunohistochemistry (IHC) is a valuable diagnostic technique that helps visualize specific bacterial antigens within tissue sections using monoclonal antibodies. The ability of IHC to localize bacterial antigens allows for better understanding of infection progression and the

efficacy of treatment strategies **(Kaldhusdal and Hofshagen, 1992).** This research focused on the isolation, identification, and assessment of the antimicrobial susceptibility of *C. perfringens* derived from chickens, as well as testing the MIC and MBC for Bacitracin Zinc. The study also examined the effectiveness of *B. licheniformis* and Bacitracin Zinc against *C. perfringens* in broiler hens, along with their role in preventing infections and reducing the severity of necrotic enteritis (NE). Multiplex PCR reaction was utilized to screen for circulating strains and their toxicity. Furthermore, it assessed the effects of probiotic and/or antibiotic supplementation on growth performance, hematological and biochemical parameters, immunological status, and immunohistochemistry (IHC), which is a valuable diagnostic approach for visualizing specific bacterial antigens in tissues.

Material and methods

Samples Collection:

- A total of 150 intestinal and liver samples were gathered from broiler chickens sourced from various farms in Sharkia Governorate. The samples were collected from chickens suspected of infection, aged between 2 to 5 weeks, that exhibited signs of diarrhea, lethargy, and suboptimal growth performance. These samples were carefully transported in ice boxes to the laboratory for the purpose of isolating and identifying *C. perfringens.*

Isolation and identification of *C. perfringens*:

Suspected samples were inoculated into tubes of cooked meat medium and then incubated at 37°C for 24 hours anaerobically **(Willis, 1977).** Neomycin sulphate (200 µg/ml) was used to streak a loopful of infected fluid media onto 10% sheep blood agar **(Smith and Holdeman, 1968; Cruickshank *et al*., 1975).** Colonies of *C. perfringens* have a double zone of hemolysis and are flat and olive in color **(Vaikosen and Muller, 2001).** Gram-positive non motile bacilli were observed under a microscope. Suspected pure isolates were acquired and identified using the **Koneman *et al*., 1992** techniques. These isolates demonstrated lecithinase activity on egg yolk agar positive **(Cruickshank *et al*., 1975),** nitrate reduction, and catalase and indole negative results.

PCR assay:

Real Time PCR targeted *cpa and cpb* genes to confirm toxin presence:

RNA extraction:

RNA extraction from tissue samples was applied using QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH) when 200 µl of the sample were added to 600 µl RLT buffer containing 10 µl β-mercaptoethanol per 1 ml, incubated at room temperature for 10 min. One volume of 70% ethanol was added to the cleared lysate, and the steps was completed according to the Purification of Total RNA protocol of the QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH).

Oligonucleotide Primers:

Primers used were supplied from Metabion **(Germany)** are listed in **Table (1)**

Toxin	Sequence	Amplified product	Reference
cp alpha	AAGAACTAGTAGCTTACATATCAACTAGTGGTG	65˚C 60 min.	**YOO *et al*., (1997)**
	TTTCCTGGGTTGTCCATTTCC		
cp beta	TGGAGCGTGAAAGAAACTGTTATTA		
	GGTATCAAAAGCTAGCCTGGAATAGA		

SYBR green rt-PCR:
Primers were utilized in a 25- µl reaction containing 12.5µl of the 2x *HERA* SYBR® Green RT-qPCR Master Mix (Willowfort, UK), 1 µl of RT Enzyme Mix (20X), 0.5 µl of each primer of 20 pmol concentration, 3 µl of water, and 5 µl of RNA template. The reaction was performed in a step one real time PCR machine in the Biotechnology Unit, Animal Health Research Institute, Zagazig Branch, Egypt.

Analysis of the SYBR green rt-PCR:
The step one program was used to determine amplification curves and ct values. Using the "ΔΔCt" method described by **Yuan *et al.* (2006),** the CT of each sample was compared with that of the positive control group using the following ratio: (2-ct) in order to determine the variation of gene expression on the RNA of the various samples.

Assays for Antimicrobial Susceptibility:
The susceptibility tests were performed using antimicrobial drugs commonly used in clinical practice, as outlined by the British Society for Antimicrobial Chemotherapy (BSAC, 2011), which describes the agar disk diffusion technique (Oxoid, UK) utilized for evaluating the antimicrobial susceptibility of isolates. The antibiotics used in the tests included Ceftriaxone, Rifampicin, Doxycycline, Lincomycin, Clindamycin, Ampicillin, Amoxicillin, Bacitracin Zinc, Vancomycin, Ciprofloxacin, Levofloxacin, Enrofloxacin, Metronidazole, Tylosin, and Tiamulin. Each isolate was cultured in Mueller Hinton agar medium (Oxoid, UK) and incubated overnight in 10% neomycin sheep blood agar. The cultures were then adjusted in saline to achieve an optical density corresponding to McFarland 0.5 standards. Following a 15-minute period, antimicrobial discs were placed on the agar. Interpretation of the results was conducted according to **Martel *et al.* (2004)** after the plates were incubated anaerobically for 24 hours at a temperature of 37°C.

Determination of MIC and MBC of antimicrobial sensitivity:
1. Bacterial Culture:
Start with a pure culture of *C. perfringens*, ideally from a fresh isolate or a reference strain. Subculture the bacteria onto Tryptose Sulfite Cycloserine (TSC) agar or reinforced clostridial medium (RCM) and incubate anaerobically. For MIC/MBC testing, select 10-20 colonies from the agar and inoculate them into fluid thioglycollate broth (FTB) for overnight growth. For more accurate results, ensure the culture is in the logarithmic growth phase by checking the optical density (OD) at 600 nm.

2. Media Preparation:
Prepare Mueller Hinton broth (MHB) and supplement it with 5% lysed horse blood. Sterilize the media by autoclaving. For *C. perfringens* testing, MHB supplemented with blood is crucial for proper growth.

3. Bacitracin Preparation
Obtain a stock solution of bacitracin with a known concentration. Prepare serial dilutions of bacitracin in MHB (e.g., 2-fold serial dilutions) to achieve a desired range of concentrations (e.g., 0.016 to 256 µg/mL).

4. Inoculum Preparation:
Prepare a bacterial suspension from the overnight FTB culture by diluting it in fresh MHB to achieve a desired cell density, typically a 0.5 McFarland standard or a concentration of 5x10^5 CFU/mL.

5. MIC Determination:
Distribute the diluted bacitracin into sterile 96-well plates. Introduce the bacterial suspension to each well containing the bacitracin concentrations. Incubate the plates in anaerobic conditions at 37°C for a duration of 24 hours. Following incubation, evaluate bacterial growth through visual observation (looking for turbidity or a clump of cells at the well's bottom) or by measuring optical density at 600 nm. The MIC is defined as the lowest concentration of bacitracin that prevents visible growth.

6. MBC Determination:
For each well that displays an MIC and concentrations exceeding it, transfer 100 µL to TSC or RCM agar plates. Incubate the agar plates anaerobically at 37°C for a duration of 24 hours. Count the number of colonies present on each plate. The MBC is defined as the

lowest bacitracin concentration that leads to a 99.9% decrease in bacterial colony count in comparison to the original inoculum.

Drugs

-Probiotic: *Bacillus Licheniformis* (GalliproTectÒ obtained commercially from Chr. Hanssen company, Denmark. *B. Licheniformis* spores administered at a dose of 500 g / ton of feed (8×10^6 CFU/g) as recommended by the producer. **(Veken *et al*., 2021).**

-Antibiotic: Zincbac 150®, Zinc bacitracin feed premix package of 1kg obtained commercially from Arab Company, Egypt, by dose 55 mg/kg feed for 5 days (recommended dose).

-Bacterial inoculum: Toxigenic *C. perfringens* type A, isolated field strain, was established by PCR, the birds were individually challenged by oral gavages with 1 milliliter of 1.5 $x10^8$ CFU at 14 days of age.

Experimental animals:

The present study was conducted at Al-Amoushi chicken farm located in Aga city, close to Mansoura Governorate, 120 Commercial Cobb chicks were kept in optimal environmental conditions concerning light, temperature, and humidity, and they had unrestricted access to water and standard commercial feed pellets throughout the duration of the experiment.

Experimental design:

In this study, a total of 120 Commercial Cobb broiler chicks were randomly divided into six equal groups: **Gp. (1)**: normal control group, **Gp. (2)**: infected control group, at 14 days old, birds infected with *C. perfringens* type A (1.5 $x10^8$ CFU/ml), **Gp. (٣** : **(**fed GalliproTect®500 g /ton of feed (8×10^6 CFU/g) from day 1 and throughout the experiment, **Gp. (4)**: fed GalliproTect®from day 1 and challenged with clostridia at 14 day old, **Gp. (5):** antibiotic treated group (infected and treated with Bacitracin Zinc (55 ppm) at 48 hours post-infection), and **Gp. (6)**: challenged at day 14 and treated with GalliproTect®from day 1 in combination with Bacitracin Zn after infection.

Table (2). Experimental design:

	Groups		No. of chicks	Type of treatment	Time of blood sampling
The experimental Commercial Cobb broilers chickens (N = 120)	**Experimental Groups broiler chickens after infection**	**Group 1**	**20**	Fed control diet without medication or bacterial challenge, (- ve control group).	Blood samples were collected from wing veins of 5 chicks per group at the age of 21 and 28 day old using sterile clean tubes
		Group 2	**20**	Challenged by *C. Perfringens* in day 14 of age at a dose of (1.5 $x10^8$ CFU/ml), and fed control diet. (+ ve control group).	
		Group 3	**20**	Non infected and fed on (GalliproTect®) at a dose of 8×10^6 CFU /g for feed (500 gm/ton) the whole feeding period).	
		Group 4	**20**	Fed on (GalliproTect®) at a dose of 8×10^6 CFU /g for feed (500 gm/ton the whole feeding period) and challenged by *C. Perfringens* at a dose of (1.5 $x10^8$ CFU/ml) at 14 days of age.	
		Group 5	**20**	Challenged by *C. Perfringens* at a dose of (1.5 $x10^8$ CFU/ml) at 14 days of age and treated with Bacitracin Zn (48 hrs after infection).	
		Group 6	**20**	Challenged by *C. Perfringens* at a dose of (1.5 $x10^8$ CFU/ml) at 14 days of age and treated with (GalliproTect®) at a dose of 8×10^6 CFU /g + Bacitracin Zn after infection.	
Total number			**120**		

Growth performance:
At the conclusion of each period, growth performance parameters, the body weight gain (BWG) and feed conversion ratio (FCR), were calculated using the body weight (BW) and the average daily feed intake (FI) to assess the impact of the treatments on growth **(Cengiz *et al*. 2015).** Additionally, clinical signs were recorded daily throughout the experimental period.

Sampling:
- A selection of five birds from each group was made to gather blood samples on the 21th and 28th days of ages. Each blood sample was separated into three parts. The first part was combined with dipotassium salt of EDTA (1 mg/1 mL of blood) for conducting hematological tests. The second part was transferred into a clean centrifuge tube without any anticoagulant to collect serum by centrifugation for biochemical testing. The third part (2 mL of blood) was moved to a sterile plastic centrifuge tube containing heparin (50 I.U/mL) to evaluate phagocytic activity. **(Xue *et al*., 2011).**
- Liver and intestinal tissue samples were gathered and prepared for immunohistochemistry using antibodies targeted at *C. perfringens* in the Faculty of Science at Zagazig University. **(Meer and Songer, 1997)**.

Hematological studies
A complete blood count was performed, measuring factors such as hemoglobin levels, percentage of packed cell volume, red blood cell count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total white blood cell count, and the differential count of white blood cells. This was accomplished using the Sysmex XT automatic cell counter (2000 IV; located at AHRI Zagazig), following the methodologies outlined by **Thrall *et al*., (2012).**

Serum Biochemistry studies:
Biochemical tests were carried out utilizing test kits from Diamond-Egypt in accordance with the manufacturer's guidelines. The assessment of serum total proteins was based on the method described by **Grant *et al*. (1987)**, while serum albumin levels were determined following the protocol established by **Doumas *et al*. (1981).** The activities of liver enzymes, such as alkaline phosphatase (ALP) as outlined by **Kind and King (1954),** and alanine aminotransferase (ALT) along with aspartate aminotransferase (AST) as per **Reitman and Frankel (1957),** were measured. Serum levels of uric acid and creatinine were evaluated using commercially available kits, following the manufacturer's instructions. The serum cortisol concentration (Monobind Inc., Lake Forest, CA, sensitivity: 0.25 mg/dl) was analyzed with NS BIOTECH Apparatus using Elisa kits sourced from Diametra company, as referenced by **Loriaux (2017)**.

Determination of serum antioxidative status:
The activities of Superoxide dismutase (SOD) and Catalase (CAT), along with the Malondialdehyde (MDA) concentration in serum, were evaluated using commercially available assay kits from the Nanjing Jiancheng Bioengineering Institute, following the manufacturer's guidelines to assess serum antioxidant capacity. The measurement of CAT activity was carried out according to the technique described by **Senthilkumar *et al*. (2021),** while the assessment of Superoxide dismutase activity was performed using the method outlined by **Oberley and Spitz (1985).** Malondialdehyde levels were quantified based on the protocol established by **Thrall *et al*. (2012).**

Immunological studies:
- Serum IgG levels were assessed using a sandwich ELISA technique according to **Lovland *et al*. (2003).**
- The evaluation of phagocytic activity, including both phagocytic percentage and phagocytic index, was conducted following the method outlined by **Platt and Fineran (2015).**

Immunohistochemistry detection of *C. perfringens* Alpha-toxin in Liver and intestinal tissues of broiler chickens:
Immunohistochemistry (IHC): Liver and intestinal tissues that had been fixed in formalin and embedded in paraffin (4–5 µm thick) were subjected to deparaffinization and rehydration. Antigen retrieval was conducted using a citrate buffer (pH 6.0) in a microwave. The activity of endogenous peroxidase was inhibited with 3%

hydrogen peroxide. The sections were incubated at 4°C overnight with a rabbit polyclonal antibody targeting C. perfringens alpha-toxin (at a 1:200 dilution). Following this, a biotinylated secondary antibody and streptavidin–HRP complex were applied, and DAB chromogen was used for visualization. Lastly, the sections were counterstained with hematoxylin and observed under a light microscope **(Meer and Songer, 1997).**

Statistical analysis:

The statistical evaluations were conducted using SPSS for Windows (Version 18.0; SPSS Inc., Chicago, IL). The differences among the experimental groups were assessed using one-way analysis of variance (ANOVA). If a significant difference was identified through the one-way ANOVA, individual group comparisons were performed with the post hoc Fisher's least significant difference (LSD) test. The results are presented as mean ± standard error of mean. A P-value of less than 0.05 was considered significant **(Kinnear and Gray, 2006).**

Results

Isolation and identification of *C. perfringens*:

Bacteriological examination revealed colonies with a characteristic double zone of hemolysis and olive coloration on blood agar**.** Microscopic evaluation showed Gram-positive non motile bacilli. Suspected pure isolates were acquired and identified. These isolates demonstrated lecithinase activity on egg yolk agar positive**,** nitrate reduction, and catalase and indole negative results.

Molecular confirmation by Real Time PCR as shown in Fig (1):

The amounts of alpha and beta toxins in various groups were displayed by the results of real-time PCR as in **Fig (1).** GalliproTect® inhibits the growth of *C. perfringens* type A and formation of its toxins.

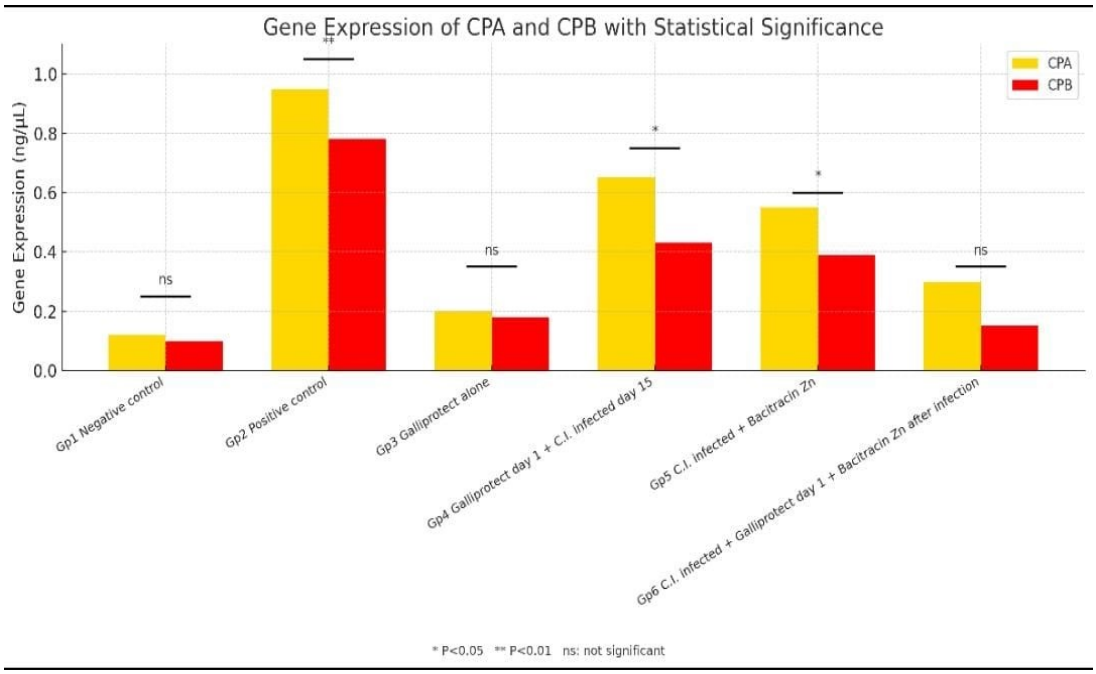

Fig. (1). Gene expression levels of *C.perfringes alpa* (cpa) & *beta* toxins (cpb) in all experimental groups Cpa& cpb expression were quantified& compared between all groups
Statistical significance as follow: ns (not significant), p< 0.05(*) and p <0.01 (**).

Table (3) Antimicrobial susceptibility of C. perfringens isolates: inhibition zones of different antibiotics MIC and MBC :

The results of the antibiotic susceptibility tests performed on *C. perfringens* were presented. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were established for four promising antibiotic drugs: Enerofloxacin, Bacitracin Zn, Lincomycin, and Clindamycin. Bacitracin Zn was identified as the preferred medication, with the concentration necessary to eliminate the bacterium represented by the MBC values, which typically exceed the MIC values. The MIC50 and MIC90 ranged from 0.25 to 0.5 µg/ml. **(NCCLS, 2004).**

Antibiotic	Disc Content (µg)	Inhibition Zone (mm)	MIC 50 (µg/mL)	MBC 90 (µg/mL)
Ceftriaxone	**30**	24	-	-
Ampicillin	**10**	22	-	-
Amoxicillin	**25**	20	-	-
Vancomycin	**30**	25	-	-
Enrofloxacin	**5**	24	0.5	1.0
Bacitracin Zinc	**10**	**30-36**	0.25	0.5
Ciprofloxacin	**5**	23	-	-
Levofloxacin	**5**	22	-	-
Metronidazole	**5**	21	-	-
Lincomycin	**15**	25	0.25	0.5
Clindamycin	**2**	20	1.0	2.0
Rifampicin	**5**	19	-	-
Tylosin	**30**	18	-	-
Tiamulin	**30**	22	-	-

MIC 50: MIC that inhibit 50% of the bacteria
MIC 90: MIC that inhibit 90% of the bacteria

Immunohistochemistry (IHC):

Immunohistochemistry staining outcomes for intestinal epithelial cells and hepatocytes revealed no positive staining in control negative group (G1), suggesting the absence of *C. perfringens*. Conversely, the positive control group (G2) displayed intense brown staining, indicating significant colonization. In the meantime, (G3) showed weak staining and (G4) exhibited moderate immunoreactivity, while (G5) and (G6) showed either no staining or very minimal, signifying effective bacterial suppression. As shown in Figures 2 and 3.

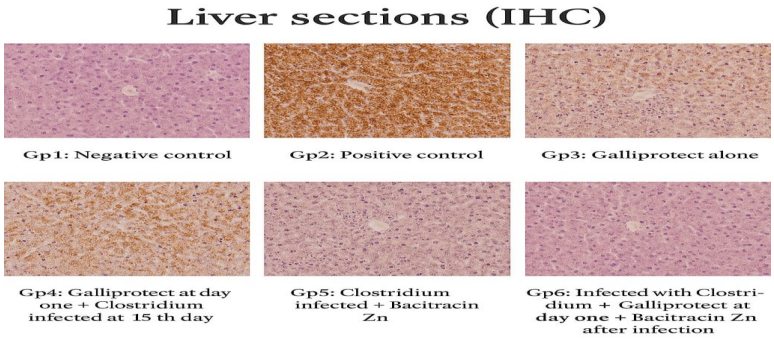

Fig. (2). liver sections in all experimental groups by (IHC)

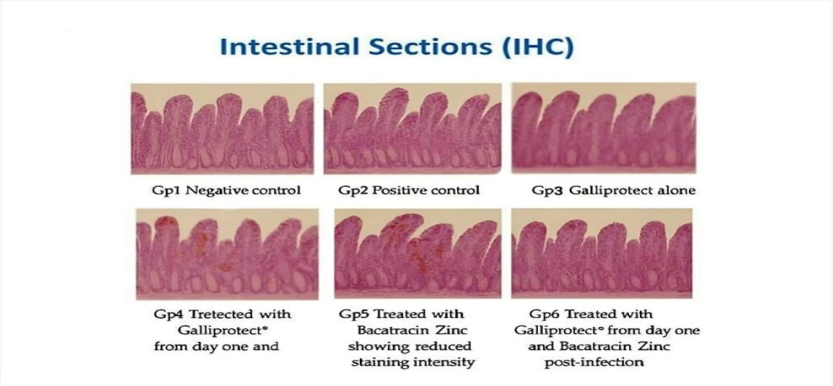

Fig. (3). Intestinal sections in all experimental groups by (IHC)

Growth Performance parameters:

Table (4). Growth performance of *C. perfringens* infected and treated chickens at 21th and 28th days old (M ± SE), (n=5). Different letters in the same raw indicate significant changes.

Groups / Parameters	Age of bird	G1 (-ve Control)	G2 *C. perfringens* (+ve Control)	G3 *B. licheniformis* alone from day one	G4 *B. licheniformis* from day one + *C. perfringens* at 15th day	G5 Infected with *C. perfringens* + Bacitracin Zn after infection	G6 Infected with *C. perfringens* + *B. licheniformis* from day one + Bacitracin Zn after infection
Initial Body weight (gm)	14th day	646.67 ±3.26^{b}	635.28 ±4.01^{b}	710.72 ±4.42^{a}	653.33 ±2.98^{b}	638.33 ±3.56^{b}	696.67 ±4.36^{a}
Body weight (gm)	21th day	1021.67 ±1.67^{b}	751.67 ±6.01^{f}	1310.32 ±3.56^{a}	823.33 ±4.41^{e}	910 ±5.77^{d}	946.67 ±6.01^{c}
	28th day	1531.67 ±6.01^{b}	965 ±2.89^{e}	1980.00 ±1.67^{a}	1090 ±5.77^{d}	1315 ±2.89^{c}	1313.33 ±12.02^{c}
Body weight gain (gm)	21th day	375 ±8.66^{b}	116.39 ±1.67^{e}	599.6 ±1.65^{a}	170 ±5.77^{d}	271.67 ±1.67^{c}	255 ±2.89^{c}
	28th day	510.67 ±6.01^{b}	213.37 ±6.01^{e}	669.68 ±2.69^{a}	266.67 ±2.89^{d}	405 ±8.82^{c}	396.67 ±6.01^{c}
Food consumption (gm)	21th day	530 ±5.77^{b}	260 ±11.55^{e}	670 ±5.77^{a}	283.33 ±8.82^{d}	430 ±11.55^{c}	423.33 ±14.53^{c}
	28th day	890 ±11.55^{b}	450 ±11.55^{e}	1150 ±11.55^{a}	533.33 ±8.82^{d}	766.67 ±8.82^{c}	770 ±11.55^{c}
Food conversion ratio (FCR)	21th day	1.41 ±0.01^{d}	2.23 ±0.05^{a}	1.12± 0.09^{e}	1.67 ±0.06^{b}	1.58 ±0.01^{c}	1.69 ±0.02^{b}
	28th day	1.75 ±0.03^{e}	2.11 ±0.04^{a}	1.72± 0.19^{e}	2.00 ±0.03^{b}	1.89 ±0.04^{d}	1.94 ±0.02^{c}

Table (5). Erythrogram of *C. perfringens* infected and treated chickens at 21th and 28th days old (M ± SE), (n=5). Different letters in the same raw indicate significant changes.

Groups / Parameters	Age of bird	G1 (-ve Control)	G2 *C. perfringens* (+ve Control)	G3 *B. licheniformis* alone from day one	G4 *B. licheniformis* from day one + *C. perfringens* at 15th day	G5 Infected with *C. perfringens* + Bacitracin Zn after infection	G6 Infected with *C. perfringens* + *B. licheniformis* from day one + Bacitracin Zn after infection
Hb (g/dl)	21th day	8.6 ± 0.03 ab	6.53 ± 0.05 d	9.10 ± 0.03 a	7.98 ± 0.019 c	7.49 ± 0.039 c	8.33 ± 0.06 b
	28th day	9.18 ± 0.40 a	6.22 ± 0.30 c	9.70 ± 0.04 a	8.38 ± 0.05 b	8.44 ± 0.08 b	8.87 ± 0.05 b
RBCs (10^6/µl)	21th day	2.40 ±0.04 a	1.87 ± 0.06 c	2.38 ± 0.04 a	2.01 ± 0.01 b	2.13 ± 0.02 ab	2.22 ± 0.01 ab
	28th day	2.56 ± 0.05 a	1.76 ± 0.04 b	2.21 ± 0.04 a	2.17 ± 0.03 a	2.32 ± 0.04 a	2.49 ± 0.03 a
PCV (%)	21th day	34.64 ± 0.53 a	28.75 ± 0.12 d	34.61 ± 0.53 a	32.42 ± 0.10 c	31.26 ± 0.24 c	33.56 ± 0.21 b
	28th day	35.53 ± 0.25 a	27.09 ± 0.38 c	35.50 ± 0.24 a	33.79 ± 0.26 b	32.07 ± 0.23 b	35.33 ± 0.28 a
MCV (fL)	21th day	144.33 ±2.10 b	153.74 ±2.80 a	145.42 ± 2.20 b	158.29 ± 2.30 a	146.76 ± 2.40 b	151.17 ±2.70 ab
	28th day	138.79 ± 2.50 c	153.92 ±2.40 ab	161.63 ± 2.90 a	155.71 ± 2.20 ab	138.23 ± 2.60 c	141.89 ± 2.80 b
MCH (pg)	21th day	36.66 ± 1.13 a	37.40 ± 0.96 a	36.60 ± 1.13 a	37.29 ± 0.40 a	37.39 ±0.63 a	37.21 ± 0.5 a
	28th day	35.92 ± 0.62 b	37.10 ± 0.86 a	35.85 ± 0.62 b	35.25 ±0.50 b	36.17 ±0.80 b	35.04 ± 0.30 b
MCHC (g/dL)	21th day	25.60 ± 0.109 a	23.80 ± 0.089 c	26.01 ± 0.11 a	24.70 ± 0.0198 b	24.01 ± 0.099 bc	24.39 ± 0.025 b
	28th day	25.69 ± 0.11 a	23.97 ± 0.25 c	26.25 ± 0.11 a	24.73 ± 0.26 bc	24.90 ± 0.21 bc	25.12 ± 0.11 b

Table (6). Leukogram of *C. perfringens* infected and treated chickens at 21th and 28th days old (M ± SE), (n=5). Different letters in the same raw indicate significant changes.

Groups / Parameters	Age of bird	G1 (-ve Control)	G2 *C. perfringens* (+ve Control)	G3 *B. licheniformis* alone from day one	G4 *B. licheniformis* from day one + *C. perfringens* at 15th day	G5 Infected with *C. perfringens* + Bacitracin Zn after infection	G6 Infected with *C. perfringens* + *B. licheniformis* from day one + Bacitracin Zn after infection
WBCS (10^3 /µl)	21th day	20.19 ±1.12^{d}	25.41 ±1.53^{a}	20.34 ±1.13^{d}	21.97 ±1.41^{c}	22.34 ±1.44^{b}	20.48 ±1.11^{d}
	28th day	19.46 ±1.13^{d}	25.75 ±1.46^{a}	19.42 ±1.16^{c}	21.89 ±1.13^{c}	22.02 ±1.18^{b}	19.43 ±1.14^{c}
Heterophils (10^3 /µl)	21th day	4.42 ± 0.05^{c}	8.46 ±0.19^{a}	3.98 ± 0.05^{d}	6.26 ± 0.44^{b}	6.85 0.05^{b}	4.75 ± 0.36^{c}
	28th day	4.32 ± 0.05^{c}	8.54 ± 0.20^{a}	3.39 ± 0.05^{d}	6.42 ± 0.44^{b}	7.08 ± 0.35^{b}	4.27 ±0.05^{c}
Lymphocytes (10^3 /µl)	21th day	11.22 ±0.12^{a}	9.19 ±0.11^{c}	11.73 ±0.02^{a}	10.88 ±0.11^{b}	10.56 ±0.11^{b}	11.02 ±0.08^{ab}
	28th day	11.19 ± 0.12^{a}	9.24 ± 0.12^{c}	11.99 ± 0.01^{a}	10.92 ± 0.11^{b}	10.38 ± 0.10^{b}	11.07 ± 0.07^{ab}
Monocytes (10^3 /µl)	21th day	2.92 ±0.01^{c}	3.95 ± 0.138^{a}	2.86 ± 0.02^{c}	3.17 0.027^{b}	3.19 0.036^{b}	3.03 ± 0.03^{bc}
	28th day	2.26 ± 0.01^{c}	3.63 ± 0.09^{a}	2.20 ± 0.01^{c}	2.82 ± 0.02^{b}	2.75 ± 0.02^{b}	2.35 ± 0.02^{bc}
Eosinophils (10^3 /µl)	21th day	1.05 ± 0.01^{a}	1.07 ± 0.01^{a}	1.14 ± 0.01^{a}	1.10 ± 0.01^{a}	1.13 ± 0.01^{a}	1.09 ± 0.01^{a}
	28th day	1.06 ± 0.01^{a}	1.08 ± 0.01^{a}	1.18 ± 0.01^{a}	1.12 ± 0.01^{a}	1.19 ± 0.01^{a}	1.11 ± 0.01^{a}
Basophils (10^3 /µl)	21th day	0.58 ± 0.01^{a}	0.56 ± 0.02^{a}	0.63 ± 0.02^{a}	0.56 ± 0.03^{a}	0.61 ± 0.01^{a}	0.59 ± 0.01^{a}
	28th day	0.63 ± 0.02^{a}	0.60 ±0.01^{a}	0.656 ± 0.016^{a}	0.61 ± 0.011^{a}	0.62 ± 0.012^{a}	0.63 ±0.013^{a}

Biochemical study:

Table (7). Biochemical parameters (liver and kidney functions, and cortisol) of *C. perfringens* infected and treated chickens at 21th and 28th days old (M ± SE), (n=5). Different letters in the same raw indicate significant changes.

Groups / Parameters	Age of bird	G1 (-ve Control)	G2 *C. perfringens* (+ve Control)	G3 *B. licheniformis* alone from day one	G4 *B. licheniformis* from day one + *C. perfringens* at 15th day	G5 Infected with *C. perfringens* + Bacitracin Zn after infection	G6 Infected with *C. perfringens* + *B. licheniformis* from day one + Bacitracin Zn after infection
AST (U/L)	21th day	47.73 ±1.72 d	84.89 ± 1.99 a	45.28 ± 1.76 e	71.34 ± 2.53 b	73.04 ±1.16 b	65.84 ±2.27 c
	28th day	46.25 ± 1.02 d	89.45 ± 1.87 a	41.33 ±1.05 e	60.17 ± 0.86 b	63.45 ± 1.66 b	55.51 ± 1.10 c
ALT (U/L)	21th day	9.17 ± 0.20 d	20.12 ± 0.46 a	7.48 ± 0.21 e	15.74 ± 0.245 c	16.48 ± 0.22b	13.07 ± 0.138 d
	28th day	9.12 ± 0.27 c	21.82 ± 0.30 a	7.08 ± 0.27 d	11.95 ± 0.216 b	12.62 ± 0.165 b	9.62 ± 0.30 c
ALP (IU/L)	21th day	52.09 ± 0.19 d	90.69 ± 0.22 a	50.23 ± 0.20 e	73.84 ± 0.86 b	75.53 ± 0.16 b	62.08 ± 1.21 c
	28th day	52.59 ± 0.24 c	91.51 ± 1.54 a	49.41 ± 0.23 d	62.47 ± 1.59 b	64.67 ± 1.30 b	53.87 ±1.05 c
Total protein (g/ dL)	21th day	4.22 ±0.0198 a	2.91 ± 0.029 c	4.44 ± 0.02 a	4.04 ± 0.029 b	4.09 ± 0.01 b	4.13 ± 0.01 ab
	28th day	4.52 ±0.10 a	2.69 ±0.02 c	4.56 ± 0.012 a	4.37 ± 0.09 b	4.29 ± 0.02 b	4.45 ± 0.01 a
Albumin (g/dL)	21th day	1.50 ± 0.05 a	1.15 ± 0.06c	1.52 ± 0.04 a	1.43 ±0.05b	1.47 ± 0.03b	1.51 ± 0.05 a
	28th day	1.59 ± 0.05 a	1.07 ± 0.06 b	1.61 ± 0.04 a	1.51 ± 0.05 a	1.54 ± 0.04 a	1.57 ± 0.05 a
Uric Acid (mg/ dL)	21th day	7.40 ± 0.40 e	11.60 ± 0.35 a	7.35 ± 0.38e	9.52 ± 0.37 c	10.34 ± 0.38b	8.48 ± 0.37d
	28th day	8.00 ± 0.40 d	11.70 ± 0.43 a	7.03 ± 0.35 e	9.08 ± 0.36c	9.11 ± 0.37 b	8.03 ±0.37 d
Creatinine (mg dL)	21th day	0.25 ± 0.02 e	0.76 ± 0.02 a	0.20 ± 0.02 e	0.44 ±0.01c	0.56 ± 0.02 b	0.35 ± 0.02 d
	28th day	0.23 ± 0.02 d	0.87 ± 0.02 a	0.17 ± 0.01e	0.36 ± 0.02 c	0.51 ± 0.02 b	0.25 ± 0.02 d
Cortisol (ng/ml)	21th day	1.82 ± 0.22 e	3.85 ± 0.22 a	1.80 ± 0.19 e	2.79 ± 0.206 c	2.84 ± 0.20 b	2.39 ± 0.18 d
	28th day	1.90 ± 0.20 c	3.92 ± 0.21 a	1.78 ± 0.09 d	2.41 ± 0.20 b	2.49 ± 0.20 b	1.92 ± 0.19 c

Antioxidants status:

Table (8). Antioxidants status of *C. perfringens* infected and treated chickens at 21th and 28th days old (M ± SE), (n=5). Different letters in the same raw indicate significant changes.

Groups / Parameters	Age of bird	G1 (-ve Control)	G2 *C. perfringens* (+ve Control)	G3 *B. licheniformis* alone from day one	G4 *B. licheniformis* from day one + *C. perfringens* at 15th day	G5 Infected with *C. perfringens* + Bacitracin Zn after infection	G6 Infected with *C. perfringens* + *B. licheniformis* from day one + Bacitracin Zn after infection
SOD (u/ml)	21th day	530.0 ± 4.97 b	350.0 ± 4.72 f	572.2 ± 4.81 a	428.4 ± 5.11 d	405.1 ± 5.22 e	460.2 ± 5.4 c
	28th day	540.0 ± 4.72 b	341.0 ± 4.60 e	610.0 ± 4.61 a	485.2 ± 4.84 d	465.0 ± 5.12 d	524.5 ± 5.01 bc
CAT (u/ml)	21th day	21.4 ± 1.34 b	14.31 ±1.02 f	23.5 ± 1.21 a	16.3 ± 1.11 d	15.4 ± 1.40 e	18.5 ± 1.22 c
	28th day	21.0 ±1.12 b	13.13 ± 0.90 d	24.4 ± 1.00 a	19.1 ± 1.00 c	18.7 ± 1.34 c	20.5 ± 1.11 b
MDA (mmol/l)	21th day	9.1 ± 0.30 e	13.6 ± 0.52 a	8.62 ±0.33 e	11.2 ± 0.50 c	12.5 ± 0.41 b	10.5 ± 0.44 d
	28th day	9.0 ± 0.24 c	14.3 ± 0.40 a	8.31 ±0.42 d	10. 0 ± 0.32 b	10.4 ± 0.31 b	9.7 ± 0.33 c

Some immunological parameters:

Table (9). Immunological parameters of *C. perfringens*

Groups / Parameters	Age of bird	G1 (-ve Control)	G2 *C. perfringens* (+ve Control)	G3 *B. licheniformis* alone from day one	G4 *B. licheniformis* from day one + *C. perfringens* at 15th day	G5 Infected with *C. perfringens* + Bacitracin Zn after infection	G6 Infected with *C. perfringens* + *B. licheniformis* from day one + Bacitracin Zn after infection
Phagocytic %	21th day	75.04 ±1.30 b	37.08 ± 0.71 f	79.44 ± 1.35 a	65.49 ± 1.17 d	61.77 ± 1.26 e	68.82 ± 2.00 c
	28th day	76.0 ± 0.96 b	41.2 ± 0.73 e	81.2 ± 1.01 a	69.09 ± 1.17 d	67.91 ± 1.27 d	75.30 ± 1.43 bc
Phagocytic Index	21th day	4.53 ± 0.13 a	1.03 ± 0.72 e	4.64 ± 0.13 a	3.19 ±0.12 c	2.82 ±0.14 d	3.79 ± 0.14 b
	28th day	4.46 ± 0.17 b	1.83 ± 0.11 d	4.98 ± 0.18 a	3.14 ± 0.07 c	3.04 ± 0.17 c	4.40 ± 0.14 b
IgG (mg/dL)	21th day	911 ± 2.12 d	953 ± 3.22 a	913 ± 1.73 d	937.80 ± 2.26 b	936.44 ± 1.42 b	924.40 ± 1.41 c
	28th day	920 ± 2.0 d	960 ± 3.51 a	926 ± 1.99 d	944.4 ± 1.58 b	941.3 ± 2.02 b	915.9 ± 1.99 cd

Discussion

This study demonstrated that *C. perfringens* type A is the primary agent responsible for NE in broilers, with alpha-toxin being central to disease severity. Infections in animals are associated with particular types of toxins produced by *C. perfringens*, indicating that variations in toxin production influence the virulence traits of the pathogen's isolates. **(Petit *et al.,* 1999).** The genes *cpa* and *cpb*, which are involved in the pathogenicity of *C. perfringens* to produce NE, and the toxin expressed on the plasmid **(Albini *et al*., 2007).** The primary toxins generated by strains of *C. perfringens* are A, B, C, and I. **(Prabhu *et al*., 2013; and Abd Al-Tawab *et al*., 2014).** *Clostridium perfringens* type A is the main culprit which responsible to both clinical and subclinical NE in chickens and found in the environment and in the intestines of warm-blooded animals **(Songer and Meer 1996; Van Immerseel *et al*., 2004).** The ability of *C. perfringens* strains to induce gas gangrene arises from their production of α-toxin, which is the only significant typing toxin generated by type A strains. **(Awad *et al*, 1995, Songer, 1996 and Rood, 2007).** Our findings, which are consistent with those of **Wages (2003), Keyburn *et al*. (2010), Yang *et al*. (2021), and Sally *et al*. (2023),** who proposed that the primary virulence factor for NE lesions found during an outbreak is alpha toxin showed an increase in toxin in both *cpa* and *cpb* gene expression, particularly in experimental groups as compared to normal control ones. In the current study's antimicrobial sensitivity profiling to 14 antibiotics of verified *C. perfringens* isolates Type A were Bacitracin Zn which showed strong antibacterial activity against *Clostridium perfringens*, followed by lincomycin, ceftriaxone, vancomycin, enrofloxacin, ciprofloxacin, ampcillin, clindamycin, tiamulin, amoxicillin, metronidazole, rifampicin and tylosin. The most promising four antibiotic medications were Bacitracin Zn, Enerofolaxcin, Lincomycin, and Clindamycin examined for MIC and MBC; Bacitracin Zn showed the highest rates of sensitivity.

The IHC findings from this study align with the established pathology of necrotic enteritis, and the detection of brown deposits in both liver and intestinal tissues verifies the presence of *C. perfringens* alpha-toxin. Treatment with Bacitracin Zn (G5) and the combination of GalliproTect® with Bacitracin Zn (G6) demonstrated a lower presence of toxins when compared to the positive control, indicating a potential protective or therapeutic role. These results align with **(Costa *et al*., 2018; Ritter *et al*., 2019 and Lin *et al*., 2013)** Who attributed this reduction due to the effect of GalliproTect® as probiotic which act as growth promoters and the main line of defense against *C. perfringes* because of their ability to prevent infection, change the gut microbiota, reduce inflammation in the gastrointestinal tract and improve GIT health. This prevents the bacterial colonies from being washed away by the peristalses of the intestinal wall because they adhere to the wall and prevent pathogenic germs from colonizing the gut wall, which stops the disease from progressing. These results endorse the application of IHC as an additional diagnostic method and emphasize the effectiveness of the treatment protocols. **(Meer and Songer, 1997**; **Kadra *et al*., 1999 and Wu *et al*., 2009).** The reduction in immunostaining in G5 and G6 highlights the therapeutic effectiveness of Bacitracin Zn, either alone or in combination with GalliproTect®. These findings are consistent with previous studies that demonstrated reduced necrotic enteritis severity following probiotic or antibiotic administration **(Knarreborg *et al*., 2002).** IHC remains a critical technique to validate infection control in histological samples.

The performance characteristics regarding growth in broiler chickens during the starter, grower (pre-infection), and finisher stages (post-infection) were analyzed. When comparing the groups that had probiotics in their drinking water, there was a notable increase in body weight (BW), body weight gain (BWG), and feed conversion ratio (FCR) compared to the other experimental groups, except for the broilers that were given GalliproTect® in their drinking water from day one, all the other experimental groups showed significant growth retardation at the end of the *C. perfringens* challenge in relation to the control positive group. These results align with those of **Kabir *et al*. (2004), Li *et al*. (2010), and Zhou *et al*. (2016),** who claimed that probiotic supplemen-

tation can improve chicken performance. The results clearly show that the experimental birds' live weight gains were substantially ($P < 0.05$) higher than those of the control group. Moreover, probiotics enhance feed conversion efficiency via several mechanisms, including alterations in intestinal flora, promotion of gram-positive and nonpathogenic facultative anaerobic bacteria that generate hydrogen peroxide and lactic acid, suppression of intestinal pathogen development, and better digestion and utilization of nutrients. **(Villagrán-de la Mora *et al*., 2020; Elleithy *et al*., 2023 and García-Vela *et al*., 2023 (.Abudabos *et al*. (2017) and Xue *et al*. (2018)** ascribed that to its involvement in necrotic enteritis, which damages intestinal function, impairs nutrient absorption, and negatively affects growth performance by destroying gut integrity, lowering villus height, and activating the immune system.

Changes in hematological parameters in broilers with necrotic enteritis may be caused by a bacterial toxin. Our results showed that broilers infected with *C. perfringens* showed a significant decrease in hemoglobin concentration and RBC count. A decrease in the erythrogram was also seen, which may be related to intravascular hemolysis brought on by *C. perfringens* **(El-Deen *et al*., 2019); Allam *et al*., (2013).** According to **Kojima *et al*. (2023) and Charitaki and Liapis (2025),** Clostridial toxins facilitate the breakdown of phospholipids in erythrocyte membranes, leading to hemolysis by damaging the circulating red blood cells. Total leukocyte count, heterophil, and monocyte levels were assessed, but lymphocyte levels significantly decreased in the infected group. The groups treated for the infection exhibited a notable rise in RBC count and hemoglobin content compared to the untreated infected group.Based on the findings of **Nagaralli *et al*. (2002).** Bacitracin ZN hinders the production of cell wall mucopeptides during the multiplication of bacteria. **Mikkelsen *et al*. (2009)** observed that the incorporation of organic acids inhibited the proliferation of *C. perfringens*. Our results align with those of **El-Gharbawy (2014), Sayed *et al*. (2016), and Sally *et al*. (2023)** who found an enhancement in erythrogram parameters in broilers infected with *C. perfringens*. The variations in leukograms identified in our research may be associated with bacterial infections and inflammation, resulting in leukocytosis, heterophilia, and monocytosis. **(Nasr El-Deen *et al*. 2019).** Our findings were consistent with those of **Gheith *et al*. (2011) and Saleh *et al*. (2011),** who revealed leukocytosis and heterophilia as characteristics of *C. Perfringens* infected broilers. The leukograms of birds treated with Bcitracin Zn or GalliproTect®, or a combination of Bcitracin Zn and GalliproTect®, improved when compared to the non-treated group**. (El-Shahat ,2014 and El-Sheikh *et al*., 2018)** reported similar findings. Probiotics have demonstrated their ability to enhance the immune system through several mechanisms, such as increased lymphocyte levels, oxidative bursts in heterophils, and the production of immunoglobulins by soothing the digestive tract and modulating the immune response, probiotics may assist in preserving a proper equilibrium between pro-inflammatory and anti-inflammatory cytokines. **(Shumaila *et al*., 2022).**

The treated groups had significantly lower levels of AST, ALT, ALP, total protein, albumin, uric acid, creatinine, and cortisol than the positive control group. Serum total protein levels in the treatment groups were significantly higher than those in the control positive group. The majority of the measures listed below reverted to normal in the treated groups when compared to the control negative group. An increase in liver enzyme levels (AST and ALT) was noted in the *C. perfringens* infected group, suggesting biliary stasis and liver damage brought on by clostridial toxins. **(El-Deen *et al*.,2019).** Serum levels of uric acid and creatinine were significantly increased in the untreated infected group, which may suggest renal damage and cellular death. The deterioration of renal tubules impaired the excretion of uric acid and creatinine, resulting in higher serum levels of these substances in the infected birds. Similar findings were noted by **Allam *et al*. (2013), El-Deen *et al*. (2019), Sally *et al*. (2023), and Saleh *et al*. (2023).** Hypoproteinemia observed in the infected non-treated group may be due to reduced feed consumption, excretion through the colon and kidneys, or liver damage caused by clostridial toxins. An increase in probiotic levels in broilers led to a significant rise in

blood uric acid levels. Additionally, cortisol levels in the blood were elevated in the infected group compared to the control group as a consequence of *C. perfringens* infection, which can have a cytotoxic impact at high concentrations, resulting in nuclear fragmentation and damage to genetic material. **(Zhang *et al*., 2017).** Considering this, our results aligned with the observations of **Zaytsoff *et al*. (2020),** who discovered changes in cortisol levels associated with *C. perfringens* infection in chickens as a physiological response to stress.

Oxidative stress is currently a significant issue in animal production across the globe. **(Sies and others, 2017).** CAT and SOD levels in treatment groups were significantly lower than those in the control positive group, indicating a decline in antioxidant status. In contrast to the control positive group, MDA significantly increased in the treatment groups. When compared to the negative control group, the majority of the markers showed reverted to normal ranges in the treated groups. This is consistent with the findings of **Surai *et al*. (2019),** who found that most stress, regardless of its cause, is associated with an imbalance in the formation of free radicals and detoxification. Additionally, *B. licheniformis* has the ability to safeguard hens from oxidative harm by managing the redox balance of the host through mechanisms such as metal ion chelation, antioxidant enzymatic activity, regulatory signaling pathways, and the modulation of gut microbiota**. (Zhang *et al*., 2017; Zhao *et al*., 2020)**. In contrast**, Lei *et al*. (2013)** showed that treatment with *B. licheniformis* does not influence the activity of antioxidant enzymes in laying hens, except for glutathione S-transferase (GST). This might be related to the role of *B. licheniformis*, which could vary based on strain and dietary concentration. Furthermore, **Pereira (2014) and Darafsh *et al*. (2020)** found a reduction in the activity of glutathione peroxidase, superoxide dismutase, and catalase following the administration of probiotics.

The obtained immunological results, it was obvious the decline in phagocytic activity, accompanied with an elevation in IgG as a consequence of *C. perfringens* infection. Our results reinfoced previous results of **Salah *et al*. (2015); El-Deen *et al*. (2019)** who expressed that IgG significantly increased in *C. Perfringens*. Additinally, *B. licheniformis* supplement significantly promoted the thymus index and spleen index, and the bursa index at different dosage and period,and these immune organ index reflects the immune function of the body **(Qin *et al*., 2024).** These findings all proved that *Bacillus* has a beneficial impact on enhancing immunological function. While, phagocytic percentage and phagocytic index were decreased. However, administration of bacitracin zn and/or GalliproTect® effectively improved the immunological parameters. These results were in harmony with those of **Kan *et al*. (2021) and Abou-Khadra *et al*., (2024),** who documented that treatments with antibiotic or probiotic or both could ameliorate the immunity in clostridial infection. Our study showed that, when compared to the control group, the inclusion of GalliproTect® significantly increased the phagocytic percentage, phagocytic index, and IgG levels.. Similarly, numerous studies **Darafsh *et al*., (2018); Pan *et al*., (2022)** who record a gradual increase in IgG levels due to *B. licheniformis* treatment, and **Elleithy *et al*., (2023)** Numerous studies show that probiotics clearly enhance the immune system, helping the body fight off harmful organisms. This effect may be linked to the fact that Bacillus has a cell barrier composed of dextran, which acts as an immunostimulant. (**Abdelqader *et al*., 2013)**.

Conclusion

The study's findings demonstrate that *C. perfringens* can cause disease in broiler chickens and has a major effect on the productivity and well-being of the birds. By administering GalliproTect® alone or in combination with Bacitracin Zn, the severity of necrotic enteritis is lessened, growth performance is improved, immunological responses are improved, and antioxidant status is improved while the infection severity is decreased. Furthermore, the presence of bacterial toxins in intestine and liver tissues was confirmed by immunohistochemistry (IHC), and Real Time PCR has been shown to be a successful technique for detecting toxin genes. With regard to avoiding and

managing necrotic enteritis in chicken husbandry, these findings collectively support the use of probiotics as a sustainable substitute or adjunct to antibiotics.

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