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Effect of lactoferrin alone or in combination with bacitracin on *Clostridium perfringens* infection in rabbits

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Abstract

This work studied *in vitro* and *in vivo* the antibacterial activity of lactoferrin and/or bacitracin against *Clostridium perfringens* type A in rabbits. *C. perfringens* was bacteriologically isolated with a total rate of 37% from rectal swabs and fecal matter of diseased rabbits besides intestine of freshly dead. After targeting alpha, beta, epsilon and iota toxins genes by molecular typing (multiplex PCR), all isolates produced only alpha toxin and typed as type A. A total of 35 rabbits were divided into 5 groups; **G1**: negative control, **G2**: infected control with *C. perfringens* type A (1×10^9 CFU/ml) orally, **G3**: infected (Bacitracin Methyline Disalicylat BMD) 10 mg/kg body weight), **G4**: infected (lactoferrin 50 mg/kg body weight) and **G5**: infected (BMD 5 mg/kg + lactoferrin 50 mg/kg). MIC and MBC were 7.812 and 125 µg/ml for bacitracin, 0.195 and 3.125 mg/ml for lactoferrin respectively. *C. perfringens* count significantly decreased by all treatments while no bacteria were re-isolated from the liver in G5. All drugs recovered most of the detected parameters especially G4 and G5 reflected a significant increase in TP, albumin, globulin, lysozyme activity, and calcium, while reduced ALT, AST, ALP, LDH, BUN, and creatinine levels than G2. Finally, lactoferrin revealed significant antibacterial activity against *C. perfringens* either alone or synergistically adjunct with BMD.

Keywords: Lactoferrin; Bacitracin Methyline Disalicylat (BMD); *Clostridium perfringens* type A; Rabbits.

1. Introduction

Rabbits are hindgut fermenters and depend greatly on their gastrointestinal microbiota for the finest digestion of plant-based diets. Pathogenic bacteria are significant contributors to monogastric dysbiosis, therefore rabbit enteritis complex considers a major cause of morbidity and mortality **[1]**, depending on the rabbit's age and management system. Clostridiosis regards as one of the major economic impact diseases that threaten commercial rabbitaries in Egypt causing enterotoxaemia to pass on to enteropathy characterized by diarrhea and sometimes sudden death **[2]**. Losses rose during the early weaning period as a result of weaning stress; ration alteration and increases in cecal pH even though it may be found in adults **[3]**. *Clostridium perfringens* is one of the most commonly spread, strictly anaerobic gram-positive spore-forming rods that naturally inhabits the gastrointestinal tract **[4]**. Reliable on its produced toxins, there are 7 strains; type A (alpha-toxin), type B (alpha, beta, and epsilon toxins), type C (alpha and beta toxins), type D (alpha and epsilon toxins), type E (alpha and iota toxins), type F (alpha and enterotoxin) and type G (alpha and NetB toxins) **[5]**. *C. perfringens* type A is the most predominant with high mortalities facing kids at post-weaning age (4-6 weeks), especially when associated with coccidia **[6]**. It provokes its pathogenicity after toxin binding, increases the intestinal epithelial barrier's permeability (primary defense line) to luminal pathogens, and transmits to other vital organs **[7]**. Enterotoxaemia can also be activated by the overuse of antibiotics that cause microbiota imbalances.

Polypeptides group including bacitracin are considered one of the highly important veterinary antimicrobials for curing enteric disorders in food-producing animals including rabbits **[8]**. Bacitracin is bactericidal against Gram-positive cocci and bacilli, chiefly against clostridia spp. It obstructs bacterial cell wall biosynthesis by restraining the pyrophosphatase involvement in transporting peptidoglycan precursors throughout membranes **[9]** with a weak lipophilic profile and bacitracin A is the main component. It synthesizes non-ribosomally through a multistep mechanism **[10]**. A significant

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increase in the mean number of rabbit daily doses consumption of bacitracin, quinolones, and sulfonamides was found **[11]**. Meanwhile, their misuse plays a key role in the epidemiology of antimicrobial resistance (AMR). Many studies **[12, 13]** reported the efficacy of bacitracin methylene disalicylate (BMD) in mitigating the negative effects of *C. perfringens* in poultry either in subtherapeutic or therapeutic doses; it beneficially advances general health production, increases short chain fatty acids production (responsible for provoking mucus secretion, standardizing T cell role, and providing energy for colonocytes) and enhances tryptophan metabolites (responsible for sustain gut immune homeostasis and decrease inflammation). On the contrary, it decreases beneficial microorganisms plus increases the susceptibility of other pathogenic ones to colonize as Campylobacter and Salmonella sp. **[14]**, besides the development of bacitracin resistance mechanism **[15]** through induction of the structural *B. subtilis* BceAB ABC transporter genes.

Based on this, antibiotics should be used judiciously in rabbits where disequilibrium of the normal intestinal flora allows pathogenic bacteria propagation as clostridia sp or coliforms with their subsequent toxins **[16]**. Furthermore, bacitracin treatment is followed by low microbial carbohydrate fermentation that severely affects rabbits causing bloating, high amino acids catabolism, and elevation of pro-inflammatory cytokines gene expression **[17]**. Therefore, attempts to eliminate Clostridium sp. using antibiotics are unlikely to be successful in the long term. Enteropathies affect errand the onset of AMR in microorganisms of public health importance besides production costs **[18]**. As well, reports from the European Medicines Agency (EMA), the European Centre for Disease Prevention and Control (ECDC), and the European Food Safety Authority (EFSA) have proposed to use antimicrobials wisely in rabbit farming problems. Hence, natural materials can improve the efficacy of the antibiotics or use them as an alternative to diminishing their side effect, especially bacterial resistance microorganisms.

In recent times, postbiotic compounds play a crucial role in pathogens' suppression. They contain antimicrobial peptides (AMPs) that are produced by living organisms as a natural barrier against Gram-positive and negative bacteria. Lactoferrin (Lf) regards as one of these compounds that put into upholding the homeostasis and control of various intestinal diseases with rife applications in many animal species **[19]**.

Lactoferrin, a safeguard multipotential and multifunctional cationic glycoprotein, is made up of amino acids in a single polypeptide chain that is highly homologous across mammalian species. It is one of the iron-binding transferrins (TF) where its chelated capacity is 300-fold greater than TF, has an immunological reaction in the innate immune defense, and has high iron binding stability even at acidic values of 3.0 pH **[20]**. So it regularly works at inflamed and infected sites where it sequestrates iron and limits its availability to microbes for growth and virulence besides interacting with the microbial components **[21]**. Throughout lactation Lf exerts a critical role in enhancing newborn immune defense in addition to promoting iron absorption and metabolism hence, kids have a lower prevalence of diarrhea related to gastrointestinal infections. Because of that, using milk components as a supplement may be valuable. The whey protein Lf is considered one of the bioactive agents with high oral bioavailability. Lf prevents host cell invasion by specific intracellular attachments. Formerly in the intestinal absorption, it interacts with the intelectin-1 receptor **[22]** and internalizes by enterocytes. Due to it being a large protein, it transports via Payer's patches by endocytosis then enterically formulated in liposome soon after it is vehiculated into the bloodstream and delivered to tissues via the portal vein and the lymphatic pathway **[23]**. Lf is a component of gastrointestinal fluids and many exocrine secretions **[24]**. As well, it incites the production of interferon-gamma (IFN-γ) and interleukin (IL-12), which polarized a Th1 cell response in the intestinal mucosa that activates neutrophils, macrophages, natural killer cells, and cytotoxic T cells. It is considered a vital element of host physiological activities with multipharmacological properties **[25]**. It owns distinctive anti-inflammatory, antimicrobial and immunomodulatory characteristics where all of these activities can be dependent or independent of its iron-binding ability. Its immunoregulatory effect is dependent on the actual host's immune status. Hence, it may activate immune cells to secrete specific cytokines that increase its anti-infectious activity, and in analogous induces others that lower excessive reactivity of immune cells **[26]** and also restricts inflammatory processes in septic inflammations. Lf supplementation is a useful method to enhance antioxidant levels, stabilize the immune response and normalize proinflammatory cytokines production, which all are associated with intestinal health **[27]**. Lately, Lf has an escalating concern to be the focus of research as prospective antimicrobial agents **[28]**. It can act as bacteriostatic against Streptococcus, Staphylococcus, Salmonella, Shigella, and Enterobacter spp., bactericidal against; *P. aeruginosa, V. cholera, S. pneumonia*, and *E. coli* **[29]**, binds endotoxin (lipopolysaccharide) or diminishes the release of toxins as Shiga toxins, interferes with the bacterial-host cells adhesion, does

not affect the host microbiota or induce any documented resistance against it **[30]**.

Searching for natural alternatives has provoked upward interest; especially after lessen of classical antibiotic effectiveness in treating common infections, where the biological hazard of multi-drug resistant pathogens has become a threat to global public health and costs impact. Therefore, on a veterinary scale, Lf offers a new source with potential pharmaceutical applications as a rich source of antimicrobial agents and one of the crucial host defense molecules **[31]**. This orally active biologic drug which retains its bioactivity even after digestion can be used as a therapeutic protein not only as a nutraceutical agent. Consequently, these promising *in vitro* findings must be confirmed *in vivo* against various bacterial infections responsible for economic loss in animals besides judging its ability to synergize with antibiotics for potentiating their antimicrobial effect.

There is a scarcity of studies that assessed Lf's therapeutic effectiveness against *C. perfringens* in rabbits either alone or together with an antibiotic. Therefore, this study aimed to investigate *in vitro* and *in vivo* the antibacterial activity of lactoferrin and/or bacitracin against field strain of *C. perfringens* type A previously isolated and defined from diseased rabbits through detecting their minimal inhibitory and bactericidal concentrations (MIC and MBC), besides evaluating their effects on *C. perfringens* count, non-specific immunity (lysozyme activity, total protein, and globulin), biochemical profiles; albumin, (alanine and aspartate aminotransferase (ALT and AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) activities), calcium (Ca), blood urea nitrogen (BUN) and creatinine levels of rabbits.

2. Materials and Methods

22.1. Sampling for *C. perfringens* isolation from rabbits:

A total of 100 samples were collected from diseased rabbits suffered from enteric problems. Sixty rectal swabs, 20 fecal matter samples, and other 20 intestinal samples from freshly dead rabbits were collected and obtained separately under aseptic condition in sterile plastic bag and transferred rabidly in ice box to the lab **[32]**.

2.2. Isolation of *C. perfringens*:

Each sample was transferred aseptically into a sterile test tube containing freshly prepared cooked meat broth for microbial enrichment and incubated at 37 ºC/24-48 hrs in anaerobic jar. A loopfull from each tube was streaked on sheep blood agar with neomycin sulfate 10% and incubated anaerobically at 37 ºC/24-48 hrs. Suspected colonies were picked up separately and inoculated on cooked meat media and then kept in the refrigerator for further identification **[33]**.

2.3. Identification of suspected coloniesof *C. perfringens* isolates:

1- Morphological identification **[34]**.
2- Microscopical examination: using Gram's stained smears **[32]**.
3- Biochemical tests: using catalase, indole, urease, sugar fermentation, and gelatin liquefaction tests **[35, 36]**. Nagler test on egg yolk agar plate **[37]** for the detection of lecithinase activity of *C. perfringens* alpha toxin.

2.4. Molecular typing of *C. perfringens* isolates using multiplex PCR:

Five pure isolates of *C. perfringens* were randomly selected and typed using multiplex PCR targeting alpha, beta, epsilon and iota toxins genes **(Table 1)**in the Reference Laboratory for veterinary Quality control on Poultry production (RLQP), Animal Health Research Institute **[38]**.

DNA extraction: DNA extraction from isolates was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C/10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Oligonucleotide Primer: the used primers were supplied from Metabion (Germany)and listed in **(Table 1)**.

PCR amplification: Multiplex PCR for toxins**,** primers were utilized in a 50 µl reaction containing 25 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentrations, 12 µl of water, and 5 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler. The products of PCR were separated by electrophoresis on 1.5% agarose gel.

2.5. Infective dose preparation

According to the guidelines **[39]**, one of the PCR confirmed field *C. perfringens* type A serotyped strain isolated in this study was purified on TSC (Tryptose sulphite cycloserine) agar plate and incubated anaerobically at 37 ºC/ 24-48 hrs, 3-5 colonies picked up and inoculated on 5 ml of Muller broth and incubated under anaerobic conditions for 2 hrs to obtain the stock inoculum for infection. Infective dose wasenumerated to achieve 1×10^{9} cfu/ml **[6]**.

Table (1): Target genes, primers sequences, amplicon sizes and cycling conditions for molecular typing of *C. perfringens* isolates using multiplex PCR.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension
				Secondary denaturation	Annealing	Extension	
Alpha	GTTGATAGCGCAG GACATGTTAAG	402	94˚C 5 min.	94˚C 45 sec.	55˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.
	CATGTAGTCATCTG TTCCAGCATC						
Beta	ACTATACAGACAG ATCATTCAACC	236					
	TTAGGAGCAGTTAG AACTACAGAC						
Epsilon	ACTGCAACTACTAC TCATACTGTG	541					
	CTGGTGCCTTAATA GAAAGACTCC						
Iota	GCGATGAAAAGCC TACACCACTAC	317					
	GGTATATCCTCCAC GCATATAGTC						

2.6. The used drugs

Two drugs were used, Bacitracin Methylene Disalicylate (BMD) antibiotic (Maxtracin ® 500 (KIROVEST, Pharmaceuticals Co.)) and Lactoferrin-100 (Pravotin ® (HYGINT Pharmaceuticals, Egypt)).

2.7. *In vitro* antibacterial activity tests

2.7.1. Inoculum standardization for sensitivity tests

The bacterial inoculum was adjusted photometrically using a spectrophotometer at 625 nm to give absorbance from (0.08- 0.1) to be approximately (1.5 x 10^8) CFU/ml equal to 0.5 McFarland's standard **[40]** to be used in agar disc diffusion and MIC assays.

2.7.2. Antimicrobial susceptibility test (AST) assay

The antibacterial activity of bacitracin and lactoferrin against *C. perfringens* was assessed using agar disc diffusion method **[41]**. Sterilized filter paper discs (6 mm in diameter) were individually impregnated at various stock solutions with concentrations of 12.5, 25, 50 and 100 mg/ml for lactoferrin besides commercial disk (10 µg) for bacitracin and placed onto surface of *C. perfringens* inoculated agar. After incubation at 37 ºC/24 hr, the inhibition zones diameters (IZD) were measured in millimeter (mm). Each antimicrobial assay was performed simultaneously in triplicate. For judging susceptibility patterns to bacitracin, the IZD resistance was defined when it < 16 mm **[42]** in standard interpretive criteria charts.

2.7.3. MIC and MBC values

The MIC as a quantitative bioassay was determined by using a micro-broth dilution test in a 96-well microplate using standard procedures **[41, 43]**. A solution of 1mg/ml concentration for bacitracin and 100 mg/ml for lactoferrin were twofold serially diluted throughout wells to give the following concentrations 500, 250, 125, 62.5, 31.25, 15.63, 7.812, 3.91, 1.96, 0.98 and 0.49 µg/ml for bacitracin and 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.195, 0.097 and 0.049 mg/ml for Lf. Both negative (only drug without bacteria) and positive (only bacteria without drug) control wells were performed. Dimethylsulfoxide (DMSO, Sigma) was used to indicate the presence of uninhibited bacterial growth in each well. Referring to the results of the MIC assay a loopful, from each clear well was streaked on the specific media for *C. perfringens* incubated at 37 ºC/24 hr, then observed for growth. The lowest concentration that showed no turbidity was recorded as the MIC and the lowest concentration showing no growth was recorded as the minimum bactericidal concentration (MBC). The experiment was performed in duplicate.

2.8. *In vivo* assessment of the used drugs

2.8.1. Experimental design

A total of 40, apparently healthy rabbit kids at weaning age (4-5 weeks) with no previous disease history were obtained from a private farm. Rabbits were acclimatized for 10 days for indoor lab

conditions before the experiment. Then, the livers of 5 kids were tested bacteriologically to ensure that they are free from any systemic *C. perfringens* infection. The remaining 35 rabbits were divided into 5 groups (7 kids/group). **G1**: negative control, **G2**: positive control infected orally with *C. perfringens* type A (1×10^9 CFU/ml/rabbit), **G3**: infected treated with (BMD 10 mg/kg body weight), **G4**: infected treated with (lactoferrin 50 mg/kg body weight) and **G5**: infected treated with (BMD 5 mg/kg + lactoferrin 50 mg/kg). All groups had the same management with free access to ration and water. The first day is the day of infection. Rabbits were observed daily for morbidity, mortality, clinical signs, and necropsy finding. All treatments were given after the appearance of the first clinical signs on the 5^{th} day post infection (PI) and lasted for 5 successive days via drinking water. The experiment lasted for 10 days post-treatment (PT). The used dose for BMD was recommended commercially while lactoferrin was calculated through dose conversion using $K_m$factor table (where Km = body weight/body surface area (BSA)). The animal studies were approved by Research Ethics Committee for environmental and clinical studies at Animal Health Research Institute (AHRI) and were carried out in accordance with Egyptian Ethics Committee Guidelines and the NIH Guidelines for the Care and Use of Laboratory Animals.

2.8.2. Sampling

2.8.2. A- Blood samples

Samples were collected post treatment (PT) and at the end of the study from the ear vein (5 rabbits/group) for serum in a clean dry centrifuge tube without anticoagulant for biochemical analysis; ALT and AST **[44]**, ALP **[45]**, and LDH activities **[46]**, Ca **[47]**, total protein (TP) **[48]**, albumin **[49]** were determined. Globulin was detected by subtraction of albumin value from TP. Kidney function tests; BUN **[50]** and creatinine **[51]** were estimated using commercial kits (Spectrum) according to the manufacturer's instructions. For immunological evaluation: Serum lysozyme activity was measured by agarose gel lysis assay based on the lysis of *Micrococcus lysodeikticus* cells (lysozyme-sensitive gram-positive bacteria) **[52]**.

2.8.2. B- Liver and intestine samples for *C. perfringens* re-isolation and count

Post treatment, 1 g of (cecum, ilium and liver) from all infected groups (3 rabbits/group) were collected under aseptic conditions and transferred to 9 ml phosphate buffer saline (PBS) for preparation of initial suspension. Tenfold serial dilutions were made, 1 ml of each dilution distributed in a sterile petri dish, then 10-15 ml of molten TSC agar poured and mixed well with the inoculum by gentle rotation and left to solidify, then another layer of TSC was poured. All plates were incubated anaerobically at 37 ^{0}C/24 hrs **[53]**.

2.9. Statistical analysis

Experimental data were assessed by One-way analysis of variance (ANOVA). Duncan Multiple Range post-hoc analysis test using IBM SPSS software statistical program (version 20.0) was used for comparison of means at significance level ($P < 0.05$). Data were expressed as mean ± SE (standard error) when (N=5).

3. Results

3.1. Incidence of *C. perfringens* isolates among examined samples

In our study after identification of isolates, *C. perfringens* was isolated with a total rate of 37 % from the examined samples of enteric diseased rabbits. They were (5/40) considered 12.5% from rectal swabs, (24/40) considered 60% from fecal matter samples and (8/20) considered 40% from intestinal samples.

3.2. Identification of pure suspected colonies of *C. perfringens* isolates

On sheep blood agar colonies appeared raised, rounded, smooth, glistening and showed double zones of hemolysis. Microscobically, Gram stained smears from the colonies, revealed gram positive, short bacilli, straight with rounded ends and parallel sides. Biochemically, isolates were negative for catalase, indole, urease tests while positive with gelatinase activity, lecithinase reaction, sugar fermentation tests (lactose, glucose, sucrose and maltose).

3.3. Molecular typing of *C. perfringens* strains by multiplex PCR

Results showed that all of the representative *C. perfringens* isolates were positive for *alpha* toxin gene only and produced amplicons at 402 bp, none of the isolates possessed *beta, epsilon or iota* toxin gene, so they were considered *C. perfringens* type A **(Figure 1)**.

3.4. Antimicrobial sensitivity tests

AST of bacitracin was (18.3 ± 0.33 c) mm IZD while for Lf were (14.33 ± 0.33 a, 16.66 ± 0.33 b, 18.00 ± 0.58 bc and 21.33 ± 0.67 d) corresponding to these concentrations 12.5, 25, 50 and 100 mg/ml respectively. Results indicated no significant difference between IZD of bacitracin and 50 mg Lf. Results of MIC (the lowest effective concentration showed no turbidity) and MBC (the lowest effective concentration showed no growth) of bacitracin were 7.812 and 125 µg/ml, while for Lf were 0.195 and 3.125 mg/ml respectively.

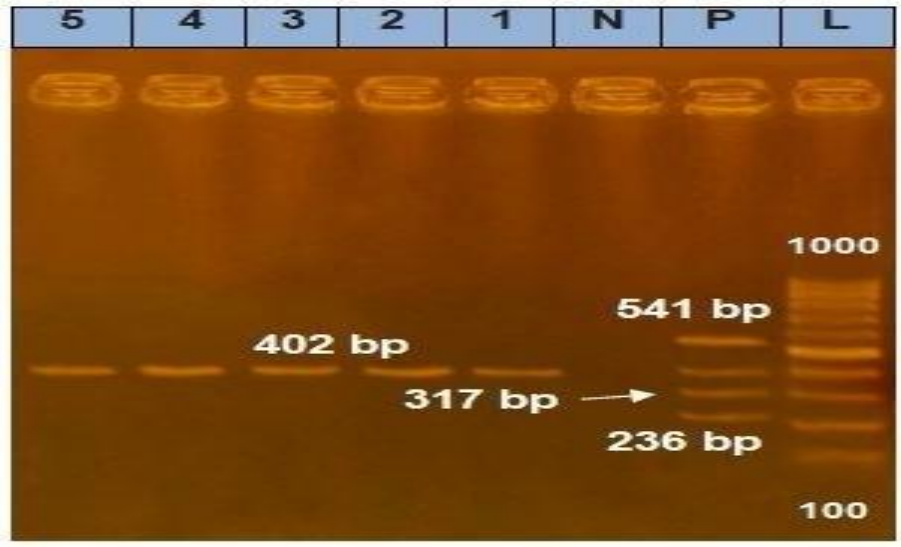

Figure (1): Agarose gel electrophoresis showing multiplex PCR for typing of *C. perfringens* isolates: Lane (L): 1000bp DNA Ladder, Lane P: Spiked samples with alpha, beta, epsilon and iota toxins served as a positive control, Lane N: negative control, Lane: 1-5 : positive *C. perfringens* isolates type A to alpha toxin at 402bp.

3.5. Clinical signs, post mortem findings and mortality rate

Infected rabbits showed the chronic form of the disease; decreased feed intake, ruffled fur, depression, polydipsia, slight swollen belly and inability to walk, intermittent doughy brownish and/or yellowish diarrhea with unpleasant odour, weight loss and mild dehydration. No mortalities were recorded. Sacrificed rabbits for *C. perfringens* count revealed dilated stomach, distention of the small intestine, enteritis with congested mesenteric blood vessels, caecal impaction with offensive odor doughy brownish contents mixed with gases, congestion of liver and kidney. Most of these findings relieved obviously by various treatments.

3.6. *C. perfringens* re-isolation and count

Post treatment **Table 2**, there was a significant decrease in the *C. perfringens* count in both liver and intestine (cecum and ilium) samples in all treated groups than infected control G2, while there is no significant difference among them. The lowest count of the intestine recorded in G5 treated with drug combination, while no bacteria re-isolated in liver.

Table (2): Effect of BMD and Lactoferrin (Lf) on *C. perfringens* count post treatment (Mean ± SE) n=3:

Sample	*C. perfrengin* count (x 10^2) CFU/gm			
	G2 (Positive control)	G3 (Infected + BMD)	G4 (Infected + Lf)	G5 (Infected + BMD + Lf)
Liver	74.00 ± 2.65 b	0.67 ± 0.33 a	1.66 ± 0.33 a	0.00 ± 0.00 a
Intestin (Cecum+ilium)	138.33 ± 1.66 b	2.00 ± 0.57 a	3.67 ± 0.33 a	0.66 ± 0.33 a

The various letters in the same row indicate statistically significant differences when (P<0.05).

3.7. Serum biochemical parameters

In **Table 3** results showed a significant decrease in TP, albumin and globulin besides increase in ALT, AST, ALP and LDH activities in infected control G2 than normal control G1. Post treatment and at the end, in a varying degrees treated groups revealed a significant increase in TP, albumin and globulin besides a significant decrease in most of the detected enzymes' activities than G2 while they showed no significant difference than G1 in most of these detected parameters at the end of the experiment especially G5.

4. Discussion

C. perfringens is the most significant cause of Clostridialenteric disease in domestic rabbits. It caused several intestinal pathologies that correspond to significant threats to survival and are associated with adverse long-term health outcomes **[54]**. Concerning finding natural alternative antibacterial agents to control the incidence and severity of enterotoxaemia to overcome multiple resistance resulting in the post-antibiotic era, Lf considers a highly conserved commercially available protein, it may guard against infection as a therapeutic

involvement in addition to the back of nutritional status **[55]**. Hence, this study assessed the *in vitro* and *in vivo* therapeutic effectiveness of Lf either alone or combined with the half dose of the BMD in rabbits against *C. perfringens* type A infection.

Table (3): Effect of BMD and Lactoferrin (Lf) on some serum biochemical parameters (Mean ± SE) n=5.

G	Periods	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	ALT (u/l)	AST (u/l)	ALP (u/l)	LDH (u/l)
G1	**PT**	6.47 ± 0.05 c	3.96 ± 0.10 c	2.51 ± 0.14 b	31.74 ± 0.89 a	64.99 ± 1.00 a	13.88 ± 1.02 a	56.08 ± 1.09 a
	AEE	6.20 ± 0.12 c	3.67 ± 0.09 b	2.53 ± 0.03 b	31.83 ± 1.01 a	66.35 ± 1.18 a	12.88 ± 0.68 a	55.92 ± 1.08 a
G2	**PT**	4.46 ± 0.12 a	2.81 ± 0.11 a	1.64 ± 0.03 a	42.58 ± 1.14 c	79.66 ± 1.20 d	24.53 ± 0.32 d	75.31 ± 0.74 d
	AEE	4.43 ± 0.12 a	2.94 ± 0.08 a	1.48 ± 0.16 a	44.20 ± 1.29 c	80.48± 1.29 d	20.39 ± 0.73 c	70.67 ± 1.76 c
G3	**PT**	5.90 ± 0.10 b	3.65 ± 0.11 bc	2.25 ± 0.16 b	35.71 ± 0.80 b	75.61 ± 0.73 c	19.00 ± 0.50 c	73.18 ± 1.33 d
	AEE	5.94 ± 0.07 bc	3.45 ± 0.12 b	2.49 ± 0.18 b	36.29 ± 0.65 b	75.73 ± 1.46 c	16.21 ± 0.87 b	66.18 ± 1.15 b
G4	**PT**	5.71 ± 0.15 b	3.39 ± 0.06 b	2.32 ± 0.26 b	35.11 ± 0.85 b	73.30 ± 0.65 c	17.13 ± 0.40 bc	69.39 ± 1.49 c
	AEE	5.73 ± 0.05 b	3.48 ± 0.19 b	2.25 ± 0.19 b	34.57 ± 0.46 ab	72.35 ± 1.21 bc	14.62 ± 0.49 ab	63.40 ± 1.08 b
G5	**PT**	5.65 ± 0.14 b	3.72 ± 0.09 c	1.93 ± 0.21 b	32.87 ± 0.59 ab	69.77 ± 0.97 b	15.65 ± 0.64 ab	64.18 ± 1.60 b
	AEE	5.84 ± 0.13 b	3.56 ± 0.02 b	2.00 ± 0.15 b	34.33 ± 0.77 ab	70.88 ± 1.20 b	14.81 ± 0.38 ab	63.15 ± 0.68 b

The various letters in the same colon of the same period indicate statistically significant differences when (P<0.05). **G1=** negative control, **G2=** infected control, **G3=** inf (BMD), **G4=** inf (Lf), **G5=** inf (BMD + Lf). **PT**= Post treatment, **AEE**= At experimental end.

In **Table 4** results indicated a significant decrease in lysozyme activity besides increase in BUN and creatinine in G2 than G1. Post treatment, only G4 and G5 showed a significant increase in Ca and lysozyme activity, while all treated groups showed a significant decrease in BUN and creatinine levels than G2.

Table (4): Effect of BMD and Lactoferrin (Lf) on lysozyme activity, calcium (Ca), blood urea nitrogen (BUN) and creatinine (Mean ± SE) n=5.

Group	Periods	Lysozyme activity (µMol)	Ca (mg/dl)	BUN (mg/dl)	Creatinine (mg/dl)
G1	**PT**	9.14 ± 0.20 b	6.43 ± 0.22 b	15.43 ± 0.32 a	0.69 ± 0.02 a
Negative control	**AEE**	9.22 ± 0.18 b	6.15 ± 0.07 a	16.67 ± 0.47 a	0.60 ± 0.04 a
G2	**PT**	8.20 ± 0.28 a	6.38 ± 0.16 b	21.38 ± 0.73 c	1.03 ± 0.04 c
Infected control	**AEE**	7.92 ± 0.04 a	5.82 ± 0.17 a	21.69 ± 0.39 b	1.05 ± 0.04 b
G3	**PT**	9.47 ± 0.26 b	6.50 ± 0.31 bc	17.74 ± 0.94 b	0.87 ± 0.06 b
Inf (BMD)	**AEE**	9.36 ± 0.39 b	6.07 ± 0.12 a	18.07 ± 0.61 a	0.72 ± 0.02 a
G4	**PT**	10.73 ± 0.21 c	7.20 ± 0.12 cd	18.78 ± 0.63 b	0.75± 0.05 ab
Inf (Lf)	**AEE**	9.96 ± 0.28 b	6.14 ± 0.07 a	18.10 ± 0.44 a	0.75 ± 0.04 a
G5	**PT**	10.61 ± 0.33 c	7.43 ± 0.27 d	17.36 ± 0.48 ab	0.72 ± 0.04 ab
Inf (BMD+ Lf)	**AEE**	11.06 ± 0.19 c	6.33 ± 0.19 a	17.20 ± 0.34 a	0.73 ± 0.03 a

The various letters in the same colon of the same period indicate statistically significant differences when (P<0.05). **PT**= Post treatment, **AEE**= At experimental end.

In our study after morphological, microscopical, and biochemical identification of isolates, *C. perfringens* was isolated with a total rate of 37% from the examined samples. Similarly, **Abdel-Rahman et al., [56]** isolated *C. perfringens* from examined rabbits at a rate of 39.3%. Meanwhile, it was isolated with a low percentage from rabbits;

13.2% by **Abdeen and Abdel latif, [57]** and 25% by **El-Helw et al., [58]**. As well, higher incidence of Clostridium spp. isolation was recorded by **Maghawry and Nasr, [59]** at 43.8% from diarrheic rabbits and 53% by **Khelfa et al., [60]**. Regarding the type of samples, it was shown that the incidence and isolation rate of C. perfringenes in our study was (5/40) equal to 12.5% from rectal swabs, (24/40) equal to 60% from fecal matter samples, and (8/20) equal to 40% from intestinal samples of dead rabbits. Another study by **El-Rahman and Atwa [61]** stated that *C. perfringens* isolation incidences were 36 and 70 % from fecal samples and intestines of dead rabbits respectively. While **Khelfa et al., [60]** isolated it at 44% from rectal swabs and 83% from the intestine of examined rabbit samples. Differences in incidence and isolation rate between our study and other previous studies may be due to changes in hygienic measures, rearing method (battery or ground), weaning age of rabbits, season of breading and type of isolated Clostridium spp. Furthermore, these differences may be due to the prophylactic use of anticoccidial drugs as it considers a major predisposing factor for clostridial enteritis. *C. perfringens* can produce 4 types of major toxins (α, β, ε, and ι) and so it is divided into 5 different serotypes, from A to E **[62]**. *C. perfringens* type A is the most communal serotype in the environment; it is the most extensively occurring pathogenic bacterium and is responsible for necrotic and diffuse enterotoxaemia in poultry and all mammals. It is also associated with food poisoning in humans **[63]**. Multiplex PCR is a reliable procedure for genotyping of *C. perfringens* strain and as a specific test for detection of its toxin genes. So we used this technique as a test for serotyping of *C. perfringens* isolates. Our molecular typing **(Fig 1)** revealed that all examined strains were *C. perfringens* type A. This agrees with **Khelfa et al., [64]** who recorded that most *C. perfringens* isolated strains from rabbit farms at different Egyptian governorates were classified as type A. **Freedman et al., [65]** also previously concluded that most *C. perfringens* enterotoxin (CPE) positive strains were classified as *C. perfringens* type A. Also **El-Helw et al., [58]** revealed that the alpha toxin gene was the only detected among major toxins in the obtained isolates from rabbits and so all stains were *C. perfringens* type A. While, **Pietro et al., [66]** showed that Type A was detected in only 63% from examined rabbit origin isolates, as well **Forti et al., [67]** recorded that the majority of *C. perfringens* isolates collected from rabbits were dispensed mainly to type A while other serotypes were actual restricted and limited.

AST of bacitracin was (18.3 ± 0.33) mm IZD while Lf ranged from (14.33 ± 0.33 to 21.33 ± 0.67) according to its concentration while no significant differences were recorded between bacitracin and 50 mg Lf IZD. Recently, the high incidence of multi-drug resistant (MDR) of *C. perfringens* type A isolates denoted the significance of periodical awareness of antimicrobial resistance (AMR) profiles, appropriate usage of antibiotics, and searching for effective alternatives. For instance, **Anju et al., [68]** found that phenotypical MDR (more than 3 antibiotics) among toxic types of *C. perfringens* isolates were 44% for gentamicin, 40% for bacitracin and erythromycin, and 26.67% for tetracycline while, genotypically, isolates revealed 41.33 % resistance genes to tetracycline, 34.66% to erythromycin and 17.33 % to bacitracin besides a weak positive relationship between tetracycline and bacitracin genes based on correlation matrix analysis. As well, **Rachid, [69]** recorded a high resistance rate against tetracycline (86.36%), erythromycin (77.27%), co-trimoxazole (68.18%), and co-amoxiclav (50%), clindamycin (36.36%), bacitracin (36.36%) and penicillin G (31.81%). Antibiogram of **Eid et al., [70]** recorded various phenotypic resistances to lincomycin (82.26%), ampicillin (72.58%), nalidixic acid (69.35%), and spectinomycin (69.35%), in addition to (79.03%) of these isolates verified MDR phenotypes. Also **Alimolaei et al., [42]** found that resistance among *C. perfringens* isolates to bacitracin, clindamycin, penicillin G, erythromycin, tetracycline, and co-trimoxazole ranged from 51 to 90%. Where 44% of isolates were MDR and 4% were resistant to all. Spotlighting the antibacterial activity of Lf, **Morrin et al., [71]** mentioned that anti-bacterial and prebiotic bioactivities of glycoproteins like; Lf and immunoglobulins are directly correlated to their glycan structures. **Leo´n-Sicairos et al., [72]** evaluated the bactericidal effect of Lf and its other synthetic peptides against gram-positive bacteria and found that Lf and LFchimera were the best bactericidal agents with different physicochemical properties. Mechanisms of action of cationic molecules as Lf protein exhibits by many aspects, it can be through interaction with anionic bacterial plasma membrane causing changes in its shape, size and membrane permeability, scattering of membrane components like; lipopolysaccharides (LPS) and the negatively charged lipid matrix in Gram-positive bacteria ended by cell death besides down-regulating its pathogenic genes. This Lf action via the intracellular mechanism allowed bacterium DNA-Lf binding which indirectly affects the expression of bacterial virulence genes. Besides this direct effect on the bacteria, Lf binds with a great affinity to the essential free iron for bacterial metabolism, inhibits bacterial adhesion and target host cell invasion, controls bacterial aggregation and biofilm expansion, affects the mucosal immune function, and supports host immune defense. Moreover, several studies **[30,**

73] coincided with Lf's microbiostatic activity through iron binding capability and microbicidal activity through strapping of lipoteichoic and teichoic acid in Gram-positive bacteria leading to cell lysis and consequently biofilm assembly.

In this study MIC and MBC of bacitracin were 7.812 and 125 µg/ml, while in Lf were 0.195 and 3.125 mg/ml respectively. In this regard, **CLSI, [74]** described MIC for studying resistance patterns of anaerobes as a helpful laboratory tool for researchers. Also **Agnoletti et al., [75]** recorded that the MIC of zinc bacitracin (ZnB) against various isolates of rabbit *C. perfringens* ranged from 0.5 to 16.0 µg/ml. In another study, **Richez et al., [76]** recorded that the threshold values of ZnB for MIC 90 and MBC 90 were 930 and 1860 µg/kg, respectively to maintain its cecal bactericidal concentrations for the entire 24hr at 420 IU/mg dose. For Lf, **Théolier et al., [77]** found that Lfcin (Lf derived peptides) MIC was 24 µg/ml for *C. perfringens* in mice. Similarly, the surveyed literature by **Cutone et al., [78] and Lepanto et al., [79]** indicated that most of the *in vitro* studies on Lf multi-functional activities showed that higher concentrations need to be used for their effectiveness *in vivo*.

C. perfringens can induce acute or chronic manifestations. Death can happen within hours from disease onset otherwise forewarning signs of diarrhea, dropping off feeding and growth rate as a result of the desquamated intestine, and reduction of digestion and nutrient absorption **[70]**. Similar chronic signs were reported **[2]**. High mortalities are primarily associated with many adverse environmental conditions, predisposing factors (high carbohydrate diet), and stressors (crowdedness). This could explain the absence of mortalities in our controlled experiment than that rabbits are exposed in nature. **Brennan et al., [80]** observed similar relief of signs post BMD treatment. **Artym et al., [81]** reported that multi-actions of Lf (anti-infective, antioxidant, immunoregulatory, and anti-inflammatory) can initiate general improvement of clinical state. As well **Gomez et al., [82]** recorded *in vivo* the protection effect of Lf (0.125 mM) on enteric signs and inflammatory intestinal response progression in rabbits. This cure may be attributed to the direct effect of Lf either on the bacteria or the gut through its anti-inflammatory properties that decrease the bacteria invasion and degradation of invasive plasmid antigens.

C. perfringens counts **(Table 2)** were performed to compare the efficacy of treatments. Results revealed a marked decrease in counts of both liver and intestine samples in this descending order G4 (Lf), G3 (BMD) then G5 (BMD + Lf). No bacteria re-isolated from the liver in G5 that indicated the absence of systemic infection. These *in vivo* results are supported by various pharmacological actions of the used drugs. BMD, as a bactericidal drug trimmed down the colonization of the general intestinal microbial community **[83]**, through reduced almost all phospholipids, the major component of bacterial cell membranes, resulting in an overall decline of bacterial load **[13]**. However, **Richez et al., [84]** reported that *C. perfringens* in growing rabbits can be still isolated even after the treatment course as in our study. For Lf, **Pammi and Abrams, [85]** recorded its protection against late-onset sepsis and necrotizing enterocolitis (NEC) that results from a variety of infections. Diminishing of *C. perfringens* count by Lf can be occurred in many ways either related to its direct destruction of pathogens or not. The occupation of Lf to its receptors on intestinal epithelial cells and lymphocytes blokes some critical entrance receptors of bacteria or its products into host cells besides competing for the infection's consequences on the host's cellular response incorporating complex biomolecular network formation **[86]**. As well, Lf receptor binding prompts different intracellular signaling pathways that enhance host immunity. **Gomez et al., [82]** reported that Lf induced both bacteriostatic activity (by iron-binding capacity) and bactericidal activity (by the iron-independent way) through its pepsin-derived fragment. Lf generates *in vivo* for host defense 2 types of peptides after oral ingestion (under acidic conditions, proteolytic enzymes, and tryptic digestion) called lactoferricin (Lfcin) and lactoferrampin (Lfampin), where both signify the physiological allegation of Lf in gut homeostasis besides their biological functions as a potent anti-bacterial and anti-inflammatory activities **[87]**. They cause bacterial membrane depolarization (like the antibiotics colistin and polymixin B) resulting in disordering and alteration of its permeability, inhibition of macromolecule biosynthesis, and cell death, besides their synergic action with host innate immunity compounds **[24]**. Other ways for Lf antibacterial activity **[88, 29]**; the direct way applied through its electrostatic adhesion with the bacterium leading to their physical bilayer membranes interruption, cytoplasm leakage and holding up of intracellular protein, nucleic acids biosynthesis, and enzyme activity. While, the indirect way may be achieved throughout; by supporting the beneficial intestinal bacteria colonization (e.g. Lactobacillus spp. and Bifidobacterium spp. which have a critical role in butyric acid production that regulates epithelial O2 utilization), providing natural and sustainable approaches to control infectious bacterial disease, strengthen intestinal homeostasis besides boosting the host health by a variety of physiological mechanisms (e.g. competitive exclusion, secretion of short-chain fatty acids, activation of the intestinal immune system, etc.) where mucosal immunity plays

an important role in host defense against pathogens. From this point of view, BMD regrettably does not differentiate between commensal or pathogenic bacteria and may upset the intestinal microbial balance and deprive it form its benefits and products **[90]**. Besides the above mentioned, **Rosa et al., [89]** reported that Lf is exclusively surefire by neutrophils degranulation in inflammation and/or infection spots where the high free iron levels. The closed iron-binding stable form (holo-Lf) is the main form that resulted from withholding iron by open iron-free unsaturated normal form (apo-Lf), limiting free radical-mediated damage and lessening the availability of the iron to pathogens. Also, **Legrand, [90]** attributed the positive effects of Lf to its structure, as a component of innate immunity, and potent immunomodulator in addition to preventing the tissues from excessive inflammatory processes through modulation of cytokine production. In such concern, the normally formed Lf by mucosal epithelial cells and secondary neutrophil granules supported by exogenous Lf couples with bacterial LPS and glycosaminoglycans **[20]**, controls the release of neutrophils' extracellular traps after killing the bacteria, boosts natural killer cell activity in immune defense and restraints the entry of infection into host cells, all these acts of immunomodulation support its antibacterial activity **[91]**.

Similarly, **Daneshmand et al., [88]** studied the effect of both 45 mg/kg ration BMD antibiotic and 20 mg LFchimera/kg (one of Lf antimicrobial peptides formed by host defense systems) against *C. perfringens* infection in broilers and concluded the ability of Lf's peptide to substitute BMD as a competent alternative. It counters wise *C. perfringens* through; recovered intestinal lesions, diminished mortalities, renewed jejunal villi morphology, epithelial barrier, regulated the expression of extra pro-inflammatory cytokines (which cause gut damage and consume high energy), junctional proteins plus mucin transcripts, and reinstated the ileal microflora balance (increase commensal spp. and decrease the colonization of invading pathogens *E. coli* and Clostridium spp.) that enhance immune function. On the other hand, BMD antibiotic could not return normal villi distinctiveness or express junctional proteins' genes besides diminishing all bacterial count non-selectively. Other *in vitro* **[72]** and *in vivo* **[92]** studies on Lf bactericidal activities on a variety of gram-positive microorganisms were reported.

Co-administrated G5 with both drugs showed the best decrease in intestinal *C. perfringens* count, where no bacteria re-isolated from the liver indicated the absence of systemic infection. This may be explained by the ability of Lf to increase the susceptibility of some bacteria to antibiotics and lysozyme after losing their membrane integrity by binding to the lipid A component of lipoteichoic and teichoic acids of gram-positive ones leading to synergy with conventional antibiotics **[93]**. As well, combination therapy targeting different mechanisms of antibacterial activity simultaneously hence, it might help in slowing the emergence of resistance **[94]**, besides their synergy with the immune response **[95]**. Clinically, bacitracin has been used in combination with other antimicrobial agents. Many previous studies tried to use Lf either as an antibiotic alternative or in co-therapy with synthetic antibiotics to treat various bacterial diseases that cause economic losses in production and they assured the effectiveness of Lf through its antibacterial and synergistic effects. For instance, co-administration of Lf with penicillin G **[96]** or with cefazolin **[97]** increased the cure rate, prevented chronic infection, and decreased illness severity and bacterial count. Likewise, **Théolier et al., [98] and Durzy´nska et al., [99]** recommended testing the combinations of milk AMPs with a greater number of antimicrobials and their systematic evaluations, as they provide new directions to combat pathogens to optimal *in vivo* therapeutic use.

Serum biochemical analysis **(Table 3, 4)** of the infected control G2 revealed impairment of liver and kidney functions by a significant decrease in TP, albumin, globulin, and lysozyme activity, and an increase in serum activities of (ALT AST, ALP, and LDH), BUN and creatinine than normal control G1. All treated groups recovered most of these parameters and improved general liver and kidney functions through a significant decrease in protein loss, tissue damage, and enzyme liberation expressed by a significant increase in TP, albumin, globulin, lysozyme activity, and Ca levels, and decrease in ALT AST, ALP, LDH, BUN and creatinine levels than G2. The chronic form of the disease permits bacteria to reach the bile duct, bloodstream, liver, and other vital organs. Findings of G2 resulted from damaged hepatocytes besides other factors; anorexia and sloughing of intestinal mucosal cells which caused liberation of the intracellular enzymes. As a fact, the not well-controlled inflammatory response provoked by pathogens resulted in progressive tissue damage. Alpha-toxin as well, considers the main cause that impetuous the virulence of *C. perfringens*; type A, it is a proteic exotoxin, acts as phospholipase, crashes cell membranes (hepatic and renal tissues), liberates the histamine (lysozyme inhibitor) plus triggers platelet aggregation and thrombosis **[100]**. Liver and kidney lesions post *C. perfringens* infection were previously confirmed **[2, 101]**. Furthermore, *C. perfringens* enterotoxin activated phospholipase A2 that lead to Ca invasion into intestinal cells, augmented its intracellular

concentration, and shifted the mitochondrial activity that ended by extended cell death besides induction of lysosome secretion in the initial stages of microbial invasion **[102, 103]**. **Scott et al., [104]** reported that LDH is released during tissue damage and is considered an indicator of common injuries and diseases. So, changes in transaminases and LDH activities could be a sign of liver structure and its cellular integrity while liver synthetic function could be measured by TP and albumin levels. Although most mammals have two isoenzymes (intestinal and a liver/kidney/bone form) of ALP, rabbits are unique in having three forms of ALP, including an intestinal form and two forms present in the liver and the kidney. As well, the hepatobiliary system could be measured by ALP. The used drugs either alone or combined improved most of the measured parameters; this may be related to their noticeable reduction in the number of proliferated *C. perfringens* that consequently, alleviated its toxin production, tissue damage, and enzyme liberation. Acting aside from the ability of Lf to agglutinate *C. perfringens* cells through the mutual action of electrostatic, Lf-specific binding proteins, and hydrophobic interactions **[105]**, there was a special activity of bacitracin was recorded in neutralizing the secreted bacterial exotoxins and suppressed toxin uptake that avoids mammalian cells intoxication with the binary enterotoxins *C. perfringens* transferase within the endosomal compartment **[106]**.

Similar results were reported **[107-109]** that ZnB or BMD did not upset the hepatic or renal functions or induced any significant changes in the AST, ALT activities and uric acid than normal control, while increased creatinine levels. In addition, **Attia et al., [110]** observed that intermittently administered ZnB at (0.083 g/rabbit/day) non significantly affect (ALT, TP, albumin, globulin, or phagocytic activity), normal morphology of hepatic, renal, or ileum tissues, while continuous treatment for 42 days led to deleterious effects on them. Moreover, **Thema et al., [111] and EL-Deep et al., [112]** recorded that bacitracin-treated rabbits showed decreased (ALT, AST, and urea), increased (TP and globulin) and non significantly affected (creatinine, albumin, and A/G ratio) than normal. They concluded that it has not any harmful impacts on the blood metabolites (proteins and lipids), liver function (ALT and AST), and renal function (urea and creatinine) while considering the increased TP as a direct result of enhanced feed utilization and metabolic function in the entire body of rabbits. While, **Manafi et al., [113]** reported that BMD decreased albumin, and increased ALP while not affecting globulin or ALT than normal or challenged controls. **Ji et al., [114]** found that bacitracin oligomer increased the numbers of Ca2+-positive macrophages hence; it depends on the Ca signaling pathway to boost immune capacities. Where, the inhibition of intracellular Ca2+ influx significantly suppressed the phagocytosis, inflammatory mediator production, and NF-κB activation. These discrepancies in blood biochemical profiles seem to differ depending on treatment dose, duration, drug form, and animal or bacterial species.

Regarding Lf treated G4, **Zimecki et al., [115]** reviewed that Lf as a bactericidal protein triggered lysozyme-mediated bacteria lysis, lessened the plasma endotoxin activity and mesenteric lymph nodes bacterial colonization, conserved gut mucosal integrity post endotoxemia, put off nitric oxide stimulation that considers a destructive agent for the gut epithelium besides defended against hepatitis advancement that resulted from activation of Kupffer cells by LPS. The cytoprotective role of exogenous Lf against induced liver injury was recorded by **Guo et al., [116] and Fan et al., [117]**, where Lf suppressed (hepatocellular death, inflammatory responses (TNF-α, IL-6, and nitric oxide) and endoplasmic reticulum stress), sustained the liver oxidative steadiness using nonenzymatic antioxidant manner, backed-up damaged hepatocytes autophagy, decreased ALT, AST, ALP, and γ-GT besides recovered hepatocyte siderosis through focusing on the hepcidin-ferroportin axis. Hepcidin acts as an antibacterial peptide synthesized in the liver and transported all over the body passing through the blood circulation to regulate the iron output of both hepatocytes and intestinal epithelial cells. Hence, all these belongings helped in restoring regular liver function. Furthermore, **Moradian et al., [118]** found that Lf had no adverse effects on the levels of TP, albumin, or globulin, reduced ALT and ALP, while increased lysozyme and bactericidal activities than the normal group. Authors suggested that connecting to immune cell receptors, supporting leukocytes function by positively enhancing natural killer cell, neutrophils, macrophages, and their phagocytic activities, bonding to bacterial cell walls, and limiting their intracellular proliferation are all means of immunomodulating properties. Interestingly, rabbit serum contains high levels of β-lysin activity that acts as a primary bactericidin, where lysozyme is an important module of the immune system emitted by tissue macrophages with major anti-gram-positive-bactericidal activity through hydrolyses the β-1,4-glycosidic bond in cell wall peptidoglycans using intracellular enzymatic digestion. Its activities are allied to phagocytic cells, monocytes, and granulocytes **[119]**. The significant increase in Ca levels in Lf treated G4 and G5 may be attributed to many hypotheses including the pivotal role of Lf high-affinity intelectin-1 receptor that participates in Ca metabolism and signaling, promotes immune functions, involves in hepatocyte lipoproteins uptake, and several cellular processes **[120]**. The reduction of intracellular Ca2+ levels suppresses phagocytosis and

assemblies of inflammatory mediators **[121]**. Also, Lf alleviated rabbit osteomyelitis **[92]**, and osteoporosis by improving bone growth through its anabolic properties and metabolic processes **[122, 28]**, besides increased bone mineral density and calcitonin post-treatment **[73]**. Furthermore, Lf advances the intestinal barrier function and normalizes colon microbiota in addition to superior the expression of colon vitamin D receptors that referee all biological actions of vitamin D and guarantees intestinal homeostasis **[26]**.

In another study **Fisher et al., [123]** verified that significant levels of the exogenous Lf were recorded in different organs, such as the liver, gall bladder, kidneys, and spleen signifying that it is efficiently delivered safely to the entire body without any side effects. Parallel to this study, **Manzoni et al., [124]** mentioned that no adverse effects were reported from daily doses of 100 to 3000 mg Lf for 1 year regarding safety parameters (mortality, liver enzymes, hematological indices, and body weight). Moreover, several studies **[125-126]** reported that Lf is classified as a "generally recognized as safe" (GRAS) substance and several tests in animals have proven its safety and tolerability, even at high doses. The authors reported a significant decrease in levels of AST, ALT, and ALP and considered these hepatic changes as possibly indicative of improved liver function. Likewise, a decrease in BUN was considered to be possibly associated with Lf-related protective effect on kidney function. As well, Lf can also serve to prevent nephrotoxicity, **[127]**. **Flores-Villaseñor et al., [128]** assessed that Lf and its peptide effectively reduced sepsis, hepatic and renal damage caused by infection, and confirmed these findings histologically besides bacterial count where bacteria not detected in the kidney or liver after 72 h. Similar studies **[129-130]** examined the renoprotective effect of Lf against induced renal tubular damage, acute or chronic kidney diseases and found that Lf reduced elevated BUN and creatinine levels and protected renal tissue histologically. Authors attributed these positive renal impacts to its functional bioactivities in maintaining the antioxidant enzyme activities, scavenging radicals, and augmenting autophagy besides its antiproliferative and anti-inflammatory effects. For illuminating Lf-interacting molecules after its oral administration, **Hsu et al., [129]** found that Lf is expressed in various organs, especially kidneys where the high levels of Lf mRNA and protein. It has an antifibrotic role in kidney tubular cells that recovered renal function in an *in vivo* study, where fibrosis considers the final result of chronic inflammatory reactions stimulated by persistent infections.

5. Conclusions

Enterotoxaemia is the most frequent problem in rabbit husbandry, particularly during the post-weaning period. Hence, seeking new promising natural, safe, active, and available alternative therapeutics is important in their *in vivo* fight. This study assessed the therapeutic and utility potential of Lf glycoprotein alone or in combination with BMD in the treatment of *C. perfringens* type A infection in rabbits. The used drugs revealed a significant *in vitro* and *in vivo* antibacterial effect besides recovering most of the detected parameters in various degrees to normal levels by a significant increase in TP, albumin, globulin, lysozyme activity and Ca, besides a significant reduction in ALT AST, ALP, LDH, BUN and creatinine levels than infected control group. Hence, Lf could be used effectively as a substitute or adjunct therapy to BMD. The treated group with both Lf and the half dose of BMD revealed the best long-lasting curative antibacterial effect; it showed the lowest *C. perfringens* count in the intestine along with the absence of liver systemic infection, besides their proper effects on the hepatic and renal functions than either Lf or BMD alone. Hence, Lf increased the bacterial susceptibility to BMD besides its antibacterial effect; targeted different mechanisms of antibacterial activity simultaneously further its lysozyme activation property that lastly led to its synergy with bacitracin. Based on this, combination therapy might help in increasing the cure rate, preventing chronic infection, decreasing illness severity and bacterial count along with providing an attractive therapeutic choice to decrease the risk of severe cases of infection. More research is eventually needed to better understand the pharmacological potentials of Lf against other infective cause or antibiotic combinations and their systematic evaluations, as they provide new directions to combat pathogens to optimal *in vivo* therapeutic use.

6. Conflicts of interest

"There are no conflicts to declare".

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