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Role of Saltose Probiotic for the Control of the Experimental Infection of the *Clostridium Perfringens* and the *Coccidia* in Chickens

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Key words

Saltose Probiotic, *Clostridium Perfringens*, *Coccidia*, Chickens

ABSTRACT:

Two hundred and forty, one day old broiler chicken were randomly divided into eight equal groups. The 1st group was control. The 2nd group was given saltose from the 1st day of age till the end of experiment at a dose 1gm / kg of ration. The 3rd group was infected intra crop with inoculums containing $0.5x10^9$ cfu/ ml *Clostridium perfringens* at 15 days of age .The 4th group infected intra crop with 75000 sporulated oocyte of *Eimeria necatrix* at 15 days of age .The 5th group was infected with inoculums containing $0.5x10^9$ cfu/ml *Clostridium perfringens* and 75000 sporulated oocyts of *Eimeria necatrix* intra crop at 15 days of age. The 6th group given saltose from the 1st day and infected with *Clostridium perfringens* as in group3. The 7th group was given saltose as in group 2 and infected with *Eimeria necatrix* as in the 4th group. The 8th group was given saltose as in group 2 and infected with *Clostridium perfringens* and *Eimeria necatrix* as in group 5. Three blood samples were collected from all groups at 21 days and at 28 days of age. The 1st blood sample was collected with anticoagulant for RBCs and differential leucocytic count. The 2nd blood sample was collected without anticoagulant for serum separation for measuring ALT, AST and alkaline phosphatase and IgA. The 3rd blood sample was collected by heart puncture under strict aseptic condition on heparin for immunological examination. Necropsy was performed and the intestinal tracts of sacrificed chickens of all challenged groups (groups 3, 5, 6, 8) at 7, 14 and 21 days post challenged were collected for bacterial count and for DNA analysis by PCR to recognize the enterotoxin gene that produced during sporulation and demonstrate the efficacy of Saltose on the cell wall microorganism . Also tissue specimens were collected from the intestine (small, large and ceci), liver, kidneys, heart and spleen, fixed in 10% buffered neutral formalin for Pathological examination.

The results showed that the probiotic Saltose reduced the severity of the necrotic enteritis due to *Clostridium perfringens* infection through the intestinal bacterial count, DNA analysis by PCR, hematological, biochemical and histopathological findings, also alleviated the pathological changes demonstrated by *Eimeria necatrix* infestation.

These results imply that the Saltose based probiotic was able to reduce the severities of necrotic enteritis (NE) and ameliorate the pathological changes associated with coccidiosis.

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1. INTRODUCTION

Necrotic enteritis (NE) in broilers is caused by type A strains of *Clostridium perfringens* (CP) that are specific to poultry with the major toxin type being alpha toxin and recent evidence suggests the involvement of a novel toxin called NetB **(**Keyburn, *et al.*,2008; Sherry, *et al.*,2013). *Clostridium perfringens* is ubiquitously found in the environment and is a Gram positive, anaerobic, spore-forming bacterium. Clinical signs of NE include rapid loss in performance, in appetence, severe intestinal damage and are often associated with high mortality (Timbermont et al.2011). Normally, healthy birds harbor a significant number of *Clostridium perfringens* in their intestinal tract. At low population levels (< 10^4cfu) the organism is non-pathogenic. The pathogenicity of the organism is associated with several toxins. Pathogenesis of the infection is determined by enterotoxins produced by *C. perfringens* strains of type A (CPE strains) (Sawires and Songer, 2006). An enterotoxin

(CPE) relates to food poisoning and is produced during sporulation in the infected host intestine, where it binds to the intestinal epithelium, forms pores and causes diarrhea (Lindstrom et al. 2011).The major lethal effects associated with α-toxin are necrotic enteritis and enterotoxaemia in animals (Siragusa et al. 2006). α-toxin (phospholipase C), can hydrolyze lecithin into phosphoryl choline and diglyceride, which leads to tissue damage (Smedley III. et al. 2004). Under specific abnormal conditions, the bacteria are able to colonize and secrete increased amounts of toxins leading to necrosis of the gut mucosa (Sheedy et al.2004 and Sherry et al.2013).The actual mechanisms of *Clostridium perfringens* pathogenesis are not well understood at this point of time. However, it is widely understood that a coccidial infection is the most pre-requisite for NE to occur. Damage to the intestinal mucosa is an important factor for *Clostridium perfringens* intestinal colonization and the presence of a coccidial infection is probably the most common causative factor facilitating *Clostridium perfringens* pathogenesis (McReynolds et al. 2004). Intestinal damage will result in the release of plasma proteins into the lumen of the intestinal tract. Because the minimal requirements for growth of *Clostridium perfringens* include more than 11 amino acids and several growth factors, leaking of plasma into the intestinal lumen can provide a necessary growth substrate for extensive proliferation of these bacteria (Dahiya et al.2006 and Sathishkumar et al.2013).

Avian coccidiosis is a parasitic disease of intestinal tract caused by single cell protozoan parasite belonging to genus *Eimeria.* It causes massive destruction of epithelial cells, which leads to bloody diarrhea, reduced weight gain and temporary reduction in egg production. Seven species have been recognized to infect poultry and each specie has its own characteristics according to site of infection, immunogenicity and pathogenicity (Sabiqaa, *et al.*,2013). The protozoan parasite of the genus *Eimeria* multiplies in the intestinal tract and causes tissue damage, resulting in the interruption of feeding, digestive processes, nutrient absorption, dehydration, blood loss, loss of skin pigmentation and increased susceptibility to other disease pathogens . Nine different species of coccidian are known: seven *Eimeria* occur in chicken—namely, *E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox* and *E. tenella* (Conway and Mckenzie, 2007).

Recent evidence that various dietary and microbial supplements can influence host immunity against enteric disease prompted us to investigate the role of a commercial probiotic on coccidiosis. This probiotic consists of live *Pediococcus acidilactici,* which belongs to the homofermentative gram-positive bacteria, able to grow in a wide range of PH, temperatures and osmotic pressures, and thus able to colonize and inhabit the digestive tract. Some commercial bacteria have been found to enhance development of both the intestinal epithelia and the gastrointestinal lymphoid system. A balanced microbial population would support the inherent defense mechanisms of a healthy intestinal tract, resulting in better control of intestinal pathogens (Pollmann *et al*.,2005 and Lee, *et al*., 2007). Probiotics play an important role in stabilizing the intestinal ecosystem of animals by enhancing nutrient digestibility (Apata, 2008), increasing performance (Kabir,*et al.*,2004) and competing with pathogenic bacteria in the intestine (Higgins, *et al.,* 2008; Vicente, *et al.*,2008 and Jerzsele, *et al.*,2012). Administration of bacteria belonging to the *Bacillus* genus have beneficial effects in several conditions, like enteritis caused by *Escherichia coli, Salmonella enterica* or *Clostridium perfringens*. Decreased *C. perfringens* colonization and lower incidence of NE in chickens treated with bacteria belonging to the normal gut flora were reported by (Barbosa et al.,2005; Jerzsele, *et al.*,2012). Saltose is one of leading probiotics which contains *Bacillus* group and unique enzymes. Saltose can prevent and treat the bacterial and parasitic diseases with the characteristics of used bacteria and enzymes, and improve the productivity for poultry and livestock

and aquaculture. Ingredients and characteristic Saltose have selected 5 strains of *Bacillus* group among more than ten thousand strains of *Bacillus* group which do not produce antibiotics, but produce unique enzymes which can break down the cell wall of bacteria and oocysts of some important parasites (PIC-BIO, 2013). Nonpathogenic and nontoxic bacterium *Pediococcus acidilactici* induce healthy intestinal conditions in pigs and may interfere with the pathogen infection sites, produce antimicrobial peptides, or induce host immune responses, thus enhancing its resistance to enteric pathogens like *Eimeria* (Guerra, et al.,2006;Lee. *et al.*, 2007).

The present work was conducted to investigate the potential protective effects of the probiotic Saltose in broiler chickens experimentally infected with *Clostridium perfringens* and *Eimeria necatrix* separately and both.

1. MATERIALS AND METHODS

Drug :-

Saltose®: Patent product produced by Poultry Industry Consultant Company **(PIC-BIO, Inc. JAPAN, 2013)** Gosaku Bld., 1-29-2 Nishigotanda, Shinagawa-ku, Tokyo, 141-0031, Japan, and distributed by Elyoser Medicine trading Company, Egypt.

Saltose composed of:- Cell Wall Lyase (patent new enzyme) 3,700 U/g *bacillus licheniformis, Bacillus subtilis, Bacillus pumilus* 1.8 x 0^9 cfu/g, *Enterococcus faecalis, Enterococcus faecium* 2.5 x 0^8 cfu/g, Protease, Lipase, Cellulase, Amylase 12,000 U/g, and Beta-Xylanase 350 U/g

Bacterial strain:

Clostridium perfringens strain was obtained kindly from Microbiology Department -Faculty of Veterinary Medicine, Cairo University.

***Eimeria necatrix* species:**

Sporulated oocyte of *Eimeria necatrix* was obtained from Poultry Department- Faculty of Veterinary Medicine, Cairo University.

Oocysts preparation of *Eimeria necatrix*:-

Oocysts were collected directly from the infected birds, scraping were made from the lesions and rinsed into a beaker with potassium dichromate solution (2.5%) to release the unsporulated oocysts, then oocysts were stored at 4°c. Oocysts must undergo sporulation before they are infective. The collected oocysts washed by distilled water 3-4 times and centrifuged on 3000 rpm for 10 minutes to remove the potassium dichromate. The oocysts were counted using the hemocytometer method (Reid, 1978).

Experimental design:-

Chickens: Two hundred and forty, one day old broiler chicken were fed balanced rations *ad libitum* with free access to water for 40 days of age and divided into eight equal groups. The first group was control, The second group was given saltose from the 1st day of age till the end of experiment by a dose 1gm / kg of ration. The third group was infected with inoculums containing $0.5x10^9$ cfu/ ml *Clostridium perfringens* intra crop at 15 days of age (Atta *et al*.,2014). The fourth infected intra crop with 75000 sporulated oocyte of *Eimeria necatrix* at 15 days of age (Hamidi *et al*., 2010).The fifth group was infected with $0.5x10^9$ cfu/ ml *Clostridium perfringens* and inoculums containing 75000 sporulated oocyte of *Eimeria necatrix* intra crop at 15 days of age. The six group given saltose from the 1st day and infected with *Clostridium perfringens* as in group 3.The seventh group was given saltose as in group 2 and infected with *Eimeria necatrix* as in the 4th group. The eighth group treated with saltose as in group 2 and infected with *Clostridium perfringens perfringens* and *Eimeria necatrix* in group 5

Vaccination:-

All groups were vaccinated against Newcastle disease with Hitchiner B1 at 7days and LaSota at 21 days of age. Also all groups were vaccinated against Gumboro disease (IBD) at 13 day of age .

Sampling :

Three blood samples were collected from all groups at 21 days and at 28 days of age . The **1st** blood sample was collected with anticoagulant for RBCs and differential leucocytic count. The **2nd** blood sample was collected without anticoagulant for serum separation for measuring ALT, AST and alkaline phosphatase and IgA. The **3rd** blood sample was collected by heart

puncture under strict aseptic condition on heparin for immunological examination. Necropsy was performed at the end of experimental period and tissue specimens from the intestine (small, large and ceci), liver, kidneys, heart and spleen were collected and fixed in neutral buffered formalin 10% for histopathological examination and another tissue specimens from intestine were collected for bacterial count and DNA analysis by PCR.

Laboratory examinations

Hematological examinations:- Blood samples were collected from all groups for RBCs count according to **(Coles,1986).** Blood smear were stained with wright'stain for differential leucocytic count and absolute values were calculated according to (Schalm 1975).

Biochemical analysis:- Aspartate and alanine amino transferase (ALT and AST) activities were determined colorimetrically according to (Reitman and Frankel 1957). Serum alkaline phosphatase activity was determined according to (Kind and King 1954).

Immunological assay :- 1- Lymphocytes transformation test (LTT) according to (Nariuchi,1989) 2-Determination of serum antibody titre*:* a goat anti chicken IgA ELISA kit (Bethylco, E30-130) was used to quantify the IgA content of serum (Lowry *et al*., 1951).

Microbiological methods:

Challenge *C. Perfringens* strain: Toxigenic strain of *C. Perfringens* type (A) was kindly obtained from Microbiology Department, Faculty of Veterinary Medicine. Cairo University. That strain of *C. Perfringens* was isolated from broiler chickens flock suffered from NE. The organism was anaerobically cultured on 10% sheep blood agar media containing 200ug/ml neomycin sulphate incubated in Gaspack anaerobic jar at 37C for 24 hours. Culture was centrifuged at 1000 r.p.m. for 10 minutes and the bacterial concentration of the culture was adjusted to a turbidity of opacity tube to 10^9colony forming units (CFU) /ml. Chickens were orally inoculated with 0.5 ml of *C. perfringens* both culture at 14 days of age (Dahiya et al., 2005).

Isolation and detection of *C.perfringenes*, according to (Willis 1977), the collected samples were inoculated into tubes of freshly prepared boiled and cooled cooked meat medium (Oxoid) and incubated anaerobically for 24 hours at 37 ^{0}c. A loopful of inoculated fluid medium was streaked onto neomycin sulphate sheep blood agar plate (Cruickshank, *et al.*,1975), and incubate anaerobically at 37 ^{0}c for 24 hours using Gaspack anaerobic jar (Brewer, and Allgeier 1966). Suspected *C. perfringens* colonies were cultured onto 2 plates of sheep blood agar and egg yolk agar. One plate was incubated aerobically and the other plate was incubated anaerobically. The colonies that grew only in anaerobic condition and lecithinase producer and showed double zone of hemolysis on blood agar were picked up and purified for identification tests (Cruickshank, *et al.*,1975 and Koneman, *et al*.,1988). Isolated colonies with a typical appearance were then biochemically tested by using a commercial biochemical panel kit (API 20 A, Bio Mérieux). All isolated strains were stored in a cooked meat medium (Oxoid, Basingstoke, UK) at –70 °C.

Count of *C.perfringenes:*

The intestinal tract of sacrificed birds of each group at 7, 14 and 21 days post challenge were collected for bacterial count (Cruickshank, *et al*., 1975). About 1-2 grams from the intestinal contents of three birds from each group were pooled. The samples were diluted in buffered peptone water to an initial 10^{-1} dilution. Ten fold serial dilutions was spread in duplicate on blood agar base containing 10%sheep blood with 200ug/ml neomycin sulphate for enumeration of *C. Perfringens*. All the plates were incubated in Gaspack anaerobic jar at 37C for 48 hours. Hemolytic colonies on blood agar plates (colonies with a typical double zone of haemolysis) were counted and and expressed as log10 CFU/g of intestinal or caecal contents. Isolated colonies were then biochemically tested by API 20A, Gram stained and microscopically examined to be confermed as C. Perfringens. All isolated strains

were stored in a cooked meat medium (Oxoid, Basingstoke, UK) at –70 °C.

Extraction of DNA from *Clostridium perfringens* isolates according to (Liu *et al*., 2002):-The bacteria were grown on *blood agar* agar plates at 37°C and up to 100 *colonies from the plates were combined. The DNA for PCR analyses was obtained by* suspending colonies of bacteria in 500 µl of PBS, pH 7.2, then washing 3 times in PBS. The cell suspension was centrifuged for 10 min at 14,000 xg .The supernatant was discarded carefully. The bacterial pellets were resuspended in 400 µ l Tris-EDTA buffer (pH 8.0) and extract bacterial DNA by phenol –chloroform method .The supernatant was transferred to a fresh tube with double volume absolute ethanol and 0.1volume 3M sodium acetate (pH 5.2) and the test tubes were kept at -20^0C for overnight. The DNA was pelleted by centrifugation at 14.000xg/ minute for 20 minutes, followed by washing with 70% ethanol and re-centrifugation at 14.000 xg /minute for 10 minutes. The DNA pellet was dried and resuspended in 20 µl sterile distilled water and stored at -20^0C till use.

Estimation of purity and concentrating of the DNA:-The concentration and purity of the DNA that had been extracted were determined by estimating the optical density at a wave length of 260 and 280nm using the spectrophotometer. The concentration was calculated as follows:

1OD. 260 nm = 50 ug/ml

The purity of DNA = OD. At 260/OD. At 280 nm.

The purity of DNA had a value of 1.8 where the optimum ranged between 1.8 – 2.

DNA amplification PCR for the *Clostridium perfringens* The amplified reactions were performed in 50 ul volumes in micro-amplification tubes (PCR tubes). The reaction mixture consisted of (DNA template (30-50ng) (5µl), Taq DNA polymerase (2.5 U/ul) (1.54 ul), 10X reaction buffer (5ul), 25mM dNTPs(4ul), 25mM MgCL2 (5 ul), Primers (2ul) for each. The mixture was placed in the thermal cycler and PCR amplification was carried out using the following cycling condition: Initial denaturation 93°C for 2 min, amplification (35 cycles of), denaturation 93°C for 30 sec, annealing 50ºC for 30 sec, Extension 72°C for 1 min and final extension phase 72°C for 10 min. The PCR products were stored in the thermal cycler at 4ºC until used.

Screening of PCR products by agarose gel electrophoresis according to the method of (Sambrook *et al.*, 1989):-Two grams agarose was added to 100 ml Tris acetate EDTA (TAE) buffer. The agarose was autoclaved for 10 minutes and 0.5 µg/ml ethidium bromide was added and then left to cool to room temperature. The gel tray was tapped and the warm agarose was poured in. The comb was inserted in the agarose which was left to polymerize. After hardening, the tray was untapped, the comb was removed and the gel was applied to the electrophoresis cell. The cell was filled with TAE buffer. 10 µl of each of the PCR product samples were applied to the gel along with 5 µl molecular weight marker after mixing each with 1µl loading buffer on a piece of par film. Each mixture was applied to a slot using 10µl micropipette. The electrophoresis cell was covered and the power supply was adjusted at 10 Volt/cm. The gel was taken out from the cell and examined under short wave UV *using digital camera (Acer CR-5130, China).*

Oligonucleotide primers used for amplification of the DNA recovered from *C. perfringens* isolates The sequence, specificities, the primer combination and the size and length of the amplified products were summarized according to (Baums *et al.*, 2004) in Table (1).

Pathological Examination:-

Specimens from the intestine (small, large and ceci), liver, kidneys, heart and spleen were collected and fixed in 10% buffered neutral formalin solution, dehydrated in gradual ethanol (70-100%), cleared in xylene, and embedded in paraffin. Five-micron thick paraffin sections were prepared and then routinely stained with

hematoxylin and eosin (H&E) dyes (Suvarna, *et al*., 2013) and then examined microscopically.

Table (1) Oligonucleotide primers used for amplification of toxin genes of targeted products *C. perfringens*

Primer name	Sequence		Amplification product
Alpha toxin	F	5'-TGG CTAATGTTACTGCCGTTGATAG-3	324 bp
	R	5 ' -ATAATCCCAATCATCCCAACT ATG-3	
Beta toxin	F	5'-AGGAG GTTTTTTTATGAAG-3'	196 bp
	R	5'-TCTAAA TAGCTGTTACTTTGT-3'	
Epsilon toxin	F	5'-TACTCATACTGTG GGAACTTCGATACAAGC-3'	655 bp
	R	5'-CT CATCTCCCATAACTGCACTATAAT TTCC-3'	
Cpe(enterotoxin)	F	GGG GAA CCC TCA GTA GTT TCA	506 bp
	R	ACC AGC TGG ATT TGA GTT TAA TG	

3. RESULTS

The present results of *C. perfringens* count in intestine of sacrificed birds at 7,14,and 21 days post challenge in (table 2) revealed that significant decrease in treated groups(6,8) rather than control positive non treated groups(3,5). However, chicken treated with saltose and infected with *C. perfringens* only showed statistically the lowest intestinal count. The re-isolated strain of C. *perfringens* from the intestine post-experimental infection showed complete identity with the inoculated C.*perfringens* as demonstrated in Fig. (A). *C. perfringens* was identified by analysis of its toxin using PCR to assure the experimental infection and to confirm the efficacy of Saltose as a probiotic on the incidence of *C. perfringens* and on releasing enterotoxin gene cpe which release during sporulation. In Fig. (B) Lanes 3, 4, 5, 7 and 10: Positive *C. perfringens* for enterotoxin gene (cpe). Lanes 6, 8, 9 and 11: Negative C. perfringens for enterotoxin .

The results of this work showed significant decrease in RBCs count in the 3rd, 4th and 5th groups during the 1st week after infection and in the 4th and 5th groups during the 2nd week after infection but the other groups showed non-significant changes (table 3) .Differential leucocytic count revealed significant eosinophilia in groups 4,5,7 and 8 in the 1st week after infection and in group 3 in the 2nd week . Lymphocytosis appear in groups 2,3,4 and 5 in the 1ST week (table 4,5) .In serum analysis in this work showed significant decrease in level of ALT enzyme in groups 3,4,5 and 8 in the 2nd week while AST and Alkaline phosphatase showed non-significant changes in the 1st and 2nd week after infection (table 6,7). Lymphocyte transformation test (LTT) revealed significant increase in group 4 and 8 in the 1st week and in group 4 and 6 in the 2nd week but the others showed non-significant changes (table 8). IgA level showed significant increase in group 4 and 5 (table 9).

Table (2): The effect of Saltose on *C. perfringens* intestinal count in infected with *C. perfringens* and infected with *C. perfringens* and coccidia (log_{10} CFU/g of intestinal content):

Weeks / Groups	1st week after infection	2nd week after infection	3rd week after infection
Clostridium	$12\times10^7\pm200.67$a	$15\times10^7\pm288.67$a	$6\times10^8\pm267.88$a
Saltose+ Clostridium	$5\times10^5\pm52.70$ d	$4\times10^4\pm57.73$ d	$2\times10^4\pm50.70$ d
Coccidia+ clostridium	$11\times10^8\pm287.6$b	$14\times10^9\pm200.7$b	$7\times10^9\pm288.67$b
saltose + coccidia + clostridium	$14\times10^6\pm208.6$ c	$5\times10^6\pm277.67$ c	$5\times10^5\pm288.8$ c

Non infected, non treated.	0	0	0

Values are means ± standard error. Mean values with different letters at the same column differ significantly at ($p \geq 0.05$).

Table(3): Results of RBCs count in all experimental groups

parameters / Groups	RBCs 10^6.mL	
	1st week after infection	2nd week after infection
control	1.58±0.17a	1.58±0.13a
saltose	1.55±0.21a	1.65±0.30a
clostridium	1.26±0.13b	1.35±0.13a
coccidia	1.25±0.13b	1.35±0.21b
coccidian + clostridium	1.27±0.15b	1.35±0.13b
saltose + clostridium	1.54±0.23a	1.56±0.13ab
saltose + coccidia	1.40±0.14ab	1.45±0.12ab
saltose+coccidia+ clostridium	1.63±0.17a	1.55±0.13ab

Values are means ± standard error. Mean values with different letters at the same column differ significantly at ($p < 0.05$).

Table (4): Results of differential leuckocytic counts in all experimental groups in the 1st week after infection

Parameters / Groups	Differential leukocytic count				
	heterophil%	eosinophil %	basophil%	Monocytes%	Lymphocytes%
control	43.40±5.00a	9.88±1.14b	5.08±1.48a	14.88±2.11a	22.7±2.71b
saltose	42.78±1.98a	11.80±2.45ab	3.73±1.73a	11.90±6.88a	34.4±4.97a
clostridium	43.73±3.90a	11.15±1.89ab	5.45±4.0a	12.75±6.89a	34.79±4.19a
coccidia	41.71±3.22a	14.60±2.45a	4.57±0.74a	16.20±2.26a	35.13±5.57a
coccidian + clostridium	45.08±4.09a	15.10±2.24a	5.49±3.29a	13.13±2.12a	34.8±7.9a
saltose + clostridium	41.06±2.55a	13.62±2.75ab	5.23±2.59a	14.86±1.75a	32.68±2.25ab
saltose + coccidia	41.87±5.96a	15.11±1.51a	3.83±1.05a	14.08±3.27a	28.68±4.63ab
saltose + coccidia + clostridium	41.45±7.69a	15.90±2.25a	6.18±2.23a	12.97±1.44a	29.67±4.62ab

Values are means ± standard error. Mean values with different letters at the same column differ significantly at ($p < 0.05$).

Table (5): Results of differential leuckocytic counts in all experimental groups in the 2nd week after infection

parameters / Groups	Differential leukocytic count				
	heterophil%	esinophil%	basophil%	Monocytes%	Lymphocytes%
control	41.83±5.99a	10.18±2.29b	3.98±1.30a	13.80±2.57a	24.03±2.73a
saltose	37.88±7.79a	11.6±4.31ab	5.00±1.4a	14.1±3.76a	27.78±2.91a
clostridium	42.30±9.05a	10.3±1.82b	6.57±1.55a	14.95±4.22a	23.85±4.24a
coccidia	41.40±2.78a	11.15±1.95ab	3.38±0.83a	14.37±0.48a	24.98±1.06a
coccidian + clostridium	45.10±3.29a	14.0±3.66a	2.65±0.55a	13.25±3.9a	24.11±1.45a
saltose + clostridium	41.10±5.73a	9.2±2.29b	3.98±1.75a	12.8±1.92a	25.80±4.53a
saltose + coccidia	39.67±5.79a	10.85±2.69b	5.25±1.28a	14.38±1.28a	26.80±4.98a
saltose + coccidia + clostridium	44.00±1.69a	11.78±2.35ab	4.63±0.65a	14.45±1.97a	26.90±7.85a

Values are means ± standard error. Mean values with different letters at the same column differ significantly at ($p < 0.05$).

Table (6): Results of some blood serum enzyme activities in all experimental groups in the 1st week after infection

Parameters / Groups	Enzyme		
	ALT	AST	ALP
control	50.25±11.62a	65.00±17.17a	1723.13±882.76a
saltose	51.25±5.62a	67.75±18.5a	3170.55±795.44a
coccidia	54.50±11.47a	62.25±12.31a	3101.62±611.32a
clostridium	55..00±14.66a	59.25±17.25a	1723.13±470.48a
coccidian + clostridium	46.50±4.35a	67.25±6.94a	2998.75±660.66a
saltose + clostridium	42.75±12.91a	59.25±14.61a	1723.40±911.33a

saltose + coccidia	47.75±9.39a	73.25±15.45a	1825.93±557.55a
saltose + coccidia + clostridium	48.25±7.14a	70.75±14.56a	2205.60±669.67a

Values are means ± standard error. Mean values with different letters at the same column differ significantly at ($p < 0.05$).

Table (7): Results of blood serum enzyme activities in all experimental groups in the 2nd week after infection

Parameters / Groups	Enzyme		
	ALT	AST	ALP
control	44.40±8.67a	69.00±22.50a	772.5±159.06a
saltose	36.00±8.50ab	71.00±26.90a	992.52±314.34a
clostridium	33.80±5.22b	90.60±34.62a	827.1±194.94a
coccidia	35.00±4.84b	71.00±15.54a	827.1±137.23a
coccidian+clostridium	34.00±5.15b	71.20±19.41a	883.04±229.25a
saltose + clostridium	39.02±7.01ab	71.20±18.67a	607.8±201.23a
saltose + coccidia	36.20±7.94ab	59.80±16.02a	992.52±404.43a
saltose + coccidia + clostridium	31.40±2.79b	70.40±23.76a	551.34±237.64a

Values are means ± standard error. Mean values with different letters at the same column differ significantly at ($p < 0.05$).

Table (8): Results of lymphocyte transformation test(LTT) in all experimental groups

Parameters / Groups	Lymphocyte transformation test	
	1st week after infection	2nd week after infection
control	54.40±5.75b	58.90±3.21b
saltose	59.93±5.82ab	59.10±4.94ab
clostridium	57.60±2.88ab	57.15±1.58b
coccidia	61.30±4.38a	62.53±2.55a
coccidian + clostridium	56.50±3.57ab	59.04±5.85ab
saltose + clostridium	59.26±3.59ab	61.15±2.33a
saltose + coccidia	57.12±3.68ab	59.38±2.84ab
saltose + coccidia + clostridium	62.80±5.19a	58.85±3.88ab

Values are means ± standard error. Mean values with different letters at the same column differ significantly at ($p < 0.05$).

Table (9): Results of blood serum IgA concentration (gL^{-1})

Parameters / Groups	IgA (gL^{-1})	
	1st week after infection	2nd week after infection
control	0.17±0.02b	0.16±0.02b
saltose	0.18±0.01ab	0.19±0.02ab
clostridium	0.19±0.02ab	0.15±0.02b
coccidia	0.20±0.01a	0.17±0.01ab
coccidian + clostridium	0.20±0.02a	0.19±0.01a
saltose + clostridium	0.18±0.02ab	0.18±0.03ab
saltose + coccidia	0.18±0.02ab	0.18±0.03ab
saltose + coccidia + clostridium	0.16±0.03b	0.18±0.03ab

Values are means ± standard error. Mean values with different letters at the same column differ significantly at ($p < 0.05$).

Pathological Findings:-

The examined organs (intestine, liver, kidneys, heart and spleen) of **control** "non-infected, non-treated" were normal with no evidence of microscopic abnormalities. However, the intestine of chickens only received saltose (probiotic) showed increased the absorptive area which represented by change the finger-like villi

to leaf or wave-like types with increased numbers of goblet cells (Fig 1). Spleen showed moderate hyperplasia in the lymphoid cells of white pulp (Fig 2). The other organs were normal. The intestine of chickens infected with clostridium showed extensive mucosal necrosis (coagulative type) in small intestine with huge numbers of leukocytes infiltrations mostly of heterophils and macrophages (Figs 3 and 4). The ceci were rarely affected and showed mucosal and lymphoid necrosis. The liver revealed severe congestion of hepatoportal blood vessels and sinusoids (Fig 5). Multiple areas of coagulative necrosis infiltrated with macrophages and few heterophils were detected throughout the hepatic parenchyma (Fig 6). Other areas showed empty cavities or contained necrotic debris and surrounded by thick zone necrotic cells and leukocytes infiltrations. The contagious portal areas were heavily infiltrated with heterophils (Fig 7). Sometimes, perihepatitis of necrosis and round cells infiltrations besides few basophilic bacterial colonies were visualized (Fig 8). In some cases, the portal areas were widened with chronic cholangitis. The latter was represented by hyperplastic epithelium, surrounded by fibrous connective tissue and numerous mononuclears (Fig 9). The kidneys revealed mild lesions of congestion and vacuolation of renal tubular epithelium (Fig 10). Some renal tubules showed coagulative necrosis in the tubular epithelium which represented by pyknosis and karyorrhexis (Fig 11). The heart showed few heterophils among the cardiac myocytes. Focal areas of Zenker's degeneration and rarely necrosis were seen. The spleen showed depletion of the lymphoid tissue of white pulp. Few subcapsular heterophils infiltrations were observed. The intestine (ceci) of chickens infected with Eimeria revealed severe necrosis, destruction and desquamation of the lining epithelium with the presence of developmental stages of Eimeria including mature schizonts and gamonts in the enterocytes and lumens with extensive extravasated erythrocytes (Figs 12 and 13). Sometimes, the necrotic mucosa was heavily infiltrated or replaced with lymphocytes and few heterophils besides hyalinization of the muscular coat. Hemorrhages, edema and necrosis were seen in the submucosa particularly near the muscular layer. Almost all the epithelial lining of the large intestine mucosa were invaded with different developmental stages of Eimeria (Fig 14). The liver showed periportal vacuolation in the hepatocytes (Fig 15) and aggregation of round cells mostly lymphocytes. The portal areas showed severe hyperplastic bile ducts, round cells aggregation and thickening in the wall of hepatic arteriole (Fig 16).The kidneys revealed few heterophils and lymphocytes infiltrations among the renal tubules with individual cell necrosis (Fig 17). The heart was normal except for mild interstitial edema among the myocytes. The spleen was normal except for mild hyperplasia in lymphoid follicles of white pulp. Few brown pigments of hemosiderosis were noticed. The chickens infected with both clostridium and Eimeria showed severe lesions more than each alone. The intestine showed the developmental stages of Eimeria in the enterocytes of the ceci and large intestine (Fig 18). Extensive necrosis was noticed in the upper parts of small intestine with hemorrhage and round cells infiltrations. Sometimes, the mucosa was replaced by round cells and few erythrocytes (Fig 19). The liver revealed extensive coagulative necrosis at the areas of portal areas with thick and hyalinized hepatic arterioles. Sometimes, the necrotic areas showed central cavitations containing necrotic debris (Fig 20). The adjacent portal areas were heavily infiltrated with round cells and few heterophils (Fig 21). The bile ducts were hyperplastic. The kidneys showed congestion of peritubular capillaries (Fig 22) and focal necrosis in the renal tubular epithelium. The heart was normal except for few vacuolation in the cardiac myocytes. The spleen showed proliferated lymphocytes around the thickened splenic arterioles and depleted at the margins.

The supplementation of saltose to these infections (clostridium, Eimeria and both) was lowered the induced lesions and alleviated the

developmental stages of Eimeria in ceci and large intestine. The intestine of chickens infected with clostridium and supplemented with saltose showed normal mucosa with finger-like villi and the submucosa showed aggregation of round cells with no evidence of necrosis (Fig 23). Focal areas revealed villi with round tips and hyperplastic lining epithelium. The submucosa of these villi showed masses of hyperplastic cells with round vesicular nuclei and few extravasated erythrocytes (Fig 24). The liver showed mild hydropic degeneration and vacuolation besides heterophils infiltrations in the portal areas (Fig 25). The kidneys showed regenerative changes in the renal tubular epithelial cells. Such cells were large with vesicular hyperchromatic nuclei and scanty basophilic cytoplasm (Fig 26). The glomerular tufts were slightly hyperplastic. The heart was normal. The spleen showed mild hyperplasia in the lymphocytes of white pulp(Figs 27). Meanwhile, the ceci and large intestines of chickens infected with Eimeria and supplemented with saltose showed few developmental stages of Eimeria with intact epithelial lining and round cells infiltrations (Figs 28). Focal hyperplasia in the lining epithelium particularly with gamonts was visualized. Hemorrhage was rarely detected. The liver revealed portal areas with huge numbers of round cells and hyperplasia in the bile ductal epithelium. These ducts contained granular eosinophilic material (Fig 29). The kidneys, heart and spleen were normal. The intestine (small, large and ceci) of chickens infected with both clostridium and Eimeria and supplemented with saltose showed areas of necrosis infiltrated with mononuclears and hetrophils. The ceci revealed areas of proliferation of fibrous tissue infiltrated with round cells and few degenerated developmental stages in the lining epithelium. The small intestine showed extensive aggregation of round cells among the intestinal villi without necrosis (Fig 30). Edema was noticed in the submucosa. The liver was nearly normal without any necrosis throughout the hepatic parenchyma. Slight congestion in hepatoportal blood vessels and mild hydropic degeneration were recorded. Some portal areas showed chronic cholangitis with biliary epithelial hyperplasia, fibroblast proliferation and round cells infiltrations. Few interstitial aggregations of round cells were noticed. The kidney revealed focal or individual cell necrosis represented by pyknosis. Mild congestion of peritubular capillaries was visualized besides extensive regenerative attempts in the renal tubules. The heart was normal except for perivascular edema and few round cells infiltrations. The spleen was normal with overcrowded round cells and few heterophils in the red pulp. The splenic capsule was focally thickened

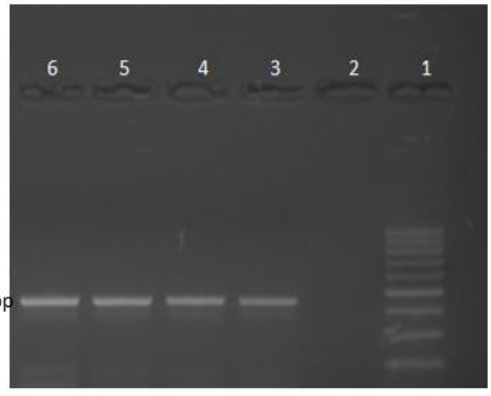

Fig (A):-Agarose gel electrophoresis showing amplification product of 324 bp fragment of alpha toxin gene of Clostridium perfringenes performed with specific primer. The results observed revealed that the gene of alpha toxin (324 bp) was present in C. perfringens type A (ATCC12917)(control positive) and the reisolated strains (complete identity) and absent in E.coli (ATCC25922) (control negative). L1:-100-1000bp DNA ladder, L2:-Negative control E.coli ATCC 25922. L3:- positive control C. perfringens type A (ATCC12917). L4:- C. perfringens reisolated from first week after infection, L5:- C. perfringens reisolated from seconed week after infection, L6:- C. perfringens reisolated from third week after infection

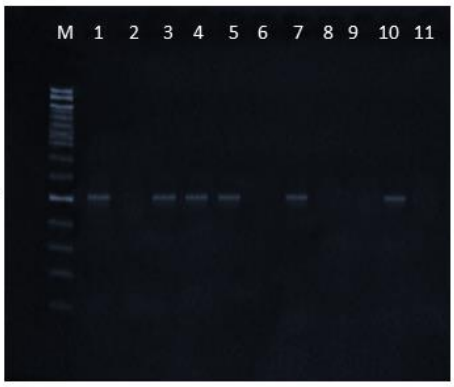

Fig (B): Agarose gel electrophoresis of PCR of cpe primer (506 bp) for characterization of specific gene of C. perfringens enterotoxin. The results observed revealed that the enterotoxin gene (cpe) gene (506 bp). L. M: 100 bp ladder as molecular size DNA marker
L. 1: Control positive for cpe gene. L. 2: Control negative for cpe gene. Lanes 3, 4, 5, 7 and 10: Positive C. perfringens for enterotoxin gene (cpe). Lanes 6, 8, 9 and 11: Negative C. perfringens for enterotoxin gene (cpe).
N.B:One week treatments occupied lanes 3, 4 and 5.
Two week treatments occupied lanes 6, 7 and 8.
Three week treatments occupied lanes 9, 10 and 11

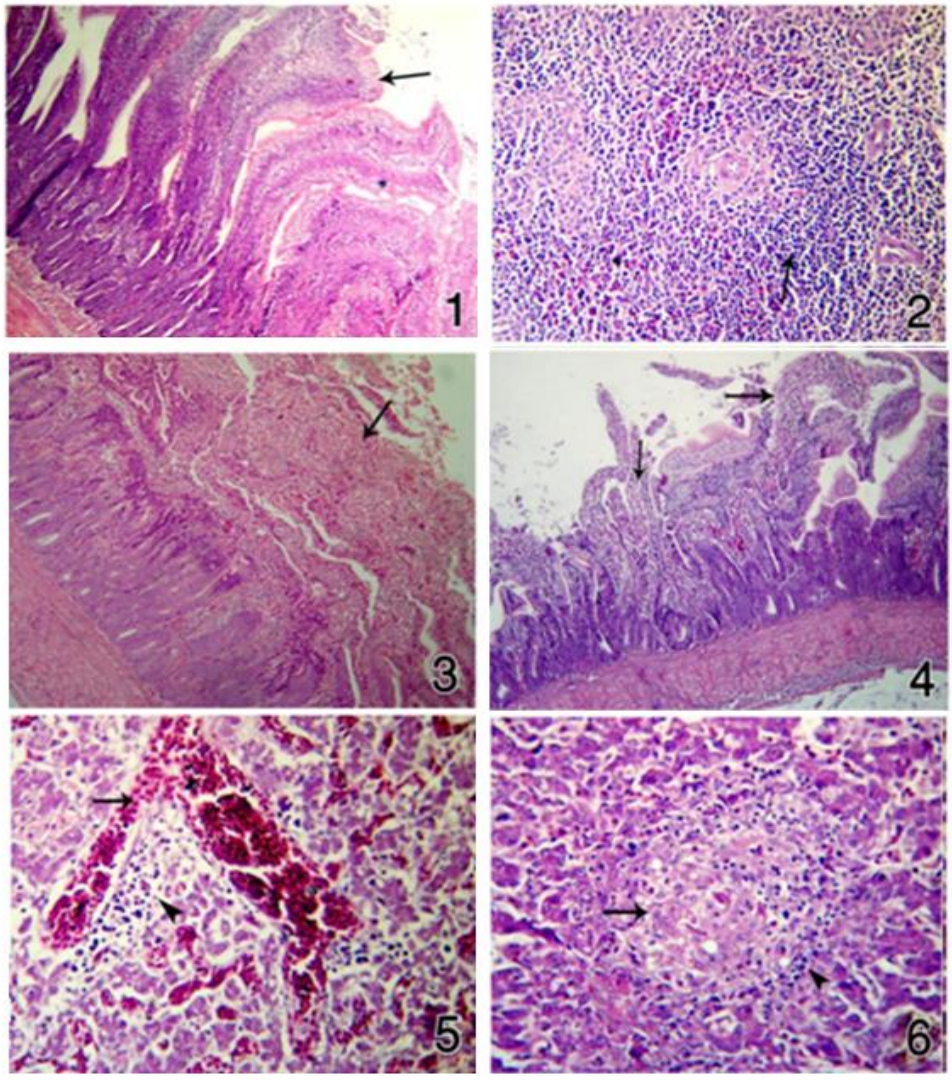

Fig (1): Intestine of saltose shows leaf or wave-like types villi and increased numbers of goblet cells (arrow). H&E x 200.
Fig (2): Spleen of saltose shows moderate hyperplasia in the lymphoid cells of white pulp (arrow). H&E x 400.
Fig (3, 4): Intestine of clostridium shows extensive mucosal necrosis (coagulative type) in small intestine with leukocytic infiltrations mostly of heterophils and macrophages (arrows). H&E x 200.
Fig (5): Liver of clostridium shows severe congestion of hepatoportal blood vessels and sinusoids (arrow). H&E x 400.
Fig (6): Liver of Clostridium shows area of coagulative necrosis infiltrated with macrophages and few heterophils (arrow). H&Ex 400.

4. DISCUSSION

In addition to nutritional and physiological studies on increasing poultry production with high-quality feeds, the exploitation of supplements to basal diets for maintaining the gut environment is also important. Recently, functional feeds such as probiotics and prebiotics have been used to enhance intestinal health and to obtain safe, reliable, and high-quality animal products without any medication or antibiotics (Sathishkumar, *et al.*,2013). Probiotics are live microbial feed additives that beneficially affect the host animal by improving its intestinal bacterial balance. Dietary probiotics have shown

improved BW gain, reduced mortality, and enhanced feed conversion, resulting in an increase of broiler productivity (Torres, *et al*., 2005 and Willis *et al*., 2007).

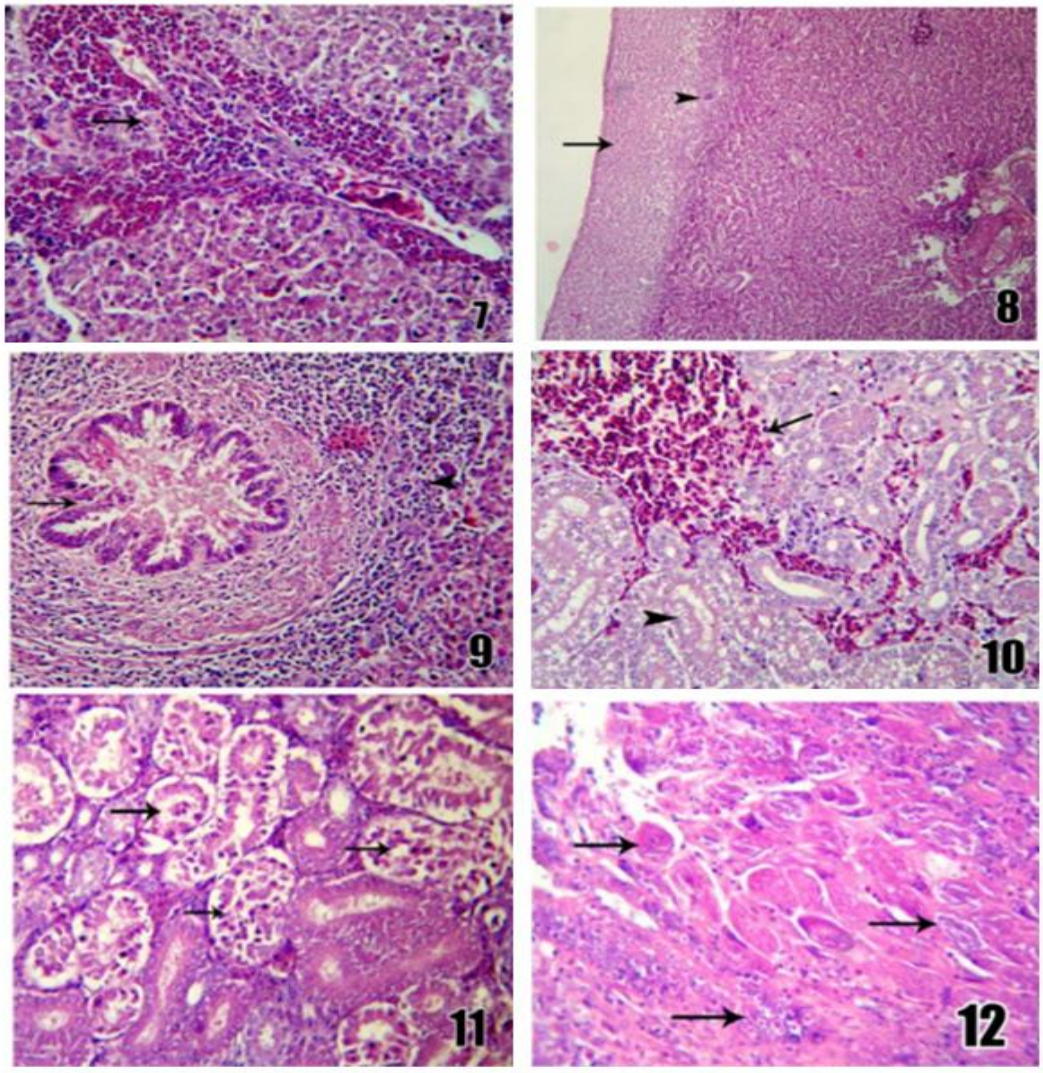

Fig (7): Liver of Clostridium shows portal areas heavily infiltrated with heterophils (arrow). H&E x 400.
Fig (8): Liver of Clostridium shows perihepatitis of necrosis and round cells infiltrations (arrow) besides few basophilic bacterial colonies (arrowhead). H&E x 200.
Fig (9): Liver of Clostridium shows portal areas were widened with chronic cholangitis (arrowhead). H&E x 400.
Fig (10): Kidney of clostridium shows congestion (arrow) and vacuolation of renal tubular epithelium (arrowhead). H&E x 400.
Fig (11): Kidney of clostridium shows coagulative necrosis in the tubular epithelium (arrows). H&E x 400.
Fig (12,13): Ceci of Eimeria shows severe necrosis, destruction and desquamation of the lining epithelium with the presence of developmental stages of Eimeria including mature schizonts and gamonts in the enterocytes and lumens (arrows). H&E, (12 x 200).

Considering the results of *C. perfringens* colonization in the present study, it was shown that either group treated with saltose and infected with C. *perfringens* or treated with saltose and infected with both *C. perfringens* and coccidian there was significantly reduced intestinal colonization of *C. perfringens*. Statistically, it was found that the group treated with saltose and infected with *C. perfringens* only showed the lowest intestinal count along the course of observation period. Moreover, the achieved results proved that chickens infected with coccidia and *C. perfringens* without treatment had the highest *C. perfringens* colonization. That explanation might be due to that coccidial infection induced severe intestinal mucosal

damage that permited *C. perfringens* to induced necrotic enteritis (Williams, 2002).

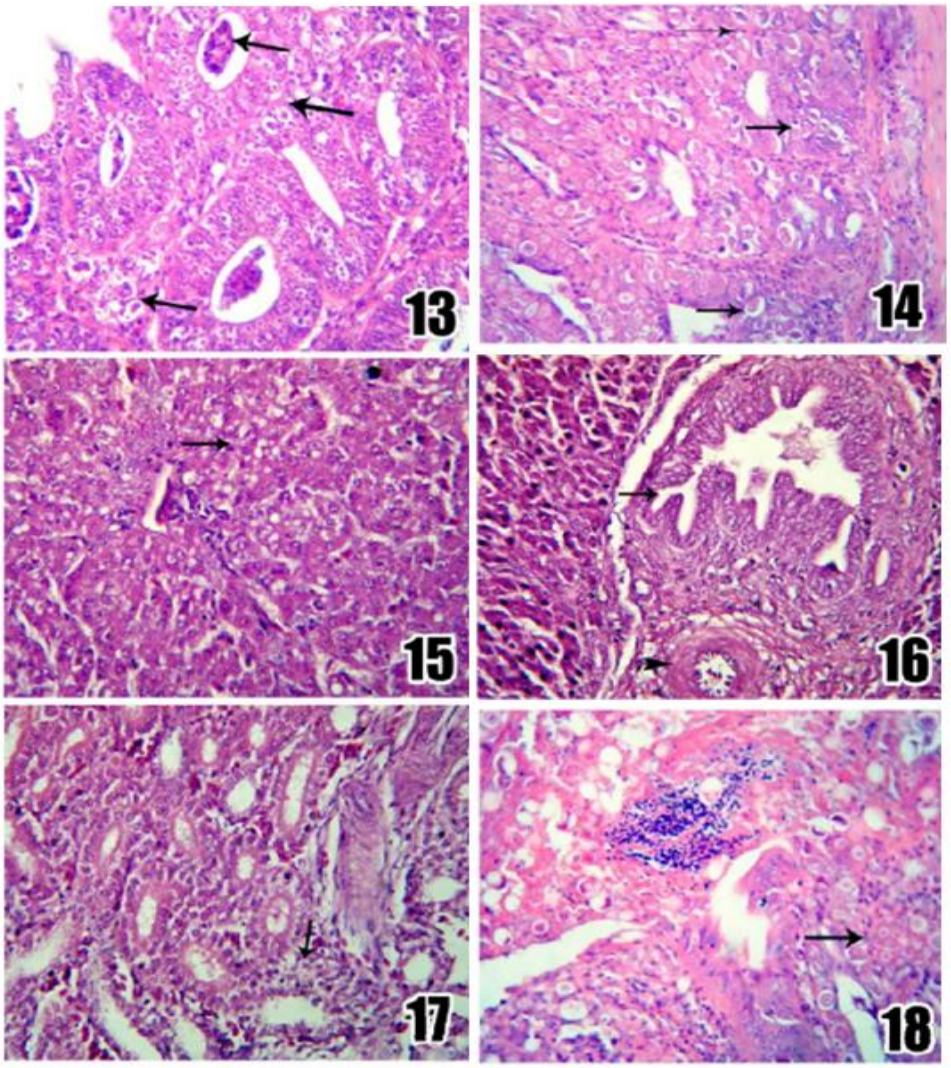

Fig (13): Ceci of Eimeria shows severe necrosis, destruction and desquamation of the lining epithelium with the presence of developmental stages of Eimeria including mature schizonts and gamonts in the enterocytes and lumens (arrows). H&E, (x 400).
Fig (14): Large intestine of Eimeria shows different developmental stages of Eimeria in the lining epithelium (arrows). H&E x 400.
Fig (15): Liver of Eimeria shows periportal vacuolation in the hepatocytes (arrow). H&E x 400.
Fig (16): Liver of Eimeria shows portal areas with severe hyperplastic bile ducts (arrow) and thickening in the wall of hepatic arteriole (arrowhead). H&E x 400.
Fig (17): Kidney of Eimeria shows few heterophils and lymphocytes infiltrations among the renal tubules with individual cell necrosis (arrow). H&E x 400.
Fig (18): Intestine of both cl. and Eim. shows developmental stages of Eimeria in the enterocytes (arrow). H&E x 400

Saltose is probiotics, contain "Cell Wall Lyase" which is a combination of unique new patent enzymes, which can damage the cell wall of oocyst of parasite (such as *Coccidiosis*, *Cryptosporidiosis*) and bacterial agents, *Salmonellosis* and *Clostridiosis*. Saltose also reduced *C. perfringens* colonization. These were evaluated comprehensively by (Hosoi *et al.*, 2000),who demonstrated that *bacillus subtills* produces different enzymes. Colonization and persistence of *C. perfringens* were suppressed (La Ragione and Woodward 2003) *B. licheniformis* spores (one of the main including bacteria in Saltose) at higher concentrations (8×10^6 and 8×10^7 cfu/g of feed), showed no differences in NE

lesion score mortality in compared with virginiamycin

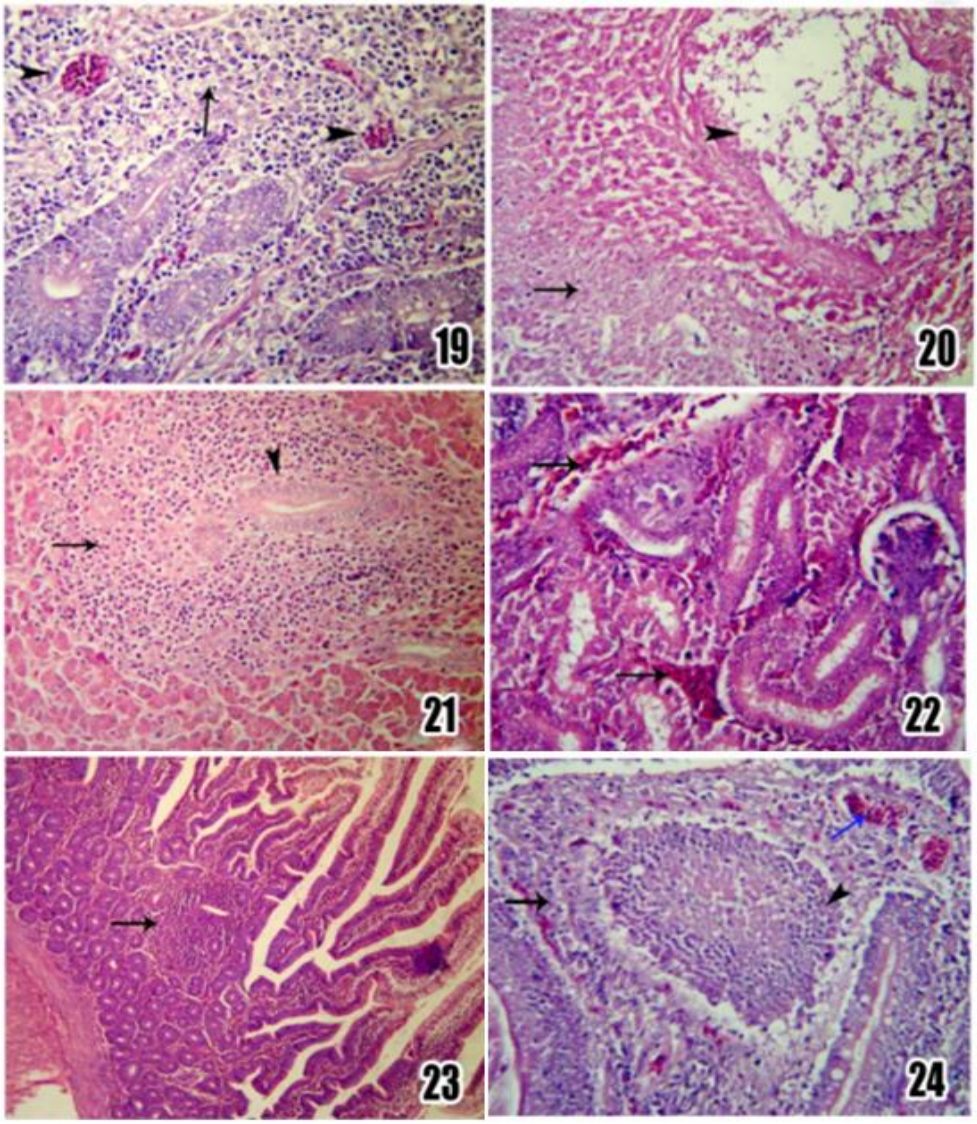

Fig (19): Intestine of both cl. and Eim. shows replacement of mucosa by round cells (arrows) and few erythrocytes (arrowheads). H&E x 400.
Fig (20): Liver of both cl. and Eim. shows cavitations (arrowhead) of the necrotic area (arrow). H&E x 400.
Fig (21): Liver of both cl and Eim shows portal area with round cells and few heterophils infiltrations (arrow) and hyperplastic bile ducts (arrowhead). H&E x 400.
Fig (22): Kidney of both cl and Eim shows congestion of peritubular capillaries (arrows). H&E x 400.
Fig (23): Intestine of cl and saltose shows normal mucosa and aggregation of round cells in the submucosa (arrow) with no evidence of necrosis. H&E x 200.
Fig (24): Intestine of cl and saltose shows round tips with hyperplastic lining epithelium (arrow), masses of hyperplastic cells in the submucosa (arrowhead)and few extravassated erythrocytes (blue arrow) . H&E x 400.

This result explained by (Knap *et al*., 2010 and Tactacan *et al.*, 2013),they reported that a strain of *Bacillus licheniformis*, inhibited the subsequent establishment of a *Clostridium perfringens* strain ingested by the animals. This inhibitory effect depended on the in vivo production by *B. licheniformis* of an antibiotic substance having a number of the characteristics of bacitracin which is effective on *Clostridium*. Probiotics of the genus *Bacillus subtilis* can be used without issue in combination with organic

acids, coccidiostats and therapeutic antibiotics (Hongh *et al*. 2005).

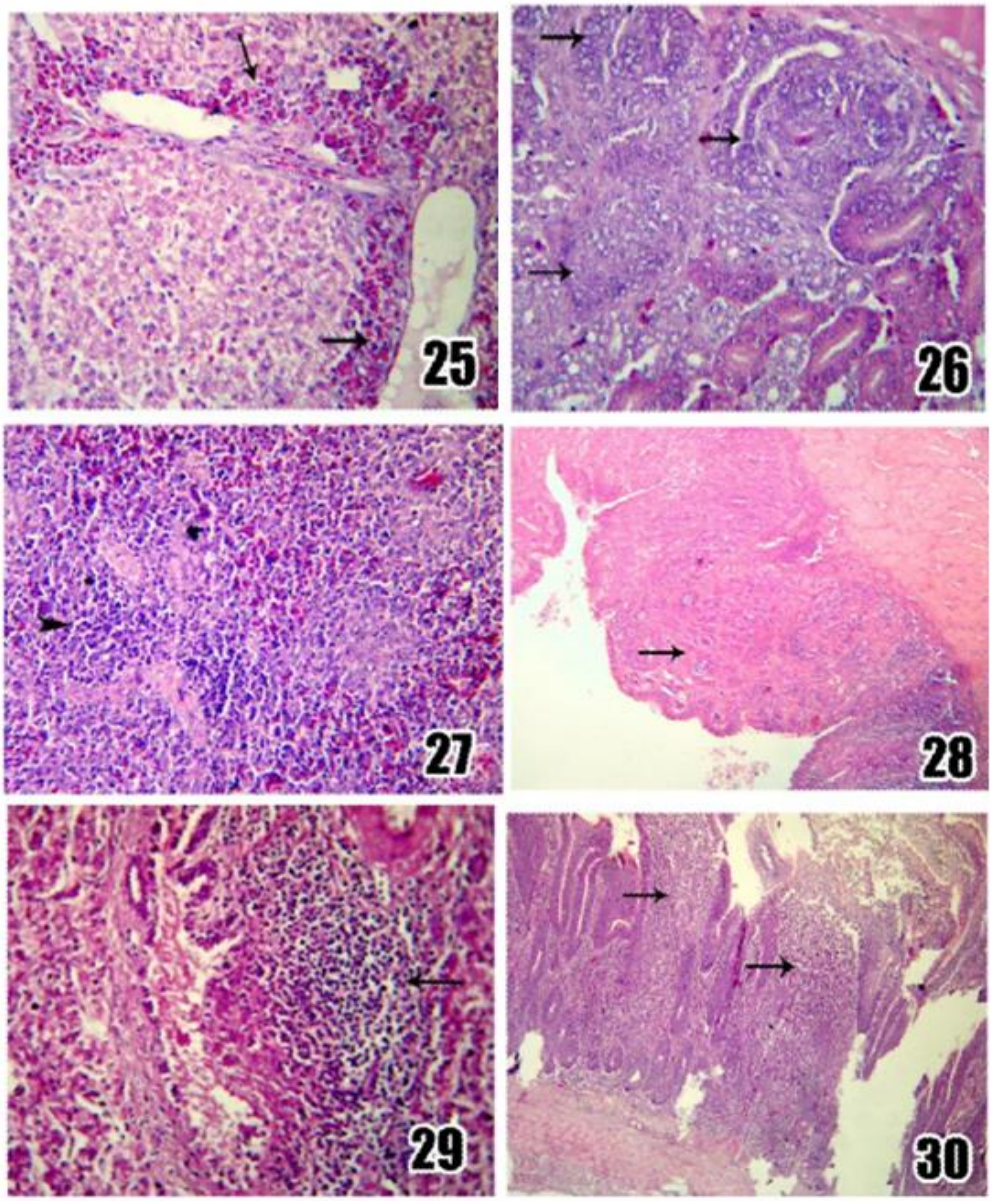

Fig (25): Liver of cl and saltose shows hydropic degeneration and vacuolation besides heterophils infiltrations in the portal area (arrows). H&E x 400.
Fig (26): Kidney of cl and saltose shows vacuolation in the renal tubular epithelial cells (arrows). H&E x 400.
Fig (27): Spleen of cl and saltose shows mild hyperplasia in the lymphocytes of white pulp (arrowhead). H&E x 400.
Fig (28): Intestine of Eimeria and saltose shows few developmental stages of Eimeria with intact epithelial lining (arrow) and round cells infiltrations (arrowhead). H&E x 150.
Fig (29): Liver of Eimeria and saltose shows portal area with huge numbers of round cells (arrow) and hyperplasia in the bile ductal epithelium (arrowhead). H&E x 400.
Fig (30): Intestine of Cl, Eim and saltose shows extensive aggregation of round cells among the intestinal villi (arrows). H&E x 200.

Teo and Tan 2005 reported the production of an antimicrobial factor typical for gram-positive bacteriocin by *B. subtilis* (PB6) which was found to be active against various strains of *Clostridium* spp. The production of antimicrobials by probiotics is considered one of the principal mechanisms that inhibit pathogenic microorganisms in the gastrointestinal tract.

Bacillus spp. are known to produce a large number of antimicrobials (Urdaci and Pinchuk. 2004) these include bacteriocins and bacteriocin-like inhibitory substances (e.g., Subtilin, Coagulin), as well as antimicrobials based on peptides and polyketides (e.g., Surfactin, Bacilysin, Difficidin, and Macrolactin). Additionally, Lactobacilli produce lactic acid, which can affect pathogenic bacteria, such as, *Salmonella, E.Coli, Campylobacter* and *Clostridiae*. On the other side the role of probiotics in reducing colonization of *C. perfringens* was studied by (Maruta *et al*., 1996 and Pascual *et al.,*1999), they estimated that lactic acid bacteria was effective in reducing *C. perfringens* intestinal colonization.

PCR has been widely used in identify the toxin genes of C. *perfringens* because of its high sensitivity. C. *perfringens* was identified by analysis of its toxin using PCR to assure the experimental infection and to confirm the efficacy of Saltose. In the present study our results suggest that the use of Saltose can protect chickens against the deleterious effects of *C. perfringens* infection. It has previously been shown that *C. perfringens* could be identified by PCR in feces samples after enrichment (Miwa *et al*., 1997). Twelve *C. perfringens* isolates were toxins typed, nine isolates from chickens treated with saltose and infected with *C. perfringens* type A (the 6th group), three isolates from chickens non treated and infected with *C. perfringens* (third group). In Fig. (A) all of suspected isolates were PCR positive for the α toxin gene in third group (the infected non-treated) which revealed that alpha toxin gene (324bp) identified by PCR belongs to the used C. *perfringens* type A (not detected beta toxin and epsilon toxin.). In addition, the re-isolated strain of C. *perfringens* from the intestine of infected non-treated showed complete identity with the previously inoculated *C. perfringens*. (Ma *et al*., 2007), who proved that this type of insertion neither affect α toxin expression or the molecular weight of the α toxin produced. The genotyping results demonstrated that there were no differences in the toxin genotype between challenge strain and isolated strain (Manfreda *et al,*. 2006).Therefore, the detection of toxin produced by bacteria is a more convenient method (Arda *et al*., 1997). CPE enterotoxin gene (506 bp) of *C. perfringens* was demonstrated in the 6th group (infected, treated with saltose from one day) in all isolates1st week post infection, not detected in 2 isolates in 2nd and 3rd week post infection Fig(B) belongs to the used of saltose protect chickens against the toxogenic effects of *C. perfringens* infection. This agrees with (Gholamiandekhordi *et al.* 2006) who reports the presence of the enterotoxin gene in 2 strains of a total of 27 healthy broiler chickens examined. In any event, genetic control must be responsible for the large amounts of enterotoxin produced during sporulation of food-poisoning strains.In the intestinal tract where upon sporulation (spore formation) CPE is released (Sawires and Songer, 2006). Some *C. perfringens* strains, in addition to a toxin, produce enterotoxin: two toxins that have been proposed as being important in the pathogenesis of intestinal disorders in animals and humans respectively (Sarker *et al*., 1999).

The results of this work revealed significant decrease in in RBCs count in group 3which infected with *C. perfringens* ,this result may be due to Alpha toxins of the *C. perfringens* type A are considered to be the major toxins involved in the disease pathogenesis. The activity of alpha toxin is lethal, necrotizing and hemolytic ((Quinn *et al*., 2004; Fatmawati *et al*., 2013). In the present study as the disease progressed, total number of red cells started to decrease . The hemolytic activity may be due to binding of alpha toxin on the red cells receptor, which activate the signaling pathway in the cell, resulted in the hemolysis. Our finding is supported by (Ombe *et al.* 2006). Also RBCs count decrease in group 4 and 5.This decline in the blood components may be due to the severe bleeding and tissue damage in the mucosa of duodenum originated from invasion of *Eimeria tenella* (Mohammed 2012). Concerning the differential WBC (leukocyte)

count on broilers infected by *C. perfringens and E. tenella*, increased numbers of lymphocytes in both infection, eosinophils in coccidial infection were obtained when compared with the reference value indicated by (Merck Veterinary Manual 2011). The present results were similar to those reported by (Rose *et al.* 1979) who indicated that the peripheral blood leukocytes (PBL) response to infection with *E. maxima* and *E. acervulina* in chicken shows the increment in the number of PBL. In primary infections, the number of PBL increased biphasically and changes were found in the count of polymorphonuclear cells, lymphocytes and large mononuclear cells. Similar findings were also mentioned by (Ricklefs and Sheldon 2007), who found the high counts of lymphocytes, heterophils and eosinophils in parasitic (malaria and haemosporidin) infected birds. The increase in the lymphocyte count may be attributed to the effect of the inflammation of the caeca and intestine. Chronic antigenic stimulation may result in a greatly expanded circulating lymphocyte pool because the primary functions of the lymphocytes are immunological response, humoral antibody formation and cell mediated immunity (Irizaary, 2004). Antibody mediated responses play a minor role in protection against coccidiosis. There is increasing evidence that cell-mediated immunity plays a major role in resistance to infection as T lymphocytes appear to respond to closteridial and coccidial infection through both cytokine production and a direct cytotoxic attack on infected cells (Lillehoj and Trout, 1996; Yun *et al.*, 2000). The present study also showed decreases in ALT and AST in *E. tenella* and *E. brunetti* infected broilers These results are similar to the result of (Mondal *et al*.,2011) who reported that ALT decreased in broiler chickens infected with a field isolate of *E. tenella*. A decrease in plasma enzyme levels is much less frequently used for clinical interpretation. However, there are a few specific cases where low plasma enzyme levels will indicate that the relevant organ is hyperplastic, atrophied or destroyed (Kerr, 2002). ALT and AST are the enzymes found in erythrocytes; therefore, the decrease in the activities of serum ALT and AST reported in the present study may be associated with the high reduction of erythrocytes because of the loss of blood into the gastrointestinal tract (Meskerem *et al.,* 2013) . Studies on the immunology of the intestinal tract of the chicken have revealed an antibody system based on an immunoglobulin with many features in common with mammalian secretory IgA (Chang-Hsin Chen 2012). Its close association with the intestine underlines its strategic importance as a first line of defense against organisms invading the mucous surface or attempting to gain access to the circulation via this route. The normal gut flora stimulates the production of IgA and the concentration of IgA in the healthy gut is maintained at a normal, equilibrium level. By analogy with this and other well defined systems (Yun et al., 2000) reported that IgA accumulated in the apical portion of the lamina propria at sites of parasite development. So,it would be expected that, in response to infection with E. tenella, there would be an increased net synthesis of secretory IgA resulting in an elevated concentration in the gut contents. The results obtained from the measurements of IgA concentration throughout the course of infection thus contribute an important line of evidence in favour of secretory IgA involvement. The current results in groups 6,7 and 8 all parameters nearly return to normal level .This results may be attributed to antibacterial and anti parasitic effect of saltose .The anti parasitic effect through unique enzyme“ Cell-Wall Lyase” which have high affinity for the lipid layer and disruptive effect on membrane integrity so it proved to be an inhibitor of cell wall biosynthesis of Gram positive bacteria, gram negative bacteria and protozoa like *Eimeria* resulting misshape and loss of infective character (El Iraqi *et al*.,2014*)* also *Bacillus subtilis* Probiotic organisms in the gut can produce products such as free radicals, hydrogen peroxide, short–chain fatty acids and other oxygen metabolites that could conceivably harm motile merozoites directly and/or affect intracellular stages thereby

reducing their numbers. Probiotics modify receptors on enterocytes (Fooks and Gibson 2002). This could either impair or destroy sporozoites and/or merozoites from penetrating an enterocyte. The results are in agreement with (Nasr, *et al.*,2014).

In the current study the pathological results on chickens infected with *C. perfringens* or *Eimeria* and both and supplemented with probiotic (Saltose), established typical necrotic enteritis model of broiler in the form of variable degree of mucosal necrosis together with leucocytic infiltration mainly heterophil and macrophages. Our results are similar to that afore described in *C. perfringens* by (Gazdinski and Julian 1992 and Filip, *et al.*,2004). On the same line our findings are in accordance with (Kerry, *et al*., 2013), he reported that, extensive mucosal necrosis and sloughing of the epithelial cells. As lesions progress, there is a sharp line of demarcation between necrotic and viable tissue due to accumulation of the inflammatory cells in the outer areas of the viable tissue. Heterophils are the dominant inflammatory cells and mononuclear cells are also present in more chronic lesions. The pathological findings on the liver of the present study, were congestion of the hepatic blood vessels together with multiple areas of necrosis in addition chronic cholangitis in some cases. The obtained results are agreement with (
On derka, *et al.*, 1990; Sasaki, *et al*., 2000 and Filip, *et al.*, 2004), they mentioned that (*C. Perfringens*)infection is associated with hepatitis or cholangiohepatitis. Also our findings on the liver lesions in ((*C. Perfringens*) infection are similar to that mentioned by (Kerry,*et al.*,2013), he observed multifocal coagulative necrosis of the liver and bile ducts. Few literature about the pathological lesions (*C. Perfringens*) infection in other organs (kidneys, heart and spleen). Early literature were reported by (AL-Sheikly and Truscatt, 1976 a,b and Filip, *et al,*,2004), these literature are coincided with our findings, they stated blood vessels congestion in the liver, spleen and kidneys on the poultry with necrotic enteritis at 12 h after inoculation of broth culture or toxins of (C. *perfringens*). Necrosis of the follicular lymphocytes in the bursa of Fabricius and other lymphoid organs are described by (Gazdinski and Julian 1992 and Filip, *et al*.,2004). The microscopic results in different organs may be attributed to *C. perfringens* α toxin (phospholipase C), can hydrolyze lecithin into phosphoryl choline and diglyceride, which leads to tissue damage (Smedley III. *et al*., 2004).

The pathological findings on the Eimeria infection in this work showed destruction and desquamation of the intestinal epithelium with the presence of developmental stages of Eimeria in addition to extensive extravassated erythrocytes. Our results are in accordance with that mentioned by (McDougald and Fitz-Coy, 2008 and Meskerem, *et al.*,2013), they concluded that, the most pathogenic stage caused by E. tenella as the second generation schizont which caused excessive tissue damage, bleeding, disruption of the cecal glands and destruction of the mucosa and muscularis layer.

The supplementation of probiotic (Saltose) to the infected groups (C. *perfringens*, Eimeria and both) showed reduced in the severity of the lesions in the most examined organs. The probiotic are live microorganisms that when administered through the digestive route is favorable to the host's health (Guillat, 1998). The microorganisms used in animal feed are mainly belonging to the type *Lactobacillus*, *Enterococcus*, *Pediococcus* and *Bacillus*(Guillat, 1998 and Badran and Lukesova, 2006). They explained the ability of some strains of probiotics to coloniz the gut of axenic and gnotoxenic chickens and they also reported that, a probiotic strain of *Enterococcus faecium* is able to colonize the axenic and gnotoxenic gut after a single administration through the different mechanisms of action suggested are : nutritional effect and sanitary effect. The nutritional effect causes a reduction of the metabolic reactions that produce the toxic substances, stimulation of indigenous enzymes and production of vitamins or

antimicrobial substances, while the sanitary effect can increases the colonization resistance and stimulation the immune response. On the same line (Brigidi, *et al.*,2001 and Filip,*et al.*,2004), mentioned that, oral uptake of *lactobacilli* and *bifidobacteria* decrease the number of intestinal C. *perfringens* bacteria and spores in human and mice. Hofacre, *et al.*,1998 and Filip,*et al.*,2004, showed that a commercial probiotic preparation reduced gross lesions of necrotic enteritis in chickens. On the other hand (Dalloul, *et al.*,2003 and Lee,*et al*., 2007), concluded that, administration of *lactobacillus*- based probiotic induced protective immunity against *Eimeria* infection. Some strains of *Pediococcus* species produce antimicrobial peptides (bacteriocins) that inhibit closely related lactic acid bacteria and other gram-positive spoilage and pathogenic bacteria (Ennahar and Des Champs 2000). These bacteriocins are designated pediocins and they have been shown to exert high antimicrobial activity against Listeria species (Ennahar,*et al.*,2000). In accordance to our findings,(Lee, *et al.*,2007) stated that probiotic (Mito Grow) consisting of live *P. acidilactici* bacteria provided some degree of defense against *E.acirvulina* and *E. tenella* infections in broiler chicken.

In conclusion, supplementation of Saltose probiotic when giving as a prophylactic, results a better overcome on *C. perfringens* induced NE and *Eimeria* infection lesions also improved gut health throw the intestinal histologic findings.

4. REFERENCES

Al-Sheiky, F and R.B. Truscott .1976 a . The pathology of necrotic enteritis of chickens following infusion of broth culture of *Clostridium perfringens* into the duodenum. Avian Dis.21: 230-240.

Al-Sheikly, F. and R.B. Truscott. 1976 b . The pathology of necrotic enteritis of chickens following infusion of crude toxins of *Clostridium perfringens* into the duodenum. Avian Dis. 21:241-255.

Apata D.F. 2008 . Growth performance, nutrient digestibility and immune response of broiler chicks fed diets supplemented with a culture of *Lactobacillus bulgaricus*. J. Sci. Food Agric. 88:1253-1258.

Arda, M., Minbay, A., LeloÛlu, N., AydÝn, N., Kahraman, M., Akay, Ilgaz, A., Üzg.r, M., Diker, K.S. 1997. zel Mikrobiyoloji. Epidemiyoloji, Bakteriyel ve Mikotik Ünfeksiyonlar. Medisan YayÝn Serisi No: 26. Medisan YayÝnevi. Ankara .

Atta, A. H., Shalaby, M. A and Saifan H Y. 2014. Efficacy of commiphora molmol extract against clostridium perfringens experimental infection in chicken World J. of Pharma. and Pharmace. Sci. 3(12): 365-380.

Badran, I., Lukesova, D. 2006. Control of coccidiosis and different coccidian of chicken in selected technologies used in tropics and subtropics. Agric. Tropica EtSubtropica. 39(1):39-43.

Barbosa, T. M., Serra, C.R., La Ragione, R.M., Woodward, M.J., Henriques, A.O. 2005. Screening for *Bacillus* isolates in the broiler gastrointestinal tract. Appl. Environ. Microbiol. 71: 968-978.

Baums, C.G., Schotte, U., Amtsberg, G., Goethe, R. 2004. Diagnostic multiplex PCR for toxin genotyping of Clostridium perfringens isolates. Vet. Microbiol. 100:11-16.

Brewer, J.H., Allgeier, D. 1966 .Safe, selfcontained carbon dioxide-hydrogen anaerobic system. Appl. Microbiol. 14: 985-988.

Brigidi, P., Vitali, B. , Swnnen, E., Bazzocchi, G and M atteuzzi, D. 2001 . Effect of probiotic administration upon the composition and enzymatic activity of human faecalmicrobiota in patients with irritable bowel syndrome or functional diarrhea. Res. in Microbiol. 152: 735-741.

Chang-Hsin Chen, B.S. 2012 . Enhancing Chicken Mucosal IgA Response against *Clostridium perfringens* α-toxin. Ph.D Thesis Texas A&M University

Coles, E.H. 1986. "Veterinary Clinical Pathology 4th Ed. W.B. Sound, P hiladelphia ,London ,Toronto ,Mexico ,Sydney ,Tokyo, Hong Kong

Conway, D.P., McKenzie, M.E. 2007. Poultry Coccidiosis*:* Diagnostic and Testing Procedures. 3rd ed. Blackwell Publishing. Ames, IA, USA. 164pp.

Cruickshank, R, Duguid, J.R. Marmion, B.P., Swain R.H.A.1975 .Text book of medical microbiology,12 ed Churchill, Livingstone, Edinburgh and New York.

Dahiya J.P., D. Hoehler, Wilkie, D.C. , Van Kessel, A.G. and Drew, M.D. 2005 . Dietary glycine concentration affects intestinal Clostridium perfringens and lactobacilli populations in broiler chickens. Poult Sci., 84: 1875-1885.

Dahiya J.P., Wilkie, D.C., Van Kessel, A.G. and Drew, M.D. 2006 . Potential strategies for controlling necrotic enteritis in broiler chickens in post-antibiotic era. Anim. Feed Sci. Technol. 129: 60-88.

Dalloul, R.A., Lillehoj, H. S., Shellem, T. A., Doerr, J.A. 2003 . Intestinal immunomodulation by vitamin A deficiency and *Lactobacillus*-based probiotic in *Eimeriaacervulina*- infected broiler chickens. Avian Dis.47:1313-1320.

El Iraqi, K.G., Melegy, T.M., Hassan, A.O. 2014 . Evaluation of New Biological Product Saltose for

controlling Coccidia and Clostridia in broiler chickens. Global Vet.12(2):257-263.

Ennahar, S., Deschamps, N. 2000. Anti-*Listeria* effect of enterocin A, produced by cheese- isolated *Enterococcus faecium* EFMO1,relative to other bacteriocins from lactic acid bacteria. J. Appl. Microbiol. 88: 449-457.

Ennahar, S., Sashihara, T., Sonomoto, K., Ishizaki, A. 2000 . Class IIabacteriocins :Biosynthesis, structure and activity. FEMS Microbiol. Rev. 24:85-106.

Fatmawati, N. N., Sakaguchi, Y., Suzuki, T., Oda, M., Shimizu, K., Yamamoto, Y., Sakurai, J., Matsushita, O., Oguma, K. 2013. Phospholipase C produced by *Clostridium botulinum* types C and D: comparison of gene, enzymatic, and biological activities with those of *Clostridium perfringens* alpha-toxin. Acta Med Okayama. 67: 9-18.

Filip Van, Immerseel, Jeroen, De B., Frank, P., Gerard, H., Freddy, H., Richard, D. 2004. Clostridium perfringens in poultry: an emerging threat for animal and public health. Avian Pathol. 33(6), 537-549.

Gazdzinski, P.,Julian RJ. 1992. Necrotic enteritis in turkeys. Avian Dis. 36: 792-798.

Gholamiandekhordi, A. R., Ducatelle, R., Heyndrickx, M., Haesebrouck, F., Van Immerseel, F. 2006. Molecular and phenotypical characterization of Clostridium perfringens isolates from poultry flocks with different disease status. Vet. Microbiol. 113: 143-152.

Guerra N.P., Bernardez, P.F., Mendez, J., Cachaldora, P., Castro L.P. 2006. Production of four potentially probiotic lactic acid bacteria and their evaluation as feed additives for weaned piglets. Anim. Feed Sci. Technol.

Guillot, J.F. 1998. Les probiotiques en alimenationanimale. Cahiers Agric. 7: 49-54.

Hamidi, H., Jahanian, R. Pourreza, J. 2010. Effect of dietary betaine on performance ,immunocompetence and gut content osmolarity of broilers challenged with a mixed coccidial infection. Asian J. Anim. Vet. Adv.

Higgins, S.E., Higgins, J.P.,Wolfenden, A.D., Henderson, S.N., Torres-Rodriguez, A.,Tellez, G. Hargis, B. 2008. Evaluation of a *Lactobacillus*-based probiotic culture for the reduction of *Salmonella enteritidis* in neonatal broiler chicks. Poult. Sci. 87:27-31.

Hofacre, C.L., Froyman,R., Gautrias, B.,George,B., Goodwin, M.A., Brown,J. 1998 . Use of Aviguard and other intestinal bio products in experimental clostridium perfringens-associated necrotizing enteritis in broiler chickens. Avian Dis. 42: 579-584.

Hongh, H., Duc, L. and Cutting, S. 2005 . The use of bacterial sporeformers as probiotics. FEMS Microbiol. Rev. 29: 813-835.

Hosoi, T., Ametani, A., Kiuchi, K., Kaminogawa, S. 2000. Improved growth and viability of lactobacilli in the presence of Bacillus subitlis (natto), catalase or subtilisin. Canadian J. Microbiol. 46: 892-897.

Irizaary-Rovira, A.R. 2004. Avian and reptilian clinical pathology (Avian hematology & biochemical analysis), Section XI, pp.282–313. *In* R.L. Cowell, (ed.). Vet. Clini. Path. Secrets. Elsevier Inc. St. Louis, MO, USA.

Jerzsele, A., Szeker, K., Csizinszky, R., Gere, E. , Jakab, C., Mallo, J.J., Galfi P. 2012 . Efficacy of protected sodium butyrate, a protected blend of essential oils, their combination and *Bacillus amyloliquefaciens* spore suspention against artificially induced necrotic enteritis in broilers. Poult. Sci. 91: 837-843.

Kabir S.M.L., Rahman, M.M., Rahman, M.B., Ahmed,S.U. 2004 . The dynamics of probiotics on groth performance and immune response in broilers. Int. J. Poult. Sci. 3: 361-364.

Kerr, M.G. 2002 . Clinical enzymology-plasma enzymes in diagnosis, pp. 135–296. *In* M.G.Kerr, (ed.). Veterinary Laboratory Medicine (Clini. Biochem. and Haemato.).

Kerry, k.C., Glenn S. J., Francisco, A.U. 2013. Diagnosing clostridial enteric disease in poultry. J. of Vet. Diagnostic Investig. Xx (x):1-14.

Keyburn A.L., Boyce J.D., Vaz P.T.L. Bannam, M.E. and Ford 2008 . "NetB" a New toxin that is associated with Avian Necrotic Enteritis caused by *Clostridium perfringens.* PLOS Pathogens4 (2): e26.

Kind, P, King E. 1954. Colorimetric determination of alkaline phosphatase J. Clinical Pathol.

Knap I., Lund B., Kehlet A. B., Hofacre C., Mathis G. 2010. *Bacillus licheniformis* Prevents Necrotic Enteritis in Broiler Chickens. Avian Dis. 54(2):931-935.

Koneman, E.W., Auen, S.D., Dowell, V.R., Sommers, H.M. 1988. Color Atlas and Text Book of Diagnostic Microbiol. 2nd Ed., J. B. Lip. Co. New York, London.

La Ragione, R M., Woodward. M. J. 2003. Competitive exlusion by spores of Salmonella enteric serotype Enteritidis and Clostridium perfringens in young chickens. Vet. Microbiol. 94: 245-256.

Lee, S.H., Lillehoj, R.A., Dalloul, D.W., Park, Y.H., Hong and Lin . 2007. Influence of *Pedoiococcus*-Based probiotic on Coccidiosis in broiler chickens. Poult. Sci. 86: 63-66.

Lillehoj, H.S., Trout, J.M. 1996. Avian gutassociated lymphoid tissues and intestinal immune responses to *Eimeria* parasites. Clin. Microbiol. Rev. 9: 349–360.

Lindstrom M., Heikinheimo A., Lahti P., Korkeala H. 2011. Novel insights into the epidemiology of *Clostridium perfringens* type A food poisoning. Food Microbiol. 28 (2): 192-198.

Liu, l.T., Coenye, J.L., Burns, P.W., Whitby, T.L., Lipuma, J.J. 2002. Ribosomal DNA-directed PCR for identification of Achromobacter (Alcaligenes) Xylosoxidans recovered from sputum samples from cystic fibrosis patients. J. Clin. Microbiol. 40:1210-1213.

Lowry,O.H., Rose brough, N.J., Farr, A.L., Randall. A 1951. Protein measurement with folin phenol reagent. J.Bil .Chem.193: 265-275.

Ma, M., Ohtani, K., Shimizu, T., Misawa, N. 2007. Detection of a group II Intron without an open reading frame in the alpha-toxin gene of Clostridium perfringens

isolated from broiler chicken. J. Bacteriol. 189:1633-1640.

Manfreda G, Bondioli V, De Cesare A, Franchini A. 2006. Quantitative evaluation of Clostridium perfringens in Italian broilers. Poult. Sci. 62 (Suppl): 91-92

Maruta, K., Miyazaki, H., Masuda, S., Takahashi, M., Marubashi, T., Tadano, Y., Takashi, H. 1996. Exclusion of intestinal pathogens by continuous feeding with Bacillus subtilis C-3102 and its influence on the intestinal microflora in broilers. Ani. Feed Sci. and Technol, 67 (3), 273-280

McDougald, L.R., Fitz-Coy. S.H. 2008. Protozoal infection, pp. 1068–1080. *In* Y.M. Saif, (ed.). Disease of Poultry.12th ed. Blackwell Publishing. Ames, IA, USA.

McReynolds J, Byrd, R. J., Anderson, R. Moore, T., Edrington .2004. Evaluation of immunosuppressants and dietary mechanisms in an experimental disease model for Necrotic Enteritis. Poult. Sci. 83, No. 12 pp.1948-1952.

Merck Veterinary Manual. 2011. Hematological and serum biochemical reference guides .

Meskerem, A., Chaiwat, B., Nirat, G., Montakan V. 2013. Hematological, biochemical and Histopathological changes caused by coccidiosis in chickens. Kasetsart J. (Nat. Sci)47(2): 238-246.

Miwa, N., Nishina, T., Kubo, S., Atsumi, M. 1997. Most probable number method combined with nested polymerase chain reaction for detection and enumeration of enterotoxigenic Clostridium perfringens in intestinal contents of cattle, pigs and chickens. J. Vet. Med. Sci., 59: 89-92.

Mohammed, K.A. 2012. Study of hematological and some biochemical values changing with administration of Salinomycin and Poultrystar probiotics in broiler chickens challenged with Cocciodsis (*Eimeria tenella*) AL-Qadisiya J. Vet.Med.Sci. Vol./11 No./1

Mondal D.K., Chattopadhyay, S., Batabyal, S., Bera, A.K. and Bhattacharya. D. 2011. Plasma biochemical indices at various stages of infection with a field isolate of *Eimeria tenella* in broiler chicken. Vet. World. 4: 404–409.

Nariuchi, H. 1989. New biochemical practise (12-I). Pages 151–152 *in* Jpn. Soc. Biochem., ed. Tokyo Kagaku doujin Tokyo, Japan. (in Japanese)

Ombe, B. M. H., Kohda, T., Mukamoto, M. and Kozaki. S. 2006. Purfication and sensitivity of *Clostridium chauvoei* hemolysin to various erythrocytes. Comparative Immunol., Microbiol. Infect. dis. 29:263-268.

Onderka, D.K., Langevin, C.C., Hanson, J.A. 1990. Fibrosing cholehepatitis in in broiler chickens induced by bile duct ligation or inoculation of clostridium perfringens . Canadian J. Vet. Res., 54:285-290.

Pascul M., Hugas, M., Badiola, J. I., Monfort, J. M. and Garriga, M.

1999. CTC2197 prevent *Salmonella enteritidis* and *Clostridium perfringens* colonization in chickens. Applied Environ Microbiol. 65: 4981-4986.

PIC-BIO (Poultry Industry Consultant Biotechnology Company). 2013. Saltose. September 20. Available from www.bio- live.com/en/static/pdf/saltose A4.pdf

Pollmann M., Nordhoff, M., Pospschil, A., Tedin, K., Wieler, L.H. 2005. Effect of a probiotic strain of *Enterococcus faecium* on the rate of natural *Clamydia* infection in swine. Infect. Immun.73:4346-4353.

Quinn, P. J., Carter, M. A. Markey, B. and Carte, G. R. 2004. Clin.Vet. Microb. Pp 191-208

Reid, W.M. 1978. Coccidiosis. In Diseases of Poultry, 7th ed., ed. Hofstad, M.S., Calnek, B.W., Helmboldt, C.F., Reid, W.M. and Yoder, H. W., Jr. Ames, IA: Lowa State University Press. pp.784-815.

Retiman and Frankel. 1957. Colorimetric method for determination of serum transaminase activity .American j .of clini. pathol. 28 ,65-68 .

Ricklefs, R.E., Sheldon. K.S. 2007. Malaria prevalence and white-blood-cell response to infection in a tropical and in a temperate thrush: The Ameri. Ornitholog.' Union

Rose, M.E., Hesketh, P., Ogilvie, B.M. 1979. Peripheral blood leukocyte response to coccidial infection: A comparison of the response in rats and chickens and its correlation with resistance to reinfection. Immunol. 36: 71–79.

Sabiqaa M., Abbas, RZ., Iqbal, Z. Mansor, MK., Sinddu, ZD., Zia, MA. and Khan, JA. 2013. Role of natural antioxidants for the control of coccidiosis in poultry. Pakistan J. 33(4):401-407.

Sambrook,J., Fritsch, E.F., Maniatis,T. 1989. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sarker M.R., Carman, R.J., McClane B.A. 1999. Inactivation of the gene (cpe) encoding Clostridium perfringens enterotoxin eliminates the ability of two cpe-positive C. perfringens type A human gastrointestinal disease isolates to affect rabbit ileal loops. Mol. Microbiol. 33:946-958.

Sasaki, J., Goryo. M., Okada, K. 2000. Cholangiohepatitis in chickens induced by bile duct ligations and inoculation of Clostridium perfringens . Avian Pathol., 29: 405-410.

Sathishkumar, J., Gokila, T., Hannah, K., Ravichandran, M., Rajalekshmi, M., Haridasan, C. 2013. *Bacillus subtilis* PB6 improves intestinal health of broiler chickens challenged with *Clostridium perfringens*- induced necrotic enteritis. Poult. Sci. 92:370-374.

Sawires Y.S., Songer J.G. 2006. *Clostridium perfringens*: insight into virulence evolution and population structure. *Anaerobe* 12:23-43.

Schalm, O.W. 1975. Veterinary haematology 3rd Ed Baillier Tindall and Assel.Ltd .London.

Sheedy S.A., Ingham, A.B., Rood, J.I. and Moore, R.J. 2004. Highly Conserved Alpha-Toxin sequences of avian isolates of *Clostridium perfringens*. J. of Clini. Microbiol. Vol.42.No.3.pp:1345-1347.

Sherryll, L.L., Xochitl, H.V., Shivaramaiah, C., Jorge, X.. Anita, M., Juan, D. L., Gopala, K., Vivek, A. K., Ross, E.

W., Raphael, L. A. F., Billy, M. H. and Guillermo, T. 2013. The effect of a *Lactobacillus*-based probiotic for the control of necrotic enteritis in broilers. Food and Nut. Sci., 4:1-7.

Siragusa, G.R., Danyluk, M.D., Hiett, K.L., Wise, M.G. Craven, S.E. 2006. Molecular subtyping of poultry-associated type a *Clostridium perfringens* isolates by repetitive-element PCr. J. Clin. Microbiol 44: 1065-1073.

Smedley, III JG, Fisher, DJ, Sayeed, S, Chakrabarti, G and Mcclane, BA 2004. The enteric toxins of *Clostridium perfringens.* rev Physiol. Biochem. Pharmacol. 152: 183-204

Suvarna, S. K., Layton, C., Bancroft, J.D. 2013. Bancroft's Theory and Practice of Histological Techniques. 7th ed., Churchill Livingstone. Elsevier, England.

Tactacan, G. B., Schmidt, J. K., Miille, M. J., Jimenez,D. R. 2013 . A *Bacillus subtilis* (QST 713) spore-based probiotic for necrotic enteritis control in broiler chickens . J. Appl. Poult. Res. 22: 825–831.

Teo, A. Y.,Tan. H., 2005. Inhibition of *Clostridium perfringens* by a novel strain of *Bacillus subtilis* isolated from the gastrointestinal tracts of healthy chickens. Appl.Environ. Microbiol. 71:4185–4190.

Timbermont, L., Haesebrouck, F., Ducatelle, R. and Van Immerseel, F. 2011. Necrotic enteritis in broilers. An Updated Review on the Pathogenesis. Avian Pathol.,.40, (4): 341-347.

Torres-Rodriguez, A., Sartor, C., Higgins, S. E., Wolfenden, A. D., Bielke, L. R. 2005. Effect of *Aspergillus* meal prebiotic *(*fermacto) on performance of broiler chickens in the starter phase and fed low protein diets. J. Appl. Poult. Res. 14:665–669.

Urdaci, M. C., and Pinchuk, I. 2004.Antimicrobial activity of *Bacillus* probiotics. Pages 171–182 in Bacterial Spore Formers: Probiotics and Emerging Applications. E. Ricca, A. O. Henriques, and S. M. Cutting, ed. Horizon Bioscience Oxford, UK,

Van Immerseel, F., De Buck, J., Pasmans, F., Huyghebaert, G., Haesebrouck, F., Ducatelle, R. 2004.*Clostridium perfringens* in poultry: an emerging threat for animal and public health. Avian Pathol. 33:537-549.

Vicente, J. L., Torres-Rodriguez, A., Higgins, S.E., Pixley, C., Tellez, G., Donoghue, A. M., Hargis, B.M. 2008. Effect of a selected *Lactobacillus* spp.-based probiotic on *Salmonella enterica* serovar Enteritidis-infected broiler chicks. Avian Dis. 52:143-146.

Williams, R.B. 2002. Anticoccidial vaccines for broiler chickens: pathways to success. Avian pathol., 31:317-353.

Willis, A.T. 1977. Anaerobic Bacteriology, Clinical and Laboratory Practice. 3rd Ed., pp: 131-133, Butter Worth, London, Boston

Willis,W.L., Isikhuemhen, O. S, Ibrahim, S.A. 2007. Performance assessment of broiler chickens given mushroom extract alone or in combination with probiotics. Poult. Sci. 86: 1856-1860.

Yun, C.H., Lillehoj, H.S., Lillehoj, E.P. 2000.Intestinal immune responses to coccidiosis. Dev. Comp. Immunol. 24: 303–324.