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Essential Oils and Organic Acid Blends Potentially Enhance Growth and Ameliorate Enteritis and Inflammatory Response After Challenge with *Clostridium perfringens* in Chickens

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Abstract: Single or combined essential oils (EOs), and organic acids (OAs) blends were used as continuous drinking water (DW) treatment for protection against *Clostridium perfringens* (*C. perfringens*) experimental infection in chickens. Random assignment of 185 one-day-old, commercial broiler chicks into 6 groups (30 chicks/group in 3 replicates) arranged as follows: a non-infected group (G1), *C. perfringens* infected (G2). In contrast, G3, and G4 were infected with *C. perfringens* and continuously supplemented with EOs, and OAs, respectively in DW from day 1. Chickens in G5 were challenged with *C. perfringens* and had a mixture of both products continuously in DW from day 1. At the same time, the birds of G6 were challenged with *C. perfringens* and treated with amoxicillin. All groups were fed a basal diet. Our results showed that combined EO and OA blends were more effective in ameliorating the devastating effects of *C. perfringens* in broilers than single treatments. In the infected-treated groups, the growth performance represented by body weight gain, and feed conversion ratio were improved ($p < 0.05$), intestinal lesion scores, and *C. perfringens* count were significantly low. Also, the intestinal histopathology and biochemical profile were improved. In addition, iNOS and IL-1β (pro-inflammatory cytokines) levels were significantly down regulated while IL-10 (anti-inflammatory cytokine) was increased ($p < 0.05$). Furthermore, the phagocytic activity was significantly ($p < 0.05$) enhanced. In conclusion, EO and OA blends significantly improved the broiler chickens' performance, inflammatory, and immune response and reduced intestinal lesions. A marked difference occurred in favor of combined than single treatments compared to antibiotic treatment facing the *C. perfringens*' infection.

Keywords: *Clostridium perfringens*; Cytokines; Broilers chickens; Essential oils; Organic acids

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1. Introduction

Maintaining intestinal integrity gut health and prevention of necrotic enteritis (NE) without antibiotic usage are major challenges for poultry production (Liu et al., 2019). Clinically, NE due to *Clostridium perfringens* (*C. perfringens*) and its extracellular toxins leads to decreased feed intake, depression, dehydration, diarrhea, and acute death in broilers. While in the subclinical form, NE generates poor growth performance and chronic intestinal mucosal damage without mortality (Van Immerseel et al., 2004, Shojadoost et al., 2012 & Cooper et al., 2013).

In the last decades, NE was highly controlled through the conventional use of therapeutic agents as a main strategy. But recently the explosion of NE predisposed by banning of antibiotic growth promoters globally shifted the research focus to exploring some other strategies to control its development (Shojadoost et al., 2012). Organic acids (OAs) and essential oils (EOs) are promising candidate alternatives for preventing NE having the advantages of safety and absence of tissue residues when compared to antibiotics (Brenes and Roura, 2010; Bassole and Juliani, 2012). Many studies showed that EOs exhibit several biological benefits such as antioxidant (Pirgozliev et al., 2019), anti-inflammatory, direct and indirect antimicrobial effect, (Liu et al., 2019), and antiviral effects (Nahed et al., 2020), improvement of growth performance (Sun et al., 2015; Peng et al., 2016; Chowdhury et al., 2018; Liu et al., 2018; Wang et al., 2019), enhancement of gut microbes (Hume et al., 2011), mitigation of the negative impacts caused by pathogenic bacteria including *C. perfringens* (Du et al., 2016; Yin et al., 2017), and significant reduction of *C. perfringens* counts in the gut and fasten the recovery of the infected birds (Mitsch et al., 2004; Du et al., 2015; Mathlouthi et al., 2012).

Organic acids (OAs) has numerous beneficial including antibacterial, intestinal integrity or gut development promotion, improvement of nutrient digestibility, higher population of beneficial microbiomes (Lactobacillus spp.), lower counts of harmful bacterial in the gut, antistress, and immune modulative which finally reflected on the animal health and productivity (Abudabos and Al-Mufarrej, 2014; Abudabos et al., 2016; Emami et al., 2017; Dittoe et al., 2018; Nguyen et al., 2020; Dai et al., 2021). Also, organic acids exert antibacterial activities against some poultry pathogens such as *E. coli* (Emami et al., 2017; Kazempour and Jahanian, 2017), *Salmonella* spp. (Kazempour and Jahanian, 2017), and *C. perfringens* (Geier et al., 2010).

Combining EOs and OAs have received considerable research attentions because these combinations are efficient on intestinal microbiome, gut health, and growth enhancement in broiler chickens (Liu et al., 2017; Yang et al., 2018, 2019; Stefanello et al., 2020). Such combinations have a lot of antimicrobial activities, against Salmonella (Cerisuelo et al., 2014; Zhang et al., 2019), *E. coli* (Basmacio glu-Malayo glu et al., 2016; Yang et al., 2019), *C. perfringens* infections (Pham et al., 2020 & 2022).

Thus, this research examines the efficacy of blend of EOs and OAs either single or combined on growth indices, intestinal health and inflammatory response of commercial broiler chickens faced *C. perfringens* infection.

2. Material and Methods

2.1. Ethical Statement

All experiment methods were conducted according to Animal Research Ethics Guidelines at Animal Health Research Institute, Egypt.

2.2. Clostridium perfringens isolate

A *C. perfringens* type A isolate (previously characterized from a broiler chicken flock) was kindly supplied by the department of bacteriology, anaerobic unit, Animal Health Research Institute, Dokki, Giza, Egypt.

2.3. Source of essential oils, organic acids and amoxicillin

The EOs used in the experiment contained carvacrol 107.25 g, thymol 29.7 g, capsaicin 4.8 g, cinnamaldehyde 10.35 g, eugenol 3.6 g, menthol 26 g, propylene glycol 125 g, glycol ricinolate 261 g, and distilled water up to 1000 ml (Virgi pharma Co., Ltd., Egypt), while the OAs consisted of formic acid 150 g, phosphoric acid 115 g, lactic acid 60 g, citric acid 50 g, copper sulphate 10 g, betaine HCL 25 g, and distilled water up to 1000 ml (Biosen chemical industries, Egypt). Also, amoxicillin trihydrate 50% (AB Pharma, Egypt) was used.

2.4. Experimental Design

Mixed sex commercial broiler chicks, Cobb 500, aged one-day old (*n*=185) were purchased from a local hatchery and used in the experimental study. Chicks were supplied with suitable basal diet (NRC, 1994) and kept in separate caged batteries under complete hygienic conditions. Randomly, 5 birds were humanly sacrificed and submitted for laboratory examination to confirm that chicks were free from bacterial (Salmonella and *E. coli*) and viral (Reo, chicken infectious anemia and adenoviruses) pathogens.

After this confirmation, 180 chicks were assigned into 6 groups (30 birds/group of 3 replicates per each). A non-infected group (G1), G2 was infected with *C. perfringens* while G3, and G4 were infected with *C. perfringens* [using a dose of *C. perfringens* was 1 ml of 1×10^8 CFU/ml for 3 successive days (15^{th}, 16^{th} and 17^{th} day old) via crop gavage (Gholamiandehkordi et al., 2007)] and continuously supplemented with EOs, and OAs, respectively in drinking water (DW) from 1 to 35 days at a dose of 0.5ml/L, G5 was infected with *C. perfringens* as mentioned above and received a combination of both products continuously via DW from 1 to 35 days, each at the same dose, and G6 was infected with *C. perfringens* and treated with amoxicillin (dose: 15 mg/kg body weight) via DW for five consecutive days after the appearance of clinical signs following experimental infection (started from the 3^{rd} day post infection, dpi).

All groups were vaccinated with live Hitchner-IB vaccine (Servac HB1, Abbasia, Egypt) at 1 day old via eye drop, an inactivated avian influenza-H9N2 plus Newcastle (H9+ND) vaccine (MEVAC, Cairo, Egypt) at 7-days old via subcutaneous injection, and a LaSota live ND vaccine (Servac LaSota, Abbasia, Egypt) at 10-days old, and a live intermediate plus infectious bursal disease (IBD) vaccine (IBD-L, Ceva Sante animal, France) at 13-days old, both via DW.

2.5. Evaluation Parameters

2.5.1. Clinical disease and mortality rate

Birds were observed daily following experimental infection till 35 days for clinical signs and mortality. Gross lesions were evaluated after death.

2.5.2. Performance traits

Body weight (BW), and feed intake (FI) were recorded weekly. Body weight gain (BWG) and feed conversion ratio (FCR) were calculated throughout the whole experiment.

2.5.3. Gross lesion scores

Lesion scoring from middle intestine (jejunum) was done on 3, 7 & 14 dpi as previously reported by Lovland et al. (2004).

2.5.4. Bacterial counting post infection

One gram of each sample (pooled jejunum and cecum) was used for the preparation of 10-fold serial dilutions in sterile PBS, then colony counting was conducted according to (Quinn et al., 1994) with some modification. Briefly, dilutions of the samples were inoculated in thioglycolate broth medium (Oxoid, Hampshire, UK) and incubated anaerobically at 37°C for 24hrs. Each dilution was streaked tryptic soya agar (TSA) agar plate and incubated anaerobically at 37°C for 24hrs for colonies counting.

2.5.5. Serum sampling

Blood samples (*n*= 6) were collected from different replicates at 31^{st} day of age. Sera were separated and different biochemical tests such as determination of serum total proteins, serum albumin, serum globulins, serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST), serum uric acid and serum creatinine were estimated using commercial kits (Stanbio, USA) (Salkie,1996).

2.5.6. Phagocytic activity and index

At 21 and 28 days-old, heparinized blood samples (*n*= 6) were collected for cellular immunity assessment. *Candida albicans* cultures (50 µL) were added to 1 mL of heparinized blood and placed in water bath with shaker at 24-26°C for three up to five hours. Then, blood smears were taken and stained with Giemsa stain. The phagocytic activity was evaluated by calculating the numbers of phagocytes containing intracellular yeast cells up to 300 macrophages and stated as percentage of phagocytic activity (PA%) using this equation; Phagocytic activity= numbers of phagocytes containing *Candida* yeast/number of Macrophages ×100. While the phagocytic index (PI) can be determined by this equation; Phagocytic index= Number of cells phagocytized divided by the number of phagocytic cells (Kawahara et al.,1991).

2.5.7. Histopathological examination

On the 31^{st} day of age, jejunum and liver tissue specimens (*n*= 3 from each group) were collected in 10% neutral buffered formalin, then routinely stained with hematoxylin and eosin (H & E) (Suvarna et al., 2013).

2.5.8. Intestinal gene expression

2.5.8.1. Sample collection (Cecal tissues)

Three random birds from each group were humanely culled at 3 dpi, their cecal tissues were collected, washed in PBS, snap-frozen in liquid nitrogen, and stored at-80 °C for quantification of gene expression.

2.5.8.2. RNA extraction

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

2.5.8.3. SYBR green real-time polymerase chain reaction (rRT-PCR)

The used primers for *β -actin*, IL-1*β*, iNOs, and IL-10 were chosen according to Abdul-Careem et al. (2008), Strong et al. (2015), Hassanpour et al. (2009), Rothwell et al. (2004) and supplied from Metabion (Germany). The total utilized reaction was a 25µl containing 10µl of the 2x *HERA* SYBR® Green RT-qPCR Master Mix (Willowfort, UK), 1µl of RT Enzyme Mix (20X), 0.5 µl of each primer of 20 pmol concentration, 5µl of water, and 3µl of RNA template. The reaction was performed in a step one rRT-PCR apparatus. Amplification curves and cycle threshold (Ct) values were determined by the step one software. To estimate the variation of gene expression on the RNA of the different samples, the Ct of each sample was compared with that of the positive control group according to the "ΔΔCt" method stated by Yuan et al. (2006) using the following ratio:($2^{-\Delta\Delta ct}$).

Table (1): Primer sequences, amplicon sizes and cycling conditions used for SYBR green rt-PCR

Target gene	Primers sequences	Reverse transcription	1st Denaturation	Amplification (40 cycles)			References
				2nd denaturation	Annealing	Extension	
B- actin	CAACACAGTGCTGTCTGGTGG	50˚C 30 min	94˚C 15 min	94˚C 15 sec.	55˚C 30 sec	72˚C 30 sec	Abdul-Careem et al. (2008)
	ATCGTACTCCTGCTTGCTGAT						
IL-1*β*	TGCTGGTTTCCATCTCGTATGTAC						Strong et al. (2015)
	CCCAGAGCGGCTATTCCA						
iNOs	AGGCCAAACATCCTGGAGGTC						Hassanpour et al. (2009)
	TCATAGAGACGCTGCTGCCAG						
IL-10	CATGCTGCTGGGCCTGAA						Rothwell et al. (2004)
	CGTCTCCTTGATCTGCTTGATG						

2.6. Statistical analysis

SPSS 20 was applied using Variance Analysis (ANOVA). Significant differences with Tukey's post hoc test were calculated at $p < 0.05$. RT-PCR data were analyzed using Graphpad prism 5.

3. Results

3.1. Clinical signs and mortalities

In the *C. perfringens* infected groups, clinical signs started at the 3rd dpi such as inappetence, depression, emaciation, ruffled feather and brownish diarrhea. Birds in infected-treated groups (G3, G4, G5, and G6) had milder clinical signs than those in the positive control (G2). The mortalities started on the 5th dpi reaching 20% in G2, 13.3% in both (G3 and G4), 10% in G5 and 6.66% in G6. No clinical signs or mortalities were observed in the non-infected control group (G1).

3.2. Chickens' performance after experimental infection

Non-significant ($p > 0.05$) differences in BW, BWG, FI, and FCR were recorded at 7th day of age in all groups but after at the end of the 2nd week, G5 supplemented with mix of EOs and OAs blends had significant ($p<0.05$) differences in BW, BWG and FCR followed by G4 and G3 as compared G1, G2 and G6 (**Table 1**). During the 3 weeks after challenge (3rd-5th week), there was significant increase ($p < 0.05$) in BW, BWG, and decreased ($p < 0.05$) in FCR in the challenged-treated groups (G3-6) as compared to G2. In addition, the final BWG was significantly ($p < 0.05$) higher in G6 followed by G5 then G4 and G3, however, all the 4 groups had a significant ($p < 0.05$) decrease in FCR compared to G2 [(non-significant difference ($p > 0.05$) between them].

3.3. Intestinal lesion score

Generally, all the infected groups showed macroscopic lesion scores in the jejunum such as hyperemia, thin wall, gases and yellowish-brown fluid. In addition, focal or diffused necrotic flakes of the intestinal mucosal surface. Birds in G6, and G5 showed the lowest significant ($p < 0.05$) scores as compared to the control group (G2) which had the highest one (**Table 2**).

3.4. C. perfringens re-isolation rates

The infected birds had a significantly ($p < 0.05$) higher count of *C. perfringens* in the intestine than those non-infected. The infected-treated birds had significant ($p < 0.05$) reduction in *C. perfringens* counts compared to G2. The most relevant and significant ($p < 0.05$) finding were in G6 followed by G5. Also, birds in G3 and G4 had the same significantly reduced *C. perfringens* count compared to G2 although they showed significantly higher numbers compared with G6 and G5 at 3, 7 and 10 dpi (**Table 3**).

3.5. Serum Biochemical Parameters

C. perfringens infection showed a decrease ($p < 0.05$) in total protein and albumin with an increase ($p < 0.05$) of globulin level in all infected groups (G2-6) as compared to G1. Moreover, increased ($p < 0.05$) serum AST, and ALT levels as well as uric acid and creatinine levels in the infected groups was recorded, however, this level was significantly ($p < 0.05$) lower in G6 (**Table 4**). The combined supplementation of EOs and OAs (G5) resulted in positive effects mitigating the adverse effects of infection and decreasing some biochemical indices towards their normal levels which was comparable to G6.

3.6. *Phagocytic activity and phagocytic index*

A significant decrease ($p < 0.05$) of phagocytic% and phagocytic index in G2 appeared when compared with G1 (**Table 5**). Phagocytic% and phagocytic index were increased ($p < 0.05$) in in all the infected treated groups as compared to G2 with the highest value were observed in G6 and G5.

Table 1. The effect of EOs, OAs and their combination on broiler performance in all groups

Parameter	G1	G2	G3	G4	G5	G6
Initial weight	41.7±0.882^{a}	42±1.154^{a}	40.3±0.333^{a}	42.3±0.33^{a}	41.7±1.2^{a}	41.3±0.882^{a}
Final BW	2005±1.52^{a}	1750±1.73^{f}	1819±1.52^{e}	1856.7±1.2^{d}	1897±2.08^{c}	1915±1.15^{b}
BWG	1963±0.882^{a}	1708.3±1.452^{e}	1777.6±0.882^{d}	1814.3±0.882^{d}	1855.3±1.763^{c}	1873.8±1.202^{b}
FI	3220±6.45	3070.7±6.83	3085±6.54	3116±7.01	3143±5.89	3187±6.61
FCR	1.64± 0.01^{c}	1.80±0.03^{a}	1.74±0.01^{b}	1.72± 0.01^{b}	1.70± 0.02^{b}	1.70± 0.02^{b}

*Different superscripts in the same row indicate significant changes ($p < 0.05$).

Table 2. Effect of EOs, OAs and their combination on intestinal gross lesion scores of broiler chickens in all groups

Days post infection	G1	G2	G3	G4	G5	G6
3	0±0.00^{c}	4±0.00^{a}	3.33±0.33^{ab}	3.33±0.33^{ab}	3±0.00$^{a\ b}$	2.3±0.33^{b}
7	0±0.00^{d}	3.3±0.33^{a}	2.6±0.38^{ab}	2±0.00^{bc}	1.7±0.33^{c}	1.3±0.33^{c}
14	0±0.00^{d}	2.3±0.33^{a}	1.6±0.33^{ab}	1±0.00^{bc}	0.7±0.33^{cd}	0±0.00^{d}

*Different superscripts in the same row indicate significant changes ($p < 0.05$).

Table 3. *C. perfringens* re-isolation rates from pooled jejunum and cecum samples of broiler chickens in all groups. Counts were calculated as log CFU/mL

Days post infection	G1	G2	G3	G4	G5	G6
3	3.23±0.012^{e}	7.22±0.012^{a}	7.16±0.014^{b}	7.12±0.023^{b}	7.04±0.04^{c}	6.94±0.015^{d}
7	3.21±0.0^{e}	7.46±0.015^{a}	6.64±0.04^{b}	6.62±0012^{b}	6.16±0.011^{c}	5.13±0.038^{d}
10	3.08±0.015^{e}	7.77±0.017^{a}	6.33±0.017^{b}	6.30±0.018^{b}	5.95±0.20^{c}	4.45±0.029^{d}

*Different superscripts in the same row indicate significant changes ($p < 0.05$).

Table 4. Some biochemical values of *C. perfringens* infected chickens in all groups at 31 days old

Parameter	G1	G2	G3	G4	G5	G6
Total protein	4.73±0.02^{a}	4.58±0.01^{d}	4.61±0.01^{d}	4.66±0.01^{c}	4.7±0.02^{b}	4.7±0.01^{b}
Albumin	2.62±0.02^{a}	2.37±0.01^{e}	2.43±0.03^{d}	2.5±0.02^{c}	2.56±0.04^{b}	2.51±0.02^{c}
Globulin	2.11±0.03^{d}	2.21±0.06^{a}	2.18±0.12^{ab}	2.16±0.01^{bc}	2.13±0.12^{cd}	2.17±0.10^{b}
A/G	1.14±0.11^{a}	1.06±0.02^{d}	1.11±0.14^{c}	1.16±0.02^{b}	1.2±0.10^{b}	1.18±0. 12^{b}
AST	48.7±1.76^{d}	84.7±1.56^{a}	65.6±1.45^{b}	60.3±1.63^{c}	58±1.73^{c}	52±1.45^{d}
ALT	9.22±0.21^{d}	20.6±0.95^{a}	13.79±0.17^{b}	13.6±0.15^{b}	11.19±0.23^{c}	10.5±0.20^{c}
Uric acid	5.21±0.0 1^{f}	11.4±0.0 4^{a}	6.91±0.02^{b}	6.51±0.07^{c}	5.51±0.05^{e}	5.89±0.07^{d}
Creatinine	0.85±0.02^{e}	1.94±0.021^{a}	1.73±0.03^{b}	1.24±0.06^{c}	0.89±0.03^{e}	0.94±0.02^{d}

*Different superscripts in the same row indicate significant changes ($p < 0.05$).

Table 5. Effect of EOs, OAs and their combination on blood phagocytic activity and index in broiler chickens of all groups at 21 and 28 days old

Parameters	G1		G2		G3		G4		G5		G6	
	21 d	28 d	21 d	28 d	21 d	28 d	21 d	28 d	21 d	28 d	21 d	28 d
Phagocytic (%)	75±1.5^{a}	77±1.7^{a}	46±1.6^{c}	50±1.5^{d}	55±1.7^{b}	59±1.2^{c}	58±1.3^{b}	62±1.7^{bc}	61±1.5^{b}	66±1.8^{b}	71±1.4^{a}	74±1.2^{a}
Phagocytic index	4.5±0.12^{a}	4.9±0.23^{a}	1.2±0.13^{e}	1.8±0.20^{d}	2.4±0.21^{d}	2.8 ±0.17^{c}	2.7±0.20^{dc}	3.1±0.17^{c}	3.2±0.13^{bc}	3.8±0.14^{b}	3.6±0.22^{b}	4.3±0.16^{b}

*Different superscripts in the same row indicate significant changes ($p < 0.05$).

3.7. Effect of Dietary supplementation of EOs and OAs on cytokine expression

The expressions of IL-1*β*, iNOS and IL-10 genes in cecal tonsils at 3rd dpc shown in **Figure 1**. There was a significant ($p < 0.05$) up regulation of pro-inflammatory cytokines IL-1*β* and iNOS and a significant ($p < 0.05$) down regulation of anti-inflammatory cytokine IL-10 in chickens of G2-6 as compared to G1. Chickens in G5 and G6 had significant ($p < 0.05$) lower pro-inflammation compared to G2-4. All the infected treated groups (G3-6) had significant ($p < 0.05$) down-regulations in IL-1*β* and iNOS and significant ($p < 0.05$) increase in IL-10 gene expression at 3rd dpc as compared to G2 with significant ($p < 0.05$) higher percent in G6 (**Figure 1**).

3.8. Histopathological examination of jejunum and liver

All intestinal layers of chickens in G1 aged 31 days old appeared within normal histology (**Figure 2a**). While the intestinal lumen of birds in G2 contained necrotic debris, RBCs sloughed mucosa, mucosal sheet and inflammatory cells. Diffuse coagulative necrosis was involved in the superficial mucosa, and villous enterocytes with mucosal inflammatory cell infiltration mainly lymphocytes and mild hyperplasia of intestinal crypts in submucosa with partial hyalinization of the muscular coat at 14 dpi (**Figure 2b**). The intestine of chickens in G3 showed moderate changes characterized by partial necrosis of intestinal villi with mild necrotic debris and desquamated epithelial sheets inside the lumen with mild hyperplasia of the intestinal glands and lymphocytic infiltration in mucosa and submucosa at 14 dpi (**Figure 2c**). The intestine of chickens in G4 had necrotic tips of intestinal villi with mild inflammatory cells infiltration and clusters of bacterial colonies (**Figure 2d**). Moreover, regenerative villous enterocytes in deeper mucosa and submucosa from proliferative intestinal crypts forming enlarged layer with goblet cells metaplasia were encountered at 14 dpi. The intestine of chickens in G5 showed mild desquamation of villous enterocytes and intense hyperplasia of submucosal intestinal crypts which extended superficially to replace villous enterocytes with mucosal and submucosal lymphocytic infiltration at 14 dpi (**Figure 2e**). A great ameliorative effect was seen, and many intestinal coats restore their normal histomorphology picture beside hyperplastic lymphoid follicles and goblet cells. Finally, chickens in G6 showed partial sloughing of superficial mucosa with intense hyperplasia of villous enterocytes and intestinal glands in which resulted in broad and thickened villi lined by several rows containing numerous goblet cells (**Figure 2f**).

The examined hepatic parenchyma of chickens in G1 was apparently normal (**Figure 3a**). The liver of chickens in G2 showed intense and diffuse necrosis mainly periportal of the hepatic parenchyma with hemorrhage. Portal leukocyte infiltrates varied from lymphocytes or heterophils with biliary epithelium and cholestasis could be seen (**Figure 3b**). The liver of chickens in G3 showed mild portal interstitial lymphocytic aggregation and hyperplastic Kupffer cells and bile ductules (**Figure 3c**). The hepatic cells of chickens in G4 were apparently normal at 14 dpi (**Figure 3d**). The liver of chickens in G5 showed dilated hepatic sinusoids and regenerative hepatic cells at 14 dpi (**Figure 3e**). Finally, the liver of chickens in G6 had mild reversible changes mainly acute cell swelling hyperplastic Kupffer cells with portal mononuclear cell aggregations and numerous bile ductules at 14 dpi (**Figure 3f**).

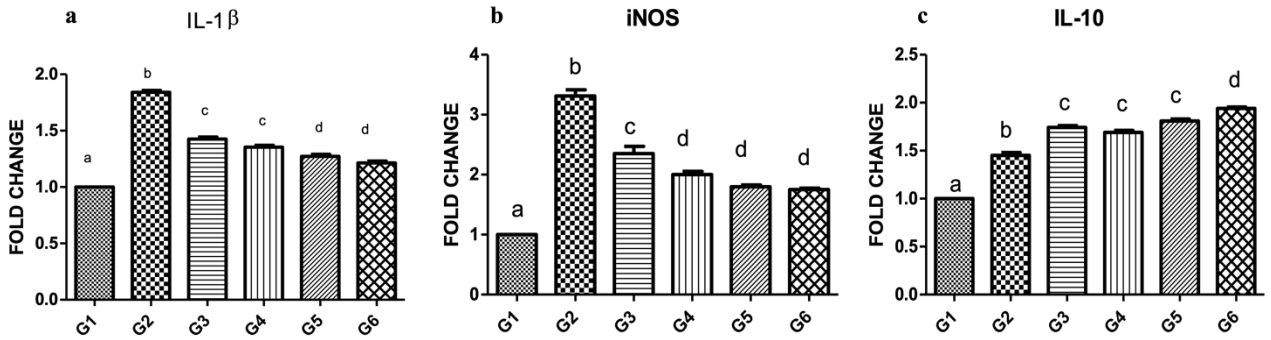

Figure 1. Gene expression of IL-1*β* (a), iNOS (b) and IL-10 (c) in the cecum tonsils of *C. perfringens* infected chickens at 3 dpi. The values are means ± SEM.

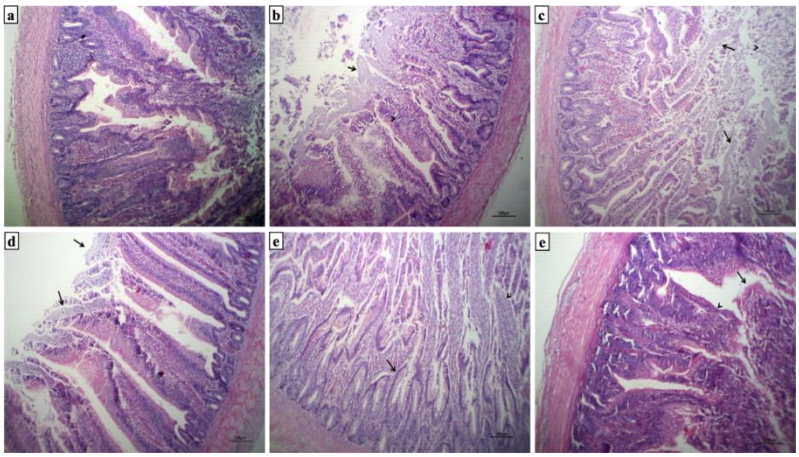

Figure 2. (a) Intestine of a chicken in G1 showing apparently normal intestinal histology at 31 days old. (b) Intestine of a chicken in G2 showing diffuse necrosis of superficial mucous and villous enterocyte (arrow) with mild inflammatory cells infiltration (arrowhead) at 14 dpi (31 days old). (c) Intestine a chicken in G3 showing necrotic changes in the upper third of the intestinal villi (arrow) with intense lymphocytic infiltrations and necrotic debris and sheets inside the lumen (arrowhead) at 14 dpi. (d) Intestine of a chicken G4 showing necrotic villus tips (arrow) containing inflammatory cells and thickened villi at 14 dpi. (e) Intestine of a chicken G5 showing intense hyperplastic intestinal crypts (arrow) replacing degenerated and necrotic villus enterocytes with mild leukocytic infiltration (arrowhead) and goblet cells metaplasia at 14 dpi. (f) Intestine of a chicken G6 showing partial sloughing of superficial mucosa (arrow) with broad and thickened deeper villi from proliferative intestinal crypts containing numerous goblet cells (arrowhead) at 14 dpi. H&E.

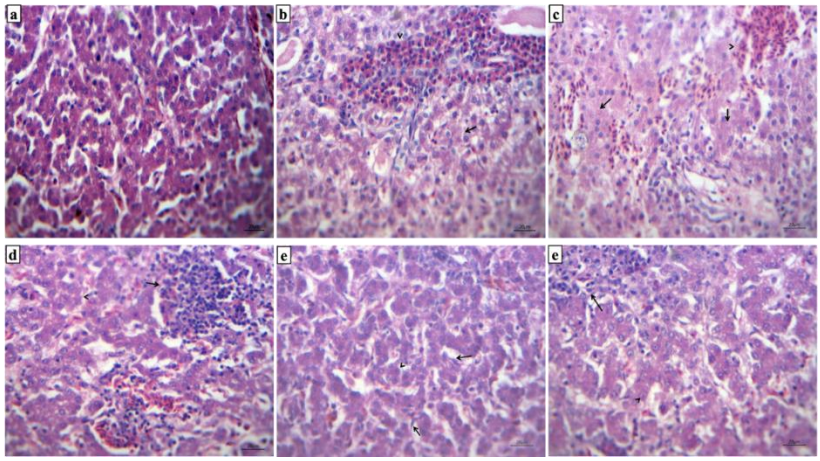

Figure 3. (a) Liver of a chicken in G1 showing apparently normal hepatic parenchyma at 31 days old. (b) Liver of a chicken in G2 showing intense degeneration and necrosis (arrow) of the hepatic cells with heterophilic aggregation (arrowhead) and cholestasis at 14 dpi (31 days old). (c) Liver of a chicken in G3 showing focal degeneration and necrosis of the hepatic cells (arrow) and hemorrhages (arrowhead) at 14 dpi. (d) Liver of a chicken in G4 showing portal and interstitial lymphocytic aggregates (arrow) and apparently normal hepatic cells (arrowhead) at 14 dpi. (e) Liver of a chicken in G5 showing hyperplastic kuffer cells (arrow) dilated hepatic sinusoids and normal hepatic cells (arrowhead) at 14 dpi. (f) Liver of a chicken in G6 showing acute cell swelling (arrowhead) of the hepatic cells and mononuclear cell aggregation (arrow) at 14 dpi. H&E.

4. Discussion

In the present study, two different blends of EOs and OAs had been tested to counteract the wasting effect of *C. perfringens*. The appeared clinical signs in the infected group may be attributed to disturbance in the metabolic activity in liver caused by *Clostridium* toxins as recorded previously by Johansson, (2006) and Saleh et al. (2011). Although, in the infected-treated chickens milder clinical symptoms and lower mortality rates appeared which might be related to efficacy of EOs and OAs blends either alone or in combination in controlling of necrotic enteritis as previously proved (Eid et al., 2018; Khodary et al., 2019; Pham et al., 2020; Kumar et al., 2021).

Regarding bird's performance, the continuous intake of birds with EOs and OAs blends in drinking water showed higher (p<0.05) BW, BWG, and better FCR values in infected treated groups (G3-6) during the whole experiment unlike those of G2. The highest BWGs were found in G6 and G5 followed by G4 and G3, respectively. On other hand, FCR was similarly recorded between all the infected-treated groups. Previous findings declared that EOs and OAs supplementation improved broiler chickens' performance and controlled the reproduction of *C. perfringens* (Pereira et al., 2015; Eid et al., 2018 and Kumar, et al., 2021). EOs enhanced FI, improved nutrient digestion and absorption which resulting higher bird performance (Khan et al., 2012; Abudabos et al., 2016; Abdelli et al., 2020). The positive impact of OAs on growth attributed to the enhanced feed utilization, inhibiting pathogenic microbes, and the suitable environment for the harmless bacterial growth (Iqbal, 2016; BaghbanKanani et al., 2019). These positive effects of both EOs and OAs may be further potentiated when these are combined (Bozkurt et al., 2012 and Abdelli et al., 2020).

Supplementation of EOs and OAs increased villus height and muscular layers of the duodenum that enhance feed efficiency (Yang et al., 2018). On the other hand, Celik and Sahin (2015) and Due et al. (2016) reported that EOs and/or OAs feed additives to the broiler rations had no beneficial effects in BW and BWG which is contradictory to our study. The difference between the research results may be due to the use of different forms or mixtures of EOs and OAs (Özsoy et al., 2017).

In this study, both OAs and EOs significantly reduced the intestinal lesion scores of treated chicken groups when compared with the infected G2. The lowest lesion score were recorded in G6 and G5 followed by G4 and G3, respectively. These findings declared the beneficial effect of these blends on intestinal health and integrity. Similar results were mentioned by (Abudabos et al., 2018; Abdelli et al., 2020; Kumar et al., 2021). Regarding the results of cecal *C.*

perfringens count, our results showed that EOs and OAs significantly reduced *C. perfringens* count at 3, 7 and 10 dpi and were comparable to the antibiotic treatment in G6. *C. perfringens* count was significantly reduced in G5, G3 and G4, respectively. While, there was no reduction in *C. perfringens* count between G3 and G4. Previous studies reported that EOs and OAs combination could decrease the *C. perfringens* colonization and reproduction in chicken guts (Çelikbilek et al., 2014). This may be due to the ability of EOs to damage the bacterial cell membrane (Lippens et al., 2005), and the capability of OAs penetrate into cytoplasm, reduce the intracellular pH and disturb the bacterial metabolism causing the death of pH sensitive bacteria, such as *E. coli*, Salmonella, and *C. perfringens*. These mechanisms characterize the synergic antibacterial action of the EOs and OAs (Zhou et al., 2007 and Bozkurt et al., 2012). Similar studies revealed that EOs and OAs could restore the homeostasis of the chickens' intestine challenged by suppressing intestinal pathogens such as *C. perfringens*, *E. coli*, and Salmonella (gluMalayo et al., 2016; Yang et al, 2019), thus reducing the gross and histopathological lesion scores (Abdelli et al., 2020; Stefanello et al., 2020) and improving gut morphological structure and integrity (Paul et al., 2007; Jerzsele et al., 2012; Çelikbilek et al., 2014; Chowdhury et al., 2018). Therefore, our results suggested that EOs and OAs had major advantages in reducing the severity of NE.

The protein profile is used as humoral immunity markers (Rosas et al., 2019). Herein, *C. perfringens* infection in broilers produced significant decrease in total protein and albumin with a significant increase of globulins. The hyperglobulinemia is mainly due to the bacterial septicemia that lead to lymphocyte stimulation, and differentiation into T and B types resulting in increase of globulins levels and acquired immune response (Coles, 1986). Also, Salah et al. (2015) and Khodary et al. (2019) reported a significant increase in the serum total protein and globulin levels in *C. perfringens*-infected chickens. In contrast, El-Sheikh et al. (2018) reported that there was a significant decrease in serum levels of globulins in *C. perfringens*-infected chickens. The decrease in albumin levels may be a result of low feed intake, the loss through the intestine and the kidneys, failure of liver to synthesize albumin due to damage by clostridial toxins (Løvland & Kaldhusdal, 1999).

Treatment of *C. perfringens* infection in broilers with EOs and OAs induced a significant improvement of serum total proteins and albumin coupled with decrease of total globulins suggesting the efficacy of treatment and the potent immunostimulant effect of these blends. Moreover, *C. perfringens* infection resulted in significant increase of AST, and ALT levels denoting hepatic damage and biliary stasis caused by clostridial toxins (Fraser et al., 1991). Also, there were significant increase in uric acid and creatinine levels in *C. perfringens*-infected chickens which may be due to cellular necrosis and degeneration of renal tubules of the kidneys caused by clostridial toxins, that prevent their excretion (Kaneko, 1980). However, these effects were alleviated and improvements of liver and kidney functions were noticed in the infected-treated groups (G3-6). This may be due to the ameliorative effects of EOs and OAs facing *C. perfringens* toxicity in liver and kidney. Our findings are in line with Abudabos et al. (2018) and Khodary et al. (2019).

Phagocytes (natural innate immune defense) play an important role in resistance to infection through the engulfment and degradation of the invading microorganisms. Also, they produce nitric oxide that kills intracellular microorganisms and secrete many different proteins such as lysosomal enzymes and cytokines that play a key role in regulating immunity as they are essential effector molecules of innate and adaptive immunity that could counteract the pathogenic microorganisms. Here, broiler chickens infected with *C. perfringens* showed a significant decrease of phagocytic % and phagocytic index which may be attributed to the negative effect of *C. perfringens* infection on the bird's immune system (Salah et al., 2015; Khodary et al., 2019). However, treatments of chickens with amoxicillin, OAs or combined EOs, and OAs improved these immunological parameters (Ryzner et al., 2013; Khodary et al., 2019).

C. perfringens infection in G2 induced an inflammatory immune response that was characterized by significant up-regulation of proinflammatory cytokines (IL-1β and iNOS) and significant down-regulation of IL-10 in the cecal tonsils, indicating induced inflammation. Previous studies demonstrated that several pro-inflammatory (IL-1β and iNOS), and anti-inflammatory (IL-10) cytokines were produced in response to *C. perfringens* experimental infections (Zhang et al., 2019; Daneshmand, et al., 2022). IL-10, a critical anti-inflammatory cytokine, acts as an inflammation feedback factor to modulate the immune response (Sanjabi et al., 2009). The continuous DW supplementation of EOs and OAs significantly reduced level of these proinflammatory cytokines, IL-1β and iNOS, which are the markers of inflammation. Therefore, the downregulation of pro-inflammatory cytokines expression levels in *C. perfringens* challenged and treated groups is indicative for the improvement in host innate immune responses and the alleviation of inflammation. The anti-inflammatory activity of the EOs and OAs blends may be associated with their antimicrobial activity (Zeng et al., 2015). The pro-inflammatory cytokines in the gut of broiler chickens challenged with *C. perfringens* was downregulated by EOs (Du et al., 2016) or OAs (Liu et al., 2019). Although, Pham et al. (2022) reported that there were no changes observed in the levels of IL-1β and IL-10, between the antibiotic growth promotors-treated broiler chickens and infected groups supplemented with different levels of blends containing EOS and OAs.

Here in this work, the intestine of chickens experimentally infected with *C. perfringens* showed different lesions including focal necrosis of superficial mucous and villous enterocyte with mild inflammatory cells infiltration and extensive round cell. Similar findings were reported by (Hussein et al., 2020; Stefanello et al., 2020). While, intestine of chickens challenged with *C. perfringens* and treated with EOs showed partial necrosis of intestinal villi with mild necrotic debris and mild hyperplasia of intestinal glands with lymphocytic infiltration in mucosa and submucosa. Eid et al. (2018) also stated that EOs can elucidate the changes of intestinal microbes can reduce necrosis in jejunum and cecum of chicks challenged with *Clostridium*. On the other hand, intestine of chicks challenged with *C. perfringens* and treated with OAs showed necrotic changes in intestinal villi with mild lymphocytic infiltrations and necrotic debris. Organic acids can inhibit pathogens in the intestinal environment and they have an important role in the development and reparation of intestinal wall (Paul et al., 2007). The combination of both EOs and OAs in G5 lead to lesser pathological changes in the intestinal mucosa rather than G3 or G4 supplemented with either EOs or OAs alone, respectively. Also, the liver of chickens in G5 had better enhancement and regeneration with lower lymphocytic aggregations, and necrosis as previously reported by Gkretsi et al. (2007), Swaggerty et al. (2018) and Hussein et al,. (2020).

5. Conclusion

The blend of EOs and OAs were effective in alleviating harmful effects of *C. perfringens* in broiler chickens, as indicated by enhanced BWG, improved the intestinal histopathology, and biochemical profile, reduced the intestinal counts, and improved the intestinal lesion scores of *C. perfringens*. Moreover, an increase immune response and improvement of phagocytic activity and modulation of the inflammatory cytokine response were recorded after EOs and OAs as compared to antibiotic treatment (amoxicillin).

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