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Opposing of Necrotic Enteritis by Phytonutrients and/or Acidifiers in Broiler Chickens

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Abbreviations: *C. perfringens=Clostridium perfringens, FCR=Feed conversion ratio, BW=Body Weight, BWG= Body Weight Gain, PC=Positive control, NC=Negative control, GIT=Gastrointestinal tract, NE=Necrotic enteritis, TAB=Total aerobic Bacteria, AGP=Antibiotic growth promoters, PI=Post infection, BF=Bursa of Fabricus, T1=Chicken group supplemented with microencapsulated phytonutrient, T2=Chicken group supplemented with an acidifiers blend, T3=Chicken group supplemented with microencapsulated phytonutrient and an acidifiers blend, PBS =Phosphate buffered saline, ND=Newcastle disease, HI=Haemagglutination inhibition.*

Abstract: *The protective effect of orally supplemented plant-derived microencapsulated phytonutrients [Capsicum oleoresin (Capsicum annuum) and Turmeric oleoresin (Curcuma longa)] and/or a protected acidifiers blend on experimentally induced NE, intestinal and caecal colonization of C. perfringens and TAB, immune status assessment, growth performance and carcass yield of broiler chickens was elucidated in the present investigation.*

Broiler chicken groups were continuously fed with a standard diet or supplemented standard diet from hatch up to slaughter (d 35 of age). Obtained results clarified that broiler diets supplemented with microencapsulated phytonutrients and/or dietary acidifiers blend significantly improved BW and BWG (P<0.05), numerically improved FCR, decreased feed consumption and mortality rate, increased production number vs. PC group. They improved carcass characteristics, reduced macroscopic and microscopic lesion scores associated with C. perfringens infection. They reduced intestinal and cecal colonization of both C. perfringens and TAB and improved the immune status assay. It could be assumed that they can make a valuable contribution to flock health and safety of food and could help in sustaining the GIT integrity damaged by C. perfringins infection and could be a tool for impedance of NE in broiler chickens.

Keywords: *Microencapsulated Phytonutrients, Dietary Acidifiers, Necrotic Enteritis, Broiler chickens.*

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I. Introduction

To reach broiler maximum potential for growth and feed efficiency, earlier establishment of their immunity and intestinal integrity were focused **(Ferket 2011).** For non-antibiotic growth promotion and antibacterial action; natural alternative concepts were highly commendable. One promising new possibility to achieve this goal was the use of phytonutrients to enhance feed efficiency, gut health, and innate immunity **(Lillehoj *et al.*, 2010, Awaad *et al.*, 2014a). Lee *et al.* (2010)** referred that *Curcuma/Capsicum* supplemented diet enhanced chicken weight gain. The auspicious effects of microencapsulated phytonutrients could be attributed to its technology that helps in preventing degradation in the proximal gut and delivers the bioactive compounds along the intestinal tract. The synergistic antimicrobial activity of *Capsicum oleoresin*, the anti-oxidant as well as the anti-inflammatory properties of *Curcuma longa* (turmeric) play a major role in this activity **(Sodsai *et al.*, 2007; Mannangatti and Narayanasamy, 2008).** On the other hand; acidifiers can be a part of the feeding concept to fill the gap from the antibiotics and to replace AGP **(Lückstädt, 2003, Awaad *et al.*, 2014 b).** It was already documented that dietary acidifiers lower the gastric pH, resulting in increased activity of proteolytic enzymes, improved protein digestibility and inhibiting the proliferation of pathogenic bacteria in GIT (**Kim *et al.*, 2005)**. The interest of using protected organic acidifiers into the feed of broiler chickens submitted to *C. perfringens* infection is considered a novel and effective alternative to antibiotics **(Awaad *et al.*, 2011)**. Because of their pH-reducing and antimicrobial effects; dietary acidifiers appeared as one

of the most feasible and functional alternative to AGP **(Eshak *et al.*, 2016, Awaad *et al.*, 2016).** Usage of gut acidifiers has been proven to be of immense help in maintaining the microbial balance of the gut and keeping it healthy **(Dhawale, 2005).**

Although *C. perfringens* belongs to the resident microbiota **(Sengupta *et al.*, 2011),** this microorganism along with predisposing factors such as mucosal damage is prerequisites for developing of NE among rapidly growing broiler chickens **(Llanco *et al.*, 2012).** The organism is relatively innocuous unless cofactors such as immunosuppressive affections occur **(Barnes, 1997).** This bacterium's pathogenicity is largely derived from its prolific ability to express protein toxins that threatens host GIT health and livability **(Kohler *et al.*, 1974, Hofarce, 1998, McClane, 2001, Craven *et al.*, 2001).** The issue of controlling NE and other enteropathogens without the use of AGP is becoming a big challenge. Accordingly; natural alternatives concepts based on natural ingredients for GIT integrity and antibacterial action became highly commendable and emerging the direction towards the use of natural control methods **(Awaad *et al.*, 2011, Awaad *et al.*, 2013)**. Organic acids and plant derivatives have a long history of use in animal nutrition as alternative to conventional AGP and growth enhancers **(Partanen and Mroz, 1999).**

NE is a dreaded disease produced by *C. perfringens* that affects the poultry industry worldwide causing serious economic losses, about of two dollar billions/year **(Kaldhusdal and Lovland; 2000, Van Immerseel et al, 2009, Cooper et al., 2009).** NE occurs in broilers aging between 2-6 weeks **(Songer, 1996 and Cooper and Songer, 2010).** It threatens GIT health and livability of many poultry flocks (**Craven *et al.*, 2001**). Many recent studies of NE have focused on finding different ways to control this disease **(Shojadoost *et al.*, 2012).**

The purpose of this study is to adopt a semi-field trial in an attempt to elucidate the effect of supplementation of a microencapsulated standardised combination of *capsicum oleoresin* and *curcuma oleoresin* and/or a protected pure distilled fatty acids blend on experimentally induced NE, intestinal and caecal colonization of both *C. perfringens* and TAB, immune status assessment, growth performance and carcass yield of broiler chickens.

II. Material And Methods

Experimental birds: Day-old male Arbor Acres plus broiler chickens (n=750) were used in this study. Duration of the trial extended from one day of age up to slaughter (35 days). These birds were allotted into 5 equal treatments (groups 1-5) consisting of 150 birds each assigned into 6 equal replicates of 25 birds. All experimented chickens were floor reared in separate pens at a density of 10 birds/m^2 with fresh wood shavings as bedding with a thickness of approximately 10 cm on a concrete floor and ran contemporaneously. Chickens of all groups were vaccinated via intra-ocular route and subcutaneous route, respectively with Hitchner B1+H120 vaccine and avian influenza inactivated H5N2 vaccine (Intervet International, B.V. BOXMEER-Holland) at 7th and 10th day of age, respectively. La Sota vaccine (Pfizer) and 228E IBDV vaccine (freeze-dried live vaccine Noblis Gumboro 228E strain from Intervet International, B.V. BOXMEER-Holland) were given at 14 and 18 days of age respectively via intra-ocular route.

Experimental Design: Chickens of all groups fed on standard diet. Those of 1st group (T1) supplemented with 100 ppm of a phytonutrient [encapsulated micro-particles containing a standardized combination of 2 appetizing spices: an oleoresin of Turmeric (*Curcuma spp.*) and an oleoresin of Chilli pepper (*Capsicum spp.*)]. Birds of 2nd group (T2) supplemented with 1000 ppm. of an acidifiers blend (pure distilled fatty acids of palm oil, coconut, rapeseed and sunflower). Those of 3rd group (T3) supplemented with both together. While chickens of 4th and 5th groups received standard diet without any supplementation. For experimental induction of NE; experimented birds were immunosuppressed by vaccination against infectious bursal disease (IBD) using 228-E vaccine (intermediate plus strain) that was given at 14th day of age **(McReynolds *et al.*, 2004)**. At the same day of age; birds of groups 1-4 were individually infected by crop gavages with 4×10^8 CFU/ml/bird of *C. perfringens* in PBS for 4 successive days after **Olkowski *et al.* (2006), Gholamiandehkordi *et al.* (2007)** and **Timbermont *et al.* (2009).** The used strain of *C. perfringens* is type A B2 NET B, isolated from cases of chicken NE. Chickens of groups 4 and 5 served as PC and NC groups respectively.

Feeding: From d 1-16 of age, the birds received a starter diet (23% crude protein; 3000 kcal/Kg ME), from d 17-28 of age a grower diet (21% crude protein; 3100 kcal/Kg ME) and from d 29-35 of age a finisher diet (19% crude protein; 3150 kcal/ Kg ME). The diet compositions are indicated in **Table 1**. No antibiotics were administrated in water or feed for the whole experimental period. Anticoccidial drug semduramycin (Pfizer) was added at a rate of 25 ppm. Birds had free access to feed and water.

Measured parameters:

1.Zootechnical performance and carcass characteristics assay: For chicken performance response variables BW, BWG, feed consumption (g/d/bird), FCR (g feed/g live body wt.) and production number were determined according to **Brady (1968), Sainsbury (1984), North (1984)** and **Timmerman *et al.* (2006).** All birds weighed individually at 1st day and at 5th wk of age. Feed consumption measured on the same days of birds weighting. Carcass characteristics (dressing %, front part %, hind part %, breast meat %, thigh drumstick %, carcass meat %, heart wt. %, gizzard wt. %, liver wt. %, giblet wt. %, and intestinal length and diameter) were measured on randomly chosen 10 birds/group at the end of the experiment.

2- Health status and Mortality assay:

During the evaluation; the health status and mortality of the birds were checked daily.

3- Macroscopic lesion scoring of gut:

Necropsy was adopted on 12 randomly selected birds (2 birds/replicate) at day 21 and 28 of age (one and 2 weeks PI) for gross pathological lesion scoring of the small intestine. The scoring system criteria are the six-point system of **Keyburn *et al.* (2006)** modified by **Shojadoost *et al.*(2012)** as follows:

0 =No gross lesions.
1 =Thin or friable walls, or diffuse superficial but removable fibrin.
2 = Focal necrosis or ulceration, or non-removable fibrin deposit 1 to 5 foci.
3 = Focal necrosis or ulceration, or non-removable fibrin deposit 6 to 15 foci.
4 = Focal necrosis or ulceration, or non-removable fibrin deposit 16 or more foci.
5 = Patches of necrosis 2 to 3 cm long variable.
6 = Diffuse necrosis typical of field cases variable, but extensive.

4. Microscopic lesion scoring of gut (Histopathological assay):

Specimens including; liver, intestine and cecum were collected from randomly sacrificed 12 chickens/group (2 birds/replicate) at the end of the experiment, fixed in 15% buffered formalin and paraffin-embedded sections were stained with Hematoxylin and Eosin **(Bancroft *et al.* 1996)** and scored for histopathological lesions according to the method described by **Rosales *et al.* (1989)**.

5. Bacterial load assessment: Randomly sacrificed 10 birds at d 14 of age (d 0 PI) as well as 12 birds of each group (2 birds/replicate) at d 21 and d 28 of age (d 7 and 14 PI) were collected, following their euthanasia, birds were necropsied and 1g of intestinal and caecal contents from each bird were weighed and serially diluted (10^{-1} to 10^{-8}) in sterile peptone saline for colonization of *C. perfringens* and TAB. All quantitative data were converted into log (CFU) values. For intestinal and caecal colonization of *C. perfringens:* 0.1 ml of each dilution of peptone saline was placed on 10 % neomycin sheep blood agar and tryptose sulfite-cycloserine (TSC) agar (supplemented by D-cycloserine) with egg yolk emulsion. These were overlaid with the same medium but without egg yolk. After anaerobic incubation at 37°C for 24 hours; typical *C. perfringens* colonies (black colonies) on TSC agar or large dome-shaped colonies with a double zone of haemolysis on blood agar plates were counted and reported as colony-forming units (CFU) per gram. The colonies picked and confirmed by criteria of **Harmon (1984)** and **Carrido *et al.* (2004), Garrido *et al.*(2006).** For TAB colonization***:*** 0.1 ml of each dilution placed onto blood agar plate and nutrient agar. The plates incubated aerobically at 37°C for 24 hours and colonies counted and calculated per gram. The bacterial colonies were enumerated, and the average number of live bacteria was calculated based on per gram of original intestinal contents.

6-Immune status assessment: The effect on relative weights of major immune organs (spleen, thymus and BF) at the end of the experiment (d 35 of age) were measured where 6 birds from each group (one bird per replicate) were slaughtered and these organs were separated and weighed. These related organ weights were calculated by the following formula: Related organ weight=organ weight (g) × 100 ÷ Live body weight (g). For determination of the effect on humoral immunity, anti- ND vaccine antibody titers were estimated by collecting blood samples at 0 and 7 days post ND vaccination from wing veins of randomly selected 12 birds/group (2 birds/replicate) and sera were subjected to HI test as described by **Swayne *et al.* (1998).** For determination of the effect on cell mediated immunity; activity of macrophages (phagocytic % and phagocytic index) on blood samples were taken at d 35 of age (21 days PI) on 5 randomly chosen birds per group after **Muller *et al*. (1995).**

Statistical analysis: Data were analyzed using the SAS statistical package **(SAS Institute 2004).** General liner model procedure with a one way ANOVA model using phytonutrients and the acidifiers blend as main effect. Mean values were compared using multiple rang test **(Duncan, 1955).** The significant level was set at 5%.

III. Results And Discussion

Experimental induction of NE was successfully achieved by oral inoculation of *C. perfringens* to immunocompromised broiler chickens (using IBDV vaccination with a hot vaccinal strain). NE infected bird groups showed variable degrees of diarrhea, inappetence, different degrees of enteritis and mortalities **(Fig.1)**. The cumulative mortality rate reached 3.32 % in NC group as compared with 7.41 % in PC group. The 3 treated groups showed numerical decrease in total mortality vs. PC group where the cumulative mortalities reached 4.32 %, 3.70 % and 4.94 % in T1, T2 and T3 groups respectively. PC group showed significant reduction in final body weight vs. BC group ($P<0.05$). Chicken performance response variables revealed significant increase in BW and BWG at the end of the trial (d 35 of age) in all NE infected supplemented groups vs. PC group ($P<0.05$). Whereas final wt. of T1 group numerically increased vs. NC group. An improve in FCR (125, 105 and 71 points less) in T1, T2 and T3 groups respectively was obtained vs. PC group. The production number recorded an increase by 41.46, 33.13 and 23.23 in T1, T2 and T3 groups respectively vs. PC group. All NE infected groups showed numerical decrease in feed consumption vs. NC group. Carcass characteristics revealed significant increase in dressing %, front part %, breast meat %, carcass meat % vs. PC group ($P<0.05$) with numerical increase in length and diameter of intestine in T1 and T2 groups vs. PC group **(Tables 2-3).** Obtained results accord with those recorded by **Lee *et al.* (2010).** The auspicious effects of our experimentally studied microencapsulated phytonutrients could be attributed to its technology as well as the synergistic antimicrobial activity of *Capsicum oleoresin* (that prepared by organic extraction of pepper fruits) **(Spices Board, 2008)** and the anti-oxidant as well as the anti-inflammatory properties of *Curcuma longa* (turmeric) **(Sodsai *et al.*, 2007; Mannangatti and Narayanasamy, 2008).** Moreover; the protected organic acidifiers supplementation to broiler chickens suffering from NE could be a novel and effective alternative to AGP **(Awaad *et al.*, 2011)**.

Macroscopic lesion scoring revealed significant reduction in T2 group with numerical reduction in T1 and T3 groups vs. PC group at d 7 PI ($P<0.05$). While at d 14 PI, there was significant reduction in gross lesion score in all treated groups over PC group ($P<0.05$) that indicates their efficacy in reducing the damage associated with NE **(Table 4)**.

Microscopic lesion scoring of different segments of intestine revealed significant decrease in duodenum, Jejunum and caecal tonsils in all treated groups ($P<0.05$) except in mucosal erosions of duodenum and dilatation of blood capillaries of jejunum of T1 group where there were numerical decrease in lesions **(Table 5).** Histopathological examination revealed typical picture of NE which was evident in the small intestine of PC group. The lesion characterized by severe necrosis of enterocytes involving large mucosal surface, massive villous fusion, severe inflammatory reaction in lamina propria with severe dilatation of blood capillaries associated with minute hemorrhages in the majority of the villi. This reaction was severe in duodenum and jejunum of this group and developed into fibrinohemorrhagic exudates in jejunum of individual cases. The picture of NE was markedly reduced in severity in T1, T2 and T3 supplemented groups as compared with the PC group as villous fusion was ranged from occasional villous fusion of 2 villi to multiple areas of villus fusion, while dilatation of blood capillaries of lamina propria was mild to moderate degrees. The inflammatory cell infiltration and necrotic changes were reduced in these treated groups. The cecal tonsils showed severe depletion and lymphocytolysis in PC group, while it was reduced in all treated groups. The hepatic histological alterations of PC group was severe including severe congestion, portal hepatitis associated with hepatocellular degeneration and necrosis. These hepatocellular changes were markedly ameliorated in all supplemented groups. Cecal lesions of PC group showed severe depletion of lymphoid elements comprising the cecal tonsils with goblet cell hyperplasia of cecal mucosa and severe lymphocytolysis and erosions of cecal mucosa. These lesions were milder in all treated groups **(Fig. 2). Lee *et al.* (2013)** mentioned that dietary supplementation of young broiler chickens with *Capsicum* and *turmeric oleoresins* increased resistance to NE.

Significant reduction in number of *C .perfringens* in intestine and ceci of experimentally infected broiler chickens has been obtained in groups T1,T2 and T3 at d 7 and d 14 PI vs. PC group ($P<0.05$) provided that the reduction was more evident in T3 group. This result indicates the potency of the used microencapsulated phytonutrients and/or acidifiers blend in ameliorating the severity of NE challenge in broilers. Moreover; at d 14 PI significant reduction in TAB count in both intestine and ceci has been also obtained in different supplemented groups vs. PC group ($P<0.05$) **(Tables 6and 7).** Aforementioned results are clearly showing that usage of these environmentally friendly alternatives can greatly assist in the control of both *C. perfringens* and TAB in broilers and could be considered a great potential as strong candidates for supporting broiler performance. Our results are on line with those reported by **Awaad *et al.* (2011). Partanen and Mroz (1999)** mentioned that organic acids and plant derivatives have been generally associated with a decreased load of pathogenic bacteria in the gut. **Mitsch *et al.* (2004)** reported on the antimicrobial efficacy of essential oils against *C. perfringens* in broiler chickens.

The immune status assessment was studied in the present investigation using the relative weight % of major immune organs, HI titers against ND vaccination and the activity of macrophages. Significant increase has been recorded in relative weight % of spleen and BF ($P<0.05$) with numerical increase in thymus gland % in

T1 group vs. PC group. T2 group showed only numerical increase in spleen % vs. PC group. T3 group showed a significant increase (P<0.05) in thymus glands % with numerical increase in spleen % vs. PC group. HI against ND vaccination showed significant increase in antibody titers in T1 group at d 7 PI vs. PC group (P<0.05) and numerical increase in T2 and T3 vs. PC group. The activity of macrophages revealed significant increase in phagocytic % in all treated groups vs. PC group (P<0.05) and significant increase in phagocytic index in T1 and T3 groups (P<0.05) with numerical increase in T2 group **(Tables 8a and 8b)**. From our results, it could be concluded that using the studied supplements modulated the cell mediated immune response rather than the humoral one. This assumption might be explained in the view of our obtained relative weights of main immune organs that showed a significant increase in weights of thymus glands in T3 group and a numerical increase in T1 group over PC group (P<0.05) as well as the significant reduction of lymphoid depletion of caecal tonsils in all supplemented groups vs. PC group (P<0.05) **(Table 5).** It is already accepted that many phytonutrients are highly effective in promoting host defense mechanisms against microbial infections **(Lee *et al.*, 2005).** *Curcuma/Capsicum* supplemented diet provides a new opportunity to utilize these dietary phytonutrients to increase local innate immunity and when they combined together increase the effectiveness on stimulating local cell–mediated immunity and enhance serum antibody levels **(Lee *et al.*, 2010)**. Macrophages are involved in the innate immune response through phagocytosis or production of a variety of compounds, like cytokines or nitric oxide **(Dempsey *et al.*, 2003)**.

C. perfringens is responsible for severe food borne enteritis in man **(Brynestad, 2002)** and its presence in foods such as meat of poultry may be unavoidable **(Rhodehamel and Harmon, 1998, Ghadban *et al.*, 1998).** Another implication of the antimicrobial action of phytogenic feed additives may be in improving the microbial hygiene of carcasses. Scanty reports are present on the beneficial effects of phytonutrients and acidifiers on the microbial load of total viable bacteria in broiler carcasses. Moreover; available data are still too limited to allow reliable conclusions on the possible efficacy of certain phytogenic feed additives to improve carcass hygiene **(Windisch *et al.*,2008).** Food safety is probably the biggest issue facing poultry production systems today and preventing contamination of poultry products with food borne pathogens remains a considerable challenge for producers and integrations (**Awaad *et al.*, 2011).** Accordingly, we assume that this important issue needs further investigation.

In conclusion; dietary phytonutrients (Capsicum and turmeric oleoresins) and/or acidifiers blend used in the present study can make a valuable contribution to flock health and safety of food as well. Regarding obtained results; it could be assumed that they could help in sustaining the GIT integrity damaged by *C. perfringins* infection and could be a tool for impedance of NE in broiler chickens.

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Conflict Of Interest

The authors declare that they have no conflict of interests.

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Table 1. Composition of the 3-phase diets (g/kg as fed) used and their calculated analysis and nutritional values.

Ingredients	**Starter**	**Grower**	**Finisher**
Yellow corn	524.5	544.2	628.5
Soybean meal 44%	332.4	299.1	221.1
Corn gluten meal 60%	70	70	66.5
Soya oil	30	43.8	40
Di-calcium phosphate	18	18	18
Lime stone	13	13	13
D.L. Methionine	2.2	2.1	2.3
Lysine hydrochloride	2.9	2.8	3.6
Sodium chloride	4	4	4
Premix*	3	3	3
Calculated analysis:			
Crude protein %	23.0	21.0	19.0
Metabolizable energy (kcal/kg)	3000	3100	3200
Calculated nutritional values			
Dry Matter	88.5	88.7	88.3
Fiber content	3.7	3.6	3.2
Ash content	6.80	6.70	6.35
Available Ca	0.99%	0.97%	0.95%
Available P	0.48%	0.47%	0.45%

*Each 3 gram of premix mixture contained: vitamin A (trans-retinyl acetate), 9,000 IU; vitamin D3 (cholecalciferol), 2,600 IU; vitamin E (dl-α-tocopheryl acetate), 16 mg; vitamin B1, 1.6 mg; vitamin B2, 6.5 mg; vitamin B6, 2.2 mg; vitamin B12 (cyanocobalamin), 0.015 mg; vitamin K3, 2.5mg; choline (choline chloride), 300 mg; nicotinic acid, 30 mg; pantothenic acid (d-calcium pantothenate), 10 mg; folic acid, 0.6 mg; d-biotin, 0.07 mg; manganese (MnO), 70 mg; zinc (ZnO), 60 mg; iron (FeSO4 H2O), 40 mg; copper (CuSO4 5H2O), 7 mg; iodine [Ca(IO3)2], 0.7 mg; selenium (Na2SeO3), 0.3 mg.

Table 2. Productive performance

Trait **Treatment**	**Final BW (g)**	**Cumulative feed intake (g/bird)**	**Final FCR**	**Wt. gain (d 1-35 of age) (g)**
1- T 1	1785.67±17.31^{a}	3191.11±47.29	1.791±0.044	1745.82±17.31^{a}
2-T 2	1736.82±17.87^{ab}	3141.90±26.26	1.811±0.041	1696.89±17.86^{ab}
3-T 3	1729.92±20.19^{b}	3188.18±55.24	1.845±0.025	1691.54±20.49^{b}
4- PC	1668.79±16.31^{c}	3190.31±22.18	1.916±0.041	1630.00±16.48^{c}
5- NC	1770.16±13.82^{ab}	3237.44±34.48	1.831±0.031	1730.20±13.81^{ab}
Probability	0.0001	0.5702	0.1942	0.0001

Means with different, lower case, superscripts, within age, are significantly different ($P \leq 0.05$).

Table 3. Carcass characteristics.

Trait / Treatment	Dressing %	Breast meat %	Front parts %	Carcass meat (%)	Proven-triculus (%)	Gizzard (%)
1- T 1	66.00±0.04^{ab*}	19.89±0.19^{a}	36.40±0.12^{a}	32.96±0.24^{ab}	0.356±0.020^{a*}	1.80±0.06^{b*}
2-T 2	65.59±0.30^{bc}	19.06±0.11^{b}	35.60±0.13^{b}	32.66±0.15^{ab}	0.326±0.013^{ab}	1.83±0.06^{ab}
3-T 3	65.03±0.21^{c}	19.30±0.15^{b}	35.85±0.09^{b}	32.44±0.17^{b}	0.312±0.013^{ab}	1.89±0.08^{ab}
4- PC	64.06±0.08^{d}	18.35±0.10^{c}	34.89±0.14^{c}	31.46±0.22^{c}	0.296±0.014^{b}	1.69±0.05^{b}
5- NC	66.50±0.25^{a}	19.91±0.20^{a}	36.57±0.18^{a}	33.16±0.22^{a}	0.299±0.015^{b}	2.02±0.08^{a}
Probability	0.0001	0.0001	0.0001	0.0001	0.0470	0.0185

Means with different, lower case, superscripts, within age, are significantly different ($P \leq 0.05$).

Table 4. Macroscopic lesion score.

Trait / Treatment	Age	
	21 days (7 days PI)	**28 days (14 days PI)**
1- T 1	2.33±0.36^{ab*}	1.00±0.35^{b}
2-T 2	1.92±0.36^{bc}	1.17±0.39^{b}
3-T 3	1.00±0.33^{cd}	0.92±0.26^{b}
4- PC	3.25±0.49^{a}	3.00±0.37^{a}
5- NC	0.67±0.22^{d}	0.83±0.39^{b}
Probability	0.0001	0.0002

* Means with different, superscripts, within age and trait, are significantly different ($P \leq 0.05$).

Table 5. Histopathological lesion score.

Lesion	Organ	Chicken groups					*Probability*
		1-T 1	**2-T 2**	**3-T 3**	**4-PC**	**5-NC**	
Villus fusion	Duodenum	1.917 ±0.229^{b*}	1.455 ±0.247^{b}	1.769 ±0.323^{b}	2.833 ±0.322^{a}	2.250 ±0.131^{ab}	0.0075
Blood Capillary Dilatation		1.583 ±0.149^{b}	1.500 ±0.195^{b}	1.750 ±0.179^{b}	2.636 ±0.310^{a}	1.667 ±0.142^{b}	0.0015
Capillary Hemorrhage		0.833 ±0.207^{b}	0.667 ±0.142^{b}	0.667 ±0.142^{b}	1.667 ±0.225^{a}	0.750 ±0.179^{b}	0.0009
Mucosal erosion		2.583 ±0.229^{a}	1.750 ±0.250^{b}	1.667 ±0.310^{b}	2.750 ±0.329^{a}	2.583 ±0.149^{a}	0.0075
Inflammatory cell infiltration		1.667 ±0.225^{b}	1.583 ±0.193^{b}	2.083 ±0.193^{b}	2.750 ±0.279^{a}	2.750 ±0.131^{a}	0.0001
Villus fusion	Jejunum	1.417 ±0.313^{b*}	0.917 ±0.336^{b}	0.750 ±0.329^{b}	3.000 ±0.213^{a}	0.667 ±0.284^{b}	0.0001
Blood Capillary Dilatation		1.500 ±0.195^{ab}	1.167 ±0.271^{b}	1.083 ±0.083^{b}	2.083 ±0.379^{a}	0.750 ±0.250^{b}	0.0070
Capillary Hemorrhage		0.500 ±0.230^{b}	0.250 ±0.179^{b}	0.167 ±0.112^{b}	1.500 ±0.151^{a}	0.333 ±0.188^{b}	0.0001
Mucosal Erosion		1.750 ±0.279^{b}	1.583 ±0.260^{bc}	1.000 ±0.174^{bc}	3.167 ±0.271^{a}	0.917 ±0.288^{c}	0.0001
Inflammatory cell infiltration		2.000 ±0.275^{b}	1.917 ±0.229^{b}	1.667 ±0.225^{b}	2.917 ±0.260^{a}	1.250 ±0.305^{b}	0.0008
Lymphoid depletion	Cecal Tonsils	0.618 ±0.127^{d*}	1.302 ±0.103^{c}	1.615 ±0.154^{bc}	2.294 ±0.098^{a}	1.711 ±0.108^{b}	0.0001

* Means with different, superscripts, within age, are significantly different ($P \leq 0.05$).

Table 6. Enumeration of *C. perfringens* after experimental induction of NE [Log (CFU) values].

Trait / Treatment	Day 7 PI		Day14 PI	
	Intestine	**Caecum**	**Intestine**	**Caecum**
1- T 1	4.78±0.14^{b*}	5.02±0.14^{b}	4.89±0.01^{b*}	4.98±0.02^{b}
2-T 2	4.72±0.02^{b}	4.90±0.00^{b}	4.38±0.08^{c}	4.56±0.01^{c}
3-T 3	3.41±0.12^{c}	3.68±0.11^{c}	3.24±0.11^{d}	3.46±0.12^{d}
4- PC	6.66±0.09^{a}	6.68±0.07^{a}	6.62±0.12^{a}	6.68±0.12^{a}
5- NC	1.68±0.17^{d}	2.38±0.15^{d}	2.73±0.19^{e}	3.04±0.20^{e}
Probability	0.0001	0.0001	0.0001	0.0001

* Means with different, superscripts, within age and trait, are significantly different ($P \leq 0.05$).

N.B.: Enumeration of *C. perfringes* of a representative sample of birds at zero day PI were 0.49±0.17 in intestine and 0.63±0.10 in caecum respectively. It was just one pool of 10 randomely chosen birds and values were shared.

Table 7. Enumeration of Total aerobic Bacteria (TAB) after experimental induction of NE [Log (CFU) values].

Trait / Treatment	Day 7 PI		Day14 PI	
	Intestine	Caecum	Intestine	Caecum
1- T 1	5.30±0.00^{b}	5.38±0.01^{b}	4.37±0.02^{d}	4.53±0.02^{d}
2-T 2	5.20±0.05^{bc}	5.27±0.03^{b}	4.73±0.03^{c}	4.95±0.03^{c}
3-T 3	4.93±0.02^{c}	5.38±0.08^{b}	4.41±0.01^{d}	4.87±0.01^{c}
4- PC	5.10±0.03^{bc}	5.31±0.01^{b}	5.11±0.01^{b}	5.39±0.01^{b}
5- NC	6.67±0.22^{a}	6.35±0.21^{a}	5.63±0.24^{a}	5.81±0.24^{a}
Probability	0.0001	0.0001	0.0001	0.0001

* Means with different, superscripts, within age and trait, are significantly different ($P \leq 0.05$).

N.B.: Enumeration of TAB of a representative sample of birds at zero day post infection were 5.90±0.18 in intestine and 6.36±0.18 in caecum respectively. It was just one pool of 10 randomely chosen birds and values were shared.

Table 8a. Immune status assessment.

Trait / Treatment	Main immune organs relative weight %			Macrophages activity	
	Spleen %	Bursa %	Thymus %	Phagocytic %	Phagocytic index
1- T 1	0.163±0.012^{a*}	0.071±0.006^{b}	0.485±0.015^{ab}	52.60±1.63^{ab*}	0.344±0.010^{b}
2-T 2	0.140±0.008^{ab}	0.047±0.002^{c}	0.430±0.009^{c}	55.40±1.60^{a}	0.260±0.12^{c}
3-T 3	0.132±0.006^{b}	0.044±0.002^{c}	0.512±0.018^{a}	56.40±1.03^{a}	0.432±0.027^{a}
4- PC	0.124±0.011^{b}	0.056±0.003^{c}	0.465±0.016^{bc}	47.40±0.93^{c}	0.224±0.005^{c}
5- NC	0.164±0.009^{a}	0.087±0.009^{a}	0.468±0.013^{bc}	50.20±0.86^{bc}	0.238±0.017^{c}
Probability	0.0088	0.0001	0.0041	0.0003	0.0001

* Means with different, superscripts, within trait, are significantly different ($P \leq 0.05$).

Table 8b. Immune status assessment.

Trait / Treatment	HI titers against ND vaccination (Days PI)			
	D 0	D 7	D 14	D 21
1- T 1	3.58±0.45	5.42±0.54^{a*}	4.67±0.48	5.25±0.45
2-T 2	3.50±0.57	3.58±0.23^{c}	4.50±0.40	4.83±0.51
3-T 3	4.08±0.47	4.00±0.25^{bc}	4.17±0.44	4.75±0.22
4- PC	4.25±0.60	3.33±0.33^{c}	4.67±0.53	5.58±0.57
5- NC	3.42±0.31	4.75±0.33^{ab}	5.17±0.58	6.00±0.37
Probability	0.6793	0.0005	0.7023	0.2404

* Means with different, superscripts, within trait, are significantly different ($P \leq$

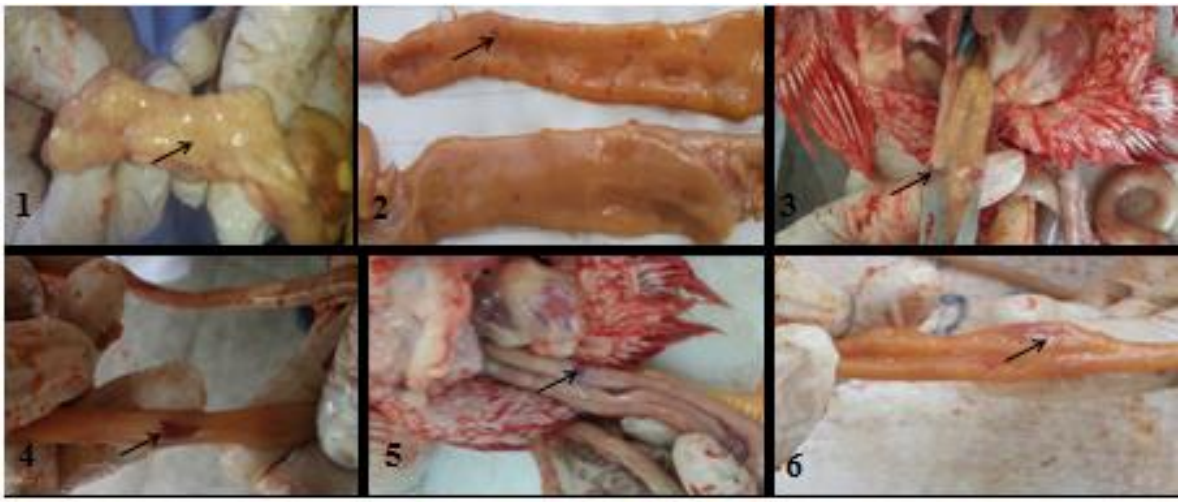

Fig.1. Post mortem lesions of experimentally induced NE in broiler chickens.

1)Diffuse superficial removable fibrin (score 1).
2- 5) Focal necrosis (score 3).
6) Patches of necrosis 2-3 cm long (score 5).
The scoring system criteria are the six-point system of Keyburn et al. (2006) modified by **Shojadoost et al. (2012).**

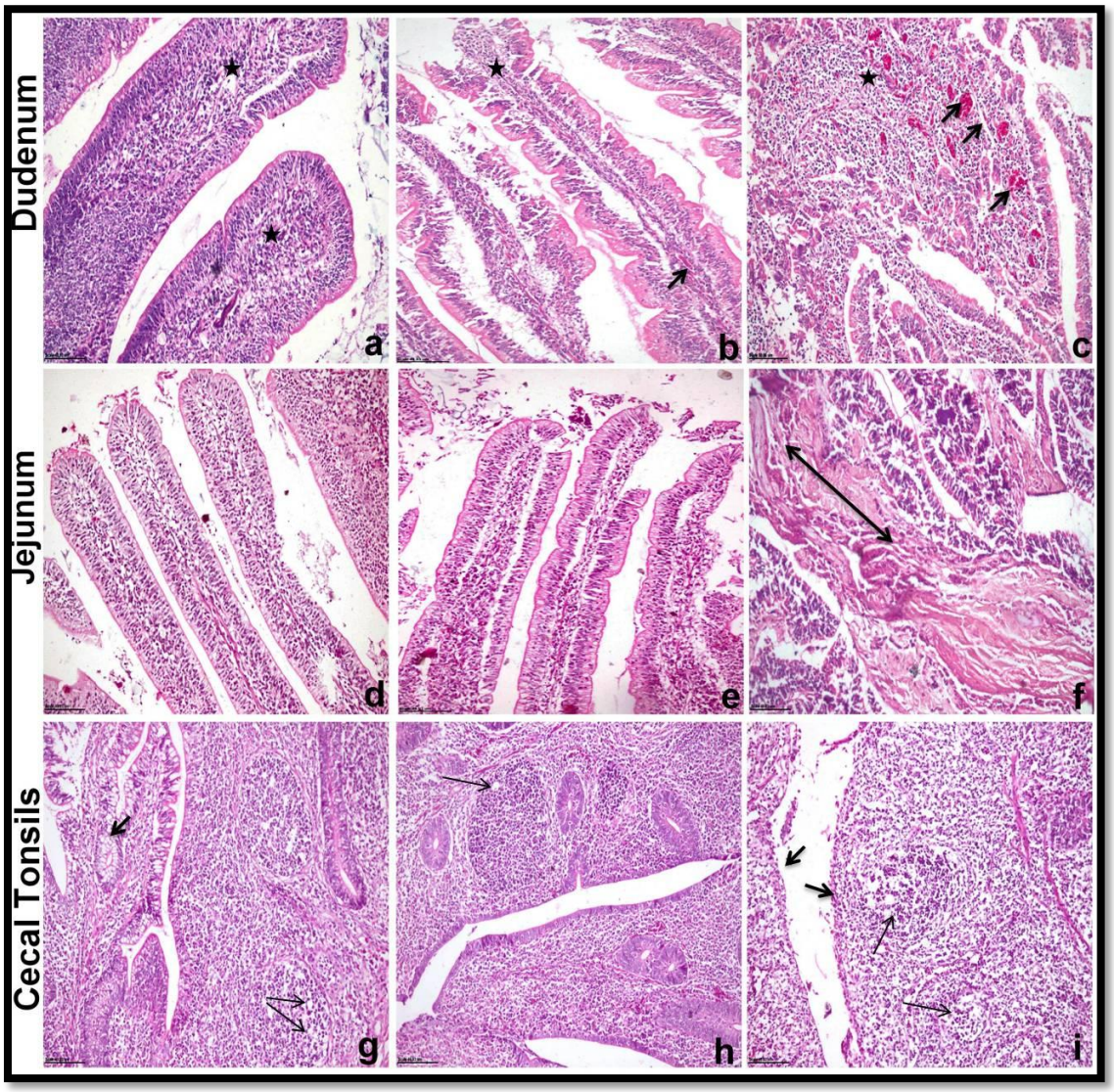

Fig2:

a-c Histological section of duodenum a) T2 group showed moderate inflammatory reaction comprising the intestinal mucosa (asterisks). b) T3 group showed mild inflammatory cells infiltration (asterisks) and dilatation of blood capillaries in the lamina prpria(arrow). c) PC group showed severe inflammatory reactions of intestinal mucosa (asterisks) with severe dilatation of blood capillaries (arrows) .

d-f) Histological section of jejunum . d) T1 group showed mild inflammatory reaction and dilatation of capillaries in lamina propriety . e) T3 group showed mild inflammatory reaction and dilatation of capillaries in lamina propria.c) PC group showed massive villous fusion and necrosis with accumulation of fibrinous exudates covering the intestinal mucosa(double head arrow).

g-i) Histological section of cecum g) T1 group showed moderate depletion of lymphoid elements comprising the cecal tonsils (thin arrows) with goblet cell hyperplasia of cecal mucosal epithelium (thick arrow). h) T3 group showed mild depletion of cecal tonsils (thin arrows) with apparently normal cecal mucosa .i) PC group 4 showed severe lymphocytolysis (thin arrows) and desquamation of cecal mucosa (thick arrow).

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