Research Article

Anticlostridial Activity of the Thyme and Clove Essential Oils against Experimentally Induced Necrotic Enteritis in Commercial Broiler Chickens

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Abstract | The objective of this study was to evaluate the efficacy of thyme and clove essential oils (EOs) as feed additive for prevention and/or control of *Clostridium perfringens* (CP) type A induced necrotic enteritis (NE) in broiler chickens. The influence of varying concentrations of both EOs was determined in vitro using the agar dilution test. Concentration as low as 0.25% and 0.125% of thyme and clove oils, respectively, were able to completely inhibit the growth of CP. An in vivo study was conducted in commercial broiler chickens, where, 2 groups of 10 day-old broilers were fed a balanced ration with addition of thyme (0.25%) or clove (0.125%). Birds were then challenged with CP (1×10^8 CFU) together with another 2 groups without EOs at 15 days of age. Starting from 3 days' post infection (DPI), the second 2 set of birds without EOs addition were fed ration containing 0.25% and 0.125% thyme and clove oils as treatment in comparison to an antibiotic control group using for 5 days. The results showed that the use of EOs improved growth performance of broiler chickens especially when used as preventive feed additive continuously. The thyme EO showed better anticlostridial activity in terms of reduced CP counts in intestine and alleviation of intestinal damage caused by infection. In conclusion, the EOs are effective feed additives to treat and/or prevent NE in poultry. Further studies on the effect of EOs on other enteric infection, intestinal microbiota, poultry feed components, and poultry meat quality are required.

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Introduction

Necrotic Enteritis (NE) is a disease of broiler chickens caused by a Gram-positive anaerobic spore-forming, rod-shaped bacterium *Clostridium perfringens* (*C. perfringens*). The *C. perfringens* is classified into 5 toxicogenic types (A, B, C, D and E), which are differentiated according to the production of four different major toxins; Alpha, Beta, Epsilon, and Iota (Quinn et al., 1994). The subclinical NE usually occurs without increase in mortality, but with clear signs of intestinal disorders, lower performance resulting in weight loss, increased feed conversion rate, and decrease in production (Cooper et al., 2013). The clinical form of *C. perfringens* infection can be developed from predisposing factors that destruct the

integrity of the gut mucosa; such as increased viscosity of intestinal digesta, high level of protein in the diet and coccidiosis infection (Collier et al., 2003; Rodgers et al., 2015).

Though *C. perfringens* strains showed susceptibility to different anti-microbial drugs in both in vitro and in vivo, the worldwide antimicrobial resistant bacteria reporting has led to the search for replacements of antibiotics (Dibner and Richards, 2005; Huyghebaert et al., 2011). New commercial additives derived from plants including aromatic plant extracts have been examined as alternative feed strategies in future. Such products have several advantages than commercial antibiotics since they are residue free and also, generally recognized as safe and commonly used items in the food industry (Brenes and Roura, 2010; Varel, 2002).

The plant extracts contain essential oils (EOs) which are volatile, natural, complex compounds characterized by a strong odor and formed by aromatic plants as secondary metabolites (Bakkali et al., 2008). In nature, essential oils play an important role in the protection of the plants as antibacterial, antivirals, antifungals, insecticides and also against herbivores by reducing their appetite for such plants. The chemical profile of the essential oil products differs not only in the number of molecules but also in the stereo-chemical types of molecules extracted according to the type of extraction method. These plant oil (e.g. thyme and clove oils) have antiseptic effect as, bactericidal, virucidal and fungicidal, and medicinal properties (Masotti et al., 2003).

Previous trials for prevention and/or control of *C. perfringens* using plant EOs have been conducted focusing mainly on the use of plant extracts containing thymol EO, Oegano anise citrus (Brenes and Roura, 2010), garlic essential oil (Kirkpinar et al., 2014) and ginger oil and carvacrol (McReynolds et al., 2009). Results showed that some of these EOs are capable to increase body weight gain and decrease C. perfringens count in jejunum especially at first half of growing period.

In this study, the efficacy of thyme and clove plant EOs against pathogenic *C. perfringens* in vitro was evaluated. Moreover, using both EOs as an in vivo continuous preventive feed additive or as treatment intervention against experimentally induced NE in commercial broiler chickens was compared.

Materials and Methods

C. perfringens isolate

The *C. perfringens* (CP) isolate was kindly obtained from Anaerobic Unit, Bacteriology Department, Animal Health Research Institute - Dokki, Giza, Egypt. The isolate is a NetB positive CP type A from intestinal specimens of chicken with NE (Ammar et al., 2013).

Essential oils (EOs)

Thyme and Clove extracted EOs (98.8% purity) were purchased from the Oil Unit at the National Research Center (NRC) at Dokki, Giza, Egypt. The thyme oil contains Thymol as ≥40%, whereas the clove oil contains Eugenol as ≥55% as indicated by the manufacturer.

C. perfringens identification and characterization

The isolate was inoculated in cooked meat media (Oxoid) for refreshment then incubated anaerobically at 37˚C for 24 hrs., then A loopful of inoculated fluid medium was streaked onto neomycin sulfate sheep blood agar plates in a concentration of 200ug/ml incubated anaerobically at 37˚C for 24 hrs (Quinn et al., 1994).

Ten white Swiss mice were classified into two group (5 mice/group). The first group as control and the second group was injected 0.5ml of culture supernatant via intraperitoneal route. The mice were observed for 72 hrs for neurological symptoms or death (Willis, 1964). The sensitivity of the CP isolate was tested by the disk diffusion technique according to (Finegold and Martin, 1982).

In vitro determination of EOs minimal inhibitory concentration (MIC)

The MIC of the tested EOs was determined using an agar dilution method (Griffin et al., 2000) with some modification. Briefly, 0.5% of tween 20 was mixed with thyme & clove oils before mixing with tryptic soya agar (TSA) medium (Oxoid). The mixtures were mixed with TSA medium to final concentrations of 1.0%, 0.50%, 0.250%, and 0.125%, and 0.063%. TSA medium vortexed for 15 seconds then poured into 9mm petri dishes. For culture preparation, 4-5 colonies were transferred using sterile loop into 5 ml of PBS. The turbidity of the inoculated

Table 1: *Experimental grouping of EOs treatments and prevention programs.*

Age	Group* (25 bird/group)						
	Negative control (NC)	Ciprofloxacin treatment (CIP)	Clove treatment (Clove-T)	Thyme treatment (Thyme-T)	Clove prevention (Clove-P)	Thyme prevention (Thyme-P)	Positive control (PC)
10 day old	Balanced feed	Balanced feed	Balanced feed	Balanced feed	Balanced feed +0.25% clove oil	Balanced feed +0.125% clove oil	Balanced feed
	Weighing all birds						
15 day old (0 DPI)	None	Challenge with *C. perfringens* (1×10^8 CFU/ml) via intra crop route					
	Weighing all birds						
20 day old (3 DPI)	3 chicks/ group were euthanized for: Colony counts in jejunum and cecum pooled samples Intestinal tissue collection for histopathology						
	None	Ciprofloxacin 1gm/liter	Balanced feed +0.25% clove oil	Balanced feed +0.125% clove oil	Balanced feed +0.25% clove oil	Balanced feed +0.125% clove oil	None
25 day old (7 DPI)	Weighing all birds 3 chicks/ group were euthanized for: Colony counts in jejunum and cecum pooled samples Intestinal tissue collection for histopathology						
28 day old (10 DPI)	Weighing all birds 3 chicks/ group were euthanized for: Colony counts in jejunum and cecum pooled samples Intestinal tissue collection for histopathology						

broth was then adjusted to match a McFarland 0.5 barium sulfate standard tube by adding sterile broth using adequate light and tube was compared against a white background with contrasting black line. The TSA containing various concentrations of EOs were inoculated with the adjusted CP inoculum. Control plates were TSA agar without essential oil and inoculated with the bacterial culture and un-inoculated TSA plates served as negative control. Test and control plates were then incubated anaerobically at 37°C for 24hrs then the colony counts were evaluated. The lowest concentration of EOs required to completely inhibit the growth of the organism was designated as the MIC.

Anti-clostridial properties of thyme and clove EOs in commercial broiler chickens

All experiments were conducted according to Animal Research Ethics Guidelines at the faculty of veterinary medicine, Beni-Suef University, Egypt.

Experimental chickens: A total of 175 day-old broiler chicken day old Hubbard chicks were obtained from commercial broiler hatchery (Cairo Poultry Company). Birds were floor reared in clean separated room and given commercial broiler starter feed without antimicrobial additives and water ad libitum. All groups received the same vaccination program including Hitchenr-IB vaccine at day old via eye drop, AI-H9+ND at 7-day old via injection, ND-LaSota at 10-day old and intermediate plus IBD vaccine at 13-day old via drinking water.

Experimental design: Birds were randomly divided into 7 groups (25 birds/group) and fed commercial balanced ration or ration with added essential oils (thyme and clove) according to the experimental design shown in Table 1. The concentration of EOs added were selected based on the in vitro MIC determination. Challenge with CP was conducted according to (Gholamiandehkordi et al., 2007) on 3 successive days via crop intubation.

Post challenge CP colony count: One gram of each sample (pooled jejunum and cecum) was diluted 1:9 (wt/vol) in sterile PBS and 10-fold serial dilution were prepared. The colony counting was conducted according to (Quinn et al., 1994) with some modification. Briefly, dilutions of the samples were inoculated in Thioglycollate medium (Oxoid) incubated anaerobically at 37°C for 24hrs. Colonies were counted after each dilution was inoculated on TSA agar plate and incubated anaerobically at 37°C for 24hrs.

Gross lesions scores: Intestinal gross lesions were re-

ported according to lesion scoring of (Lovland et al., 2004).

Histopathological samples: Jejunum and cecum tissue specimens were collected in 10% neutral formalin at room temperature for at least 48 hours before processing. The tissues were routinely stained with hematoxyline and eosin.

Statistical analysis

The differences between groups in parameters measured (body weight) were estimated using One-way ANOVA with Tukey's post-test through GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

Results and Discussion

NE is described as a disease of high economic importance affecting the health and performance of broilers resulting in weight loss, decreased feed conversion rate, and decrease in production. The disease is caused by specific (NetB positive) strains of CP type A (Ammar et al., 2013; Keyburn et al., 2010).

CP isolate microbial and pathogenicity confirmation: In this study, a NetB positive CP type A isolate from broiler chickens suffered from NE was used to induce NE experimentally. Cultivation of the CP on blood agar showed typical double zone of hemolysis and the intraperitoneal inoculation of culture supernatant (Toxoid) in Swiss mice caused death after 24hrs (Quinn et al., 1994). To identify the toxogenic type of the isolate, the classical toxicity testing in Swiss mice indicated that the CP isolate is type A as it caused death after 24 hrs (Willis, 1964). The antimicrobial susceptibility test showed that the organism is sensitive to Ciprofloxacin and Enrofloxcin. An intermediate sensitivity was observed for Amoxicillin and Doxycycline. Meanwhile the bacterium was resistant to the remaining antibiotics tested (data not shown). Therefore, ciprofloxacin was selected to be the control antibiotic to be compared to the EOs in vivo studies.

In vitro efficacy of tested EOs against CP type A isolate

The efficacy of two EOs (thyme and clove) against pathogenic CP was evaluated in vitro. Results revealed that the MICs of the tested EOs were 0.250% and 0.125% for thyme and clove oils, respectively. Thyme EO was reported specifically to inhibit CP in vitro with MIC 0.240 mg/ml (Timbermont et al., 2010). Though the used EOs were not chemically analyzed, it is well known that the thyme EOs antimicrobial activity attributed to its phenolic compounds content especially thymol and terpene hydrocarbons (≥75%) (Boruga et al., 2014). Also, the eugenol as a major components in the clove oil (74.6%) showed an anti-candidal activity compared to fluocanazol (Alshaikh and Perveen, 2017; Chaieb et al., 2007).

In vivo anti-clostridial properties of thyme and clove EOs

Based on the MICs of thyme and clove EOs determined, the in vivo studies was designed to include the use of both oils as preventive feed additive (i.e. the oils added from 10-day old till the end of experiment) or as treatment feed additive compared to antibiotic treatments (i.e. starting from 3 DPI for 5 successive-days).

Table 2: *Body weight gain of broiler chickens before and after the C. perfringens experimental infection and treatment approaches.*

Age	Group*						
	NC	CIP	Clove-T	Thyme-T	Clove-P	Thyme-P	PC
10-day old	122.1±3.2	125.5±2.7	126.6±3.6	126.9±4.5	123.2±4.4	124.8±5.5	127.0±1.9
15-day old (0 DPI)	342.7±15.6	333.5±16.5	349.0±15.9	331.4±14.4	337.3±12.8	346.5±13.0	339.3±19.2
25-day old (7 DPI)	865.3±61.27^{a}	935.1±78.38^{b}	818.5±68.98^{a}	874.0±75.24^{a}	834.6±121.5^{a}	956.1±80.05^{b}	756.1±115.2^{c}
28-day old (10 DPI)	903.2±83.99^{ab}	946.3±100.6^{ab}	858.5±108.7^{b}	899.7±79.72^{ab}	891.7±119.1^{ab}	972.8±99.04^{a}	770.1±91.60^{c}

*NC: *negative control;* CIP: *Ciprofloxacin treatment; Clove-T: Clove treatment; Thyme-T: Thyme treatment;* Clove-P: *Clove prevention;* Thyme-P: *Thyme prevention;* PC: *positive control. * Body weight expressed as mean ± SD. NC, negative control;* CIP: *Ciprofloxacin treatment;* Clove-T: *Clove treatment;* Thyme-T: *Thyme treatment;* Clove-P: *Clove prevention;* Thyme-P: *Thyme prevention;* PC: *Positive control.*

** Groups followed by the different superscript small letters are statistically significant (p<0.05).*

Table 3: *Feed conversion ratios of broiler chickens with EOs as feed additive and after challenge with C. perfringens.*

Age	Feed conversion ratio (FCR)*						
	NC	CIP	Clove-T	Thyme-T	Clove-P	Thyme-P	PC
15-day old (0DPI)	0.90	0.84	0.98	0.93	0.89	0.82	1.04
28-day old (10 DPI)	1.93	2.08	2.25	2.25	2.02	1.88	2.84

** FCR was calculated before challenge and 10 DPI. NC: negative control; CIP: Ciprofloxacin treatment;* **Clove-T:** *Clove treatment;* **Thyme-T:** *Thyme treatment;* **Clove-P:** *Clove prevention;* **Thyme-P:** *Thyme prevention;* **PC:** *Positive control.*

Experimental chicken's performance

Body weights of broiler chick groups at different time points are shown in Table 2. The use of thyme as a preventive anti-clostridial feed additive prevention significantly improved the body weight at 22 and 25 days of age (7 and 10 DPI, respectively), reduced the effect of CP challenge on the chick's performance and was comparable to the antibiotic Ciprofloxacin treatment. As compared to the challenge control group, all treatment groups showed better performance as indicated by both weight gain and feed conversion rates (Table 3). Generally, the use of both EOs as a continuous preventive feed additive showed improved body weight gain especially with thyme oil as compared to the antibiotic or EOs treatment for 5 days. Previous findings indicated that dietary supplementation with essential oil improve performance of broiler chickens and controls the proliferation of CP (Bakkali et al., 2008; Du et al., 2009; Jerzsele et al., 2012; Mitsch et al., 2004).

The mechanism of action EOs was suggested to be stimulation of the endogenous digestive enzymes and enhancing nutrient digestibility (Cross et al., 2007; Tiihonen et al., 2010). Furthermore, the enhanced antioxidant capacities of some plant extracts containing EOs (e.g Yucca schidigera extract) beside growth performance improvement was suggested to be an additional benefit of the EOs use in broiler chickens especially during finisher period (Sun et al., 2017).

Though comparison of different EOs demonstrated that most of EOs (such as oils of basil, rosemary, marjoram, peppermint, thyme and anise) have antimicrobial properties against CP, however, the efficacy was variable (Bakkali et al., 2008; Lopez et al., 2005). Similarly, other in vivo studies showed thyme and clove up to 200g/ton has no effect on weight gain and/or reduction of CP count in ileum and cecum compare to control positive group during the starter period (Oviedo-Rondon et al., 2006). The quality, chemical profiles, extraction methods of the EOs and the synergism between the plant extract components can play crucial rule in the determination of the antimicrobial activity (Radaelli et al., 2016). Meanwhile, the geographic origin, soil composition, and the age and season when the EOs were extracted from their plants can greatly affect the plant extract composition (Bakkali et al., 2008).

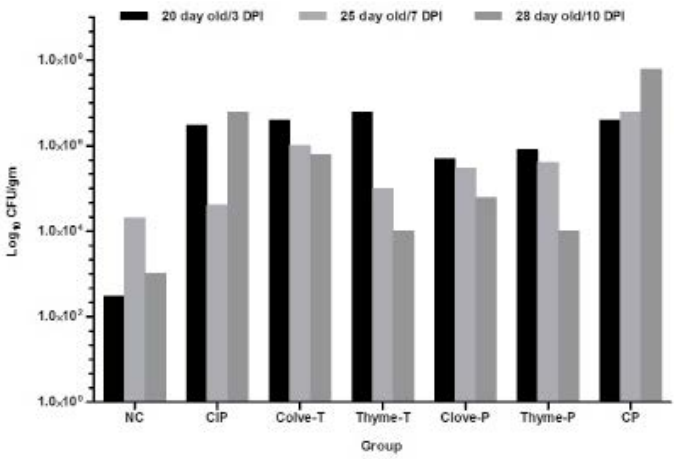

Figure 1: *Comparative C. perfringens re-isolation rates from pooled jejunum and cecum samples of broiler chickens in different experimental groups.*
NC: *negative control;* **CIP:** *Ciprofloxacin treatment;* **Clove-T:** *Clove treatment;* **Thyme-T:** *Thyme treatment;* **Clove-P:** *Clove prevention;* **Thyme-P:** *Thyme prevention;* **PC:** *positive control.*

C. perfringens re-isolation rates in EOs treated broiler chickens

The total bacterial count of pooled jejunum and cecum samples of different groups showed thyme and clove oils as preventive feed additive reduced the colony counts of CP at 3DPI and were comparable to the antibiotic treatment group followed by thyme as treatment feed additive. The EOs treatment and prevention groups maintained lower colony counts as compared to the antibiotic treatment group (Figure 1). Noticeably, the antibiotic treatment group showed elevated colony counts by 10 DPI. Though not studied, the antibiotic effect on the beneficial bacteria in the gut may explain the relapse observed after the stop of the treatment (Mookiah et al., 2014). Several studies indicated that EOs inhibit pathogenic bacteria, variable effects on beneficial microbiota in intestine were reported (Liu et al., 2010; Liu et al., 2017;

Table 4: *Intestinal gross lesion scores in broiler chickens with experimentally induced NE and EOs treatments.*

Age	Jejunum							Cecum						
	NC*	CIP	Clove-T	Thyme-T	Clove-P	Thyme-P	PC	NC	CIP	Clove-T	Thyme-T	Clove-P	Thyme-P	PC
10 day old	-	-	-	-	-	-	-	-	-	+	+	-	-	+
20-day (3DPI)	+/-	++	++	++	-	-	+++	-	+++	++	+++	+	+	+++
25-day (7 DPI)	-	+	+	++	+	+	+++	+/-	++	+	+	++	+	+++
28-day (10 DPI)	-	++	++	+	+	+	++	-	++	+	+	+	+/-	+++

***NC:** *negative control;* **CIP:** *Ciprofloxacin treatment;* **Clove-T:** *Clove treatment;* **Thyme-T:** *Thyme treatment;* **Clove-P:** *Clove prevention;* **Thyme-P:** *Thyme prevention;* **PC:** *Positive control.*

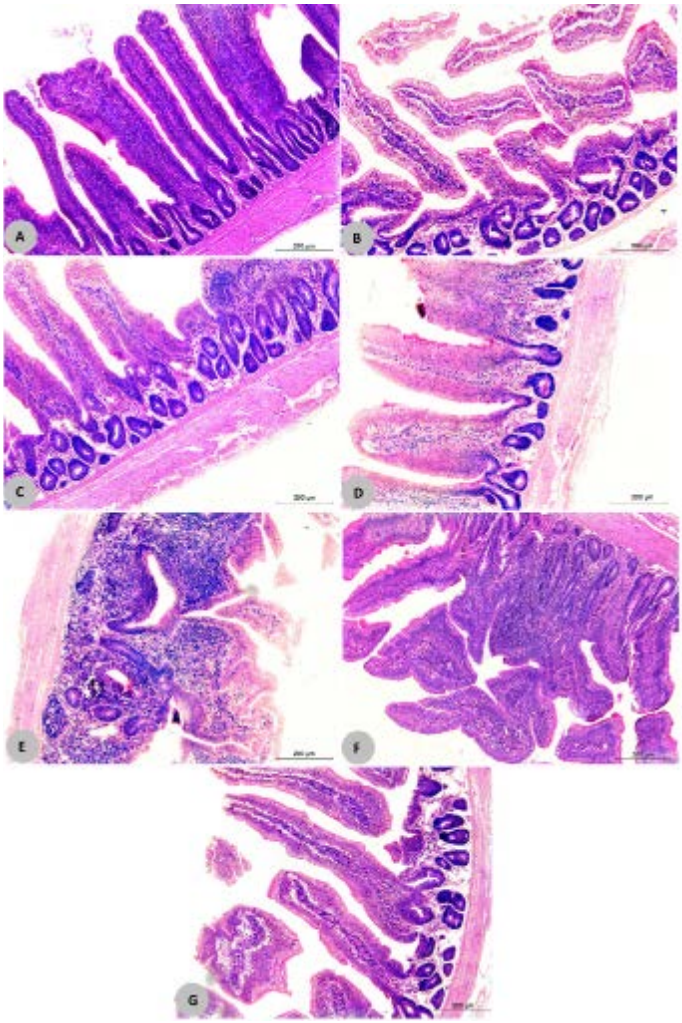

Figure 2: *Jejunum histopathology of CP experimentally inoculated EOs treated chicks at 20 days of age (3DPI). (A) Thyme prevention, (B) Clove prevention, (C) Thyme treatment, (D) Clove treatment, (E) Ciprofloxacin treatment, (F) negative control, (G) positive challenge control.*

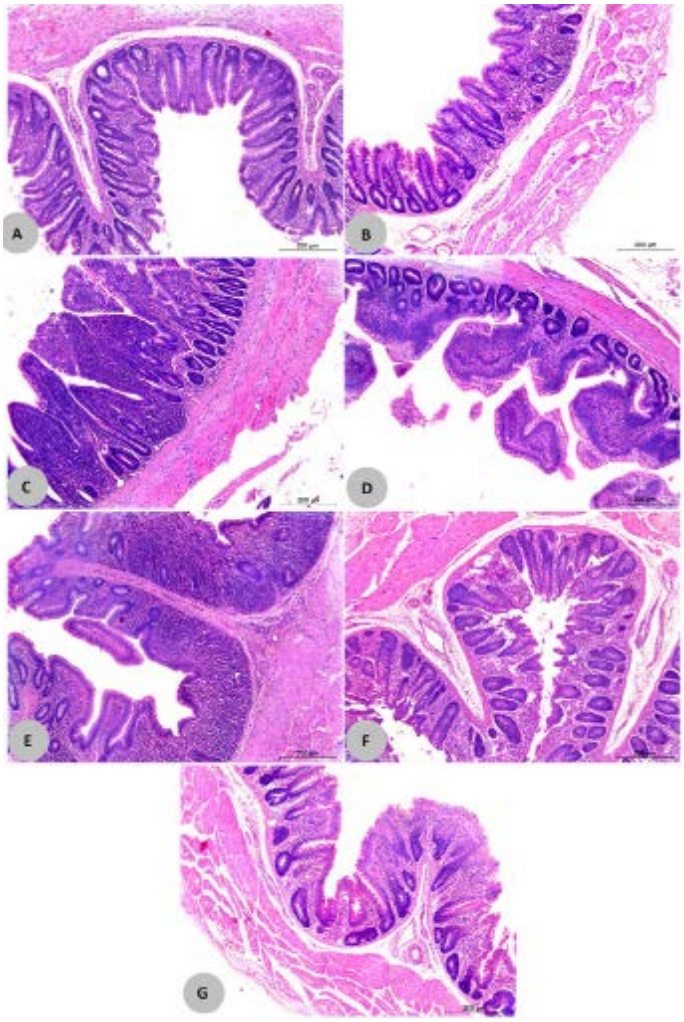

Figure 3: *Cecum histopathology of CP experimentally inoculated EOs treated chicks at 20 days of age (3DPI). (A) Thyme prevention, (B) Clove prevention, (C) Thyme treatment, (D) Clove treatment, (E) Ciprofloxacin treatment, (F) negative control, (G) positive challenge control.*

Tiihonen et al., 2010). Detailed investigations should be conducted to elucidate the changes in intestinal microbiota upon the EOs feed supplementation.

Gross intestinal pathology in EOs treated broiler chickens

Both thyme and clove EOs significantly reduced the gross pathology scores in intestine of treated chickens especially when used as preventive feed additive. Meanwhile typical gross lesions were observed in the challenge control group (Table 4).

Jejunal and cecal histopathological lesions in EOs treated broiler chickens

At 3 DPI, the two groups with thyme and clove as preventive feed additives, the jejunum showed minimal necrosis while cecum showed mild hyperplasia in

goblet cell in mucosa, mild leukocytic infiltration and mild congestion in sub mucosa. The cecum in both groups showed moderate degenerative changes, moderate hyperplasia in lining epithelium with moderate hyperplasia in goblet cell in mucosa while in sub mucosa showed moderate intestinal gland degenerative changes, moderate intestinal gland hyperplasia, moderate leukocytic infiltration with moderate congestion while in musclosa showed moderate degenerative changes (Figure 2 and 3).

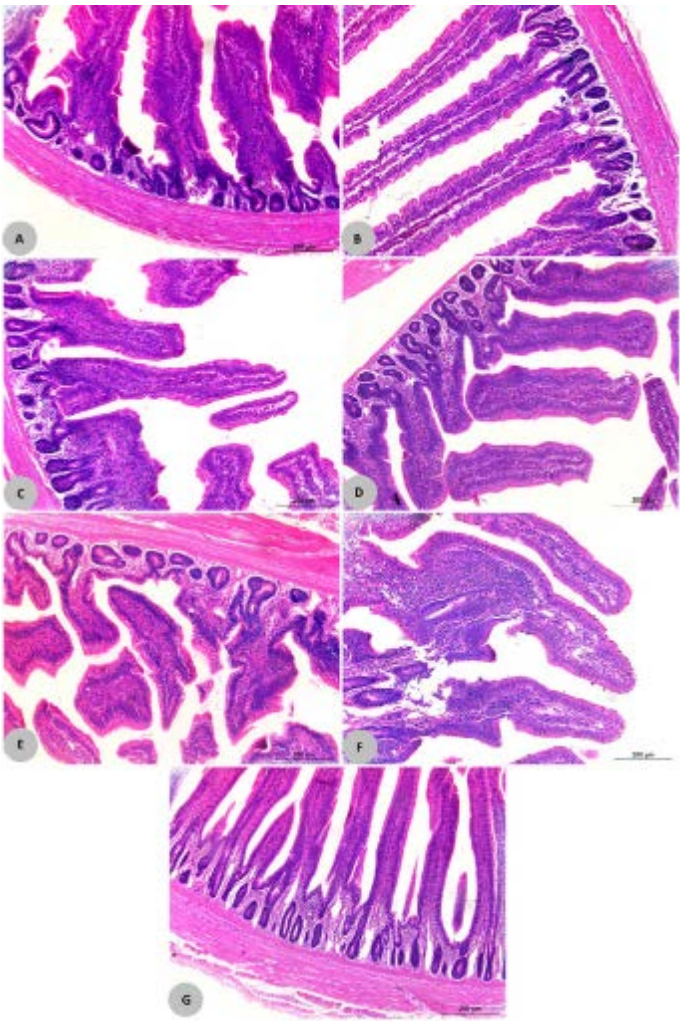

Figure 4: *Jejunum histopathology of CP experimentally inoculated EOs treated chicks at 25 days of age (7DPI). (A) Thyme prevention, (B) Clove prevention, (C) Thyme treatment, (D) Clove treatment, (E) Ciprofloxacin treatment, (F) negative control, (G) positive challenge control.*

By 7 DPI, the differences in histopathology were more prominent between all prevention and treatment groups and the positive challenge control groups with the minimal lesions observed in the antibiotic CIP treated group, clove and thyme treatment groups in the jejunum. The lesions included mucosal mild degenerative changes, mild necrosis, moderate hyperplasia in lining epithelium with mild hyperplasia in goblet cell. In the sub mucosa mild intestinal gland degenerative changes, moderate intestinal gland hyperplasia, mild leukocytic infiltration and mild congestion were noticed (Figure 4).

However, in the cecum the minimal lesions were observed in the antibiotic CIP, clove treatment and thyme prevention groups. The lesion in these groups were mild degenerative changes, mild necrosis, moderate hyperplasia in lining epithelium with mild hyperplasia in goblet cell in the jejunum mucosa. In the sub mucosa found that mild intestinal gland degenerative changes, moderate intestinal gland hyperplasia, mild leukocytic infiltration with mild congestion. The cecum mucosa showed mild degenerative changes, mild necrosis, mild hyperplasia in lining epithelium with no hyperplasia in goblet cell. The sub mucosa minimal intestinal gland degenerative changes, mild intestinal gland hyperplasia, mild leukocytic infiltration with mild congestion (Figure 5).

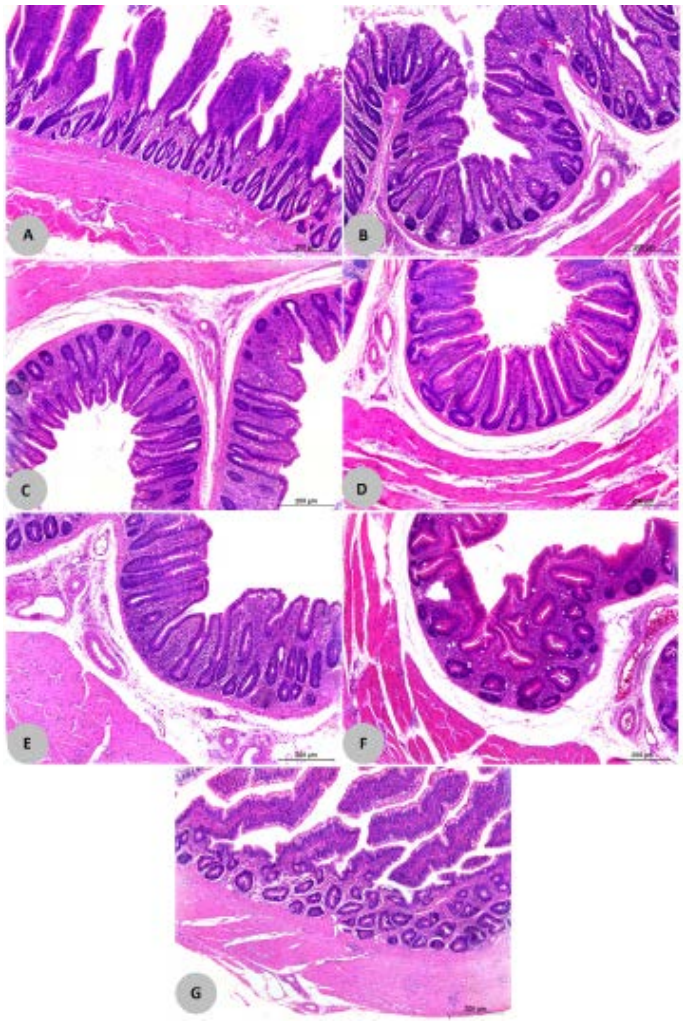

Figure 5: *Cecum histopathology of CP experimentally inoculated EOs treated chicks at 25 days of age (7DPI). (A) Thyme prevention, (B) Clove prevention, (C) Thyme treatment, (D) Clove treatment, (E) Ciprofloxacin treatment, (F) negative control, (G) positive challenge control.*

After 10 DPI, all the EOs treatment groups showed prominently less intestinal histopathology as compared to the antibiotic CIP treatment group. The lesions were mild degenerative changes, mild necrosis, mild hyperplasia in lining epithelium with mild hyperplasia in goblet cell in the jejunum mucosa. The cecum mucosa also showed moderate degenerative changes, no necrosis, moderate hyperplasia in lining epithelium with moderate hyperplasia in goblet cell. In the cecum sub mucosa moderate intestinal gland degenerative changes, mild intestinal gland hyperplasia, mild to moderate leukocytic infiltration with mild congestion (Data not shown).

The ability of EOs to alleviate experimental NE infection was also confirmed by reduced lesion scores in jejunum and cecum. It is well known that the maximum absorption and digestion capacities provided by a large luminal area with villus height and mature enterocytes which was essential to poultry development (Cera et al., 1988). Birds with EOs as feed additives were reported to have longer ileal villi with excellent gut health, high absorptive efficiency and healthier intestinal tract (Alfaro et al., 2007). Recent studies reported that dietary supplementation of EOs (contained thymol and carvacrol) reduced intestinal lesions, improved the intestinal histomorphology and enhanced the specific immune response in the CP-challenged broiler chickens (Yin et al., 2017). Therefore, the reduced lesion observed in EOs treated birds can also explain the better feed conversion rates and performance of birds.

Conclusions

The present study provides an evaluation of thyme and clove as feed additives against CP infection in broiler chickens. The two EOs improved growth performance of broiler chickens especially when used as preventive feed additive continuously starting from 10 days of age. The tested EOs reduced CP counts in intestine and alleviated intestinal damage caused by infection. Further studies of the possibility of using EOs directly to treat and/or prevent NE and other enteric diseases are needed. Moreover, the effects of EOs on intestinal microbiota, poultry feed components, and poultry meat quality need to be elucidated.

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Author's Contributions

ENM, AA, AMD and BS designed the experimental protocol, performed the experiments and wrote the manuscript. EE conducted the histopathological examination. All authors reviewed and approved the manuscript.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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