The effect of essential oils blends on the inhibition of the *Clostridium perfringes* and the rate of intestinal epithelium proliferation in broilers

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Abstract: The present study was conducted to study the effect of essential oils blends on the inhibition of the *Clostridium perfringes* and the rate of intestinal epithelium proliferation in broilers. The experiment was carried out on 12000 1-d-old unsexed Ross chickens of the same origin in experimental and control groups. All birds were vaccinated against infectious bronchitis and infectious bursal disease. To avoid mortality from yolk sac infections birds were treated with enrofloxacin per water from d 2 to 4 of age. All birds were raised in enclosed houses with forced air ventilation. Feed was provided with pan feeders. The whole broiler houses were heated with a gas-fired system. The essential oils blends (curcumin, sage extract and piperine), while, the antibiotic used were (ampicillin, streptomycin, lincomycin and amoxycillin). This study concluded that, using of the essential oils blends in the broiler feeding for the inhibition of the clostridium perfringes can improve the weight and weight gain of the broilers with reduction in mortality rate and percentage due to improvement of feed intake and improving the intestinal epithelium growth and improving the health condition of the intestinal villi. That improve the economic return obtained from the broilers than the addition of antibiotics.
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1. Introduction

Clostridium perfringes is a Gram-positive spore forming anaerobic bacterium that is commonly found in the environment and in the gastrointestinal tract of birds and humans as part of the normal gut microbiota **(Songer, 1996).** *C. perfringens* strains are classified into five types (A to E) on the basis of the production of four major toxins known as the alpha, beta, epsilon and iota toxins **(Petit *et al.*, 1999).** Although *C. perfringens* type A is frequently found in the gastrointestinal tract of healthy poultry, it is also associated with necrotic enteritis in broilers **(Gholamiandekhordi *et al.*, 2006).**

Clostridium perfringes (Cp) type A or C is considered to be the main causative agent of necrotic enteritis (NE). Cases of NE have been reported from most areas of the world where poultry are produced. This economically relevant disease is clinically characterized by depression of the birds, decreased appetite, and reluctance to move. Birds suffer from diarrhea, they have ruffled feathers, and mortality increases in affected flocks. Typical gross lesions are confined to the small intestine, primarily the jejunum and ileum. This disease is often accompanied by hepatitis or cholangiohepatitis **(Ficken and Wages, 1997).**

The disease can occur in two forms; it may present as acute clinical disease or as sub-clinical disease. The acute clinical form of the disease is characterized by a sudden increase in flock mortality, often without premonitory signs. In the sub-clinical form, the clinical signs are milder and usually there is no peak mortality. Intestinal damage leads to production losses due to decreased digestion and absorption, reduced growth rate and increased feed-conversion ratio. (**Nauerby *et al.*, 2003).**

The antibacterial effect of EO *in vitro* is well established. Clove oil, with its active principle eugenol, inactivates Cp and other bacteria **(Briozzo *et al.*, 1988).** Numerous reports exist about the antibacterial effects of *Origanum vulgare*, *Piper nigrum*, *Syzygium aromaticum*, and *Thymus vulgaris*, and the EO components thymol, carvacrol, and eugenol against *Clostridium sporogenes* **(Kaldhusdal and Skjerve, 1996).**

Development of necrotic enteritis depends on the presence of predisposing factors, two of the most important being mucosal damage caused by coccidial pathogens and feed containing high protein levels **(Williams, 2005 and Dahiya *et al.*, 2006).** Proteolytic enzymes are thought to play a role in the early stages of lesion development **(Olkowski *et al.*, 2008).** Recently, a novel pore-forming toxin, net B has been identified in a *C. perfringens* outbreak strain **(Keyburn *et al.*, 2008 and Van Immerseel *et al.*, 2009).** It was shown that this toxin is essential to induce necrotic enteritis. Almost none of the *C.*

perfringens strains isolated from healthy poultry and almost every isolate.

Candidate feed additives for preventing necrotic enteritis are short-chain and medium-chain fatty acids and essential oils. The antibacterial activity of fatty acids has been known for a long time, among these, lauric acid has high antimicrobial activity against *C. perfringens* **(Skrˇivanova´ *et al.*, 2005).** In contrast, butyrate does not inhibit *C. perfringens* but is considered a stimulant of villus growth and could therefore be important for the prevention or regeneration of the epithelial lesions. Some blends of essential oils have been shown to improve broiler performance when given as dietary supplements **(Suk *et al.*, 2003; Herna´ndez *et al.*, 2004; Cross *et al.*, 2007).** It is known that thymol, cinnamaldehyde and eucalyptol have antimicrobial activity against *C. perfringens* **(Candan *et al.*, 2003; Mitsch *et al.*, 2004; Jujena & Friedman, 2007).** It has also been shown that some essential oil blends reduce *C. perfringens* colonization and proliferation **(Mitsch *et al.*, 2004).** Moreover, specific blends of essential oils appear to control coccidial infections and consequently may reduce necrotic enteritis **(Giannenas *et al.*, 2003).**

The present study was conducted to study the effect of essential oils blends on the inhibition of the *clostridium perfringes* and the rate of intestinal epithelium proliferation in broilers.

2. Materials and Methods

Chickens and Housing:

The experiment was made on 12000 1-d-old unsexed Ross chickens of the same origin in experimental and control groups. All birds were vaccinated against infectious bronchitis and infectious bursal disease. To avoid mortality from yolk sac infections birds were treated with enrofloxacin per water from d 2 to 4 of age.

All birds were raised in enclosed houses with forced air ventilation. Feed was provided with pan feeders. The whole broiler houses were heated with a gas-fired system.

Conditions such as temperature and humidity throughout the growing period corresponded to the instructions for Ross broiler chickens and were checked several times a day. Bird density was similar for each group. Cleanouts were completed between all flocks, and for each flock there was fresh litter (straw). Water was provided adlibitum through nipple waterers. Birds were slaughtered between 34 and 46 d of age.

Feed:

Experimental and control groups were fed the same commercial corn-based diets in pelleted form adlibitum. Other dietary ingredients were wheat, peas, soybean, and rapeseed meal. The nutrient content was 21.5 to 22% CP, 6.4 to 6.5% crude fats and 13.0 MJ of ME/kg. Monensin / Natrium were the coccidiostat in all diets. From d 30 until slaughter all birds were fed a commercial finisher diet without coccidiostat**.**

Experimental Design

The experimental design take the form cleared in Table (1):

Table (1): Experimental design.

	Antibiotic treated birds	**EOs treated birds**
Number of birds	6000	6000
In days of 8-9-10	Ampicillin and streptomycin	EOs for 12 hrs
Weight in day 14		↑Than flock treated with antibiotic by 90 gm
In days 18-19-20	Lincomycin	EOs for 12 hrs
In days 28-29-30	Amoxycillin	EOs for 8 hrs
Weight in days 33	1820 gm	2000 gm
In days 33	Cefotaxim, streptomycin and lincomycin	Enrofloxacin and tylosin
In days 37	Antiacid	Clear water

Specific Blends of EO Components

Experimental group A was treated with CRINA poultry (blend A) with its main component thymol, the main constituent of the EO from *Thymus vulgaris*. In the blend for group B, half of the thymol was replaced by carvacrol from *Origanum vulgare*. Other components, used at the same concentration in both blends, were eugenol (*Syzygium aromaticum*, also part of *Cinnamomum zeylanicum*), curcumin (*Curcuma zanthorrhiza*), and piperin (*Piper nigrum*). Blends A and B were mixed to the feed for a dosage of 100 ppm from the first day of age until slaughter in experimental groups.

EO Components

The quantitative analysis of EO components from blends A and B in feed samples was performed by gas chromatography **(International Organization for Standardization, 1985).** For greater sensitivity and specificity, an ion trap mass spectrometer in the

electron impact mode was used as a gas chromatographic detector **(Ragunathan *et al.*, 1999).**

Sampling Procedure*:*

Samples were taken on d 14, 21, and 30. On each sampling day, 3 birds from each flock were euthanized by cervical dislocation. The carcasses of the birds were opened, and 1g of intestinal contents from jejunum, cecum, and cloaca were transferred to sterile plastic bags and stored at 7 ^{o}C. Furthermore on each sampling day, 5 fecal samples (experiments 1 to 7) or 10 fecal samples (experiments 8 to 12) from each flock were collected in plastic bags and stored at 7 ^{o}C. A total of 45 samples from the feed of all groups were also taken. All samples were processed within 2 d.

Bacteriology:

One gram of each sample was diluted 1:9 (wt/vol) in sterile saline. All samples were subjected to 10 sequential dilutions 1:9 (vol/vol). One milliliter from each dilution was inoculated in Crossley Milk Medium4 **(Ko¨ hler, 1992; Quinn *et al.*, 1994)** and incubated at 37 ^{o}C for 24 to 48 h.

Statistical Analysis:

The statistical analysis was made using analysis of variance (ANOVa) for comparing between the different samples in its content with *Clostridium perfrengins*. Also, the Chi^2-test were used for comparison between the group that, treated with antibiotic and that treated with essential oil blend in its level of mortality rate, total returns and net profits according to **(SAS, 2004).**

3. Results and Discussion

The results cleared in Table (2) indicated that, the birds treated with essential oil blends achieve lower mortailty rate, higher feed intake and lower costs with a higher net return than the birds treated with antibiotics.

The results in (Table, 3) cleared that, the clostridium perferenges level decreased significantly ($P < 0.05$) than the groups treated with Ampicillin + Streptomycin and lincospectin and the higher level of clostridium peferenges observed in the samples collected from cloaca, jejunum, fecal matter and cecum, respectively. This results attributed to The EO components were thymol, eugenol, curcumin, and piperin for blend A and thymol, carvacrol, eugenol, curcumin, and piperin for blend B. Specific components of EO inhibit in vitro growth of many bacteria, including various strains of Clostridia such as Cp **(Briozzo *et al.*, 1988; Dorman and Deans, 2000)**. A further effect of EO is the stimulation of digestive enzymes where digestibility of nutrients can be improved **(Platel and Srinivasan, 2000; Williams and and Losa, 2001).** We consider all of these effects a major contribution to better regulation and stabilization of the gut microflora. In the normal intestinal microflora Cp is detected irregularly and in small numbers **(Gerlach, 1994).** There is evidence that the normal gut microflora in healthy birds inhibits the pathogenicity of Cp (**Fukata *et al.*, 1988, 1991).** Furthermore, digestive enzymes such as trypsin inactivate the α-toxin of Cp type A and the β-toxin of Cp type C **(Baba *et al.*,1992).** Thus we believe that the EO antibacterial effect in vitro and effects of stimulation of digestive enzymes, stabilization of the intestinal microflora, and inactivation of Cp toxins may reduce the Cp colonization in the broiler gut.

Histopathological changes:-

At 14 das old chick the main histological changes cleared that, in the groups treated with ampicillin and streptomycin, the micrograph of jujenum (J) is showing many glands (G) and villi (V) undergoing repairing process (Fig, 1).

While, the micrograph in the group treated with lincomycin, the jujenum (J) showing intestinal villi (V) undergoing slow repairing process (arrow) (Fig, 2).

But in the group treated with essential oils blends the Micrograph of jujenum (J) showing many glands (G) with extremely long intestinal villi (V) and numerous goblet cells (Fig, 3).

At the 26 days old chickwhile

Micrograph of the group treated with ampicillin and streptomaycin cleared that, the jujenum (J) is showing glands (G) and degenerative intestinal villi (DV) and numerous goblet cells (Fig, 4).

While, the micrograph of jujenum (J) in the group treated with lincomycin showing intestinal villi (V) undergoing repairing process (arrow) (Fig, 5).

But in the group treated with essential oils blends the Micrograph of jujenum (J) is showing many glands (G) with extremely long intestinal villi (V) and numerous goblet cells (Figs, 6 and 7).

Our results attributed to strong antibacterial effects of essential oils as lauric acid and other medium-chain fatty acids have been documented, especially against Gram-positive bacteria **(Skr˘ivanova´ *et al.*, 2006).** *C. perfringens* strain 56 was also very sensitive to lauric acid. Such molecules most probably prevent necrotic enteritis lesions due to growth inhibition or killing of *C. perfringens* in the gut. Since butyric acid has no significant antimicrobial effect against *C. perfringens*, its action is most probably due to effects on the host. Butyric acid has multiple effects on the gut mucosa that may play a role in the host pathogen interaction. Butyrate possesses anti-inflammatory effects **(Place *et al.*,2005)** and, at low concentrations, it reinforces the colonic defense barrier.

This study concluded that, using of the essential oils blends in the broiler ration for the inhibition of the *clostridium perfringes* can improve the weight, weight

gain of the broilers with reduction in mortality rate and percentage due to improvement of feed intake and improving the intestinal epithelium growth and improving the health condition of the intestinal villi. That improve the economic return obtained from the broilers than the addition of antibiotics.

Table (2): Return parameters obtained from feeding birds on blended essential oils instead of antibiotics.

Item	Antibiotic treated birds	EOs treated birds
Mortality rate	4 %	2 %
Losses due to mortality (EP)	4000	2000
Feed intake	No increase in feed intake	↑ Than another flock by ¾ ton feed
Total returns	No increase in total returns	The profit ↑than another flock after calculating total cost for this flock by 18000EP

$Chi^2 = 10.55$** ** = significant at ($P < 0.01$).

Table (3): *Clostridium perfringens* (Cp) in intestinal and fecal samples in different treatment groups at 14 and 26 days of experiment.

Time	Treatment	Number of birds	Fecal matter	Jejunum	Cecum	Cloaca
After 14 - Days	Ampicillin + Streptomycin	1000	Ac 3.95±0.09	Ab 4.88±0.02	Aa 3.15±0.05	Aa 5.11±0.05
	Lincomycin	2000	Bc 3.33±0.03	Bb 4.21±0.02	Bd 3.13±0.03	Ba 5.05±0.05
	Essential oil blend	3000	Cc 2.55±0.05	Cb 3.11±0.03	Cd 2.11±0.02	Ca 5.00±0.05
After 26-Days	Ampicillin + Streptomycin	1000	Ac 3.21±0.03	Ab 4.12±0.02	Ad 3.10±0.01	Aa 5.10±0.05
	Lincomycin	2000	Bc 3.10±0.01	Bb 4.50±0.06	Bd 2.11±0.01	Ba 5.06±0.05
	Essential oil blend	3000	Cc 2.11±0.02	Cb 3.00±0.02	Cd 2.00±0.01	Ca 4.90±0.04

-Capital litters: Indicated that: Means within the same column of different litters are significantly different at ($P < 0.05$).
-Small litters: Indicated that: Means within the same row of different litters are significantly different at ($P < 0.05$).
-Values in parentheses are the mean log10 Cp concentration per gram of sampled material ± standard deviation

At 14 das old chick

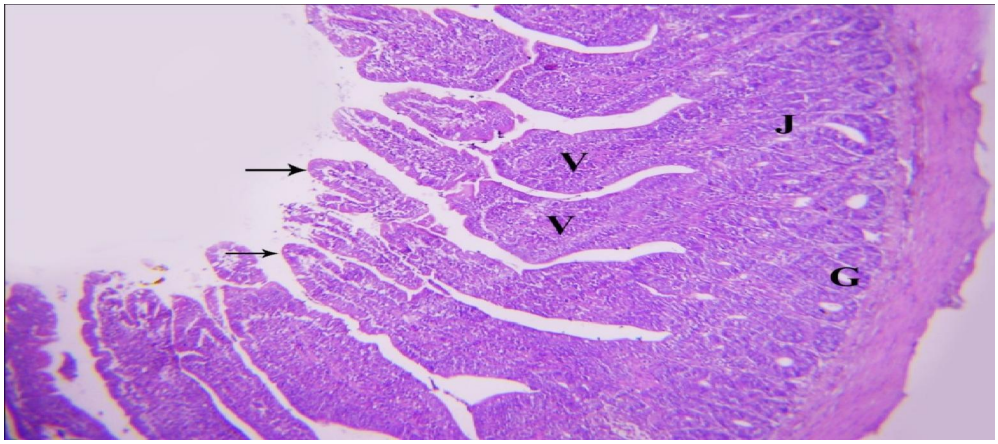


Fig (1): Micrograph of jujenum (J) is showing many glands (G) and villi (V) undergoing repairing process (arrow). Using ampicillin and streptomycin on 14 days old bird.

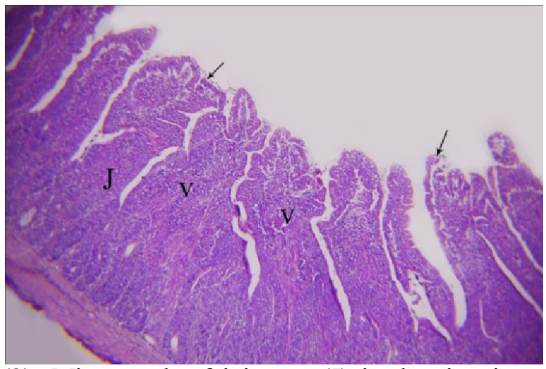
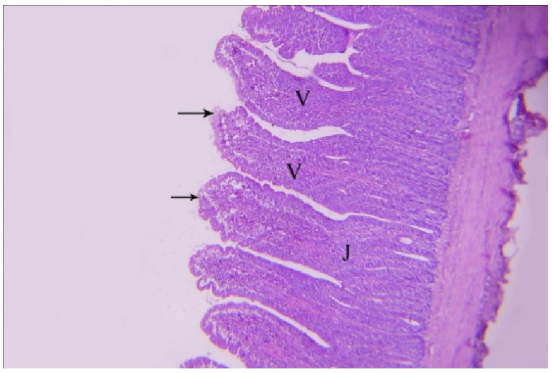


Fig (2): Micrograph of jujenum (J) is showing intestinal villi (V) undergoing repairing process (arrow). Using lincomycin on 14 days old bird.



Fog (5): Micrograph of jujenum (J) is showing intestinal villi (V) undergoing repairing process (arrow). Using amoxycillin on 26 days old bird.

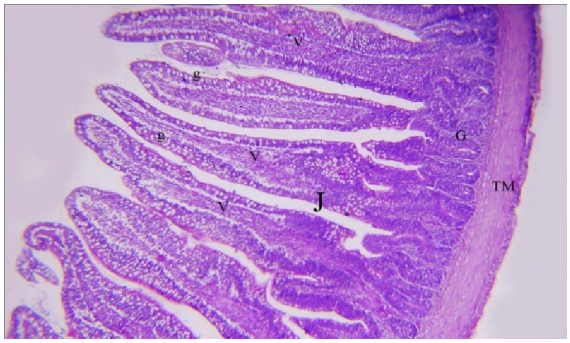


Fig (3): Micrograph of jujenum (J) is showing many glands (G) with extremely long intestinal villi (V) and numerous goblet cells. Tunica muscularis (TM). Using essential oils blends on 14 days old bird.

At the 26 days old chick

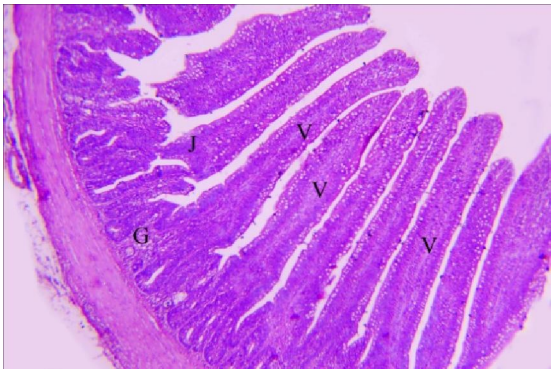


Fig (6): Micrograph of jujenum (J) is showing many glands (G) with extremely long intestinal villi (V) and numerous goblet cells. Using essential oils blends on 26 days old bird.

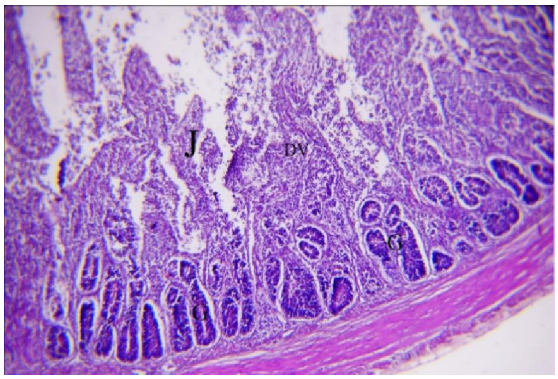


Fig (4): Micrograph of jujenum (J) at 26 days old chick is showing glands (G) and degenerative intestinal villi (DV) and numerous goblet cells.

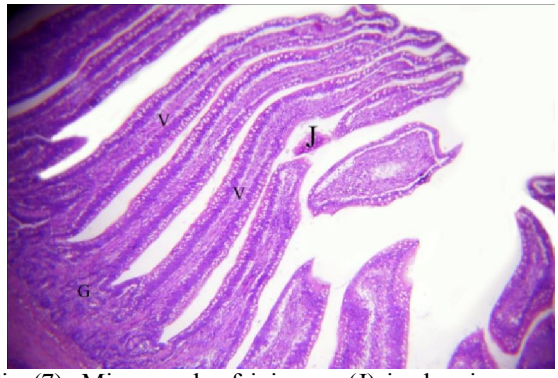


Fig (7): Micrograph of jujenum (J) is showing many glands (G) with extremely long intestinal villi (V) and numerous goblet cells. Using essential oils blends on 26 days old bird.

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