The Effect of Two Different Blends of Essential Oil Components on the Proliferation of *Clostridium perfringens* in the Intestines of Broiler Chickens

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ABSTRACT The effect of 2 different blends of essential oils on *Clostridium perfringens* (Cp) in the intestine and feces of broiler chickens was tested in 6 field trials for each blend. One hundred parts per million of the blends were mixed in a commercial corn-based diet throughout the entire growing period for experimental flocks. Samples from the jejunum, cecum, cloaca, and feces were taken on d 14, 21, and 30 from experimental and control flocks and tested quantitatively for Cp via blood agar plate, litmus milk medium, and ELISA. Blend A reduced ($P \leq 0.05$) the average Cp concentration in the feces on all sampling days, in the jejunum and cecum on d 14 and 21, and in the cloaca on d 14. Blend B effected a significant reduction of Cp concentration in the jejunum on d 14 and 30 and in the cloaca on d 14. The percentages of specimens from the control group that tested positive for Cp were 83.3% for feces, 88.0% for jejunum and cloaca, and 82.6% for cecum. Specimens from the feces and 3 sections of the intestine were Cp positive in groups treated with blend A (60.8, 64.6, 47.9, and 70.8%) and with blend B (65.9, 63.6, 63.6, and 72.7%). Our results indicate that specific blends of essential oil components can control Cp colonization and proliferation in the gut of broilers and therefore may be of help to prevent problems with Cp and necrotic enteritis.

(*Key words*: broiler chicken, *Clostridium perfringens*, essential oil, necrotic enteritis)

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INTRODUCTION

Clostridium perfringens (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 (Köhler, 1992; Vissiennon et al., 1994a,b; Ficken and Wages, 1997).

Although Cp is part of the normal intestinal flora, it is only detected irregularly and in small numbers in the gut of apparently healthy birds (Barnes et al., 1972; Gerlach, 1994). In flocks with median Cp counts above 10^6/g content of the small intestine, there is a higher risk for birds to suffer from NE (Kaldhusdal et al., 1999). Increasing numbers of Cp in the gut of broilers may also lead to lower growth rate and increased feed conversion rate (Balauca et al., 1976; Stutz et al., 1983; Stutz and Lawton, 1984; Kaldhusdal and Hofshagen, 1992). There are many reports about different ways of controlling the number of Cp. In most cases antibiotics or ionophore anticoccidials were used (Stutz et al., 1983; Stutz and Lawton, 1984; Hofshagen and Kaldhusdal, 1992; Elwinger et al., 1998).

Some workers found Cp strains to be resistant to bacitracin, colistin, tetracycline, and other antibiotics (Watkins et al., 1997). Due to the development of antibiotic resistance, concern about the effect of growth promoting antibiotics in animal feed on public health, and the approaching ban of nutritional antibiotics from feed in the European Union (EU), we need alternate methods to control the proliferation of Cp in the digestive tract of poultry.

For many years herbs and spices and their essential oils (EO) have been used as pharmaceuticals in alternative medicine and as a natural therapy (Mitscher et al., 1987). The antibacterial effect of EO in vitro is well established.

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Abbreviation Key: Cp = *Clostridium perfringens*; EO = essential oils; NE = necrotic enteritis.

TABLE 1. Experimental design showing farm number, barn used, number of birds, essential oils (EO) blend assignment, and time of each trial

Trial	Farm	Number of birds in control flocks/used barn	EO blend[1]	Number of birds in experimental flocks/used barn	Months trial was conducted
1	1	25,100/a	A	16,300/b	April/May
2	2	18,300/a	A	16,300/b	April/May
3	3	17,400/c	A	12,600/a	March/April
4	3	17,400/c	B	30,000/b	March/April
5	4	13,800/a	A	15,000/b	June/July
6	1	16,000/b	B	24,200/a	September/October
7	4	14,500/b	B	14,500/a	August/September
8	2	17,300/b	A	17,800/a	November/December
9	4	15,000/b	B	13,800/a	October/November
10	1	25,100/a	B	16,300/b	March/April
11	4	13,800/a	B	15,000/b	February/March
12	4	15,000/b	A	14,000/a	April/May

[1]EO components in blend A: thymol, eugenol, curcumin, and piperin; EO components in blend B: thymol, carvacrol, eugenol, curcumin, and piperin.

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* (Paster et al., 1990; Dorman and Deans, 2000) and other bacteria such as *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhimurium* (Juven et al., 1994; Cosentino et al., 1999). The alcohol extract and the EO from *Curcuma longa* inhibit the growth of *Clostridium septicum*, *Clostridium novyi*, and *Clostridium sporogenes* (Lutomski et al., 1974). However, all these tests were performed in vitro with only a limited number of tests performed in animals (Losa and Köhler, 2001).

The present study was conducted to assess the effect of specific blends of EO components on the intestinal colonization and proliferation of Cp in broilers. Moreover, we tested the potency of the effect of 2 different blends on Cp in the gut and feces.

MATERIAL AND METHODS

Experimental Design

Two blends of EO components were used (blend A and B). Each blend was tested in 6 field trials. In each trial there was one experimental group with EO mixed into the feed and one negative control group. Trials were conducted on 4 commercial farms. Each farm had 2 barns (a and b) except farm 3, which had 3 barns (a, b, and c). On each farm (except farm 3) houses for control and experimental groups were changed after every trial. Details of the experimental design are in Table 1. The time between trials 1 and 12 was 14 mo.

Chickens and Housing

Each experiment started with 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. Birds in control groups in trials 1 and 6 were treated with penicillin from d 15 to 17 because of increased mortality. For other flocks no further medicinal treatment was necessary.

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 ad libitum 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 ad libitum. 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 fat, and 13.0 MJ of ME/kg. Monensin/Natrium was the coccidiostat in all diets. From d 30 until slaughter all birds were fed a commercial finisher diet without coccidiostat.

Specific Blends of EO Components

Both blends[3] were provided in powder form with a total concentration of active EO components of about 30%. Experimental group A was treated with CRINA poultry (blend A) with its main component thymol, the

[3]Crina SA, Gland, Switzerland.

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.

Quantitative Determination of 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 euthanatized by cervical dislocation. The carcasses of the birds were opened, and 1 g of intestinal contents from jejunum, cecum, and cloaca were transferred to sterile plastic bags and stored at 7°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°C. A total of 45 samples from the feed of all groups was also taken. All samples were processed within 2 d. Coccidiosis is a factor that favors an outbreak of NE, therefore the fecal specimens from each flock and sampling day were pooled, 5 g of these specimens were mixed with 100 mL of 40% zinc sulfate, and centrifuged for 5 min at 3,000 rpm, and a specimen taken from the surface was examined microscopically for coccidia. The number of dead birds was recorded for all experiments except experiment 7.

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 Medium[4] (Köhler, 1992; Quinn et al., 1994) and incubated at 37°C for 24 to 48 h. One milliliter of each dilution was inoculated in thioglycollate medium,[5] incubated at 37°C for 24 h, and then inoculated on sheep blood agar base plate[4] with 0.06 μg/mL neomycin supplement.[4] All plates were incubated overnight in anaerobic atmosphere (GENbag anaer[6]) at 37°C. The highest step of dilution with a stormy clot reaction in Crossley milk medium and with colonies surrounded by a typical double-zoned hemolysis on blood agar plates was considered to be adequate to estimate the number of Cp per gram of sample. Furthermore colonies were examined microscopically by Gram stain (Cp is a short, fat, nonmotile gram-positive rod) and tested by ELISA[7] according to the producer's instructions for confirmation of bacterial and toxin identity.

Birds that died were not necropsied unless mortality of the flock was increased (control group in experiments 1 and 6 on d 14 and 15), and no further examinations were performed on these birds.

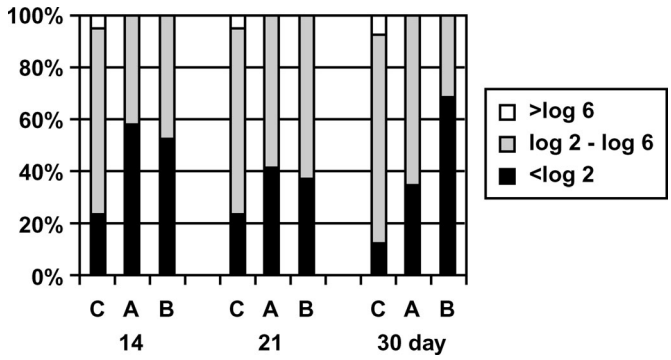

FIGURE 1. Relative frequencies of *Clostridium perfringens* concentrations per gram of sample in the feces of the control group (C) and groups with essential oils blend A (thymol, eugenol, curcumin, and piperin) and B (thymol, carvacrol, eugenol, curcumin, and piperin) on d 14, 21, and 30.

Statistical Analysis

The Cp counts were transferred to the common logarithm. Mean counts from each intestinal region and fecal samples and each sampling day of each flock were calculated. The data of each group (groups A, B, and control) were normally distributed (Kolmogorov-Smirnov-Test).

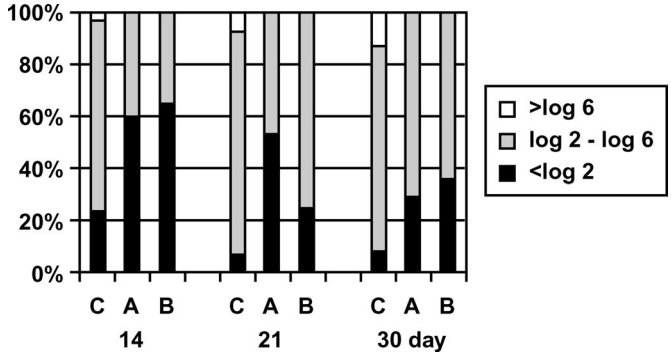

FIGURE 2. Relative frequencies of *Clostridium perfringens* concentrations per gram of sample in the jejunum of the control group (C) and groups with essential oils blend A (thymol, eugenol, curcumin, and piperin) and B (thymol, carvacrol, eugenol, curcumin, and piperin) on d 14, 21, and 30.

[4]Oxoid, Basingstoke, Hampshire, England.
[5]Biokar Diagnostics, Beauvais Cedex, France.
[6]BioMerieux, Marcy l'Etoile, France.
[7]Cypress Diagnostics, Langdorp, Belgium.
[8]SPSS 9.0 for Windows, SPSS Inc., Chicago, IL.

TABLE 2. *Clostridium perfringens* (Cp) in intestinal and fecal samples in control group, group with essential oils (EO) components blend A and B on d 14

Sample source	Control	A[1]	B[2]
	Positive[3]/count[4]	Positive[3]/count[4]	Positive[3]/count[4]
Feces	65/80 (3.33 ± 1.48)[5,a]	21/40 (1.50 ± 0.43)[b]	22/45 (1.85 ± 1.15)[a,b]
Jejunum	25/31 (3.39 ± 1.64)[a]	8/17 (1.06 ± 0.93)[b]	7/17 (1.17 ± 1.07)[b]
Cecum	24/31 (2.53 ± 1.10)[a]	6/17 (0.86 ± 0.65)[b]	7/17 (1.22 ± 1.15)[a,b]
Cloaca	26/31 (3.68 ± 1.34)[a]	9/17 (1.08 ± 0.83)[b]	9/17 (1.50 ± 1.03)[b]

[a,b]Means within rows with no common superscript differ significantly ($P \leq 0.05$).

[1]EO components in blend A: thymol, eugenol, curcumin, and piperin.

[2]EO components in blend B: thymol, carvacrol, eugenol, curcumin, and piperin.

[3,4]Number of Cp-positive samples/total samples.

[5]Values in parentheses are the mean $\log_{10}$ Cp concentration per gram of sampled material ± standard deviation.

Each parameter per day was analyzed with a one-way ANOVA using SPSS 9.0 software.[8] Means were compared using the multiple range test (Scheffé test). All statements of significance are based on a level of probability of less than 0.05.

RESULTS

Bacteriology and EO Determination

The control group consistently showed the highest average concentration of Cp in the feces and the intestine (Tables 2, 3, and 4). The concentration of Cp in the control group was higher ($P \leq 0.05$) than in the experimental group A on d 14 (in jejunum, cecum, cloaca, and feces), d 21 (jejunum, cecum, and feces), and d 30 (feces). The group fed with the EO components blend B had significantly lower Cp counts than the control group on d 14 (jejunum and cloaca) and d 30 (jejunum). There were no differences between groups A and B. Tables 2, 3, and 4 also show the number of Cp-positive specimens out of the total number of tested specimens. The percentage of Cp positive specimens was lower in groups A and B than in the control group through the entire growing period, even though the differences became smaller from d 14 to 30.

As shown in Figures 1 to 4, Cp concentrations below 10^2/g of sample were generally more frequent in groups with EO components in the feed, whereas in the control group more samples had a concentration between 10^2 and 10^6 and above 10^6/g. In the jejunum, for example, 3, 7, and 13% of all Cp counts on d 14, 21, and 30, respectively, were above 10^6/g in the control group. In groups A and B the Cp concentrations were always lower than 10^6/g (Figure 2). The samples with the highest individual Cp concentration were also found in the control group with concentrations of up to 10^8 and 10^9/g in the feces on d 14 and 10^{10}/g in the jejunum on d 21 in experiment 6. The Cp concentration detected in the feces and cloaca generally reflected the concentrations found in the jejunum. ELISA tests showed that all of our Cp strains were type A. Most of the feed samples were Cp negative (17/21 in the control group, 9/12 in group A, and 11/12 in group B). In the positive feed samples there were low Cp concentrations of about 10^1 to 10^2/g of sample. Dosages of both blends of EO in the feed were analyzed and ranged from 72 to 109 ppm in the experimental flocks. No EO component was detected in the control groups.

Mortality and Parasitology

Mortality was low in all groups. From d 1 to 34, 2.21% of the control, 2.03% of group A, and 2.04% of group B birds died. In the control group in experiments 1 and 6, the mortality rate per day was more than doubled from d 14 through 16. Intestinal gross lesions in these birds included scattered necrotic foci and hyperemia in the small intestinal mucosa and right-side cardiac enlarge-

TABLE 3. *Clostridium perfringens* (Cp) in intestinal and fecal samples in control group, group with essential oils (EO) components blend A and B on d 21

Sample source	Control	A[1]	B[2]
	Positive[3]/count[4]	Positive[3]/count[4]	Positive[3]/count[4]
Feces	63/80 (3.25 ± 0.67)[5,a]	24/40 (2.05 ± 0.50)[b]	28/45 (2.33 ± 1.03)[a,b]
Jejunum	27/29 (4.18 ± 1.53)[a]	11/17 (1.67 ± 0.92)[b]	12/16 (2.67 ± 1.91)[a,b]
Cecum	24/29 (3.42 ± 1.12)[a]	9/17 (1.17 ± 1.03)[b]	12/16 (2.53 ± 1.97)[a,b]
Cloaca	26/29 (3.68 ± 1.20)[a]	14/17 (2.14 ± 0.78)[a]	12/16 (2.67 ± 1.18)[a]

[a,b]Means within rows with no common superscript differ significantly ($P \leq 0.05$).

[1]EO components in blend A: thymol, eugenol, curcumin, and piperin.

[2]EO components in blend B: thymol, carvacrol, eugenol, curcumin, and piperin.

[3,4]Number of Cp-positive samples/total samples.

[5]Values in parentheses are the mean $\log_{10}$ Cp concentration per gram of sampled material ± standard deviation.

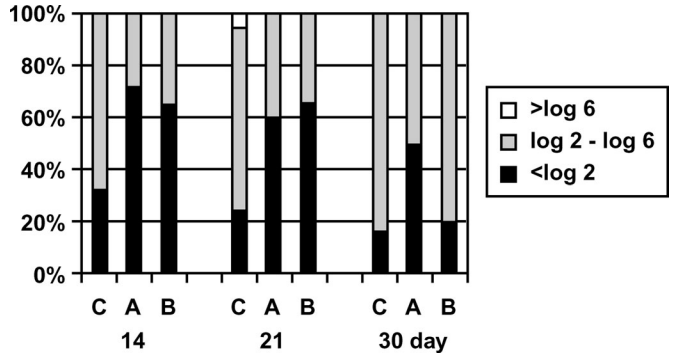

FIGURE 3. Relative frequencies of *Clostridium perfringens* concentrations per gram of sample in the cecum of the control group (C) and groups with essential oils blend A (thymol, eugenol, curcumin, and piperin) and B (thymol, carvacrol, eugenol, curcumin, and piperin) on d 14, 21, and 30.

ment. Average mortality from d 14 to 21 was 0.34% in the control group and 0.25 and 0.33% in groups A and B, respectively. No coccidial oocysts were observed in any fecal specimen.

DISCUSSION

The control group not only showed the significantly highest average Cp concentration in all 3 portions of the intestine and the feces but also a greater percentage of Cp-positive specimens. The reduction of the Cp concentration in the gut and feces of broilers by EO components was significant for both blends through the entire growing period. The strongest effect however was found in the first half of the growing period on d 14 and 21.

Blends A and B were tested once directly against each other. Growing conditions and equipment in all barns were comparable. The Cp counts from the control groups from all trials were homogeneous. Therefore we compared blends A and B directly. The inhibitory effect on the proliferation of Cp seemed to be stronger for blend A.

Our findings correspond to those of Losa and Köhler (2001). Giving a supplementation of 50 ppm CRINA poultry in the feed, they found a reduction of the average log × concentration of Cp/g of content of intestine and a lower rate of detection of Cp in the ileum, rectum, and colon on d 5, 18, and 32. Nevertheless the number of Cp and the rate of detection in the intestine found by Losa and Köhler were lower than in our experiment. This finding may be due to different infection pressure in the feed and environment, a different feed composition (wheat-based diet with meat and bone meal), and the use of zinc-bacitracin in the control group by these authors.

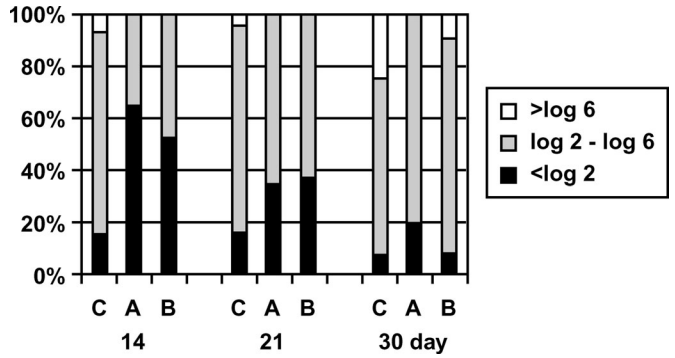

FIGURE 4. Relative frequencies of *Clostridium perfringens* concentrations per gram of sample in the cloaca of the control group (C) and groups with essential oils blend A (thymol, eugenol, curcumin, and piperin) and B (thymol, carvacrol, eugenol, curcumin, and piperin) on d 14, 21, and 30.

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, whereby digestibility of nutrients can be improved (Platel and Srinivasan, 2000; Williams 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 (Barnes et al., 1972; 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 (Niilo, 1965; Arbuckle, 1972; 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.

High numbers of Cp in the gut can cause clinical NE. Kaldhusdal et al. (1999) suggested that median counts of Cp above one million per gram predict a high probability of concurrent NE-specific gut lesions. Other authors found Cp concentrations ranging from 10^5 to 2×10^8/g of sample in the small intestine of birds that died from NE (Köhler et al., 1974; Long et al., 1974; Kondo, 1988). Kaldhusdal and Hofshagen (1992) proposed the term subclinical NE for broiler chickens with no clinical signs of a problem but with higher numbers of Cp and macroscopically visible, focal necrotic lesions in the small intestine. In the present study samples with Cp concentrations above one million per gram predominantly occured in the control groups.

There was no case of clinical NE in experimental groups with EO components in the feed. In the control group in experiments 1 and 6, mortality rate per day was more than doubled at the age of 2 wk. The $\log_{10}$ average Cp concentrations in the jejunum for these control groups were 4.7 on d 14 in experiment 1 and 6.7 on d 21 in experiment 6. In individual specimens, Cp concentrations in the jejunum reached 10^7/g of sample on d 14 and 10^{10}/g on d 21. Besides increased mortality there was no clinical sign of a flock problem. Necropsy of birds that died revealed scattered necrotic foci and hyperemia in the small intestinal mucosa and right-heart failure. The increased mortality rate and the gross lesions indicated a beginning

TABLE 4. *Clostridium perfringens* (Cp) in intestinal and fecal samples in the control group and groups with essential oils (EO) components blend A and B on d 30

Sample source	Control	A[1]	B[2]
	Positive[3]/count[4]	Positive[3]/count[4]	Positive[3]/count[4]
Feces	72/80 (3.95 ± 1.18)[5,a]	28/40 (2.27 ± 0.78)[b]	39/45 (2.75 ± 1.22)[a,b]
Jejunum	22/24 (4.98 ± 1.57)[a]	12/14 (2.97 ± 1.72)[a,b]	9/11 (2.50 ± 1.67)[b]
Cecum	21/24 (3.73 ± 1.68)[a]	8/14 (2.08 ± 1.82)[a]	9/11 (2.86 ± 1.56)[a]
Cloaca	22/24 (5.00 ± 1.88)[a]	11/14 (2.97 ± 1.34)[a]	11/11 (3.69 ± 1.85)[a]

[a,b]Means within rows with no common superscript differ significantly ($P \leq 0.05$).

[1]EO components in blend A: thymol, eugenol, curcumin, and piperin.

[2]EO components in blend B: thymol, carvacrol, eugenol, curcumin, and piperin.

[3,4]Number of Cp positive samples/total samples.

[5]Values in parentheses are the mean $\log_{10}$ Cp concentration per gram of sampled material ± standard deviation.

of NE and were caused by the high numbers of Cp and its toxin. After a treatment with penicillin, mortality decreased to a normal rate.

Although Cp concentrations in the other control groups were also relatively high, there were no increased mortality or signs of NE or subclinical NE. This finding may be due to the absence of factors such as coccidiosis, infectious bursal disease, or dietary stress, which would favor an outbreak of NE (Ficken and Wages, 1997). Nevertheless high numbers of Cp in the intestine are a strong indicator of an occurence of NE (Kaldhusdal et al., 1999). The effect of EO on Cp is, in our opinion, correlated with an effect on the risk of the occurence of NE.

The Cp counts in our experiment agreed with those of Stutz et al. (1983) and Stutz and Lawton (1984). Antibiotics decreased the rate of detection and level of carriage of Cp in cecal samples (Elwinger et al. 1998) similar to what EO did in our experiments. In a situation with a comparable infection pressure, these findings show that EO together with a coccidiostat may reduce the number of Cp similar to that of antibiotics, despite different bacteriological actions and different feed composition and environment.

In addition some reports provide evidence that Cp is a causative agent for growth depression in broilers (Balauca et al., 1976; Köhler et al., 1977; Stutz and Lawton, 1984; Kaldhusdal and Hofshagen, 1992). Reduction of Cp in the small intestine by antibiotics resulted in improved weight gain and feed efficiency in birds fed a soybean-protein-and-sucrose-based diet (Stutz et al., 1983), a practical diet (Stutz and Lawton, 1984), or a barley-based diet (Hofshagen and Kaldhusdal, 1992). Francesch et al. (1999) noted improved feed efficiency in broilers on a wheat-and-barley-based diet with addition of CRINA poultry.

Our results clearly show that specific blends of EO components can control the proliferation of Cp in the broiler intestine. Moreover it appears that different compositions of EO blends may have different efficiencies in this respect. Even though in the present study EO significantly reduced the number of Cp in the intestine and feces of broilers and therefore may have reduced the risk of NE, further studies on the effect of EO on Cp toxins, weight gain, and feed efficiency are necessary.

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