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Control of *Clostridium perfringens*-induced necrotic enteritis in broilers by target released butyric acid, fatty acids and essential oils

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The efficacy of target released butyric acid, medium chain fatty acids (C₆-C₁₂ but mainly lauric acid) and essential oils (thymol, cinnamaldehyde, essential oil of eucalyptus) micro-encapsulated in a poly-sugar matrix, to control necrotic enteritis was investigated. The minimal inhibitory concentrations of the different additives were determined *in vitro*, showing that lauric acid, thymol, and cinnamaldehyde are very effective in inhibiting the growth of *Clostridium perfringens*. The *in vivo* effects were studied in two trials in an experimental necrotic enteritis model in broiler chickens. In the first trial, four groups of chickens were fed a diet supplemented with A) butyric acid, B) essential oils, C) butyric acid in combination with medium chain fatty acids or D) butyric acid in combination with medium chain fatty acids and essential oils. In all groups except for the group receiving only butyric acid, a significant decrease in the number of animals with necrotic lesions was found compared to the infected, untreated control group. In the trial the same products were tested but in a higher concentration. An additional group was fed a diet supplemented with only medium chain fatty acids. In all groups except for that receiving butyric acid in combination with medium chain fatty acids and essential oils, a significant decrease in the number of animals with necrotic lesions was found compared to the infected, untreated control group. These results suggest that butyric acid, medium chain fatty acids and/or essential oils may contribute to the prevention of necrotic enteritis in broilers.

Introduction

Clostridium perfringens is a Gram-positive spore-forming anaerobic bacterium that is commonly found in the environment and in the gastrointestinal tract of animals and humans as part of the normal gut microbiota (Hatheway, 1990; Songer, 1996). *C. perfringens* strains are classified into five types (A-E) on the basis of the production of four major toxins known as the alpha, beta, epsilon and iota toxins (McDonel, 1980; 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 (Nauerby *et al.*, 2003; Gholamiandekhordi *et al.*, 2006). The disease can occur in two forms, it may present as acute clinical disease or 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. There is a general consensus that, although clinical outbreaks of necrotic enteritis may cause high levels of mortality, the sub-clinical form of the disease is more important than the clinical form because it may persist in broiler flocks without overt clinical manifestations. Since the disease is undetected and birds remain untreated, sub-clinical necrosis causes the greatest economic losses in the poultry production industry (Dahiya *et al.*, 2006).

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 (Kaldhusdal & Skjerve, 1996 ; Williams, 2005 ; 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, netB, has been identified in a *C. perfringens* outbreak strain (Keyburn *et al.*, 2008; 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 from diseased chickens carry the gene encoding for this toxin (Van Immerseel *et al.*, 2009). It is thus speculated that only a subpopulation of strains can cause disease, and the presence of such strains in a poultry house is another essential factor for induction of necrotic enteritis. We provided further evidence in support of this hypothesis (Timbermont *et al.*, 2008), showing that outbreak isolates, but not normal microbiota isolates, can cause disease in experimentally infected chickens.

Since the ban on supplementing growth-promoting antibiotics in animal feed in the European Union, necrotic enteritis caused by *C. perfringens* is reemerging in broilers (Grave *et al.*, 2004; Van Immerseel *et al.*, 2004; Williams, 2005). In the main it is the sub-clinical form of the disease, leading to severe production losses, that is the major problem (Vandersluis, 2000; Hermans & Morgan, 2007). Conventional therapeutic antimicrobial agents can be used to control necrotic enteritis. However, this approach is in conflict with the objective to reduce and minimize the use of antibiotics in animal production. Therefore, the poultry industry is currently in need of alternative management or dietary strategies to prevent and control the incidence and severity of this disease.

Candidate feed additives for preventing necrotic enteritis are short- and medium-chain fatty acids and essential oils. The antibacterial activity of fatty acids has been known for a long time (Nieman, 1954). Among these, lauric acid has high antimicrobial activity against *C. perfringens* (Skřivanová *et al.*, 2005). In contrast, butyrate, does not inhibit *C. perfringens* but is considered to be a stimulant of villus growth (Kien *et al.*, 2007) 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; Hernandez *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 *et al.*, 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).

In the present study the efficacy of butyric acid, medium chain fatty acids (C₆-C₁₂ but mainly lauric acid) and essential oils (thymol, cinnamon and essential oil of eucalyptus) or a combination of these products to control necrotic enteritis was investigated.

Materials and Methods

Strains and vaccines. *C. perfringens* strain 56 was isolated from the intestine of a broiler chicken with severe necrotic gut lesions. It is a type A strain (no beta2 or enterotoxin genes), and produces moderate amounts of alpha toxin *in vitro* (Gholamiandehkordi *et al.*, 2006). The strain is netB positive and has been used previously to induce necrotic enteritis in an *in vivo* model (Gholamiandehkordi *et al.*, 2007; Timbermont *et al.*, 2008). Before inoculation of the chickens, the bacteria were cultured anaerobically in brain heart infusion broth (BHI, Oxoid, Basingstoke, England) for 24 h at 42 °C.

The commercial vaccine Nobilis Gumboro D78 (Intervet – Schering-Plough Animal Health, Brussels, Belgium) and the anticoccidial vaccine Paracox-5TM (Schering-Plough Animal Health, Brussels, Belgium), containing live, attenuated oocysts of *Eimeria acervulina*, *Eimeria maxima* (two lines), *Eimeria mitis* and *Eimeria tenella*, were used in this study.

Minimal inhibitory concentrations of additives. Minimal inhibitory concentrations (MICs) were determined for the additives at pH 6. Butyric acid, lauric acid, thymol, cinnamaldehyde and essential oil of eucalyptus (all products from Sigma, St. Louis, Mo.) were tested as serial two-fold dilutions in 96-well microplates in BHI. Concentrations ranged from 220 mg/ml to 0.86 mg/ml for butyric acid, from 0.5 mg/ml to 0.0078 mg/ml for lauric acid, from 3.76 mg/ml to 0.029 mg/ml for thymol, from 1mg/ml to 0.008 mg/ml for cinnamaldehyde and from 10.67 mg/ml to 0.67 mg/ml for essential oil of eucalyptus. Bacterial suspensions were diluted in phosphate buffered saline (PBS) to a density of McFarland 0.5. An inoculum of 5 μ l of this suspension was added to 195 μ l medium in a microwell plate. These suspensions were incubated anaerobically for 20 h at 42 ° and bacterial growth was then assessed visually. Experiments were carried out in triplicate.

Birds and housing. *In vivo* necrotic enteritis trials using experimental inoculations were performed with Ross 308 broiler chickens that were obtained as one-day-old chicks from a commercial hatchery. The birds were kept in isolation. All treatment groups were housed in the same room, in cages of 1 m², with litter on the floor. All cages were separated by solid walls to prevent contact between birds from different treatment groups. Before each trial, the rooms were decontaminated with peracetic acid and hydrogen peroxide (Hygiasept® vaporizer climasept, SARL Hygiasept, Sevrey, France) and a commercial anticoccidial disinfectant (OO-CIDE, DuPont Animal Health Solutions, Wilmington, US). In the first trial, 175 chickens were divided in seven groups of 25 and in the second trial, 200 chickens were divided in two groups of 22 (negative control group and group receiving only coccidia) and six groups of 26. They were given drinking water and feed *ad libitum*. A 23 h/1 h light/darkness program was applied. The animal experiments were carried out according to the recommendations and following approval of the Ethical Committee of the Faculty of

Veterinary Medicine, Ghent University.

***In vivo* necrotic enteritis model.** Trials were performed as described previously (Gholamiandehkordi *et al.*, 2007). In short, broilers were fed a wheat/rye-based (43%/7.5%) diet, with soybean meal as protein source. The feed composition is described in Gholamiandehkordi *et al.* (2007). Gumboro vaccine was given in the drinking water on day 18 in all groups. From day 19 onwards, the same diet was used with the exception that fishmeal (30%) was used as protein source. All groups were orally challenged using a plastic tube (three times a day) with approximately $4 \cdot 10^8$ colony-forming units (cfu) *C. perfringens* bacteria on days 19, 20, 21 and 22. On day 20 all birds, except those in the control group, were orally inoculated with a 10-fold dose of Paracox-5TM. On days 23, 24 and 25, eight animals of each group were euthanized by intravenous injection of T61 (Intervet, Mechelen, Belgium). Necrotic enteritis lesion scoring and sample collection was performed, as described below.

In the first trial, four groups of chickens were fed a diet supplemented with butyric acid (group but), butyric acid in combination with medium chain fatty acids (C₆-C₁₂ but mainly lauric acid) (group but/fat), butyric acid in combination with medium chain fatty acids and essential oils (thymol, cinnamon, and essential oil of eucalyptus) (group but/fat/eo) and essential oils alone (group eo). The concentrations of the additives are shown in Table 1.

In the second trial, the same products were tested but in a higher concentration. Moreover, an extra group fed a diet supplemented only with medium chain fatty acids (group fat) was included (Table 1).

Three control groups were included in each trial: a non-medicated, non-infected group (negative control), a non-medicated group receiving only the 10-fold dose of the Paracox-5TM vaccine (only coccidia) and a non-medicated, infected group (positive control).

Macroscopic lesion scoring. Lesions in the small intestine (duodenum to ileum) were scored as described by Keyburn *et al.* (2006) as follows: 0 = no gross lesions; 1 = congested intestinal mucosa; 2 = small focal necrosis or ulceration (1-5 foci); 3 = focal necrosis or ulceration (6-15 foci); 4 = focal necrosis or ulceration (16 or more foci); 5 = patches of necrosis 2-3 cm long; 6 = diffuse necrosis typical of field cases. Lesion scores of 2 or more were classified as necrotic enteritis positive.

Statistical analysis. The data were analyzed with SPSS 16 software using the multivariate logistic regression method to compare the number of necrotic enteritis positive animals (lesion score ≥ 2) in the test groups with the number of necrotic enteritis positive animals in the positive control group. Significance was determined at $P < 0.05$.

Results

Minimal inhibitory concentrations of additives. MICs are shown in Table 2. Lauric acid was most effective in inhibiting the growth of *C. perfringens* strain 56 followed by thymol and cinnamaldehyde. Essential oil of eucalyptus and butyric acid were least effective.

In vivo trials. Table 3 summarizes the number of birds with necrotic lesions in all trials. In both trials, all test products resulted in a decrease of the number of birds having macroscopic necrotic lesions in comparison to the infected, untreated control group (positive control). The mean lesion scores also decreased. These lesions presented as multiple foci, mostly in the duodenum and jejunum. No necrotic enteritis lesions were observed in the control groups, i.e. the negative control group and the group only inoculated with a 10-fold dose of Paracox (only coccidia).

In trial 1, in all groups except for that receiving only butyric acid, a statistically significant decrease in the number of animals with necrotic lesions was found compared to the infected, untreated control group (positive control). The combination of butyric acid with medium chain fatty acids and essential oils gave the best protection against the induction of necrotic enteritis lesions.

In trial 2, addition of butyrate, medium chain fatty acids or essential oils alone gave a statistically significant reduction in the number of animals developing necrotic lesions in the gut. The combination of butyrate and medium chain fatty acids gave the best protection. Supplementation of the feed with butyric acid, medium chain fatty acids and essential oils did not result in a significant decrease in the number of animals having macroscopic necrotic enteritis lesions.

When the results of the two trials were combined and evaluated for statistical differences taking the difference in concentration into account, all groups differed significantly from the positive control group. Overall, the concentration of the additives had no significant effect on the efficacy of the product. Only in group but/fat/eo, did the concentration of the additive have a significant effect.

Discussion

Essential oils probably prevent necrotic lesions by their direct antimicrobial effect on *C. perfringens*. It has been reported that the use of essential oils improves chicken performance and controls the proliferation of *C. perfringens* (Mitsch *et al.*, 2004; Cross *et al.*, 2007). Growth of *C. perfringens* strain 56 was indeed inhibited *in vitro* by low concentrations of thymol and cinnamaldehyde (Table 2). Moreover, an anticoccidial effect of essential oils has been reported (Giannenas *et al.*, 2003). Essential oils can thus potentially have effects on necrotic enteritis by direct antibacterial and anticoccidial effects.

Strong antibacterial effects of lauric acid and other medium-chain fatty acids have been documented, especially against Gram-positive bacteria (Skřivanová *et al.*, 2006). *C. perfringens* strain 56 was also very sensitive to lauric acid (Table 2). Such molecules most likely 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* (Table 2), its action is most likely 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

defence barrier by increasing production of mucins and host antimicrobial peptides (Barcelo *et al.*, 2000; Schauber *et al.*, 2003). Moreover, butyric acid decreases intestinal epithelial permeability by increasing the expression of tight junction proteins (Mariadason *et al.*, 1997; Peng *et al.*, 2007). Finally, butyrate is an energy source for epithelial cells and helps in the maintenance of intestinal villus structure (Kien *et al.*, 2007). It is also possible that effects of butyric acid on other microbial (or protozoal) populations can play a role. For example, butyric acid was reported to have anticoccidial effects; Leeson *et al.* (2005) showed that birds previously fed butyrate can better withstand the stress of coccidial challenge. The decrease in necrotic lesions seen in our study may thus partially be explained by a reduction of the effects of the inoculation with a 10-fold dose of the Paracox-5TM vaccine. As a consequence, no nutrients are leaking in the lumen and *C. perfringens* is probably unable to proliferate to high concentrations.

The results of the second trial showed an additional or synergistic effect of butyric acid and medium chain fatty acids (Table 3). This may be because the predisposing factor, *Eimeria* lesions, as well as *C. perfringens* bacteria themselves are eliminated, thus dealing with two aspects of the pathology.

Butyric acid in combination with medium chain fatty acids and essential oils reduced the number of birds with macroscopic lesions significantly in the first trial but not in the second. In the second trial, higher concentrations of all additives were used. It is known that butyrate at low concentrations is beneficial in promoting mucosal barrier function whereas excessive butyrate may disrupt mucosal barrier function (Barcelo *et al.*, 2000; Peng *et al.*, 2007). Little information is available on the effective dose of essential oils that can be used in animals without inducing toxic effects (Acamovic & Brooker, 2005) and it is possible that the concentration of the additives used in the second trial in this group was too high and therefore a detrimental effect was seen instead of a beneficial effect.

In conclusion, it is shown that butyric acid, medium chain fatty acids (mainly lauric acid) and essential oils (thymol, cinnamaldehyde and essential oil of eucalyptus) or combinations of these additives can be used to control necrotic enteritis in broiler chickens.

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Table 1. *Concentration of test products used in each trial*

Trial	Group	Feed additive	Concentration in starter feed (g/ton)	Concentration in grower feed (g/ton)
1	but	butyric acid	235	176
	but/fat	butyric acid	188	141
		fatty acids	150	112.5
	but/fat/eo	butyric acid	164.5	123
		fatty acids	150	112.5
		essential oils	90	67.5
	eo	essential oils	150	150
2	but	butyric acid	330	250
	fat	fatty acids	400	300
	but/fat	butyric acid	330	250
		fatty acids	360	270
	but/fat/eo	butyric acid	330	250
		fatty acids	360	270
		essential oils	240	180
	eo	essential oils	200	150

Table 2 Minimal inhibitory concentrations (mg/ml) of additives at pH 6 against *C. perfringens* strain 56 grown in BHI

Additive	MIC (mg/ml)
butyric acid	6.88
lauric acid	0.063
thymol	0.24
cinnamaldehyde	0.33
essential oil of eucalyptus	5.33

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Table 3. Number of birds with macroscopic necrotic enteritis lesions and mean lesion scores^a on the three sampling days in each trial

Trial	Group	Day 23	Day 24	Day 25	Total	Total (%)	Mean score
1	negative control	0/8	0/8	0/7	0/23	0	0
	only coccidia	0/8	0/8	0/7	0/23	0	0.48
	positive control	2/8	5/8	5/8	12/24	50	1.63
	but	1/8	3/8	4/8	8/24	33.3	1.33
	but/fat	1/8	1/8	4/9	6/25	24 ^A	0.96
	but/fat/eo	0/8	1/8	3/9	4/25	16 ^A	0.84
	eo	2/8	2/8	1/8	5/24	20.8 ^A	0.83
2	negative control	0/7	0/7	0/8	0/22	0	0
	only coccidia	0/7	0/7	0/7	0/21	0	0.43
	positive control	3/8	5/8	5/10	13/26	50	1.73
	but	0/8	2/8	4/10	6/26	23 ^B	1.19
	fat	0/8	4/8	2/8	6/24	25 ^B	1
	but/fat	0/8	1/8	1/9	2/25	8 ^B	0.4
	but/fat/eo	2/8	4/8	5/10	11/26	42	1.77
	eo	1/8	2/8	3/9	6/25	24 ^B	1.16

^aBirds with macroscopic necrotic enteritis lesions are those with a lesion score of 2 or above.

Values with superscripts differ significantly from the positive control ($P < 0.05$). The negative control group and the group receiving only coccidia were not included in the statistical analysis since there were no animals with lesions (score ≥ 2) in these groups.