Efficacy of protected sodium butyrate, a protected blend of essential oils, their combination, and *Bacillus amyloliquefaciens* spore suspension against artificially induced necrotic enteritis in broilers

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ABSTRACT Necrotic enteritis caused by *Clostridium perfringens* leads to serious economical losses to the poultry industry. There is a growing need to find effective, nontoxic, antibiotic alternatives to prevent and cure the disease. In our study, the efficacy of protected sodium butyrate at 1.5 g/kg (BP70), a *Bacillus amyloliquefaciens* spore suspension with 10^9 cfu/g (BAL; Ecobiol), a protected blend of essential oils (1%) at 1.5 g/kg (EO), and a combination of sodium butyrate with essential oils (1%) protected with vegetable fat at 1.5 g/kg (BP70+EO; Natesse) was investigated in an artifical *C. perfringens*-infection model. Body weight gain, gross pathological and histopathological lesion scores, villus lengths, and villus length:crypt depth ratio was determined and compared with the control group. Broilers infected with *C. perfringens* and treated with essential oils or the combination of sodium butyrate and essential oils showed significantly better BW gain ($P < 0.05$), increased villus length and villus length:crypt depth ratio ($P < 0.001$), and decreased gross pathological and histopathological lesion scores ($P < 0.05$) compared with the control. Sodium butyrate alone and *B. amyloliquefaciens* spore suspension had no beneficial effects on the course of the disease in this study. According to our results, the protected combination of sodium butyrate and essential oils, as well as the protected essential oils, can be potential candidates for the prevention and treatment of necrotic enteritis in broiler chickens.

Key words: broiler chicken, necrotic enteritis, sodium butyrate, essential oil, probiotic

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INTRODUCTION

Necrotic enteritis (**NE**) is a commonly diagnosed disease in poultry flocks, causing severe economical losses. *Clostridium perfringens* is a gram-positive, spore-forming bacterium that grows only under anaerobic conditions. Alpha-toxigenic strains are involved in the pathogenesis of NE, which mainly affects 2- to 5-wk-old chickens and requires several predisposing factors to develop. Such factors are coccidiosis (Al-Sheikly and Al-Saieg, 1979; Jackson et al., 2003), high protein content of the feed (Kocher et al., 2003; Palliyeguru et al., 2010), high animal density, stress, and immunosuppression (Lee et al., 2011).

Future restrictions in the usage of anticoccidial drugs in the European Union might certainly increase the incidence and severity of NE in poultry farms (Elwinger et al., 1992). Administration of antibiotics against clostridia in these cases can result in drug residues in the meat, spreading of resistance among pathogenic bacteria, and further economic losses because of drug costs.

Thus, antibiotic alternatives, like organic acids, essential oils, and probiotics have an increasing importance in the prevention and treatment of NE. Recently, volatile short-chain fatty acids, like butyric acid, play an important role in the poultry industry because they can reduce the shedding and number of gram-negative bacteria in the intestinal tract (Hirshfield et al., 2003). Similarly to other organic acids, butyric acid can be used for the treatment of several intestinal bacterial infections, like salmonellosis (Van Immerseel et al., 2005; Fernández-Rubio et al., 2009). Timbermont (2009) demonstrated that sodium butyrate decreased the incidence of NE in broilers. Undissociated sodium butyrate can penetrate a bacterial cell wall and dissociate to H^+ and anions inside the cell, lowering pH and resulting in energy deficiency and osmotic problems in the organism. This leads to a bacteriostatic or bacteri-

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cidal effect (Dahiya et al., 2006). In addition, sodium butyrate can increase BW gain, feed conversion ratio, and, more importantly with respect to NE, it amends villus morphology in chickens (Hu et al., 2007; Mallo et al., 2010). Numerous beneficial effects of the essential oils were reported in the last 10 yr. Barbosa et al. (2009) presented the MIC_{90} (minimum inhibitory concentration 90%) of ginger oil as 0.56% (vol/vol) against gram-negative bacteria but did not examine activity against *C. perfringens*. Oviedo-Rondón et al. (2006) reported beneficial effects of essential oils in the control of coccidiosis predisposing to NE in chickens. Essential oil blends used in that study decreased lesion scores in chickens artificially infected with *Eimeria*. Essential oils can stimulate the secretion of digestive enzymes and might also have an in vitro antimicrobial activity against several pathogenic bacteria. Briozzo et al. (1989), Dorman and Deans (2000), and Mitsch et al. (2004) reported antibacterial activity of some essential oils, namely thymol, carvacrol, curcumin, piperine, and eugenol, against various strains of *C. perfringens*. McReynolds et al. (2009) reported beneficial effects of essential oils, mainly, but not exclusively, citrus, oregano, and annase, in the treatment of NE in broilers by decreasing mean lesion scores and mortality.

Probiotics play an important role in stabilizing the intestinal ecosystem of animals by enhancing nutrient digestibility (Apata, 2008), increasing performance (Kabir et al., 2004), and competing with pathogenic bacteria in the intestine (Higgins et al., 2008; Vicente et al., 2008). Administration of bacteria belonging to the *Bacillus* genus have beneficial effects in several conditions, like enteritis caused by *Escherichia coli*, *Salmonella enterica*, or *C. perfringens* (Barbosa et al., 2005). Craven et al. (1999) reported decreased *C. perfringens* colonization and a lower incidence of NE in chickens treated with bacteria belonging to the normal gut flora. McReynolds et al. (2009) reported beneficial effects of a probiotic-containing *Enterococcus* sp., *Pediococcus* sp., *Bifidobacterium* sp., and *Lactobacillus* spp. against NE.

In this study, efficacy of 4 different compounds was tested for the treatment of NE in an artificial infection model in broiler chickens. The effect of concentrated and protected sodium butyrate (**BP70**), a protected blend of essential oils (ginger oil and carvacrol) at 1% (**EO**), a protected combination of sodium butyrate with 1% essential oils (ginger oil and carvacrol) (**BP70+EO**), and a *Bacillus amyloliquefaciens* (**BAL**) probiotic spore suspension on NE incidence was investigated in this experiment. A concentrated and vegetable fat-protected sodium butyrate product, BP70 (Norel SA., Madrid, Spain), that releases the active substance slowly, thus having an effect along the whole gastrointestinal tract, was used in this study. The BP70 combined with the essential oils is a highly effective compound bearing the above-mentioned mechanism of action of sodium butyrate and diverse beneficial effects of the essential oil blend containing ginger oil and carvacrol.

MATERIALS AND METHODS

Bacterial Strain, Feed Additives, and Vaccines

A strain of *Clostridium perfringens* was obtained from the Hungarian National Collection of Medical Bacteria, National Center of Epidemiology (Budapest, Hungary). This strain is of poultry origin and is identical to a reference strain having an American-type culture collection code of 13124 and is a type-A, α-toxigenic strain. The *C. perfringens* A was propagated in reinforced clostridial medium (Merck, Darmstadt, Germany) at 37°C for 24 h in anaerobic jars with the use of the Anaerocult A system (Merck). The bacterium culture was pelleted by centrifugation (3,000 × *g* for 10 min at 5°C; Universal 320R Centrifuge, Hettich Zentrifugen, Tuttlingen, Germany) and resuspended in sterile PBS solution. The *C. perfringens* A titer was 3 to 4 × 10^8 cfu/mL. Feed additives used in the study (all from Norel S. A., Madrid, Spain) were BP70 (a highly concentrated and protected sodium butyrate, Ecobiol), a *Bacillus amyloliquefaciens* spore suspension (BAL) in a concentration of 10^9 cfu/g, protected essential oils alone at 1% (EO; ginger oil and carvacrol), and a protected combination of sodium butyrate and the same essential oils at 1% (BP70+EO; Natesse). The commercial bursal disease vaccine CEVAC Gumbo (Ceva-Phylaxia, Budapest, Hungary) and the anticoccidial vaccine Paracox-5 (Ceva-Phylaxia) containing live, attenuated oocysts of *Eimeria acervulina*, *Eimeria brunetti*, *Eimeria maxima*, *Eimeria mitis*, *Eimeria necatrix*, *Eimeria praecox*, and *Eimeria tenella* were used in this experiment.

Experimental Animals and Study Design

Experimental animals used in the study were conventional nonvaccinated broiler chickens (Bábolna Hybrid, Bábolna, Hungary) acquired at one day of age. The birds were kept in strict isolation on deep litter with a density of 25 animals/m^2, according to the method of Gholamiandehkordi et al. (2007). Litter and the drinking and feeding troughs were sterilized by autoclaving. Ambient temperature was set to 32 ± 4°C for the first 5 d and then decreased continuously to 24 ± 4°C for 3 wk. Animals were fed with a drug-free, high-protein content diet containing 25% fishmeal. The composition of the basal diet is shown in Table 1. No coccidiostats were present in the feedstuff. Sterilized tap water was at their disposal ad libitum. The investigation was authorized by the Hungarian Animal Welfare Commitee of the Szent István University Faculty of Veterinary Science of Hungary.

Experimental animals (n = 160) were divided into 5 groups, originally each group consisted of 32 animals. Animals in group A served as a control and were fed with the basic diet without any feed additives. Animals in the other groups were administered a drug and

Table 1. Composition of basal diet

Item	Amount
Feed ingredient (%)	
Corn	64
Rye	8
Fishmeal	25
Vitamin and mineral premix[1]	3
Calculated feed analysis (per kg of diet)	
ME (kcal)	3,173
CP (%)	31.2
Fat (%)	6.8
Fiber	2.9
Met	0.8
Lys	1.5
Ca	1.2
P	0.8
Coccidiostatics	None

[1]KPB-512 vitamin and mineral premix for broilers (Babolna Hungary Ltd., Babolna, Hungary) containing methionine, 20 g/kg; calcium, 230 g/kg; phosphorus, 65 g/kg; sodium, 35 g/kg; vitamin A, 171,450 IU/kg; vitamin D, 57,150 IU/kg; vitamin E, 571.5 mg/kg; and vitamin K_3, manganese, zinc, selenium, iron, copper, cobalt, vitamin B_1, vitamin B_2, vitamin B_6, vitamin B_{12}, pantothenic acid, niacin, choline, folic acid, biotin, without coccidiostatics.

coccidiostat-free feedstuff supplemented with different feed additives: (B) 1.5 g/kg of BP70, (C) 1.0 g/kg of BAL (corresponding to 10^9 cfu/kg dosage), (D) 1.5 g/kg of EO, and (E) 1.5 g/kg of BP70+EO.

All birds were treated with bursal disease vaccine administered via drinking water on d 16 to cause immunosuppression, according to the method of Gholamiandehkordi et al. (2007). Inoculation with *C. perfringens* A was done on d 18, 19, 20, and 21 three times a day (at 0800 h, 1200 h, and 1600 h). Animals were challenged with 2 mL of *C. perfringens* A suspension (6–8 $\times$ 10^8 cfu) orally via gastric tube each time. On d 19, a 10-fold dose of a commercial live attenuated vaccine (Paracox-5) was presented to the animals as a predisposing factor to clinical disease to mimic the detrimental effect of coccidiosis (Gholamiandehkordi et al., 2007). On d 25, all animals were killed by an intravenous injection of pentobarbital (Euthasol 40%, AST Beheer, the Netherlands). Body weight of the animals was measured and compared during the experiment on d 3, 7, 10, 14, 16, 20, and 25.

Gross Pathological and Histopathological Examination and Morphometric Evaluation

The killed animals were submitted to gross pathological and histopathological examinations. In NE, major gross pathological observations are present in the small intestine and the liver (Lovland and Kaldhusdal, 2001). The locations of sample taking were the duodenal flexure (duodenal sample), 10 cm proximally from the Meckel's diverticulum (jejunal sample), and 5 cm proximal to the iliocecal junction (ileal sample). Liver samples were taken consistently from the right lobe. Gross macroscopic lesions in the small intestine (duodenum to ileum) were scored according to the method of Gholamiandehkordi et al. (2007) with some modifications. The following lesion scores were applied: 0 = negative; 1 = inflammation of the intestine; 2 = focal necrosis (1–2 cm) or degenerative changes in the mucosa; 3 = patchy necrosis (3–4 cm); and 4 = diffuse necrosis, thick intestinal wall. Localization, size, severity, depth of necrosis, and severity and extent of inflammation were also noted. Pathologic alterations in the liver, spleen, and pancreas were also noted and scored as none (0), mild (1), moderate (2), or severe (3). Scores for the intestinal and hepatic alterations were summarized and evaluated as total. For the histopathological examination, samples were collected from the liver, spleen, duodenum, jejunum, ileum, and pancreas. Microscopic lesions were scored taking into account lympho-histiocytic infiltration, necrosis, villus fusion, capillary dilation, capillary hemorrhages, epithelial cell defects, and blood and proteinaceous material in the intestinal lumen. Each attribute was evaluated with a score between 0 and 3 (0, none; 1, mild; 2, moderate; and 3, severe); these were summarized, and mean scores were attached to each sample. Jejunal samples were subjected to morphometric analysis; villus length and crypt depth (15/animal) were measured, and the villus length:crypt depth ratio (**V:C**) was calculated. According to Gholamiandehkordi et al. (2007), the V:C ratio is appropriate for analyzing the impact of *C. perfringens* in subclinical NE.

Statistical Analysis

Statistical analysis of BW, gross pathological and histopathological results, villus lengths, and V:C ratio was carried out with Statistica 2009 software (StatSoft, Tulsa, OK). Differences between means were evaluated throughout the study by ANOVA where data were of normal distribution and homogeneity of variances was confirmed. A P-value of < 0.05 was accepted to indicate statistical significance. For pairwise comparisons of means, Tukey's post hoc test was used.

RESULTS

Broiler BW Gain and Mortality

Because the artificial infection was very low, one animal died on d 20 in group B, in which NE was confirmed pathologically. The BW data of this animal was omitted during statistical evaluation. Groups B, C, D, and E did not show significant increase in performance on d 16 compared with the control. On d 25, the beneficial effects of BP70+EO and also EO led to significantly higher BWgain in groups D and E ($P < 0.001$). Groups B and C did not show significant increase in weight gain on d 25 compared with that of the control group (Figure 1).

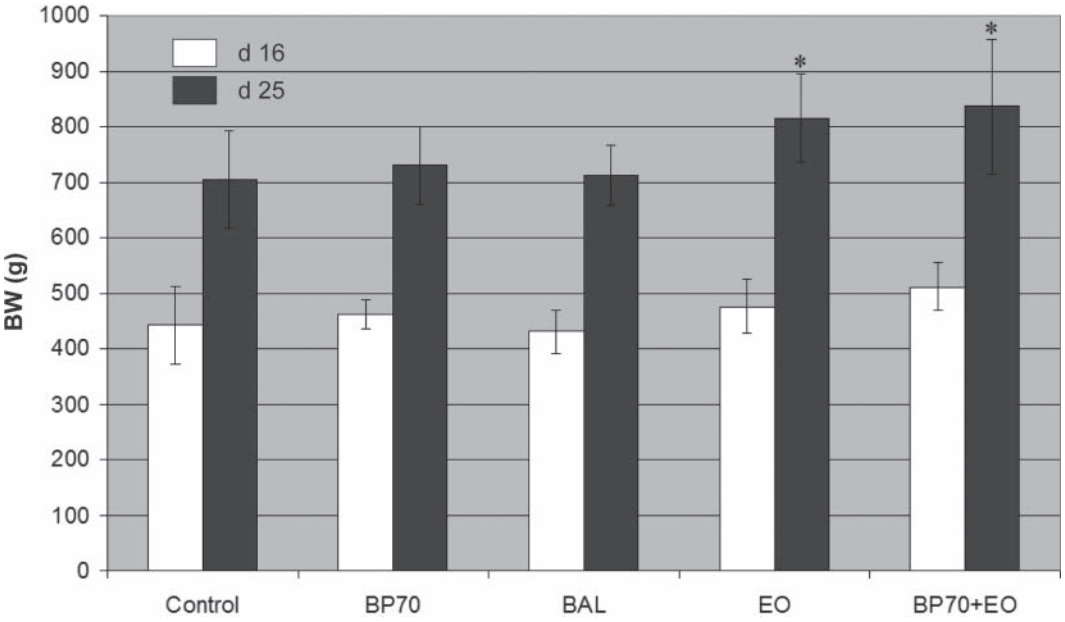

Figure 1. Broiler BW on d 16 (white columns) and d 25 (black columns). Values are present in mean values ± SD. Asterisk (*) represents significant difference ($P < 0.05$) compared with the control group. BP70 = protected sodium butyrate, BAL = *Bacillus amyloliquefaciens* spore suspension, EO = essential oils, and BP70+EO = protected sodium butyrate and essential oils.

Gross Pathological and Histopathological Examination and Morphometric Evaluation

A high percentage of the animals showed the clinical signs of NE: hemorrhagic dark diarrhea, apathy, anemia, dehydration, inappetence, and death. One animal in group B (BP70) died naturally because of the infection, which was confirmed pathologically; but because autolysis rendered objective scoring impossible, its pathological/histopathological scores were excluded from further data analysis. All of the animals were subjected to gross pathological and histopathological examinations after euthanasia. In the control group (A), all of the animals developed mild, moderate, or severe signs of NE with a gross and histopathological score of 3.0 ± 0.18 and 3.71 ± 0.30 (mean ± SEM), respectively. Pathological signs included extensive catarrheal hemorrhagic inflammation, grease-like appearance of the intestinal mucosa, and focal to generalized superficial necrosis in the distal duodenum, jejunum, and ileum. One animal in group B showed no gross pathological signs characteristic to the disease. In groups C, D, and E, 2, 3, and 2 animals showed negative results, respectively. The results of the histopathological examinations were mainly in agreement with the gross pathological signs. No pathological signs were found in the pancreas and the spleen. A summary of the macroscopical, histopathological scores, and morphometric measurements is shown in Table 2.

A significant decrease in gross pathological and histopathological signs was observed in animals treated with 1.5 g/kg of EO ($P < 0.05$) compared with that of the control, whereas a highly significant deviation ($P < 0.005$) was observed in the case of BP70+EO supplementation. No significant decrease in either macroscopic or microscopic changes was found in groups B and C compared with that of the control ($P > 0.05$). Morphometric evaluation showed a significant increase in villus lengths in group B treated with BP70 ($P < 0.05$) and in groups D and E treated with EO and

Table 2. Mean villus lengths, villus length:crypt depth ratios, gross pathological and histopathological scores, and number of animals with no signs of necrotic enteritis

Group[1]	Animals with negative pathological results	Gross pathological score (mean ± SEM)	Histopathological score (mean ± SEM)	Villus length (μm; mean ± SEM)	Villus:crypt ratio (mean ± SEM)
A	0	3.0 ± 0.18	3.71 ± 0.30	844.8 ± 39.35	5.23 ± 0.26
B	1	3.17 ± 0.29	3.67 ± 0.36	978.5 ± 31.42*	5.48 ± 0.26
C	2	2.2 ± 0.33	4.4 ± 0.38	892.1 ± 34.20	5.28 ± 0.28
D	3	2.3 ± 0.22*	2.5 ± 0.17*	1,108.0 ± 28.07**	6.29 ± 0.29**
E	2	1.1 ± 0.09**	2.2 ± 0.20**	1,028.9 ± 24.81**	5.73 ± 0.25*

[1]Basic diet with no feed additives (A), protected sodium butyrate at 1.5 g/kg (BP70; B), *Bacillus amyloliquefaciens* spore suspension at 10^9 cfu/kg (C), essential oils at 1.5 g/kg (D), and BP70+essential oils at 1.5 g/kg (E). Number of parallel evaluations in group A (control), n = 32; in group B, n = 30; in group C, n = 30; in group D, n = 29; and in group E, n = 30.

*Significant at $P < 0.05$; **significant at $P < 0.001$ compared with the control.

BP70+EO, respectively ($P < 0.001$), compared with that of the control (group A). In group C, no significant deviation was observed. Significantly higher V:C ratios were noticed in groups D and E, whereas no significant difference in V:C ratio was experienced in groups B and C compared with that of the control (group A).

DISCUSSION

In this study, the effects of BP70, *B. amyloliquefaciens* spore suspension, mix of essential oils at 1%, and essential oils combined with BP70 were investigated on the development and pathological signs of NE in broilers. The BP70, a sodium butyrate product protected with vegetable fat, showed no beneficial effect on BW gain in our experiment. However, taking into account the specific environmental and iatrogenic factors in this study, such as high protein content of the diet, high animal density, and immunosuppressive vaccinations, it cannot be excluded that sodium butyrate might have a beneficial effect on broiler weight gain in animals kept under normal conditions.

Our data were mainly in agreement with the results of Leeson et al. (2005), who detected no improvement in BW gain of broiler chickens receiving 0.1, 0.2, and 0.4% sodium butyrate supplementation. In our study, BP70 did not show any significant beneficial effects on the development of gross pathological and histopathological lesions associated with NE and no significant increase in V:C ratio compared with that of the control. The antibacterial effect of sodium butyrate was thoroughly investigated and proved to be effective against gram-negative bacteria, primarily salmonellae (Hirshfield et al., 2003; Van Immerseel et al., 2005; Fernández-Rubio et al., 2009). Timbermont (2009) observed beneficial effects of sodium butyrate in the control of NE in chickens, which was not in accordance with our results and remains an issue to be confirmed by additional in vitro and in vivo experiments. Timbermont et al. (2010) presented a dose-dependent activity of sodium butyrate against NE. Their results showed no significant decrease in necrotic lesions at 235 g/ton of dosage but a significant reduction at 330 g/ton of dosage of sodium butyrate. Indecisive results derived from previous and present studies concerning efficacy of sodium butyrate might be explained by relatively high minimum inhibitory concentration against *C. perfringens* (Timbermont et al., 2010) and numerous indirect effects of the substance (Mallo et al., 2010), including an increase in mean villus length compared with the control in our study.

Several authors reported beneficial effects of direct-fed microbials (probiotics) in the control of NE in broilers (Craven et al., 1999; Hofacre et al., 2003; Barbosa et al., 2005; McReynolds et al., 2009), with bacteria belonging to the *Lactobacillus* genus of the highest efficacy. No studies about the effects of *B. amyloliquefaciens* in the control of NE are available to this date. The presence of a closely related organism, *Bacillus subtilis*, was associated with a significant reduction of *C. perfringens* colonization in the distal gastrointestinal tract according to the results of La Ragione and Woodward (2003). In our study, no significant beneficial effect could be noticed in pathological lesion scores. In addition, no increase in mean villus length and V:C ratio could be found. Lack of efficacy might be explained by the different characteristics of the 2 genera. Clostridia are strictly anaerobic bacteria, whereas *Bacillus* spp. grow only under aerobic conditions. As the primary mode of action of probiotics is competitive exclusion, it necessitates co-colonization of the pathogen with the probiotic strain. In this case, it is hardly possible due to their different biochemical nature. In contrast, probiotics containing *Lactobacillus* spp., *Enterococcus* spp., or *Bifidobacterium* spp. proved to be highly effective against *C. perfringens* infections (McReynolds et al., 2009). Mountzouris et al. (2010) reported beneficial effects of a complex probiotic containing *Lactobacillus reuteri*, *Lactobacillus salivarius*, *Enterococcus faecium*, *Bifidobacterium animalis*, and *Pediococcus acidilactici*. Administration of that complex probiotic increased BW gain and improved cecal microflora composition.

In the current study, supplementation with a mixture of 1% essential oils (ginger oil and carvacrol) had significant favorable effects on BW gain and pathological signs. Body weights on d 25 showed a significant increase compared with the control ($P < 0.05$) and a decrease in gross pathological and histopathological lesions ($P < 0.05$ and $P < 0.005$, respectively). A highly significant increase in villus length and V:C ratio can be an explanation for better BW gain compared with control animals. Our results were in agreement with previous studies. McReynolds et al. (2009) reported good efficacy of a combination of essential oils (citrus, annase, and oregano) and fructooligosaccharides for improving lesion scores in experimental NE. Mitsch et al. (2004) presented the effects of 2 different blends of essential oils consisting of thymol, carvacrol, piperine, eugenol, and curcumin on *C. perfringens* colonization in intestines of broiler chickens. Chao et al. (2000) found that ginger and piperine showed small disc diffusion inhibition zones in the case of certain pathogenic bacteria. *Clostridium perfringens* was not investigated in this study and anticlostridial effects of these substances has to be established. In addition to possible antibacterial activity, antioxidant (Botsoglou et al., 2004), digestive stimulant (Platel and Srinivasan, 2004), and antitoxigenic (Ultee and Smid, 2001) effects of essential oils might contribute to their beneficial effects in this experiment.

In the combination, BP70 and essential oils might exert a synergistic action as there is a significant difference in gross pathological scores ($P < 0.05$) between EO and EO+BP70 groups. As BP70 alone did not have any beneficial effects in this study, it can be hypothesized that the essential oils potentiate the effect of

sodium butyrate, although this phenomenon has to be confirmed in vitro. The explanation for this synergistic action might be that sodium butyrate improves epithelium regeneration, thus diminishing the amount of α-toxigenic clostridia that can attach to the surface and produce the toxin that finally causes NE. No significant differences were found between EO and EO+BP70 in the histopathological scores and BW gain after 25 d ($P > 0.05$).

According to our results, it can be ascertained that EO and BP70+EO supplementation have important beneficial effects when evaluating pathological signs of subclinical NE.

In conclusion, broilers infected with *C. perfringens* and treated with EO and BP70+EO showed significantly better BW gain, increased villus length and V:C ratio, and decreased gross pathological and histopathological lesion scores compared with the control. In our study, no beneficial effect in the case of BP70 or *B. amyloliquefaciens* spore suspension supplementation was observed, except a significant increase in villus length in animals receiving 1g/kg of BP70. Essential oils (1%) were primarily responsible for the better performance and higher resistance to NE, and it seems that BP70 has synergistic effect with essential oils. Taking into account the complexity of the disease, additional in vitro and in vivo studies have to be conducted to evaluate precisely the effect of these substances in poultry NE.

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