The impact of a specific blend of essential oil components and sodium butyrate in feed on growth performance and *Salmonella* counts in experimentally challenged broilers

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ABSTRACT Essential oils (EO) and short-chain fatty acids have potential antimicrobial activity in broilers. This study aimed to investigate the effect of a specific blend of EO and a combination of this blend of EO with sodium-butyrate on growth performance and *Salmonella* colonization in broilers. A total of 480 one-day-old male broilers were distributed into 5 treatments (8 pens per treatment and 12 birds per pen) and reared during 42 d in experimental conditions. Dietary treatments consisted of the addition of different doses of EO (0 mg/kg, control; 50 mg/kg, EO50 and 100 mg/kg, EO100) or a combination of EO with 1 g/kg of sodium-butyrate (B; EO50 + B, EOB50 and EO100 + B, EOB100) to a basal diet. All birds were orally infected with 10^8 cfu of *Salmonella* Enteritidis on d 7 of study. Individual BW and feed intake per pen were measured at arrival and on a weekly basis. The prevalence and enumeration of *Salmonella* in feces was determined per treatment at 72 h postinfection and on d 23 and 37 of study. At slaughter, cecal content and liver samples from 16 birds per treatment were cultured for *Salmonella* and cecal pH was measured. No differences were observed on growth performance among treatments. All fecal samples analyzed were positive for *Salmonella* from d 10 to the end of the rearing period. At slaughter, *Salmonella* contamination (positive samples) in cecum was lower in birds fed EOB50 compared with the other treatments ($P < 0.05$), whereas birds fed the control diet showed the highest colonization rates. The pH of the cecal content was not different among treatments. Thus, EO or its combination with sodium-butyrate did not affect growth performance. However, a clear effectiveness of these products was observed in *Salmonella* control, especially when low doses of EO were combined with sodium-butyrate (EOB50).

Key words: broiler, essential oil, sodium-butyrate, growth performance, *Salmonella* Enteritidis

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INTRODUCTION

Salmonella is one of the most important causes of foodborne infections in humans in the European Union. Food safety concerns have urged the poultry industry and governments to introduce measures to control this pathogen in the whole production chain (EFSA, 2012). Different strategies have been proposed to prevent or to mitigate *Salmonella* intestinal colonization of chickens at the primary production level, including the use of feed additives with varying degrees of success (Van Immerseel et al., 2009).

Short-chain fatty acids such as butyrate and recently also essential oils (**EO**) have been suggested as effective against *Salmonella* colonization in broilers (Fernández-Rubio et al., 2009; Van Immerseel et al., 2009; Tiihonen et al., 2010). Additionally, some blends of EO might improve broiler performance when given as dietary supplements (Jamroz et al., 2003; Cross et al., 2007). Although their mechanisms of action are not yet fully understood, their effects on digestive enzymes, nutrient digestibility, ecosystem of gastrointestinal microbiota, and villus integrity might be implied on these effects (Kien et al., 2007; Windisch et al., 2008). However, there are still insufficient in vivo data and the efficacy of these compounds is not always demonstrated, because results are often controversial among studies.

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Factors such as the type and amount of additives used, health conditions of the flock, diet composition, and environmental conditions are likely to be important in the assessment of the final effects of these additives. Additionally, investigations on combining EO and butyrate in broiler feeds, with expected complementary improvement of growth and inhibitory activities against *Salmonella* are scarce in the literature. Hence, a study was conducted to analyze the effect of a specific blend of EO based on a mixture of cinnamaldehyde and thymol (4.5 g of cinnamaldehyde/100 g of blend and 13.5 g of thymol/100 g of blend) alone or in combination with sodium butyrate (Na-butyrate) on growth performance and *Salmonella* colonization in experimentally infected broilers.

MATERIALS AND METHODS

Birds, Housing, and Diets

The experimental protocols used in this study received previous approval from the Animal Protocol Review Committee of the Instituto Valenciano de Investigaciones Agrarias. A total of 480 newly hatched male broiler chicks (line Hubbard) of 41.7 ± 3.43 g were split in 2 environmentally controlled rooms and allocated randomly in a total of 40 floor group pens of 1.3 m^2 each (20 pens per room and 12 birds per pen). The pens contained wood shavings to a depth of 10 cm. Room temperature was controlled, decreasing from 37°C on d 1 to 20°C on d 42 of rearing. Light program provided consisted in 1 h of darkness followed by a period of 23 h light on d 1 and a progressive increase in the time of darkness until reaching 8 h of darkness on d 14 of the study. Each pen was provided with a single feed trough. A nipple watering line was provided for each 10 pens, with 3 nipples per pen.

Before the arrival of the birds, all pens were separated by plastic walls, allowing visual but no direct physical contact of the birds to minimize cross-contamination among cages according to Van Immerseel et al. (2004). At the day of the arrival, pens were assigned to 5 groups of treatment equally distributed in the 2 rooms (4 pens per treatment and room). The dietary treatments consisted on a basal diet (control) containing mainly of maize, wheat, and soybean meal and its supplementation with different doses of a commercial blend of cinnamaldehyde and thymol [50 mg/kg (**EO50**) and 100 mg/kg (**EO100**)] and their combination with 1 g/kg of Na-butyrate (**B**), respectively [E50 + B (**EOB50**) and E100 + B (**EOB100**)]. Three feeds per treatment were formulated to cover all the fattening period (starter, grower, and finisher). The first feed (starter) was provided from 0 to 14 d, the second feed (grower) from 14 to 35 d, and the last feed (finisher) from 35 to 42 d. The basal diets were formulated following NRC (1994) recommendations for poultry, and the nutrient composition is shown in Table 1.

All diets were formulated and produced at the Compound Feed Plant at the Universitat Politècnica de València. The specific blend of EO (Enviva EO 101 G) was provided by Danisco Animal Nutrition (Madrid, Spain) in powder form with a total concentration of active EO components of about 18%. Its main components are cinnamaldehyde and thymol in their nature-identical form (4.5 g/100 g blend and 13.5 g/100 g blend, respectively). The Na-butyrate (VFA C4 protected) was provided in a powder form by Novation (Coslada, Madrid, Spain).

All the experimental diets were given to the birds from d 1 of the study. Feed and water was provided ad libitum during the experimental period. The trial was conducted until 6 wk of age, a standard age for marketing commercial broiler chickens in Spain.

Challenge with Salmonella

Chicks were orally challenged (by pipette) on d 7 of the study with 10^8 cfu of *Salmonella* Enteritidis (3934-yhjL-Km, kanamycin-resistant strain; Solano et al., 2002) per chick. The strain was supplied by the Instituto de Agrobiotecnología y Recursos Naturales and the Departamento de Producción Agraria, Universidad Pública de Navarra-CSIC (Spain). Bacterial inoculum was prepared from a frozen suspension of the strain. Bacterial strain was grown on nutrient agar plates (Scharlau, Barcelona, Spain) for 24 h at 37°C. Cells were then harvested and diluted in 20 mL of buffered peptone water (**BPW**, Scharlau, Barcelona, Spain) and incubated for 24 h at 37°C. Finally, this solution was diluted in 500 mL BPW bottles and incubated at 37°C during 24 h to obtain the final inocula. *Salmonella* cfu counts in the inocula were confirmed before the challenge.

Feed Analysis

Basal diets were analyzed for DM, ash, CP, and ether extract (**EE**) following AOAC International (2003) procedures. Neutral detergent fiber (**NDF**) and acid detergent fiber (**ADF**) were analyzed sequentially (Van Soest et al., 1991). Also, the final content of botanicals (cinnamaldehyde and thymol) in feed samples was analyzed by Danisco Animal Nutrition using gas chromatography as described in Tiihonen et al. (2010).

Growth Performance

Individual broiler weight and the amount of feed offered and refused by pen were measured weekly over the whole trial. Then, ADG and ADFI by pen were calculated. Gain-to-feed ratio was also calculated per pen dividing the weight gained and the amount of feed consumed in a period of time. Additionally, the CV within each pen for the individual weights was calculated per treatment at the beginning and at the end of the study. For each pen, different individual weighing

Table 1. Composition of the basal diet (as-fed basis unless otherwise indicated)

Feed	Starter diet	Grower diet	Finisher diet
Ingredient (%)			
Maize	15.0	0	0
Wheat	46.9	60.2	66.0
Soybean meal	22.3	29.0	24.1
Fish meal	5.9	0.73	0
Soybean oil	6.0	3.0	3.0
Lard	0	3.0	3.0
Calcium carbonate	1.0	0.97	1.1
Dicalcium phosphate	1.4	1.7	1.4
Sodium chloride	0.25	0.35	0.32
Sodium bicarbonate	0.03	0	0
Avizyme 1300[1]	0.10	0.10	0.10
L-Lysine	0.28	0.23	0.26
DL-Methionine	0.25	0.22	0.17
L-Threonine	0.11	0.08	0.07
Vitamin-mineral premix[2]	0.50	0.50	0.50
Analyzed nutrient composition (% of DM)			
DM	90.8	90.8	90.9
CP	20.9	21.6	19.6
Ether extract	8.9	8.1	8.0
Neutral detergent fiber	20.5	19.8	19.5
Acid detergent fiber	2.9	4.1	3.5
Ash	6.8	6.4	6.1
Calculated composition			
AME[3] (kcal/kg)	3,000	3,100	3,150
Lysine (%)	1.34	1.20	1.07
Methionine + cysteine (%)	0.98	0.90	0.79
Threonine (%)	0.87	0.81	0.71
Calcium (%)	0.95	0.90	0.86
Available phosphorus (%)	0.45	0.43	0.38

[1]Enzymatic complex containing xylanase and protease was provided by Trouw Nutrition (Nutreco, Madrid, Spain).

[2]Trace element and vitamin premix.

[3]Apparent metabolizable energy values for broiler.

cages were used and disinfected in each weight control to avoid cross contamination among the different replicates. Also, other materials such as gloves or cellulose swab-socks attached to boots used to handle the birds were replaced in each pen. Birds' health status and mortality were monitored daily. Birds that died over the experiment were weighed and mortality was taken into account when calculating growth performance.

Sampling for Bacteriological and Laboratory Analysis

Before placement, the housing facilities and feeds were sampled and analyzed for the presence of *Salmonella spp.* A different number of samples were taken from empty feeders, water dispensers, litter, feed and surfaces using sterile wet gauze pads with disinfectant neutralizer (AES Laboratories, Bruz Cedex, France) to investigate the presence of *Salmonella* according to Marin et al. (2009).

Fresh fecal samples were collected from pens at 72 h after the experimental infection (d 10 of study) and on d 23 (wk 4) and 37 (wk 6) of the study, and cultured to determine the presence (n = 40; 8 pens/treatment) and counts (n = 20; 4 pens/treatment) of *Salmonella* Enteritidis. The day before feces collection, a clean and disinfected plastic inlay was placed on the bottom drawer of the pens (above the bedding material) to guarantee collection of fresh fecal samples (from the last 24 h). Feces collection was always carried out the day after weighing the birds.

At the end of the study, all birds were euthanized by stunning and exsanguination. Both the cecum and the liver were aseptically removed from 80 birds (2 birds/pen/room) and sampled for *Salmonella* detection. One cecum was cut longitudinally and the cecal content was aseptically removed, weighed, and diluted for *Salmonella* detection and quantification. The content of the second cecum was diluted 1:2 in distilled water and pH was determined with a glass electrode. A piece of approximately 25 g of each liver was diluted 1:10 with BPW and homogenized using a stomacher for *Salmonella* detection.

The presence/absence of *Salmonella* in facilities, feeds, cecal content, and liver samples was analyzed according to ISO 6579:2002 (Annex D). Samples were diluted 1:10 and incubated at 37°C in BPW during 24 h for preenrichment of *Salmonella*. After, samples were plated in semisolid Rappaport Vassiliadis agar (Difco, Le Pont de Claix, France) and incubated 24 h at 42°C. Finally, positive samples were transferred to 4 selective media for *Salmonella* [XLD and XLT4 (Difco), brilliant green agar (Liofilchem, Roseto degli Abruzzi, TE, Italy) and ASAV (AES Chemunex, Bruz, France)].

Salmonella enumeration in fresh fecal samples (approximately 25 g) and the cecal content (approximately 4 g) was determined by homogenization and dilution 1:10 BPW of samples followed by serial decimal dilution until 10^{-7}. Each dilution was then plated in duplicate onto brilliant green agar with 50 μg/mL Kanamycin (Kanamycin sulfate, Sigma-Aldrich, Steinheim, Germany). Kanamycin was added to the media to facilitate the selection of the antimicrobial-resistant *Salmonella* Enteritidis. Colonies were counted after 24 h of incubation at 37°C.

In both cases (*Salmonella* detection and enumeration), 5 randomly selected *Salmonella* colonies were confirmed biochemically throughout a biochemical test API (API-20, bioMerieux, Madrid, Spain).

Statistical Analysis

Data were analyzed using SAS system software (version 9.1, SAS Institute Inc., Cary, NC). Average daily gain, ADFI, G:F, and cecal pH data were analyzed using GLM procedures, with the pen as the experimental unit and room as a block factor included in the model. Means were compared using the multiple comparison Tukey test. Mortality data by treatment were analyzed using a chi-squared test (FREQ procedure).

To assess the effects of diets on *Salmonella* counts in feces, data on *Salmonella* enumeration were subjected to a log-transformation and analyzed with a repeated measures model (MIXED procedure), in which the pen was the experimental unit. Data on *Salmonella* enumeration in cecum were also subjected to a log-transformation and analyzed according to an ANOVA (GLM procedure). The percentage of positive samples from cecum and liver was analyzed using a chi-squared test (FREQ procedure). The individual was the experimental unit in the cecum and liver analyses.

RESULTS

Feeds and EO Analyses

The basal feed contained the expected amount of the analyzed nutrients (Table 1). The analyzed levels of thymol and cinnamaldehyde in the diets are shown in Table 2. No EO were detected in the control group. The expected levels of thymol and cinnamaldehyde in the diets including 50 mg/kg and 100 mg/kg of EO were 6.75 mg of thymol/kg of feed and 2.25 mg of cinnamaldehyde/kg of feed and 13.5 mg of thymol/kg of feed and 4.5 mg of cinnamaldehyde/kg of feed, respectively. Results in Table 2 show that the final analyzed levels of botanicals were slightly higher than expected but similar between groups of the same dosage of EO.

Health Status and Mortality

In general, health status of the birds was optimum during all the experimental period. No signs of intestinal disorder were recorded in any of the birds before or after the experimental infection. A total of 40 birds died during the trial, and the percentage of birds dead was 11.5, 6.3, 10.4, 5.2, and 8.3% for control, EO50, EO100, EOB50, and EOB100 treatments, respectively ($P = 0.471$). The control group showed the highest percentage of dead birds and the group EOB50 the lowest, although differences were not statistically significant ($P > 0.05$). Regarding the distribution of chick mortality by treatment over the experimental period, the highest mortality rates were observed during the first week of the experiment in all 5 treatments. The percentage of birds dead was maintained lower than 5% during this week in all treatments with the exception of treatment control (control = 5.2%, EO50 = 4.2%, EO100 = 3.1%, EOB50 = 3.1%, EOB100 = 3.1%; $P = 0.921$). During the third week of study (1 wk after the experimental infection), the percentage of dead birds increased again, but this increase was especially evident in the control group compared with the groups fed with EO (control = 3.4%, EO50 = 1.1%, EO100 = 2.2%, EOB50 = 0%, EOB100 = 2.2%; $P = 0.489$). Among treatments, differences in mortality by week were not significant. The causes of death could not be identified in all cases. In about 80% of cases (equally distributed by treatment), dead birds showed impaired growth (lower daily gains or even BW losses) at the time of death.

Growth Performance

Table 3 shows the results obtained on BW, ADG, ADFI, and G:F over the study (global) and in the different periods of growth (starting, 0 to 14 d; growing, 14 to 35 d; finishing, 35 to 42 d). No differences were observed among treatments in any of the parameters studied. The initial (around 40 g) and final (around 2,800 g) BW of birds was similar for all treatments.

Table 2. Analyzed concentrations of thymol and cinnamaldehyde in the diets (mg/kg of diet)[1]

Dietary treatment	Starter diet	Grower diet	Finisher diet
Control			
Thymol	0	0	0
Cinnamaldehyde	0	0	0
EO50			
Thymol	10	9	8.8
Cinnamaldehyde	2.7	2.9	2.8
EO100			
Thymol	19	19	18
Cinnamaldehyde	4.9	6.0	5.7
EOB50			
Thymol	7.6	9.9	7.1
Cinnamaldehyde	1.9	3.1	2.3
EOB100			
Thymol	17	19	16
Cinnamaldehyde	5.1	5.9	5.2

[1]Birds were fed with a standard broiler feed supplemented or not with essential oil [EO; 0 mg/kg (control), 50 mg/kg (EO50), and 100 mg/kg (EO100)] or a combination of EO with 1 g/kg of sodium-butyrate (B; EO50 + B, EOB50 and EO100 + B, EOB100).

Table 3. Body weight, ADG, ADFI, and G:F ratio of broiler chicks by periods (starter, grower, and finisher) and globally during the fattening period[1,2]

	Dietary treatment					
Item	Control	EO50	EO100	EOB50	EOB100	SEM
Global (0 to 42 d)						
Initial weight (g)	41.8	41.5	41.1	41.7	42.1	0.353
Final weight (g)	2,850.8	2,872.6	2,816.5	2,804.0	2,865.8	30.31
Initial CV[3] (%)	7.01	7.67	8.30	8.07	8.77	0.725
Final CV[3] (%)	11.05	12.16	12.12	13.28	12.20	1.987
ADG (g/d)	65.7	66.9	64.5	65.4	66.2	1.03
ADFI (g/d)	108.6	108.0	105.1	106.5	108.9	1.35
G:F	0.51	0.55	0.53	0.51	0.50	0.018
Starter phase (0 to 14 d)						
ADG (g/d)	30.4	30.7	29.8	30.6	30.4	0.535
ADFI (g/d)	55.6	55.6	55.3	55.3	56.0	0.287
G:F	0.55	0.55	0.54	0.55	0.54	0.008
Grower phase (14 to 35 d)						
ADG (g/d)	82.4	81.7	80.6	81.2	83.2	1.43
ADFI (g/d)	123.3	121.4	118.7	119.8	122.8	1.75
G:F	0.67	0.67	0.68	0.68	0.68	0.005
Finisher phase (35 to 42 d)						
ADG (g/d)	89.5	96.3	90.1	88.5	89.9	4.19
ADFI (g/d)	175.3	174.6	171.3	170.7	178.2	4.30
G:F	0.51	0.55	0.53	0.51	0.50	0.018

[1]Birds were fed with a standard broiler feed supplemented or not with essential oil [EO; 0 mg/kg (control), 50 mg/kg (EO50), and 100 mg/kg (EO100)] or a combination of EO with 1 g/kg of sodium-butyrate (B; EO50 + B, EOB50 and EO100 + B, EOB100).

[2]n = 96 for all treatments.

[3]CV calculated within each pen.

Additionally, no differences were observed concerning initial and final CV among treatments.

Salmonella Isolation

Before initiation of the study, facilities and feed were confirmed to be free of *Salmonella*. Thus, the only known sources of exposure to this organism in this study were the birds and the intentional challenge dose. Regarding the infection dose, *Salmonella* cfu counts in the inocula were confirmed to be 2.83×10^8 cfu/mL of solution before administration.

Fecal Shedding of Salmonella. Results on the incidence of shedding (% positive fecal cultures) and bacterial counts ($\log_{10}$ cfu/g of feces) over the study are reported in Table 4. All the pens were positive for *Salmonella* (100% prevalence) from 72 h after infection until the end of the trial. Results on *Salmonella* enumeration in feces (Table 4) showed that, in general, the degree of shedding decreased from the 72 h postinfection to d 23 (wk 4 of the study), except in the control group, and increased thereafter (d 37, wk 6) in all treatments. At the end of the study (d 37, wk 6), the group EOB50 showed the lowest degree of fecal shedding of *Salmonella* and this was statistically different from EO50 but not from the other groups of treatment.

Salmonella Detection in Cecum and Liver at Slaughter. After euthanasia, no lesions were found in the intestine, liver, or legs for any of the treatment groups. Only one chick showed adherences in the ab-

Table 4. Prevalence of *Salmonella* (% positive fecal cultures) and *Salmonella* counts ($\log_{10}$ cfu/g of feces) in samples of fecal material over the experimental period by treatment[1,2]

	Dietary treatment					
Item	Control	EO50	EO100	EOB50	EOB100	SEM
Prevalence (% positive)						
d 10 (72 h postinfection)	100	100	100	100	100	—
d 23	100	100	100	100	100	—
d 37	100	100	100	100	100	—
Bacterial count[3] ($\log_{10}$ cfu/g of feces)						
d 10 (72 h postinfection)	3.66	4.12	4.47	4.71	4.33	0.467
d 23	4.11	3.82	3.69	4.22	4.10	0.54888
d 37	5.77[ab]	6.86[a]	5.00[b]	4.59[b]	6.03[ab]	0.749

[a,b]Means within a row lacking a common superscript are significantly different ($P < 0.05$).

[1]Birds were fed with a standard broiler feed supplemented or not with essential oil [EO; 0 mg/kg (control), 50 mg/kg (EO50), and 100 mg/kg (EO100)] or a combination of EO with 1 g/kg of sodium-butyrate (B; EO50 + B, EOB50 and EO100 + B, EOB100).

[2]Prevalence data, n = 8; bacterial count data, n = 4 for all treatments.

[3]Bacterial counts ($P_{treatment}$: 0.625; P_{time}: < 0.001; $P_{treatment*time}$: 0.210).

dominal and thoracic cavity that affected the pericardium and mesentery. Results on the presence of *Salmonella* in cecum and liver and cecal pH are summarized in Table 5. According to the chi-squared analysis, the percentage of positive cecum for *Salmonella* was significantly different among treatments ($P = 0.020$). The EOB50 treatment showed the lowest contamination level (6.3%) compared with the other treatments ($P < 0.05$). Among the rest of the treatments, the control group (with no addition of EO or EO + Na-butyrate) presented the highest percentage of birds positive for *Salmonella* (68.8%), although the differences were not statistically significant with EO50, EO100, and EOB100. In the liver, the percentage of positive samples was variable among treatments (from 0% in EOB100 to 19% in EO100) but no significant differences were found. It is important to mention that the liver was colonized at a lower rate than the cecum in all treatments. Considering samples of cecum and liver altogether, differences of *Salmonella* colonization among treatments were also significant ($P = 0.046$), with control and EO100 having the highest contamination rates and the EOB50 treatment the lowest prevalence of *Salmonella*.

Regarding enumeration of *Salmonella* in cecal samples, this microorganism could only be counted with success in a few samples by treatment (less than 6 samples from a total of 16). The number of samples by treatment in which *Salmonella* was isolated and countable under the direct plate method was in relation to the percentage of samples positive to *Salmonella*, being the lowest in treatments EOB50 and EOB100 (1 sample) and the highest in the control treatment (5 samples). However, due to the low number of countable samples, these data are not representative and should not be taken into account for interpretation.

The pH of the cecal content showed no differences among treatments.

DISCUSSION

Nonantibiotic feed additives like EO from aromatic plants have received attention as growth and health promoters. Essential oils may enhance lipid metabolism, stimulate digestion of nutrients, and exert antioxidant and antimicrobial properties and antiinflammatory potential (Windisch et al., 2008; Brenes and Roura, 2010). Additionally, multiple effects on the gut mucosa that may play a role in the host-pathogen interaction have been described for butyrate. At low concentrations, butyrate reinforces the colonic defense barrier by increasing production of mucins and host antimicrobial peptides and decreasing intestinal epithelial permeability (Barcelo et al., 2000; Peng et al., 2007). Butyrate also acts as an energy source for epithelial cells and helps in the maintenance of intestinal villus structure (Kien et al., 2007). Short-chain fatty acids such as butyrate have been used extensively in the field to control *Salmonella* in broilers with demonstrated antimicrobial effects (Leeson et al., 2005; Van Immersel et al., 2006; Fernández-Rubio et al., 2009). In recent years, several studies have also been conducted to assess the effects of EO on broiler performance and the modulation of microbial population yielding variable effects. Although some studies report improvements in bird performance with the addition of EO blends containing thymol, cinnamaldehyde, or both (Jamroz et al., 2003; Cross et al., 2007; Tiihonen et al., 2010; Mathlouthi et al., 2012), others indicate no effects in broiler chickens (Lee et al., 2003; Jang et al., 2007). However, few studies have been performed to investigate the effects of the combination of EO and other feed additives such as organic acids on broiler performance and antimicrobial activity against *Salmonella*, although complementary activities could be expected (Hashemi et al., 2012).

In the present study, a commercial blend of cinnamaldehyde and thymol was used alone and in combination with Na-butyrate. No differences in growth performance were observed with the inclusion of 50 or 100 mg/kg feed of EO or the combination of these 2 doses with 1 g/kg of Na-butyrate. It has been widely recognized that the in vivo response on growth performance to the supplementation of EO in birds may be associated with the background health conditions of the flock, the basal diet composition and digestibility, hygienic standards, and environmental conditions (Lee et al., 2003; Jamroz et al., 2006; Tiihonen et al., 2010). Additionally, the quality and quantity of active substances in the EO might also determine its potential effects in vivo. Other studies in which a blend of thymol and cinnamaldehyde was included in broiler feed at a similar level than in the present study demonstrated improve-

Table 5. Prevalence of *Salmonella* (% positive cultures) in samples of cecal material and liver and pH at slaughter by treatment[1,2]

	Dietary treatment					
Item	Control	EO50	EO100	EOB50	EOB100	SEM
Prevalence in cecum (% positive)	68.8[a]	37.5[a]	43.8[a]	6.3[b]	43.8[a]	—
Prevalence in liver (% positive)	12.5	6.7	18.8	6.3	0	—
Total prevalence (ceca + liver, % positive)	40.6[a]	21.9[ab]	31.3[a]	6.3[b]	21.9[ab]	—
pH cecal content	6.16[ab]	6.29[ab]	5.99[b]	6.19[ab]	6.15[ab]	0.104

[a,b]Means within a row lacking a common superscript are significantly different ($P < 0.05$).

[1]Birds were fed with a standard broiler feed supplemented or not with essential oil [EO; 0 mg/kg (control), 50 mg/kg (EO50), and 100 mg/kg (EO100)] or a combination of EO with 1 g/kg of sodium-butyrate (B; EO50 + B, EOB50 and EO100 + B, EOB100).

[2]Prevalence in cecum, n = 16; prevalence in liver, n = 16; total prevalence, n = 32 for all treatments.

ments in ADG and final BW of birds fed with the EO (Tiihonen et al., 2010; Amerah et al., 2012). Thus, it seems probable that in the present study, health and hygienic conditions of the flock or the diet digestibility were good enough to mask EO effects, as suggested by Lee et al. (2003). In fact, in the present study, growth parameters were optimal throughout the experimental period, in spite of the experimental infection.

Butyric acid and recently also EO have demonstrated their activity against *Salmonella* colonization based on different mechanistic explanations (Fernández-Rubio et al., 2009; Van Immerseel et al., 2009). Short-chain fatty acids such as butyrate are able to prevent *Salmonella* colonization of crop and cecum (Thompson and Hinton, 1997; Fernández-Rubio et al., 2009). The antimicrobial action of butyrate can be due to effects directly on bacteria or on the host itself (Fernández-Rubio et al., 2009; Timbermont et al., 2010). In the case of EO, the exact mode of action in the organism is not well established but seems to be related to the stabilization of the ecosystem of gastrointestinal microbiota (Jang et al., 2007; Windisch et al., 2008). In fact, one of the most evident intrinsic effects of plant extracts is their in vitro antimicrobial activity (Peñalver et al., 2005). Cinnamon and thyme have both shown antimicrobial properties against foodborne pathogens such as *Escherichia coli*, *Salmonella* Enteritidis, *Campylobacter jejuni*, *Staphylococcus aureus*, and *Listeria monocytogenes* (Smith-Palmer et al., 1998; Peñalver et al., 2005). Generally, the strongest antibacterial properties against foodborne pathogens are shown with EO containing high percentage of phenolic compounds such as carvacrol, eugenol, and thymol (Juliano et al., 2000; Lambert et al., 2001; Peñalver et al., 2005), although Mathlouthi et al. (2012) found that cinnamaldehyde has also a strong antimicrobial activity. However, only a limited number of trials assessing antimicrobial effects of EO are performed in vivo; for this reason in vivo studies are relevant. Some in vivo studies suggested that EO, especially those including thymol, could have a selective antimicrobial effect for certain potentially harmful microbes and that enterobacteria are especially affected (Jang et al., 2007; Tiihonen et al., 2010), including *Salmonella* (Amerah et al., 2012). However, Cross et al. (2007) showed no effects of the inclusion of culinary herbs or their EO on the intestinal microbiota population.

In the present study, the addition of different doses of EO with or without the addition of Na-butyrate decreased the prevalence of *Salmonella* Enteritidis in cecum from 68.8% in birds fed with the control diet to a maximum of 43.8% of prevalence in birds fed with a diet including EO (Table 5). However, the combination of a low dose of EO (50 mg/kg) and 1 g/kg of Na-butyrate (EOB50) was the most efficient to reduce the amount of birds positive for this *Salmonella* strain, which has been confirmed as one of the most prevalent serotypes isolated during rearing and after transport in broiler (Marin and Lainez, 2009). Birds from the EOB50 treatment showed the lowest cecal colonization with *Salmonella* ($P < 0.05$) and also the lowest *Salmonella* counts in feces at the end of the study (Table 4), although the differences were not statistically significant. From the results of the present study, cecal pH seemed not to be related with this effect. In practice, it is unlikely that the use of feed additives alone can eliminate *Salmonella*. However, reductions of *Salmonella* prevalence in the flock and the number of bacteria in the intestine can decrease the number of contaminated carcasses, and thus transmission to humans (Corry et al., 2002; Marin and Lainez, 2009). Therefore, from the results of this study, the combination of a low dose of EO (50 mg/kg) and 1 g/kg of Na-butyrate (EOB50) can help in improving food safety, and could be relevant for the broiler industry and governments.

The results of the present study also show that when EO and butyric acid are given in combination, their effects against *Salmonella* might be additive and would depend on EO doses, the lowest being the most effective. Cross et al. (2007) also suggested that the highest doses of EO are not always the most effective. Phytogenics are a relative new class of feed additives and little information is available on their modes of action and aspects of their application, such as the effective doses that can be used in birds without inducing toxic effects (Acamovic and Brooker, 2005). It is possible that, in combination with butyrate, the concentration of the EO in the diet EOB100 was too high and therefore a detrimental instead of a beneficial effect was observed. In fact, this effect was also seen by Timbermont et al. (2010) in the control of *Clostridium perfringens* in chickens. Also, Toghyani et al. (2010) and Abdel-Wareth et al. (2012) suggested that a high dosage of thyme in the diet may reduce feed efficiency in broilers probably through a gut microbiota-mediated effect.

Thus, plant extracts or their EO in diets may therefore affect gut microbiota, although the amount or chemical composition of the extract appears to be important in obtaining optimal effects.

In conclusion, the addition of a commercial blend of cinnamaldehyde and thymol or its combination with Na-butyrate in feed did not affect growth performance in this study. However, the combination of a low dose of EO (50 mg/kg) and 1 g/kg of Na-butyrate decreased *Salmonella* prevalence in the cecum, thus suggesting that this combination contribute to food safety by lowering the incidence of meat to human transmission. More information should be pursued with respect to the specific dose responses of EO in studies in vivo and their effect in combination with different commercial feed formulations, other feed additives, and different rearing conditions to better explain their practical applications in broilers.

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