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The effect of feeding essential oils on broiler performance and gut microbiota

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Abstract 1. In this study the effect of a blend of essential oils (EO) comprising 15 g/tonne thymol and 5 g/tonne cinnamaldehyde on the performance and intestinal microbiota of broilers was investigated.
2. A total of 720 male Ross broilers were divided into two dietary treatments with 12 replicate pens per treatment. Broilers were given a control soybean-wheat-based diet with or without added EO in two diet phases (0–21 d and 22–42 d).
3. The blend of EO increased body weight gain of broilers from 0 to 42 d by 4·5%.
4. Caecal microbiota were affected by the EO blend; in particular increases in the proportions of *Lactobacillus* and *Escherichia coli* at 41 d was observed.
5. The EO blend had major effects on caecal metabolites. The proportion of caecal butyrate at 20 and 41 d of age increased, whereas the proportion of caecal acetic acid at 20 d, and propionic acid and isovaleric acid at 41 d, decreased with the EO blend. In addition, the caecal proportion of spermine increased and tyramine decreased at 41 d of age with the EO treatment.
6. The present study shows that EO supplementation exerts a positive effect on intestinal microbiota with a concomitant enhancement in growth performance. The study suggests that modulation of broiler gut microbiota composition and activity through the administration of EO offers an effective means for improving broiler performance.

INTRODUCTION

Essential oils (EO) are volatile, aromatic compounds synthesised by plants for antimicrobial, antifungal, and antiviral purposes; and to deter herbivorous insects and animals. The antimicrobial properties of EO are well recognised (Kalemba & Kunicka, 2003) and they are utilised in a variety of food protection applications and also to help regulate odour emissions from cattle and swine manure (Varel, 2002; Varel *et al*., 2007). The antimicrobial properties of essential oils arise primarily from phenols, aldehydes and terpenes that are able to disturb the integrity and function of bacterial cell membranes (Di Pasqua *et al*., 2007).

In recent years animal production in many countries has faced restrictions in the use of antibiotic growth promoters and, therefore, alternative means to support animal performance and health and to control pathogenic microbes or protozoa are needed. Essential oils have shown promising results in inhibiting various infections in experimental models. Coccidiosis is a common enteric infection in broiler chicks, caused by protozoa of the genus *Eimeria*. Hume *et al.* (2006) and Oviedo-Rondón *et al.* (2006) have reported that EO may control the adverse effects of coccidial infection on the enteric microbiota. Coccidiosis is often associated with necrotic enteritis, a secondary bacterial infection caused by opportunistic pathogens

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such as *Clostridium perfringens*. Mitsch *et al.* (2004) found that specific blends of EO can control intestinal colonisation and proliferation of *C. perfringens*, and may thus help to prevent necrotic enteritis in broiler chicks. Hafez and Hauck (2006) reported that herbal product with extracts from cinnamon, garlic, lemon, and rosemary partly protected turkey poults against experimental histomoniasis, which is caused by a protozoan *Histomonas meleagridis*. Furthermore, the potential usefulness of dietary EO in supporting the intestinal health and immune system in weanling pigs has been proposed by Stein and Kil (2006).

The aim of the present study was to investigate the effect of a specific EO blend, comprising thymol and cinnamaldehyde in a maltodextrin matrix, on the performance and immunity, as well as the intestinal microbiota in broiler chicks at two distinct time points. The activity of the intestinal microbiota indicated by the short chain fatty acid (SCFA), branched chain fatty acid (BCFA) and biogenic amine concentrations as well as their association with performance was also studied.

MATERIALS AND METHODS

Husbandry and diets

A total of 720 male Ross broilers were obtained from a hatchery (P.D. Hook hatcheries Ltd. Cote, Brampton, Oxfordshire, UK), and randomly allocated to two dietary treatments. Each treatment used 12 floor pens, equipped with wood shaving litter, with 30 birds each. The trial lasted 42 d. The chicks were fed with a starter diet (0–21 d) and a finisher diet (22–42 d) *ad libitum* and had free access to drinking water *via* bell drinkers. The ventilation rate was adjusted automatically and temperature was gradually reduced from approximately 30–17°C according to industry practice. Ethical approval of the study was by the Agricultural Development Advisory (ADAS) Gleadthorpe Ethical Review Committee.

The starter and finisher diets were manufactured by Service, ADAS Gleadthorpe (UK) to a commercial specification and were fed in a mash form (Table 1). The treatments were either a control diet or the same diet supplemented with a blend of two EO, thymol and cinnamaldehyde (Danisco Animal Nutrition, Marlborough, UK), in their nature-identical form. The two EO were included in the supplemented diet at 15 g/tonne thymol and 5 g/tonne cinnamaldehyde, respectively.

Measurements and sample collection

The birds were weighed in their pen groups at 0, 21 and 42 d. The feed intake of the birds was measured on a pen basis between 0–21 and 22–42 d. The mortality and the weight of birds that died or were culled during the trial were recorded. In addition, at 20 and 41 d of age, one chick per pen was killed by cervical dislocation and sampled for ileal and caecal digesta, yielding 12 digesta samples per treatment and time point. From the 12 digesta samples collected, digesta from every two chicks was pooled into one sample, yielding 6 replicate digesta samples per treatment and time point. The samples were placed immediately on ice and stored at −20°C until analysis.

EO analyses

To extract the EO from the feed, 4 g of ground feed samples were weighed into a centrifuge tube with 2·5 ml of water, 1 ml of carvacrol as internal standard (200 mg/l), and 1 ml of ethanol. The calibration samples were prepared from control feed as described above and supplemented with standard solutions of cinnamaldehyde and thymol at five different concentrations (100, 40, 20 10 and 5 mg/l in ethanol) or unsupplemented ethanol as a blank. The samples were mixed and allowed to stand for 15 min. Then, 12 ml of diethyl ether was added, the samples were shaken for 16 h and centrifuged at 15,000 × *g* for 5 min. To analyse the extracts, 1 µl of each supernatant was injected into the gas chromatograph with flame ionisation detector. Gas chromatographic analyses were performed using a GC 6890 system (Agilent Technologies, USA) equipped with a flame ionisation detector and HP5 (30 m × 0·32 mm ID, 5% phenyl methylsilicone; phase thickness 0·5 µm) capillary column. Helium was used as the carrier gas at a flow rate of 1·5 ml/min. Sample injection was carried out in splitless mode at 250°C with splitless time of 1 min. Temperature of the detector was 250°C. Oven temperature was maintained initially at 55°C for 2 min, then raised at a rate of 10°C/min to 125°C and maintained for 10 min, then raised at a rate of 25°C/min to 240°C and maintained for 10 min. The 5-concentration linear calibration curves were calculated using the internal standards. Using the peak heights the concentrations (mg/kg) of the analytes in the samples were calculated from the calibration curves.

Biochemical analyses

The concentrations of short chain fatty acids (SCFA; acetic, propionic, butyric and valeric acids), branched chain fatty acids (BCFA;

Table 1. *Diet specification, calculated nutrient provision, composition of vitamin/mineral premix and analysed levels of thmol and cinnamaldehyde in the diets*

	Starter		Finisher	
Ingredients, g/kg	Control	EO	Control	EO
Wheat	578·6	578·5	634·4	634·3
Rapeseed meal	50·0	50·0	60·0	60·0
Peas	100·0	100·0	80·0	8·0
Soybean meal 48	158·0	158·0	101·0	101·0
Wheat middlings	50·0	50·0	50·0	50·0
Soy oil	19·0	19·0	37·0	37·0
Sodium chloride	3·9	3·9	3·9	3·9
DL Methionine	2·5	2·5	1·8	1·8
L-Lysine HCl	3·4	3·4	2·9	2·9
L-Threonine	1·0	1·0	0·8	0·8
Limestone	8·2	8·2	9·2	9·2
Dicalcium phosphate	20·4	20·4	14	1·4
Vitamin/mineral premix[1]	5·0	5·0	5·0	5·0
Essential oils (EO)[2]	0	0·1	0	0·1
Formulated nutrient content, g/kg except when stated otherwise				
Crude protein	210·0	210·0	190·0	190·0
ME, MJ/kg	11·7	11·7	12·61	12·61
Calcium	9·2	9·2	8·0	8·0
Total phosphorus	8·0	8·0	6·7	6·7
Available phosphorus	4·5	4·5	3·5	3·5
Fat	35·9	35·9	54·7	54·7
Crude fibre	35·4	35·4	33·6	33·6
Methionine	5·5	5·5	4·6	4·6
Methionine+cystine	9·4	9·4	8·3	8·3
Lysine	12·3	12·3	10·4	10·4

[1]Composition of vitamin/mineral premix included at 5 kg per tonne of feed.

	Starter	Finisher
Vitamin A (retinyl acetate) (g)	13·6	3·6
Vitamin D3 (cholecalciferol) (g)	0·125	0·125
Vitamin E (α-tocopherol)(g)	75·00	75·00
Vitamin K (K_3, menadione)(g)	6·00	5·00
Folic acid (g)	2·50	2·00
Niacin (g)	80·00	55·00
Calcium pantothenate (g)	15·00	10·00
Riboflavin (B2) (g)	8·00	6·00
Vitamin B12 (g)	0·03	0·02
Thiamine (B1) (g)	3·00	2·00
Pyridoxine (B6) (g)	5·00	3·00
Biotin (g)	0·25	0·15
Cobalt (carbonate) (g)	0·50	0·50
Selenium (sodium) (g)	0·20	0·20
Iodine (calcium iodate) (g)	2·00	2·00
Molybdenum (sodium) (g)	0·50	0·50
Antioxidant BHT, citric (g)	25·00	25·00
Copper (g) (sulphate)	20·00	20·00
Iron (g) (sulphate)	50·00	50·00
Zinc (g) (oxide)	100·00	80·00
Manganese (g) (oxide)	100·00	80·00
Choline Chloride (g)	250·00	200·00
Calcium (%)	29·10	29·669
Ash (%)	89·88	90·655

[2]Analysed concentrations of thymol and cinnamaldehyde in the diets (g/tonne diet).

		Starter	Finisher
Control	Thymol	0	0
	Cinnamaldehyde	0	0
Essential oils	Thymol	14·0	14·3
	Cinnamaldehyde	3·7	4·9

isobutyric, 2-methybutyric and isovaleric acids) and lactic acid in both ileal and caecal digesta samples were determined by capillary gas chromatography as described by Holben *et al.* (2002). Briefly, 1 g of sample was suspended in 1 ml of an internal standard (20 mM pivalic acid) and 5 ml of milli-Q water and mixed thoroughly. The sample was centrifuged (5000 × *g*, 5 min, 22°C) and the supernatant was collected. An aliquot of 500 µl of the supernatant was mixed with 250 µl of saturated oxalic acid and incubated at 4°C for 60 min, followed by centrifugation (16 000 × *g*, 5 min, 22°C). The supernatant was collected and analysed by gas chromatography using a glass column packed with 80/120 Carbopack B-DA/4% Carbowax 20 M stationary phase (Supelco, Bellefonte, PA, USA) equipped with a flame ionisation detector and helium as the carrier gas.

Biogenic amines (methylamine, ethylamine, tryptamine, butylamine, phenylethylamine, 2-methylbutylamine, putrescine, piperidine, cadaverine, histamine, tyramine, spermidine, spermine) were determined according to Saarinen (2002). Briefly, heptylamine was added to the sample as an internal standard and the biogenic amines were extracted by shaking with 0·4 M perchloric acid for 10 min. After centrifugation the biogenic amines in the supernatant were derivatised with dansyl chloride. The derivatives were separated on a C18 reversed phase column (Spherisorb ODS-2 column, 250 × 3 mm, Waters, Milford, MA) using gradient elution with a solvent mixture of A: 0·04 M ammonium acetate and acetonitrile (70:30) and B: 0·02 M ammonium acetate and acetonitrile (15:85) as eluent at 45°C. The compounds were detected with UV-detector at 254 nm. The results were calculated using the internal standard method.

The results were expressed as µmol of SCFA and BCFA/g digesta (wet weight) and as nmol of biogenic amine/g digesta. The proportions of acetic, propionic, butyric and valeric acid were expressed as % of sum of SCFA. Similarly, the proportions of isobutyric, 2-methybutyric and isovaleric were expressed as % of sum of BCFA, and the proportions of each biogenic amine as % of the sum of biogenic amines.

Microbial analyses

The total bacterial cell counts in digesta samples were determined by flow cytometry (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ, USA) as previously described (Apajalahti *et al.*, 2002). The bacterial fractions were recovered by suspending digesta samples in a 50 mM Na phosphate buffer (pH 9·0), followed by centrifugation (48 000 × *g*, 30 min, 22°C) and washing. The cell samples were diluted, fixed and stained with the fluorescent nucleic acid binding dye SYTO 24 (Molecular Probes, Leiden, the Netherlands). The results were expressed as the quantity of bacteria/g fresh digesta weight.

DNA was extracted from the washed bacteria using the method described by Apajalahti *et al.* (1998) whereby bacteria were subjected to 5 freeze–thaw cycles and subsequently treated with lysozyme (17·5 mg/ml, 4 h, 37°C) and proteinase K (0·1 mg/ml, 1 h, 37°C). The recovered bacterial DNA was used to quantify the genus *Bifidobacterium* as described by Mäkivuokko *et al.* (2005), *E. coli* as described by Kaclikova *et al.* (2005), *Lactobacillus* as described by Byun *et al.* (2004) and *C. perfringes* as described by Tiihonen *et al.* (2008).

The primers and probe for the detection of *Campylobacter jejuni* were designed by using the putative oxidoreductase subunit (genbank accession no. CAL34564) with PrimerExpress software (Applied Biosystems, Foster City, USA). In quantitative real-time PCR (qPCR), 1 µg of isolated bacterial DNA was amplified for the analysis for total bifidobacteria, *C. perfringens* and *C. jejuni*, with 300 mM forward and reverse primers with 200 nM probe, and for the analysis of total *E. coli* 900 nM forward and reverse primers were used with 200 nM probe using TaqMan Universal PCR Master Mix (Applied Biosystems, Bridgewater, NJ, USA). For the analysis of total lactobacilli, 900 nM forward and reverse primers were used with SYBR Green PCR Master Mix (Applied Biosystems, Bridgewater, NJ, USA). The assays were run on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Bridgewater, NJ, USA) using the instrument's default settings for thermal cycling and fluorescence measurements. To prepare standard curves for absolute quantification, isolated chromosomal DNAs either from *Bifidobacterium adolescentis* (DSM 20083, DSMZ, Braunschweig, Germany) for bifidobacteria, *E. coli* strain B (Sigma-Aldrich, St. Louis, MO, USA) for *E. coli*, *C. perfringens* (ATCC 13124, LGC Standards AB, Borås, Sweden) for *C. perfringens*, *L. gallinarum* (ATCC 33199), *L. crispatus* (ATCC 33820) and *L. aviarius* (ATCC 43234, LGC Standards AB, Borås, Sweden) for lactobacilli and *C. jejuni* (ATCC 33560) for *C. jejuni* were used. The results are expressed as the quantity of bacteria/g wet digesta.

The %G + C profiling was performed as described previously (Holben *et al.*, 1988; Apajalahti *et al.*, 1998). The method yields a profile of the total microbiota of the sample based on the characteristic guanine + cytosine (%G + C) content of the DNA of the bacterial components. Briefly, 100 µg of isolated caecal DNA was subjected to 1 µg of AT-dependent DNA binding dye bisbenzimidazole in 40 ml

caesium chloride. Samples were ultra centrifuged (100 000 × *g*, 72h, 25°C) to obtain the differential density gradient. The gradient was pumped through UV detector and the highly-purified and protein-free DNA was quantified based on A_{280} to minimise the background absorbance resulting from caesium chloride and unbound bisbenzimidazole. Regression analysis ($r^2 > 0{\cdot}99$) of density gradient of standard samples containing DNA of bacteria with known G + C composition (*C. perfringens*, *E. coli*, *Micrococcus lysodeikticus*) was used to determine the G + C content of the unknown samples. Profiles of the caecal bacterial community were presented as percent G + C content versus relative abundance.

Immunoglobulin A analyses

The digesta samples were treated with 1% bovine serum albumin at 20°C for 60 min in order to extract immunoglobulin A (IgA) from the digesta. The samples were centrifuged (48 000 × *g*, 30 min, 20°C) and the IgA concentrations of the supernatants were determined with commercial chicken IgA ELISA kit (Cat no. E30-103, Bethyl Laboratories, Montgomery, AL, USA) in accordance with the manufacturer's instructions.

Statistics

The effect of EO supplementation was studied at two different time points (21 and 40 d) and digesta samples were taken from both the ileum and caecum. Pen served as the experimental unit so that there were 12 pens per diet. Within the treatments, samples from two birds were pooled to give 6 replicate samples from both time points and intestinal compartments (ileal and caecal). For digesta sample data there were total of 30 measured parameters and 4 factorial variables (compartment pen, time point, and treatment group). For performance data there were a total of 16 measured parameters and two factorial variables (pen, treatment group).

All the data were analysed using statistical programming language R version 2·6·0 (R Development Core Team, 2008). The summary statistics, i.e. mean and standard error of the mean (SEM), were calculated for each measured parameter, in each combination of compartment and treatment groups. After performing the initial analysis, the effects of the treatments were studied separately for each parameter by comparing the mean levels of treatment groups using either the *t*-test or Wilcoxon-test, depending on whether the data exhibited normal distribution or not. Both tests were performed as un-paired and two-tailed, the normality of distribution was tested using Shapiro-Wilk test. *P*-values less than 0·05 were considered statistically significant. Before analysing the differences in microbial numbers, the data were first subjected to log transformation. Measurements under the detection limit were replaced with a value of half of the known detection limit. If all measurements of a parameter in a treatment group were under the detection limit, they were treated as missing values. The total amounts of SCFA, BCFA and biogenic amines were calculated using only the values originally above the detection limit.

Linear models were used to study the microbial metabolites and IgA to get a general overview of the effects of age, intestinal compartment and treatment on the data. Accordingly, these variables were used as factors for the model. The significance of model coefficients were determined using t-statistic and corresponding (two-sided) *P*-value.

To get more insight on relationship between performance and microbial metabolites, the linear associations between the parameters were studied. This was done using Pearson's correlation coefficient which was calculated between all parameters (total 21 946 of correlations) irrespective of treatment groups. The Pearson's correlation test statistic is known to follow a *t*-distribution, which was used to define the significance of correlation.

RESULTS

In the EO group, birds had a significantly higher average ($P < 0{\cdot}05$) BWG from 0 to 21 d than the birds fed with the control diet (Table 2). Furthermore, the body weight gain (BWG) with the EO treatment was significantly higher ($P < 0{\cdot}05$) over the whole trial period (0–42 d) compared with the control diet. A trend for improved feed conversion ratio (FCR) over the whole trial period with the EO treatment versus the control diet was observed ($P = 0{\cdot}06$). The poor bird weights and FCR observed in this study, as compared to breed standards in the first 21 d, may suggest the presence of a sub-clinical problem in this flock of birds. The mortality over the whole study period was 9·7% in the control group and 5·0% in the EO group.

In the ileum, irrespective of the treatment, the sums of SCFA and BCFA concentrations were significantly lower whereas the lactic acid concentration was higher than in caecum ($P < 0{\cdot}001$, from linear models, data not shown). Results of the SCFA and BCFA analyses from the ileal and caecal samples are presented in Tables 3 and 4, respectively. The inclusion of EO did not have any significant effect on the sums of ileal SCFA at 20 and 41 d. In the caecum the

Table 2. *Effect of EO supplement on the growth performance and mortality of broilers*[1]

Variable	Treatment		*P*-value[2]
	Control	EO	
Starter period, d 0–21 of age			
BWG, kg	0·68 ± 0·02	0·72 ± 0·01	0·033
Average daily feed intake, g	57·35 ± 1·69	59·89 ± 1·54	0·387
Feed conversion, feed/gain	1·83 ± 0·05	1·76 ± 0·03	0·285
Mortality, %	6·67 ± 3·51	2·22 ± 0·95	0·30
Overall, d 0–42 of age			
BWG, kg	2·53 ± 0·05	2·65 ± 0·03	0·043
Average daily feed intake, g	117·24 ± 2·68	121·81 ± 1·66	0·094
Feed conversion, feed/gain	2·04 ± 0·02	2·00 ± 0·01	0·058
Mortality, %	9·72 ± 3·22	5·00 ± 1·33	0·12

[1]Results are means ± SEM with $n = 12$ per treatment.
[2]All variables were analysed using Wilcoxon test, except for feed intake (0–21, 0–42 d) which was analysed using *t*-test.

Table 3. *Effect of EO supplement on the sum of short chain fatty acid (SCFA) concentrations, branched chain fatty acid (BCFA) concentrations and proportions of individual components in broiler ileum*[1]

Variable	Age (d)	Treatment		*P*-value[2]
		Control	EO	
Sum of SCFA (μmol/g wet weight)	20	3·45 ± 0·48	3·20 ± 0·35	0·68
	41	3·54 ± 0·46	4·91 ± 0·65	0·12
Propionic acid (% of sum of SCFA)	20	2·2 ± 0·6	1·7 ± 0·2	0·40
	41	2·5 ± 0·3	1·8 ± 0·4	0·19
Butyric acid (% of sum of SCFA)	20	1·6 ± 0·2	2·6 ± 0·9	0·33
	41	1·6 ± 0·2	1·1 ± 0·2	0·16
Acetic acid (% of sum of SCFA)	20	99·1 ± 0·9	98·9 ± 1·1	1·00
	41	98·4 ± 0·7	99·0 ± 0·6	0·53
Valeric acid (% of sum of SCFA)	20	ND[3]	ND	
	41	ND	ND	
Total BCFA (μmol/g wet weight)	20	0·193 ± 0·041	0140 ± 0·011	0·30
	41	0·148 ± 0·017	0·155 ± 0·005	0·71
Isobutyric acid (% of sum of BCFA)	20	ND	ND	
	41	ND	ND	
2-Methylbutyric acid (% of sum of BCFA)	20	ND	ND	
	41	ND	ND	
Isovaleric acid (% of sum of BCFA)	20	100	100	
	41	100	100	

[1]Results are means ± SEM with $n = 5$–6 per treatment.
[2]All variables were analysed using *t*-test, except butyric acid (d 20) and acetic acid (d 20) which were analysed using Wilcoxon test.
[3]ND, not detectable. Detection limit 0·1 μmol/g wet weight.

sum of SCFA was higher in the control group at 20 d ($P < 0·01$). The proportion of butyrate (20 d and 41 d; $P < 0·01$) increased whereas the proportion of acetic acid (20 d; $P < 0·001$) propionic acid (41 d; $P < 0·05$) and isovaleric acid (41 d; $P < 0·01$) decreased in the EO group.

The overall sums of biogenic amine concentrations were lower in the ileum than in the caecum ($P < 0·002$, from linear models, data not shown). There were no differences in the sums of ileal or caecal biogenic amines between the treatment groups (Tables 5 and 6). The only significant difference in the ileum was the higher concentration of tyramine ($P < 0·05$) in the treatment group at 20 d of age. The proportion of spermine was higher (41 d; $P < 0·01$) and tyramine was lower (41 d; $P < 0·05$) with the EO group.

The average numbers of total bacteria in the caecum ranged between 2·33 and $3·49 \times 10^{11}$ cells/g (Table 7). The caecal qPCR measurements of microbes showed higher proportion of *Lactobacillus* and *E. coli* ($P = 0·06$) in the EO group compared to the control group at 41 d. No *Campylobacter jejuni* was found in the samples and only low numbers of *C. perfringens* were detected in both treatment groups. Bifidobacteria were present in reasonably high numbers, ranging between 5·98 and $6·35 \times 10^9$ cells/g, but no differences between the treatment groups were found.

Table 4. *Effect of EO supplement on the sum of short chain fatty acid (SCFA) concentrations, branched chain fatty acid (BCFA) concentrations and proportions of individual components in broiler caecum*[1]

Variable	Age (d)	Treatment		*P*-value[2]
		Control	EO	
Sum of SCFA (μmol/g wet weight)	20	126·4 ± 5·3	86·5 ± 7	0·008
	41	102·9 ± 13·4	125·7 ± 6·5	0·167
Propionic acid (% of sum of SCFA)	20	4·4 ± 0·4	6·3 ± 1·8	0·841
	41	13·3 ± 1·1	10·1 ± 0·7	0·042
Butyric acid (% of sum of SCFA)	20	16·1 ± 0·3	23·6 ± 1·6	0·008
	41	15·2 ± 1·6	21·7 ± 1	0·009
Acetic acid (% of sum of SCFA)	20	78·7 ± 0·5	68·9 ± 1·4	0·001
	41	70 ± 2·3	66·5 ± 0·9	0·206
Valeric acid (% of sum of SCFA)	20	0·8 ± 0·1	1·2 ± 0·2	0·150
	41	1·5 ± 0·2	1·7 ± 0·2	0·609
Total BCFA (μmol/g wet weight)	20	1·00 ± 0·1	1·05 ± 0·1	0·757
	41	1·91 ± 0·62	1·41 ± 0·16	0·937
Isobutyric acid (% of sum of BCFA)	20	50·8 ± 2·7	47·4 ± 4·9	0·557
	41	50·9 ± 2·3	55·4 ± 1·7	0·144
2-Methylbutyric acid (% of sum of BCFA)	20	13·4 ± 1·8	7·3 ± 2·0	0·095
	41	20·1 ± 1	19·5 ± 1·1	0·716
Isovaleric acid (% of sum of BCFA)	20	37·4 ± 1·9	49·3 ± 6·3	0·134
	41	29·2 ± 1·4	25·3 ± 0·8	0·009

[1]Results are means ± SEM with $n = 5$–6 per treatment.
[2]All variables were analysed using *t*-test, except total SCFA (d 29), propionic acid (d 20), total BCFA (d 41), 2-methylbutyric acid and isovaleric acid (d 41) which were analysed using Wilcoxon test.

Table 5. *Effect of EO supplement on the sum of biogenic amine concentrations and proportions of individual components in broiler ileum*[1]

Variable	Age (d)	Treatment		*P*-value[2]
		Control	EO	
Sum of biogenic amines (nmol/g wet weight)	20	697·8 ± 297·7	454·6 ± 46·3	0·937
	41	776·0 ± 266·6	1933·1 ± 958	0·240
Mehylamine (% of sum of biogenic amines))	20	ND	ND	
	41	11·1 ± 5·7	1·9 ± 1·8	0,175
Tryptamine (% of sum of biogenic amines)	20	14·8 ± 3·2	4·1 ± 1·9	0,065
	41	11·0 ± 4·8	11·5 ± 5·1	0,940
Phenylethylamine (% of sum of biogenic amines)	20	ND[3]	ND	
	41	ND	ND	
Putrescine (% of sum of biogenic amines)	20	13·0 ± 1·6	13·1 ± 1·7	0,818
	41	11·3 ± 3·6	9·4 ± 2·8	0,690
Piperidine (% of sum of biogenic amines)	20	ND	ND	
	41	2·5 ± 0·8	3·0 ± 1·3	1,000
Cadaverine (% of sum of biogenic amines)	20	29·0 ± 6	19·2 ± 2·7	0,485
	41	54·6 ± 8·3	62·8 ± 4·7	0,415
Histamine (% of sum of biogenic amines)	20	7·2 ± 4·6	4·6 ± 2·4	0,589
	41	3·3 ± 0·5	4·3 ± 0·9	0,400
Tyramine (% of sum of biogenic amines)	20	12·9 ± 4·3	33·9 ± 7·1	0,035
	41	3·1 ± 0·8	2·3 ± 0·6	0,310
Spermidine (% of sum of biogenic amines)	20	16·0 ± 3·5	17·4 ± 1·5	0,724
	41	9·8 ± 2·2	7·7 ± 2·4	0,537
Spermine (% of sum of biogenic amines)	20	9·6 ± 2·6	11·4 ± 0·8	0,528
	41	3·2 ± 1·1	3·1 ± 1·7	0,394

[1]Results are means ± SEM with $n = 5$–6 per treatment.
[2]All variables were analysed using *t*-test except total biogenic amines, tryptamine (d 20), putrescine (d 20), piperidine (d 41), cadaverine (d 20), histamine (d 20), tyramine (d 41) and spermine (d 41) which were analysed using Wilcoxon test.
[3]ND, not detectable. Detection limit 20 nmol/g wet weight except for putrescine 10 nmol/g wet weight and 5 nmol/g wet weight for spermidine and spermine.

The %G + C profiles of both treatments at 41 d are presented in the Figure. At 20 d, the %G + C profiles did not differ between the groups (data not shown). However, within the range of %G + C of 46–52% at 41 d, the control group had a higher ($P < 0·05$) relative abundance of microbes compared to the EO group. Moreover, between %G + C of 70–78%,

Table 6. *Effect of EO supplement on the sum of biogenic amine concentrations and proportions of individual components in broiler caecum*[1]

Variable	Age (d)	Treatment		*P*-value[2]
		Control	EO	
Sum of biogenic amines (nmol/g wet weight)	20	1911 ± 430	2294 ± 600	0·615
	41	2893 ± 1262	3150 ± 745	0·485
Methylamine (% of sum of biogenic amines)	20	8 ± 0·9	8·7 ± 2·1	0·752
	41	19·9 ± 4	13·3 ± 2·5	0·202
Tryptamine (% of sum of biogenic amines)	20	0·7 ± 0·2	1 ± 0·4	0·548
	41	4·6 ± 1·9	0·4 ± 0·1	0·082
Phenylethylamine (% of sum of biogenic amines)	20	2·7 ± 1·1	4·4 ± 1·9	0·454
	41	ND[3]	ND	
Putrescine (% of sum of biogenic amines)	20	6·3 ± 2·3	7·2 ± 2·3	1·000
	41	12·2 ± 5·7	6·2 ± 2·4	0·310
Piperidine (% of sum of biogenic amines)	20	ND	ND	
	41	ND	ND	
Cadaverine (% of sum of biogenic amines)	20	21·4 ± 7·6	19 ± 8·9	0·842
	41	18·2 ± 6	26·9 ± 10·1	0·483
Histamine (% of sum of biogenic amines)	20	1·3 ± 0·5	1·7 ± 0·6	0·669
	41	1·2 ± 50·5	1·9 ± 0·3	0·255
Tyramine (% of sum of biogenic amines)	20	7·7 ± 2·6	7·6 ± 3·3	0·986
	41	3·7 ± 1	0·5 ± 0·1	0·025
Spermidine (% as a sum of biogenic amines)	20	51·2 ± 5·8	48·8 ± 8·4	0·822
	41	40·5 ± 6	50·4 ± 6·9	0·304
Spermine (% of sum of biogenic amines)	20	1·6 ± 0·1	2·2 ± 0·6	1·000
	41	0·3 ± 0·1	1·3 ± 0·1	0·004

[1]Results are means ± SEM with $n = 5$–6 per treatment.
[2]All variables were analysed using *t*-test except total biogenic amines (d 41), tryptamine (d 20), putrescine, and spermine which were anlysed using Wilcoxon test.
[3]ND, not detectable. Detection limit 20 nmol/g wet weight except for putrescine 10 nmol/g wet weight and 5 nmol/g wet weight for spermidine and spermine.

Table 7. *Effect of EO supplement on the caecal microbes of broilers*[1]

Variable	Age (d)	Treatment		*P*-value[2]
		Control	EO	
Total bacterial counts (cells/g wet weight)	20	$2{\cdot}72 \times 10^{11} \pm 1{\cdot}86 \times 10^{10}$	$2{\cdot}33 \times 10^{11} \pm 1{\cdot}72 \times 10^{10}$	0·155
	41	$3{\cdot}26 \times 10^{11} \pm 3{\cdot}52 \times 10^{10}$	$3{\cdot}49 \times 10^{11} \pm 2{\cdot}77 \times 10^{10}$	0·621
Campylobacter jejuni	20	<50	<50	
	41	<50	<50	
Clostridium perfringens	20	$1{\cdot}59 \times 10^{6} \pm 1{\cdot}24 \times 10^{6}$	$8{\cdot}41 \times 10^{5} \pm 5{\cdot}84 \times 10^{5}$	0·699
	41	$3{\cdot}67 \times 10^{7} \pm 2{\cdot}51 \times 10^{7}$	$1{\cdot}99 \times 10^{6} \pm 8{\cdot}53 \times 10^{5}$	0·699
E coli	20	$2{\cdot}84 \times 10^{8} \pm 8{\cdot}27 \times 10^{7}$	$4{\cdot}33 \times 10^{8} \pm 2{\cdot}57 \times 10^{8}$	0·699
	41	$3{\cdot}63 \times 10^{8} \pm 1{\cdot}8 \times 10^{8}$	$1{\cdot}45 \times 10^{8} \pm 9{\cdot}2 \times 10^{7}$	0·093
Bifidobacterium group	20	$5{\cdot}98 \times 10^{9} \pm 1{\cdot}47 \times 10^{9}$	$9{\cdot}13 \times 10^{9} \pm 1{\cdot}88 \times 10^{9}$	0·217
	41	$6{\cdot}85 \times 10^{9} \pm 1{\cdot}04 \times 10^{9}$	$6{\cdot}35 \times 10^{9} \pm 8{\cdot}55 \times 10^{8}$	0·589
Lactobacillus group	20	$2{\cdot}76 \times 10^{7} \pm 1{\cdot}61 \times 10^{7}$	$4{\cdot}30 \times 10^{8} \pm 2{\cdot}48 \times 10^{8}$	0·180
	41	$6{\cdot}42 \times 10^{8} \pm 2{\cdot}4 \times 10^{8}$	$1{\cdot}67 \times 10^{8} \pm 9{\cdot}03 \times 10^{7}$	0·093

[1]Results are means ± SEM with $n = 6$ per treatment, except *Campylobacter jejuni* that was below detection limit of 50 cells/g wet weight.
[2]All variables were analysed using Wilcoxon test, except for total bacterial counts and *Bifidobacterium* group (d 20) which were analysed using t-test.

the control group had a lower relative abundance of microbes compared to the EO group.

Both age and intestinal segment was observed to have a significant effect on IgA ($P < 0{\cdot}001$, from linear models, data not shown). The concentration of IgA increased with age on average from 6·5 to 7·40 mg/g in the ileum, and on average from 7·6 to 9·1 mg/g in the caecum, and was higher in the caecum than in ileum. The EO did not affect IgA concentration.

DISCUSSION

Trials assessing the effect of essential oil or herbal extract blends on broiler chicks have yielded variable effects on bird performance or nutrient digestibility (Botsoglou *et al.*, 2002; Lee *et al.*, 2003; Hernández *et al.*, 2004; Cross *et al.*, 2007). However, the performance effects are likely to be dependent on the quality and quantity of essential oils used in each study, and

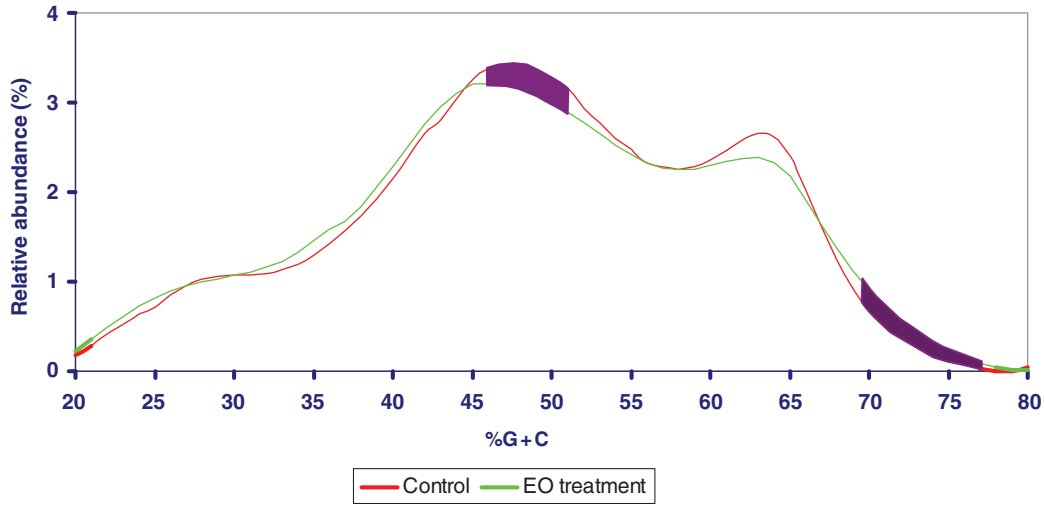

Figure. *Effect of EO supplementation on the average %G + C profiles of broiler caecum samples at d 41. The green line represents the EO treatment group and the red line represents the control group. The violet area represent the % G + C range in which the difference between the treatments is statistically significant (P < 0·05). Differences were observed in the relative abundance of bacteria with %G + C content of approximately 50% (typical for* Escherichia coli *and* Salmonella*). The shoulder of the peak of the bacteria with high %G + C content also differed between the groups.*

detailed information on the blends used is not always reported. The variation in responses may also be associated with the type of EO present in the blend and their potential synergistic, additive or counteractive effects. Other factors that affect the response in growth performance to the supplementation of EO are the hygienic conditions in which the animals are kept, the background health condition of the flock, and the type of ingredients present in the diets. This study assessed the effects of an EO treatment, comprising of a blend of cinnamaldehyde and thymol, on broiler performance and gut microbiota composition and activity. The EO blend increased bird BW significantly at 20 d of age and improved the BWG statistically significantly over the whole study period. Positive performance results by dietary EO supplementation were also demonstrated by Alcicek *et al.* (2003) who showed that EO combination from wild herbs increased the BW and improved FCR of broilers. Denli *et al.* (2004) observed that thyme EO increased BWG and feed efficiency in Japanese quails.

Several authors have suggested that dietary essential oils improve bird performance because these substances stimulate the secretion of endogenous digestive enzymes which then increases nutrient digestion, gut passage rate or feed intake (Salam *et al.*, 2002; Lee *et al.*, 2003, 2004*a*, 2004*b*; Jamroz *et al.*, 2005; Jang *et al.*, 2007; Muhl & Liebert, 2007). These studies have given variable results. In the present study, we chose another view and addressed the importance of gut microbiota and intestinal immunity to the broiler chick performance. The intestinal microbes and the microbial fermentation profiles of broiler chicks have recently reviewed by Rehman *et al.* (2007). The results of the present %G + C profiling suggested that while the majority of the caecal microbiota were unaffected by the EO treatment, certain specific changes in the profile of the microbiota were observed. The most notable difference in the microbiota composition was observed between the %G + C values of 46 to 52. Within this range, which includes potentially harmful organisms such as *E. coli* and *Salmonella* (Apajalahti *et al.*, 1998), the proportion of bacteria was lower in the EO treatment group compared to the control group. This observation was supported by the qPCR analysis, where the levels of *E. coli* tended to be lower in the EO treatment group. Thus, the EO treatment may beneficially influence the caecal microbiota of chickens by lowering the proportion of *E. coli* in caecum. The EO treatment also increased the relative proportion of the microbes at the high end of the %G + C range, the range which includes microbes such as bifidobacteria and propionibacteria. However, the qPCR analysis showed no dietary effects on the total amount of *Bifidobacterium*. While the diet apparently had no direct bifidogenic effect in the caecum, it may have increased the relative proportion of the high %G + C bacteria by reducing the numbers of certain other bacteria.

The antimicrobial effect of the EO treatment appears to be selective for certain potentially harmful microbes, as indicated by our *in vitro* work where thymol or cinnamaldehyde at the level of 50 mg/l decreased the growth of *E. coli* yet beneficial bacteria such as bifidobacteria and lactobacilli were not negatively affected to the same degree (Ouwehand *et al.*, 2010). Similarly,

Helander *et al.* (1998) found that *E. coli* was sensitive to both cinnamaldehyde and thymol and that *Salmonella typhimurium* was more sensitive to thymol. The reduction of *E. coli* may be explained by the ability of EO to disrupt the bacterial cell membrane. Moreover, it has been shown that EO stimulates the release of mucus into the small intestine which reduces the adhesion of pathogens to the epithelium (Jamroz *et al.,* 2006). Positive effect of EO on gut microbiota, associated with improved growth performance, has also been observed by others (Jamroz & Kamel, 2002; Jamroz *et al*., 2005) with the feeding of capsaicin, carvacrol and cinnamaldehyde.

Not only the microbial community composition, but also the microbial metabolites may have an effect on the nutritional status of the broiler chick and the function and health of its gastrointestinal tract. Microbial fermentation products are derived either from carbohydrates (i.e. SCFAs) or proteins (i.e. BCFAs, biogenic amines). In this study EO treatment increased the proportion of butyrate. Butyrate provides energy to colonic mucosa (Roediger, 1980) and thus has important implications on intestinal health (for reviews see Scheppach *et al*., 2001; Hamer *et al*., 2008). Moreover, EO treatment decreased the proportion of acetic acid, isovaleric acid and tyramine proportions but increased caecal spermine proportions. The physiological roles of BCFA and biogenic amines in the gastrointestinal tract are not fully understood. Sousadias and Smith (1995) report increased weight gain and feed efficiency when low concentrations of spermine are fed to broilers**,** although at high supplemental levels spermine is found to be toxic.

Correlations were studied between the intestinal biomarkers and performance indicators irrespective of the treatment group (data for correlations is not shown). Of the intestinal biomarkers, ileal spermine and spermidine at d 20 were found to be prognostic for improved performance at 42 d; high ileal concentrations of spermidine and spermine at d 20 were associated with decreased mortality at d 42 ($r = -0{\cdot}739$, $P < 0{\cdot}01$ and $r = -0{\cdot}768$, $P < 0{\cdot}05$, respectively). Of the caecal microbes, the number of bifidobacteria at d 20 positively correlated with weight at d 42 ($r = 0{\cdot}679$, $P = 0{\cdot}051$). The concentration of the sum of biogenic amines in the ileum at d 20 was associated with an increase in mortality at d 42 ($r = 0{\cdot}849$, $P < 0{\cdot}001$), but inversely associated with weight at d 42 ($r = -0{\cdot}665$, $P < 0{\cdot}05$). It is unclear to what extent the associations observed between the early intestinal microbial environment and the subsequent weight gain or mortality can explain the performance of the broilers. However, there are several reports describing the impact of intestinal microbes on broiler health and growth (Miles *et al*., 2006; Mountzouris *et al.* 2007). To our knowledge, this is the first time a connection between the small intestinal microbial environment at an early age and the subsequent growth at older age has been demonstrated. Our results encourage future research to focus more on intestinal microbiology when improved growth and health are targeted.

In conclusion, the present study showed a significant increase in broiler chick body weight gain by the dietary EO amendment. Concomitant changes in the caecal microbiota and microbial metabolites indicate that EO supplementation may induce beneficial changes in the intestinal microflora and also to the health and performance of the birds. The current results provide a rationale for treatments aimed at the modulation of gut microbiota composition and activity in order to improve broiler performance and health. Further research in this area is required to fully understand the associations between gut microbiota and broiler performance.

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