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Effect of *Origanum* chemotypes on broiler intestinal bacteria

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ABSTRACT Essential oils have been proposed as alternatives to antibiotic use in food animal production. This study evaluated 3 chemotypes of the *Origanum* genus, containing varying amounts of secondary metabolites carvacrol, thymol, and sabinene, in the broiler chicken diet. Aerial parts of *Origanum vulgare* L. (OL), *O. vulgare* L. ssp. *hirtum* (OH), and *O. majorana* (OM) were collected from a greenhouse located in the high altitude Sabana de Bogotá (Savanna of Bogotá) and *O. vulgare* L. ssp. *hirtum* (OG) produced and ground in Greece. Oregano essential oils (OEO) from these plants were obtained by steam distillation and analyzed by gas chromatography coupled to a mass spectrometer. Six treatments were evaluated: 200 mg/kg of OEO from OH, OL, and OM, 50 mg/kg of OEO from OG, 500 mg/kg of chlortetracycline, and without additives. Broiler chicks were maintained at 2,600 m above sea level, placed in brooder cages under a completely randomized design. Template DNA was isolated from duodenal, jejunal, ileal, and cecal contents in each group and bacterial 16S rDNA patterns were analyzed by denaturing gradient gel electrophoresis. Dendrograms of denaturing gradient gel electrophoresis band patterns revealed 2 main clusters, OEO-treated chicks and nontreated control chicks, in each intestinal segment. Band patterns from different gut compartments revealed major bacterial population shifts in the foregut (duodenum, jejunum, and ileum) compared with the hindgut (cecum and colon) at all ages evaluated ($P < 0.05$). The OEO groups showed less shift (62.7% similarity coefficient) between these 2 compartments versus the control groups (53.7% similarity coefficient). A reduction of 59% in mortality from ascites was seen in additive-supplemented groups compared with the control group. This study represents the first work to evaluate the effects of the 3 main chemotypes of *Origanum* genus in broilers.

Key words: essential oil, carvacrol, thymol, oregano, denaturing gradient gel electrophoresis

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INTRODUCTION

The use of antibiotics as growth promoters in animal feed has been decreasing over the last few years. This shift has stimulated the search for alternative supplements for use in animal production and plant essential oils are among these targeted alternative growth promoters (Williams and Losa, 2002; Lee et al., 2003a; Hernández et al., 2004). Plant extracts are characterized by several effects including antimicrobial activity (Aligiannis et al., 2001; Baricevic and Bartol, 2002; Çetin et al., 2011; Kačániová et al., 2012), stimulation of appetite and secretion of endogenous enzymes, increased digestibility, and having antioxidant and immunostimulant properties in humans and other animals (Bakkali et al., 2008). These properties in oregano essential oils (**OEO**) were attributed to the presence of a hydroxyl group in their phenolic components, thymol and carvacrol (Ben Arfa et al., 2006). Antimicrobial activity in vitro of OEO, as well as 2 of their main constituents, thymol and carvacrol, has been well documented (Hammer et al., 1999; Dorman and Deans, 2000; Aligiannis et al., 2001). The free hydroxyl group and the hydrophilic character of these molecules increase outer membrane permeability, dissipate internal pH, allowing ions and other components to leave the cytoplasm (Helander et al., 1998; Lambert et al., 2001). Carvacrol and thymol present a broad spectrum of antibacterial activity in vitro against gram-negative and gram-positive bacteria and other physiological ef-

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fects (Dorman and Deans, 2000; Aligiannis et al., 2001; Mathlouthi et al., 2012). Despite the additive effects of OEO, more attention and importance have been given to carvacrol and Greek oregano due to its high content of carvacrol. However, experimental results following supplementation of high carvacrol OEO to broiler diets are controversial due to the fact that OEO improved growth in some cases (Giannenas et al., 2003), whereas other studies demonstrated no effects on broiler growth (Botsoglou et al., 2002).

Essential oil mixtures have been shown to play a significant role in the modulation of gut microbiota (Hume et al., 2006; Oviedo-Rondón et al., 2006) and the colonization of pathogenic bacteria (Jamroz et al., 2003). The normal intestinal microbiota is regarded as a first defense against exogenous and endogenous, potentially pathogenic microorganisms, and is associated with broiler chicken health and production (Guarner and Malagelada, 2003; Amit-Romach et al., 2004). Gut integrity may be associated with susceptibility to disorders such as those presented with ascites. Solis de los Santos et al. (2005) showed that the addition of a prebiotic to the diet of chickens reduced ascites mortality. Ascites syndrome in broilers is a metabolic disorder and results in significant economic losses (Pakdel et al., 2005). The most important environmental factors causing the development of ascites in broilers are high altitude and cold temperatures (Hernández, 1987; Shlosberg et al., 1996; Özkan et al., 2010). Very limited attention has been given to the effect of OEO composition on intestinal microbial communities and incidences of ascites at high altitude. The pathogenesis of ascites can originate with a high metabolic basal rate induced by several factors (Currie, 1999). The gastrointestinal tract is a highly metabolically active organ with a high oxygen requirement (Yen et al., 1989) and can be negatively influenced by inflammation, pathogens, environment, or a high metabolism resulting in ascites (Ivatury et al., 1996). Additionally, the effect of oregano cultivation at high altitude on the composition of OEO and the effects of different chemotypes on intestinal microbial communities have been given little study.

The current study explored the effects of 3 chemotypes from *Origanum* genus grown at high altitude on i) carvacrol and thymol content compared with the conventional Greek variety produced and ground in Greece; ii) the effect of OEO supplementation on intestinal microbiota of broiler chickens as examined by denaturing gradient gel electrophoresis (**DGGE**), and iii) the incidence of ascites in broilers supplemented with OEO and an antibiotic.

MATERIALS AND METHODS

Essential Oil Extraction

Aerial parts of 3 types of oregano were collected (50 kg each) from a greenhouse in the Sabana de Bogotá, Colombia. The plants were identified by the Herbarium of National University Science faculty (Bogotá, Colombia). Essential oils from these plants were obtained by steam distillation for 3 h using a Clevenger-type apparatus. The steam and oil mixture was cooled through a condenser and the condensate was collected to allow phase separation (Vazquez and Dunford, 2005). Oregano essential oil was collected in dark containers and kept at 4°C before further analysis.

Essential Oil Analysis

The OEO were sent to the Chromatography Laboratory of the Universidad Industrial de Santander, Bucaramanga, Santander, Colombia, for GC/MS analysis using an Agilent Technologies (Santa Clara, CA) 6890 Plus gas chromatograph coupled with an ion trap detector mass spectrometer equipped with a flame ionization detector and a DB-5 capillary column (Agilent Technologies), 60 m × 0.25 mm, film thickness 0.25 um (5%-phenyl-methylpolysiloxane column). Injector and detector temperatures were at 240°C, split ratio, 5:1 mL/min. The essential oil constituents were identified by comparison of their relative retention times and mass spectra using the NIST (National Institute of Standards and Technology) database and Wiley Registry of Mass Spectral Data. A standard solution of n-alkanes was used to obtain the retention indices.

Bird Husbandry and Treatments

Seven hundred fifty 1-d-old commercial Hybro male broiler chicks, maintained at 2,600 m above sea level of the Sabana de Bogotá, were placed in 30 brooder cages under a completely randomized design. Chicks were fed ad libitum a corn-soy meal diet that met or exceeded NRC (1994) requirements. A prestarter feed was provided for the first 7 d and was replaced by starter feed until broilers were 21 d old. Grower feed was provided for the remainder of the grow-out to 42 d (Table 1). Six treatments were evaluated: 200 mg/kg of OEO from each of the 3 OEO chemotypes, *O. vulgare* L. spp. *hirtum* (**OH**); *O. vulgare* L. (**OL**) and *O. majorana* (**OM**); 50 mg/kg of EO from *O. vulgare* L. spp. *hirtum* grown in Greece (**OG**); 500 mg/kg of chlortetracycline (**AB**); and control with no treatment. The essential oils were added to the diets through palm oil, and all additives were preblended with a portion of basal diet before being added to the final mixture. All diets were fed as mash. Body weight, feed intake, feed conversion, and percent mortality were determined. Mortality was recorded daily, and all of the dead birds were examined throughout the study for lesions of heart failure and ascites. Ascites mortality was evidenced by the presence of plasma in the thoracic-abdominal cavity.

Intestinal Contents Collection

Five chicks randomly selected at each sampling period (21 and 42 d) from each replicate were killed by

Table 1. Basal experimental diets

Item	Prestarter (1–7 d)	Starter (7–21 d)	Growth 21–42 d
Ingredient, g/kg			
Corn	539.1	566.5	621.8
Soybean meal, 49% CP	277.1	254.0	203.2
Extruded soybean	100.0	100.0	100.0
Palm oil	17.9	21.7	18.8
Fish meal	20.0	15.0	15.0
Dicalcium phosphate	14.3	13.6	12.0
Calcium carbonate	12.2	11.7	10.9
Vitamin-trace mineral premix[1]	1.0	1.0	1.0
Salt	3.5	3.5	3.5
Sodium bicarbonate	3.0	3.0	3.0
L-Lysine HCl	1.9	1.2	1.8
DL-Methionine	2.1	1.5	1.5
L-Threonine	1.1	0.3	0.6
Choline Cl 60%	1.0	1.0	1.0
Calculated composition			
ME, kcal/g	3.00	3.05	3.10
Fat, %	6.67	6.95	6.50
Calcium, %	0.96	0.90	0.82
Available phosphorus, %	0.48	0.45	0.41
DEB,[2] mEq/kg	257	247	224
Digestible lysine, %	1.28	1.15	1.07
Digestible methionine, %	0.53	0.45	0.44
TSAA, %	0.81	0.72	0.70
Digestible threonine, %	0.83	0.74	0.70
Analyzed composition, %			
DM	87.1	88.6	87.3
CP	21.5	20.2	18.4
Crude fiber	4.1	3.8	4.3
Ether extract	6.3	6.7	6.5
Ash	6.8	7.0	7.5

[1]Supplied the following per kilogram of diet: vitamin A, 12,000 IU; vitamin D_3, 2,125 IU; vitamin K_3, 2.65 mg; thiamine, 2.25 mg; riboflavin, 5.1 mg; pyridoxine, 2.475 mg; cyanocobolamin, 14.375 g; calcium pantothenate, 14.5 mg; niacin, 27.425 mg; folic acid, 0.800 mg; choline, 187.5 mg; Zn, 90.74 mg; Fe, 82.25 mg; Cu, 18.12 mg; Mn, 79.137 mg; I, 1.839 mg; Se, 0.363 mg; Co, 2.498 mg.

[2]DEB: dietary electrolyte balance.

cervical dislocation, and digestive tracts were aseptically removed. Duodenum (duodenal loop), jejunum (from duodenal loop end to Meckel's diverticulum), ileum (from Meckel's diverticulum to the ileocecal junction), cecum, and colon were removed, and the contents from each section were collected aseptically. Samples of intestinal contents from corresponding sections were pooled to reduce individual within group variation (Zhou et al., 2007). From the pooled contents of each section, a total of 1.35 g was suspended in 12.15 mL of sterile Butterfield's buffer (0.62 m*M* KH_2PO_4, pH 7.2) and stored at −20°C for further analysis.

Bacterial DNA Isolation

The DNA was extracted from 1 mL of stored intestinal samples using a modified protocol (Denman and McSweeney, 2005), which combines freezing and bead-beating with SDS and lysozyme treatment. Briefly, pellets were collected by centrifugation at 20,000 × *g* for 5 min and resuspended in 1 mL of lysis buffer [2% (wt/vol) SDS, 100 m*M* Tris HCl, 5 m*M* EDTA, 200 m*M* NaCl, pH 8.0] and 100 μL of potassium acetate solution, pH 5.5 to 6.0 [29.4% (wt/vol) of potassium acetate, 11.5% (vol/vol) of glacial acetic acid]. Suspensions were frozen in liquid nitrogen and thawed for 3 min in boiling water. The samples were bead-beaten for 2 min at maximum speed on a Mini-Bead Beater (BioSpec Products, Bartlesville, OK) using 1.0-mm-diameter glass beads (0.25 g). The freezing-thawing and bead-beating steps were repeated 2 additional times. Lysozyme (0.15 mg/mL final concentration) was added, and the mixture was incubated at 37°C for 1.5 h. Subsequently, DNA samples were precipitated by adding glass milk [16.7% (wt/vol) of silica (Sigma-Aldrich, St. Louis, MO) 3 *M* guanadinium isothyocianate] and washed with 100% ethanol. Finally, the DNA was suspended in 10 m*M* Tris-1 m*M* EDTA (pH 8.0) buffer and stored at −20°C.

Amplicons

Concentrations and purity measurements of DNA were conducted by using a Nanodrop ND-100 spectrophotometer (Nanodrop, Wilmington, DE). Denaturing gradient gel electrophoresis was run according to methods of Muyzer et al. (1993) and Hume et al. (2003). Primers for the domain *Bacteria* were used for amplification of the highly variable V3 region on the 16S rDNA gene, and PCR was performed in a total volume of 25 μL.

Sample DNA (250 ng) was added to Jump Start Red-Taq Ready Mix (Sigma-Aldrich) containing 25 pmol of each primer [primer 2, 5′-ATTACCGCGGCTGCTGG-3′, and primer 3 with a 40-base G-C clamp (Sheffield et al., 1989; Muyzer et al., 1993), 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGC ACGGGGGGCCTACGGGAGGCAGCAG-3′]. This primer part corresponds to position 341 to position 534 in 16S rDNA of *Escherichia coli* and results in a 233-bp amplicon. The reaction was carried out on a PTC-200 Peltier Thermal Cycler (MJ Research Inc., Waltham, MA) with the following program: 1) denaturation at 94°C for 2 min; 2) subsequent denaturation at 94°C for 1 min; 3) annealing at 67°C for 45 s, −0.5°C per cycle [touchdown to minimize spurious by-products (Don et al., 1991; Wawer and Muyzer, 1995)]; 4) extension at 72°C for 2 min; 5) repeat steps 2 to 4 for 17 cycles; 6) denaturation at 94°C for 1 min; 7) annealing at 58°C for 45 s; 8) repeat steps 6 to 7 for 12 cycles; 9) extension at 72°C for 7 min; and 10) hold at 4°C.

DGGE

Samples from PCR were mixed with an equal volume of 2 × loading buffer [0.05% (wt/vol) bromophenol blue, 0.05% (wt/vol) xylene cyanol, and 70% (vol/vol) glycerol] and run on polyacrylamide gels [8% (vol/vol) acrylamide-bisacrylamide ratio 37.5:1, Bio-Rad Laboratories, Richmond, CA] cast with a 35 to 60% urea-deionized formamide gradient (100% was 7 *M* urea and 40% deionized formamide). A DCode Universal Mutation Detection System (Bio-Rad Laboratories) was

used for electrophoresis with 1 × TAE buffer [20 m*M* Tris (pH 7.4), 10 m*M* sodium acetate, 0.5 *M* EDTA] at 59°C for 17 h at 60 V. Gels were stained with SYBR Green I (Sigma) diluted 1:10,000; fragment pattern relatedness was determined with Molecular Analysis Fingerprinting Software, version 1.6 (Bio-Rad Laboratories). Dendrograms were constructed based on the unweighted pair group method using arithmetic averages for clustering.

Statistical Analysis

The DGGE profiles were digitized and converted to a binary matrix using a script (available on request) written in the Python programming language (Python Software Foundation, Hampton, NH). The binary matrix was further analyzed with the R statistical software (Foundation for Statistical Computing, Vienna, Austria; www.R-project.org) using various packages. Correlation of treatment groups and digestive tract sections with richness was calculated using the MGCV package (Wood, 2011). Correlation with ordination was calculated using envfit functions available in the Vegan package (Oksanen et al., 2010; Community ecology package, version 1.17). The significance of the correlations was evaluated with 10,000 permutations with a level of significance of $P < 0.05$.

Coefficients of similarity for principal clusters were computed by: OEO treated and nontreated; foregut and hindgut; and period, 21 and 42 d. The results were subjected to 1-way ANOVA with 2 × 2 × 2 factorial arrangement, 2 periods, 2 sections of gastrointestinal tract (**GIT**; foregut and hindgut), and 2 treatments with OEO. Five replicate brooder cages (25 birds/pen) were assigned to each treatment in a completely randomized design. Feed intake, BW, feed conversion, and mortality were analyzed under 1-way design. Percentages of similarity coefficient (**SC**) data were transformed by square root function (X + 1) for normalization before analysis, and final data are presented as natural numbers. Treatment means were tested for significance using the GLM procedure of SAS software (SAS Institute Inc., 2002). Group differences for productive performance were determined by Tukey's test, and differences were considered significant at $P < 0.05$.

RESULTS

Classification and Composition of OEO

The plants were identified by the Herbarium of National University Science as OL, OH, and OM. The main components found in the OEO analyzed are presented in Table 2. Major compounds present in the *Origanum* spp. were aromatic mono-terpenes, as thymol, carvacrol, and their precursors, *p*-cymene, and γ-terpinene. *Origanum vulgare* L. ssp. *hirtum* showed the highest carvacrol concentration, 50.8%. Other compounds found were thymol (7.7%), *p*-cymene (19.0%) and γ-terpinene (5.0%). *Origanum majorana* was represented mainly by γ-terpinene (14.1%) and *cis*- and *trans*-sabinene hydrates (17.1%), but also included sabinene (4.3%), p-cymene (3.0%), thymol (10.0%), and carvacrol (3.7%). In contrast, the major volatile compounds found in OL were thymol (21.5%), γ-terpinene (20.3%), *p*-cymene (21.0%), and carvacrol (4.3%).

Bacterial Communities

Generalized additive model was used to fit the richness calculated from DGGE profiles to parametric groups of treatment and digestive tract sections. The model explained 76.1% of deviance with $R^2 = 0.69$. Significance levels for group comparisons are presented in Tables 3 and 4. The significance levels show that richness in AB treatment was not significantly different ($P > 0.05$) from control, but was different from OG ($P < 0.01$), OH ($P < 0.001$), OL ($P < 0.05$), and OM ($P < 0.05$; Table 3). A significant difference in composition of digestive tract sections was observed (Table 4). Richness in colon contents was not significantly different from cecal contents as a reference. However, cecal

Table 2. Main components of different oregano chemotypes

	Relative amount[1] (% of total compounds)			
Component	OH[2]	OM[2]	OL[2]	OG
Sabinene	0.6	4.3	0.4	ND
β-Myrcene	0.1	2.4	4.1	2.1
α-Terpinene	0.1	5.2	5.9	1.8
p-Cymene	ND[3]	3.0	21.1	19.0
γ-Terpinene	15.5	14.0	20.3	5.0
cis-Sabinene hydrate	ND	2.6	0.3	0.4
trans-Sabinene hydrate	0.5	14.5	0.4	0.3
Terpinen-4-ol	1.6	6.0	1.2	0.6
Carvacrol	21.7	3.7	4.3	50.8
Thymol	4.7	10.0	21.5	7.7

[1]OH = *Origanum vulgare* L. ssp. *hirtum* (low carvacrol), OL = *O. vulgare* L. (high thymol), OM = *Origanum majorana* (low carvacrol and thymol), and OG = *O. vulgare* L. ssp. *hirtum* (high carvacrol).

[2]From green house on the Sabana de Bogotá (Colombia).

[3]ND = not detected.

Table 3. Mean richness in treatment groups compared with the chlortetracycline reference

Treatment[1]	Richness	*P*-value
AB	34.4 ± 3.21	
CON	29.6 ± 8.14	0.180
OG	44.8 ± 5.45	0.009
OH	49.2 ± 9.04	0.000
OL	43.0 ± 10.46	0.029
OM	44.2 ± 7.73	0.014

[1]AB = chlortetracycline; CON = control, no additives; OG = *Origanum vulgare* L. ssp. *hirtum* (high carvacrol); OH = *O. vulgare* L. ssp. *hirtum* (low carvacrol), OL = *O. vulgare* L. (high thymol), and OM = *Origanum majorana* (low carvacrol and thymol).

Table 4. Mean richness values in digestive tract sections compared with the cecum

Section	Richness	*P*-value
Cecum	46.8 ± 8.75	
Colon	47.0 ± 9.35	0.966
Duodenum	38.2 ± 7.93	0.022
Ileum	38.2 ± 10.45	0.022
Jejunum	34.2 ± 7.70	0.001

content was determined to be different from duodenal ($P < 0.05$), ileal ($P < 0.05$), and jejunal ($P < 0.001$) content. The principal component analysis of DGGE profiles showed no significant differences ($P > 0.05$) between bacterial compositions of contents from different treatment groups (Figure 1).

Dendrograms resulting from amplicon profiles of duodenal, jejunal, ileal, cecal, and colonic bacterial community DNA revealed 2 main clusters, OEO-treated broilers and nontreated control broilers, in each intestinal segment (Figure 2). When the clusters duodenum-jejunum and ileum with respect to cecum-colon were analyzed, different similarity coefficients were observed for OEO-treated and nontreated groups. Additionally, a similarity coefficient of 68.1% was found for nontreat-

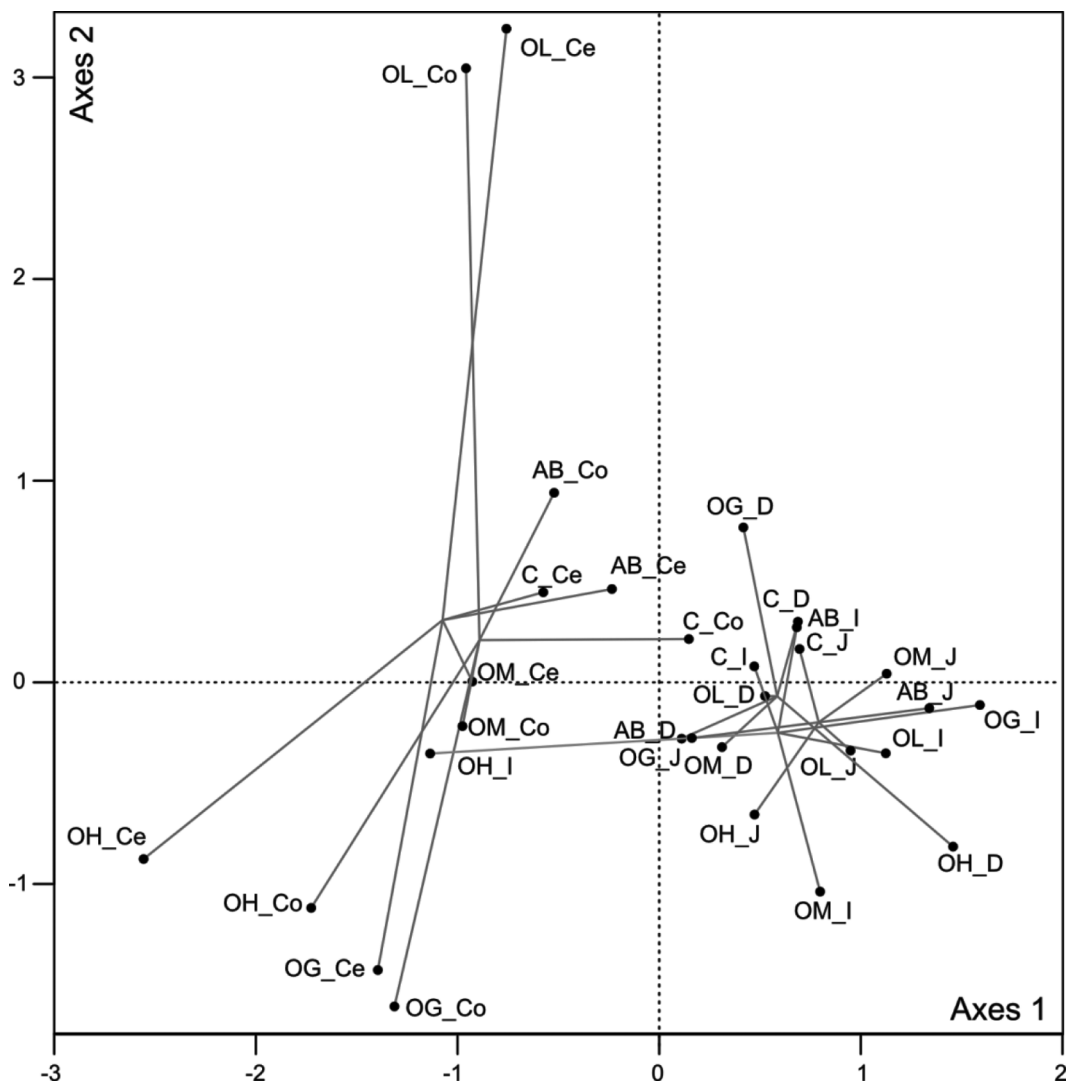

Figure 1. Principal component analysis ordination plot of denaturing gradient gel electrophoresis profiles. Solid lines connect sample digestive tract groups (centroids). Duodenum (A), jejunum (B), ileum (C), and cecum (D) of broiler chickens fed oregano essential oils. OH = *Origanum vulgare* L. ssp. *hirtum* (low carvacrol); OL = *O. vulgare* L. (high thymol); OM = *Origanum majorana* (low carvacrol and thymol); OG = *O. vulgare* L. ssp. *hirtum* (high carvacrol); AB = chlortetracycline; and C = control, no additives.

ed groups AB and control with respect to 81.5% SC for all OEO-treated groups ($P < 0.01$).

Comparison of bacterial DNA profiles from different gut compartments revealed major bacterial population shifts in the foregut (duodenum, jejunum, and ileum) compared with the hindgut (cecum and colon) at the 2 ages evaluated (Table 5). Higher mean similarity coefficients were seen within cecum-colon (80.6%) and duodenum-jejunum-ileum (78.7%) clusters compared with the lower SC observed between the 2 clusters, 57.8% SC ($P < 0.0001$). Significant interaction between treatments and clusters could be explained because groups treated with OEO showed higher similarity (91.5% SC) between cecum-colon versus the control groups, AB and control (68.5% SC; $P < 0.01$).

A high similarity coefficient (90.0%) was found collectively for OM and AB in the jejunum (21 d), ileum (3 and 21 d), cecum (3, 7, and 21 d), and colon (7 and 21 d; data not shown). Although the group fed essential oil from *O. majorana* had similar behavior for BW and gut microbial composition, compared with the group given the antibiotic, this effect was not associated with the content of carvacrol and thymol, because *O. majorana* had the lowest content of carvacrol and thymol.

Production Parameters

The OM, AB, and control groups had significantly higher BW at 21 d ($P < 0.05$): 778 ± 12.6 g, 779 ± 12.1 g, and 770 ± 9.1 g, respectively, compared with OH and OG groups, 739 ± 10.2 g and 724 ± 8.3 g, respectively (Table 6). No differences were observed between treatments for BW at 42 d and average feed conversion ratio (Table 6). However, when a correlation analysis was made, a negative correlation coefficient (−0.50) between the BW in respect to carvacrol intake at 42 d of age was found ($P < 0.05$). In contrast, a positive correlation coefficient (0.37) between BW and thymol intake was observed in broilers at 42 d of age ($P < 0.05$).

The OEO and antibiotic-supplemented groups presented the lowest ascites mortality ($P < 0.05$) with an average of 5.4% mortality in OEO and AB treatment groups in respect to 13.3% in the control group (Table 6). Mortality in the group given OL was only 2.2%, whereas that for the OG was 8.3%, which was at the high range for the OEO treatments.

DISCUSSION

OEO Composition

This study confirmed that subspecies *O. vulgare* L. ssp. *hirtum* is more carvacrol-rich than thymol-rich (Kokkini and Vokou, 1989; Başer et al., 1994; Kokkini, 1997). The subspecies *hirtum* is widely used as a spice under the name Greek oregano and is a type of oregano considered to have the highest quality in the market. The essential oil composition of this subspecies, however, was not homogenous. Oregano cultivated at the high altitude of Sabana de Bogotá showed high content of precursors and low carvacrol. In contrast, oregano cultivated in Greece showed a high content of carvacrol. It is known that the regulation of essential oil production and synthesis of metabolites is integrated into the plant physiology and depends on the metabolic state and its adaptation to the ecosystem (Sangwan et al., 2001; Toncer et al., 2009), the genotype, the ontogenic development, and the environmental and growing conditions (Piccaglia et al., 1991). Based on the composition of essential oils at the genus level, *Origanum* can be divided into 3 intraspecific chemotypes: type carvacrol, type thymol, and type terpinen-4-ol (Skoula and Harborne, 2002). This classification was confirmed with results from the current study, which identified the 3 chemotypes, OH with a high proportion of carvacrol, OL with a high proportion of thymol, and OM with a high content of compounds derived from sabinene. Reports in the literature reveal that OH is most commonly carvacrol-rich and less commonly thymol-rich (Kokkini and Vokou, 1989; Başer et al., 1994; Kokkini, 1997). As a quality parameter, it is considered that an OEO should contain at least 55% carvacrol + thymol and the carvacrol:thymol ratio should be more than 10 (Nitsas, 2000). Interestingly, carvacrol has received more attention, although both carvacrol and thymol have antioxidant and antibacterial effects in vitro.

The chemotypes evaluated in this study were cultivated at 2,600 m above sea level and showed a low content of carvacrol + thymol and high content of precursors, *p*-cymene and γ-terpinene, compared with oil from *O. vulgare* L. ssp. *hirtum* cultivated in Greece. These differences would be related to agro-ecological factors and culture conditions related to each cultivar. In the current study, the *O. majorana* essential oil showed a similar composition to a Tujan Oregano Group studied by Skoula and Harborne (2002), where *cis*- and *trans*-sabinene, and γ-terpinene exhibited a major concentration, whereas *p*-cymene, thymol, and carvacrol were less abundant. These compounds are together referred to as sabinil compounds, whereas carvacrol and thymol and their precursors are referred to as cimil compounds (Skoula and Harborne, 2002). When a plant, such as *O. majorana*, presents sabinil compounds, usually the cimil compounds are in a lower concentration (Başer et al., 1994). It has been suggested that when the cimil pathway is activated in *Origanum* spp., the sabinil metabolic route is repressed, but not completely absent (Skoula and Harborne, 2002).

Under the OEO inclusion levels used in the current study, high carvacrol OEO did not have the expected positive effects. Lee et al. (2003b) found that an inclusion of 200 mg/kg of carvacrol to the broiler diet exerted an adverse response on feed intake and BW gain. Fabian et al. (2006) also found a cytotoxic effect from high doses of commercial OEO (55% of carvacrol) on Caco-2 cells. The dilution at which the OEO and the vehicle used are important factors to consider in future studies.

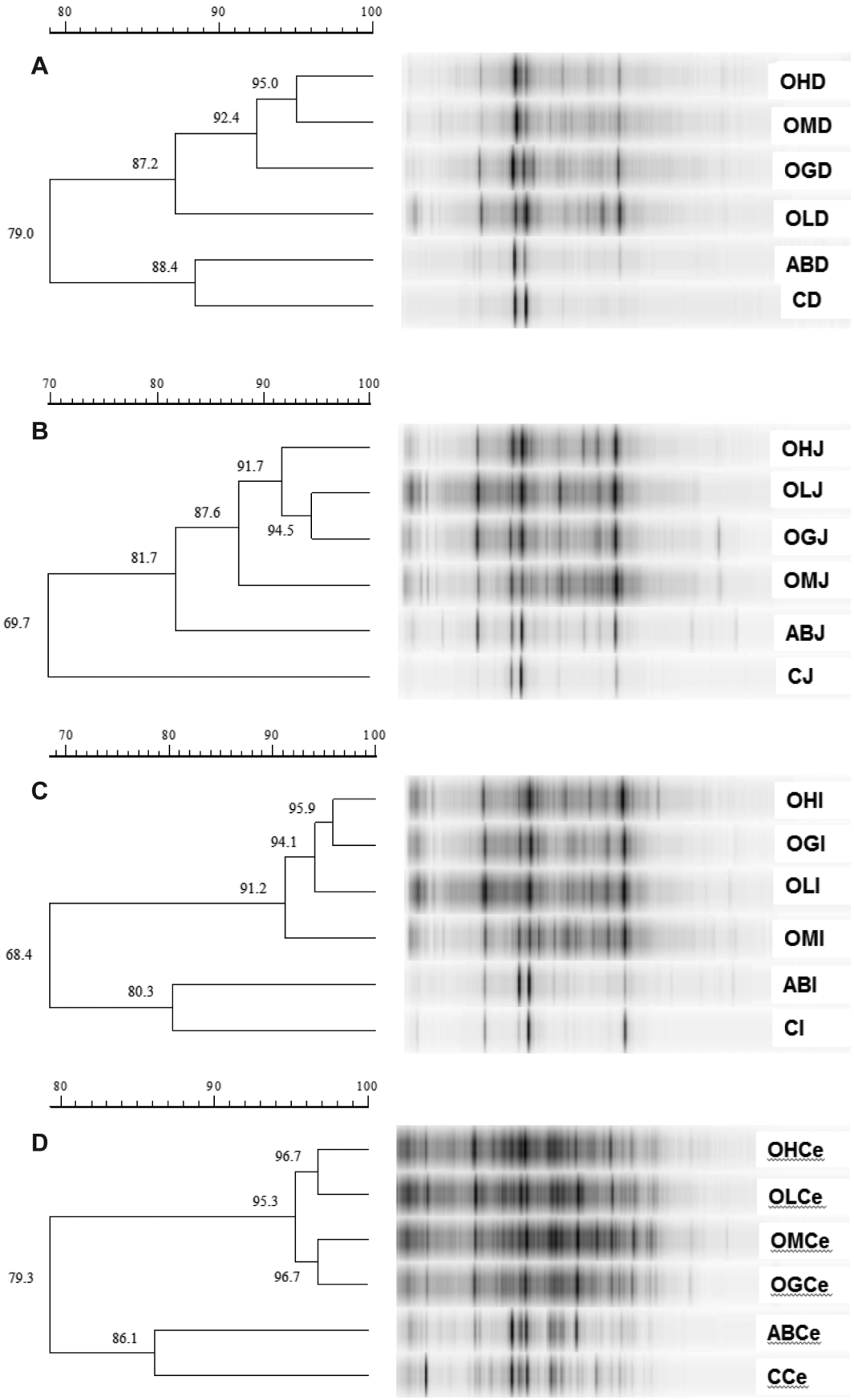

Figure 2. Dendrograms of intestinal community of the duodenum (D; panel A), jejunum (J; panel B), ileum (I; panel C), and cecum (Ce; panel D) of broiler chickens feed oregano essential oils. OH = *Origanum vulgare* L. ssp. *hirtum* (low carvacrol); OL = *O. vulgare* L. (high thymol); OM = *Origanum majorana* (low carvacrol and thymol); and OG = *O. vulgare* L. ssp. *hirtum* (high carvacrol); AB = chlortetracycline; and C = control, no additives.

Table 5. Similarity coefficients (%) from foregut and hindgut dendrogram clusters of microbial communities among oregano essential oil (OEO)-treated and nontreated broiler chickens

Treatment	Age (d)	DJI[1]	CeCo[1]	DJI versus CeCo
OEO	21	73 ± 2.3[2]	88 ± 7.3	53 ± 3.9
	42	83 ± 4.2	95 ± 8.4	65 ± 6.4
No OEO	21	84 ± 5.3	61 ± 1.0	57 ± 6.9
	42	75 ± 4.4	76 ± 1.9	58 ± 6.9

Effect	Treatments	Age	Clusters	T × C[3]	T × A[3]	T × A × C
P-value	0.009	0.045	0.000	0.002	0.257	0.327

[1]DJI = duodenum-jejunum-ileum cluster. CeCo = cecum-colon cluster.
[2]Mean ± SE, n = 9.
[3]T = treatments; C = clusters; A = age.

Intestinal Microbial Communities

The current study applied DGGE as the molecular approach to monitor the intestinal microbiota in broiler chickens fed different chemotypes of OEO. Molecular techniques such as DGGE, adapted to study microbial ecology of complex communities (Muyzer and Smalla, 1998; Sinéad et al., 2007; Hume et al., 2012) allows detection of changes in intestinal microbiota by the effect of additives in the diet (Hume et al., 2003, 2012; Pedroso et al., 2005; Oviedo-Rondón et al., 2006; Rehman et al., 2008). In the current study, DGGE allows visualization of changes in microbial communities in different compartments of the intestinal tract as effects of supplementation with chemotypes of OEO to the broiler chick diet. Several studies have found changes in intestinal microbial communities following use of essential oils as feed additives in broiler chickens. Hume et al. (2012) showed, using DGGE, a modulating effect of a commercial mixture of essential oils as feed additives following coccidia challenge and the avoidance of drastic changes in microbial communities. However, there are no known reports on the implementation of DGGE to monitor the effect of different OEO chemotypes on microbial populations of the GIT of broilers.

An important effect observed in this study was a reduction of microbial community profile differences between foregut (duodenum, jejunum, and ileum) and hindgut (cecum and colon) in broiler chickens OEO-supplemented when they are compared with control groups, AB and control. It has been suggested that differences in microbial composition between gut compartments are expected, and as the distance between sections increases, the differences between microbiota compositions become larger (Hume et al., 2003). Furthermore, each compartment of the gut develops its own microbiota community as the bird matures. Using genomic libraries, Lu et al. (2003) found that the microbial composition of the ileum and cecum did not differ when the birds were 3 d old. Additional, they found that during the first 14 d of age the cecal microbiota was a subset of the ileal microbiota. After this time, the composition of the ileum and cecum microbiota gene libraries differed significantly.

Results from a previous studies showed that these OEO chemotypes presented a strong antibacterial effect in vitro against pathogenic bacteria and lower antibacterial effect against beneficial bacteria (Betancourt et al., 2012). Inferences can be made that varied OEO antibacterial efficacy and selectivity toward intestinal tract bacterial pathogens and beneficial bacteria may be expressed in vivo and, potentially, could account for a reduction in microbiological profile differences between foregut and hindgut in broiler chickens. Si et al. (2006) showed also a high efficacy of OEO against *S. typhimurium* DT104, *E. coli* O157:H7, and *E. coli* K88 with little inhibition toward lactobacilli and bifidobacteria. However, is not clear at present the ideal bacterial composition for broiler health and production or the optimum composition that can fight diseases.

Table 6. Body weight, feed intake, feed conversion ratio, and mortality of broilers treated with oregano essential oil chemotypes

Item	OH[1]	OL	OM	OG	AB	CON	SEM
BW, g							
d 7	135[b]	142[a]	143[a]	135[ab]	138[ab]	140[ab]	2.61
d 21	739[b]	768[ab]	778[a]	724[b]	779[a]	770[a]	13.22
d 42	2,359	2,347	2,453	2,385	2,390	2,394	65.44
Feed intake, g							
1–42 d	3,595	3,830	3,764	3,747	3,772	3,790	77.18
Feed conversion ratio (feed intake:total weight gain)							
1–42 d	1.52	1.63	1.53	1.57	1.58	1.58	0.45
Mortality, %							
1–42 d	5.5[b]	2.2[b]	6.7[b]	8.3[b]	4.4[b]	13.3[a]	0.65

[1]OH = *Origanum vulgare* L. ssp. *hirtum* (low carvacrol); OL = *O. vulgare* L. (high thymol); OM = *Origanum majorana* (low carvacrol and thymol); OG = *O. vulgare* L. ssp. *hirtum* (high carvacrol); AB = chlortetracycline; and CON = control, no additives.

Reduced mortality caused by ascites (59%) in broiler chickens kept at 2,600 m above sea level was observed in the current study. The reduced mortality was seen in OEO- and antibiotic-supplemented groups when they are compared with control group without additive. A stimulation of maturation and increased gastrointestinal tract efficiency has been associated with ascites incidence reduction effects in broiler chickens given diet additives (Solis de los Santos et al., 2005). The same authors showed that the addition of a prebiotic in the diet reduced ascites mortality. Another possible explanation is based on a reduction of oxygen consumption by the GIT; energy demand in the gut is between 23 to 36% of total energy intake (Dibner and Richards, 2005).

The effects of the antibiotic and OEO are seen in the reduction of the populations of pathogenic microorganisms in the GIT, reduced metabolic activity and production of toxic compounds (e.g., ammonia, phenols, indoles, and biogenic amines such as the histamine and cadaverine) from amino acid catabolism, increased intestinal epithelial cell turnover, and increased intestinal wall thickness, (Knarreborg et al., 2002; Dibner and Richards, 2005). Further testing will be needed to determine if gut oxygen demand and predisposition to ascetic syndrome can be correlated with gut microbial profile as well as with community profile modifications seen following administration of OEO as feed additives.

Our study represents the first work to evaluate the 3 main chemotypes of *Origanum* as additives in broiler diets. Results revealed a high concentration of carvacrol and thymol precursors in OEO grown at 2,600 m above sea level and a low content of carvacrol and thymol. When these OEO were included in the diet of broilers, they increased percentage SC of the microbial community profiles between the foregut and hindgut and significantly reduced mortality from ascites at this high altitude.

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