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THE EFFECTIVENESS OF THE USE OF OREGANO AND LAUREL ESSENTIAL OILS IN CHICKEN FEEDING*

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Abstract

The objective of this experimental study was to investigate the effects of essential oils of oregano, laurel and their combination on growth performance, intestinal microbiota and intestinal morphology as replacers of antibiotic growth promoters, as well as on the antioxidant capacity and mineral content of breast and thigh meat. A total of 256 day-old broiler chickens were randomized into 4 groups with 4 replicates. Control group received a basal corn-soybean diet, whereas the other experimental groups received the basal diet plus 25 mg/kg oregano essential oil, 2.5 mg/kg laurel essential oil or their combination, respectively. Chickens had free access to water and feed. Body weight gain and feed to gain ratio was calculated for the total fattening period and mortality was daily recorded. Intestinal microbiota was enumerated by conventional techniques with selective agar media at the end of the trial at both ileum and caecum. Also, evaluation of intestinal morphology was carried out in small intestine and caecum. At the end of the trial, birds were slaughtered, their carcasses were processed and samples of breast and thigh meat were analyzed for moisture, fat and protein content. Total phenolic content was determined in feeds and breast and thigh meat in order to assess its antioxidant capacity. Mineral content of breast and thigh meat was evaluated by ICP-MS. The results of the trial showed that the group that received oregano or the mixture of oregano and laurel presented better BW and FCR and mortality compared to control group. Bacterial counts for the *Lactobacilli* and *Bifidobacteria* were higher in the experimental groups compared to the control group at both ileum and caecum, and total coliforms were lower in caecum in the experimental groups compared to control. Higher values for villus height were

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found for the oregano supplemented groups compared to control or laurel ones. Oregano supplemented groups showed higher antioxidant capacity of breast and thigh meat compared to control and laurel groups, however no changes in mineral content were noted among the different groups. In conclusion, oregano essential oil alone or as a mixture with laurel essential oil can be used to improve growth performance and gut health in broiler chickens.

Key words: chicken, oregano, laurel, intestinal bacteria, intestinal morphology, trace elements

The widespread use of antibiotics in human and veterinary medicine is considered to have major contribution to the development of antibiotic resistance (Hamer and Gill, 2002; Wegener, 2003). In order to limit the spread and development of antibiotic resistance the use of antimicrobial growth promoters has been banned in the European Union since 2006. Because of this, there is considerable interest in alternative feeding strategy to replace antibiotic as growth promoters in poultry production and several non-antibiotic feed additives such as probiotics (Nousiainen et al., 2004), organic acids, prebiotics, synbiotics, enzymes, organic minerals (Fulton et al., 2002) and plant essential oils (Cross et al., 2007) have been tested. The essential oils of aromatic plants can have different modes of action, including enhanced feed consumption and flavour, stimulation of the digestive enzymes' secretion, increased gastric and intestinal mobility, antimicrobial, antiviral, antiparasitic, antifungal, immunomodulating, antioxidant and anti-inflammatory activities (Giannenas et al., 2013).

The primary mode of action of plant essential oils as feed additives arises from beneficially affecting the ecosystem of gastrointestinal microbiota through controlling potential pathogens. The main functions of the essential oils cover pathogen control including antimicrobial activity (Azaz et al., 2002; Cowan, 1999; Dorman and Deans, 2000), antioxidant activity (Botsoglou et al., 2002, 2004), digestion aid including stimulation of endogenous enzyme activity (Platel and Srinivasan, 1996; Wenk, 2003); and nitrogen absorption (Gill, 2001) and inhibition of odour and ammonia control (Varel, 2002). Their antimicrobial mode of action consists of interactions with cell membranes that change the permeability for cations such as H+ and K+ (Di Pasqua et al., 2007; Kamel, 2000; Kamel et al., 2001; Ouwehand et al., 2006; Ultee et al., 1999). Moreover, essential oils have anticoccidial, antifungal or antioxidant properties (Cruickshank, 2001; Wenk, 2003); and stimulate the immune response (Vidanarachchi et al., 2005; Wang et al., 2011; Windisch et al., 2008).

Numerous studies have documented the benefits of essential oils on the performance of poultry (Brenes and Roura, 2010; Franz et al., 2010; Giannenas et al., 2014 a, b). Bassett (2000) and Alçiçek et al. (2003) reported that the supplementation of the oregano essential oil to the broiler diet or drinking water increased body weight and feed conversion ratio. Windisch et al. (2008) reviewed 11 papers with poultry and reported that the average improvement in weight gain, feed intake and feed conversion induced by essential oils were 0.5, −1.6 and −2.6%, respectively. Aromatic herbs and essential oils are often claimed to improve the flavor and palatability of feed, thus increasing voluntary feed intake resulting in improved weight gain. However, in a choice feed experiment conducted in growing chickens by Symeon et al. (2010),

the classification of oregano essential oil as flavor additive or as 'appetite promoter' in chicken diets was questioned because it decreased feed intake.

Despite the fact of the early start of the use of aromatic plants and their extracts in animal nutrition, several decades ago, since 1989 (Vogt et al., 1989), knowledge regarding their modes of action and aspects of application are still rather rudimentary (Franz et al., 2010; Giannenas et al., 2013; Windisch et al., 2008; Zeng et al., 2015). However, aromatic plants and their extracts are known to possess strong antimicrobial properties due to their phenolic and aromatic substances. This is the explanation of the traditional use of the antimicrobial protection offered by aromatic plants, their extracts, herbs and spices by mankind for thousands of years ago in Mesopotamia, Egypt, India, China and old Greece (Greathead, 2003). In this study, we used essential oils of Greek endemic plants such as *Origanum vulgare hirtum* and *Laurus nobilis* as a source of functional ingredients after dietary supplementation on growth performance and gut health. The second objective was to investigate the effects of oregano and laurel oils on antioxidant capacity and trace mineral content of breast and thigh meat, since the effect of either oil on mineral content of breast and thigh meat has not been published in the literature.

Material and methods

Experimental design

The trial protocol was approved by the Institutional Committee for Animal Use and Ethics of The Technological Institute of Epirus, Department of Animal Production (Nr: 11SYN_3_47-01/03/2013). Throughout the trials, the birds were handled in compliance with local laws and regulations and in accordance to the principles and guidelines for poultry welfare (National Research Council, 1996). All groups were housed in rice hulls litter. The stocking density was found to be 16 birds per m^2. During the trial, commercial breeding and management procedures were employed, natural and artificial light was provided on a basis of 23 h for the first 2 days, 16 hours from day 3 to day 14, 21 h from day 15 to the slaughter days, and ambient temperature was controlled. All birds were vaccinated against Marek disease after hatching; and against Newcastle disease, Infectious Bronchitis and Gumboro during the second week of their life. Feed and drinking water were offered to all birds *ad libitum* throughout the experiment. All birds were weighed at the time of their placing into the poultry house and at slaughter age. Feed consumption within each group was recorded during the experimental period and feed conversion ratio was finally calculated. Mortality was also daily recorded. The trial was conducted in Arta (39°09'38"N; 20°59'07"E), Epirus, Greece. Two hundred fifty six broiler chickens (Ross 308) were divided into 4 groups with 4 replicates of 16 chicks (eight males and eight females) and reared for 42 days in a commercial farm (Agricultural Poultry Farmer Cooperative, Arta). Control diet contained no anticoccidal or antimicrobial growth promoters – CON group, whereas the diet of the second group was further supplemented with oregano essential oil 5% (Ecodiar® powder at 500 g/tn) – OREG group, the third group was further supplemented with laurel essential oil 0.5% (Pan-

aroma® powder at 250 g/tn) – LAUR group, and the fourth group received a blend of oregano 5% (Ecodiar® powder) at 500 g/tn and laurel essential oils (Panaroma® powder) at 250 g/tn – ORLA group. The composition of the basal diet for the trial is presented in Table 1. Each group received the supplemented diet throughout the trial. The main constituents of oregano and laurel essential oils are presented in Table 2 and 3, respectively. Analysis of essential oils was made by gas chromatography and provided by the supplier company. The products with essential oils were in form of powder, whereas final feeds were in mash form to assure fine mixing.

Table 1. Composition of diets in the trial

Ingredients	Control			
	Composition (g kg^{-1})			
	1–12 day	13–24 day	25–36 day	37–42 day
1	2	3	4	5
Maize	600	618	625	632
Soybean meal (47.0% CP)	335	310	297	290
Soybean oil	25	25	25	25
Coconut fat	-	15	25	25
Limestone	14	11	10	10
Dicalcium phosphate	11	9	7	7
L-Lysine, hydrochloride	3.5	3.0	2.5	2.5
DL-Methionine	2.5	1.5	1.5	1.5
Sodium bicarbonate	2.5	1	1	1
Salt	2.5	2.5	2.0	2.0
Vitamin, mineral and enzyme premix[1]	4	4	4	4
Calculated analysis				
Crude protein (%)	22.0	21.0	20.0	20.0
Ether extract (%)	6.2	6.6	6.8	6.8
Crude fiber (%)	3.5	3.6	3.6	3.6
Ash (%)	4.7	4.6	4.6	4.6
Lysine (%)*	1.3	1.2	1.1	1.1
Phosphorus (%)	0.71	0.69	0.66	0.64
Methionine+Cystine (%)*	1.0	0.96	0.94	0.94
Metabolisable energy (kcal /kg)*	3100	3180	3220	3220
Trace element analysis by ICP-MS				
Calcium (%)	91.2	90.4	90.2	89.8
Mg (%)	0.41	0.38	0.37	0.37
K (%)	0.99	0.96	0.97	0.96
Na (%)	0.33	0.32	0.32	0.32
Mn (mg/kg)	69.5	69.3	69.2	69.1
Cu (mg/kg)	21.8	21.6	21.8	21.5
Fe (mg/kg)	65.9	64.5	64.6	64.2
Zn (mg/kg)	84.5	83.5	84.2	82.5
Co (mg/kg)	0.06	0.06	0.06	0.06
Mo (mg/kg)	0.31	0.30	0.30	0.29
Ba (mg/kg)	0.17	0.16	0.16	0.16

Table 1 – contd.

1	2	3	4	5
B (mg/kg)	4.56	4.52	4.58	4.54
Se (mg/k)g	0.03	0.03	0.03	0.03
Pb (mg/kg)	ND[2]	ND	ND	ND
As (mg/kg)	LQD[3]	LQD	LQD	LQD
Cd (mg/kg)	ND	ND	ND	ND

[1]Supplying per kg feed: 12,000 IU vitamin A, 5,000 IU vitamin D_3, 30 mg vitamin E, 3 mg vitamin K, 5 mg thiamin, 6 mg riboflavin, 6 mg pyridoxine, 0.02 mg vitamin B_{12}, 60 mg niacin, 15 mg pantothenic acid, 1.5 mg folic acid, 0.25 biotin, 10 mg vitamin C, 500 mg choline chloride, 100 mg Zn, 120 mg Mn, 20 mg Fe, 15 mg Cu, 0.2 mg Co, 1 mg I, 0.3 mg Se, and phytase in recommended quantities per kg of diet.

*Calculated values

[2]ND: Values were below the analytical capacity of detection limit.

[3]LQD: Values were detected higher than lowest detection limit but lower than quantification limit.

Table 2. Main compounds of the oregano essential oil

Compound	%
Carvacrol	77.95
p-cymene	5.43
γ-terpinene	4.65
Thymol	3.02
β-caryophyllene	1.65
β-myrcene	1.22
β-bisabolene	1.13
α-terpinene	1.00
α-thujene	0.84
α-pinene	0.58
terpinen-4-ol	0.46
β-phellandrene	0.38
carvacrol methyl ether	0.31
1-octen-3-ol	0.30
borneol	0.28
cis-sabinene hydrate	0.20
α-humulene	0.15
camphene	0.14
α-phellandrene	0.13
β-pinene	0.10

Table 3. Main compounds of the laurel essential oil

Compounds	%
1.8-cineol	47.04
α-terpinyl acetate	9.93
sabinene	7.08
linalool	5.75
α-pinene	4.63
β-pinene	3.91
α-terpineol	3.10
terpinen-4-ol	2.85
methyl eugenol	2.74
p-eugenol	1.71
γ-terpinene	1.07

Sampling

At the end of the trial, 4 chickens from each subgroup (16 samples per group) were sacrificed by cervical dislocation to sample either for breast and thigh meat or intestinal parts.

Meat chemical analysis

From each group of animals, parts of breast (*Pectoralis major*) and thigh (*Biceps femoris*) meat without skin were analyzed for moisture, crude protein and fat content, by NIR spectroscopy using a FoodScan™ Lab (FOSS, Denmark). The total content of phenolic compounds of meat (breast and thigh) was determined using the Folin--Ciocalteau procedure as modified by Chatzilazarou et al. (2010) using a Shimadzu UV-1700 Spectrophotometer (Shimadzu Co., Japan). Briefly, 4 ml of methanolic extract was transferred into a 25 mL calibration flask, 0.5 ml of Folin-Ciocalteu reagent was added. After 3 min 1 mL of saturated sodium carbonate solution was added and made up to the mark with distilled water. After 1 h, solutions were centrifuged at 4500rpm for 10 min and absorbance at 750 nm was measured against a reagent blank. Calibration curves were prepared using working solutions of gallic acid (2–10 ppm).

Intestinal measurements

The contents of the crop, gizzard, ileum, caeca and rectum were quantitatively collected. The ileum was defined as the small intestinal segment caudal to the Meckel's diverticulum. The pH in the contents of all gastrointestinal segments was measured with a combined glass/reference electrode portable pH meter BT-600 (BOECO, Germany). To determine bacteria populations, fresh weighed digesta samples from ileum and caecum were mixed homogeneously at a ratio of 1 g sample with 9 ml of peptone water (0.1% v/v) in the universal bottle for bacterial enumeration such as total aerobes, total anaerobes, *Lactobacilli spp, Bifidobacteria spp.* and total Coliforms by conventional microbiological techniques using selective agar media. Subsequently, serial decimal dilutions were made, avoiding aeration, using the medium as previously described (Giannenas et al., 2011). Morphometrical analysis of the small intestine was evaluated according to Giannenas et al. (2011). During necropsy of the selected birds, the gastrointestinal tract was removed and the small intestine was divided into three parts: duodenum (from the gizzard outlet to the end of the pancreatic loop), jejunum (from the pancreatic loop to Meckel's diverticulum) and ileum (from Meckel's diverticulum to the ileo-caeco-colic junction). One cm long segments were taken from the center of each part and fixed in 10% buffered formalin for morphometrical studies under light microscopy, with a Nikon microscope coupled with NIS Elements imaging software analysis system (Nikon Eclipse 200, Tokyo, Japan). Images were viewed (4×) to measure morphometric parameters of intestinal architecture. For this purpose, villous height (VH) was estimated by measuring the vertical distance from the villous tip to villous-crypt junction level for 10 villi per section and crypt depth (CD) by the vertical distance from the villous-crypt junction to the lower limit of the crypt. Caecum morphology was also evalueated.

Feed buffering capacity analysis

In order to explain our results further *in vitro* test was performed to determine the buffering capacity of the experimental diets and their ingredients using a WTW pH meter (Weilheim, Germany). A portion of 10 g feed was placed in a beaker and 100 ml of distilled water were added. The solution was kept for about 30 min, and then titrated with 0.1 N HCl, under continuous stirring, to reach pH 4 (Giannenas et al., 2014 a). The microliters of the acid consumed were used as the units for expressing the buffering capacity of the feeds.

Major and trace element analysis

Inductively coupled plasma mass spectrometry (ICP-MS) was used to determine certain trace or major elements were determined in control finisher feed and meat samples, according to Nisianakis et al. (2009) using an Agilent 7500s instrument (Agilent Technologies, Waldbronn, Germany). Samples were treated in triplicates and each sample was measured three times.

START D Microwave digestion system (Milestone Srl Sorisole (BG), Italy) was used for sample preparation. Processed meat samples were homogenized by Ultra-Turrax type Yellowline by IKA DI 18 Basic Homogenizer (IKA Werke GmbH & Co., Staufen, Germany) working at 5,000 rpm.

Table 4. Instrumental parameters for the ICP-MS

	Parameters
Frequency (MHz)	27.12
Reflect Power (Weber et al., 2012)	1.55
Reflect Matching (Van Overmeire et al., 2006)	1.62
Sampling Depth (Van Overmeire et al., 2006)	6.8
Torch-H (Van Overmeire et al., 2006)	0.1
Torch-V (Van Overmeire et al., 2006)	0.3
Carrier gas (L/min)	1.20
Nebuliser pump (rps)	0.10
S/C Temperature (°C)	2
Oxide ions (156/140)	0.67 %
Doubly charged (70/140)	1.6
Nebuliser type	concentric

The major elements calcium (Ca) magnesium (Mg), potassium (K), sodium (Na) and trace elements iron (Fe), selenium (Se), zinc (Zn), manganese (Mn), copper (Cu), cobalt (Co), molybdenum (Mo), boron (B), barium (Ba), arsenic (As), lead (Pb) and cadmium (Cd) were determined in feeds and trace elements iron (Fe), selenium (Se), zinc (Zn), manganese (Mn), molybdenum (Mo), boron (B), barium (Ba), arsenic (As) and cadmium (Cd) were determined in meat samples. Feed samples were collected prior to feeding milled through a 1 mm sieve. Meat samples were homogenized with Ultra-Turrax DI-18 disperser (IKA-Werke, Staufen, Germany) at 5,000 rpm for three minutes.

Table 5. Performance characteristics for the ICP-MS

Metal	Recovery % spiking level 10 ng/g	Detection limits in sample (ng/g)	Quantification limits in sample (ng/g)	Analytical mass of examined element	Internal standard
Fe	105	1.280	3,840	56	^{45}Sc
Na	98	1.262	3.784	23	^{45}Sc
K	98	0.286	0.859	39	^{45}Sc
Mg	96	1.980	5.942	24	^{45}Sc
Ca	114	2.828	8.484	44	^{45}Sc
Se	110	0.012	0.036	82	^{72}Ge
Zn	91	0.005	0.016	66	^{45}Sc
Mn	99	0.001	0.003	55	^{45}Sc
Cu	94	0.001	0.003	63	^{45}Sc
Co	104	0.001	0.003	59	^{45}Sc
Mo	105	0.004	0.013	95	^{89}Y
B	110	0.018	0.053	11	^{6}Li
Ba	104	0.001	0.003	137	^{159}Tb
As	95	0.003	0.008	75	^{72}Ge
Cd	98	0.001	0.003	111	^{115}In
Pb	102	0.001	0.003	208	^{209}Bi

Elements were determined using an ICP-MS, Agilent 7500s-Agilent Technologies (Waldbronn, Germany). The instrumental settings and operative conditions are reported in Table 4. Recovery and detection limits of the analytical methodology used in the current study are presented in Table 5.

For digestion aliquots of 1 g homogenized sample were accurately weighed using a Teflon vessel. After the addition of 8 mL of concentrated HNO_3 (65%) and 2 mL H_2O_2 30% w/w, the digestion vessel was closed and heated in the microwave digestion system. The temperature was increased gradually up to 200°C in 10 min and remained constant for another 10 min. The obtained solutions were allowed to cool at room temperature, and were quantitatively transferred into a glass volumetric flask of 50 mL (class A) and completed to volume with ultrapure deionized water. Analysis was performed by ICP-MS, following external calibration. Filtration was not necessary since the resultant digesta was clear enough.

Standard solutions were obtained from High Purity Standards (Merck, Germany), single element solutions (Ca, Mg, Fe, Se, Zn, Mn, Co, Cu, Mo, Cr, Ni, As and Cd) and used to get calibration curves. Several standard reference materials were used to validate the analytical procedure; these included the standard element solutions. All chemicals used were of analytical grade. Nitric acid (Hiperpur) was purchased from Merck and internal standards (Sc, In, Ge, Bi) from Agilent.

Statistical analysis

Statistical analysis was performed by one-way analysis of variance using SPSS for Windows (Version 20, Chicago, USA). The homogeneity of the variances was tested and bacteria numbers were log transformed and then analyzed in order to have better homogeneity of variance. When significant treatment effects were disclosed at probability level of $p<0.05$, the Duncan's test was applied in order to determine statistical differences between means.

Results

Dietary supplementation with oregano essential oil and oregano and laurel oils significantly affected ($P<0.05$) the average body weight, FCR and mortality rate compared to control group (Table 6). The effects of the three experimental diets on the chicken breast and thigh meat chemical composition are presented in Table 7. No significant ($P>0.05$) differences were found for moisture, crude protein and fat. The effect of the different diets on the phenolic content of the refrigerated breast and thigh meat are presented in Table 8, respectively. Significant ($P>0.05$) differences were found in both breast and thigh meat at all time points of refrigerated storage; where the control group had lower ($P<0.05$) total phenolic content values compared to the other groups, but this effect was more pronounced at day 1 for the oregano supplemented group.

Table 6. Performance of broiler chickens, pH values in the digestive tract and feed and buffering capacity and phenolic content of the feeds[1]

Item	Dietary treatment[2]					
Days	CON	OREG	LAUR	ORLA	SEM[3]	P-value
Body weight (g), 42 d	2254 b	2544 a	2513 a	2543 a	52.5	0.042
FCR (kg/kg), 42 d	1.78 a	1.65 b	1.66 b	1.66 b	0.04	0.004
Mortality (%)	4.7 a	1.5 b	3.1 ab	1.5 b		0.005
Digesta pH						
Gizzard	3.23	3.21	3.24	3.24	0.04	NS
Ileum	6.82	6.76	6.76	6.76	0.14	NS
Caeca	7.11	7.02	7.03	7.02	0.26	NS
pH values of the feeds						NS
0–14	6.35	6.34	6.22	6.21	0.15	NS
15–28	6.23	6.22	6.16	6.16	0.08	NS
29–35	6.21	6.19	6.18	6.18	0.19	NS
36–44	6.18	6.13	6.11	6.11	0.12	NS
Buffering capacity of the feeds (mL[4])						NS
0–14	44.2	45.4	43.4	43.4	4.61	NS
15–28	42.5	44.1	41.1	41.1	3.23	NS
29–35	41.2	42.2	40.2	40.2	2.23	NS
36–42	40.5	41.1	40.1	40.3	2.61	NS
Total phenolic content[5]	18.5 a	44.5 b	36.5 b	45.5 b	2.51	0.005

a, b – Mean values in a row with a different letter differ significantly at $P\leq0.05$.

[1] Results are given as means of groups (n=4=subgroups).

[2] Group of birds fed the basal diet or the basal diet supplemented with oregano, laurel essential oil or their combination.

[3] S.E.M.: standard error of the mean.

[4] mL 0.1 N HCl required to acidify 10 g feed dispersed in 100 ml distilled water to pH 4.

[5] Total Phenolic content measured as mg/L gallic acid equivalents of feed during the finishing period 36–42 days.

The composition of the microflora was determined at the day of slaughter. In ileum and caecum significantly higher values of lactic acid and bifidobacteria were noted for the oregano supplemented groups, where, in caecum, reduced counts of coliform bacteria ($P<0.05$) were also noted compared to control group (Table 9).

Table 7. Effect of dietary supplementation of oregano essential oil, laurel essential oil and combination on the chemical composition of chicken breast and thigh meat[1]

Groups[2]	Moisture		Crude protein		Fat	
	breast %	thigh %	breast %	thigh %	breast %	thigh %
CON	71.9	70.5	22.2	20.0	5.2	8.1
OREG	71.7	70.3	22.3	19.8	5.1	7.9
LAUR	71.5	70.1	22.2	20.1	5.1	7.7
ORLA	71.5	70.7	22.5	20.2	5.1	8.3
SEM3	0.2	0.1	0.2	0.2	0.1	0.2
P-value	NS	NS	NS	NS	NS	NS

[1] Results are given as means of groups (n=4=subgroups).
NS: Not significant (P>0.05).
[2] Group of birds fed the basal diet or the basal diet supplemented with oregano, laurel essential oil or their combination.
[3] S.E.M.: standard error of the mean.

Table 8. Total Phenolic Content (mg/L gallic acid) of raw breast and thigh meat during refrigerated storage of chickens fed oregano essential oil, laurel essential oil, and their combination[1]

	Day 1	Day 3	Day 6
		Breast	
Groups[2]			
CON	3.18 a	3.11 a	2.11 a
OREG	5.44 b	5.03 b	4.64 b
LAUR	4.95 b	4.75 b	4.44 b
ORLA	5.41 b	4.77 b	4.52 b
SEM[3]	0.2	0.1	0.2
P-value	0.005	0.005	0.005
		Thigh	
Groups			
CON	4.12 a	3.22 a	2.32 a
OREG	6.38 b	6.22 b	5.55 b
LAUR	6.19 b	6.01 b	5.51 b
ORLA	6.22 b	5.99 b	5.54 b
SEM	0.2	0.1	0.2
P-value	0.005	0.005	0.005

[1] Results are given as means of groups (n=4=subgroups).
a, b – values in the same column with different superscript differ significantly P<0.05.
[2] Group of birds fed the basal diet or the basal diet supplemented with oregano, laurel essential oil or their combination.
[3] S.E.M.: standard error of the mean.

The diet supplementation with oregano gave also higher values (P<0.05) of intestinal villous height (Table 10) in duodenum, jejunum and ileum. No morphological lesions or any inflammatory response were found in the examined intestinal parts of both caecum and small intestine.

Table 9. Intestinal microbiota on ileum and caecum of 42-day-old broiler chickens fed oregano essential oil, laurel essential oil, and their combination[1]

	CON[2]	OREG[2]	LAUR[2]	ORLA[2]	SEM[3]	P-value
Ileum (log cfu/ g)						
total aerobes	6.16	6.60	6.45	6.62	0.125	0.215
total anaerobes	6.06	6.14	6.22	6.15	0.075	0.922
total coliforms	3.24	3.12	3.16	3.13	0.065	0.175
Lactobacilli spp	5.456	6.22 a	6.18 a	6.21 a	0.102	0.005
Bifidobacteria spp	5.86 b	6.15 a	6.24 a	6.267 a	0.102	0.005
Caecum (log cfu/g)						
total aerobes	8.25	8.44	8.41	8.22	0.123	0.555
total anaerobes	7.14	6.78	6.89	7.11	0.249	0.127
total coliforms	6.67 a	5.32 b	5.44 b	5.11 b	0.107	0.006
Lactobacilli spp	6.55 b	7.78 a	7.58 a	7.68 a	0.086	0.005
Bifidobacteria spp	6.48 b	7.55 a	7.67 a	7.74 a	0.101	0.001

[1] Results are given as means of groups (n=4=subgroups).
a, b – values in the same row with different superscript differ significantly P<0.05.
[2] Group of birds fed the basal diet or the basal diet supplemented with oregano, laurel essential oil or their combination.
[3] S.E.M.: standard error of the mean.

Table 10. Intestinal morphology of 42-day-old broiler chickens fed oregano essential oil, laurel essential oil, and their combination[1]

	CON[2]	OREG[2]	LAUR[2]	ORLA[2]	SEM[3]	P-value
Duodenum						
villous height (μm)	1804.4 b	1899.4 a	1812.1 ba	1889.2 a	41.5	0.015
crypt depth (μm)	179.4	185.0	181.1	182.4	9.56	0.236
villous height to crypt depth ratio	10.06	10.27	10.01	10.36	0.66	0.264
Jejunum						
villous height (μm)	1532.6 b	1665.3 a	1569.3 ba	1641.6 a	33.3	0.024
crypt depth (μm)	159.9	171.8	162.8	167.8	15.2	0.233
villous height to crypt depth ratio	9.58	9.69	9.64	9.78	0.29	0.221
Ileum						
villous height (μm)	932.3 b	1034.5 a	1004.5 ba	1029.5 a	23.6	0.031
crypt depth (μm)	120.5	129,6	126.3	129.3	10.2	0.276
villous height to crypt depth ratio	7.74	7.98	7.95	7.96	0.66	0.217

[1] Results are given as means of groups (n=4=subgroups).
a, b – values in the same row with different superscript differ significantly P<0.05.
[2] Group of birds fed the basal diet or the basal diet supplemented with oregano, laurel essential oil or their combination.
[3] S.E.M.: standard error of the mean.

The effects of the experimental diets on trace element concentrations in the chicken breast and thigh meat are given in Table 11. No significant (P>0.05) differences were found for elements Cu, Zn, Se, Mn and B. Elements As and Cd were detected at levels higher than the lowest detection limit, but lower than the quantification limit. The values for element Ba were below the analytical capacity of the detection limit.

Table 11. Effect of dietary supplementation of oregano essential oil, laurel essential oil and combination on the trace element concentrations (mg or μg/kg) of chicken breast and thigh meat[1]

Groups[2]	Breast							
	Cu μg/kg	Zn mg/kg	Se μg/kg	Mn μg/kg	As μg/kg	Cd μg/kg	B μg/kg	Ba μg/kg
CON	52.5	3.3	145.6	133.5	LOQ5	LOQ	2.3	ND[6]
OREG	52.8	3.2	147.3	132.5	LOQ	LOQ	2.1	ND
LAUR	51.9	3.1	142.2	135.6	LOQ	LOQ	2.2	ND
ORLA	53.1	3.3	143.4	135.1	LOQ	LOQ	2.1	ND
SEM[3]	0.3	0.1	0.3	0.5	LOQ	LOQ	0.1	ND
P-value	NS	NS	NS	NS	-	-	NS	-
	Thigh							
Groups	Cu μg/kg	Zn mg/kg	Se μg/kg	Mn μg/kg	As μg/kg	Cd μg/kg	B μg/kg	Ba μg/kg
CON	64.4	4.1	125.5	97.1	LOQ	LOQ	3.1	ND
OREG	66.1	4.1±0.7	126.2	96.7	LOQ	LOQ	3.0	ND
LAUR	61.5	4.3±0.4	124.7	89.6	LOQ	LOQ	3.1	ND
ORLA	61.9	4.3±0.4	127.5	89.4	LOQ	LOQ	3.3	ND
SEM[2]	0.6	0.2	0.8	0.4	-	-	0.1	-
P-values	NS	NS	NS	NS	-	-	NS	-

[1] Results are given as means of groups (n=4=subgroups).
NS: Not significant (P>0.05).
[2] Group of birds fed the basal diet or the basal diet supplemented with oregano, laurel essential oil or their combination.
[3] S.E.M.: standard error of the mean.
[5] LQD: Values were detected higher than lowest detection limit but lower than quantification limit.
[6] ND: Values were below than analytical capacity of detection limit.

Discussion

The relatively high in-feed inclusion of pure oregano essential oil was effective compared with literature inclusion levels (Windisch et al., 2008). The potent antimicrobial effect of both oregano and laurel oils may be the basic explanation of this effect (Baratta et al., 1998). Similarly, it has been shown that oregano essential oil improved performance of chickens at various levels of supplementation (Giannenas et al., 2003, 2005, 2013).

Plant essential oils are well known to exert antibacterial, antifungal and antiviral activity in *in vitro* experiments (Windisch et al., 2008). It is generally accepted that essential oils are slightly more active against gram-positive than gram negative bacteria (Brenes and Roura, 2010). The essential oils were shown to affect cell integrity, as measured using propidium iodide, of gram-positive bacteria; however, growth inhibition of gram-negative bacteria, in contrast, occurred mostly without cell integrity loss (Thapa et al., 2012). Comparable *in vivo* studies also found inhibiting effects against pathogens such as *C. perfringens*, *E. coli* or *Eimeria* species (Bozkurt et al.,

2013). The controlled pathogen load also contributed to healthy microbial metabolites, improved intestinal integrity and protection against enteric disease (Bozkurt et al., 2013). Attention should also be paid to the potential negative effects induced by essential oils on healthy intestinal bacteria. Horošová et al. (2006) reported that oregano essential oil exhibited a strong bactericidal effect against Lactobacilli isolated from fecal samples of chickens fed diets with oregano. In contrast, Giannenas et al. (2014 a, b) found a promoting effect on intestinal Lactobacilli by a blend that contained aromatic compounds mainly with carvacrol and a mixture of this product with organic acids. In an *in vivo* anti-bacterial study, Thapa et al. (2012) concluded that beneficial commensal *Faecalibacterium rausnitzii* were sensitive to essential oil at similar or even lower concentrations than the pathogens. In addition, Cross et al. (2007) and Muhl and Liebert (2007) reported that essential oils had no effect on the microbial population and composition in the digestive tract or fecal excretions of broilers. Our results concerning the effects of essential oils of Greek endemic aromatic plants on intestinal microbiota are in agreement with findings of Jamroz et al. (2006) who investigated the influence of diet type (corn vs. wheat and barley) on the ability of plant extracts (100 mg/kg containing 5% carvacrol, 3% cinnamaldehyde and 2% of capsicum oleoresin).

Our results are also in agreement with findings of Jamroz et al. (2006) about morphological and histochemical modification of the stomach and jejunal walls in chickens, after dietary essential oils. These authors showed significantly higher jejunal wall villi in chickens fed the maize diet supplemented with plant extracts. The incorporation of carvacrol, cinnamaldehyde, and capsicum oleoresin promotes positive and negative changes in digestive function, intestinal epithelium, microbial ecology, and fermentation in weaned pigs depending on the amount of protein included in the diet (Manzanilla et al., 2009). The intestinal villous can be regarded as the capacity of the bird to absorb nutrients from the feed. Longer villi are typically associated with excellent gut health and high absorptive efficiency. Cook and Bird (1973) reported a shorter villus and a deeper crypt when the counts of pathogenic bacteria increased in the gastrointestinal tract (Schneeman, 1982). Changes in intestinal morphology, such as shorter villi and deeper crypts have been associated with the presence of toxins or higher tissue turnover (Miles et al., 2006).

Another explanation for our results on the effects of essential oils applications especially in terms of growth performance might be also due to differences in the buffering capacity value of the used diets. The buffering capacity value indicating the amount of acid needed to lower the pH of a feed to a certain value is important because it affects the course of digestion. High buffering capacity values in feeds pose higher risks for young animals, which have limited capacity to secrete gastric acid. When using feeds with high buffering capacity, the gastric pH will remain high, impairing protein digestibility. Undigested protein will reach the lower digestive tract where excessive protein fermentation may occur, leading to formation of toxic biogenic amines. In addition, poultry feeds with high buffering capacity may result in proliferation of non-beneficial bacteria in the digestive tract. The buffering capacity of poultry diets can be readily manipulated by varying the type and proportion of the inorganic mineral sources (Partanen et al.,

2002). Such an approach might deserve some further investigation as it could be a low-cost alternative to reduce the buffering capacity of feeds (Giannenas et al., 2014 a). However, the pH values in the digestive tract were similar among the experimental groups for the several intestinal parts.

The use of herbs or extracts and essential oils obtained from herbs can be important under situations of debilitating stress, including the presence of unfavorable environmental conditions, compromised health and/or low nutrient content of the diet (Jang et al., 2006). Further scientific research is therefore needed to use essential oils effectively in poultry feeding. In addition, investigating potential synergistic or antagonistic effects of essential oils with other feed additives, including enzymes, organic acids and probiotics, represents a fertile area, particularly in antibiotic growth promoter-free feeds. The efficacy of essential oils mixture also depends upon the compatibility with the other ingredient(s) of the mixture in the feed.

The information on the composition of chicken meat in micro minerals in the literature is not abundant. Despite the increased interest, the difficulty in simultaneous determination of several elements was a main obstacle. The inductively coupled plasma mass spectrometry (ICP-MS) overcomes many of these issues. It is well established as a method for multi elemental analysis and the determination of isotope ratios. It enables simultaneous analysis of a wide range of trace elements in the same sample, a task that could not be achieved by previous methodologies. As a consequence, past measurements of individual trace elements required significant time and cost effort. Moreover, the technique has lower detection limits than those of other atomic absorption spectroscopy techniques. For example, ICP-MS can detect trace minerals at the sub-ng/mg level, (lower than 0.001 ppb), whereas the common atomic absorption (Weber et al., 2012) spectroscopy (Graphite Furnace AA) can detect between 0.01 to 0.005 ppb, Hydride Generation AA between 0.05 to 0.01 ppb, ICP Emission Axial between 0.1 to 0.01 ppb, ICP Emission Radial higher than 0.01 ppb, and Flame above the 1 ppb level, respectively. The ICP-MS method for simultaneous trace element determination in biological samples has prevailed as the most suitable methodology because of its rapidity, detection limits and minimum sample quantity needed for analysis and direct calibration against aqueous standards (Nisianakis et al., 2009; Giannenas et al., 2009).

As far as consumption of meat by humans is concerned, chicken is increasingly recognized as an important source of nutrients, including micro minerals (Surai and Sparks, 2001) and information on their trace mineral composition is being sought after. The development of 'designer meat' has suggested that their composition may be manipulated to meet the specific needs of human diets.

Our findings about the levels of trace elements in chicken breast and thigh meat are in agreement with published data (Gerber et al., 2009). Similar to our results, several workers found that examined trace minerals such as Cu, Zn, Se and Mn did not differ in any of the examined tissues, among the experimental groups, but their concentrations varied between different cuts from the same species (Gerber et al., 2009). Boron content of breast and thigh meat was low, whereas Ba was not detected showing that breast or thigh meat was clear from any ground bone material. Boron is an integra-

tive element supporting the function of Ca, Mg and vitamin D in the body and plays an important role in mineral metabolism and development and maintenance of normal bone (Bozkurt and Kucukyilmaz, 2015 a, b).

Concentrations of heavy metals found in the meat of our study (Table 11) were far below the toxic limits reported in the literature (Yneyama et al., 2007). Other researchers have found increased concentrations of heavy metals and organochlorine compounds in chicken meat or eggs from hens kept in free range systems (Van Overmeire et al., 2006).

Our work did not intend to expand upon the toxicological risks from trace minerals in meat. In the case that increased contents of heavy metals are detected, determination of possible toxic activity of heavy metals would be crucial, and research should focus on mineral speciation by separating heavy metals such as arsenic in two forms (i.e. organic and non organic). Methodologies that combine ICP-MS with other techniques that allow mineral speciation, such as High Performance Liquid Chromatography (HPLC)-ICP-MS or Ion Chromatography (IC)-ICP-MS offer this possibility (Heitkemper et al., 2001) would allow the distinction between the two metal forms. In our work there was detectable variation in the concentration of meat in trace elements like Zn and Se, as well as Pb and Cd, despite similar values in feeds. These differences were achieved despite the fact that the concentration of the diets in trace minerals was not different. The trace mineral content of the essential oils did not seem to increase the trace element content of meat. Body mineral reserves may have been used as sources of the trace minerals lacking in the diets (Leeson, 2008), although chickens were kept and fed their respective diets for 6 weeks. We assumed that this six week period of feeding on the diets was sufficiently long to 'stabilize' the body reserves of the chickens.

Nutritionists have focused their research on the effects of supplementing the diet of the chicken with various levels of trace elements. However, there is no clear answer as to which level and source of trace elements supplementation is optimal for poultry (Wilson et al., 1992; Pappas et al., 2005); further research is needed to clarify this point (Surai, 2002). Our study contributes to the resolution of such a debate, since it shows that the feed additives may marginally influence the composition of meat in trace minerals, even beyond differences expected due to the diet. Whether the differences observed in trace mineral meat composition could contribute to the enrichment of human diet is beyond the scope of our study (Fisinin et al., 2008). A more systematic study would be required to address the consequences of husbandry system on meat composition.

Conclusions

Our study showed that dietary supplementation with 25 mg/kg oregano essential oil or combination of 25 mg/kg oregano and 2.5 mg/kg laurel essential oils may offer an effective tool to improve the growth performance and modulate intestinal microflora and gut integrity to a more favorable balanced status.

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