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Effects of oregano (*Origanum vulgare* L.) and rosemary (*Rosmarinus officinalis L.*) aqueous extracts on broiler performance, immune function and intestinal microbial population

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ABSTRACT

A 57-day study was performed to determine the effects of two aqueous extracts (AEs) on broiler performance, immune function and intestinal microflora. Four groups of 75 one-day-old female broilers (Ross308) received one of the following treatments: (1) a standard commercial feed (C); (2) C supplemented with 2 g/kg rosemary AE (R); (3) C supplemented with 2 g/kg oregano AE (O); (4) C supplemented with 1 g/kg oregano AE + 1 g/kg rosemary AE (OR). Individual body weight, average daily gain, average daily feed intake and feed conversion efficiency were determined at 1, 11, 22, 36 and 57 days. Sample collections for IgG titration and intestinal microflora examination were performed at 22 and 57 days. The addition of oregano AE alone or in combination with rosemary AEs improved body weight up to 36 days of age ($P < .01$). A time effect was recorded for total serum IgG in all groups ($P < .001$) and the percentage increase of the value was positively ($P < .05$) influenced by the AE supplementation. Lactobacilli raised ($P < .001$) in ileum and cecum of all groups supplemented with AEs. *Staphylococcus* spp. population was constantly lower in both intestinal tracts of the AE supplemented groups. On the basis of our results, AEs could improve broiler performance and immune function and contribute to a balanced gut microflora, essential for the digestion process and protection against enteropathogenic organisms.

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Aqueous extracts; broiler performance; immune function; intestinal microflora; oregano; rosemary

1. Introduction

Since 2006 the banning of the use of antibiotics by the European Commission has focused the attention of the scientific community on the development of alternative products, able to replace antibiotics while achieving the same productivity. In this view, aromatic plants and extracts obtained from these plants have become increasingly important due to their several positive effects on animals. Currently, the chemical components of most plant extracts are generally recognized to be safe and are commonly used in the food industry (Varel 2002), because they are well-accepted by consumers who perceive the final product to be healthier. Oregano (*Origanum vulgare* L.) and rosemary (*Rosmarinus officinalis* L.) have been particularly studied for their properties. Specifically the oregano essential oils (EOs) possess *in vitro* antimicrobial (Lambert et al. 2001), antifungal (Kalemba & Kunicka 2003), insecticidal (Karpouhtsis et al. 1998) and antioxidant (Botsoglou et al. 2004) properties, due to carvacrol and thymol (Adam et al. 1998). Another Labiatae species with significant antioxidative properties is rosemary; its antioxidative activity arises from phenolic terpenes, such as rosmarinic acid and rosmarol (Cuppett & Hall 1998). Several studies reported an improvement in the performance of chickens fed with a combination of plant extracts. The higher average daily gain (ADG) and greater feed conversion efficiency (FCE) achieved could be attributed to the positive effects of extracts on nutrient digestibility (Alçiçek et al. 2003; Hernandez et al. 2004; Jamroz et al. 2006). Wenk (2002) stated that herbs develop their initial activity by adding flavour and, therefore, influencing the eating pattern, secretion of digestive fluids and total feed intake. The growing attention, devoted to environmental issues, led to new techniques capable of extracting plant bioactive compounds without using non-renewable resources (i.e. solvents) with a consequent reduction in the environmental impact of wastes because the plant material, remaining after solvent extraction, is considered hazardous waste (Setti & Zanichelli 2009). The obtained products are defined as aqueous extracts (AEs) and, in addition to being solvent-free and more environmentally friendly, these products contain the complete phytocomplexes instead of the oily fraction alone. However, there is a lack of studies concerning the use of AEs in animal nutrition (Javed et al. 2009; Ogbe & Affiku 2012; Onu 2012) if compared to those concerning EOs. On the basis of these considerations, the aim of the present study was to give a contribute on the effects of dietary supplementation with oregano and rosemary AEs, used alone or in combination, on broiler chicken performance, intestinal microbial population and immune function.

2. Materials and methods

2.1. *Experimental design*

The study was carried out at the experimental farm of the Department of Veterinary Medicine Perugia (Italy), according

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Table 1. Ingredient and chemical composition of the experimental diets.

	Starter	Grower–Finisher
Ingredients (kg/100 kg)		
Maize	53.99	58.99
Wheat middlings	6.00	6.50
Corn gluten	1.15	1.00
Soybean meal, 46% CP	32.00	25.00
Extruded soybean	–	3.00
Soybean oil	2.00	1.50
Calcium carbonate	1.00	.50
Dicalcium phosphate	1.50	1.25
Sodium chloride	.35	.30
Vitamin and mineral mix[a]	.50	.50
Lysine	.15	.10
Methionine	.20	.20
Fatty acid supplement[b]	1.16	1.16
Composition, g/kg		
Analysed		
Dry matter	90.21	89.82
Crude protein	20.44	19.82
Crude fat	4.59	5.07
NDF	10.20	11.83
ADF	2.19	2.18
Lignin (s.a.)	.49	.63
Crude ash	5.99	5.62
Starch (%)	41.85	42.39
Total calcium	1.20	1.20
Total phosphorus	.70	.60
Available phosphorus	.52	.44
Calculated		
Lysine	1.20	1.00
Methionine	.50	.52
Methionine + Cystine	.88	.83
Threonine	.80	.74
Tryptophan	.23	.21
Arginine	1.35	1.22
Leucine	1.86	1.73
Isoleucine	.88	.79
Valine	.98	.90
Histidine	.55	.50
ME (Mcal/Kg)	3.03	3.09

[a]Supplied per kilogram of diet: vitamin A, 12,500 I.U. (retinol); vitamin D3, 3,000 I.U.; vitamin E, 50 mg (tocopheryl acetate); vitamin K3, 2 mg; thiamine, 2 mg; riboflavin, 4 mg; pyridoxine, 1 mg; cyanocobalamin, .015 mg; pantothenic acid 15 mg; folic acid, 50 mg; biotin, 10 mg; choline chloride, 60; iodine, 3 mg; selenium, 20 mg; iron, 3 mg; manganese, 12, mg; copper, 1,5 mg; zinc, 5 mg.
[b]Supplied per kilogram of diet: C18:2 9c,11t, 2.5 g; C18:2 10t, 12c, 2.5 g; C14:0, .16 g; C16:0, .45 g; C20:3, .02 g; C20:5, .02 g; C22:6, .63 g; and others, .36 g.

to the European Directive 2010/63/EU on animal welfare for experimental and other scientific purposes. A total of 300, one-day-old female broilers (Ross 308), were divided into four groups of 75 birds, each fed a starter diet from 1 to 21 days and a grower–finisher diet from 22 to 56 days (Table 1). The experimental diets were based on a standard commercial feed used as control (C group) which was supplemented with 2 g/kg rosemary AE (R group), 2 g/kg oregano AE (O group) and 1 g/kg oregano + 1 g/kg rosemary AEs (OR group). The composition of the rosemary and oregano AEs is indicated in Table 2. The diets, formulated according to the nutritional requirements for broiler chickens (Larbier & Leclerq 1992), were administered in mash form ad libitum. The trial was performed during the months of November and December. Birds were submitted to standard management usually adopted in industrial broiler production. All subjects were vaccinated at the hatch against Newcastle disease (ND), Marek disease (MD), infectious bronchitis (IB) and coccidiosis ND, IB vaccines were again administered in the farm at 21 days. The individual body weight (BW) was measured at 1, 11, 22, 36 and 57 days and feed consumed per group was recorded daily. ADG, average daily feed intake (ADFI) and FCE of all birds were calculated.

Blood samples from five chickens/group were collected for IgG titration at 22 (T1) and 57 days (T2) by wing vein puncture, and serum samples were stored at −20°C until analysis. At the same time intervals, eight subjects from each group were humanely killed for intestinal microflora examination.

Table 2. Mean and SEM of the composition of the oregano (O) and rosemary (R) AEs.

AE	Antioxidant capacity (ORAC/L)	Total polyphenols (g/L)	Reducing sugars (g/L)
O	17780 ± 260	2.5 ± .2	3.3 ± .2
R	10460 ± 160	2.3 ± .2	2.9 ± .1

Note: AEs: aqueous extracts.

2.2. *Feed analysis*

Dry matter was evaluated following AOAC (2000) method 934.01. Crude protein was determined by measuring the total nitrogen according to AOAC (1990) Kjeldahl method 954.01 and converting this value to the protein content by multiplying by 6.25. Crude fat and ash were determined by the 920.39 and 942.05 methods, respectively (AOAC 1990). Neutral detergent fibre (NDF), acid detergent fiber (ADF) and lignin were analysed according to Van Soest et al. (1991). The calcium and phosphorous concentrations were determined according to Julshamn et al. (1998) and AOAC (1996), respectively. Starch was analysed by the polarimetric method according to ISO 10520:1997 and Community methods of analysis for the official control of feeding stuffs (European Directive 72/1999/CEE).

2.3. *Extraction and analysis of AEs*

The AEs were obtained by a process of bio-liquefaction based on enzyme bio-catalysis (Setti & Zanichelli 2009). The plant material was placed in boiling water, and a specific enzymatic preparation was then added after cooling. After four hours of hydrolysis, the plant material was filtered. The AEs obtained were analysed to quantify antioxidant capacity, total polyphenols and reducing sugars (Table 2). The antioxidant activity of the oregano and rosemary AE was measured in terms of radical scavenging ability using the stable radical DPPH (Donglin & Yasunori 2004), and the values were expressed in ORAC/L using trolox® as a reference compound (Davalos et al. 2004). Total polyphenols were evaluated using the Folin-Ciocalteu reagent (Ainsworth & Gillespie 2007). The content of the reducing sugars was evaluated using the ADNS method (Bailey et al. 1992).

2.4. *IgG titration*

The chicken serum IgG value was determined by a commercially available ELISA kit (Cat. No. E33–104. Bethyl Laboratories, Inc., USA) and an automated washing and reader instrument (Mago4S, Diamedix Corporation, Miami, FL, USA). The procedures for sample assays were performed according to the manufacturer's instructions, and the colorimetric reactions

Table 3. Body weight (g) of broilers fed with the experimental diets up to 57 days.

	Age (days)				
Diets	1	11	22	36	57
C	44.16	179.79^{B}	389.69^{B}	1001.18^{B}	2447.20
R	43.76	183.91^{B}	466.57^{AB}	1124.01^{AB}	2437.29
O	43.90	221.40^{A}	493.92^{AB}	1157.18^{A}	2168.64
OR	48.05	173.24^{B}	504.23^{A}	1204.47^{A}	2431.37
SEM	2.05	5.69	30.98	37.13	83.18
P	N.S.	<.001	<.05	<.01	N.S.

Note: C: control diet; R: diet supplemented with rosemary AE (.2 mg/kg); O: diet supplemented with oregano AE (.2 mg/kg); OR: diet supplemented with rosemary AE (.1 mg/kg) and oregano AE (.1 mg/kg).
Means within a column lacking a common superscript differ.
N.S.: not significant.

Table 4. Average daily gain (g) in experimental groups up to 57 days.

	Age intervals (days)			
Diets	1–11	11–22	22–36	36–57
C	12.33^{B}	18.82^{B}	44.04^{B}	65.85^{A}
R	13.52^{B}	24.88^{AB}	48.52^{AB}	54.91^{AB}
O	16.13^{A}	24.49^{AB}	50.18^{AB}	47.65^{B}
OR	12.39^{B}	29.50^{A}	53.84^{A}	56.30^{AB}
SEM	.47	2.75	2.38	3.53
P	<.001	<.05	<.05	<.01

Note: C: control diet; R: diet supplemented with rosemary AE (.2 mg/kg); O: diet supplemented with oregano AE (.2 mg/kg); OR: diet supplemented with rosemary AE (.1 mg/kg) and oregano AE (.1 mg/kg).
Means within a column lacking a common superscript differ.

were read at the 450 nm wavelength. All samples were assayed three times.

2.5. *Microbiological analyses*

The caecum and ileum (between the Merkel's diverticulum and a point 40 mm proximal to the ileocecal junction) from eight birds per group were carefully removed and pooled to obtain four samples for each intestinal region. A sterile stick was used to place 1 g of intestinal contents into a sterile test tube containing 2 mL of .9% sterile saline solution. The stool was pressed and mixed in this solution, and the tube was brought to volume (10 mL) with .9% sterile saline solution. Each pooled sample (.1 mL) was 10-fold serially diluted (from 10^{-1} to 10^{-10}). Violet red bile agar and KF streptococcus agar were used for the enumeration of Coliforms and Enterococci, respectively. Mannitol salt agar was used for the enumeration of Staphylococci. All the plates were aerobically incubated at 37°C for 24–48 h, and the number of colonies was counted. For the enumeration of anaerobic bacteria, reinforced clostridial agar enriched with 5% sheep blood and 1 mg/mL vitamin K1, was used as anaerobe blood agar. Anaerobic incubation was performed in anaerobic jars (Oxoid) at 37°C for 48 h. Anaerobic conditions were obtained using Anaerogen (Oxoid) and checked using methyl blue strips as oxidation reduction indicators. For the enumeration of Lactobacilli, Rogosa agar (Oxoid) was used. The plates were incubated for three days at 35°C under microaerophilic conditions. All data were expressed as CFU × log/g.

2.6. *Statistical analysis*

Performance data were subjected to statistical analyses with the GLM (General Linear Model) procedures of SAS (SAS Institute Inc 2010) according to a model for repeated measurements, where the independent factor was the dietary treatment (four levels). Microbiological data were analysed using a completely randomized design with factorial arrangement 2 × 2 × 4 with factors A (ileum and cecum), B (sampling time) and C (dietary treatment). Mean separation and comparison were conducted using Tukey's test. The microbiological data were adjusted using a $\log_{10}$ transformation. The chicken serum IgG concentrations were analysed according to a model where the independent factors were dietary treatment (four levels), sampling time (two levels) and their interaction. The estimated marginal means of IgG increases (T2 vs. T1) were analysed by pairwise comparisons. The IgG percentage increases were calculated with the following formula: IgG increase = [(IgG values at T2–IgG values at T1)/IgG values at T1], and they were expressed as percentage (%). The level of statistical significance was set at $P < .05$.

3. Results and discussion

The BW means for all experimental groups are summarized in Table 3. There were no differences in BW means at the beginning of the trial. AE supplementation significantly affected the BWs of birds at 11, 22 and 36 days of age, while no effects were observed at the end of the experiment. The general trend recorded for BW was confirmed by the ADG (Table 4) and the ADFI (Table 5), with the exception of the data obtained at the end of the trial. A numerically lower FCE was observed at 36 and 57 days in birds fed diet with the AEs (Table 5).

Table 5. Feed conversion efficiency (FCE) and average daily feed intake (ADFI, g/die) in experimental groups up to 57 days.

	Age intervals (days)							
Diets	1–11		11–22		22–36		36–57	
	ADFI	FCE	ADFI	FCE	ADFI	FCE	ADFI	FCE
C	24.04	1.95	62.12	3.62	76.19^{B}	1.73	183.72^{A}	2.79
R	27.31	2.02	66.18	2.66	95.10^{AB}	1.96	93.90^{B}	1.71
O	25.08	1.60	59.75	2.44	90.83^{AB}	1.81	92.44^{B}	1.94
OR	25.40	2.05	60.77	2.06	104.45^{A}	1.94	88.39^{B}	1.57
SEM	.84	.05	4.05	.06	6.69	.07	12.32	.06
P	N.S.	N.S.	N.S.	N.S.	<.05	N.S.	<.05	N.S.

Note: C: control diet; R: diet supplemented with rosemary AE (.2 mg/kg); O: diet supplemented with oregano AE (.2 mg/kg); OR: diet supplemented with rosemary AE (.1 mg/kg) and oregano AE (.1 mg/kg).
Means within a column lacking a common superscript differ.
N.S.: not significant.

Table 6. Effect of the different diets on total serum IgG (mg/mL) at 22 (T1) and 57 days (T2).

Diets	T1	T2	% of increase T2–T1
C	3.7	11.7	2.2^{A}
R	4.1	14.9	2.7^{A}
O	3.3	19.8	5.0^{B}
OR	3.4	11.9	2.4^{A}
SEM	2.1	2.1	.5
P	N.S.	N.S.	<.05

Note: C: control diet; R: diet supplemented with rosemary AE (.2 mg/kg); O: diet supplemented with oregano AE (.2 mg/kg); OR: diet supplemented with rosemary AE (.1 mg/kg) and oregano AE (.1 mg/kg).
Means within a column lacking a common superscript differ.
N.S.: not significant.

Previous studies have already reported the beneficial effects of some plant extracts (Hernandez et al. 2004; Windisch et al. 2008), indicating reduced feed intake and higher ADG or BW, responsible for improved FCE. Alçiçek et al. (2004) investigated the effects of dietary supplementation in the diet of broilers with EO mixture, alone or in association with organic acid and probiotic, showing higher yields in the group fed with EO alone. Hernandez et al. (2004) tested two different types of plant extract mixtures, including oregano and rosemary, in a 42-day trial. They found no differences in feed intake or FCE for the entire experimental period, but broilers fed with plant extracts grew faster than the controls. Hashemipour et al. (2013) evaluated the effects of supplementation of different levels of thymol and carvacrol on performance in broiler chickens. However, Papageorgiou et al. (2003) demonstrated that a mixture of EOs did not improve the growth performance of broilers. Phytogenic feed additives were also reported to stimulate the intestinal secretion of mucus in broilers, which impairs the adhesion of pathogens and contributes to the stabilization of the microbial flora in the guts of the animal, improving digestive processes (Jamroz et al. 2003).The different effects of plant extracts on broiler performance may be due to intrinsic and extrinsic factors, mainly including the experimental approaches used to test the suitability of these substances as growth-promoting additives for broilers (Windisch et al. 2008). With respect to studies on AEs, Javed et al. (2009) analysed the effects of AEs derived from different plants administered in drinking water in broiler chicks. Their results showed positive effects of AEs on ADG, FCE, dressing percentage, weight of some organs and feed intake. Onu (2012) evaluated the effect of different levels of *Telfairia occidentalis* AEs on the performance and haematological indices of starter broilers and observed a positive influence on BW, ADG and FCE.

Although the dietary treatments did not appear to significantly affect mean serum IgG values (Table 6) in terms of absolute numbers, their increases (expressed in percentages) from T1 to T2 revealed a significant ($P < .05$) diet effect with the highest value in the O group.

In this study, the increase in the total IgG in the O and R groups at T2 may indicate a more intense immune response to vaccine antigen in the treated subjects. The total IgG values observed at the end of our trial were greater than those reported in the literature (Larsson et al. 1993) although they could be related to the different procedures followed.

However, dietary immunomodulators can amplify or decrease the magnitude of the reaction to a challenge through their effects on other immune cells (Dietert et al. 1991).

Hashemipour et al. (2013) found an improved immune response in broilers fed with a diet supplemented with thymol and carvacrol, characterized by an enhancement of hypersensitivity response and an increase of total IgG and IgG anti-sheep red blood cells with decreased heterophil to lymphocyte ratio. Varshney et al. (2013) examined the effects of *Ocimum sanctum* and *Argemone Mexicana* AEs in chickens, reporting higher antibody titres and improved cell-mediated immune response compared to control. With respect to the microbiological investigations, this study was mainly undertaken to detect possible differences in some bacterial populations following the administration of diets supplemented with AEs. We focused on *Lactobacillus* spp., *Enterococcus* spp., Coliforms and *Staphylococcus* spp. and finally Anaerobics (including *Clostridium* spp.).

The results produced by the experimental diets on the intestinal microflora composition are shown in Tables 7 and 8. In our investigation the effects on the intestinal microflora population were encouraging though not conclusive.

At T1, the values for Coliforms were significantly lower ($P < .001$) in the cecum of the O and R groups than the C group. The Coliform values increased in the ileum of all groups with age though the O group showed the lowest values. Coliforms are a large population of bacteria whose effects are not always qualified as positive, including several potential pathogenic agents (Amit-Romach et al. 2004). Globally *Staphylococcus* spp. population was lower in the cecum and ileum in all groups fed diets enriched with AE at both sampling times; in particular, the R and O groups showed the lowest ($P < .001$) count in the ileum and caecum at T1, whereas the O group presented the lowest values in the ileum at T2($P < .001$).

Staphylococcus spp. is not considered a beneficial species in the microbial gut population. Most infections in chickens are

Table 7. Effect of the diets on different bacterial populations in the ileum tract at 22 (T1) and 57 days (T2).

	Coliforms		*Staphylococcus* spp.		*Enterococcus* spp.		Total anaerobics		Lactic acid bacteria	
Diets	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2
C	4.47^{E}	6.80^{B}	3.78^{D}	4.80^{A}	7.64^{BC}	7.50^{CD}	11.18^{B}	9.40^{D}	10.36^{A}	6.5^{E}
R	3.58^{G}	6.09^{C}	3.52^{E}	4.03^{C}	7.31^{D}	7.83^{B}	9.35^{D}	9.72^{C}	4.23^{F}	9.53^{B}
O	3.26^{H}	4.20^{F}	3.36^{E}	3.86^{D}	8.28^{A}	8.38^{A}	8.69^{E}	12.52^{A}	6.64^{E}	7.22^{D}
OR	5.41^{D}	7.34^{A}	4.52^{B}	4.12^{C}	7.31^{D}	8.50^{A}	9.73^{C}	12.57^{A}	7.94^{C}	9.40^{B}
SEM	.051		.034		.057		.032		.042	
P	<.001		<.001		<.001		<.001		<.001	

Note: C: control diet; R: diet supplemented with rosemary AE (.2 mg/kg); O: diet supplemented with oregano AE (.2 mg/kg); OR: diet supplemented with rosemary AE (.1 mg/kg) and oregano AE (.1 mg/kg).
Means within each bacterial population lacking a common superscript differ.

Table 8. Effect of the diets on different bacterial populations in the cecum tract at 22 (T1) and 57 days (T2).

	Coliforms		*Staphylococcus* spp.		*Enterococcus* spp.		Total anaerobics		Lactic acid bacteria	
Diets	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2
C	6.20^{C}	5.86^{D}	6.28^{A}	4.83^{B}	9.26^{B}	8.24^{D}	13.15^{A}	11.18^{E}	11.16^{A}	7.86^{E}
R	4.21^{F}	6.96^{A}	3.35^{E}	3.78^{D}	7.55^{E}	8.91^{C}	12.78^{BC}	10.52^{F}	7.58^{F}	9.20^{D}
O	4.60^{E}	6.21^{C}	3.38^{E}	4.03^{D}	8.18^{D}	9.72^{A}	12.68^{C}	12.41^{D}	7.17^{G}	7.54^{F}
OR	6.58^{B}	4.10^{F}	3.40^{E}	4.42^{C}	8.16^{D}	9.40^{B}	12.90^{B}	12.35^{D}	9.70^{C}	10.16^{B}
SEM	.044		.060		.067		.031		.036	
P	<.001		<.001		<.001		<.001		<.001	

Note: C: control diet; R: diet supplemented with rosemary AE (.2 mg/kg); O: diet supplemented with oregano AE (.2 mg/kg); OR: diet supplemented with rosemary AE (.1 mg/kg) and oregano AE (.1 mg/kg).
Means within each bacterial population lacking a common superscript differ.

caused by coagulase-positive staphylococci, particularly *S. aureus,* and coagulase-negative staphylococci (Jordan 1996; McNamee et al. 1998). Staphylococci are frequently found in poultry products for human consumption (Rosec et al. 1997; Manie et al. 1998) and can thus spread to humans through the food chain. With regards to *Enterococcus* spp., diets supplemented with oregano produced the highest count ($P < .001$), particularly in the caecum tract, and its value increased in all groups at T2 supporting the hypothesis of a time-dependent positive effect. Antagonistic effect of *Enterococcus faecium* against the human and poultry pathogenic *Salmonella* spp. was reported by Audisio et al. (1999), though its high level of bile salt hydrolase activity may be responsible for growth depression in chickens (Garrido et al. 2004).

Globally, total anaerobes were higher in the cecum than ileum, with higher values ($P < .001$) in the O and OR groups in both intestinal tracts at T2. At T1 their values were significantly higher in the C group. Among the AE-treated groups, R showed the lowest counts in both intestinal tracts at T2. Barnes et al. (1972) reported that the majority of bacteria in the cecum, detected by culture-based methods, were strictly anaerobic, and their number and diversity were influenced with age. Anaerobes constitute a large intestinal population of which the clostridia, including *C. perfringens*, represent a significant part. An increase in *C. perfringens* could determine the occurrence of clinical and subclinical necrotic enteritis in birds (Kaldhusdal et al. 1999; Williams et al. 2003).

In our work the influence of rosemary, added either alone or in combination with oregano, on *Lactobacillus* spp. should be emphasized. Birds in the C group showed the highest values for Lactobacilli in the ileum and cecum tracts at T1. Values increased ($P < .001$) in all groups fed the AE diets at T2, especially in the R group, showing, also in this case, a potential time-dependent effect. The stimulation of beneficial bacteria, such as Lactobacilli, could contribute to balanced gut microflora and provide a favourable condition for digestion processes and protection against enteropathogenic organisms. The role of this bacterium in protecting the intestinal environment against invasions by pathogens, such as *C. perfringens, Campylobacter jejuni* subsp. *jejuni*, *Salmonella* spp. and pathogenic strains of *E. coli* (Mead 2000), has been known for a long time. Various members of the *Lactobacillus* spp. are also able to modulate chicken cytokine and chemokine gene expression (Haghighi et al. 2008; Brisbin et al. 2010).

In conclusion several limitations are related to culture-based methods in the identification of the intestinal ecosystem because up to 99% of bacteria fail to grow under artificial conditions (Amann et al. 1995; Hanson & Henson 1996). In particular, traditional methods were not always capable of isolating the anaerobic bacteria and maintaining viability (Mead 1997). However, Wang et al. (1996) reported a good correlation between PCR-based and culture-based methods for bacterial species requiring no particular enrichment media. Although these results are in agreement with others (Garrido et al. 2004; Cross et al. 2007), further studies are needed to ensure the actual effectiveness of AE on the performance and welfare of broiler chickens.

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Disclosure statement

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