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INFLUENCE OF OREGANO EXTRACT ON THE INTESTINE, SOME PLASMA PARAMETERS AND GROWTH PERFORMANCE IN CHICKENS

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The effect of oregano essential oil on growth performance, intestinal alkaline phosphatase (IAP) level, enterocyte proliferative ability, plasma proteins, and plasma minerals (calcium and magnesium) were studied in 1 day-old Ross 308 hybrid broiler chickens under commercial conditions during 42 experiment. Chickens fed oregano oil supplemented diet (0.707 g.kg^{-1}) had significantly higher body weight gain (BWG) in the grower (19-29 d) and finisher (30-42 d) periods. Total plasma immunoglobulin levels were lower while mineral levels were higher during the finisher period. Significant decrease of IAP activity was demonstrated in animals fed oregano oil supplemented diet on 29, and 42 days of the experiment. Proliferative activity of enterocytes significantly increased in the finisher period along duodenal villi in animals treated with essential oil extracted from oregano.

We suppose that higher body performance was probably the result of higher digestibility in the digestive tract. It is suggested that the antibacterial effect of carvacrol caused a lower number of intestinal bacteria, decrease of immunoglobulins and increase of investigated plasma proteins.

Key words: alkaline phosphatase, biochemistry, chickens, extract, oregano, essential oils, PCNA

INTRODUCTION

Essential oils (EO) are volatile oily liquids obtained from plant material. Detailed compositional analysis of essential oils is achieved by gas chromatography (Marcin *et al*., 2006). Major components can constitute up to 85% of the EO, whereas other components are present only in a traces (Senatore, 1996). The phenolic components are chiefly responsible for the antibacterial properties of EOs (Cosentino *et al*., 1999). There is some evidence that minor components have a critical part to play in antibacterial activity, possibly by producing a synergistic effect between other components. This has been found to

be the case of oregano (Paster *et al*., 1995). The composition of EOs from a particular species of plant can differ between harvesting seasons, geographical sources, and composition of soil (Kokkini *et al*., 1997).

Mechanisms for the interaction of plants including *Oregano* spp. with the host organism may be related to intestinal and extraintestinal effects. Intestinal effects may be explained by the effects on the microflora (Taylor, 2001) or intestinal mucosal system (Garcia *et al*., 2007). Microflora can influence the activities of certain enzymes involved in the uptake of nutrients and incorporation of dietary nucleic acid components by enterocytes (Whitt and Savage, 1988).

Proliferative activity of enterocytes is the sign of a healthy tissue turnover and maintenance (Garcia *et al*., 2007). Unlike mammals, chickens' enterocyte proliferation is not localized only in the crypt region and the site of enterocyte proliferation is not precisely localized (Uni *et al*., 1998). Duodenal brush-border membrane, localized on the surface of enterocytes, has multiple highly expressed ectoenzymes with extracellular catalytic sites including intestinal alkaline phosphatase (IAP). Function of IAP remains somewhat speculative, particularly with regard to its apparently non-physiological pH optimum. Activity of IAP, however, helps to regulate the intestinal lipid absorption and hydrolyzis of ingested organic phosphates (Akiba *et al*., 2007). Feeding a high-fat diet produced changes the brush-border membrane lipid composition and increased intestinal AP activity (Kaur *et al*., 1996; Mozeš *et al*., 2007).

Dietary effect of oregano oil has already been studied during *in vitro* (Burt, 2004) and *in vivo* (Botsoglou *et al*., 2002; Garcia *et al*., 2007) conditions. Numerous works have been devoted to antioxidant effects (Botsoglou *et al*., 2002), anti-inflammatory activity (Juhas *et al*., 2007) and antibacterial properties (Burt *et al*., 2005).

One of our objectives was to study the IAP level and enterocytes proliferative ability in relation to growth promoting effects after application of relatively high doses of oregano oil in commercial diets. Another object was to determine the plasma protein and immunoglobulin level, as well as some macrominerals in studied chickens, and the possible *in vivo* effect of carvacrol.

MATERIAL AND METHOD

Plant aromatic oil

The plant aromatic oil (100% v/v) was isolated from oregano tops (*Origanum vulgare* L., family *Lamiaceae*) by steam distillation of plant material in Calendula joint-stock company (Nová L'ubovna, Slovak Republic). The percentual range of the main components was analysed by gas chromatography (GC) using Hewlet-Packard 5890 Series II (injection input split splitless, capillary column HP-5, detector FIF, automatic injector HP 7673) with nitrogen as carrier gas (Pavlišinová and Danielovič, 2007). The percentual ranges of the main components of the aromatic oil utilized in the experiment were carvacrol 60%, y-terpinene 12% and p-cymene 6.4%.

Chickens and diets

Sixty 1-day-old Ross 308 hybrid broiler chickens (mixed sex in ratio 1:1) were obtained from a commercial hatchery. Individually weighed chickens were divided at random into experimental (P, n=30) and control (C, n=30) groups. The chicks were housed in two floor pens located in a commercial broiler chickens fattening farm (Michalovce, Slovak Republic). The pens were identical, with the same direction and covered area (0.12 m^2/broiler chicken). The animals had constant access to feed and water from identical fixtures. The broiler chicken flock studied during 42 days was fed three commercial diets (BR1, BR2, BR3, Pol'nonákup DOMICA, Plešivec, Slovak Republic) with anticoccidicum salinomycin sodium and without antibiotics or growth promoters. The diets corresponded to the standards for broiler chickens in Feeding Norms for Poultry in Slovakia (code of laws and decrees No. 440/2006). The essential oil isolated from oregano (*Origanum vulgare* L., family *Lamiaceae*) was included into the mash diet of the experimental group of chickens by admixing in a dosage calculated according to the carvacol content (707.195 ppm), as well as according to the results of chemical and palatability tests. The carvacrol content in diet silica (1.242 $mL.kg^{-1}$) was 0.707 $g.kg^{-1}$ (707.195 ppm).

Performance parameters

During the experiment the body weight of chickens was monitoring daily. Feed was prepared and weighed daily to evaluate the feed consumption and feed conversion ratio.

Feed analysis

The experimental diets were dried at +102 ^oC for 16h in a forced air oven and analysed for dry matter, crude protein, crude fat, crude fibre, and ash by methods of Javorský *et al*. (1987). The dietary amino acid analyses was done by AAA400 amino acid analyser (INGOS, Czech Republic) according to Regulation 1998/64/EC. Lysine was determined after hydrolysis with 6 M HCl, while methionine and cystine were determined after oxidative hydrolysis. The content of calcium, and sodium were determined by atomic absorption spectrophotometry (AAS) with Shimadzu AA-6200 (Javorský *et al*., 1987). The content of phosphorus was determined spectrophotometrically with Jenway 6400 (Javorský, 1983). The nutrient analyses of the experimental diet is given in Table 1.

Blood and tissue samplings

Ten randomly-chosen chickens from each group at 2, 4, and 6 weeks of age were anaesthetized with intraperitoneal injection of xylazine (Rometar 2%, SPOFA, Czech Republic) and ketamine (Narkamon 5%, SPOFA, Czech Republic) at doses 0.6 and 0.7 $mL.kg^{-1}$ body weight, respectively. After laparotomy, blood was collected into tubes by intracardial punction, samples for sera analyses were frozen and stored at -20 ^oC. During the following necropsy the samples from the intestine (duodenum, jejunum) were stored in formalin for PCNA analysis, and in nitrogen for histochemical analysis.

Table 1. Composition of the experimental diets (g.kg^{-1} dry matter)

Ingredients	Experimental diets		
	BR1 days 1–21	BR2 days 22–35	BR3 days 36–42
Crude protein	347.23	258.73	240.84
Crude fat	16.18	25.05	52.01
Crude fibre	57.09	40.82	35.16
Crude ash	85.45	67.15	62.12
Starch	385.92	447.08	463.84
Total sugar	48.67	67.23	58.13
Reducing sugar	8.73	30.45	12.62
Calcium	12.69	10.55	8.87
Phosphorus	13.01	10.04	9.28
Sodium	3.63	2.90	2.58
Methionine	4.44	5.12	4.71
Lysine	11.39	15.04	13.42
Cystine	3.38	3.21	2.90

Biochemistry of proteins and minerals

Total plasma protein concentration (g.L^{-1}) was determined spectrophotometrically by the Bradford method (Bradford, 1976).

Determination of total plasma immunoglobulins was performed with zinc sulphate turbidity (ZST) reaction in modification of McEvan's method (McEvan *et al*., 1970). Briefly, 25 μL of tested sera were mixed with 1.7 mL 0.7 mmol.L^{-1} 5.8 pH zinc sulphate (208 mg.L^{-1}; Merck, Germany). The mixture was shaken, incubated for 2h at room temperature and light absorption was measured photometrically at 590 nm (Spekol 11, Carl Zeiss, Jena, Germany). Blood serum mixed with PBS in the same ratio was used as the blank. The immunoglobulin's content of the tested sera was derived from a calibration curve plotted on the basis of turbidity values corresponding to four dilutions of the standard serum samples (Precinorm Protein, Roche Diagnostics, USA). Zinc sulphate turbidity reaction – the visual readings of the density of turbidity were performed by a comparative method. The turbidity of the tested samples, obtained by the zinc sulphate reactions, were compared to the turbidity of five dilutions of the standard serum (1.44, 2.16, 2.88, 3.59, 4.31 g.L^{-1}).

Serum content of calcium, phosphorus and sodium were determined by atomic absorption spectrophotometry (AAS) with Shimadzu AA-6200 (Javorský *et al*., 1987).

Histochemical assay

The frozen tissue was cut (7 μm) in a cryocut at -25 °C, then tissue sections were transferred to supporting glass slides and air-dried. Demonstration of

alkaline phosphatase activity was performed by using a modified simultaneous azocoupling method (Lojda *et al*., 1979). The incubation medium contained naphtol AS-BI phosphate (Sigma, Germany), Fast blue BB (Aldrich, Germany) and veronal acetate buffer (pH 9.2). The samples were incubated at 37 ^{o}C for 10 min, using a substrate concentration of 2.0 mmol.L^{-1} at pH 8.9. Enzyme activity was cytophotometrically analyzed with a Vickers M85a microdensitometer. The measurements were performed using a magnification of 400x, an effective scanning area of 28.3 μm^2 and scanning spot of 0.5 μm. The integrated absorbance was measured at a wavelength of 480 nm. The mask was set over at least 30 brush border areas along the villus length in five sections of the jejunum. The IAP activity was calculated as the absorbance values recorded by the instrument/min/μm^3 brush border and their mean values were referred to one animal.

Proliferating cell nuclear antigen (PCNA) assay

Samples were taken from the caudal part of the duodenum and medial part of jejunum, fixed in 10% neutral buffered formalin and embedded in paraffin. The examination of PCNA was carried out at 5 μm tissue sections with commercial Animal Research Kit (ARK) according to special included protocol (DAKO, Denmark). Kit contained monoclonal mouse anti-PCNA antibody (Clone PC10; DAKO, Denmark) and all components (peroxidase block, streptavidin HRP, blocking reagent, biotinylation reagent and DAB tablets), needed for examination. Negative controls were obtained omitting the primary antibodies. The positively-stained cells in three duodenal sections/per animal were counted in a standardized area at the tip, middle, and at the base of the villi. Five areas were selected at random from each of these sites with the use of an ocular graticule LTD 0.25 mm IdXD rd (Tonbridge, Kent, UK). Measurements were performed with the use of a light microscope (Nikon, Type 104, Japan) at a magnification of x400. Mean values and standard deviations were calculated.

Statistical analysis

All data were expressed as means ± standard deviation (SD) (SAS, Version 8.2; SAS Institute Inc., 1999, Cary, NC USA). The results were compared by one-way analysis of variance and Tukey-Kramer multiple comparison test. Significance was declared at $p<0.05$, $p<0.01$, and $p<0.001$.

RESULTS

Significant increase of body weight gain (BWG) was found in chickens fed with oregano extract in starter (1–16 days of age), grower (17–29 days of age), and finisher feed (30–42 days of age) (Table 2). Average daily feed intake (FI) and feed conversion (FE) were not significantly different and are presented in Table 3.

Level of total plasma protein and total plasma immunoglobulins are demonstrated in Table 4. The changes of total plasma protein were determined on day 29 of experiment with a significant increase in animals fed oregano extract. Total plasma immunoglobulins demonstrated a significant decrease in chickens

fed oregano extract on day 29 of experiment. Significant increase in calcium and magnesium was seen in animals after the application of oregano extract on day 42 of experiment (Table 5).

Table 2. The effect of oregano extract on body weight gain (mean ± SD)

Group	Initial body weight (g)	Final body weight (g)	Body weight gain ($g.day^{-1}$) Age (day)		
			1–16	17–29	30–42
Control	38.9 ± 6.7	1747 ± 341	14.6 ± 3^{a}	36.9 ± 13^{a}	69.5 ± 18^{a}
Oregano	39.3 ± 8.3	1840 ± 231	17.2 ± 4^{c}	46.6 ± 11^{c}	80.8 ± 8^{b}

Means with different superscript letters in the same column differ significantly $^{ab}p<0.05$; $^{ac}p<0.001$

Table 3. The effect of oregano extract on growth performance

Group	Average daily feed intake ($g.day^{-1}$) Age (day)			Feed conversion (g.g) Age (day)		
	1–16	17–29	30–42	1–16	17–29	30–42
Control	29.4	80.5	160.6	2.02	2.18	2.31
Oregano	32.8	91.4	160.4	1.90	1.96	1.99

Table 4. Values of protein and immunoglobulin in blood serum of broiler chickens (mean ± SD)

Age (d)	Group	Total protein ($g.L^{-1}$)	Total Ig ($mg.dL^{-1}$)
16	Control	154.85 ± 4.9	0.41 ± 0.1
	Oregano	154.52 ± 4.7	0.39 ± 0.1
29	Control	115.08 ± 5.7^{a}	1.25 ± 0.5^{a}
	Oregano	128.94 ± 15.7^{b}	0.99 ± 0.2^{b}
42	Control	176.88 ± 12.2	1.14 ± 0.4
	Oregano	174.24 ± 5.4	1.03 ± 0.1

Means with different superscript letters in the same column differ significantly $^{ab}p<0.05$; $^{ac}p<0.01$; $^{ad}p<0.001$

Activity of IAP in jejunal microvilli is shown in Table 6. Application of oregano extract to the experimental group of chickens resulted in a significant decrease of jejunal IAP activity on 16, 29, 42 days of the experiment.

Proliferative activity of enterocytes was significantly increased along the duodenal villi and insignificantly along the jejunal villi of chickens in the finisher period (42 days) after feeding of diet supplemented with oregano extract (Table 7).

Table 5. Values of macroelements in blood serum of broiler chickens (mean ± SD)

Age (d)	Group	Phosphorus ($mg.dL^{-1}$)	Potassium ($mg.dL^{-1}$)	Calcium ($mg.dL^{-1}$)	Magnesium ($mg.dL^{-1}$)
16	Control	nd	nd	nd	nd
	Oregano	nd	nd	nd	nd
29	Control	15.41 ± 3.1	19.07 ± 1.4	11.70 ± 3.3	3.09 ± 0.4
	Oregano	15.69 ± 2.3	18.24 ± 3.1	9.73 ± 2.7	2.48 ± 0.5
42	Control	14.54 ± 2.7	20.32 ± 2.7	9.03 ± 2.5^{a}	2.68 ± 0.5^{a}
	Oregano	13.16 ± 1.2	21.39 ± 2.0	15.10 ± 0.5^{b}	3.48 ± 0.2^{b}

Means with different superscript letters in the same column differ significantly $^{ab}p<0.05$

Table 6. Alkaline phosphatase (AP) activity in jejunal microvillous zone (mean ± SD)

Age (d)	Group	AP activity
16	Control	4.53 ± 0.14^{a}
	Oregano	3.66 ± 0.24^{b}
29	Control	4.87 ± 0.07^{a}
	Oregano	4.42 ± 0.08^{c}
42	Control	4.91 ± 0.13^{a}
	Oregano	4.45 ± 0.14^{c}

Means with different superscript letters in the same line differ significantly $^{ab}p<0.001$; $^{ac}p<0.01$

Table 7. Numbers of enterocytes in duodenum and jejunum measured by PCNA assay (mean ± SD)

Age (d)	Group	Duodenum	Jejunum
16	Control	15.87 ± 1.40	25.90 ± 5.80
	Oregano	23.88 ± 4.10	24.67 ± 0.90
29	Control	17.75 ± 0.40	23.27 ± 3.30
	Oregano	22.50 ± 4.30	23.57 ± 2.10
42	Control	14.70 ± 2.70^{a}	18.52 ± 2.20
	Oregano	21.09 ± 0.80^{b}	24.20 ± 2.00

Means with different superscript letters in the same line differ significantly $^{ab}p<0.01$

DISCUSSION

The experiment was performed in a commercial farm in order to modulate the stress conditions of large-scale production. Very important is the microbiological stress on the animal which can be different in experimental cages than the one encountered under normal commercial conditions (Bedford, 2000).

Our result demonstrated positive effects of oregano extract supplemented diet on BWG during feeding. The beneficial effect of growth promoter substances on performance is related to a more efficient use of nutrients which in turn results in an improved FCR (Elvinger *et al*., 1993). In our experiments, however, there was only a tendency for improving FCR in the experimental group. Botsoglou *et al*. (2002) did not find positive effects on performance when administered oregano oil at 50 or 100 mg.kg^{-1} of feed. Garcia *et al*. (2007) after feeding plant extract (200 ppm) based on a blend of oregano, cinnamon, and pepper essential oils, found the positive effect only in feed conversion in the treated group. Our results compared to other observations suggest that an important role in body performance may be played by the dose and blend of oil extracts.

Our experiment also showed an increased level of plasma total protein in experimental chickens. On the contrary, in the same feeding period the total immunoglobulin decreased in the blood plasma. Blood plasma proteins, are synthesized mainly in the liver such as albumins, clotting proteins, and globulins which have a significant extrahepatic role (Grieninger and Granick, 1975).

Generally, the EOs possessing the strongest antibacterial properties against foodborne pathogens contain a high percentage of phenolic compounds such as carvacrol (Burt *et al*., 2005). Carvacrol-containing essential oils are biostatic and/or biocidal against many bacteria, yeast, and fungi in laboratory media (Burt, 2004; Kisko and Roller, 2005). Degradation of antigens in the gut can reduce exposure to foreign antigens, and consequently reduce the effect of the immune system on bird performance (Korver, 2006; Yegani and Korver, 2008). It is suggested that the administered concentration of oregano in our experiment showed antibacterial activity in the gut what could be the result of decreased immunoglobulins in the plasma of chickens fed the supplemented oregano oil. On the other hand, activation of the immune response to the overloading of gastrointestinal antigenic stimulation could have negative impacts on feed efficiency which are energetically expensive, and divert nutrients away from production (Korver, 2006).

The results demonstrated that oregano oil improved bio-availability of minerals calcium and magnesium in the finisher period in experimental animals. Increased absorption of these two minerals can indicate a decrease of viable intestinal microflora (Bandaru *et al*., 1969) caused probably by antibacterial effects of carvacrol. Similarly, increased levels of minerals can be the consequence of increased availability of fatty acids for incorporation into biliary micelles and, thus improved fat digestibility (Xu *et al*., 1998).

Demand for energy and protein for gut maintenance is higher compared to other organs. Fast growing broilers devote about 12% of the newly synthesized proteins to the digestive tract (Xu *et al*., 2003). Factors that are present in the digesta can lead relatively quickly to changes in the intestinal mucosa due to the close proximity of the mucosal surface to the intestinal content. Activity of nutrient assimilation is in close connection with the proliferative activity of enterocytes enhancing healthy tissue turnover and maintenance (Garcia *et al*., 2007). The significant increase of enterocyte proliferation along the duodenal villi during the finisher period and tendency for increased proliferative activity in other samplings

found in our trial demonstrate the benefit properties of oregano extract on the proliferative ability of intestinal epithelial cells.

The current experiment demonstrated a significant reduction of mucosal alkaline phosphatase activity in chickens fed oregano extract supplemented diet. Earlier studies suggest that IAP activity helps to regulate intestinal lipid absorption (Kaur *et al*., 1996; Akiba *et al*., 2007). The decreased level of IAP in enterocytes in the current trial can be related to an improved digestibility of the dietary fat. Emulsification and absorption of dietary lipid is less efficient by deconjugated bile salts (Knarrerborg *et al*., 2004; Guban *et al*., 2006). Enzymes, known as bile salt hydrolases are expressed by the number of gastrointestinal bacteria including *Bacteroides*, *Clostridium*, *Enterococcus*, *Bifidobacterium*, and *Lactobacillus* (Whitt and Savage, 2001). On the other hand, oregano EO demonstrated antibacterial activity against a number of bacteria (Burt, 2004). This fact is supported by *in vitro* inhibition effect of oregano oil against the probiotic (Marcin *et al*., 2006). More recent research has shown that oregano oil, and its main component carvacrol, may inhibit ATPase activity when added in sub-lethal concentrations and this may play a role in the inhibition of bacterial growth (Gilla and Holley, 2006). Decrease in AP found in experimental animals indicates the down-regulation in the expression of this enzyme.

In contrary, increase in IAP activity may preceed the development of obesity (Mozeš *et al*., 2000), and there is evidence which indicates to the inverse relation between IAP and the rate of fat absorption in obese animals (Mozeš *et al*., 2007).

In conclusion, the results after administration of oregano (707.195 ppm) demonstrated decreased level of IAP which can be the consequence of strong antibacterial properties and improved fat digestion. Higher body performance could be influenced by the decrease of intestinal microflora, followed by the decrease of total plasma immunoglobulin. Future studies could investigate the determination of bacterial numbers in the ileum of birds and evaluate conjugated bile salts in the ileal content after administration of higher doses of oregano oil. The balance between levels of immune system, antibactericidal effects of carvacrol and animal feed efficiency performance in poultry is essential.

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UTICAJ EKSTRAKTA ORIGANA NA CREVA, VREDNOSTI NEKIH PARAMETARA KRVNE PLAZME I PRIRAST PILIĆA

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SADRŽAJ

U ovom radu su izneti efekti esencijalnog sastojka iz ulja origana na rast, aktivnost intestinalne alkalne fosfataze (IAP), proliferativnu sposobnost enterocita, koncentraciju ukupnih proteina i minerala krvne plazme (kalcijuma i magnezijuma) kod brojlerskih pilića hibrida Ross 308. Ogled je izveden pod komercijalnim uslovima tokom 42-dnevnog eksperimenta. Pilići koji su bili na ishrani sa origanovim uljem (0.707 gkg^{-1}) su imali značajno veći prirast u periodu rasta (19-29 dan) i završnog tova (30-42 dan). Koncentracija ukupnih imunoglobulina u krvnoj plazmi je bila manja, dok su koncentracije ispitivanih minerala bile veće tokom završnog perioda tova. Značajno opadanje aktivnosti intestinalne alkalne fosfataze je primećeno kod životinja na ishrani sa dodatkom ulja origana 29. i 42. dana eksperimenta. Proliferativna sposobnost enterocita duodenalnih resica je bila značajno veća u završnom periodu kod jedinki ogledne grupe.

Može se pretpostaviti da su bolji rezultati tova nastali usled povećanja svarljivosti hrane. Takođe se pretpostavlja da je antibakterijski efekat karvakrola imao za posledicu smanjenje broja crevnih bakterija, smanjenje koncentracije imunoglobulina i povećanje koncentracije ukupnih proteina krvne plazme.