The effect of feeding thyme, sage and rosemary oil on laying hen performance, cholesterol and some proteins ratio of egg yolk and Escherichia Coli count in feces

Einfluss des Einsatzes von Thymian-, Salbei- und Rosmarinöl im Futter auf Leistung, Dotter-Cholesteringehalt, Anteile einiger Dotterproteine und E. coli/Keimzahlen im Kot von Legehennen

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Material and Methods

Introduction

The use of antibiotics as growth promoters in animal feed has led to some unwanted advances in antibiotic resistance in certain bacterial pathogens (Botsoglou and Filetouris, 2001; Madrid et al., 2003; Moser et al., 2003). Since 2006 the use of antibiotic growth promoters is prohibited in the European Union (Nasir and Grashorn, 2006). Therefore, many studies were conducted to find alternatives for antibiotics (Langhout, 2000; Mellow, 2000; Wenk, 2000; Kamel, 2001). It was reported that essential oils derived from spices and herbs, prebiotics, probiotics, organic acids etc could be successfully used as growth promoters. In the present decade, Langhout (2000) and Williams and Losa (2001) discovered that essential oils have a stimulating effect on animal digestive systems. They postulated that these effects could be due to the increased production of digestive enzymes and the improved utilization of digestive products through enhanced liver functions. Therefore, specific essential oils and combinations of them provided a totally new approach for improving feed digestion.

Cowan (1999) and Faliero et al. (2003) reported that essential oils had antimicrobial actions in vitro. It has been reported that oregano, laurel, sage and myrtle included natural antimicrobial compounds such as carvacrol, thymol, limonene and cineol (Rebaud et al., 1997; Ultee et al., 2002). Some studies have shown that essential oils of rosemary (Rosmarinus officinalis), sage (Salvia sclarea), thyme (Thymus vulgaris) in this respect were the most active against strains of E. coli (Smith-Palmer et al., 1998; Hammer et al., 1999; Dorman and Deans, 2000).

Jambroz et al. (2003) determined that plant extract (carvacrol, cinnamaldehyde and capsaicin) reduced the total E. coli and Clostridium perfringens numbers in the intestines of broiler chickens. Mitisch et al. (2004) reported that blends of essential oil components can control Clostridium perfringens colonization in the intestine and feces of broiler chickens. Tucker (2002) reported that the supplementation of a mixed herbal product containing garlic, anise, cinnamon, rosemary and thyme to commercial pig diets significantly inhibited the number of E. coli in the digestive tract. Also, in vitro studies have shown essential oils to have antibacterial properties against Listeria monocytogenes, Salmonellatyphimurium, Escherichia coli, Bacillus cereus and Staphylococcus aureus (Cosentino et al., 1999). In addition to their antimicrobial activity, they possess biological activities as hypcholesteroleemics (Craig, 1999). The hypocholesterolemic effect of essential oils has been reported for chickens (Case et al., 1995; Bölükbaşi et al., 2007) and humans (Elson et al., 1989).

It was reported that the pure components of essential oils inhibit hepatic 3-, hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity which is a key regulatory enzyme in cholesterol synthesis (Crowell, 1999). Also, Qureshi et al. (1983) reported that a correlation between HMG-CoA reductase activity and either total or LDL cholesterol in chicken, but not with HDL cholesterol. All lipids of egg yolk are associated with proteins to form lipoproteins, which are commonly classified in low-density lipoproteins (LDL) and high-density lipoproteins (HDL) (Anton, 1998).

Unfortunately, little information has been published on the effects of the essential oil supplements on cholesterol and triglyceride ratios in egg yolk and plasma of laying hens. Therefore, the present study was conducted to test the antibacterial effects of thyme, sage and rosemary oils and their effects on laying hen performance, ratio of triglyceride and cholesterol in serum and egg yolk, some proteins of egg yolk and E. coli content of feces.

Experimental design and animals

Sixty four, 24- wk-old Lohman-LSL hybrid laying hens were used in this experiment. Birds were randomly assigned to 4 groups of 16 hens, each of which were distributed to 4 cages (50 x 46 x 46 cm) with four animals. The four dietary treatments included a control (basal diet), basal diet + 200 mg /kg thyme oil, basal diet + 200 mg /kg thyme oil and basal diet + 200 mg /kg thyme oil.
sage oil, and basal diet + 200 mg/kg rosemary oil. Composition of the experimental diets is presented in Table 1. During the experiment (12 wk) hens were fed and watered ad libitum. Feed intake, egg production and feed conversion were recorded daily. Egg quality characteristics including egg weight, Haugh units, and ratio of albumen, yolk and shell were measured biweekly using ten eggs from each dietary treatment.

At the end of the experiment, eight blood and egg samples were taken from each treatment in order to determine the ratio of triglyceride, egg yolk proteins and cholesterol. Also, feces samples were taken from each replicate in order to determine total Coliform and E. coli counts.

Collection of blood samples. Blood samples were taken from wing vena into blood tubes containing clot activator. Tubes were centrifuged at +4°C, 3000xg for 5’ and supernatant collected.

Isolation and homogenization of hen-egg yolk. Egg shells were broken manually, yolks were carefully separated from the white, washed gently with distilled water and rolled on Whatman 3 MM filter paper to remove any adherent egg white. The yolk membrane was then punctured with a disposable pasteur pipette and egg yolk was transferred into pre-weighted falcon tubes. 2 volumes of 20% SDS was added onto each g of isolated egg yolk in falcon tubes and homogenized at 1000 rpm for 2 min using an UltraTurrax homogenizer. The homogenate was aliquoted and used for SDS-PAGE and HPTLC analysis.

Total Lipid Extraction. Each volume of serum and egg yolk lipids was shaked vigorously with 1 volume of a mixture of n-hexane/2-propanol (3/2) (Merck, Darmstadt/Germany). After, centrifugation of suspension at +4°C, 2000 x g for 10’ upper phase was aspirated and used for HPTLC analysis (HARA and RADIN, 1978).

HPTLC. For separation and identification high performance thin-layer chromatography (HPTLC) plates (20x10 cm) (Merck, Darmstadt/Germany) were used. At the end of the plate five al portions of extracted lipids of egg-yolk and serum were spotted with a micropipette 2 cm from the bottom of HPTLC plates. The lipids were developed 6 cm from application point using a mobile phase of n-hexane: diethyl ether: formic acid (80:20:2 (v/v/v)) (Merck, Darmstadt/Germany). To visualize lipid classes, the entire plate was sprayed with a 10% CuSO₄ (w/v) in 8% H₃PO₄ (v/v) (Merck, Darmstadt/Germany) and charred at 180°C. After cooling HPTLC plates were evaluated by Phoretix 1D (TL120) software (DAMYANOVA, 2002).

SDS-PAGE. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SE 260 vertical electrophoresis unit and EPS 301 power supply, Amersham Biosciences Piscataway/USA) carried out by using Laemmli 4% stacking and 12% resolving gel. The serum and egg yolk extracts were diluted with 2x sample buffer (Sigma-Aldrich Chemie GmbH, Germany) and were applied to the gel. The electrophoresis was carried out at 20 mA/gel constant current for 90’. Proteins were visualized by silver staining (HEUKESHOVEN and DERNICK, 1988) and after drying evaluated by Phoretix 1D (TL120) software (LAEMMLI, 1970).

Bacteriology

Fecal samples were blended in a stomacher (Stomacher 400: AJ Seward, London, England) for 2 min in 50 mL of 0.85% (w/v) salt water. A series of fermentation tubes containing Fluorocult Lauryl sulfat broth (Merck, Germany) were inoculated with water and incubated for 48 hours at 35°C. The fermentation tube contained an inverted tube to trap gases produced by the Coliform bacteria. After 48 hours, the fermentation tube was examined for gas production. After that the tubes were examined under a 366-nm UV Lamp (Lampe UV, 4W/366nm; Merck, Germany) for E. coli. A table of most probable numbers was used to estimate the Coliform content of dilutions that showed positive results for Coliform and E. coli. The results were reported as most probable number (MPN) of Coliform and E. coli per g (ANONYMOUS, 1992).

Statistic analysis

Differences between groups were analysed by one-way analysis of variance (ANOVA), using the statistical package SPSS FOR WINDOWS (1999), version 10.0. Significantly different means were subjected to a multiple comparison test (Duncan).

Results

In this present study, significant differences were observed between the groups with respect to feed intake, feed conversion and egg weight (Table 2). All of the additive treatments significantly (P < 0.01) reduced feed intake compared with the control. Feed conversion ratios were significantly (P < 0.05) improved by the supplementation of the essential oils. Supplementing thyme, sage and rosemary oil to the basal diet increased (P < 0.05) egg weight compared to the control. The thyme oil and rosemary oil groups exhibited the highest egg weight compared to the other groups.
The effect of dietary treatments on some egg quality characteristics is shown in Table 3. There were no significant differences (P < 0.05) in proportion of albumen among the dietary treatments. Essential oils treatment significantly reduced proportion of yolk compared with the control. On the other hand, supplementing thyme, sage and rosemary oil to the basal diet increased (P < 0.05) proportion of egg shell above the control. The thyme oil group exhibited the highest shell rate compared to the other groups. Although rate of shell increased significantly, reason for this situation could not be found and explained in the present study.

Haugh unit was significantly influenced by treatment groups. The addition of sage oil to the basal diet led to a significantly higher (P < 0.05) Haugh unit than the addition of the other essential oils. Whereas, the groups receiving thyme oil and rosemary oil showed significantly lower Haugh unit compared to control group. Although rate of shell increased significantly, reasons for this situation could not be found and explained in the present study.

Control group showed the highest average count of Coliform and E.coli in the feces (Table 4). Supplementing thyme, sage and rosemary oils to the basal diet reduced (P < 0.05) total Coliform count compared to the control. The E. coli count was significantly higher (P < 0.05) than the addition of the other essential oils. Whereas, the groups receiving thyme oil and rosemary oil showed significantly lower E. coli count compared to control group.

The effect of dietary supplementation of thyme, sage and rosemary oil to the basal diet increased (P < 0.05) apovitellin Va and α-livetin/apovi-tellenin III, LDL proteins and apovitellin 3+4 and apolipoprotein CII, HDL proteins and α-livetin and phosphovitin increased very significantly compared to control group. The thyme oil and rosemary oil groups exhibited the lowest apovi-tellenin Vb compared to the control and sage oil groups. Apovitellin 7 was maximum in sage oil group. Apovitellin 7 and β-livetin were significantly influenced by treatment groups. Apovitellin 7 and β-livetin were very low (P < 0.01) in treatments with the addition of essential oils (Table 6).
laying hens, cholesterol and triglycerides ratio of egg yolk and *Escherichia coli* (*E. coli*) count in feces. Supplementation of essential oils reduced feed intake but improved feed conversion and egg weight of animals in this study. This may be caused by the stimulating effects of the oils on digestive enzymes as reported by LANGHOUT (2000) and WILLIAMS and LOSA (2001). In contrast to our results, BOTSOGLU et al. (2005) reported that the addition of rosemary, oregano and saffron to a layer diet had no significant effect on egg production, feed intake and feed conversion rate. Similarly, FLOROU-PANERI et al. (2005) reported that the supplementation of the oregano oil did not significantly affect egg weight, feed intake and feed conversion rate of laying hens. On the other hand, HERTRAMPF (2001) reported that essential oils derived from spices and herbs could be successfully used as growth promoters, since they increased the feed intake due to their aromatic characteristics in chickens. Although the egg productions did not differ between the groups in the present study, ÇABUK et al. (2006) reported that the addition of essential oil resulted also in a significantly higher (P < 0.05) egg production than for control diet.

In accordance with the present findings, BOTSOGLU et al. (2005) reported that rosemary oil lowered Haugh unit in laying hen. Contrasting results have been presented by FLOROU-PANERI et al. (2005) who reported that a diet supplemented with oregano oil had no effect on Haugh unit, proportion of yolk and shell.

Sage oil was less effective in antimicrobial activity (*E. coli*) than it was either thyme oil or rosemary oil in this study. Since, to our knowledge, no data dealing with the antimicrobial effect of essential oils supplemented to layer diets are available in literature present findings have importance with respect to the use of these aromatic plants in hen feeding. Therefore, the present findings were compared with other studies mainly conducted with broilers.

In accordance with the present findings, JAMROZ et al. (2003) reported that plant extract (carvacrol, cinnamaldehyde and capsaicin) reduced total *E. coli* numbers in intestine of broiler chickens. TUCKER (2002) also reported that supplementation of a mixed herbal product containing garlic, anise, cinnamon, rosemary and thyme to commercial pig diets significantly inhibited the number of *E. coli* in the digestive tract. However, HAGMULLER et al. (2006) published data that supplementation of thyme had no significant effect on the rate of *E. coli* in feces of piglets. Also, in vitro studies have shown that thyme (*Thymus vulgaris*) was most active against strains of *E. coli* (COSENTINO et al., 1999; DORMAN and DEANS, 2000; HAMMER et al., 1999; SMITH-PALMER et al., 1998).

Egg yolk cholesterol and triglyceride levels were not reduced by supplementation of essential oils (thyme, rosemary and sage). But, significant changes were observed in cholesterol and triglyceride levels in this study. Since, to our knowledge, no data dealing with the antimicrobial effect of essential oils supplemented to layer diets are available in literature present findings have importance with respect to the use of these aromatic plants in hen feeding. Therefore, the present findings were compared with other studies mainly conducted with broilers.

### Table 5. Ratios of cholesterol and triglyceride as a percentage of total lipids in serum and egg yolk (*n* = 10)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Egg yolk (%)</th>
<th>Serum (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Triglyceride</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>Control</td>
<td>58.30</td>
<td>14.90</td>
</tr>
<tr>
<td>Thyme oil</td>
<td>60.20</td>
<td>14.57</td>
</tr>
<tr>
<td>Sage oil</td>
<td>64.16</td>
<td>15.71</td>
</tr>
<tr>
<td>Rosemary oil</td>
<td>62.18</td>
<td>14.54</td>
</tr>
<tr>
<td>SEM</td>
<td>1.70</td>
<td>0.57</td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

*P* < 0.05; **P** < 0.01; NS: not significant; a,b,c: Column means with no common superscript differ significantly

### Table 6. Percentage compositions of some egg yolk proteins (*n* = 10)

<table>
<thead>
<tr>
<th>Names of yolk proteins</th>
<th>Control</th>
<th>Thyme oil</th>
<th>Sage oil</th>
<th>Rosemary oil</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apovitellenin Va³</td>
<td>3.75c</td>
<td>5.54a</td>
<td>4.91b</td>
<td>5.49ab</td>
<td>0.88</td>
<td>**</td>
</tr>
<tr>
<td>Apovitellin 3+4²</td>
<td>0.67c</td>
<td>1.35a</td>
<td>0.81bc</td>
<td>0.97b</td>
<td>0.27</td>
<td>**</td>
</tr>
<tr>
<td>Apovitellenin Vb³</td>
<td>2.06a</td>
<td>1.26b</td>
<td>1.87a</td>
<td>1.08b</td>
<td>0.11</td>
<td>**</td>
</tr>
<tr>
<td>Apovitellenin V¹</td>
<td>1.95b</td>
<td>1.72c</td>
<td>2.30a</td>
<td>1.42d</td>
<td>0.09</td>
<td>**</td>
</tr>
<tr>
<td>α-livetin</td>
<td>12.32a</td>
<td>14.87b</td>
<td>14.41c</td>
<td>17.45a</td>
<td>0.55</td>
<td>**</td>
</tr>
<tr>
<td>Phosvitin</td>
<td>3.96c</td>
<td>4.67b</td>
<td>4.03c</td>
<td>5.05a</td>
<td>0.14</td>
<td>**</td>
</tr>
<tr>
<td>α-livetin/apovitellenin III¹</td>
<td>22.99d</td>
<td>26.23c</td>
<td>26.81a</td>
<td>26.58b</td>
<td>0.46</td>
<td>**</td>
</tr>
<tr>
<td>Apovitellin 7²</td>
<td>1.36a</td>
<td>0.81bc</td>
<td>0.78c</td>
<td>0.94b</td>
<td>0.07</td>
<td>**</td>
</tr>
<tr>
<td>β-livetin</td>
<td>43.06c</td>
<td>21.48d</td>
<td>28.04b</td>
<td>24.55c</td>
<td>2.51</td>
<td>**</td>
</tr>
<tr>
<td>Apolipoprotein CIP²</td>
<td>2.35d</td>
<td>11.40b</td>
<td>9.88c</td>
<td>12.87a</td>
<td>1.22</td>
<td>**</td>
</tr>
</tbody>
</table>

³: LDL apoproteins; ²: HDL apoproteins; **P** < 0.01; a,b,c: Means within the same row with no common superscript differ significantly
thone, menthol, fenchone, fenchyl and, alcohol have been shown to suppress hepatic HMG-CoA reductase activity (Clegg et al., 1980; Middleton and Hu, 1982; Yu et al., 1994). It has also been reported that thymol, carvacrol and beta-ionone might induce a putative regulatory non-sterol product(s) (Case et al., 1995; Elson, 1996). Bölükbasi et al. (2007) reported that thyme oil (200 or 300 mg/kg) lowered plasma cholesterol and triglyceride in laying hen. Controversially, Sareca et al. (2006) reported that total plasma cholesterol concentrations were not significantly changed by supplementation of dietary thyme (1 g/kg) in broilers.

It is well known that all lipids of egg yolk are associated with proteins to form lipoproteins, low-density lipoproteins (LDL) and high-density lipoproteins (HDL) (Anton, 1998). Bernardi and Cook (1960) have shown that the HDL fraction of egg yolk (lipovitellin) consists of two forms, α- and β-lipovitellin (apovitellin). Lipoproteins contain apoproteins in their outer shell, which have important roles in lipid transport and metabolism. The effects of essential oils on apoproteins were tested in the present study. Although, it was found that some HDL apoproteins (Apovitellenin Vb, Apovitellenin V) and LDL apoprotein (apovitellin 7) decreased. As to our knowledge, no publication exists on this topic in literature no comparison could be made with other studies. Further work is needed in order to give more detailed information on this topic in chickens.

In conclusion, the data of the present study showed that feeding laying hens with diet containing thyme, sage and rosemary oil improved egg weight and feed conversion. The addition of sage oil to the laying hens’ feed led to a significant increase in the Haugh unit of the egg. It was also determined that E. coli concentration of feces samples was reduced significantly (P < 0.05) with usage of thyme oil and rosemary oil in laying hens diets. Thyme oil and rosemary oil exhibited a higher antimicrobial activity than sage oil. Results showed that there were no significant (P < 0.05) differences in the ratio of triglyceride and cholesterol of egg yolk among the dietary treatments.

Acknowledgement
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Summary
The aim of the present study was to investigate the effect of dietary supplementation of thyme, sage and rosemary oil on performance of laying hens, cholesterol and some proteins ratio of egg yolk and Escherichia coli (E. coli) count in feces. Sixty four Lohman LSL laying hens with 24 weeks of age were randomly and equally assigned to four groups (n = 16). Each treatment consisted of 4 replications of 4 hens. The control group received a basal diet. In addition to the basal diet, the three experimental diets included one of the following supplements: 200 mg/kg thyme oil, 200 mg/kg sage oil, 200 mg/kg rosemary oil.

Feed conversion of animals was improved by supplementation of thyme, sage and rosemary oil. Birds that were fed the 200 mg /kg thyme oil and 200 mg/kg rosemary oil diets, exhibited the largest egg weight in this study. The addition of sage oil to the laying hens feed led to a significant increase in the Haugh unit of the egg. It was also determined that E. coli concentration of feces samples was reduced significantly (P < 0.05) with usage of thyme oil and rosemary oil in laying hens diets. Thyme oil and rosemary oil exhibited a higher antimicrobial activity than sage oil. Results showed that there were no significant (P < 0.05) differences in the ratio of triglyceride and cholesterol of egg yolk among the dietary treatments.

Key words
Layer, thyme oil, sage oil, rosemary oil, E. coli, egg production, cholesterol

Zusammenfassung
Einfluß des Einsatzes von Thymian-, Salbei- und Rosmarinöl im Futter auf Leistung, Dotter-Cholesteringleichheit, Anteile einiger Dotterproteine und E. coli Keimzahlen im Kot von Legehennen


Stichworte
Legehennen, Thymianöl, Salbeiol, Rosmarinöl, E. coli, Legeleistung, Cholesterin

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