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EFFECTS OF FEEDING THYMOL AND ISOEUGENOL ON PLASMA TRIGLYCERIDES AND CHOLESTEROL LEVELS IN JAPANESE QUAIL

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ABSTRACT

Dietary supplementation can be considered one of the main strategies to include new additives with beneficial effects into animal products. Thymol supplementation has shown to improve oxidative stability of eggs and meat during storage and also to increase the relative composition of polyunsaturated fatty acids. Feed supplementation with isoeugenol has shown to increase hatching success. The present study evaluates supplementation with thymol and isoeugenol on plasma lipid profile in Japanese quail. At 4 weeks of age, 48 males and 96 females were housed in groups of 1 male and 2 females and assigned during 10 weeks to 1 of 4 diet treatments: vehicle or 400 $mgkg^{-1}$ of butylated-hydroxytoluene (controls), and 400 mg kg^{-1} of thymol or isoeugenol. Body weight, growth rate, and egg production were determined. At 14 weeks of age, plasma lipids (total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides) were also determined. Results demonstrated that supplementation with thymol and isoeugenol increased female triglycerides compared to males and to control females. No triglyceride changes were induced by butylated-hydroxytoluene. Body weight, growth rate and egg production were not affected by dietary treatments. The increased plasma triglycerides observed in female birds may have biological relevance to better sustain the metabolic needs during laying period. These results contribute to show the potential usefulness of these essential oil main components as natural feed supplement alternatives for poultry.

Keywords: Essential oil component, diet, poultry, phytogenic feed additive, plasma lipid.

INTRODUCTION

Dietary supplementation can be considered one of the main strategies to include new additives with beneficial effects into animal products. In the past decades, the consumption and production of poultry meat and eggs, as well as the consumer´s concern on the quality of these products has been greatly increased (Min and Ahn, 2005). Furthermore, recent public concern about the use of synthetic compounds in animal diets to enhance health and performance coupled with changes in some countries´ regulations on the use of synthetic medicaments, has stimulated the interest and research on the use and effects of phytochemicals in the diets of farmed animals (Acamovic and Brooker, 2007). In this regard, it has been proposed that herbs and spices could help to sustain the good health and welfare of animals and to improve their performance (Brenes and Roura 2010; Krishan and Narang 2014; Rezaeipour *et al*. 2015; Akbari *et al*. 2016).

Essential oils are complex mixtures of secondary plant metabolites consisting of low-boiling-phenylpropenes and terpenes (Brenes and Roura, 2010; Krishan and Narang, 2014) which are commonly used in the flavors and fragrances market (Van de Braak and Leijten, 1999). Several recent reports on various beneficial effects of essential oils or their main components when supplemented in poultry diets have been well summarized (Lee *et al*., 2004; Brenes and Roura, 2010; El-Hack *et al*., 2016). A number of biological activities has been observed including antimicrobial (Dorman and Deans, 2000; Rota *et al*., 2004; Ahmad *et al*., 2016), antioxidant (Botsoglou *et al*. 1997; Bulbul *et al*. 2015), digestive stimulant (Platel and Srinivasan, 2004), hatching success and fertility promoter (Cetingul *et al*., 2009; Luna *et al*., 2012), anti-stress (Labaque *et al*., 2013) and egg production enhancer (Yesilbag *et al*., 2013). Because of the wide range of biological properties mentioned above, further research on the potential beneficial use of these compounds as feed supplements may be of practical relevance.

Because lipids are required for the biogenesis of cell membranes and components, and also to provide the avian embryo with almost all energy needed to sustain development within the egg (Speake *et al*., 1998) changes in serum triglyceride or cholesterol levels can not only affect the quality of the bird's products but also their performance. Sex differences in lipid metabolism have been reported with laying hens showing an estrogen enhanced hepatic lipogenesis in order to meet the demand for vitellogenesis (Hermier, 1997). Vitellogenesis is characterized by increased levels of plasma triglycerides (TG) (and limited low density lipoprotein; LDL catabolism), with subsequent higher levels in laying hens compared to males (Walzem *et al*., 1994) and immature hens (Griffin *et al*., 1982; Walzem *et al*., 1994; Hermier, 1997; Schneider, 2016). Cholesterol (chol) is first biosynthesized in the liver of birds, secreted into the plasma and distributed to tissues and oocytes by very low density lipoproteins and LDL, and accompany the oocyte growing through lipid deposition with a coordinated intervention and function of several members of the low density lipoprotein receptor gene family (Schneider, 2007; Osorio and Flores, 2011, Schneider, 2016), which are also recognized triglyceride rich lipoproteins (Griffin *et al*., 1982; Walzem *et al*., 1994; Hermier, 1997).

It has been shown that the biological activity and the essential oil chemical composition may vary with the geographic location, growth conditions and the part of the plant used to extract these oils, as well as the extraction and isolation methods used to obtain them (Faleiro *et al*., 2005). Indeed, those oil chemical variations may underlie the lack of consistency found between some of the studies that evaluate feed supplementation of chemically similar essential oils (Cetingul *et al*., 2009; Christaki *et al*., 2011; Yesilbag *et al*., 2013). Therefore, evaluating the effects of single essential oil components would help to discriminate which molecules are involved in the observed bioactive effects. This study focuses on the effects of the natural phenols thymol (2-Isopropyl-5-methylphenol) and isoeugenol (4-hydroxy-3-methoxy-1-propenylbenzeneclove) which are the main components of essential oils including oregano, thyme, and clove respectively. Thymol (THY) has the "generally recognized as safe" (GRAS) status, endorsed by the Flavor and Extract Manufacturers' Association (FEMA) and Food and Drug Administration (FDA) of the USA with safety levels calculated in a wide range of animal species by the European Food Safety Authority (FEEDAP 2012). Furthermore, THY has been characterized and authorized as a food additive and is commonly used in the food industry. In birds, THY has shown to improve the oxidative stability of chicken eggs (Botsoglou *et al*., 1997), meat during storage (Luna *et al*., 2010) and it also increased polyunsaturated fatty acids when used in combination with its isomer carvacrol (Hashemipour *et al*., 2013). Specifically in quail, thymol diet supplementation has also shown a potential for increasing hatchability and fear reducing properties (Luna *et al*., 2012; Labaque *et al*., 2013). Hypocholesterolemic effect of dietary THY was also recently reported by Saadat Shad *et al*. (2016) on broiler chickens exposed to heat stress. On the other hand, little is known about the use of isoeugenol as a feed additive for animals. A few reports have provided information about the potential antioxidant activity of isoeugenol administration (Rajakumar and Rao, 1993; Tuckey *et al*., 2009).

The objective of this study was to evaluate the effects of a feed supplementation with thymol and isoeugenol on plasma lipid profile in Japanese quails. This enables us to address three main questions. Firstly, can the supplemented phenols alter the circulating levels of triglycerides and cholesterol? Secondly, are those effects similar in magnitude? Thirdly, considering the gender differences in lipid metabolism mentioned above, can these supplements differentially affect males and females?

Experimental Animals and Husbandry*:* All experiments were carried out in the Biological and Technological Research Institute (CONICET-UNC), in the Poultry Science Group, National University of Cordoba, Argentina, in accordance with *international standards of care and use of laboratory animals* and our Institutional Committee on Care and Use of Animals. Japanese quail (Coturnix coturnix) were used in the present study not only because they are considered an important agricultural species for meat and egg production in many countries (Minvielle, 2004) but also because they are considered a useful animal model for the extrapolation of data to chickens and other commercially important poultry species (Minvielle, 2004). Egg incubation, chick brooding, and lighting procedures were according to previous studies (Shanaway, 1994; Luna et al., 2012). Brooding temperature was set at 37.8°C during the first week of life, with a weekly decline of 3.2°C until final room temperature of 25 ± 2°C was achieved. Water and a starter feed ration (240 g Crude Protein (CP) and 12 MJ ME/Kg) were provided ad libitum. Feed composition (Marcelo E. Hoffman e Hijos S.A., Entre Ríos, Argentina) included corn meal, soybean meal, wheat shorts, sunflower meal, limestone, sodium chloride, dicalcium phosphate, vitamins and minerals. At 28 days of age, quail were sexed by plumage coloration, individually weighted and wing banded for later identification. Forty eight males and ninety six females with similar body weight were randomly housed in groups of one male and two females into cages measuring 20 × 45 × 25 cm (length × width × height). At this time, birds were also switched to a breeder ration (200 g CP and 12.14 MJ ME/kg) with feed and water continued ad libitum. Birds were subjected to a 14-hours light (between 06.00 to 20.00 hours; approximately 180 cd), 10 hours dark cycle. Daily maintenance and feeding chores were carried out at the same time each day (09.00 hours).

Treatments and traits measured: At 4 weeks of age, all quail within each cage were assigned to one of four feed treatments: Control (basal diet with vehicle), 0.40 g of BHT per kg of basal diet, 0.40 g of thymol per kg of basal diet, or 0.40 g of isoeugenol per kg of basal diet.

Doses were selected following Luna *et al* (2012). Supplements were prepared in a 5 gL^{-1} ethanol (vehicle) solution that was uniformly sprayed weekly over fresh commercial feed (Luna *et al*., 2010, 2012). The chemicals used in this study were reagent grade commercial products. Feed supplements were obtained commercially: thymol (SAFC®, ≥99%, FCC, USA) and Isoeugenol (SAFC®, ≥99%, mixture of cis and trans, FCC, USA). The thymol and isoeugenol doses were selected considering that same doses produced favorable changes egg´s hatchability (Luna *et al*., 2012).

Individual body weights were measured prior to starting feed supplementations (4 weeks of age) and at the end of the study (14 weeks of age). Growth rate during the supplementation period was registered. Daily egg production was recorded from 6 to 11 weeks of age to calculate a cumulative hen-day-egg production (HDEP).

After the 10 weeks of supplementation, blood samples were collected in labeled sterile test tubes and centrifuged at 3000 x g for 10 minutes to isolate serum. Total cholesterol, HDL-cholesterol and triglycerides (TG) were determined by colorimetric enzymatic commercial kits (Wiener Lab). In concordance with Bölükbaşi *et al.* (2006), low density lipoprotein cholesterol (LDL-cholesterol) levels were estimated using the Friedewald equation (Friedewald *et al*., 1972).

Statistical Analysis: A Two-way ANOVA was used to determine the effects of dietary supplementation (control, BHT, THY and isoeugenol), quail sex (male and female) and their interaction on TG, Total cholesterol, LDL-cholesterol, HDL-cholesterol, final body weight and growth rate. A One-way ANOVA was used to determine the effects of dietary supplementation (control, thymol, isoeugenol and BHT) on hen day egg production (HDEP). Fisher-LSD tests were used for post-hoc comparisons. To test the hypothesis, a P<0.05 was considered statistically significant.

RESULTS

Plasma TG male and female response to dietary supplementation with thymol, isoeugenol and BHT are shown in Figure 1. ANOVA showed a significant effect of sex ($P < 0.001$) as well as a significant interaction ($P < 0.05$) between sex and dietary supplementation. Post-hoc analysis showed that, while males were not affected by feed supplementation, females supplemented with thymol or isoeugenol showed a significant ($P< 0.05$) increase in TG levels compared to both males of the same supplemented treatment and to control females. Females supplemented with BHT showed intermediate values that did not significantly differ from their controls or from the thymol or isoeugenol supplemented groups.

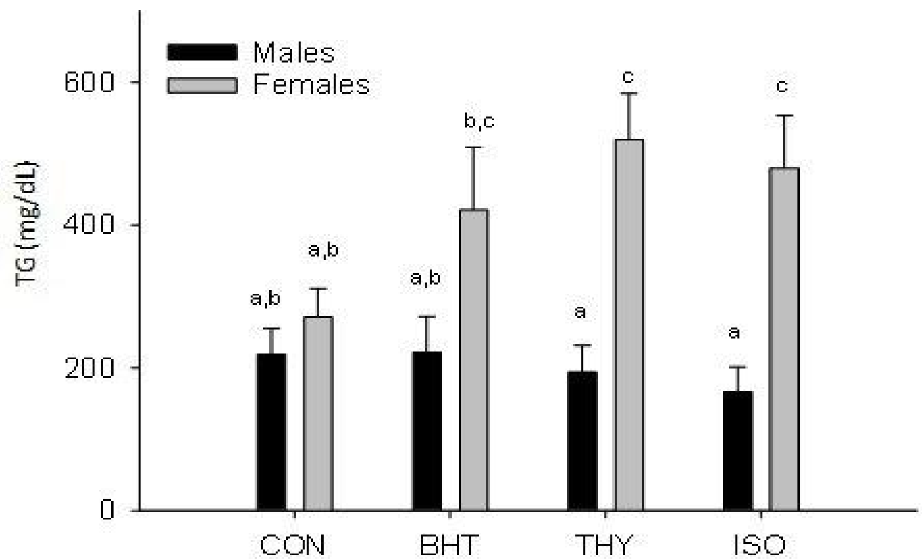

Figure 1. Total plasma triglycerides (TG) in male and female quail supplemented with vehicle (control), butylated hydroxy toluene (BHT), thymol (THY) or isoeugenol (ISO) (0.4 g/kg feed). [a-c] Bars not sharing a common letter differ statistically at P<0.05.

No significant effects of sex or dietary supplementation were either detected for cholesterol parameters evaluated (Total cholesterol, HDL-cholesterol, LDL-cholesterol, and LDL/HDL cholesterol ratio) (Table 1).

Table 1. Mean (± SEM) total cholesterol (Chol), HDL-chol, LDL-chol and LDL/HDL cholesterol ratio in male and female quail supplemented with vehicle (control), butylated hydroxy toluene (BHT), thymol or isoeugenol (400 mg/kg of feed).

	Control		BHT		Thymol		Isoeugenol	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Total Chol (mg/dL)	144.2 ± 17.0	144.9 ± 25.1	134.6 ± 7.3	149.7 ± 16.6	162.6 ± 18.4	145.7 ± 16.6	121.5 ± 6.8	129.0 ± 10.0
HDL-chol (mg/dL)	116.8 ± 15.8	111.2 ± 15.4	108.7 ± 6.3	112.3 ± 10.9	126.8 ± 16.2	106.9 ± 11.5	98.6 ± 4.9	105.7 ± 8.6
LDL-chol (mg/dL)	27.5 ± 2.8	33.5 ± 10.4	25.8 ± 3.6	30.7 ± 6.3	35.8 ± 4.6	31.3 ± 4.7	22.8 ± 2.3	23.3 ± 2.8
LDL/HDL	0.26 ± 0.04	0.27 ± 0.04	0.25 ± 0.04	0.28 ± 0.04	0.31 ± 0.04	0.31 ± 0.04	0.23 ± 0.02	0.23 ± 0.03

As expected for this animal model, final body weight and growth rate were significantly higher ($P < 0.05$) in female quail compared to males, whereas final body weight (FBW), growth rate (GR), and cumulative HDEP were not altered by dietary supplementation (Table 2).

Table 2. Mean (± SEM) final body weight (FBW), growth rate (GR) and hen-day egg production (HDEP) in male and female quail supplemented with vehicle (control), butylated hydroxy toluene (BHT), thymol or isoeugenol (400 mg/kg of feed).

	Control		BHT		Thymol		Isoeugenol	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
FBW	**225.4^a ± 7.9**	**267.7^b ± 6.1**	**229.6^a ± 7.2**	**260.5^b ± 5.2**	**229.9^a ± 6.1**	**260.1^b ± 6.7**	**225.5^a ± 6.9**	**256.1^b ± 7.2**
GR	2.62^a ± 0.12	2.94^b ± 0.08	2.57^a ± 0.12	3.04^b ± 0.06	2.56^a ± 0.08	2.84^b ± 0.07	2.58^a ± 0.07	2.88^b ± 0.09
HDEP	--	0.88 ± 0.02	--	0.82 ± 0.04	--	0.85 ± 0.02	--	0.82 ± 0.07

Note: $^{a-b}$ Means within rows with different superscripts differ at $P < 0.05$

DISCUSSION

Females supplemented with thymol or isoeugenol showed an increase in TG levels compared to both males of the same supplemented treatment and to control females. Females treated with BHT showed intermediate TG values. These results are in accordance with Bölükbaşi *et al*. (2006), who reported increases in serum TG when 100 or 200 ppm of Thyme oil were included into broilers diet. On the other side when THY was supplemented to broilers under heat stress, no changes in serum TG were reported (Saadat Shad *et al*. 2016). Considering that in our study the changes in TG levels after feed supplementation were only observed in females but not in males, the more plausible explanation is that the supplemented natural phenols are involved in changes at certain levels of the lipid metabolic pathways that are also hormonally regulated (a regulation that differs between males and females). As mentioned, sex differences in lipid metabolism have already been reported, with laying hens showing an estrogen enhanced hepatic lipogenesis in order to meet the demand for vitellogenesis (Hermier, 1997). Considering that, during the egg formation process, a quail laying hen needs to provide each egg with about 2-3 g of lipids, and at a very high laying frequency (approximately 9 eggs every 10 days) (Shanaway, 1994), the observed increase in TG levels with thymol and isoeugenol supplementation may represent a biological advantage to better sustain egg production. To better visualize the magnitude of the female metabolic challenge that needs to be overcome during egg formation, it may be relevant to mention that the laying process involves an average daily egg fat deposition that represents between 0.8 to 1.25 % of the total female body weight. Sarica *et al*. (2009) reported that oregano essential oil (EO) supplementation was able to decrease plasma TG in male quail. Similar findings were also reported by Khaksar *et al*. (2012), when dietary supplementation with thyme EO lowered serum TG in male Japanese quail. However, our study using thymol (a main component of oregano and thyme EO) and isoeugenol supplementation did not show any significant changes in male TG levels. Differences in doses, potential EO compounds interactions, and experimental conditions used between studies could explain this inconsistency. On the other hand, BHT did not show significant effects on TG levels neither on female nor in male suggesting *a priori* that supplementation with this synthetic antioxidant is not affecting the lipid TG metabolic pathways or their absorption.

Al Ankari *et al*. (1998) showed a direct correlation between plasma TG levels and egg production. This direct correlation is not being supported by our results, where the increased levels of female plasma TG as a consequence of feed supplementation did not affect egg production. Moreover, no negative effects on growth performance were detected either for thymol or isoeugenol dietary supplementation, with same result on the BHT supplemented group. The absence of significant changes on those productive parameters is consistent with previous study by Luna *et al*. (2012) where no effects of thymol and isoeugenol feed supplementation were found on egg production and body weight. Cetingul *et al*. (2007) and Christaki *et al*. (2011) reported similar results when oregano leaves were used as supplement quail diets. On the other hand, Yesilbag *et al*. (2013), showed improvements in egg production as a consequence of rosemary EO supplementation and Bampidis *et al.* (2005), reported potential growth promoting effects with oregano leaves supplementation. This information allows us to hypothesize that the potential improvements observed in those studies are due to the either other EO components and/or their interaction with thymol. More research should be conducted in order to identify which compound/s is responsible for the observed results.

Lee *et al*. (2004) and Mo & Elson (2006) reviewed hypocholesterolemic effects of some essential oils and main components on poultry diets. On the other hand, Cetingul *et al*. (2007) and Cetingul *et al*. (2009) reported no effect of dietary oregano (10-50 g dried natural oregano leaf/kg feed) on plasma cholesterol in laying quail which suggests that the effects on cholesterol levels may be dependent on the particularities of experimental situation and the species evaluated. In our study, no effects of dietary supplementation were detected on any of the plasma cholesterol parameters evaluated (total cholesterol, LDL-cholesterol, HDL-cholesterol or LDL/HDL cholesterol) when thymol (the main component of oregano EO) or isoeugenol were supplemented to male or female quail in doses of 0.4 g/kg of feed. If we consider that oregano essential oil contains approx. 30% of thymol (Dambolena *et al*., 2010) and that oregano leaves contain approx. 0.1% of essential oil, the thymol doses supplemented per Kg feed in this study represents the thymol from approximately 1.3 kg of oregano leaves. Our results would suggest that the effects of EO (containing thymol) reported in other studies (Khaksar *et al*., 2012; Yesilbag *et al*., 2013) may be dependent on other components of the EO mixture and/or the interaction of some of the components rather than on thymol itself. This hypothesis is also consistent with the lack of effects of thymol supplementation on cholesterol levels observed both in domestic quail and chickens (Lee *et al*., 2003; Cetingul *et al*., 2009). Due to the higher amount and high structural diversity of components of oregano and clove EOs, the studies supplementing herbs, EO, extracts or pure components should not be compared directly. Nevertheless, it is important to recall the significance of developing studies using single components that would help to elucidate the mechanism of action of each particular supplemented compound.

Results demonstrated that supplementation with thymol and isoeugenol increased female triglycerides compared to males and to control females. Cholesterol levels, body weight, growth rate and egg production were not affected by dietary treatments. The increased plasma triglycerides observed in female birds may have biological relevance to better sustain the metabolic needs during laying period. These results contribute to show the potential usefulness of these essential oil main components as natural feed supplement alternatives for poultry.

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