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Effects of fermented *Camilla sinensis*, Fuzhuan tea, on egg cholesterol and production performance in laying hens

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Fuzhuan tea, which is a Chinese dark tea produced by fermentation with the non-pathogenic fungus *Eurotium cristatum*, has reportedly demonstrated lipid lowering effects in rat models. These published studies suggest that fuzhuan tea may be a suitable feed additive for laying hens to reduce egg cholesterol levels. Therefore, a 5 week feeding study was conducted to determine the effects of tea-supplementation on egg cholesterol and production performance in laying hens. A total of 136 laying hens were randomized into three diet groups and fed *ad libitum* a basal diet (control), or diets supplemented with milled fuzhuan tea or its water-soluble extracts(treatment groups). Tea supplemented hens demonstrated significant improvement in laying performance with increased egg production of 36% (milled tea) and 24% (tea extracts) compared to control animals. Both treatments also resulted in decreases in serum total cholesterol (TC) and egg yolk cholesterol. No significant differences between groups were observed with respect to egg weight, yolk ratio or other serum lipid parameters. In conclusion, supplementation of chicken feed with fuzhuan tea may be a safe and effective means of improving feed efficiency and production in layer hen operations while resulting in eggs with a lower cholesterol content.

Key words: layer hen, Fuzhuan tea, *Camellia sinensis*, HPLC, total cholesterol, egg production, feed efficiency

INTRODUCTION

Eggs are one of the most widely consumed animal food products and are generally considered to be an important source of unsaturated fats, essential amino acids, folate and other B vitamins. There is also some evidence to suggest that consumption of eggs can decrease blood glycemic index (Pelletier *et al.*, 1996) and raise high-density lipoprotein cholesterol (HDL) levels compared to carbohydrate rich diets (Packard *et al.*, 1983; Schnohr *et al.*, 1994). A 20 ounce egg contains between 186-213 mg of cholesterol, and some studies have linked high dietary cholesterol to increased risk of cardiovascular disease (CVD) (Krauss *et al.*, 1996). However, recent investigations suggest that diets high in saturated fats rather than high in cholesterol have a greater impact on CVD risk, and that about 75% of the population show little or no increase in plasma cholesterol levels when challenged with a high cholesterol diet (Fernandez, 2010). Despite new findings, the American Heart Association's Nutrition Committee still recommends limiting dietary cholesterol intake to 300 mg per day or 200 mg per day for people with heart disease or diabetes (Lichtenstein *et al.*, 2006). As egg consumption has tripled worldwide over the past 40 years, a new market for "designer" eggs with value added traits such as increased omega-3 fatty acids or decreased cholesterol has emerged (Hernandez *et al.*, 2009). To meet this

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Table 1. Chemical major components of Fuzhuan tea. Table adapted from data on Fuzhuan tea chemical composition in previously published work (Wu *et al*., 2010)

Fuzhuan Tea Components	Amount (mg/g)
Catechins	30
Gallocatechin	9
Epigallocatechin	4
epigallocatechin gallate	8
gallocatechin gallate	3
epicatechin gallate	3
(±)-catechin	1
Epicatechin	3
Total Polyphenols	65
Polysaccharides	80
Amino Acids	20
Organic Acids	50
oxalic	6
Pyruvic	1
D-malic	7
a-ketoglutaric	3
Ascorbic	3
Lactic	6
Acetic	9
Citric	8
L-malic	6
Succinic	3

demand, new approaches to alter the nutritive properties of eggs are being developed, including genetic selection of laying hens and exploring feed additives that convey positive egg traits (Wang and Tong, 2002).

Tea (*Camellia sinensis* L.) is the second most consumed beverage world-wide and contains bioactive compounds such as polyphenols, caffeine, theanines, and vitamins. Numerous health benefits have been attributed to these phytochemicals including anti-oxidant and anti-cancer properties as well as the regulation of blood lipid metabolism (Zhao, 2003; Chen, 2009; Yung *et al*., 2008; Alshatwi *et al*., 2010). Fuzhuan tea (aka Fu Brick tea, PHatea), a Chinese tea whose leaves undergo a unique process of fermentation with the fungus *Eurotium cristatum* (Raper and Fennell) Malloch and Cain, has distinct phytochemical profiles compared to other teas (Wu *et al*., 2010; Luo *et al*., 2012; Fu *et al*., 2008). Several studies suggest that consumption of fuzhuan tea has significant human health effects on metabolic regulation of blood lipids (Xiao, 2007; Fu *et al*., 2011), and thus could potentially lower egg cholesterol levels when used as an additive in chicken feed. Therefore, the objective of this study was to test milled fuzhuan tea and its water-soluble extracts as feed additives for laying hens to determine their effects on production parameters and egg traits.

MATERIALS AND METHODS

Raw Material and Sampling. Fuzhuan tea: Special package *"Golden Xiangyi" Brick Tea* produced in 2007 was obtained from Yiyang Tea Manufacturer in Changsha, Hunan, China. Milled samples were produced by pulling apart compressed tea bricks by hand and milling with a comminutor. Water-soluble extracts were produced by brewing loose leaf fuzhuan tea with sterile distilled water for 45 minutes at 90°C at a ratio of 1:8 (w/v) followed by a second extraction of 1:7 (w/v). The two fuzhuan tea extracts were combined and then concentrated to powder by lyophilization. A phytochemical analysis of the fuzhuan tea based on data obtained from previously published reports (Wu *et al*., 2010) is shown in Table 1.

Animals and Diets. One hundred thirty six 22-week old Roman brown laying hens with a mean body weight of

Table 2. Ingredient and nutrient composition of the basal diet fed to laying hens

Ingredient	g/kg	Nutrient composition	g/kg
Maize	545.0	Crude protein	162.6
Rice bran	100.0	Crude fat	35.7
Soybean meal	180.0	Calcium	36.0
Rapeseed meal	60.0	Total phosphorus	6.5
$CaHPO_4$	8.0	Non-phytate phosphorus	2.9
Milled limestone	87.0	Lysine	7.3
*Premix	20.0	Crystine	6.5
Total	1000.0	Threonine	5.6

Metabolizable energy (kcal/kg)= 2,507

* provides (mg/kg diet): retinol 2.4, cholecalciferol 0.075, DL-α tocopherol acetate 20, menadione 2, thiamin 1.5, riboflavin 6, pyridoxol 3.5, cobalamin 0.01, niacin 25, panthotenic acid 8, folic acid 1, d-biotin 0.03, ascorbic acid 30, choline chloride 600; Mn 80, Fe 60, Zn 60, Cu 5, I 1, Co 0.2, Se 0.15

1500-1725 grams were individually housed in wire cages at Hunan Animal Husbandry Veterinarian's Research Institute under approved animal handling protocols. All the animals were fed a pelleted basal dry diet consisting primarily of corn, soybean meal, and rice bran twice per day (Table 2). Hens were randomly allocated into 3 treatment groups: 1) basal diet amended with milled fuzhuan tea at a concentration of 11.2 g /kg of feed (n=48), 2) basal diet amended with 5.7 g of water-soluble tea extracts per kg of feed (n=40), or 3) a control group fed unamended basal diet (n=48). Tea concentrations used in this study were determined by preliminary experiments and previously published studies in other animal models (Alshatwi *et al.*, 2010). The experiment was conducted over a 5-week period with all the laying hens kept under uniform management conditions throughout the experimental period. Temperature of 25-34℃ and a photoperiod of 16.5 h light were maintained.

Production traits. Egg production was recorded daily for each individual hen and was calculated every week on a bird/day basis. Eggs from each group were collected daily and stored at 4ºC until further analysis. The eggs produced each week were weighed and the average daily weight of the eggs produced was calculated as grams/hen/day. A total of 1162 eggs were analyzed from hens in the milled tea group, while 813 eggs were analyzed in the tea extract group, and 512 eggs from hens fed control diets. Feed intake was recorded weekly and calculated as grams per hen per day. The value of feed efficiency was calculated as a ratio of grams of feed: grams of egg.

Egg Traits. At the end of the experimental period, total egg weight for each of the three groups was obtained and the mean egg weight for each group was calculated. Six eggs were chosen at random from each treatment group on a weekly basis and used to determine yolk size. Each egg was weighed individually and then the yolks were separated from the albumen and weighed. The yolk ratio was calculated as grams per yolk: grams per egg.

Yolk cholesterol. Eight eggs were collected weekly from each group for determination of cholesterol levels in egg yolks. Cholesterol in the yolk was extracted and measured by High Pressure Liquid Chromatography (HPLC) according to published protocols (Wang and Tong, 2002; Wang, 2002). Eggs were weighed and then the egg whites were removed and the egg yolk/total egg weight ratio was calculated. Egg yolk suspensions were prepared by dissolving 6 g of egg yolk in distilled water in 10 mL volumetric flask. One milliliter of egg yolk suspension was added into a 5 mL centrifuge tube and 110 µl ethanol containing 210 mol/L KOH was added and mixed thoroughly. The mixture was saponified by incubating at 55ºC for 30 min, shaking every 5 min. The mixture was cooled and 210 µl *n*-hexane: isopropanol (4:1) solution was added and mixed prior to centrifugation at 3000 rpm for 3 min. The supernatant was removed and the extraction procedure was repeated 4 times per sample, combined and allowed to air dry. Concentrated extracts were resuspended in methanol and passed through a 0.45uM filter prior to HPLC analysis. Ten microliter injections of sample were analyzed on a Shimadzu SCL-10ATVP system equipped with a Shimadzu LC-10ATV pump, SPD-M20A diode array detector, and LC-solution data system (Shimadzu Corporation, Japan) using an Ultimate C-18 reverse-phase column with pore size of 4.6x150mm (Welch

Table 3. Production performance of laying hens in three dietary groups.

Diet Group	Production (%)	Egg weight （g/egg)	Daily egg weight (g/egg/day)	Feed intake (g/hen /day)	Feed Conversion (kg feed/kg egg)	Yolk ratio (%)
Control	50.8±6.7*	60.2±3.2	31.1±4.4	70.9±4.5	2.4±0.1	26.0±2.9
Milled Tea	69.2±6.4[a]	60.1±1.9	40.9±2.8[a]	90.3±2.7[a]	2.2±0.1[a]	26.1±2.1
Tea Extract	63.0±4.8[a]	58.1±2.4	37.0±2.5[a]	87.3±2.3[a]	2.4±0.2	25.3±2.7

*(± standard deviation)

[a]: denotes a significant difference from control values at P＜0.05

Table 4. Effects of Fuzhuan tea on laying hen serum lipid parameters

	Mg/dL				Ratios	
Diet	**LDL**	**HDL**	**triglyceride**	**cholesterol**	**HDL/TC**	**HDL/LDL**
Control	34.8 ±3.1	54.1 ±18.5	572.2 ±101.9	97.8 ±42.5	0.55	1.55
Milled Tea	35.9 ±13.9	55.3±21.7	517.3 ±84.2	99.0±20.5	0.56	1.54
Tea Extract	37.5 ±16.6	57.2 ±31.3	513.3 ±77.0	95.5 ±23.6	0.60	1.52

TC=total cholesterol, TG=Triglycerides, HDL=high density lipoprotein, LDL=low density lipoprotein

Materials, Shanghai, China). The column temperature was maintained at 38ºC and a mobile phase of 100% methanol was delivered isocratically at a flow rate of 1.0 mL/min. All solvents and chemicals used were HPLC grade and a commercially purchased cholesterol standard (purity=99%, RMHot, Beijing, China), showing a retention time of 9.3 min under the described conditions, was used to generate a standard curve used to quantify cholesterol.

Serum parameters. Blood lipid parameters were obtained from blood samples collected at slaughter from six randomly selected laying hens from each group. Collected blood was centrifuged to separate out the serum for determination of blood lipid levels. Total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL-C) and low density lipoprotein (LDL-C) were assayed using commercial kits (Jiancheng Biochemical Company, Nanjing, China) and measured on a MINDRAY Auto Chemistry Analyzer, BS-200 (Mindray, Shenzhen, China).

Statistical analysis. All statistical analyses were done using SPSS 17.0 program (IBM China Company, Ltd., Beijing, China). The difference between means of the treatment and control groups was tested for significance by Student’s t-test limit set at $p<0.05$ (significant) or $p<0.01$(highly significant).

RESULTS

The effects of milled fuzhuan tea and its extracts on production performance and egg traits are shown in Table 3. Compared with the control group, the inclusion of milled fuzhuan tea in the diet of laying hens resulted in significant increases in egg production (36.8%, $p<0.01$), average daily egg weight (31.6%, $p<0.01$) and average daily feed intake (27.3%, $p<0.01$). Feed conversion ratio in these animals was also increased by 8.3% ($p<0.01$). A linear regression of feed intake and egg production showed a significant positive correlation between feed intake and egg production (R^2 =0.683, $P<0.05$). Less pronounced effects were reported for inclusion of fuzhuan tea extracts compared to the milled tea; however, significant benefits in production and efficiency were observed. Hens fed fuzhuan tea extracts showed increased production, higher average daily egg weight and average daily feed intake by 24.1% ($p<0.05$), 19.1% ($p<0.05$) and 23.1% ($p<0.01$) respectively. No significant difference in the values of egg weights and yolk ratio was observed among the groups, suggesting that basic egg traits were not affected by the dietary treatments.

Levels of total serum cholesterol (TC), triglycerides (TG) and high and low density lipoproteins (HDL-C, LDL-C) in laying hens fed basal diet or milled fuzhuan tea and its water soluble extracts were measured at the completion of the trial and are shown in Table 4. No significant differences were observed among the three groups in any of the lipid parameters measured.

Despite a lack of change in serum lipid levels of the laying hens, there were significant decreases in yolk total cholesterol as a result of milled fuzhuan tea and its water soluble extracts. The mean total cholesterol in yolks from the control group was 190.7 ± 26.6 mg/ dL, while yolk TC for milled fuzhuan tea and its water-soluble extracts were 178.0 ± 23.9 mg/dL and 170.3 ± 29.0 mg/dL^{-1}, respectively; which is well below the average egg cholesterol of 186-213 mg/dL average (Krauss *et al*., 1996). Thus, supplementation with milled fuzhuan tea lowered yolk TC by 6.7% ($p<0.05$), and the water soluble extracts decreased TC by 10.7% ($p<0.05$) compared to hens fed basal diet alone.

DISCUSSION

Modifying egg composition by supplementing the diet of laying hens has been used to enhance levels of omega-3 fatty acids (Kirubakaran *et al*., 2011); and similar attempts have been made in producing egg yolks with lower cholesterol (Wang and Pan, 2003). We observed a significant reduction in egg total cholesterol levels by incorporating fuzhuan tea and its water-soluble extracts into the diets of laying hens. Supplementation of basal chicken feed with milled tea and its extracts also improved egg production, and increased feed intake and a higher feed conversion ratio, but did not demonstrate any negative effects on egg weight and yolk ratio, suggesting that supplementing chicken feed with this tea may be a feasible approach to reduce overall costs for egg production while resulting in a value-added final product, eggs with less cholesterol.

Fuzhuan tea has a unique chemical profile compared to other teas because it undergoes a microbial fermentation. When compared with unfermented teas, fuzhuan tea is lower in polyphenols (catechins), caffeine, and amino acids but has an increased level of organic acids (Fu *et al*., 2008; Wu *et al*., 2010). Organic acids reduce intestinal pH to inhibit the growth of microbial pathogens, improve the absorption and conversion of nutrients in the body, and improve overall gastric function (Park *et al*., 2009; Roth and Kirchgessner, 1998). This may account for the increased feed intake observed in this study in the tea supplemented animals. The increased food intake was positively correlated with increased egg production and improved feed conversion.

Cholesterol is present in the yolk of eggs as very low density lipoprotein cholesterol (VLDL-C). Unlike in mammals, where TC and TG are transformed into LDL-C and HDL-C, (Chen and Zhang, 2003) in chickens, egg cholesterol is biosynthesized in the liver (Andrews *et al*., 1968), secreted into the plasma as VLDL-C (Burley *et al*., 1984), and transported to the ovaries (Nimpf and Schneider, 1991). Some reports have suggested that a strong relationship between serum total cholesterol and egg cholesterol is lacking (Shivaprasad and Jaap, 1977; Wang and Pan, 2003), while others suggest that a reduction in serum TC results in reduced yolk cholesterol (Kurtoglu and Nizamlioglu, 2004; Azeke and Ekpo, 2008; Khan *et al*., 2007). In the present study, there were no significant changes in serum lipid levels, but yolk cholesterol was lower in the tea fed animals, in concurrence with published reports that suggest that the serum and egg cholesterol levels are unrelated. An inverse ratio between serum HDL/LDL ratio and total cholesterol in eggs has also been noted (Wang and Pan, 2003) and may be an important parameter to target for reduced egg cholesterol, although this inverse relationship was not noted in our study (Table 3). However, conflicting reports regarding the relationship between serum and egg lipids suggest that more research is needed and that genetic differences in the breeds of laying hens, which affect egg cholesterol levels, may play an important role.

Antioxidant compounds were previously shown to reduce the content of cholesterol in egg powders (Du and Ahn, 2000; Khan *et al*., 2007), and tea polyphenols and other compounds have long been utilized as natural antioxidants (Zhao, 2003). The action of tea polyphenols on lipid metabolism in laying hens and reducing egg cholesterol content has also been observed and reported (Wang and Tong, 2002; Lou *et al*., 2004; Khan *et al*., 2007). While it is possible that tea polyphenols contribute to the results seen in this study, these compounds are reduced in fuzhuan tea compared with other teas suggesting that unique compounds resulting from the fungal fermentation might be involved in observed anti-lipidemic effects, particularly organic acids with anti-oxidant activity (Purvis, 2001; Nath *et al*., 1995), that occur in higher levels in this tea and are highly water soluble. In fact, the solubility of these organic acids may account, in part, for the lower egg cholesterol reported for water soluble extracts of fuzhuan tea.

The results of the present study suggest that supplementing the diet of laying hens with fuzhuan tea could significantly improve egg production and feed efficiency and reduce egg cholesterol. Both hen serum and egg cholesterol can vary dramatically with breed and age of laying hen and future studies directed toward identifying the optimal dose for fuzhuan tea-mediated reduction of egg cholesterol taking these additional variables into consideration are needed. Fuzhuan tea also has reported anti-microbial properties (Liu *et al*., 2010), and it would be worth exploring affects of feed supplementation on reduction of *Salmonella* and other potential pathogens that decrease the health of hens or that enter the human food supply in eggs.

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