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Dietary Tea Tree (*Melaleuca alternifolia*) Oil Supplementation Improves Growth Performance, Cecal Microflora, Immunity, and Antioxidant Capacity of Partridge Shank Chickens

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The aim of this study was to investigate the effects of tea tree oil (TTO) supplementation on the growth performance, cecal microflora composition, immunity, and antioxidant status of Partridge Shank chickens. A total of 144 one-day-old chicks were allocated into three treatments with six replicates of eight chicks each and fed with a basal diet supplemented with 0 (Control group), 500, and 1000 mg/kg TTO for 50 days. Compared with the control group, the broilers fed with the basal diet supplemented with 1000 mg/kg TTO exhibited an increase in average daily gain from 22 to 50 days ($P=0.035$) and in both relative thymus weight ($P<0.001$) and *Lactobacillus* colonies in the cecal contents ($P=0.045$) at 50 days of age, but a reduction in the feed/gain ratio during 1 to 50 days ($P=0.048$). Additionally, dietary TTO supplementation, irrespective of dosage, increased the relative spleen weight ($P=0.003$) and total antioxidant capacity in the jejunum ($P=0.049$) and ileum ($P=0.001$) at 21 days, but decreased the malondialdehyde content in the ileum at both 21 ($P=0.003$) and 50 days ($P<0.001$) and in the jejunum at 50 days ($P=0.012$). The results suggested that TTO supplementation could improve the growth performance, cecal microflora composition, immunity, and antioxidant capacity of Partridge Shank chickens.

Key words: antioxidant capacity, broilers, cecal microflora, growth performance, immunity, tea tree oil

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Introduction

Essential oil is extracted from the flowers, stems, leaves, and roots of plant (Baldissera *et al*., 2014). In the last two decades, essential oils have been increasingly widely used as feed additives in animal nutrition for the improvement of growth performance, immune function, and antioxidant status in animals (Westendarp *et al*., 2006; Faix *et al*., 2009; Hassan and Gökçe, 2014; Haselmeyer *et al*., 2015). Tea tree oil (TTO) is a kind of essential oil, which is scientifically extracted from *Melaleuca alternifolia*, a plant native to Australia (Hart *et al*., 2000). It has been reported that the TTO treatment could induce loss of cytoplasmic contents and tolerance to sodium chloride, format mesosomes, and impair membrane integrity in cells, eventually resulting in microbe death (Carson *et al*., 2002). As a result, TTO is well known as an antimicrobial agent, which is able to kill a wide range of bacteria, fungi, and viruses (Carson *et al*., 1995; Mondello *et al*., 2006; Carson and Riley, 2010; Oliva *et al*., 2010; Hammer *et al*., 2012; Zeng *et al*., 2015). The TTO has also been demonstrated to stimulate lymphocyte proliferation and suppress the production of proinflammatory cytokines, thus exhibiting its anti-inflammatory activity (Hart *et al*., 2000; Brand *et al*., 2002). In addition, the available studies have illustrated that TTO could activate nuclear factor-erythroid 2-related factor 2-antioxidant responsive element pathway, an essential antioxidant signaling pathway (Lee *et al*., 2017), thus improving antioxidant status (Wang, 2017) and ameliorating oxidative damage in animals (Souza *et al*., 2018). Based on its characteristics and function, studies on TTO have also been performed in animals. It has been reported that TTO can improve growth performance, organ development, meat quality, antioxidant status, and immunity in animals (Nogueira *et al*., 2014; Feng *et al*., 2017). Partridge Shank chickens, a native poultry strain in China, not only provide exquisite and nutrient-rich meat for Chinese consumers, but also exhibit strong adaptability and disease resistance. Therefore, owing to their advantages, Partridge Shank chickens have attracted much attention of consumers, and their breeding volume has been continuously expanding for meeting the current consumer demand (Wang *et al*., 2014; Shen, 2016). However, no study has investigated the effects of dietary TTO supplementation on Partridge Shank

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chickens. Therefore, in the present study, we investigated the effects of TTO on the growth performance, cecal microflora composition, intestinal antioxidant capacity, and immune function of Partridge Shank chickens.

Materials and Methods

Animals, Diets, and Treatments

All experimental conditions and animal procedures were approved by Nanjing Agricultural University Institutional Animal Care and Use Committee.

A total of 144 one-day-old male chicks (Partridge Shank chickens) with similar hatching weight were obtained from a commercial hatchery and raised from 1 to 50 days. The chicks were randomly distributed into three dietary treatments consisting of six replicates. A replicate included a cage with eight chicks so that each treatment had a total of 48 chicks. The chickens were fed a basal diet supplemented with 0 (Control group), 500, and 1000 mg/kg TTO powder (Anhui Zhengzheng Feed Technology Co. Ltd., Bengbu, Anhui province, P. R. China). The ingredient composition and nutrient level of the basal diet are given in Table 1. The main component of TTO is terpinen-4-ol, whose concentration was estimated to be over 4000 mg/kg. Birds were allowed free access to water and mash feed in 3-level cages in a room with controlled environmental conditions. The temperature of the chicken house was maintained at 32 to 34℃ for the first 3 days, and then reduced by 2 to 3℃ per week to a final temperature of 20℃. Birds were exposed to natural light during daytime, and the light intensity at night was adjusted to approximately 10 lx. At 21 and 50 days of age, birds were weighed after feed deprivation for 12 h, and the feed intake of each replicate (cage) was recorded to calculate the average daily gain (ADG), average daily feed intake (ADFI), and feed/gain ratio (F/G). Birds that died during the experiment were weighed, and the data were included in the calculation of F/G.

Sample Collection

At 21 and 50 days of age, one bird was randomly selected from each cage (replicate) to collect samples and weighed. The birds were weighed after feed deprivation for 12 h (providing enough water). Subsequently, the birds were euthanized by cervical dislocation and necropsied immediately. The bursa of Fabricius, thymus, and spleen were then excised and weighed. The weights were recorded to calculate the relative organ weights using the following formula: relative weight of immune organ (g/kg)＝immune organ weight (g) /body weight (kg). The whole gastrointestinal tract was rapidly removed and placed on a chilled stainless steel tray. The cecum samples (left side) were quickly excised aseptically, and the contents were removed and cultured to de-

Table 1. **Composition and nutrient level of basal diet (g/kg, as fed basis unless otherwise stated)**

Items	1 to 21 days	22 to 50 days
Ingredients		
Corn	576.1	622.7
Soybean meal	310	230
Corn gluten meal	32.9	60
Soybean oil	31.1	40
Limestone	12	14
Dicalcium phosphate	20	16
L-Lysine	3.4	3.5
DL-Methionine	1.5	0.8
Sodium chloride	3	3
Premix[1]	10	10
Calculated nutrient levels[2]		
Apparent metabolizable energy (MJ/kg)	12.56	13.19
Crude protein	211	196
Calcium	10.00	9.50
Available phosphorus	4.60	3.90
Lysine	12.00	10.50
Methionine	5.00	4.20
Methionine + cystine	8.50	7.60

[1] Premix provided per kilogram of diet: vitamin A (transretinyl acetate), 10,000 IU; vitamin D_3 (cholecalciferol), 3,000 IU; vitamin E (all-rac-α-tocopherol), 30 IU; menadione, 1.3 mg; thiamin, 2.2 mg; riboflavin, 8 mg; nicotinamide, 40 mg; choline chloride, 400 mg; calcium pantothenate, 10 mg; pyridoxine·HCl, 4 mg; biotin, 0.04 mg; folic acid, 1 mg; vitamin B_{12} (cobalamin), 0.013 mg; Fe (from ferrous sulfate), 80 mg; Cu (from copper sulphate), 8.0 mg; Mn (from manganese sulphate), 110 mg; Zn (from zinc oxide), 60 mg; I (from calcium iodate), 1.1 mg; Se (from sodium selenite), 0.3 mg.

[2] Calculated according to the tables of feed composition and nutritive values in China (2012).

termine the populations of *Salmonella*, *Escherichia coli*, and *Lactobacillus*. The jejunum (from the end of pancreatic loop to the Meckel's diverticulum) and ileum (from Meckel's diverticulum to the ileocecal junction) were excised free of the mesentery, and then opened longitudinally. The jejunal and ileal mucosa was scratched carefully using a sterile glass microscope slide, which was rapidly frozen in liquid nitrogen and stored at −80℃ for further analysis.

Microflora Population Measurement

The *Salmonella, Escherichia coli*, and *Lactobacillus* colonies were determined according to methods described by Wang *et al.* (2012). Approximately 0.2 g cecal contents were placed in Bio-Clean centrifuge tubes aseptically and diluted in 2 mL sterilized saline solution (concentration, 154 mmol/L), and then three 10-fold serial dilutions were prepared from the diluted cecal contents (10^{-3}, 10^{-4}, and 10^{-5} for *Salmonella*; 10^{-4}, 10^{-5}, and 10^{-6} for *Escherichia coli* and *Lactobacillus*). A 0.1-mL portion of the last three dilutions was spread evenly on the agar plates. The *Salmonella* and *Escherichia coli* colonies were determined on the *Salmonella-Shigella* agar plates (Qingdao Hope Bio-Technology Co. Ltd., Qingdao, Shandong, P. R. China) and MacConkey agar plates incubated at 37℃ for 24 h, respectively. The *Lactobacillus* colonies were enumerated on the de Man, Rogosa, and Sharpe (MRS) agar medium cultured at 37℃ for 48 h. The colony-forming unites (CFU) from the plates with countable colonies were enumerated and averaged to express 1 g CFU per gram of cecal contents.

Measurement of Mucosal Antioxidant and Immune Parameters

Approximately 0.3 g of jejunal and ileal mucosa samples were homogenized (1:9, wt/vol) with ice-cold 154 mmol/L sodium chloride solution using an Ultra-Turrax homogenizer (Tekmar, Cincinnati, OH, USA) and centrifuged at 4450×*g* for 15 min at 4℃. The supernatant was then collected and stored for assaying the mucosal antioxidant and immune parameters. The total protein concentration, total antioxidant capacity (T-AOC), total superoxide dismutase (T-SOD) activity, and malondialdehyde (MDA) content were measured using diagnostic kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, P. R. China) according to the manufacturer's instructions. Briefly, the total protein concentration of mucosa was measured by a Coomassie brilliant blue protein assay kit. The T-AOC was determined using the ferric reducing ability methods. The activity of T-SOD was measured using the hydroxylamine method (Oyanagui, 1984). One unit of T-SOD activity was defined as the amount of enzyme per milligram protein of mucosa that would produce 1/2 inhibition of the rate of nitrite production at 37℃. The MDA content was determined by the barbiturate thiosulfate assay. The concentrations of immunoglobulin G (IgG), immunoglobulin M (IgM), and secretory immunoglobulin A (sIgA) were determined in appropriately diluted mucosal samples by enzyme-linked immunosorbent assay (ELISA) using microtiter plates and chicken-specific IgG, IgM, and sIgA ELISA quantitation (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, P. R. China). All results were normalized against the total protein concentration in each sample for conducting an inter-sample comparison.

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) using the SPSS (2008) statistical software (Ver. 16.0 for windows, SPSS Inc., Chicago, IL, USA) with a pen (cage) as the experimental unit. Differences among treatments were examined using the Tukey-Kramer's multiple range tests, which were considered significant when the *P*-value was less than 0.05. The means and standard error of means (SEM) were presented.

Results

Growth Performance

Compared with the control group (Table 2), the birds fed the basal diet supplemented with 1000 mg/kg TTO exhibited an increase in ADG from 22 to 50 days ($P<0.05$), but a decrease in F/G during 1 to 50 days ($P<0.05$). However, the similar effect was not observed in the birds given the diet supplemented with 500 mg/kg TTO ($P>0.05$). The treatments did not alter ADFI from 1 to 50 days ($P>0.05$).

Relative Weight of Immune Organs

Compared with the control group (Table 3), the birds fed the diets supplemented with either 500 or 1000 mg/kg TTO showed a significant increase in their relative spleen weights at 21 days ($P=0.003$), but this effect was not observed at 50 days ($P>0.05$). Moreover, the supplementation of 1000 mg/kg rather than 500 mg/kg TTO increased the relative thymus weight at 50 days ($P<0.001$). No significant difference was observed in the relative weight of the bursa of Fabricius among treatments ($P>0.05$).

Microflora Population

The supplementation of 1000 mg/kg TTO increased the number of cecal *Lactobacilli* at 50 days compared with the control group (Table 4, $P<0.05$), but this effect was not observed when its dosage was 500 mg/kg ($P>0.05$). The populations of the cecal *Escherichia coli* and *Salmonella* colonies were not affected by TTO inclusion ($P>0.05$).

Intestinal Immunoglobulins

The broilers given the basal diet supplemented with TTO did not alter the concentrations of immunoglobulins in their jejunal and ileal mucosa (Table 5, $P>0.05$).

Intestinal Oxidative Status

Compared with the control diets (Table 6), the diets containing either 500 or 1000 mg/kg TTO significantly increased the jejunal ($P<0.05$) and ileal ($P=0.001$) T-AOC activity, but reduced MDA accumulation in the ileum ($P<0.05$) at 21 days. Besides, the jejunal ($P<0.05$) and ileal ($P<0.001$) MDA accumulation was reduced at 50 days by TTO inclusion. However, the T-SOD activity in both jejunum and ileum was similar among treatments ($P>0.05$).

Discussion

Studies have documented the benefits of essential oils on the growth performance of poultry and swine. Najafi and Torki (2010) reported that the chicks fed with the essential

Table 2. **Effects of dietary TTO[1] supplementation on the growth performance of Partridge Shank chickens**

Items[2]	TTO (mg/kg diet)			SEM[3]	*P*-value
	0	500	1000		
1 to 21 days					
ADG (g/d)	18.45	17.72	18.42	0.22	0.344
ADFI (g/d)	29.50	28.39	29.26	0.25	0.159
F/G	1.61	1.60	1.59	0.02	0.979
22 to 50 days					
ADG (g/d)	39.94[b]	41.31[ab]	44.10[a]	0.68	0.035
ADFI (g/d)	102.62	102.01	103.92	0.82	0.626
F/G	2.57	2.47	2.39	0.04	0.139
1 to 50 days					
ADG (g/d)	31.30	31.41	33.02	0.34	0.051
ADFI (g/d)	72.37	70.11	71.94	0.56	0.219
F/G	2.32[a]	2.24[ab]	2.18[b]	0.02	0.048

[a, b] means within a row with different superscripts are different at $P<0.05$.
[1] TTO, tea tree oil.
[2] ADG, average daily gain; ADFI, average daily feed intake; F/G, feed/gain ratio.
[3] SEM, standard error of means ($n=6$).

Table 3. **Effects of dietary TTO[1] supplementation on relative weights of the immune organs of Partridge Shank chickens (g/kg)**

Items	TTO (mg/kg diet)			SEM[2]	*P*-value
	0	500	1000		
21 days					
Thymus	5.39	5.24	5.87	0.18	0.382
Spleen	1.41[b]	1.68[ab]	1.90[a]	0.07	0.003
Bursa of Fabricius	2.68	3.04	2.82	0.13	0.570
50 days					
Thymus	2.24[b]	2.07[b]	3.25[a]	0.16	<0.001
Spleen	2.55	2.69	2.37	0.14	0.706
Bursa of Fabricius	1.02	1.25	1.17	0.11	0.724

[a, b] means within a row with different superscripts are different at $P<0.05$.
[1] TTO, tea tree oil.
[2] SEM, standard error of means ($n=6$).

Table 4. **Effects of dietary TTO[1] supplementation on microflora population in the cecal contents of Partridge Shank chickens (lg CFU/g)**

Items	TTO (mg/kg diet)			SEM[2]	*P*-value
	0	500	1000		
21 days					
Lactobacillus	7.57	7.79	7.87	0.08	0.351
Escherichia coli	7.86	7.25	6.83	0.19	0.099
Salmonella	7.90	7.78	7.74	0.16	0.932
50 days					
Lactobacillus	6.85[b]	7.55[ab]	7.83[a]	0.18	0.045
Escherichia coli	7.20	6.89	7.28	0.20	0.745
Salmonella	6.62	6.30	6.44	0.19	0.823

[a, b] means within a row with different superscripts are different at $P<0.05$.
[1] TTO, tea tree oil.
[2] SEM, standard error of means ($n=6$).

Table 5. **Effects of dietary TTO[1] supplementation on the intestinal immunoglobulin concentrations of Partridge Shank chickens (mg/g protein)**

Items[2]	TTO (mg/kg diet)			SEM[3]	*P*-value
	0	500	1000		
21 days					
Jejunum					
sIgA	0.88	0.93	0.84	0.05	0.765
IgG	15.78	17.51	11.60	1.47	0.261
IgM	0.80	0.85	0.74	0.05	0.690
Ileum					
sIgA	0.89	1.01	1.13	0.07	0.372
IgG	13.75	16.51	20.36	1.90	0.416
IgM	0.79	0.87	1.02	0.09	0.391
50 days					
Jejunum					
sIgA	0.76	0.84	1.17	0.08	0.050
IgG	12.59	12.53	14.22	1.21	0.841
IgM	0.69	0.68	0.77	0.05	0.714
Ileum					
sIgA	1.13	0.93	0.97	0.05	0.210
IgG	18.56	16.43	19.09	1.60	0.819
IgM	0.96	0.91	0.97	0.05	0.922

[1] TTO, tea tree oil.
[2] sIgA, secretory immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M.
[3] SEM, standard error of means ($n=6$).

Table 6. **Effects of dietary TTO[1] supplementation on the intestinal antioxidant status of Partridge Shank chickens**

Items[2]	TTO (mg/kg)			SEM[3]	*P*-value
	0	500	1000		
21 days					
Jejunum					
MDA (nmol/mg protein)	1.44	1.01	0.83	0.12	0.089
SOD (U/mg protein)	156.18	161.24	161.63	5.52	0.926
T-AOC (U/mg protein)	0.34[b]	0.45[a]	0.45[a]	0.02	0.049
Ileum					
MDA (nmol/mg protein)	1.63[a]	1.00[b]	0.95[b]	0.10	0.003
SOD (U/mg protein)	156.18	157.60	159.79	5.05	0.962
T-AOC (U/mg protein)	0.46[c]	0.68[b]	0.93[a]	0.06	0.001
50 days					
Jejunum					
MDA (nmol/mg protein)	1.43[a]	0.80[b]	0.78[b]	0.11	0.012
T-SOD (U/mg protein)	170.76	154.24	177.25	5.31	0.217
T-AOC (U/mg protein)	0.32	0.35	0.39	0.02	0.255
Ileum					
MDA (nmol/mg protein)	1.43[a]	0.94[b]	0.82[b]	0.08	<0.001
T-SOD (U/mg protein)	160.77	164.30	151.71	5.28	0.623
T-AOC (U/mg protein)	0.49	0.55	0.58	0.03	0.379

[a, b] means within a row with different superscripts are different at $P<0.05$.
[1] TTO, tea tree oil.
[2] MDA, malondialdehyde; T-SOD, total superoxide dismutase; T-AOC, total antioxidant capacity.
[3] SEM, standard error of means ($n=6$).

oils extracted from thyme showed decreased F/G and improved body weight gain in broilers during the growth period. Similar results were also observed by Zeng *et al*. (2015), who reported that essential oils could decrease F/G in broilers and increase body weight in pigs. In addition, Feng *et al*. (2017) reported that dietary TTO supplementation into a basal diet could significantly increase the final weight and ADG of finishing pigs. In the present study, we found that the chickens fed the diets supplemented with 1000 mg/kg TTO had a higher ADG from 22 to 50 days, whereas a lower F/G during 1 to 50 days. Diet supplementation with TTO has a beneficial effect on ADG and F/G of broilers probably because essential oils can not only enhance the secretion of digestive enzymes and immunity (Franz *et al*., 2010), but also inhibit pathogenic bacteria selectively to improve the intestinal microecological balance (Haselmeyer *et al*., 2015; Zeng *et al*., 2015). In addition, the beneficial consequences of TTO on growth performance in broilers during 22 to 50 days was more pronounced than that during 1 to 21 days probably because of the increased feed intake that resulted in relatively high amount of TTO ingestion, as well as the cumulative effect of TTO on the body.

The thymus, spleen, and bursa of Fabricius are important immune organs of poultry and the main sites of the growth and proliferation of various immunological cells (Fan *et al*., 2013). The measurement of immune organ relative weight is a common method to evaluate the immune status of chickens (Heckert *et al*., 2002). It has been demonstrated that the immunopotentiator epimedium polysaccharide-propolis flavone resists immunosuppression by significantly enlarging immune organ index (Fan *et al*., 2013). In this study, diet supplementation with TTO increased the relative weight of spleen and thymus at 21 and 50 days, respectively. Similarly, Han (2017) reported that the thymus and spleen index of mice were increased significantly by feeding a diet supplemented with TTO that increases spleen lymphocyte proliferation, which, in turn, is beneficial to the growth and development of spleen. Thymus is one of the most important immune organs of poultry and the main site for T-cell proliferation and maturation (Sehra and Dent, 2006). Therefore, we speculated that diet supplementation with TTO caused an increase in the relative weight of thymus because TTO likely stimulates T-cell proliferation and maturation, which in turn suggested a possibility of using TTO as an immunopotentiator. This was supported by the findings of Hart *et al*. (2000), who illustrated that TTO could stimulate immunocyte proliferation.

In the current study, TTO supplementation increased the number of *Lactobacillus* colonies in the cecal contents at 50 days. Similar results were also found by Du (2013), who reported that TTO administration increased the number of *Lactobacillus* colonies in the cecal contents of broilers. It has been identified that terpinen-4-ol, the main component of TTO, has the ability to selectively kill intestinal pathogenic bacteria *in vitro* (Carson and Riley, 2010; Oliva *et al*., 2010), indicating that the increased *Lactobacillus* population in the cecum resulting from TTO addition is beneficial to regulate cecal microflora composition.

Reactive oxygen species (ROS) are produced during normal metabolism in cells (Yu, 1994). However, the concentrations of ROS exceeding the antioxidant protection levels of cells can damage carbohydrates, nucleic acids, lipids, and proteins and impair their biological functions (Birben *et al*., 2012). The SOD is regarded as an important antioxidant enzyme in removing the oxygen free radical and superoxide, and protecting cells from the damage caused by ROS (Limón-Pacheco and Gonsebatt, 2009). The MDA is the main end-product of the lipid peroxidation caused by ROS, and the accumulation of MDA is usually considered a marker of lipid peroxidation (Ayala *et al*., 2014). It has been reported that the broilers that received a diet supplemented with essential oils extracted from ginger and C*innamomum zeylanicum* exhibited increased T-SOD and T-AOC activities, but decreased MDA concentration in the liver and serum (Faix *et al*., 2009; Habibi *et al*., 2014). In this study, we observed a similar effect that the diet supplemented with TTO improved the intestinal oxidative status of broilers by increasing the jejunal and ileal T-AOC activity at 21 days and by reducing MDA accumulation at 21 days in the ileum and at 50 days in both ileum and jejunum. The improved intestinal oxidative status induced by TTO supplementation might be attributed to its constituents, such as terpinen-4-ol, γ-terpinene, α-terpinolene, and α-terpinene, which have promising antioxidant capacities, as described in previously published papers (Ruberto and Baratta, 2000; Kim *et al*., 2004). The compromised immune system, digestive function, and antioxidant capacity of young chicks make them more prone and sensitive to adverse factors and various stresses (such as the oxidative and immune stress), which might explain why only the 21-day T-AOC activity was affected in this study (Bottje *et al*., 1998; Karadas *et al*., 2011).

In conclusion, the supplementation of TTO can promote growth performance, stimulate growth and development of spleen and thymus, balance cecal microorganism, and improve intestinal antioxidant status in Partridge Shank chickens.

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