Dietary fresh lemon improves the albumen quality, immune status and lipid metabolism of Jingfen laying hens during the late laying period

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ABSTRACT
With the appeals for antibiotic-free production and the attention to natural feed additives in poultry, the effects of fresh lemon (FM) supplementation on older laying hens (79–96 weeks) and its comparison with citric acid (CA) and flavomycin (FLA) were evaluated in the present study. Female Jingfen chickens (n = 540) were randomly distributed into 5 dietary treatments: 0 (control), 20 g/kg (2%) and 40 g/kg (4%) FM, 0.7 g/kg CA and 0.005 g/kg FLA. Production performances were recorded daily and egg quality traits were measured biweekly. Serum biochemical indices and antibody titres were detected every 4 weeks from 84 to 96 weeks of age. Compared with the control diet, FM, CA and FLA increased the laying rate (p < .05) and average daily feed intake (ADFI), and reduced the feed to egg ratio (FER), indicating positive effects on production performance. Albumen height and HU were increased by 2% and 4% FM groups compared to the control, while albumen height was decreased in CA and FLA groups. FM improved serum antioxidant enzyme activities (p < .05) and reduced serum HDL, LDL, TG and T-CH compared to other groups, indicating favourable effects on antioxidative status and lipid metabolism. Antibody titres against H5N1, H9N2 and NDV were higher in FM groups than that in other groups during most experimental periods (p < .05). In conclusion, FM supplementation enhanced the production performance and antioxidant capacity of laying hens during the late laying period and was superior to the CA and FLA in improving albumen quality, immune status and lipid metabolism.

HIGHLIGHTS
- FM supplementation in layers’ diets had positive effects on production performance, egg albumen quality and lipid metabolism, and could enhance antioxidant capacity and immune levels.
- Adding FM had better effects on the albumen quality, immune status and lipid metabolism of laying hens than adding CA and FLA during the late laying period.

Introduction
Antibiotics have long been added as feed supplements for poultry to improve performance traits and for therapeutic and prophylactic purposes (Tabo et al. 2013). Despite the useful aspects of antibiotics, the definitive mechanism underlying their growth-promoting effect remains unknown (Kumar et al. 2018). Additionally, the prolonged use of antibiotics may lead to the development of bacteria resistant to drugs, which can be transferred to humans (Costa et al. 2018); thus, the elimination of the use of antibiotics as feed additives is desirable. Therefore, alternate approaches to replace antibiotics in poultry production are urgently needed.

Several antibiotic alternatives, such as organic acids, probiotics, prebiotics and enzymes, have recently been reported to be beneficial for layers and broilers, as they improved weight gain (Haque et al. 2010), feed conversion efficiency (Al-Sharafat et al. 2009; Koivunen et al. 2016), immune status (Al-Khalifa et al. 2019), carcass weight and carcass quality (Islam et al. 2012).
With the appeals for antibiotic-free production and attention to natural feed additives, various natural plants have been used as dietary supplements in animal production. Dietary supplementation with 0.15% ginger was reported to improve the growth performance of Ross 308 chicks from 1 to 42 days of age (Qorbanpour et al. 2018), while supplementation with 0.5% to 1% rosemary showed positive effects on ileal microbiota at 42 days of age (Norouzi et al. 2016). Supplementation with 10 to 15 g/kg ginger powder was reported to improve egg yolk antioxidant status and enhance dietary oxidation stability in 27-week-old Hy-Line brown chickens (Zhao et al. 2011). Adding 5% Mulberry leaves to the feed could modulate the antioxidative status of 22-week-old laying hens and improve their production performance and egg quality (Lin et al. 2017).

Lemon (Citrus limon L.), the third most important Citrus species, is rich in phenolic compounds as well as vitamins, minerals, dietary fibre, essential oils and carotenoids (Papoutsis et al. 2017), and its compositions have been reported to enhance the oxidation resistance and lipid metabolism of animals and humans (Gonzalez-Molina et al. 2010). Dietary supplementation with 1.5% lemon pulp had desirable effects on reducing abdominal fat and blood low density lipoproteins of Ross 308 chicks across the entire production period (Nobakht 2013). Being given the freshly squeezed pure lemon fruit juice at 30 mL/day could significantly lower the serum uric acid levels in humans with hyperuricaemia (Wang et al. 2017). Supplementation with 300 g dried lemon per animal per day could increase the digestible organic matter concentration of the urea-treated straw-based diets of goats (Madrid et al. 1996). In general, dried lemons were considered to be more suitable for feed material compared to FM, because they were better for storage and transport, but their nutrients would be lost during the drying process (Mehmet et al. 2020). However, few studies have investigated the use of fresh lemon (FM) as an additive in poultry feed, and to our knowledge, the effects of dietary FM on older laying hens have not been tested.

The continuous decline in laying rate, egg quality and immune function of chickens during the later laying period have badly hindered the forming profits of poultry farmers (Liu et al. 2018; Chen et al. 2020). In the current study, we hypothesised that the addition of FM may enhance the production performance of older laying hens and help extend the laying cycle. Therefore, different levels of FM were used in standard commercial diets to investigate its effects on Jingfen laying hens and to compare its advantages with antibiotics and organic acids during the late laying period.

Materials and methods

Ethical statement

The study protocol was approved by the Committee for the Care and Use of Experimental Animals at Anhui Academy of Agricultural Science located in Hefei, Anhui, China, under permit No. A11-CS06.

Birds, diets, and management

Jingfen chickens, which originated from North China, are used as dual-purpose breeds. A total of 540 female Jingfen laying hens (78 weeks of age) were obtained from a commercial farm (Anhui Wanki Poultry Development Co., Ltd., Luan, China). The chickens were distributed randomly into five dietary treatments supplemented with the following: 0 g/kg FM (control), 20 g/kg (2%) FM, 40 g/kg (4%) FM, citric acid (CA) and flavomycin (FLA). Each treatment included 36 replicates with 3 birds each, and the birds were raised in cages. The cultivar of FM is Eureka, which was provided by Yunnan Academy of Agricultural Science (Kunming, Yunnan), and its nutritional ingredient and antioxidant activity were shown in Supplementary Table S1. FM was directly sliced and mashed, and then mixed well with the basal diet (Table 1). CA was purchased from Nature Biological Group Co., Ltd. (Rizhao, China; purity 98.0%) and was added to the basal diet in premix.

Table 1. Ingredient composition and nutrient levels of the basal diet

| Ingredient         | Content (g/kg, as fed basis) | Nutrient level (calculated value) |
|--------------------|------------------------------|---------------------------------|
| Ingredient         |                              |                                  |
| Soybean meal       | 251.0 g/kg                  | Crude protein (g/kg) 152.3       |
| Soybean oil        | 14.0 g/kg                   | Crude fibre (g/kg) 31.5          |
| DL-Methionine      | 2.0 g/kg                    | Phosphorus (g/kg) 5.2            |
| Limestone          | 62.0 g/kg                   | Calcium (g/kg) 34.0              |
| Dicalcium phosphate| 17.6 g/kg                   | Lysine (g/kg) 10.5               |
| Calcium carbonate  | 10.4 g/kg                   | Methionine (g/kg) 5.2            |
| Salt               | 3.0 g/kg                    | Methionine + cystine (g/kg) 7.6  |
| Premixb            | 13.0 g/kg                   |                                  |
| Total              | 1000.0 g/kg                 |                                  |

*bVitamin-mineral premix provided the following per kg of diet: Cu, 10 mg; Fe, 65 mg; Mn, 75 mg; Zn, 65 mg; Se, 0.5 mg; retinyl acetate, 2.7 mg; cholecalciferol, 0.08 mg; tocopheryl acetate, 16.7 mg; menadione, 0.5 mg; thiamine, 4 mg; riboflavin, 2 mg; cyanobalamin, 0.02 mg; pyridoxine, 5.3 mg; biotin, 1.2 mg; folacin, 1.5 mg; pantothenic acid, 12 mg; and nicotinic acid, 36 mg.
at the concentration of 0.7 g/kg (the content of citric acid was equal to 4% FM). FLA was purchased from Henan Yong’en Biotechnology Co., Ltd. (Zhengzhou, China; purity 98.0%) and was added to the basal diet at a concentration of 0.005 g/kg. The dosages applied for CA and FLA were added according to the manufacturer’s instructions. The 2% and 4% FM dosages were chosen according to our previous experimental results. All experimental chickens have been homogenised before the trial to avoid the initial differences between groups. All experimental diets were formulated to meet all nutrients recommendations by the National Research Council (Nick 1994) for laying hens.

All chickens were reared in the same environmentally controlled house, and the photoperiod was 16 h light: 8 h dark. Crumbled feed was supplied in troughs placed in front of each cage two times every day (130 g/d per hen), and water was provided from nipple drinkers. All birds had free access to water and standard feed. There was a one-week preliminary feeding period, and then the experimental period lasted for 18 weeks, during which the chickens were 79 to 96 weeks of age.

**Production performance**

All eggs were collected in the afternoon. Then, the eggs from each replicate were counted and weighed every day to calculate the laying rate, average egg weight and broken egg ratio. The average daily feed intake (ADFI) was also recorded on a per replicate basis every day, and the feed to egg ratio (FER) was calculated.

**Measurement of egg quality**

From 79 weeks of age, 30 randomly collected eggs from each treatment were measured within 2 h after egg laying every two weeks. Egg weight was measured using an electronic scale with an accuracy of 0.01 g. Shell breaking strength was measured with an eggshell force gauge (EGG-0503, Robotmation Co., Ltd., Tokyo, Japan). Shell thickness was measured at the eggshell equator in three places using a micrometer gauge (FHK Co., Ltd., Tokyo, Japan). Albumen height and Haugh unit (HU) were measured using an automatic egg multimeter (EMT-5200, Robotmation Co., Ltd., Tokyo, Japan).

**Measurement of serum biochemical indices and antibody titers**

Thirty birds from each group were randomly selected for blood sampling at 84, 88, 92 and 96 weeks of age. A 4-mL blood sample was collected from the wing vein of the chickens into 2 heparinised tubes (2 mL in each tube). The time between catching the bird and obtaining the blood sample did not exceed 60 s. Samples were placed in an ice bath immediately after collection and then transported to the laboratory for processing. Blood serum was separated by centrifugation for 10 min (3000 × g) at 4°C and stored at −20°C until analysis.

Serum glutathione peroxidase (GSH-Px) activity, superoxide dismutase (SOD) activity, malondialdehyde (MDA) level, triglyceride (TG) level, total cholesterol (TC) level, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) level were determined by commercial analytical kits (Sigma, Thermo Fisher Scientific, Shanghai, China) with an autoanalyzer (Hitachi Ltd., Tokyo, Japan).

Antibody titres against the avian influenza viruses H5N1 (strain Re-5) and H9N2 (strain Re-2) and against Newcastle disease virus (NDV) were determined with enzyme-linked immunosorbent assay (ELISA) kits (Mibio Biotech Co., Shanghai, China) according to the manufacturer’s protocol. Antibody titre data were logarithmically transformed (base 2) prior to analysis.

**Statistical analysis**

The data were subjected to analysis of variance (ANOVA) using the general linear model (GLM) command in SAS version 9.3 statistical software (SAS Institute Inc., Cary, NC, USA). Tukey’s multiple comparison was used to test the significance of the differences between treatment means; significance was declared at $p < .05$. All data are presented as the mean and standard error of the mean.

**Results**

**Production performance**

The production performance of laying hens fed different dietary supplements from 79 to 96 weeks is presented in Table 2. Compared with the control diet, supplementation with FM, CA and FLA increased the laying rate and reduced the ratio of broken eggs during most experimental periods. There were no significant differences in laying rate among FLA, 2% FM and 4% FM groups, while the CA group had the highest laying rate throughout the experiment ($p < .05$). The ratio of broken eggs was significantly lower in FM groups than in CA and FLA groups from 79 to 90 weeks of age ($p < .05$). The ADFI of birds was higher in the treatment groups than in the control group.
The addition of FM at any level decreased the FER compared to the control diet; this effect was significant during 79 to 84 weeks of age and 91–96 weeks of age \((p<.05)\). The FER was also decreased in the CA and FM groups compared with the control group.

Egg quality traits

The egg quality traits of each group from 79 to 96 weeks of age are provided in Table 3. The egg weight was lower in FM groups compared to the control, but was higher in CA and FLA groups. No significant effects on shell thickness and strength were found among the five treatments. The 2% and 4% FM supplementation significantly increased the albumen height \((p<.05)\) and improved the HU, especially for the greater increase in 4% FM. Additionally, HU was slightly increased in the CA group compared with the control group.

Blood serum index

The blood biochemical parameters of different dietary supplementation treatments are shown in Table 4. The activities of antioxidant enzymes, namely, GSH-Px and SOD, and the level of MDA were significantly increased in the 2% FM, 4% FM and CA treatment groups compared with the control group \((p<.05)\). Moreover, the serum concentrations of HDL and LDL were significantly decreased in the 2% FM, 4% FM and CA treatment groups \((p<.05)\) but slightly increased in the FLA group compared with the control group. The serum concentrations of TG and T-CH were lower in all the treatment groups than that in the control group, and the T-CH concentration was further decreased in the 2% and 4% FM treatment groups \((p<.05)\).

Immune response

The effects of different dietary supplementation treatments on serum antibodies against the avian influenza viruses H5N1 and H9N2 and against NDV are presented in Figure 1. The antibody titres against H5N1, H9N2 and NDV were increased in the birds from the 2% FM group compared with those from the control group \((p<.05)\), with the exception of H5N1 and NDV at 88 weeks of age. Additionally, the levels of antibody...
tittres against H5N1, H9N2 and NDV were increased in the 4% FM treatment group compared with the control group (p < .05), with the exception of NDV at 88 weeks of age. However, compared with the control group, birds fed the diets supplemented with CA and FLA exhibited lower levels of H5N1 from 84 to 92 weeks of age and lower levels of H9N2 and NDV from 92 to 96 weeks of age.

**Discussion**

Diet composition is widely recognised to play a major role in balancing nutrition to promote body development. FLA is poorly absorbed after oral administration and is thus used as a feed additive to promote growth in livestock animals (Pfaller 2006) as FLA acts on intestinal microbes to improve metabolic activity and immune function (Dibner and Richards 2005). CA is an organic acid that increases the digestibility of protein and fibre (Atapattu and Nelligaswatta 2005) and has been reported to improve the growth performance of chickens (Denli et al. 2003; Martinez-Amezcua et al. 2006). The present study found that CA and FLA supplementation increased the laying rate and ADFI and improved the FER of chickens compared with the control diet, which showed positive effects on the production performance of chickens and was in accordance with the previous findings. However, the removal of antimicrobial growth promoters (AGP) from poultry diets has triggered a search for suitable natural alternatives.

Plants contain an extensive variety of phytochemical compounds with antimicrobial activity (Cowan, 1999), which may have either beneficial or detrimental effects in animals (Acamovic and Brooker 2005), depending on both the compound used and its concentration. In the present study, improvements in the laying rate, ADFI and FER were found in the 2% and 4% FM treatment groups, which suggested that FM provided the same beneficial effects as CA and FLA during the late laying period, and could enhance the conversion of digested feed into eggs. The health-promoting effects and properties of FM may associate with its contents, namely, vitamin C and flavonoids, which could promote digestion and resist oxidation and were conducive to growth (Vinson et al. 2001).

The HU, which describes the height of thick albumen relative to the egg weight, is generally used to assess albumen quality (Nematinia and Mehdizadeh 2018). Albumen height was decreased in CA and FLA groups and HU was decreased in the FLA group compared to the control and FM groups, which indicated negative effects on albumen quality of CA and FLA. Relatively larger increases of albumen height and HU were found in the FM groups, especially for the 4% FM supplementation, which indicated that the effects of FM were superior to that of CA and FLA. Compared to the industrial synthesis of CA and FLA, FM contained a variety of natural compound organic acids such as citric acid and malic acid as well as other flavour substance, which could promote nutrient absorption and facilitate protein synthesis and improve the palatability of feed (Gonzalez-Molina et al. 2010; Yakhnesi et al. 2014). This implied that FM supplementation was conducive to the protein secretion in the magnum of the oviduct of laying hens, which resulted in an improvement in egg albumen quality.

Blood serum biochemistry parameters reflect the condition of organism; GSH-Px, SOD and MDA are related to oxidative stress, and TC, LDL-C, HDL-C, and TG are related to lipid metabolism. Some studies reported that dietary supplementation with natural plants increased serum GSH-Px and SOD activities (Chen et al. 2009; Zhao et al. 2011) and MDA levels (Lin et al. 2017) in chickens. Support for finding was also found in the present study, that 2% and 4% FM groups had higher SOD and FLA activity and MDA levels than the control group, indicating an increased capacity of laying hens to remove oxygen free radicals. The enhanced antioxidant status induced by FM supplementation is likely due to its antioxidant compounds as mentioned above. The levels of HDL,
LDL, TG and T-CH were significantly decreased by 2% and 4% in FM groups compared to the control group, which showed that FM could promote lipid metabolism. Much more reduction of lipid levels in the 4% FM group indicated greater lipid-lowering effects with the increase of FM dosage. In addition, Supplementation with CA improved the antioxidant parameters and reduced total lipid level, while supplementation with
FLA had weak effects on oxidation resistance or lipid metabolism of laying hens. However, the much larger decreases of HDL and T-CH in the FM groups indicated that FM is more effective in lipid metabolism than CA and FLA.

Natural plants enhance many immune system activities, such as lysozyme, antiprotease, peroxidase, phagocytosis and antibody production (Provenza and Villalba 2010). In the present study, antibody levels were all significantly higher in the 2% and 4% FM groups than that in the other treatment groups during most of the experimental period, indicating the improved immune function of FM-treated birds. Similar results were reported by Kadam et al. (2009), who found that C. limon juice had an additional beneficial effect improving the immune response in broilers during heat stress. The antibody titre of NDV in the 2% FM group at 88 weeks of age was extremely low, which could be caused by human factors. Haque et al. (2010) found that CA and FLA supplementation increased the density of the lymphocytes in lymphoid organs, enhancing the non-specific immunity of broiler chicks. However, birds fed CA and FLA had lower antibody titres compared to the control in the present study, which may be due to the older age (84–96 weeks) of chickens; this result indicated that the addition of CA and FLA had a relatively weak effect on immune performance of laying hens during the late laying period.

Conclusions
The difference in advantages between 2 and 4% FM supplementation was not great in the present study. Supplementation with FM improved production performance and antioxidative status of Jingfen laying hens during the late laying period, and was superior to the CA and FLA in improving albumen quality, immune status and lipid metabolism. Further studies are needed to explore the optimum dosage of FM supplemented in feed during other production periods in different chicken strains.

Ethical approval
This research did not involve the introduction of any intervention on hens. The data collection was obtained with humanly handled, which according of animal care and welfare standard of The People’s Republic of China. Animal experimentation procedures were approved by the Committee for the Care and Use of Experimental Animals at Anhui Academy of Agricultural Science located in Hefei, Anhui, China.

Disclosure statement
No potential conflict of interest is reported by the author(s).

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