Effects of dietary supplementation with red yeast rice on laying performance, egg quality and serum traits of laying hens

Hong Sun, Yifei Wu, Xin Wang, Yong Liu, Xiaohong Yao, Jiangwu Tang
Institute of Plant Protection and Microbiology, Zhejiang Academy of Agricultural Sciences, Hangzhou, China

Abstract

To determine the influence of red yeast rice on production performance, egg quality and serum traits of laying hens, 288 laying hens (40-week-old) were randomly assigned into four groups with 6 replications of 12 birds each. Hens were provided with diets containing 0, 0.5, 1 and 5 g/kg red yeast rice for 8 weeks. Results showed that dietary supplementation of red yeast rice had no effect (P>0.05) on laying performance over the entire feeding period. Albumen height (quadratic, cubic, P<0.05) and Haugh units (quadratic, P<0.05) were increased with gradient addition of red yeast rice. Moreover, albumen height and Haugh units were higher (P<0.05) in 1 and 5 g/kg red yeast rice groups, with an exception of albumen height in 1 g/kg group at day 28. Yolk cholesterol content was lower (P<0.05) in 5 g/kg red yeast rice-supplemented group at day 56. There were decreased (P<0.05) contents of serum cholesterol and triglyceride, but higher (P<0.05) concentration of serum calcium in the 1 and 5 g/kg red yeast rice-treated hens compared with the control group. Serum level of very-low-density lipoprotein was also reduced (P<0.05) in 5 g/kg red yeast rice-treated hens. The results suggest that red yeast rice could lower serum and egg yolk cholesterol levels and improve the egg quality in laying hens.

Introduction

Chicken eggs are recognized as a perfect source of protein, lipids, vitamins and other valuable nutrients, but eggs also contain a high level of cholesterol, which is strongly associated with cardiovascular diseases. The current recommended level for daily intake of cholesterol is less than 300 mg and people often limit their egg consumption to avoid increases in the blood cholesterol levels (AHA, 1996; Carrillo-Dominguez et al., 2005). Therefore, approach to reducing the cholesterol content in eggs not only helps to improve public health efforts, but it can also be beneficial for the egg industry. Recently, dietary supplementation of probiotic strains (Mikulski et al., 2012; Abdelqader et al., 2013; Lei et al., 2013) and fermented feed ingredients (Loh et al., 2009; Zhao et al., 2013) have already been used to regulate the egg yolk cholesterol concentration.

The fermented products of Monascus species have been used in Chinese and Asian traditional medications and foods for thousands of years. Monascal rice (red yeast rice, red koji) is one of the most important Monascus-fermented product made by inoculating cooked rice with the Monascus spp. Monascal rice and its isolated components have several beneficial effects in both mouse/rat models and humans, including antioxidiant activities (Yang et al., 2006; Rajasekar and Kalaivan, 2011), antihyper-tensive effects (Wu et al., 2009), and cholesterol synthesis-inhibiting properties (Chen et al., 2008; Rajasekar and Kalaivan, 2011; Lee et al., 2013). Previous research suggested that the cholesterol-lowering ability of red yeast rice was mainly attributed to lovastatin (monacolin K), one of the secondary metabolite from Monascus species (Endo, 1979). Monacolin K functions as a specific inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase in the biosynthesis of cholesterol (Lin et al., 2008). Thus, red yeast rice could be applied as a food additive or medicine to control the concentration of blood cholesterol. Moreover, red yeast rice has also been used as feed supplements for chickens (Wang and Pan, 2003), rabbits (Wei et al., 2003) and fish (Thongprajaukaraw et al., 2014). Wang et al. (2006) demonstrated lower serum and meat cholesterol levels in broiler chickens fed red yeast rice-supplemented diets.

Despite these findings, the application of red yeast rice in the diets of layers has not been fully documented. The aim of the current study was to evaluate the effects of dietary supplementation with red yeast rice on laying performance, egg quality and selected serum traits of laying hens.

Materials and methods

Microorganism and culture condition

Monascus purpureus GM-039 was a laboratory strain obtained from the Institute of Plant Protection and Microbiology, Zhejiang Academy of Agricultural Sciences, Hangzhou, PR China. The strain was stored on slants of PDA medium (CM0139, Oxoid, Hampshire, UK) at 28°C and transferred monthly.

Seed culture of M. purpureus GM-039 was prepared by transferring a loop of spores from the potato-dextrose agar slant into a 500-mL flask containing (g/L) glucose 100, peptone 10, KNO3 20, NH4H2PO4 20, MgSO4 5.0, and CaCl2 1.0 at pH 6.0 according to the medium reported by Kumari et al. (2009). The medium was kept on a reciprocal shaker at 30°C for 48 h at 160 r/min. After inoculation, 5% (w/v) seed culture suspension was used as the start inoculum in the fermentation process of red yeast rice.

Preparation of red yeast rice

Rice, soybean powder and rice bran were obtained from a local market and were used as the substrates for the production of red yeast rice. The fermentation medium had 100 g of rice, soybean powder and rice bran (3: 1: 1, w/w/w) and 50 mL of nutritive salt solution (glycerol 8 g, NaNO3 0.5 g, (NH4)2SO4 1 g, MgSO4·7H2O 0.3 g, KH2PO4 0.2 g). After mixing...
thoroughly, the fermentation medium was placed in plastic bottles (20 cm long × 10 cm diameter) and was autoclaved for 20 min at 121°C. 5% (w/v) of the _M. purpureus_ GM-039 seed was added into the sterilized substrate for an aerobic fermentation. The fermentation of red yeast rice lasted for 12 days at 30°C. At the end of the fermentation, the fermented substrate was oven-dried and subsequently milled to pass through a 0.25-mm mesh. The crushed substrate (red yeast rice) was used as the supplement to the basal diet of laying hens. The ingredients and chemical composition of _M. purpureus_ red yeast rice is shown in Table 1. The total concentration of carbohydrate was measured based on the anthrone-sulphuric acid method reported by Brink et al. (1960). The crude protein (method 976.06), moisture (method 934.01) ash (method 942.05) and fat (method 920.39) contents of red yeast rice were determined by AOAC (1999) procedures. The contents of monacolin K and citrinin in red yeast rice were determined by HPLC method described by Kumari et al. (2009) using Waters e2695 separations module (Milford, MA, USA). Standard monacolin K and citrinin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The total phenolic content was measured by the method of Beh et al. (2012). The concentration of γ-aminobutyric acid was determined according to the method of Yap et al. (2014).

**Birds, diets and experimental design**

A total of 288 commercial laying hens (Lohmann Brown) at the age of 40 weeks were used for the 8-week study. Birds were randomly divided into four dietary treatments with 6 replicates of 12 birds per replication. Hens in each replication were kept in 3-tier wire cages (60 cm length × 60 cm width × 40 cm height) in three levels (upper, middle and lower) with four birds per cage. Cages were kept in a ventilated room with a temperature approximately 21°C. Birds were allowed to acclimate to the diets and environment for 7 days before the start of the experiment. Diets were offered twice daily (9:00 and 16:00) and hens had free access to mash feed and water through the experiment. A photoperiod of 16 h was maintained throughout the experimental period. The experimental was approved by the Animal Care and Use Committee of Zhejiang Academy of Agricultural Sciences (Hangzhou, China).

Hens in each treatment were randomly fed with one of the four experimental diets: a basal diet as control and three experimental diets supplemented with 0.5, 1 and 5 g/kg red yeast rice in the basal diet. The control diet was a corn-soybean meal based diet formulated to meet or exceed the nutrient requirements of the NRC (1994). Table 2 shows the chemical composition of the basal diet. Crude protein (method 976.06), available phosphorus (method 993.31), calcium (method 927.02) and amino acid composition (method 994.12) were analyzed according to the method of AOAC (1999). The dietary monacolin K concentrations for the red yeast rice-supplemented groups are equivalent to 0.005, 0.009 and 0.046 g/kg, respectively.

**Performance**

Mortality and health status of hens were observed daily during the experimental period. Eggs from each replicate were counted and weighed daily to measure laying rate and average egg weight. Feed consumption was recorded weekly in each replicate and calculated as g per day per hen. Feed conversion ratio was expressed as g feed per g egg. Egg weight and the egg production rate were also recorded for all treatments in the acclimation period to guarantee the similar pre-experimental values. No significant differences were detected between treatments on egg production and egg weight before the experiment.

**Egg quality, egg-yolk cholesterol and triglyceride**

On the last day of every 4 weeks, 36 eggs (no cracks or shell defects) were randomly collected from each treatment (6 eggs per replicate). Eggshell thickness, breaking strength of eggshells, egg albumen height, Haugh units and yolk color score were determined by a tester (DET6000, Nabel Co., Ltd., Kyoto, Japan). The eggshell thickness was detected based on the average thickness of the rounded end, middle and pointed end of the egg without the inner membrane. After the determination of egg quality, yolks were separated, homogenized by a digital disperser (Ultra Turrax T25, IKA, Staufen, Germany) and kept at -20°C prior to analysis for yolk cholesterol and triglyceride.

To detect yolk cholesterol and triglyceride, yolk (1 g) was diluted with 19 mL of chloro-

| Table 1. Proximate composition of _M. purpureus_ red yeast rice (as dry matter basis). |
|-----------------------------------|-------|
| Ingredient                        | Content |
| Crude protein, g/kg               | 204.8  |
| Carbohydrate, g/kg                | 687.9  |
| Crude lipid, g/kg                 | 35.2   |
| Ash, g/kg                         | 31.0   |
| Monacolin K, g/kg                 | 9.23   |
| γ-aminobutyric acid, g/kg         | 0.72   |
| Total phenolic content, mg Gallic acid equivalence (GAE)/g | 9.86   |
| Citrinin, μg/kg                   | 58.0   |

| Table 2. Ingredients and chemical composition of the basal diets (g/kg as fed basis unless otherwise stated). |
|-----------------------------------|-------|
| Ingredient                        | Content |
| Corn                              | 630    |
| Soybean meal, 470 g/kg crude protein | 250    |
| Limestone                         | 95     |
| Calcium hydrogen phosphate        | 12.0   |
| Sodium chloride                   | 3.0    |
| Vitamin mineral premix*           | 10.0   |
| Total                             | 1000   |

*Provided per kilogram of diet: vitamin A, 15,000 IU; vitamin D₃, 3500 IU; vitamin E, 20 IU; vitamin K₃, 3.9 mg; vitamin B₁₂, 5 mg; vitamin B₆, 10 mg; vitamin B₉, 5 mg; vitamin B₅, 0.02 mg; niacin, 40.3 mg; pantothenic acid, 15 mg; biotin, 0.45 mg; copper, 8 mg; iron, 80 mg; manganese, 60 mg; zinc, 40 mg; selenium, 0.3 mg. Analytical values except for ME that were calculated based on NRC (1994).
form-methanol (2:1, v/v). The cholesterol and triglyceride contents in the yolk solution were detected using commercial kits as previously reported (Deng et al., 2012). The yolk cholesterol and triglyceride were reported as mg of cholesterol/triglyceride per g of egg-yolk.

### Blood sampling and serum parameter measurement

At the end of the experiment (day 56), six hens were selected from each treatment (1 from each replicate) and were fasted for 12 h. Fresh blood samples were collected from brachial vein and left at room temperature for 1 h for clotting. The collection process was limited to less than 2 min to minimize the impact of handling stress. After centrifugation at 2000 x g for 15 min, the serum was obtained and kept at -20°C until further analysis.

Total protein, alanine aminotransferase (ALT), albumin, aspartate aminotransferase (AST), triglycerides, total cholesterol and calcium in serum samples were detected by commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions. One international unit of AST/ALT activity is defined as the amount of enzyme that catalyzed the transformation of one μmol of substrate (aspartate/alanine) per min under given assay conditions. The contents of very-low-density lipoprotein (VLDL) and high-density lipoprotein (HDL) were measured by a commercial kit according to the manufacturer’s instructions.

### Statistical analysis

Data were analyzed by one-way ANOVA (SPSS 13.0, Chicago, IL, USA). Results are expressed as means and standard error of means (SEM). Variances between different groups were determined by Tukey’s HSD test.

| Table 3. Effects of different levels of *M. purpureus* red yeast rice on the egg production of laying hens over the 8-week feeding period. |
|---|---|---|---|---|---|---|---|
| | Red mould rice addition, g/kg | SEM | L | Q | C |
| 1-4 week | | | | | | |
| Feed intake, g | 132.32 | 133.05 | 133.27 | 133.04 | 0.98 | 0.73 | 0.87 | 0.72 |
| Egg weight, g | 65.39 | 64.88 | 65.83 | 64.96 | 1.60 | 0.43 | 0.13 | 0.16 |
| Laying rate, % | 80.62 | 83.96 | 80.87 | 81.92 | 1.96 | 0.81 | 0.92 | 0.90 |
| Feed/egg ratio, g/g | 2.51 | 2.45 | 2.55 | 2.50 | 0.12 | 0.56 | 0.39 | 0.87 |
| 5-8 week | | | | | | |
| Feed intake, g | 131.69 | 129.81 | 130.15 | 134.35 | 2.33 | 0.42 | 0.17 | 0.23 |
| Egg weight, g | 64.12 | 64.34 | 65.67 | 64.90 | 0.91 | 0.46 | 0.33 | 0.68 |
| Laying rate, % | 78.69a | 80.10ab | 81.56ab | 82.76a | 1.00 | 0.05 | 0.14 | 0.07 |
| Feed/egg ratio, g/g | 2.61 | 2.48 | 2.43 | 2.52 | 0.19 | 0.48 | 0.18 | 0.56 |
| 1-8 week | | | | | | |
| Feed intake, g | 131.92 | 132.45 | 132.30 | 133.75 | 1.86 | 0.36 | 0.81 | 0.98 |
| Egg weight, g | 64.45 | 64.55 | 65.31 | 64.91 | 1.12 | 0.46 | 0.51 | 0.80 |
| Laying rate, % | 79.03 | 82.11 | 81.97 | 81.66 | 1.98 | 0.70 | 0.43 | 0.32 |
| Feed/egg ratio, g/g | 2.59 | 2.48 | 2.49 | 2.51 | 0.08 | 0.61 | 0.64 | 0.78 |

L, linear; Q, quadratic; C, cubic effect of supplemental red mould rice; standard error of means (n=6). *Means in the same row not sharing a common superscript differ significantly (P<0.05).

| Table 4. Effects of different levels of *M. purpureus* red yeast rice on the egg quality of laying hens over the 8-week feeding period. |
|---|---|---|---|---|---|---|---|
| | Red mould rice addition, g/kg | SEM | L | Q | C |
| Day 28 | | | | | | |
| Shell thickness, mm | 0.38 | 0.37 | 0.37 | 0.37 | 0.01 | 0.68 | 0.39 | 0.55 |
| Shell breaking strength, kg/cm² | 3.74 | 3.59 | 3.57 | 3.47 | 0.11 | 0.26 | 0.13 | 0.34 |
| Albumen height, mm | 7.32a | 7.73ab | 7.57ab | 7.94a | 0.19 | 0.39 | 0.03 | <0.01 |
| Haugh units | 83.40a | 86.56ab | 92.06a | 91.26a | 1.90 | 0.09 | <0.01 | 0.22 |
| Yolk colour score | 68.34 | 66.78 | 61.34 | 60.78 | 8.9 | 0.85 | 0.89 | 0.66 |
| Yolk cholesterol, mg/g | 12.78 | 12.56 | 12.49 | 12.11 | 0.32 | 0.97 | 0.54 | 0.78 |
| Day 56 | | | | | | |
| Shell thickness, mm | 0.50 | 0.48 | 0.47 | 0.49 | 0.01 | 0.55 | 0.06 | 0.43 |
| Shell breaking strength, kg/cm² | 3.59 | 3.96 | 3.81 | 3.32 | 0.27 | 0.49 | 0.53 | 0.61 |
| Albumen height, mm | 6.44a | 6.72ab | 6.90a | 6.81a | 0.12 | 0.17 | 0.03 | 0.01 |
| Haugh units | 75.05a | 79.33ab | 80.34a | 80.11a | 1.47 | 0.27 | <0.01 | 0.23 |
| Yolk colour score | 7.0 | 7.1 | 7.0 | 7.2 | 0.09 | 0.85 | 0.43 | 0.66 |
| Yolk triglyceride, mg/g | 170.34 | 164.34 | 160.11 | 151.34 | 7.40 | 0.44 | 0.34 | 0.59 |
| Yolk cholesterol, mg/g | 12.98a | 12.80ab | 12.70a | 12.01b | 0.28 | 0.02 | 0.62 | 0.97 |

L, linear; Q, quadratic; C, cubic effect of supplemental red mould rice; standard error of means (n=6). *Means in the same row not sharing a common superscript differ significantly (P<0.05).
The linear, quadratic and cubic effects of red yeast rice supplementation among treatments were analyzed with contrasts among the means. P<0.05 were regarded as significant.

**Results**

**Laying performance**

Hens were keeping healthy throughout the trial and were not subjected to any medical intervention. No significant difference was observed among treatments in feed intake, egg weight, laying rate or feed/egg ratio over the whole feeding period (P>0.05) (Table 3). However, supplementation with red yeast rice increased (linear, P<0.05) the laying rate in the later 4 weeks, to a greater extent (P<0.05) in hens fed diets containing 5 g/kg red yeast rice than in those fed the control diet.

**Egg quality**

Dietary supplementation of red yeast rice increased albumen height (quadratic, cubic, P<0.05) and Haugh units (quadratic, P<0.05) at day 28 and 56 (Table 4). The albumen height in the 5 g/kg red yeast rice group was higher (P<0.05) compared with the control group at day 28 and day 56, this improvement could also be detected for the supplementation dose of 1 g/kg at day 56. The Haugh units were higher (P<0.05) in eggs derived from 1 and 5 g/kg red yeast rice groups compared with those in the control group. The lowering effect on serum calcium concentration was also observed in 1 and 5 g/kg red yeast rice groups. On the other hand, the level of serum calcium increased (P<0.05) linearly in response to dietary addition of red yeast rice. The concentration of serum calcium was higher (P<0.05) in 1 and 5 g/kg red yeast rice groups in contrast to the control group. The VLDL concentration decreased (P<0.05) linearly as the addition of red yeast rice increased. The VLDL content was lower (P<0.05) in hens fed a diet with 5 g/kg red yeast rice than the control group. No differences in the concentrations of serum total protein, serum albumin, HDL, ALT and AST activities were detected among treatments (P>0.05).

**Discussion**

The result of the current study showed the potential of dietary red yeast rice at the level of 5 g/kg to improve egg production in the later 4 weeks of the experimental period. Sabrina and Latif (2012) also reported a higher level of egg production in Monascus fermented-product-fed laying hens. These results may partly due to the increase in feed intake of laying hens (data not shown), resulting from the improvement of palatability of diets after red yeast rice supplementation. Obviously, the present study had a lower dietary concentration of monacolin K (up to 0.046 g/kg), compared with those (0.6 g/kg) in the previous studies (Elkin and Yan, 1999; Wang and Pan, 2003). However, the level of monacolin K was higher than the dietary supplementation content of 0.015 g/kg reported by Mori et al. (1999). The high level of red yeast rice supplementation could increase the concentration of citrinin, a toxic ingredient that had serious side effects (Lin et al., 2008). Meanwhile, the monacolin K itself may also induce several side effects on hepatic cells (Yap et al., 2014).

Lower supplementation of red yeast rice brings a decreased content of citrinin in diets, thereby reducing the risks for liver and/or kidney toxicity in laying hens (Kumari et al., 2009). Our finding may suggest that diets supplemented with a low content of red yeast rice have advantages in improving egg production of laying hens.

Interestingly, the current study demonstrated that red yeast rice supplementation (1 and 5 g/kg) could significantly increase the albumen height and Haugh units of eggs. Red yeast rice contains a large amount of water-soluble polysaccharides that could be beneficial to layer growth by enhancing intestinal functions similar to prebiotics (Zhao et al., 2013). Previous study also has reported growth promoting effects of red yeast rice extracts on UMR 106 cells (Wong and Rabie, 2008). Therefore, the supplementation of red yeast rice might improve the protein synthesis and secretion in ovaries of laying hens, further increasing the scores of Haugh units. This study also showed a significant lower content of yolk cholesterol for red yeast rice at 5 g/kg supplementation levels at day 56. The results were consistent with a similar study on the supplementation of red yeast rice by Wang and Pan (2003) on laying hens. Elkin and Yan (1999) and Mori et al. (1999) also demonstrated a decreased egg cholesterol concentration in monacolin K-fed hens. The reduction in egg

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**Table 5. Effects of different levels of *M. purpureus* red yeast rice on the serum characteristics of laying hens on day 56.**

| Serum biochemical traits | Red mould rice addition, g/kg | SEM | L | Q | C |
|--------------------------|-------------------------------|-----|---|---|---|
| Total protein, g/L        | 64.49                         | 0   | 2.53 | 0.34 | 0.83 | 0.12 |
| Albumin, g/L              | 29.15                         | 0.71 | 0.71 | 0.51 | 0.32 | 0.43 |
| Aspartate aminotransferase, U/L | 305.49               | 10.04 | 10.04 | 0.64 | 0.41 | 0.51 |
| Alanine aminotransferase, U/L | 5.58                | 0.61 | 0.61 | 0.52 | 0.24 | 0.09 |
| Triglycerides, mmol/L     | 22.90                         | 1.61 | 1.61 | 0.05 | <0.01 | 0.14 |
| Cholesterol, mmol/L       | 3.38a                        | 0.22 | 0.22 | 0.15 | 0.04 | 0.23 |
| VLDL, mg/dL               | 47.4a                        | 2.07 | 2.07 | 0.01 | 0.34 | 0.88 |
| HDL, mg/dL                | 34.1                         | 1.16 | 1.16 | 0.34 | 0.19 | 0.10 |
| Calcium, mmol/L           | 3.74a                        | 0.04 | 0.04 | 0.01 | 0.56 | 0.66 |

L, linear; Q, quadratic; C, cubic effect of supplemental red mould rice; standard error of means (n=6); VLDL, very-low-density lipoprotein; HDL, high-density lipoprotein. *Means in the same row not sharing a common superscript differ significantly (P<0.05).
yolk cholesterol concentration may be attributed to the suppression of HMG-CoA reductase activity that regulates the cholesterol biosynthesis in the liver. The inhibition of cholesterol production causes a lower intracellular supply, thereby reducing of cholesterol content in egg yolks (Gonzalez-Esquerra and Leeson, 2000).

Importantly, the lowering of yolk cholesterol was achieved by a small amount of monacolin K supply, compared with the previous studies (Elkin and Yan, 1999; Wang and Pan, 2003). Fujimoto et al. (2012) compared the effects of red yeast rice with different monacolin K levels and suggested that γ-aminobutyric acid and various peptides could help to strengthen the hypocholesterolemic effect of red yeast rice. Yeap et al. (2014) also suggested that the hypocholesterolemic effect of red yeast rice could attributed to other bioactive compounds. In the present study, a high level of phenolic compounds and γ-aminobutyric acid was detected in the red yeast rice. Phenolic compound such as flavonoids and isoflavones can suppress the intestinal absorption of lipids (Francis et al., 2002; Rao and Gurﬁnkel, 2000) and reduce serum lipid proﬁle (Afonso et al., 2012). Thus it was not surprising to find a similar hypocholesterolemic effect of red yeast rice with a low content of monacolin K. Our results showed reduced contents of serum cholesterol and serum total triglyceride in 1 and 5 g/kg red yeast rice treatments. These findings are consistent with the results of Wang et al. (2006) on broiler chickens at dose of 20 g/kg, Lee et al. (2013) on rats. The signiﬁcantly lower content of serum cholesterol could be also ascribed to the inhibition of the HMG-CoA reductase activity, resulting in a lower intracellular supply of cholesterol. Wang and Pan (2003) reported that red yeast rice could strengthen the direct absorption of VLDL in the liver and reduce the hepatic production of VLDL. Our results also showed signiﬁcant reductions of serum VLDL levels in the 5 g/kg red yeast rice-treated group compared with the control group. This ﬁnding may support the view that the impaired hepatic lipogenesis induced by red yeast rice causes reductions in serum triglyceride concentrations. Interestingly, our result indicated an increased level of serum calcium at day 56 in response to increased levels of red yeast rice. No comparison could be made due to the shortage of related studies. The increase in serum calcium could be attributed to the flavonoids exist in the red yeast rice which possess phyto-oestrogens properties and promote calcium absorption in intestine (Setchell and Lydeking-Olsen, 2003).

Conclusions

To sum up, the results suggest that an appropriate dietary supplementation with red yeast rice can lower serum and egg yolk cholesterol levels and improve the egg quality in laying hens without impairment of laying performance. The optimal dosage of red yeast rice could be 5 g/kg under the current experimental conditions, although further studies are still needed to elucidate the effects of red yeast rice on poultry nutrition in different additional levels.

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