Effects of Alfalfa Extract on Antioxidant Capacity, Lipid Metabolism, and Related Gene Expression in Laying Hens during the Late Laying Period (Postprint)

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Abstract

The present experiment was conducted to investigate the effects of different levels of alfalfa extract on antioxidant capacity, lipid metabolism, and related gene expression in laying hens during the late laying period. A total of 324 healthy Hy-Line Brown laying hens aged 69 weeks were randomly allocated into 3 groups with 6 replicates per group and 18 hens per replicate. The control group was fed a basal diet, while the experimental groups were supplemented with 500 and 1,000 mg/kg alfalfa extract based on the basal diet. The experiment consisted of a 2-week pre-trial period and an 8-week formal trial period. The results showed that, compared with the control group: 1) The egg production rate of hens in the experimental groups was significantly increased (P<0.05), and the feed-to-egg ratio was significantly decreased (P<0.05). 2) 500 mg/kg alfalfa extract significantly increased eggshell strength (P<0.05), extremely significantly decreased liver triglyceride content (P<0.01), and significantly decreased liver total cholesterol content (P<0.05). 3) 1,000 mg/kg alfalfa extract extremely significantly increased plasma glutathione peroxidase (GSH-Px), total superoxide dismutase (T-SOD), and liver GSH-Px activities (P<0.01), significantly increased plasma total antioxidant capacity (T-AOC) and decreased malondialdehyde (MDA) content (P<0.05), and extremely significantly decreased total cholesterol and triglyceride contents in liver and yolk (P<0.01); it also extremely significantly increased hepatic mRNA expression levels of thioredoxin reductase 1 (TrxR1), cholesterol 7α-hydroxylase (CYP7A1), and sterol regulatory element-binding protein-1c (SREBP-1c), and extremely significantly decreased mRNA expression level of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) (P<0.01), while showing no significant effect on GSH-Px mRNA expression level (P>0.05). In conclusion, dietary supplementation of alfalfa

extract can decrease yolk cholesterol and triglyceride contents, improve production performance, eggshell strength, and antioxidant capacity and cholesterol metabolism in laying hens during the late laying period, with 1,000 mg/kg alfalfa extract showing better effects.

Full Text

Effects of Polysavone on Antioxidant Capacity, Lipid Metabolism, and Related Gene Expression in Laying Hens during the Late Laying Period

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Abstract

This experiment was conducted to investigate the effects of different dietary polysavone supplementation levels on antioxidant capacity, lipid metabolism, and related gene expression in laying hens during the late laying period. A total of 324 healthy 69-week-old Hy-Line Brown laying hens were randomly divided into 3 groups with 6 replicates per group and 18 hens per replicate. The control group was fed a basal diet, while the experimental groups were fed the basal diet supplemented with 500 or 1,000 mg/kg polysavone. The experiment consisted of a 2-week preliminary period followed by an 8-week formal trial period. The results showed that compared with the control group: (1) dietary polysavone supplementation significantly increased laying rate and decreased feed-to-egg ratio ($P<0.05$); (2) supplementation with 500 mg/kg polysavone significantly increased eggshell strength ($P<0.05$), extremely significantly decreased liver triglyceride content ($P<0.01$), and significantly decreased liver total cholesterol content ($P<0.05$); (3) supplementation with 1,000 mg/kg polysavone extremely significantly increased plasma glutathione peroxidase (GSH-Px), total superoxide dismutase (T-SOD), and liver GSH-Px activities ($P<0.01$), significantly increased plasma total antioxidant capacity (T-AOC) and decreased malondialdehyde (MDA) content ($P<0.05$), extremely significantly decreased total cholesterol and triglyceride contents in both liver and egg yolk ($P<0.01$), extremely significantly increased hepatic mRNA expression of thioredoxin reductase 1 (TrxR1), cholesterol-7α hydroxylase (CYP7A1), and sterol regulatory element binding protein-1c (SREBP-1c) ($P<0.01$), and extremely significantly decreased mRNA expression of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) ($P<0.01$), while having no significant effect on GSH-Px mRNA expression ($P>0.05$). These findings indicate that dietary polysavone supplementation can reduce egg yolk cholesterol and triglyceride contents, improve production performance, eggshell strength, and antioxidant and cholesterol metabolism capacity in late-period

laying hens, with 1,000 mg/kg polysavone showing the best effects.

Keywords: polysavone; laying hens during late laying period; antioxidant; lipid metabolism; gene expression

Introduction

Eggs are a common daily food, but their high cholesterol content significantly affects consumer psychology regarding egg and egg product consumption [1]. The main active components of polysavone are flavonoids ($ 5 15 $5%), which have been shown to exert hypolipidemic, anti-atherosclerotic, antioxidant, and immune-enhancing effects [2-3]. Previous research has demonstrated that dietary supplementation with different levels of alfalfa saponins can enhance glutathione peroxidase (GSH-Px) activity in the liver and serum of broiler chickens while reducing serum malondialdehyde (MDA) content, thereby protecting animals from lipid peroxidation damage [4]. Deng et al. [5] found that aqueous alfalfa extract significantly reduced total cholesterol (TC) in serum and liver and decreased lipid content in egg yolk, serum, and liver, but had no significant effect on serum low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) levels. Zhou et al. [6] further analyzed the cholesterol-lowering effects of alfalfa saponin extract in laying hens using digital gene expression profiling and found that supplementation at 120 mg/kg was most effective. Additionally, dietary polysavone supplementation has been shown to significantly reduce cholesterol content in whole eggs [7] and promote broiler growth through a pattern of early inhibition followed by later promotion [8], suggesting that polysavone may be more effective under conditions of long growth cycles or serious disease risks. While polysavone possesses both antioxidant and cholesterol-lowering properties, research has primarily focused on its application, with limited investigation into its underlying mechanisms. Therefore, this study examined the effects of different dietary polysavone levels on production performance, antioxidant capacity, cholesterol metabolism, and related gene expression in late-period laying hens, with preliminary exploration of its molecular mechanisms, aiming to determine the optimal supplementation level and provide a theoretical basis for feed resource development and sustainable development of the egg industry.

Materials and Methods

1.1 Experimental Period and Location

The experiment was conducted from June 11 to August 21, 2015, at Jianxin Breeding Farm in Datong County, Datong City, Shanxi Province, and at the Provincial Key Laboratory of Animal Genetics, Breeding and Nutrition at Shanxi Agricultural University.

1.2 Experimental Materials and Design

Polysavone, a natural plant extract from alfalfa with active components of 15% polysaccharides, 5% flavonoids, and 5% triterpenoid saponins, was purchased from Deyang Sanfengyuan Technology Co., Ltd., Sichuan Province. A total of 324 healthy 69-week-old Hy-Line Brown laying hens were randomly divided into 3 groups with 6 replicates per group and 18 hens per replicate. The control group received a basal diet, while the experimental groups received the basal diet supplemented with 500 or 1,000 mg/kg polysavone. The experiment included a 2-week preliminary period and an 8-week formal trial period. The composition and nutrient levels of the basal diet are presented in Table 1 .

1.3 Management

Hens were housed in three-tier step cages with 3 hens per cage. They were fed 4 times daily with free access to feed and water. Natural light was supplemented with artificial lighting to provide 16 hours of light daily. Ventilation consisted of natural ventilation combined with longitudinal negative pressure ventilation. Other management practices followed conventional farm procedures.

1.4 Measurements

1.4.1 Production Performance During the experimental period, daily egg number and total egg weight were recorded for each group. Feed consumption was measured weekly to calculate average daily feed intake. Laying rate and feed-to-egg ratio were then calculated for each group.

1.4.2 Egg Quality On days 14, 28, 42, and 55 of the experiment, 3 eggs were randomly selected from each replicate to measure egg weight, egg shape index, eggshell strength, albumen height, Haugh unit, yolk color, and eggshell thickness, with average values calculated. At the end of the experiment, 2 eggs per replicate were weighed, and after breaking, the yolk was separated from the albumen. Yolk TC and TG contents were measured using assay kits purchased from Nanjing Jiancheng Bioengineering Institute.

1.4.3 Plasma and Tissue Indices At the end of the experiment, one hen with good health and average body weight was randomly selected from each replicate. Blood (5 mL) was collected from the wing vein after fasting and centrifuged at 3,500 r/min for 10 minutes to prepare plasma, which was stored at -20°C. The hens were then euthanized by jugular venous exsanguination, and liver tissue from the same anatomical location was rapidly collected under sterile conditions, snap-frozen in liquid nitrogen, and stored at -80°C for subsequent analysis. Plasma GSH-Px and total superoxide dismutase (T-SOD) activities, total antioxidant capacity (T-AOC), and contents of MDA, TC, and TG, as well as liver GSH-Px activity and TC and TG contents, were measured using assay kits from Nanjing Jiancheng Bioengineering Institute.

1.4.4 Liver RNA Extraction and Reverse Transcription Total RNA was extracted from liver tissue using the Trizol method. RNA integrity, concentration, and purity were assessed using 1% agarose gel electrophoresis and a nucleic acid-protein analyzer. cDNA was synthesized according to the reverse transcription kit instructions and stored at -20°C.

1.4.5 Primer Design Primers for GSH-Px, cholesterol-7α hydroxylase (CYP7A1), sterol regulatory element binding protein-1c (SREBP-1c), thioredoxin reductase 1 (TrxR1), and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) were designed based on sequences from the GenBank database (Table 2) and synthesized by BGI (Beijing).

1.4.6 Real-Time Quantitative PCR Using the synthesized cDNA as template, real-time quantitative PCR was performed in a 20 L reaction system using SYBR® Premix Ex Taq™ II kit (TaKaRa, Dalian) on an ABI 7500 Real-Time PCR System (USA). The reaction conditions were: pre-denaturation at 95°C for 30 s, followed by 45 cycles of denaturation at 95°C for 5 s and annealing/extension at 60°C for 34 s. Melting curve analysis was performed at 95°C for 10 s, 60°C for 1 min, followed by a temperature increase to 95°C at a rate of 0.5°C/10 s. Relative quantification was performed using β-actin as the internal reference gene, and target gene mRNA expression levels were calculated using the 2^-ΔΔCt method.

1.5 Data Processing and Statistical Analysis

Data were analyzed using one-way ANOVA with SPSS 19.0 statistical software. All data are expressed as "mean $\pm$ standard deviation." Duncan' s multiple range test was used to compare differences among groups, and LSD test was used for comparisons of extremely significant differences.

Results

2.1 Effects of Polysavone on Production Performance

As shown in Table 3 , dietary polysavone supplementation significantly increased laying rate and decreased feed-to-egg ratio (P<0.05). The 1,000 mg/kg group achieved the highest laying rate (9.13% higher than control) and the lowest feed-to-egg ratio (5.98% lower than control), while average egg weight and average daily feed intake showed no significant differences (P>0.05).

2.2 Effects of Polysavone on Egg Quality

Table 4 shows that dietary polysavone supplementation had no significant effects on albumen height, Haugh unit, egg shape index, yolk color, or eggshell thickness (P>0.05), but did affect eggshell strength, with the 500 mg/kg group showing the highest value that was significantly greater than the control group (P<0.05).

2.3 Effects of Polysavone on Antioxidant Capacity

As presented in Table 5 , compared with the control group, dietary supplementation with 1,000 mg/kg polysavone extremely significantly increased plasma GSH-Px, T-SOD, and liver GSH-Px activities ($P<0.01$), significantly increased plasma T-AOC, and decreased MDA content ($P<0.05$). Supplementation with 500 mg/kg polysavone had no significant effects on plasma GSH-Px activity, T-AOC, MDA content, or liver GSH-Px activity ($P>0.05$), but extremely significantly increased plasma T-SOD activity ($P<0.01$).

2.4 Effects of Polysavone on Lipid Metabolism

Table 6 demonstrates that compared with the control group, dietary supplementation with 1,000 mg/kg polysavone extremely significantly decreased TC and TG contents in both liver and egg yolk ($P<0.01$), while supplementation with 500 mg/kg polysavone extremely significantly decreased liver TG content ($P<0.01$) and significantly decreased liver TC content ($P<0.05$). Dietary polysavone supplementation had no significant effects on plasma TC and TG contents ($P>0.05$).

2.5 Effects of Polysavone on Hepatic mRNA Expression of Antioxidant and Lipid Metabolism-Related Genes

As shown in Table 7 , compared with the control group, dietary supplementation with 1,000 mg/kg polysavone extremely significantly increased hepatic mRNA expression levels of TrxR1, CYP7A1, and SREBP-1c ($P<0.01$), extremely significantly decreased HMGCR mRNA expression ($P<0.01$), and had no significant effect on GSH-Px mRNA expression ($P>0.05$).

Discussion

3.1 Effects of Polysavone on Production Performance

The main active components of polysavone are flavonoids, polysaccharides, and saponins. Previous studies have shown that dietary supplementation with 600 mg/kg polysavone significantly reduced feed-to-egg ratio [9]. Mansoub [10] reported that supplementation with 100, 150, and 200 mg/kg polysavone in Hy-Line W-36 laying hens resulted in the highest laying rate and egg production at 200 mg/kg and the best feed conversion at 150 mg/kg. Xie [11] found that supplementation with 500 and 1,000 mg/kg polysavone during both early and late laying periods significantly increased laying rate and average egg weight while decreasing average daily feed intake and feed-to-egg ratio during early period, and increased laying rate while decreasing feed-to-egg ratio during late period, without significantly affecting average egg weight or daily feed intake. The present study demonstrated that polysavone supplementation significantly increased laying rate and decreased feed-to-egg ratio, consistent with previous findings, and delayed the decline in laying rate after 71 weeks of age. This effect may be attributed to flavonoids in alfalfa stimulating the pituitary gland to release follicle-stimulating hormone and luteinizing hormone, thereby promoting follicular growth and maturation and ovarian granulosa cell proliferation, ultimately improving laying performance [12].

3.2 Effects of Polysavone on Egg Quality

Key indicators for evaluating egg quality include eggshell thickness, eggshell strength, egg shape index, albumen height, Haugh unit, and yolk color. Eggshell strength and thickness directly affect breakage rates, while Haugh unit reflects egg freshness and albumen quality [13]. Previous research indicates that polysavone can significantly improve eggshell strength during late experimental periods without affecting eggshell thickness [11]. Hou et al. [14] found that dietary supplementation with 60 mg/kg alfalfa saponins significantly increased eggshell thickness and Haugh unit while reducing cholesterol content in yolk and whole egg. Liang et al. [15] reported that polysavone supplementation affected various egg quality parameters without consistent patterns. The current study showed that polysavone supplementation enhanced eggshell strength to varying degrees but caused no regular changes in eggshell thickness, possibly because alfalfa saponins promote secretion from the shell gland, while polysaccharides may enhance intestinal calcium absorption by regulating calcium ion concentration [16]. Although Haugh unit showed no significant changes, an increasing trend was observed, suggesting that active compounds in polysavone enhanced protein metabolism and deposition [17].

3.3 Effects of Polysavone on Plasma and Liver Antioxidant Capacity and Related Gene Expression

Antioxidant capacity is a crucial factor affecting animal health, with indices such as GSH-Px, T-SOD, T-AOC, MDA, and TrxR reflecting systemic antioxidant status. Studies have demonstrated that active compounds in alfalfa enhance serum antioxidant properties, reduce MDA content, and prevent cardiovascular disease. Different levels of alfalfa saponins can increase GSH-Px activity in liver and serum of broiler chickens while decreasing MDA content, protecting animals from lipid peroxidation damage [4]. The present results showed that dietary supplementation with 1,000 mg/kg polysavone extremely significantly or significantly increased plasma GSH-Px and T-SOD activities, T-AOC, liver GSH-Px activity, and TrxR1 mRNA expression, while decreasing plasma MDA content and showing a trend toward increased liver GSH-Px mRNA expression. These findings indicate that polysavone supplementation enhances antioxidant capacity by upregulating antioxidant gene expression.

3.4 Effects of Polysavone on Lipid Metabolism

Avian cholesterol is primarily synthesized in the liver, with egg yolk containing nearly all the cholesterol in eggs. As a major active component of polysavone, saponins can reduce bile acid absorption in the intestine, and as cholesterol excretion increases, cholesterol deposition in eggs decreases [18]. Dietary supplementation with 1,000 mg/kg polysavone has been shown to significantly reduce egg yolk TG content in 58-65-week-old Hy-Line Brown hens [7], while 500 mg/kg polysavone significantly reduced serum TG and TC contents in broiler chickens [3]. The current study demonstrated that 1,000 mg/kg polysavone extremely significantly reduced TC and TG contents in egg yolk without significantly affecting plasma TC and TG levels, indicating no necessary correlation between blood cholesterol and egg cholesterol [19]. This may be because egg cholesterol content is determined by lipoprotein content in yolk rather than blood cholesterol levels, so changes in blood cholesterol do not necessarily affect egg cholesterol trends [20].

Research has shown that CYP7A1 is the rate-limiting enzyme in bile acid synthesis and plays an important role in maintaining cholesterol homeostasis. The SREBP-1c gene belongs to the sterol regulatory element-binding protein family and promotes fatty acid synthesis and cholesterol ester formation from cholesterol when intracellular cholesterol levels are high, thereby consuming cholesterol [21]. HMGCR is the rate-limiting enzyme in endogenous cholesterol synthesis [22-23]; inhibiting HMGCR activity reduces endogenous cholesterol synthesis. Zhou et al. [6] evaluated the molecular mechanism of alfalfa saponin extract in reducing egg cholesterol using digital gene expression profiling and found that feeding 120 mg/kg alfalfa saponins for 60 days continuously decreased egg yolk cholesterol content while upregulating hepatic CYP7A1 gene expression. Li et al. [1] reported that dietary supplementation with 600, 900, and 1,200 mg/kg polysavone significantly reduced HMGCR mRNA expression in laying hens, suggesting that regulating HMGCR mRNA expression can control serum and egg yolk cholesterol contents. The present study found that polysavone supplementation significantly increased hepatic CYP7A1 and SREBP-1c mRNA expression while decreasing HMGCR mRNA expression, suggesting that polysavone reduces liver TC and TG contents and consequently egg yolk TC and TG contents through multiple pathways including promoting cholesterol excretion, inhibiting cholesterol synthesis, and affecting reverse cholesterol transport.

Conclusion

Dietary polysavone supplementation can reduce egg yolk TC and TG contents, improve production performance, eggshell strength, and antioxidant and cholesterol metabolism capacity in late-period laying hens, with an optimal supplementation level of 1,000 mg/kg.

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