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Original Article

Dietary Supplementation with Ferula Improves Productive Performance, Serum Levels of Reproductive Hormones, and Reproductive Gene Expression in Aged Laying Hens

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ABSTRACT

This study investigated the effects of dietary Ferula supplementation on productive performance, egg quality, follicular development, serum levels of reproductive hormones, and reproductive gene expression in aged laying hens. A total of 300 Dawu pink laying hens (65 weeks old) were assigned to four treatments with six replicates per treatment and 10 hens per replicate. The birds were individually housed in wire cages and fed a corn-soybean meal-based diet with added Ferula at doses of 0, 50, 100, and 200 mg/kg for 12 weeks. The results showed that the laying rate in the 100 mg/kg Ferula-supplemented group was higher than in birds of the control group during weeks 1 to 12 ($p<0.05$). The average egg weight in the 100 mg/kg Ferula-supplemented group was significantly higher than in the other groups ($p<0.01$), while the feed-to-egg ratio was significantly lower than in other groups ($p<0.01$). The numbers of small yellow follicle, middle white follicle, and small white follicle were higher in the 100 mg/kg Ferula-supplemented birds than in the other groups ($p<0.01$). On weeks 69, the serum levels of estradiol, follicle-stimulating hormone, and luteinizing hormone were significantly higher in the 100 mg/kg Ferula-supplemented group than in the other groups ($p<0.05$). Additionally, expressions of ERα, FSHR, and LHR in the ovarian tissue were up-regulated by Ferula supplementation, especially in the 100 mg/kg group ($p<0.01$). These results indicate that the Ferula supplementation can significantly improve productive performance, egg quality, reproduction of hormonal profile, and reproductive gene expression of aged laying hens.

INTRODUCTION

The production performance of laying hens during their late laying period is rapidly decreasing. This late period represents nearly 50% of the whole laying period, and laying hens are generally eliminated at about 500 days of age. However, laying hens still maintain a laying rate of 60-70% at this time; therefore, the production performance of flocks during this late laying period directly affects the economic benefits of farmers. Therefore, prolonging the utilization period of laying hens in the late laying period is of great significance, and reproductive aging has received increasing attention in recent years. Both the volume and weight of reproductive organs decrease during aging, and levels of reproductive hormones decrease. The ovary is the most important organ to regulate reproductive aging. Poultry ovaries use follicles as basic functional unit, and the number of follicles is closely related to the egg production rate. Therefore, delaying ovarian aging and reducing excessive consumption of the original follicle bank after the peak period of laying eggs are particularly important factors for improving the production performance of poultry.

Ferula is a genus of flowering plants that belongs to the family Umbelliferae, which consists of 130 species distributed throughout the Mediterranean and Central Asia. Ferula plants have important medicinal value because of their phytoestrogen properties, which have been linked to a number of beneficial health effects such as improved animal reproductive performance. Over the years, research explored Ferula´s taxonomy, the anatomy of its nutritional and reproductive organs, as well as the phytochemistry, pharmacology, and potential clinical applications of plants. Ferutinin, a natural phytoestrogen molecule, has been isolated from Ferula by the feed additive producing company Pancosma Feed Additive Co., Ltd., in Switzerland, and its biological activity has been explored. This sesquiterpene molecule has a daucane core, which has been identified as an important part of the chemical structure of ferutinin. Double bonds within this core have been shown to play a key role in the biological activity of ferutinin. Ferutinin is entirely stable in human plasma, which appears to be due to its ability to bind to serum albumin, thus preventing its hydrolyzation. The presence of an ester bond between the daucane core and the p-hydroxybenzoyl moiety indicates that ferutinin can serve as a substrate for esterases and as a template for estrogen receptor binding. Ferula has been shown to improve the binding of estrogen to the estrogen receptor alpha (ERα). Furthermore, the affinity of ferutinin for this receptor is higher than that of the well-known phytoestrogen genistein, whilst its affinity to ERα and ERβ is estimated to be 10% of that of estradiol. Overall, it is considered to be one of the most biologically active phytoestrogens.

Ferula consumption has been linked to improved animal growth and reproductive performance, as well as increased quality and yield of animal products such as meat, eggs, and milk. However, most existing research has focused on the chemical composition of ferutinin, and only limited research focused on its effects on the growth and reproductive performance of animals. It is well known that laying hens face a series of problems at the later laying period, including lower laying performance, lower number of follicles in the ovary, higher rate of egg breaking, apoptosis and follicular atresia , and extended interval of average egg production. In addition, delaying ovarian aging and reducing the excessive consumption of the primordial follicle pool after the peak of egg production are particularly important for improving the performance of aging laying hens. The addition of plant extracts can increase production performance. Existing studies have focused on the effect of dietary supplementation with Ferula on productive performance of broilers, quail, and rats. However, these studies have not specifically evaluated the effects on follicle growth and hormone production, nor have they considered the effects on other types of livestock. The aim of the present study was to evaluate the putative effect and mechanism underlying the action of Ferula on productive performance, egg quality, follicular development, hormone production, and reproductive genes in aged laying hens.

MATERIAL AND METHODS

Ethics statement

This experiment strictly followed the recommendations of the relevant national and local animal welfare bodies. Protocols were approved by the Animal Care and Use Committee of Hebei Agricultural University.

Birds and Experimental Design

Three hundred 65-week-old Dawu Pink laying hens with almost the same body weight (1.4 ± 0.2 kg) were obtained from a commercial breeder (Breeding Poultry of Hebei Dawu Group, Baoding, China). Birds were randomly assigned to one of five treatments, with 10 hens/replicate and six replicates/treatment, and were housed individually in wire cages. Environmental conditions included a 16 h Light: 8 h Dark lighting regime, temperature of 22 ± 1 °C, and optimized ventilation. Birds were provided with water ad-libitum and a commercial basal corn-soybean meal-based diet (Table 1) during the 12-week experimental period. Treatment groups comprised a control (non-supplemented) group and three experimental groups, which received dietary supplementation with Ferula at 50 mg/kg, 100 mg/kg, and 200 mg/kg from 65 to 77 weeks of age. The product was obtained from Pancosma Shanghai Feed Additives Co., Ltd. (N60-0200, Geneva, Switzerland).

Production Performance

Layer performance (including egg-laying rate, average egg weight, average daily feed intake, and feed conversion ratio) was determined on a weekly basis during the 12-week experimental period. Daily records of egg production and feed intake, as well as weekly records of feed consumption were maintained. Eggs were collected and weighed at the same time every day to calculate the daily egg production per hen and the egg weight. Feed consumption was recorded weekly and calculated as g per day per bird. The value of feed efficiency was calculated as g feed per g egg.

Table 1 – Ingredients and nutrient composition of the basal diet (%, as fed basis).

Item	Content
Ingredient	
Corn	62.61
Soybean meal	24.00
Cottonseed meal	1.20
Corn gluten meal	0.20
Limestone powder	9.00
Calcium bicarbonate	0.90
Salt	0.30
Zeolite powder	1.24
DL-Me99t	0.21
Bacillus subtilis probiotic	0.01
Ethoxyquin	0.01
Phytase	0.01
Vitamin mix[b]	0.02
Mineral mix[b]	0.20
Choline	0.09
Calculated nutrients[a]	
ME (MJ/kg)	10.91
CP (%)	15.98
Crude fat (%)	2.59
Total lysine (%)	0.80
Methionine +cysteine (%)	0.45
Ca (%)	3.63
Na (%)	0.34
Total P(%)	0.48
Available P (%)	0.27

aBased on composition of ingredients provided by NRC, 1994.

bProvided per kilogram of diet: vitamin A, 11,000 IU; vitamin D3, 3,000 IU; vitamin E, 12 IU; vitamin K, 3 mg; vitamin B1, 2 mg; vitamin B2, 6 mg; vitaminB6, 3 mg; vitamin B12, 0.012 mg; biotin, 0.04 mg; folic acid, 0.6 mg; niacin,20 mg; pantothenic acid, 10 mg; chromium picolinate, 0.6 mg; Cu(CuSO4·5H2O), 10 mg; I (KI), 0.35 mg; Fe (FeSO4·7H2O), 65 mg; Mn(MnSO4·H2O), 120 mg; Se (NaSeO3), 0.25 mg; Zn (ZnO), 65 mg.

Egg Quality Measurements

On the 4th, 8th, and 12th weeks, four eggs were selected at random from each treatment replicate for the determination of egg quality (total of 24 eggs/week). The egg shape index was measured using an egg form coefficient measuring instrument (NFN385, FHK Corp., Tokyo, Japan) and was calculated by dividing the short diameter by the long diameter of each egg and multiplying by 100 (i.e., long diameter / short diameter * 100). Randomly selected eggs from the control group and the experimental group were weighed individually by an egg multitester (EA-01, ORKA FOOD TECHNOLOGY LTD., Bountiful, UT, USA); albumen height, Haugh unit, and yolk color score were also evaluated. The egg shell breaking strength was determined by an egg force reader (EFR-01, ORKA FOOD TECHNOLOGY LTD., Bountiful, UT, USA), and the eggshell was broken to determine other quality parameters. The shells of the broken eggs were cleaned and weighed using an electronic balance to an accuracy of 0.01 g. Yolks were separated and weighed using an electronic balance to an accuracy of 0.01 g. The number of eggs was recorded every day.

Numbers of Follicular Development

On the 12th week, two birds from each treatment replicate were euthanized by cervical dislocation, the ovaries were removed, and the number and size (i.e., diameter), of the follicles were recorded. Follicle sizes were determined based on the following parameters: 1) preovulatory follicle, POF: >10 mm; 2) small yellow follicle, SYF: 8–10 mm; 3) large white follicle, LWF: 6–8 mm; 4) medium white follicle, MWF: 4–6 mm; 5) small white follicle, SWF: 2–4 mm; 6) primary follicle, PF: <2 mm.

Determination of Reproductive Hormones

On the 4th, 8th, and 12th weeks, two hens from each treatment replicate were euthanized as above and 5 ml blood was collected from the lower wing vein (total of 24 hens per week). Blood samples were centrifuged at 3000 r/min for 15 min. The supernatant was extracted and stored at -20 °C for the determination of blood parameters. Levels of estradiol (E2) (JL15972), progesterone (P4) (JL21702), follicle-stimulating hormone (FSH) (JL11312), and luteinizing hormone (LH) (JL13001) were measured by Enzyme Linked Immunosorbent Assay (ELISA) using commercial kits (Shanghai Jianglai Biotechnology Co., Ltd., Shanghai, China), following the manufacturer's instructions. The assay limits of detection, limits of quantification, intra-assay coefficients of variation (%), and inter-assay coefficients of variation (%) for the measured factors were: FSH (0.1 mIU/ml, 0.25 mIU/ml, 10%, and 15%), LH (1.0 ng/ml, 5 ng/ml, 10%, and 15%), E2 (1.0 pg/ml, 10 pg/ml, 10%, and 15%), and P4 (1.0 pmol/ml, 37.5 pmol/ml, 10%, and 15%), respectively.

mRNA Expression of Reproductive Genes in Ovaries

On the 12th week, two hens from each treatment replicate were euthanized, and the ovaries (removal of follicles) were immediately removed and frozen at -80 °C (total of 24 hens per week). Expressions of ERβ, ERβ, FSHR, and LHR genes in the tissue samples were measured using real-time qRT-PCR. The primer sequences were designed to span an intron to avoid genomic DNA contamination that can otherwise occur when using Primer 5.0. Total RNA was isolated from ovaries using a total RNA extraction kit (Biotech Biotechnology, Beijing, China), following

the manufacturer's instructions. The quality and concentration of the extracted RNA were determined by agarose gel electrophoresis and nucleic acid quantification, respectively. The latter was performed using a nucleic acid quantification analyzer (SmartSpec Plus BIO-RAD). The total cDNA was synthesized using a cDNA synthesis kit (Trans Gen Biotechnology, Beijing, China), following the manufacturer's instructions. Briefly, a 20-µl sample of reaction mixture containing 5 µl total RNA, 4 µl 5 × Prime Script Buffer, 1 µl Prime Script RT Enzyme MixI, 1 µl Oligo dT Prime (50 µmol/L), 1 µl random hexamer-primer 6-mers (10 µmol/L), and 8 µl RNase Free dH_2O, were added. The mixture was incubated under the following conditions: reverse transcription at 37 °C for 15 min, followed by inactivation of reverse transcriptase at 85 °C for 5 min until the temperature decreased to 4 °C. Successful cDNA synthesis was confirmed by amplifying the β-actin amplicon by PCR. The synthesized cDNA was amplified using a 20-µl PCR reaction system containing 1 µl cDNA, 10 µl 2× SYBR Green PCR Mix (TaKaRa Biotechnology, Dalian, China), 0.5 µl PCR Forward Primer, 0.5 µl PCR Reverse Primer (Huada Biological Engineering Technology & Service, Beijing, China), and 8 µl ddH_2O. The PCR conditions were as follows: initial denaturation at 94 °C for 2 min, denaturation at 94 °C for 15 s, followed by PCR reaction comprising 40 cycles of 72 °C for 30 s, and 60 °C for 15 s, followed by melting curve analysis at 95 °C for 15 s, 60 °C for 2 s, and 95 °C for 15 s. The PCR products were verified by electrophoresis on a 1% agarose gel, and subsequent DNA sequencing. Standard curves were generated using pooled cDNA. The primer sequences and parameters are listed in Table 2. The relative expression levels of each gene were calculated in triplicate for each sample using the $2^{-\Delta\Delta Ct}$ method.

Table 2 – Primers used for analysis of gene expression in chicken ovaries.

Gene	Primer sequences	GenBank No.	Product size (bp)
β-actin	F: TATGTGCAAGGCCGGTTTC R: TGTCTTTCTGGCCCATACCAA	NM_205518.1	110
ERα	F: TATTGATGATCGGCTTAGTCTGGC R: CGAGCAGCAGTAGCCAGTAGCA	NM_205183	145
ERβ	F: CTCAGCACAGTCAGTCCAGAACA R: TCAGGGACATCATCATGGAGG	NM_204794	118
FSHR	F: TACCCGTCGTCCATAAGG R: CATCCAGGCAGGTTCCAT	ENSGALG00000009100	186
LHR	F: CAAAAACCTCAGGCGGAT R: GGCGGCAGTCTCTTCAGT	ENSGALG00000009095	162

ERα, Estrogen receptor α; ERβ, Estrogen receptor β; FSHR, follicle-stimulating hormone receptor; LHR, luteinizing hormone receptor.

Statistical Analysis

The analysis was conducted by SPSS 21.0 software (version 13, SPSS Inc., Chicago, IL, USA). Statistical significance and significance levels were determined by one-way ANOVA. Differences at the 0.05 level because of treatments were separated by Duncan's multiple range tests.

RESULTS

Productive Performance

As shown in Table 3, during weeks 1-4 and 5-8, no significant differences were found between the groups. At weeks 9-12, the feed-to-egg ratio of 100 mg/kg Ferula-supplemented groups was significantly lower than that of the control group ($p<0.05$). During weeks 1 to 12, the laying rate of the 100 mg/kg Ferula-supplemented group was significantly higher than that of the control group ($p<0.05$). The average egg weight in the 100 mg/kg Ferula-supplemented group was significantly higher than in other groups ($p<0.01$). The feed-to-egg ratio of the 100 mg/kg Ferula-supplemented group was extremely lower than that of the 50 mg/kg group ($p<0.01$), and significantly lower than that of the 200 mg/kg group ($p<0.05$).

Egg Quality Measurements

Ferula supplementation did not significantly affect egg quality on weeks 4, 8, and 12 of the experiment (Table 4). However, the eggshell strength and eggshell thickness was higher in the Ferula-treatment group than in the control group ($p>0.05$).

Follicular Development Numbers

The effects of Ferula treatment on follicle development numbers are summarized in Figure 1. The numbers of SYF of birds supplemented with 50 mg/kg and 100 mg/kg Ferula were higher than those of both the control group and the group supplemented

Table 3 – Effect of Ferula on the productive performance in aged laying hens.

Period	Items	Ferula supplementation (mg/kg, as fed basis)				
		0	50	100	200	*p* Value
Week 1-4	ADFI (g)	119.87±4.72	119.31±1.52	118.67±5.09	117.38±4.51	0.765
	AEW (g)	64.63±2.34	64.19±1.12	65.60±1.88	65.70±0.54	0.215
	LR (%)	70.15±6.76	74.46±5.77	74.14±4.32	75.04±6.34	0.63
	FER	2.66±0.21	2.50±0.16	2.47±0.18	2.38±0.22	0.198
Week 5-8	ADFI (g)	125.72±6.88	128.39±8.68	121.69±5.79	127.56±5.34	0.931
	AEW (g)	63.90±2.28	63.05±1.11	65.21±1.98	65.05±0.60	0.287
	LR (%)	66.97±12.73	71.16±12.08	73.93±1.96	73.32±3.47	0.814
	FER	2.71±0.32	2.48±0.29	2.39±0.28	2.52±0.15	0.589
Week 9-12	ADFI (g)	127.68±9.84	125.66±6.87	122.78±7.06	127.18±10.47	0.626
	AEW (g)	65.65±3.07	63.39±3.24	66.27±2.23	65.89±1.96	0.06
	LR (%)	58.92±16.05	62.08±12.92	65.61±7.36	65.52±6.46	0.651
	FER	3.19±0.79^{a}	3.12±0.69	2.44±0.30^{b}	2.49±0.28	0.021
Week 1-12	ADFI (g)	122.80±14.69	120.74±114.48	117.96±12.08	121.37±12.75	0.491
	AEW (g)	64.74±2.47^{Ba}	63.69±2.06^{Bb}	65.99±1.85^{A}	64.54±1.25^{B}	0.001
	LR (%)	64.17±7.80^{b}	66.74±13.97	68.66±8.87^{a}	67.03±7.19	0.044
	FER	2.90±0.46^{A}	2.85±0.54^{A}	2.31±0.37^{Bb}	2.70±0.37^{a}	0.001

ADFI , Average daily feed intake; AEW, Average egg weight; LR, Laying rate; FER ,Feed-egg ratio.

Laying rate = egg number/the number of hen-days × 100%. Average egg weight = total egg weight/total egg number. Daily intake = total feed intake/feeding days. Feed-egg ratio = daily feed consumption/average egg weight.

Values are means ± S.D. ^{a,b}Means within each row that possess different superscripts differ significantly (p< 0.05, n=60). ^{A,B}Means within each row that possess different superscripts differ extremely significantly (p<0.01, n=60). ^{a,b}Means within each row that possess different superscripts differ significantly (p<0.05, n=60). If there is no marked letter, the difference is not significant.

Table 4 – Effect of Ferula on egg quality in aged laying hens.

	Items	Ferula extract supplementation (mg/kg, as fed basis)				
		0	50	100	200	*p* value
Weeks 4	ESI	1.28±0.01	1.31±0.01	1.32±0.04	1.28±0.04	0.378
	EST (mm)	0.34±0.02	0.35±0.04	0.33±0.02	0.35±0.03	0.927
	EYRW(%)	26.21±1.65	27.03±2.21	27.53±1.95	26.86±1.69	0.843
	ESRW(%)	13.13±0.56	13.48±0.96	13.67±0.89	13.25±0.56	0.956
	EW (g)	62.30±5.40	64.25±2.52	65.25±5.23	64.56±3.38	0.753
	AH (mm)	5.35±0.99	5.45±1.62	5.20±0.98	4.51±1.09	0.814
	HU	69.95±9.33	68.93±15.62	67.36±9.86	61.23±12.04	0.458
	ESS (N)	36.49±5.92	36.64±3.99	38.14±3.37	38.34±1.87	0.543
Weeks 8	ESI	1.29±0.01	1.30±0.02	1.30±0.04	1.29±0.02	0.489
	EST (mm)	0.35±0.02	0.34±0.04	0.34±0.04	0.34±0.03	0.814
	EYRW(%)	26.32±1.09	26.68±2.08	26.79±1.61	25.34±1.55	0.829
	ESRW(%)	13.31±1.83	13.74±0.68	13.71±0.67	13.34±1.27	0.943
	EW (g)	65.65±6.04	67.33±3.17	65.83±3.06	66.08±2.41	0.331
	AH (mm)	5.80±2.13	4.76±1.91	5.23±1.89	5.65±2.16	0.628
	HU	69.83±14.72	57.71±23.86	59.83±13.89	67.51±20.17	0.544
	ESS (N)	37.76±5.11	33.74±6.04	35.41±6.14	34.47±7.34	0.825
Weeks 12	ESI	1.31±0.06	1.30±0.06	1.30±0.04	1.30±0.04	0.952
	EST (mm)	0.35±0.03	0.35±0.03	0.35±0.02	0.33±0.04	0.772
	EYRW(%)	27.55±2.53	27.60±2.20	26.92±2.57	25.61±2.38	0.446
	ESRW(%)	12.55±1.35	12.63±0.61	12.67±1.31	12.72±1.17	0.604
	EW (g)	63.87±2.20	62.91±5.41	65.00±2.57	67.27±4.00	0.509
	AH (mm)	5.60±0.93	5.80±1.00	6.51±1.25	5.95±0.53	0.725
	HU	71.73±7.45	73.65±8.46	78.15±8.34	73.80±4.89	0.747
	ESS (N)	30.64±7.90	34.76±9.32	33.73±9.66	31.02±6.20	0.488

ESI, Egg-shape index; EST, Eggshell thickness; EYRW, Egg yolk relative weight; ESRW, Eggshell relative weight; EW, Egg weight; AH, Albumen height; HU, Haugh unit; ESS, Eggshell strength.

Values are means ± S.D. ^{a,b}Means within each row that possess different superscripts differ significantly (p<0.05, n=24). ^{A,B}Means within each row that possess different superscripts differ significantly (p<0.01, n=24).If there is no marked letter, the difference is not significant.

with 200 mg/kg (p<0.05). The numbers of MWF in the 50 mg/kg and 100 mg/kg Ferula-supplemented group were higher than that in the 200 mg/kg supplemented group (p<0.05). The numbers of SWF in the 100 mg/kg supplemented group were higher than that of the 200 mg/kg supplemented group (p<0.05).

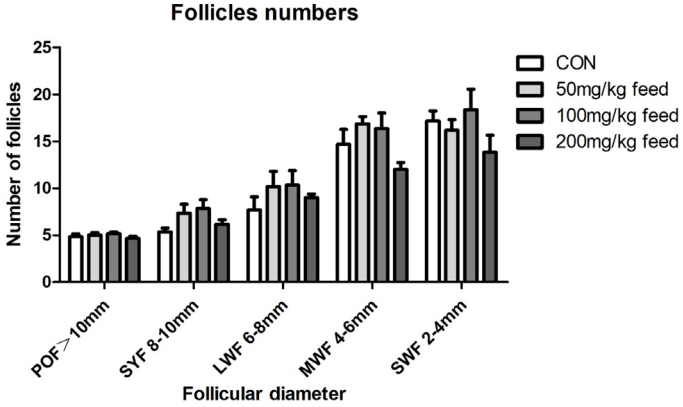

Figure 1 – Effect of Ferula on follicle Numbers in aged laying hens. Values are means ± S.D. [a–b]Bars with different superscripts are statistically different (p<0.05; n = 12).

Reproductive Hormones Levels

The effects of the treatment on the concentration of reproductive hormones in the serum are summarized in Table 5. On week 4, the concentrations of E2 in the 50 mg/kg and 100 mg/kg Ferula-supplemented groups were higher than in the control group (p<0.05). In addition, the levels of P4 were higher in the control group than in the 50 mg/kg supplemented group (p<0.05). The levels of FSH in the 100 mg/kg supplemented group were higher than those in the control, the 50 mg/kg, and the 200 mg/kg supplemented groups (p<0.05, p<0.01, and p<0.01, respectively). The LH serum concentrations in the control birds and birds supplemented with 100 mg/kg and 200 mg/kg Ferula were higher than that of birds supplemented with 50 mg/kg (p<0.01). In week 8, E2 concentrations were higher in 100 mg/kg supplemented birds than in 50 mg/kg supplemented birds (p<0.05). On week 12, the concentration of P4 in 100 mg/kg supplemented birds was higher than in 200 mg/kg supplemented birds (p<0.05), and FSH level was higher in 100 mg/kg supplemented birds than in 200 mg/kg supplemented birds (p<0.05).

Table 5 – Effect of Ferula on reproduction hormone levels in aged laying hens.

	Items	Ferula supplementation (mg/kg, as fed basis)				
		0	50	100	200	p value
Weeks 4	E2(pmol/L)	72.35±2.10^{b}	88.39±14.97^{a}	85.34±12.87^{a}	77.99±13.74	0.045
	P4(pmol/L)	493.26±17.61^{A}	335.33±45.92^{B}	486.94±0.45^{A}	426.06±48.81	0.006
	FSH (U/L)	7.99±2.19^{b}	7.31±0.23^{B}	9.68±0.73^{Aa}	6.93±0.72^{B}	0.001
	LH (ng/L)	52.42±9.64^{A}	41.56±2.41^{B}	60.73±1.32^{A}	55.76±0.23^{A}	0.001
Weeks 8	E2(pmol/L)	80.36±11.06	73.74±10.88^{b}	89.74±19.61^{a}	84.44±16.30	0.028
	P4(pmol/L)	388.74±65.95	427.59±84.22	434.29±66.43	408.84±74.76	0.779
	FSH (U/L)	8.09±1.78	7.67±0.90	8.52±1.34	8.18±1.41	0.856
	LH (ng/L)	48.73±9.14	54.05±8.08	52.96±9.14	49.68±8.35	0.56
Weeks 12	E2(pmol/L)	84.36±14.78	80.22±14.22	89.37±12.54	83.95±14.85	0.087
	P4(pmol/L)	411.14±54.20	409.03±74.72	434.62±69.77^{a}	367.74±88.17^{b}	0.029
	FSH (U/L)	7.88±1.38	7.59±1.49	8.67±0.89^{a}	6.74±0.74^{b}	0.019
	LH (ng/L)	47.09±5.57	47.62±9.16	51.88±9.68	47.99±8.25	0.851

E2, estradiol; P4, progesterone; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

Values are means ± S.D. [a,b]Means within each row that possess different superscripts differ significantly (p<0.05, n=12). [A,B]Means within each row that possess different superscripts differ extremely significantly (p<0.01, n=12). [a,b]Means within each row that possess different superscripts differ significantly (p<0.01, n=12). If there is no marked letter, the difference is not significant.

Expression of Reproductive Genes

As shown in Figure 2, the expression of ERα in the ovaries of the birds supplemented with 100 mg/kg Ferula was extremely higher than in other treatment groups (p<0.01). Expressions of ERα in the 50 mg/kg and 200 mg/kg Ferula groups were significantly higher than in the control group (p<0.01). The expression of ERβ was higher among birds supplemented with 50 mg/kg Ferula compared with other treatment groups (p<0.01). In addition, the expression of FSHR in the ovaries of birds supplemented with 100 mg/kg Ferula was higher than that of all other treatment groups (p<0.01). The expression of LHR in the 100 mg/kg Ferula supplementation group was extremely higher than in the other treatment groups (p<0.01), and higher in the 200 mg/kg Ferula supplement group than in the control group and 50 mg/kg groups (both p<0.05).

DISCUSSION

The present study has shown that dietary supplementation with Ferula exerted a significant effect

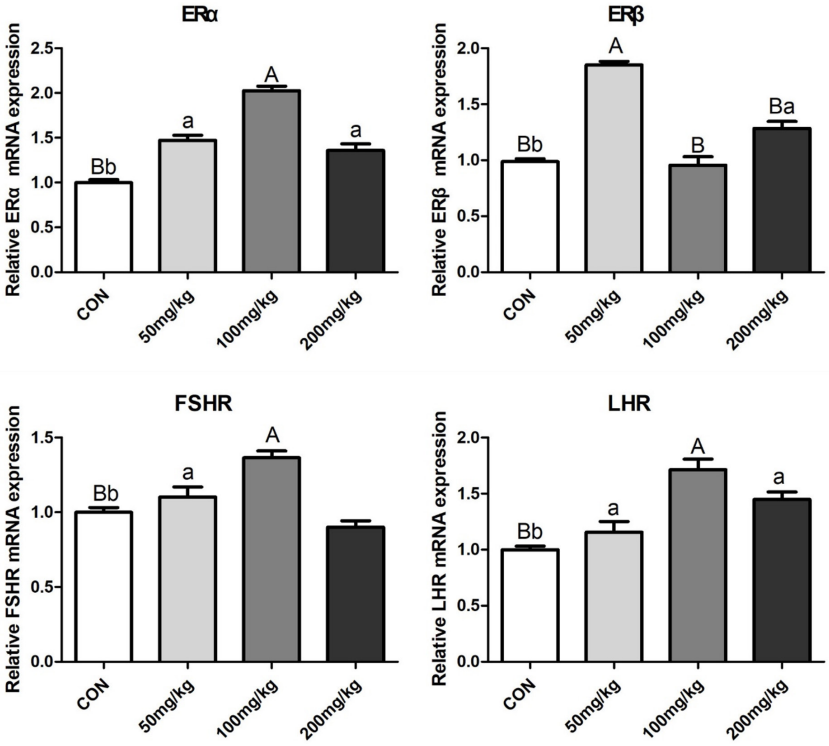

Figure 2 – Effect of Ferula on mRNA expression of ERα , ERβ, FSHR and LHR in the ovarian stroma of laying hens. Values are means ± S.D. $^{a-b}$Bars with different superscripts are statistically different ($p<0.05$; n = 12). $^{A-C}$Bars with different superscripts are statistically different ($p<0.01$; n = 12).

on the productive performance of aged laying hens. Furthermore, the decline in the egg laying rate that occurred over time in all birds was numerically smaller in the Ferula-supplemented groups compared with the control group. Ferula-supplemented at 100 mg/kg yielded the highest egg laying rate and the lowest feed-to-egg ratio compared with other treatment groups. Other authors have also observed the same effect of Ferula on production performance in laying hens, ducks and geese. The structure of phytoestrogens resembles that of endogenous estrogens. Phytoestrogen molecules are capable of binding to ERα and ERβ receptors because of their unique phenolic ring structure, thus improving endogenous estrogenic activity during late egg production, which improves production performance. Similar effects have previously been described for other plant derived products such as daidzein and quercetin. The findings of the present study suggest that Ferula supplementation increased the egg production rate at least in part because of its estrogen-like effects.

Egg production and shell strength decrease as hens age. Older hens in production lay larger but fewer eggs than younger birds; the incidence of soft and broken shells is higher. The present study found that dietary supplementation with Ferula (at specific dose levels) resulted in higher eggshell strength in week 12. It has been shown that intestinal calcium absorption decreases with age in chickens, and estrogen substances promote intestinal calcium absorption. Moreover, soy isoflavone supplementation significantly improves bone mineral density and the serum calcium level in quails during the late laying period. Chickens supplemented with daidzein have also been shown to exhibit higher serum calcium concentrations; it has also been suggested that daidzein supplementation may increase the rate of bone formation and eggshell quality of chickens, possibly because of improved intestinal calcium absorption. The changes in egg production and shell quality are also attributable to changing hormone profiles, decreased sensitivity of tissues to hormone action, and diminished ability of the hen to transport calcium at the duodenum. The results of the current study indicate that supplementation with Ferula improved egg quality to some extent.

Compared with mammals, birds have no regular estrus cycle; therefore, follicles within the ovaries can develop and ovulate continuously. Although a large number of follicles are contained within the ovaries of birds, only few of these have the capacity to maturate and ovulate. In general, there are 4-7 mature follicles of different sizes in the ovary cortex of poultry, which can be divided into different grades according to their decreasing volume. These are denoted F1, F2, F3, etc., and are referred to as preovulatory follicles (POF). Other large numbers of non-invasive follicles are collectively referred to as pre-grade follicles. Only one POF follicle matures and ovulates per day, and the other follicles are apoptotic or blocked. It is also known that the egg production rate of aged laying hens rapidly declines, as follicular development is inhibited and follicular atresia and abnormal ovulation increase. Comparing the number of graded follicles in the ovaries of birds of different ages showed that the average number of graded follicles was 7.8 at 28 weeks of age, which decreased to 6.9 at 44 weeks of age and 6.3 at 76 weeks of age. Only few of these follicles can mature and ovulate, while more than 99% will generate atresia. Notably, in the present study, the numbers of SWF, MWF, and SYF in the 100 mg/kg Ferula-supplemented group were higher than in other treatment groups. This is consistent with the

findings of who reported that 10 mg/kg daidzein (fed to 13-month-old ISA laying hens during the post-peak period of egg laying) markedly increased the numbers of SYFs and LWFs compared with the control groups. Shi et al. (2013) similarly found that the addition of 10 mg/kg or 50 mg/kg daidzein increased the number of SYFs and LWFs, whilst the addition of 100 mg/kg decreased the numbers of these follicles. In the present study, Ferula supplementation increased the numbers of follicles in the ovary, indicating that Ferula supplementation reduced follicular atresia and thus improved the egg production performance of aged laying hens.

The hypothalamus is part of the pituitary-ovary axis. The reproductive axis of poultry mainly consists of gonadotropin-releasing hormone (GnRH) secreted by the hypothalamus, Luteinizing (LH), Follicle stimulating hormone (FSH), secreted by the pituitary gland, and both progesterone and estradiol secreted by the ovary. After the peak egg production period, laying hens exhibit reduced endogenous estrogenic activity. Estrogen plays an important role in the regulation of the reproductive axis, mainly as a feedback regulator of the gonad to the brain, regulating both the synthesis and secretion of hormones such as GnRH and LH. Ketones stimulate the release of FSH and LH from the pituitary gland and thus accelerate follicle maturation and ovulation. In the present study, Ferula altered the level of hormone secretion in aged laying hens. Compared with the control diet, the serum E2 content increased by Ferula supplementation at 50 or 100 mg/kg. Furthermore, the FSH content produced by Ferula supplementation at 100 mg/kg was higher than that produced by 200 mg/kg and this dose of Ferula also increased the serum LH content to a level above that of all other treatment groups. A similar effect on LH modulation was previously reported in male rats and it has also been observed that ferutinin can increase the levels of gonadotropin in the blood. Studies on the effects of dietary supplementation with quercetin and daidzein have reported similar effects. These effects may result from the known estrogen-like effects of Ferula, which, at 100 mg/kg, promote hormone secretion and increase follicular development, thus leading to an increased egg production rate. In contrast, the observed decrease in hormone levels in the serum among birds supplemented with 200 mg/kg may have been caused by high levels of internal estrogen; a high-dose of Ferula may inhibit the development of reproductive organs. Overall, the results showed that the estrogenic effects of Ferula altered hormone secretion in aged laying hens in a dose-dependent fashion.

Estrogens are key regulators of reproductive functions such as gonadal differentiation and development, reproductive behavior, synthesis of egg yolk proteins in the liver, egg white proteins in the oviduct, and mobilization of calcium for eggshell formation. It has been shown that in chicken, ER decreases with age. There are two different types of intracellular receptors: ERα and ERβ. ERα is predominantly involved in estrogen effects on ovarian functions in chicken. In contrast, the mammalian ovary expresses mainly ERβ. The present study found that the relative expression levels of ERα and FSHR in the ovaries of birds supplemented with 100 mg/kg Ferula were higher than those in other treatment groups. This indicates that addition of this plant extract at this dose level increased the sensitivity of ERα receptors to some extent, while it did not affect ERβ receptors. These results are consistent with those of Gu who reported that the expression of ER and FSHR in the basal tissues of the ovaries of birds fed 10 mg/kg or 50 mg/kg daidzein increased significantly. Liu et al. reported that treatment with daidzein (50 mg/kg) resulted in increased mRNA expression of FSHR in follicle granulosa layer. This was also consistent with the results of the present study. The mechanism of action of phytoestrogen involves its binding to the estrogen receptor inside cells. Phytoestrogens possess the phenolic ring necessary to bind to the estrogen receptor, have a similar molecular weight to that of estradiol, and can act as agonists and antagonists of estrogen receptors. Several studies have demonstrated that the phytoestrogen ferutinin can act as an estrogen agonist by binding to the estrogen receptors and generating estrogen-induced responses. Ferutinin is one of the most potent natural phytoestrogens, which has agonistic activity on estrogen receptors, particularly on the ERα receptor. Ferula may act as an agonist by occupying the empty binding site of estrogen, leading to an increase in the magnitude of systemic estrogen effects, and upregulating gonadotropin receptor mRNA expression. The results of the present study support this hypothesis by demonstrating that Ferula supplementation upregulated reproductive gene expression in aged laying hens.

CONCLUSION

Dietary supplementation of Ferula to laying hens promoted the production of reproductive hormones and increased the expression of the reproductive genes ERα and FSHR in aged laying hens. These effects were beneficial for ovarian function by promoting follicle development and ovulation, thus improving

production performance (i.e., egg weight and feed to egg ratio) during the post-peak egg laying period. The strongest beneficial effects were observed with a Ferula supplementation dose of 100 mg/kg.

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