

« Research Note »

## Egg Production, Egg Quality, Blood Profiles, Cecal Microflora, and Excreta Noxious Gas Emission in Laying Hens Fed with Fenugreek (*Trigonella foenum-graecum* L.) Seed Extract

Jae-Hong Park, Yong-Min Kim and In-Ho Kim

Department of Animal Resource and Science, Dankook University, Cheonan, 31116, Republic of Korea

This study was designed to investigate the effects of dietary fenugreek seed extract (FSE) supplementation on egg production, egg quality, blood profiles, cecal microflora, and excreta noxious gas emission in laying hens. A total of 384 laying hens (26-weeks old, Hyline-brown) were fed three different levels of FSE (0, 0.05, and 0.1%) in a corn-soybean diet for 6 weeks. The inclusion of FSE in the laying hen diet did not affect egg production, feed intake, or feed conversion among treatments; however, egg weight, eggshell breaking strength, eggshell thickness, and yolk color increased in FSE-fed groups (linear,  $P < 0.05$ ). Supplemental FSE decreased the serum total cholesterol concentration, whereas the HDL-cholesterol concentration increased in the FSE fed-groups (linear,  $P < 0.05$ ). FSE led to an increase in cecal *Lactobacillus* number (linear,  $P < 0.05$ ), and a decrease in *Escherichia coli* number (quadratic,  $P < 0.05$ ) and excreta ammonia gas emission (linear,  $P < 0.05$ ). These results suggest that the addition of FSE does not increase egg production, but may affect egg quality, serum total- and HDL-cholesterol concentration, and cecal microflora. FSE also decreased ammonia gas emission in laying hen excreta.

**Key words:** ammonia gas, cholesterol, eggshell, egg weight, intestinal microbiota

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### Introduction

Phytogenic feed additives (PFA) have attracted a lot of attention in recent years. When used in diets, these substances exhibit antioxidative properties and antimicrobial activity, improve nutrient absorption, and could ultimately improve animal performance (Hernandez *et al.*, 2004; Khalaji *et al.*, 2011; Park *et al.*, 2014). PFA are less toxic, have fewer side effects, and are residue-free compared with synthetic feed additives, and are thought to be ideal feed additives in animal production (Hashemi *et al.*, 2008). Therefore, many phytogenic compounds have been recommended for use as feed additives.

Fenugreek (*Trigonella foenum-graecum* L) belongs to the Leguminosae family and is cultivated predominantly in India, West Asia, the Mediterranean, North Africa, and Canada (Kaviarasan *et al.*, 2007). The seeds are generally used for condiments in various food preparations, are regarded to have great nutritive value and restorative properties, and have been used in folk medicine for centuries for

their hypoglycemic, anthelmintic, antibacterial, anti-inflammatory, antipyretic, and antimicrobial properties (Vats *et al.*, 2002; Bash *et al.*, 2003; Vyas *et al.*, 2008). The major active components of fenugreek seeds are 4-hydroxyisoleucine, trigonelline, galactomannan with flavonoids, carotenoids, coumarins, and saponins, which confer pharmacological activity and beneficial effects (Udayasekhara and Sharma, 1987; Basch *et al.*, 2003).

Recently, the modes of PFA action in vivo, including aromatic plants, plant extracts, their single active components, or their blended additives, have been investigated in several studies (Yan *et al.*, 2011; Amouzmehr *et al.*, 2012; Park *et al.*, 2014; Park *et al.*, 2015). However, in laying hens, a comparatively small number of studies have investigated the effects of fenugreek seed on production performance and egg quality. Therefore, in this study, an attempt was made to evaluate the fenugreek seed as natural growth promoter in laying hen diets. The evaluation included biochemical changes in egg production, egg quality, blood profiles, cecal microflora, and excreta noxious gas emission.

### Materials and Methods

#### Ethical Considerations

The experimental protocols describing the management and care of animals were reviewed and approved by the

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Correspondence: In-Ho Kim, Department of Animal Resource and Science, Dankook University, Cheonan, 31116, Republic of Korea.

(E-mail: inhokim@dankook.ac.kr)

Animal Care and Use Committee of Dankook University.

### Experimental Design, Animals, and Housing

A total of 384, 26-week-old (Hyline-brown) laying hens were used in this 6-week trial. Laying hens were randomly assigned to one of three treatments with eight replicates (16 hens/replicate). The experimental treatments were: control, basal diet (FSE 0%); fenugreek seed extract (FSE) 0.05%, basal diet + 0.05% FSE; FSE 0.1%, basal diet + 0.1% FSE. The FSE used in our study was Nutrifen<sup>®</sup> (Emerald Seed Products Ltd., Avonlea, Canada), which contains  $\geq 15.0$  mg/g saponin. Nutrifen<sup>®</sup> was composed of 3584 kcal/kg ME, 10.4% moisture, 28.0% CP, 9.9% crude fiber, 10.4% crude fat, and 4.6% crude ash. It also contained macrominerals as follows; 0.34% calcium, 0.28% sulfur, 0.74% phosphorus, 0.26% magnesium, 36.2 mg/kg zinc, 21.4 mg/kg manganese, and 36.2 mg/kg iron. Laying hens were provided ad libitum access to water and feed. All the diets were formulated in mash form to meet or exceed the NRC (1994) nutrition requirement (Table 1). Treatment additives were included in the diet by replacing the same amount of corn. Laying hens were allowed to adjust to the environment for 5 days prior to beginning the feeding trial, during which they were fed a basal diet. They were raised in an ambient-

regulated house, in which the temperature was maintained below 23°C and the light regime was set on a 16:8-light: dark cycle throughout the entire experiment. Birds were individually reared in adjacent steel cages fitted with a nipple drinker, feeder, and an egg collecting plate.

### Laying Production, Performance, and Egg Quality

The hen-day egg production and egg weights were recorded daily, while feed consumption was measured weekly. The feed conversion ratio was calculated as the feed consumption per hen divided by egg weight per day per hen. For each treatment, 40 normal eggs (five eggs/cage) were collected randomly at 32 weeks and used to determine the egg quality. Eggshell color scores were determined using an eggshell color fan on a 1–15 scale (1=light to 15=dark brown) by a single trained evaluator. Haugh units, albumen height, and yolk color were determined, using an egg multi tester (Touhoku Rhythm Co., Ltd., Tokyo, Japan). Eggshell breaking strength was evaluated, using an Eggshell force gauge, model II (Robotmation Co., Ltd., Tokyo, Japan), and eggshell thickness was measured, using a dial pipe gauge (Ozaki Mfg. Co., Ltd., Tokyo, Japan).

### Blood Profile

At the end of the experiment, 16 laying hens were randomly selected from each treatment (two hens/replication) and blood samples were taken from the jugular vein by a sterilized syringe with needle. Then, the samples were transferred to either a vacuum or K<sub>3</sub>EDTA vacuum tube (Becton Dickinson Vacutainer Systems, FranklinLakes, NJ, USA). The blood samples were centrifuged at 2000 × g at 4°C for 15 min to separate the serum. High-density lipoprotein (HDL), low-density lipoprotein (LDL), and total cholesterol, and immunoglobulin G (IgG) concentrations in the serum were then analyzed using an automatic biochemistry blood analyzer (HITACHI747, Tokyo, Japan). Whole blood samples from the K<sub>3</sub>EDTA vacuum tube were analyzed immediately to determine the white blood cells (WBC), red blood cells (RBC), and lymphocyte concentrations using an automatic blood analyzer (ADVIA 120, Bayer, Tarrytown, NY, USA).

### Cecal Microflora

At the end of the experiment, samples of cecal contents were collected from 16 laying hens randomly selected from each treatment, then placed on ice for transportation to the laboratory, where analyses were immediately performed using the method described by Wang and Kim (2011). One-gram of pooled cecal content sample was diluted 1:9 (wt/vol) with phosphate buffer saline solution (PBS; 0.1M, pH 7.0). Then, 10-fold serial dilutions (10<sup>-3</sup> to 10<sup>-6</sup>) of cecal content samples were generated with PBS and placed onto MacConkey (Difco Laboratories, Detroit, MI, USA) and *Lactobacillus-Rogosa* agar plates (Difco Laboratories) to isolate the *Escherichia coli* and *Lactobacillus*, respectively. The *Lactobacillus-Rogosa* and MacConkey agar plates were then incubated for 24 h at 37°C under anaerobic and aerobic conditions, respectively. After incubation, *Lactobacillus* and *E. coli* colonies were counted immediately using a colony counter, and are reported as colony-forming units (CFU) log<sub>10</sub> per g.

Table 1. Formula and chemical composition of basal diet (as-fed basis)

Item	(%)
<b>Ingredients</b>	
Corn	50.40
Soybean meal (CP 46%)	18.70
Wheat grain	10.00
Corn gluten meal	2.00
Wheat bran	5.00
Animal fat	4.40
Limestone	7.50
Dicalcium phosphate (P 18%)	1.40
Salt	0.30
DL-Met (50%)	0.10
Vitamin premix <sup>1</sup>	0.10
Trace mineral premix <sup>2</sup>	0.10
Total	100
<b>Calculated values</b>	
ME (kcal/kg)	2,904
CP (%)	15.02
Lys (%)	0.78
Met + Cys (%)	0.65
Ca (%)	3.25
P (%)	0.61

<sup>1</sup> Provided per kilogram of premix: 125,000 IU vitamin A; 2,500 IU vitamin D<sub>3</sub>; 10 mg vitamin E; 2 mg vitamin K<sub>3</sub>; 1 mg vitamin B<sub>1</sub>; 5 mg vitamin B<sub>2</sub>; 1 mg vitamin B<sub>6</sub>; 15 mg vitamin B<sub>12</sub>; 500 mg folic acid; 35,000 mg niacin; 10,000 mg Ca-Pantothenate and 50 mg biotin.

<sup>2</sup> Provided per Kg of diet: 8 mg Mn (as MnO<sub>2</sub>); 60 mg Zn (as ZnSO<sub>4</sub>); 5 mg Cu (as CuSO<sub>4</sub>·5H<sub>2</sub>O); 40 mg Fe (as FeSO<sub>4</sub>·7H<sub>2</sub>O); 0.3 mg Co (as CoSO<sub>4</sub>·5H<sub>2</sub>O); 1.5 mg I (as KI), and 0.15 mg Se (as Na<sub>2</sub>SeO<sub>3</sub>·5H<sub>2</sub>O).

### Excreta Noxious Gas Emission

Fresh excreta from laying hens were collected from eight cages per treatment to determine excreta noxious gas emission according to the method described by Cho *et al.* (2008). Excreta samples (300 g) were stored in 2-L plastic boxes. The samples were allowed to ferment for 1 day at room temperature (28°C). After the fermentation period, the gases that formed were determined using a Gastec (model GV-100) gas sampling pump (Gastec Corp., Gastec detector tube No. 3L and 3La for ammonia; No. 4LL and 4LK for hydrogen sulfide; No. 70 and 70L for total mercaptan, Gastec Corp, detector tube, Ayase, Japan) from approximately 5 cm above the excreta samples.

### Statistical Analysis

Data were statistically analyzed via ANOVA using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC) for a randomized complete block design. The linear and quadratic effects of FSE among treatments were analyzed using a contrast statement. The mean values and standard error of means (SEM) were reported. Probability values less than 0.05 were considered significant.

## Results

### Egg Production Performance

Laying hens fed diets supplemented with FSE during weeks 27–32 showed significant differences in egg weight compared with those fed the control treatment, as dietary FSE increased from 0.05 to 0.1% (linear,  $P=0.012$ ). However, throughout the experimental period, there was no significant difference in egg production, feed intake, and feed

conversion in laying hens fed different levels of FSE in their diet (Table 2).

### Egg Quality

Eggshell breaking strength and eggshell thickness were found to be increased in the FSE groups compared with the control group (linear  $P<0.05$ ). As the dietary levels of FSE increased, a linear increase in the intensity of yolk color was observed ( $P=0.001$ ). There was no significant difference in eggshell color, Haugh units, or albumen height of laying hens fed different levels of FSE in their diet (Table 3).

### Blood Profiles

Serum levels of HDL-cholesterol were elevated in the FSE treatment group compared with the control group (linear,  $P=0.017$ ). The total cholesterol concentration decreased as dietary FSE increased in laying hens compared with laying hens fed the control diet (linear,  $P=0.042$ ). However, serum LDL-cholesterol and IgG levels were not affected by the addition of FSE. Furthermore, FSE treatment had no significant effect on WBC, RBC, or lymphocytes (Table 4).

### Cecal Microflora

FSE supplementation linearly increased *Lactobacillus* numbers in the cecum, compared with the control treatment ( $P=0.012$ ). Cecal *E. coli* decreased quadratically as dietary FSE increased ( $P=0.010$ ) (Table 5).

### Excreta Noxious Gas Emissions

Excreta ammonia emissions were decreased as dietary FSE supplementation increased (linear,  $P=0.020$ ). However, FSE supplementation did not affect total mercaptan and hydrogen sulfide emissions, compared with the control treatment (Table 6).

Table 2. The effects of dietary fenugreek seed extract (FSE) on productivity in laying hens<sup>1</sup>

	FSE, %			SEM <sup>2</sup>	P-value	
	0	0.05	0.1		Linear	Quadratic
Egg production, %	96.0	97.9	96.5	1.21	0.677	0.115
Egg weight, g	63.1	64.2	66.4	0.89	0.012	0.642
Feed intake, g	115	115	117	1.93	0.659	0.698
Feed conversion, g:g	1.833	1.797	1.757	0.02	0.052	0.952

<sup>1</sup> Each treatment mean represents 8 replicates (16 hens/replicate).

<sup>2</sup> Standard error of mean

Table 3. The effects of dietary fenugreek seed extract (FSE) on egg quality in laying hens<sup>1</sup>

	FSE, %			SEM <sup>2</sup>	P-value	
	0	0.05	0.1		Linear	Quadratic
Eggshell color	11.25	11.35	11.3	0.19	0.855	0.752
Eggshell breaking strength, kg/cm <sup>2</sup>	4.390	4.483	4.661	0.06	0.012	0.588
Eggshell thickness, mm <sup>-2</sup>	35.41	36.36	36.73	0.43	0.038	0.304
Haugh unit	82.52	83.03	82.62	1.41	0.959	0.788
Albumen height, mm	7.55	7.84	7.68	0.19	0.635	0.355
Yolk color	7.0	7.1	7.8	0.14	0.001	0.091

<sup>1</sup> Each treatment mean represents 40 replicates (5 eggs/replicate).

<sup>2</sup> Standard error of mean

Table 4. The effect of dietary fenugreek seed extract (FSE) on blood profiles in laying hens<sup>1</sup>

	FSE, %			SEM <sup>2</sup>	P-value	
	0	0.05	0.1		Linear	Quadratic
White blood cells, 10 <sup>3</sup> /μl	27.0	27.3	31.5	3.92	0.365	0.636
Red blood cells, 10 <sup>6</sup> /μl	2.3	2.6	2.3	0.15	0.906	0.141
Lymphocyte, %	70.4	60.7	66.2	9.92	0.745	0.495
HDL-cholesterol, mg/dL	33	41	44	2.05	0.017	0.503
LDL-cholesterol, mg/dL	63	62	61	6.24	0.848	0.987
Total cholesterol, mg/dL	222	172	184	11.14	0.042	0.067
Immunoglobulin G, mg/dL	446	469	452	106.45	0.968	0.883

<sup>1</sup> Each treatment mean represents 16 replicates (2 hens/replicate).

<sup>2</sup> Standard error of mean

Table 5. The effects of dietary fenugreek seed extract (FSE) on cecal microbiota in laying hens<sup>1</sup>

	FSE, %			SEM <sup>2</sup>	P-value	
	0	0.05	0.1		Linear	Quadratic
<i>Lactobacillus</i> , log <sub>10</sub> cfu/g	6.98	7.33	7.42	0.10	0.012	0.298
<i>E. coil</i> , log <sub>10</sub> cfu/g	6.57	6.14	6.37	0.08	0.151	0.010

<sup>1</sup> Each treatment mean represents 16 replicates (2 hens/replicate).

<sup>2</sup> Standard error of mean

Table 6. The effects of dietary fenugreek seed extract (FSE) on excreta gas emission in laying hens<sup>1</sup>

	FSE, %			SEM <sup>2</sup>	P-value	
	0	0.05	0.1		Linear	Quadratic
Total mercaptan, ppm	1.4	0.9	1.0	0.17	0.058	0.134
Ammonia, ppm	48	33	30	4.40	0.020	0.277
Hydrogen sulfide, ppm	2.4	2.4	2.4	0.07	0.987	0.892

<sup>1</sup> Each treatment mean represents 8 replicates (16 hens/replicate).

<sup>2</sup> Standard error of mean

## Discussion

Laying performance of the FSE-treated groups did not significantly differ over 27–32 weeks in our study. PFA has been investigated in numerous studies on poultry with varying responses, depending upon the plant species, disease challenges, and different environmental conditions used. Results have also varied depending on the specific plant-derived compound used, or stress type (Demir *et al.*, 2003; Griggs and Jacob, 2005; Cabuk *et al.*, 2006; Cho *et al.*, 2014). Several studies have reported that dietary supplementation with extracts of some plants increased egg production, feed conversion ratio, and egg weight in laying hens (Uganbayar *et al.*, 2005; Nadia *et al.*, 2008; Radwan *et al.*, 2008). Recent reports have also suggested an improvement in the early growth performance of broilers and pigs, when supplemented with FSE (Hossain *et al.*, 2015; Park and Kim, 2016). In contrast, previous studies have shown that neither PFA nor FSE exhibited significant effects on feed intake,

feed efficiency, and/or egg production of laying hens (Botsoglou *et al.*, 2005; Deng *et al.*, 2012; Al-Aqil, 2016). Growth responses of broilers, or the egg production performance of laying hens supplemented with PFA remain controversial, and the mechanism of FSE action on poultry performance has not yet been clearly established. Our finding suggests that FSE had no direct beneficial effect on egg production, feed intake, or feed conversion. However, a significant improvement in egg weight, eggshell strength, and eggshell thickness was observed when FSE was included in the diet. The results of our study were similar to those reported recently by El-Shafei *et al.* (2012), who showed significant effects on the eggshell thickness, eggshell weight, and specific gravity between control and fenugreek powder groups. Qureshi *et al.* (2016) reported that fenugreek seeds in the diet may enhance nutrient absorption due to greater villus height and crypt depth, thereby improving intestinal health. Similarly, Amad *et al.* (2011) found that the apparent ileal digestibility of crude ash, calcium, and phosphorus

increased linearly with increasing dose of PFA in the diet. Moreover, fenugreek seeds naturally contain high levels of minerals, including calcium, iron, and zinc (Hooda and Jood, 2003). The beneficial effect on egg weight, eggshell breaking strength, and eggshell thickness might be attributable to favorable alterations in the intestinal environment and function, which may have increased intestinal calcium absorption. Therefore, FSE may help calcium utilization for eggshell formation, although no significant differences were observed in egg production performance between the FSE groups and the control. In our study, the addition of FSE increased the intensity of the yolk color. Fenugreek seeds are also widely used as a spice and food colorant in food preparations because of their light yellow-brown color (Ciftci *et al.*, 2011). An increase in the intensity of yolk color is likely to be due to the action of the yellow color agent present in FSE on the yolk following the addition of FSE. Thus, FSE could be supplied as a component of pigment in the diets in order to influence egg yolk yellowness.

Serum lipid metabolites in relation to PFA and their extracts have been extensively studied. The addition of PFA could affect serum triglyceride and cholesterol concentrations and effectively prevent the progress of hypercholesterolemia and cholesterol accumulation in the liver induced by a high cholesterol diet in rats (Fukushima *et al.*, 2001). PFA also significantly decreased blood lipid fractions and increased high-density lipoprotein in poultry (Abdel-Azeem, 2005). Results obtained in the present study show that FSE increases serum HDL-cholesterol, and decreases total cholesterol, regardless of LDL-cholesterol levels. Some studies have indicated that fenugreek seeds have hypocholesterolemic activity, which may reduce the risk of heart disease (Sharma *et al.*, 1991; Khosla *et al.*, 1995). Saponins, which are a major active constituent of the fenugreek seeds, have beneficial effects on blood cholesterol and triglyceride concentrations in rats, rabbits, and humans (Morehouse *et al.*, 1999; Sowmya and Rajyalakshmi, 1999; Afrose *et al.*, 2009). Additionally, non-starch polysaccharides, which provide the major soluble fiber content in fenugreek seeds, include galactomannan (Brummer *et al.*, 2003), which has been reported to reduce blood lipids (Yamamoto *et al.*, 2000; Srichamroen *et al.*, 2001). The presence of these compounds in particular could increase the viscosity of the digested food and decrease serum total cholesterol levels by inhibiting bile salt reabsorption in the small intestine (Kumar *et al.* 2012). Therefore, the decreased concentration of blood lipid metabolites observed in the present study due to the inclusion of dietary FSE may be related to the saponins or galactomannan fiber content in FSE.

Several PFAs have long been recognized for their antimicrobial actions against major pathogens including coccidium and fungi (Giannenas *et al.*, 2003; Windisch *et al.*, 2008). An in vitro study by Du *et al.* (2015) found that carvacrol and thymol inhibited the growth of *E. coli*, *Clostridium perfringens*, and *Salmonella* strains. Gutierrez *et al.* (2008) reported that in vitro, essential oils including basil, lemon balm, marjoram, oregano, rosemary, sage, and thyme,

strongly inhibited the growth of *B. cereus*, *P. aeruginosa*, *E. coli*, and *L. monocytogenes*. In an in vivo study, Guo *et al.* (2004) demonstrated that the herb extracts were largely associated with reduced cecal *Bacteroides* spp., enterococci, and *E. coli* numbers, but increased numbers of bifidobacteria and lactobacilli, relative to the control and antibiotic groups in chickens. In addition, Zentek *et al.* (2013) noted that a fenugreek diet caused higher *Lactobacillus* and *Clostridium* concentrations and lower *Escherichia*, *Hafnia*, and *Shigella* concentrations in the small intestine of piglets. Many investigators have reported an antibacterial effect of fenugreek seeds (Dash *et al.*, 2011; Alwhibi and Soliman, 2014; Majumdar and Alluri, 2014). Such an effect seems to be related to the presence of certain molecular compounds, usually in the form of secondary metabolites, such as flavonoids, saponins, and phenolic compounds (Dash *et al.*, 2011). In our study, laying hens fed the FSE diets had a higher population of cecal *Lactobacillus* and a lower population of *E. coli*. Therefore, decreased cecal *E. coli* counts in laying hens fed dietary FSE may be explained by the antibacterial activity of FSE against different pathogenic bacteria. These effects of FSE on cecal microflora have led to a reduction in ammonia gas emission in laying hen excreta. In other words, a positive effect of FSE is exerted by suppressing harmful cecal *E. coli*, and favoring beneficial *Lactobacillus*, which may be reflected in the ammonia gas emissions of the excreta. This result is in line with the reports of Ushild *et al.* (2002) and Sung (2013), who determined that supplemental PFA decreased noxious gas emission, including ammonia concentrations, in pigs and laying hens. Furthermore, decreased levels of excreta ammonia gas found in the FSE groups may be associated with intestinal pH. Denli *et al.* (2004) observed that supplementation of quail diets with PFA (thyme and black seed essential oils) significantly decreased intestinal pH. Similarly, Zentek *et al.* (2013) indicated that fenugreek seed reduced the pH in the cecum and colon of piglets, due to an increase in the number of lactate-producing bacteria or their relevant metabolic activity. Therefore, the decrease in ammonia gas emission observed in excreta following the addition of FSE to laying hen diets is thought to be induced by the maintenance of beneficial microbiota or a reduction in intestinal pH.

In conclusion, based on these findings, FSEs are a valuable natural feed additive for laying hens, particularly with regard to egg weight, egg quality, serum cholesterol, cecal lactobacilli number and excreta ammonia gas emission.

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