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Effects of Germinated and Fermented Unmarketable Soybean on Laying Performance and Egg Quality in Laying Hens

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발아, 발효 처리한 비상품성대두 급여가 산란계의 생산성과 계란의 품질에 미치는 영향

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Abstract

This study was conducted to investigate the effects of germinated and fermented unmarketable soybean (GFS) on laying performance and egg quality in laying hens. A total of two hundred laying hens were divided into 5 groups (5 treatment × 4 replication × 10 birds each) and fed with the experimental diets for 8 wk as follows: control, GFS free; T1, GFS 0.15%; T2, GFS 0.3%; T3, GFS 1%; T4, GFS 2%. The laying performance, egg quality, blood profiles, cecal microbial population, isoflavone content in egg yolk were investigated. There were no significant differences laying performance, relative liver and spleen weights, egg yolk color, eggshell color among groups. Eggshell strength in groups fed with diets containing GFS increased, but not significantly. Eggshell thickness significantly increased in the GFS-supplemented group. No significant differences were observed in the blood profiles and intestinal microflora after supplementation. The decrease of Haugh unit during storage was alleviated by feeding of GFS ($p<0.05$). The concentrations of malondialdehyde in groups fed with GFS were decreased as compared with control ($p<0.05$). Isoflavones in the egg yolk were detected in group fed with diet containing 2% GFS. These results showed that unmarketable GFS could be used as a favorable feed additive and feedstuff for production of quality enhanced and isoflavone fortified eggs.

Key words : unmarketable soybean, fermentation, germination, egg quality, isoflavone

INTRODUCTION

Legumes play an important role in the traditional diets of many regions throughout the world. Soybeans in particular have been consumed as an important protein source to complement grain protein in Asian countries for a long time. They contain various nutritious and functional components such as isoflavones, phytic acids, saponins, and oligosaccharides (Anderson *et al.*, 1995; Oh *et al.*, 2002).

Various products have been produced from soybeans in Asian countries. There are several traditional fermented soybean foods such as Tofu, Miso, Tempeh, Chungkookjang, Doenjang, and soy sauces. It has been established that dietary intake of soybean foods is effective in reducing the risk of cardiovascular disease and cancer, and its effect is

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seen particularly among Asian populations, where consumption of soybean foods is high, compared with Western populations (Persky *et al.*, 2002).

Isoflavones (ISF) have been shown to be effective in lowering blood cholesterol (Crouse *et al.*, 1999; Greaves *et al.*, 2000), and reducing the oxidative susceptibility of low-density lipoprotein (Tikkanen *et al.*, 2000), and these are implicated in reducing the incidence of atherosclerosis. ISF may be an effective feed supplement to decrease fat deposition in animals because of this estrogen-like function. Fatty acids, fat-soluble vitamins, and other fat-soluble nutrients can be transferred from the feed to the egg yolk (Michella *et al.*, 2000). Nutritionally enriched eggs, such as high vitamin E-containing eggs, have been developed by using this phenomenon (Meluzzi *et al.*, 2000; Scheideler *et al.*, 1996). Saitoh (2004) reported that in laying hens daidzin, a soy ISF-glycoside, in the diet was transformed into equol, and was absorbed, and transported in circulating peripheral blood. Daidzin was also show to be, preferentially accumulated into egg yolk in its conjugated form. Many studies have revealed that the biological effects of ISF are not due to the glycoside form but instead the effects are mainly from their aglycones, such as daidzein and genistein (Hendrich, 2002; Kawakami *et al.*, 2005). However, most of ISFs in nature food materials exist as a glycosylated form and cannot be easily absorbed in the intestines (Izumi *et al.*, 2000; Kawakami *et al.*, 2005). Most of them exist as β-glycoside, the malonylglycoside, and acetylglycoside forms. The bioavailable ISF (aglycones) are formed by the hydrolysis of glycosides through β-glycosidase present in soybean. Fermentation, heat treatments, and chemical and enzymatic hydrolysis were reported to induce changes in composition of ISF profile (Ikeda *et al.*, 1995; Matsuura *et al.*, 1995; Cook *et al.*, 1998; Pandjaitan *et al.*, 2000a; Pandjaitan *et al.*, 2000b; Xie *et al.*, 2003).

Germination processes have been developed to overcome the disadvantages of soybean seed used in food products. These shortcomings include undesirable flavor and odor due to lipoxygenase activity and the presence of anti-nutritional factors such as trypsin inhibitors, phytates, and flatulence. Many studies showed the benefits of germinated soybeans, including improvement of nutritional quality by prevention of lipid oxidation, increasing the ascorbic acid and riboflavin contents, hydrolysis of raffinose and stachyose that are responsible for flatulence problems, and decreased levels of trypsin inhibitors (McGrain *et al.*, 1989; Sattar *et al.*, 1990; Ryu *et al.*, 1996; Ahmad *et al.*, 2000).

The objectives of this research were to evaluate the utilization of germinated and fermented unmarketable soybean on laying performance, egg quality and transfer of soy ISF into the chicken eggs.

MATERIALS AND METHODS

Preparation of unmarketable soybeans

Unmarketable soybean varieties commonly grown in Yonchon, Korea, were obtained for use in this study. Unmarketable soybeans were washed three times in distilled water at ambient temperature for 10 hr. The soaked soybeans were drained and germinated in an incubator at 20°C for about 38 hr and then cooked in an autoclave at 121°C for 20 min. Cooked soybeans were cooled to room temperature and inoculated with *Bacillus subtilis* var. *natto* KCCM 11315, *Aspergillus oryzae* KCCM 60241, and *Rhizopus oligosporus* KCCM 11605, mixed, and fermented in an incubator for 48 hr. Fermented samples were dried in a hot-air oven at 80°C for 24 hr. After drying, germinated and fermented soybean (GFS) were stored at 4°C until used.

Experimental design and diets

All procedures were performed at the Department of Food Science and Biotechnology of Animal Resources, Konkuk University and met required departmental approvals. 30 wk old Hy-line Brown layers were randomly placed in three replicate pens with 10 birds each per treatment (total of 200 birds) in wire cages. The birds were fed with one of the following five diets containing GFS 0%, GFS 0.15%, GFS 0.3%, GFS 1% or GFS 2% for 8 wk, respectively. The experimental diets were formulated to meet or exceed nutrient requirements of NRC (1994). GFS was substituted in place of basal diet 0.15, 0.3, 1, or 2% levels on weight basis. The composition of the basal experimental diet is shown in Table 1. Animal facilities and husbandry were similar to conditions described by An *et al.* (2003). The experimental diets and water were provided for *ad libitum* intake. A room temperature of 22±3°C and a photoperiod of 16/8 hr light/dark cycle were maintained throughout the experimental period.

Egg productivity and the analyses of egg quality

The number of total, dirty, and broken and shell-less eggs and mortalities were recorded daily by replicate. Feed intake was recorded on the last day of bi-weekly. Egg production was recorded daily and experimental diets were freshly added. The eggs laid for the last three days of experiment were used for analysis of egg qualities according to the method of Hayirli *et al.* (2005), with some modification. The

Table 1. Composition of the experimental diet

Ingredients	Starter
Yellow corn	50.40
Wheat bran	11.27
Soybean meal	22.48
Corn gluten meal	2.51
Tallow	1.35
Vit.+Min. mixture[1)]	0.42
L-lysine HCl (98%)	0.05
DL-methionine (99%)	0.07
Dicalcium phosphate	0.91
Limestone	10.19
Choline-Cl (50%)	0.07
Salt	0.25
Phytase	0.03
Total	100
Calculated values	
TMEn[2)], kcal/kg	2,776
Crude protein, %	17
Ether extract, %	4.01
Crude fiber, %	3.17
Ca, %	4.0793
Available P, %	0.2536
Lysine, %	0.79

[1)] Vit.+Min. Mineral mixture provided the following nutrients per kg of diet: vitamin A, 18,000 IU; vitamin D_3, 3,750 IU; vitamin E, 30 IU; vitamin K_3, 2.7 mg; vitamin B_1, 3.0 mg; vitamin B_2, 9.0 mg; vitamin B_6, 4.5 mg; vitamin B_{12}, 30.0 μg; niacin, 37.5 mg; pantothenic acid, 15 mg; folic acid, 1.5 mg; biotin, 0.07 mg; Fe, 75.0 mg; Zn, 97.5 mg; Mn, 97.5 mg; Cu, 7.5 mg; I, 1.5 mg; Se, 0.2 mg.

[2)] TMEn : nitrogen corrected true metabolizable energy.

collected eggs were kept in storage temperature of 18°C during 7 or 14 d to observe changes in Haugh units.

Blood profiles

At the end of the experimental period, eight hens from each treatment were selected and weighed individually. The blood was drawn from the jugular vein using a syringe for determination of the concentration of various lipid fractions and components. At necropsy, the liver and spleen were immediately removed and weighted. The serum was separated from each blood sample by centrifugation and stored at -30°C until use. The concentration of total cholesterol (Total-C) and high-density lipoprotein cholesterol (HDL-C), the activity of glutamic oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) in serum were measured according to the colorimetric method using cholesterol diagnostic-kits (Cholesterol E kit and HDL-cholesterol kit, Youngdong Medical Corporation, Korea) and GOT-GPT assay kit (GOT-GPT assay kit, Youngdong Medical Corporation, Korea).

Cecal microbial population

The cecal digesta homogenates in PBS were serially diluted from 10^{-1} to 10^{-7}. Dilutions were subsequently plated on duplicate selective agar media for enumeration of target bacterial strains. Total microbes, coliforms and *Lactobacillus* spp. were enumerated using nutrient agar, MacConkey agar, and MRS agar, respectively, using traditional methods (Tuohy *et al.*, 2002). Each plate was incubated at 37°C, for 24 to 72 hr aerobically or anaerobically, and colonies were then counted. Results obtained were presented as base-10 logarithm colony-forming units per gram of cecal digesta.

Determination of MDA in egg yolk

Lipid oxidation was assessed on the basis of the malondialdehyde (MDA) formed during refrigerated storage. MDA, the used as an index of lipid peroxidation, was determined by a selective third-order derivative spectrophotometric method (Botsoglou *et al.*, 1994). In brief, yolk samples were homogenized (Philips HP 2870, Philips, Eindhoven, Netherlands) in the presence of 8 mL 5% aqueous trichloroacetic acid (Sigma Chemical, Co., USA), and 5 mL of 0.8% butylated hydroxytoluene (Sigma) in hexane, and the mixture was centrifuged. The top was discarded, and a 2.5 mL aliquot from the bottom layer was mixed with 2 mL of 0.8% aqueous 2-thiobarbituric acid (Sigma), to be further incubated at 70°C for 30 min. Following incubation, the mixture was cooled under tap water and submitted to conventional spectrophotometry (Beckman Coulter, Inc., USA) in the range of 400-650 nm. Third-order derivative spectra were produced by digital differentiation of the normal spectra using a derivative wavelength difference setting of 21 nm. The concentration of MDA in analyzed samples (μg/g yolk) was calculated on the basis of the height of the third-order derivative peak at 521.5 nm by referring to slope and intercept data of the computed least-squares fit of a standard calibration curve prepared using 1,1,3,3,-tetraethoxypropane (Sigma), the precursor of MDA.

Content of ISF in egg yolk

ISF were extracted using a modified method of Xie *et al.* (2003): 30 g of egg yolk dried in a freeze-drying machine, finely ground samples were mixed with 300 mL of 80% aqueous methanol solvent, heated at 60 for 4 hr, and centrifuged at 2000×g for 10 min to extract ISFs. The supernatants were filtered through a 0.2 μm PVDF Target Syringe Filter (National Scientific, USA). ISF were quantified by the Agilent Liquid Chromatograph model 1200 series equipped with a diode array ultraviolet detector. The column used was ZORBAX Eclipse XDB-C18 and absorbance of the effluent

was monitored at 262 nm. The mobile phase was consisted of solvent A, HPLC-grade water, and solvent B, omnisolve-grade methanol. The flow rate was 1.0 mL/min and column temperature was maintained at 37°C. The initial solvent condition was 20% solvent B. A gradient was set to increase solvent B from 20 to 100% within 10 min in 5 min. A sample size of 5 μL was injected for the HLPC analysis. The concentrations of ISF in the sample were calculated from standard curves calibrated using the 12 ISF standards.

Statistical analysis

All statistical analyses were computed using the GLM procedures of SAS software (SAS Institute, 2001). A software program using Duncan's multiple range test to compare treatment means was applied. A $p<0.05$ was considered statistically significant. One replicate was considered as the experimental unit for each performance parameter. The experimental unit was on bird for the other parameters. All data were expressed as mean ± SD.

RESULTS AND DISCUSSION

There were no significant differences in egg production rate, egg weight, and feed intake in laying hens fed with experiment diets as shown in Table 2. Some experiments using older hens with decreased estrogen synthesis might show some effect of dietary isoflavones on hens' physiological properties such as egg-laying ability (Saitoh *et al.*, 2001). When their diets were supplemented with daidzein, the laying performance of Shaoxing duck breeders improved significantly during the postpeak but not the prepeak laying stage (Zhao *et al.*, 2005). Nevertheless, the effect observed in chickens has not been elucidated and the mechanism is still unclear.

The effects of GFS supplementation on egg shell strength, egg shell thickness, egg shell color, egg yolk color and Haugh unit are shown in Table 2. Supplementation of GFS did not affect the egg shell color or yolk color. However, eggshell thickness significantly increased in the GFS-supplemented group. GFS supplementation increased egg shell strength and Haugh unit slightly compared with control, but this increase was not statistically significant.

The proximate analyses of liver and spleen weight were not influenced by dietary treatment (Table 3). The total requirement for nutrients to support growth of the different digestive organs in birds fed GFS might have exceeded dietary intake. The mobilization of body reserves to meet the needs of the rapidly growing tissues could increase the liver's activity, thus causing hypertrophy. Viveros *et al.* (2001) added chickpeas to chicken diets and observed an increase in the relative weight of the liver. In broiler chickens, the length and weight of the small intestine increase when diets with lower digestibility are fed (Smits, 1996). The presence of large amounts of unabsorbed material in the intestine exerts a trophic effect on the intestinal mucosa. Feeding soluble NSP from barley, field peas, fava bean, chickpeas, and lupins was shown to increase the length and absolute and relative weights of the small intestine in broilers (Viveros *et al.*, 1994; Brenes *et al.*, 2002) as well as the intestinal viscosity (Hughes *et al.*, 2000). The dietary treatment did not have significant effects on the activities of GOT and GPT (Table 3). Serum GOT and GPT levels are the most sensitive indicator of tissue damage in avian species (Lumeij, 1997). This result suggests that GFS in chicken feed did not have negative effects.

The concentrations of various lipid fractions in the serum in hens fed with experimental diets are shown in Table 3. No significant differences were observed in the concentration of

Table 2. Dietary effects of germinated and fermented soybean on the laying performance and egg quality in laying hens[1)]

Items	Control	GFS			
		0.15%	0.3%	1.0%	2.0%
Feed intake (g/day/bird)	128.80±3.64	126.56±4.36	130.52±4.87	127.32±3.75	123.74±1.01
Egg production (%)	93.29±0.72	94.11±0.84	92.89±0.88	93.56±0.62	92.41±0.83
Egg weight (g/egg)	63.67±0.29	63.85±0.25	63.13±0.23	63.63±0.24	63.88±0.29
Daily egg mass	59.42±0.57	60.11±0.64	58.12±0.64	59.55±0.50	59.07±0.69
Eggshell strength (kg/cm^2)	3.24±0.09	3.43±0.10	3.42±0.08	3.49±0.07	3.35±0.08
Eggshell thickness (mm/100)	34.84±0.26^b	36.12±0.36^a	35.97±0.32^a	36.28±0.24^a	36.26±0.33^a
Egg shell color	26.02±0.53	25.07±0.63	27.95±0.44	25.09±0.53	25.20±0.52
Egg yolk color (R.C.F)	7.27±0.11	7.24±0.13	7.25±0.13	7.33±0.13	7.11±0.11
Haugh unit	92.94±1.08	93.77±1.06	93.30±1.27	94.07±1.13	94.37±1.06

[1)] GFS : germinated and fermented soybean; R.C.F : Roche color fan.
Values are presented as Mean±S.D.
[a, b]Mean±S.D. values in a same row with no common superscripts are significantly different ($p<0.05$).

Table 3. Dietary effects of germinated and fermented soybean on the blood biochemical parameters and relative organs weight in the laying hens[1)]

Items	Control	GFS			
		0.15%	0.3%	1.0%	2.0%
Liver (g/100 g BW)	2.08±0.08	2.10±0.08	2.08±0.09	1.95±0.09	1.92±0.05
Spleen (g/100 g BW)	0.08±0.01	0.09±0.01	0.08±0.01	0.08±0.01	0.09±0.01
GOT (U/ dL)	117.22±5.57	124.76±5.21	119.06±3.78	125.54±2.39	124.66±3.64
GPT (U/ dL)	16.30±1.84	15.08±2.00	14.82±1.85	14.82±1.01	15.50±0.83
Total-C (mg/dL)	172.20±5.13	173.48±4.21	171.32±5.51	170.08±5.33	170.87±4.57
HDL-C (mg/dL)	71.10±6.74	64.12±6.49	69.64±4.90	69.08±5.74	71.64±5.07

[1)] GFS : germinated and fermented soybean; Total-C, total cholesterol; HDL-C, high density lipoprotein-cholesterol; GOT, glutamic-oxaloacetic transaminase; GPT, glutamic-pyruvic transaminase.
Values are presented as Mean±S.D.

Total-C and HDL-C. Hypercholesterolemic effects with dietary phospholipid may be attributed to the decreased cholesterol secretion from the liver, or increased uptake of HDL into the liver. In rats, dietary soybean phospholipid reduced the secretion of apoprotein A-1 and cholesterol from the liver (Murata *et al.*, 1983). The HDL cholesterol is particularly important because of its relationship to coronary heart disease (Fielding and Fielding, 1995). Childs *et al.* (1981) reported a significant increase in HDL cholesterol after feeding soybean lecithin compared to an equal amount of corn oil. The information on GFS activity in avian species is very few, the interpretation of the present result has remained difficult.

The concentrations of cecal microbes laying hens fed with the experimental diets are shown in Table 4. No significant differences were observed in the concentrations of total microbes, coliform bacteria, and lactic acid bacteria.

Changes in the Haugh unit and MDA during storage are shown in Table 5. The Haugh unit, an indicator of most widely accepted measure of internal egg quality, tends to decrease according to the elapsed time of storage (Williams, 1992). The Haugh unit of groups fed a diet containing 2% GFS was highest among the eggs stored for 14 d. With an increase in dietary GFS, the Haugh unit increased linearly during storage ($p<0.05$). General nutrients in layer feed did not appear to have any beneficial effect on the Haugh unit (Naber, 1979), but it has been suggested that certain natural antioxidants such as vitamin C, vitamin E, and selenium may be beneficial to albumen quality due to its antioxidant properties (Keshavarz, 1996; Sahin *et al.*, 2003).

Table 4. Dietary effects of germinated and fermented soybean on the profiles of cecal microflora in the laying hens[1)]

Items	Control	GFS			
		0.15%	0.3%	1.0%	2.0%
Total microbes (Log_{10} CFU/g)	7.88±0.07	8.09±0.08	8.10±0.09	7.99±0.29	8.05±0.37
Coliform bacteria (Log_{10} CFU/g)	4.73±0.11	4.30±0.20	4.54±0.07	4.36±0.18	4.52±0.12
Lactic acid bacteria (Log_{10}CFU/g)	7.40±0.06	7.29±0.19	7.52±0.14	7.48±0.09	7.55±0.04

[1)] GFS : germinated and fermented soybean.
Values are presented as Mean±S.D.

Table 5. Dietary effects of germinated and fermented soybean on content of isoflavone in yolk and the change of Haugh unit and malondialdehyde content during storage[1)]

Items	Control	GFS			
		0.15%	0.3%	1.0%	2.0%
Haugh unit					
1 d	92.94±1.08	93.77±1.06	93.30±1.27	94.07±1.13	94.37±1.06
7 d	70.89±1.37^{c}	71.50±1.55^{bc}	71.71±1.45^{bc}	72.46±0.63^{ab}	73.23±1.42^{a}
14 d	63.80±1.00^{c}	65.37±1.21^{ab}	65.29±1.06^{b}	65.65±1.31^{ab}	66.49±1.38^{a}
MDA (μg/g)	0.075±0.01^{a}	0.069±0.01^{b}	0.068±0.02^{b}	0.065±0.01^{c}	0.064±0.01^{c}
Isoflavone (μg/100g)	ND	ND	ND	ND	7.24

[1)] GFS : germinated and fermented soybean; MDA, malondialdehyde; ND, not detected.
Values are presented as Mean±S.D.
[a-c] Mean±S.D. values in a same row with no common superscripts are significantly different ($p<0.05$).

GFS supplementation significantly decreased the MDA levels compared with control. The extent of lipid peroxidation by reactive oxygen species can be monitored by monitoring MDA levels (Sumida *et al.*, 1989). Young *et al.* (2003) reported that MDA production decreased in the *pectoralis major* of ascorbic acid-α-tocopherol-supplemented chickens. Decreased lipid oxidation in chicken muscle following supplements with a high content of antioxidants originating from plants, e.g., tea catechins (100 to 300 mg/kg; Tang *et al.*, 2000) and rosemary-sage extracts (500 mg/kg; Lopez *et al.*, 1998), has previously been shown. Soybean IFSs are capable of suppressing formation of plasma lipid oxidation products *in vivo* (Tikkanen *et al.*, 1998; Wiseman *et al.*, 2000; Chen, 2001). For example, the concentrations of MDA in plasma and tissue are significantly decreased by ISF treatment in male rabbits (Yousef *et al.*, 2004). In this study, MDA production of the yolk decreased by adding GFS to the diet (above 150 mg/kg) of laying hens. The ISF content of egg yolks is shown in Table 5. The conjugated ISF were detected in birds fed a diet containing 2% GFS. Saitoh (2004) reported that laying hens were fed with experimental diets containing at 1 to 9 d intervals. HPLC analyses revealed that most of the ISF and metabolite, equol, were present in blood and egg yolk in conjugated form.

These results indicate that GFS in chicken feed can improve egg quality. Thus, it is possible that GFS can be used for fortifying ISF levels in eggs.

In this study, we have demonstrated that there were no adverse effects on egg laying performance, egg quality, and physiological response, when 0.15-2% GFS was added to the feed of laying hens. There were no significant differences in the concentration of total cholesterol and other lipid fractions and the pathway of cholesterol biosynthesis. These results raise the possibility that feeding domestic animals soy-based fodder may produce animal-based foods rich in a more active form of phytoestrogens.

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