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Effect of golden needle mushroom (*Flammulina velutipes*) stem waste on laying performance, calcium utilization, immune response and serum immunity at early phase of production

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Objective: This experiment was conducted to evaluate the effects of golden needle mushroom (*Flammulina velutipes*) stem waste (FVW), on organic eggs production, calcium utilization, antibody response, serum immunoglobulin, and serum cytokine concentration at early phase of production in laying hens.
Methods: A total 210, 19 weeks old aged ISA Brown layers were randomly assigned into 5 equal treatment groups, with 7 replications of 6 hens each. Dietary treatment included a standard basal diet as control; antibiotic (0.05% flavomycin); 2% FVW; 4% FVW; and 6% FVW. The experimental duration was 10 weeks.
Results: There was no significant differences (p>0.05) on hen day egg production, egg weight, egg mass, feed intake, and feed conversion ratio (FCR) among experimental groups. Unmarketable eggs were significantly lower (p<0.05) both in 4% FVW and 6% FVW fed groups than control group. The calcium retention and calcium in egg shell deposition were significantly higher (p<0.05) in FVW inclusion groups than control and antibiotic groups. Antibody titers against Newcastle diseases were significantly higher (p<0.05) in 6% FVW fed group (except combined with 4% FVW at day 147) and infectious bronchitis were significantly higher (p< 0.05) in FVW fed groups (except 2% FVW and 4% FVW at day 161) than control and antibiotic groups. Serum immunoglobulin sIgA was significantly higher (p<0.05) in all levels of FVW and IgG was significantly higher (p<0.05) in 4% FVW than control and antibiotic groups. Serum cytokine concentration interleukin-2 (IL-2) was significantly higher (p<0.05) in 6% FVW; IL-6 and tumor necrotic factor-α were significantly higher (p<0.05) both in 4% FVW and 6% FVW than control and antibiotic groups; IL-4 was significantly higher (p<0.05) in antibiotic, 2% FVW and 4% FVW fed groups than control.
Conclusion: *F. velutipes* mushroom waste can be used as a novel substitute for antibiotic for organic egg production and sound health status in laying hens.

Keywords: Laying Hens; Mushroom Waste; Laying Performance; Calcium Utilization; Antibody Response; Serum Immunity

INTRODUCTION

Golden needle mushroom (*Flammulina velutipes* [*F. velutipes*]) is one of the main edible mushrooms long recognized for its nutritional value and delicious taste in Asian countries especially China, Japan, Vietnam and Korea. *F. velutipes* fungus belonging to the family Physalacriaceae is also known as winter mushroom or lily mushroom or velvet stem or enoki mushroom among different countries [1]. Having nutritional values, folk medicine has long recognized this mushroom for wide spectrum of therapeutic and prophylactic uses [2]. Many bioactive constituents from a range of families have been isolated from different parts of the mushroom, including carbohydrates, protein, lipids, glycoproteins, phenols, and sesquiterpenes [3]. In addition, bioactive substances like sterol, sesquiteropenoids and its compound sterpurol, flammulinol-A, enokipodin isolated from *F. velutipes* mushroom have strong antioxidant, cholesterol lowering effects, anti-inflammatory, immune modulatory, antibacterial, antifungal, and cytotoxic effects [4,5]. Low calories, good fiber food, protein with all the essential amino acids, adequate minerals and vitamins in *F. velutipes* were reported by Wu et al [6]. During the last several decades, antibiotic has been widely used in the poultry industry to promote growth. Moreover, the extensive use of antibiotic has the possibility to generate antibiotic resistant bacteria in animal products [7]. Usage of antibiotic as an animal growth promoter in animal diets has been banned or limited in many countries [8]. Due to recent ban of using antibiotic as growth promoters in poultry diet, fungi or herbs that have been used as feed supplements for centuries in veterinary medicine [9] and mushroom waste could be a proper substitute for antibiotics [10]. Cultivation of mushroom has become popular which leads stem waste material available but its utilization is still limited.

However, there is little information available till date about the effects of mushroom waste on layer production and health parameters. Based on the previously reported favorable effects of mushroom in an *in vitro* study, the current study was designed to evaluate the effects of *F. velutipes* stem waste (FVW) on egg production, calcium utilization, immune response, serum immunity, and serum cytokine concentration in layers at the early phase of production.

MATERIALS AND METHODS

Experimental hens and diets

A total 210 ISA Brown laying hens of 19 weeks old were randomly assigned into 5 equal treatment groups, with 7 replications of 6 hens for each treatment. The experiment was carried out at the animal shed building and handling with experimental hens was approved by the animal care and use committee of Jilin Agricultural University. Three hens were kept together in one cage and house temperature was maintained at approximately 24°C. The hens were exposed to a 16 hour light and 8 hour dark period throughout the experiment period of 10 weeks, from 19 weeks to 29 weeks.

Hens were given a standard basal diet considered as control group; antibiotic (0.05% flavomycin) group; 2% FVW fed group; 4% FVW fed group; and 6% FVW fed group respectively. Feed and water were offered *ad libitum* during the whole experimental period of 70 days. The *F. velutipes* stem was collected from a domestic mushroom farm in Changchun, China. The collected mushroom stem was properly dried under sun and transferred to feed mill for further uses. Mushroom and antibiotic were mixed with layer diet by a feed mixer in Feed Mill (Jilin Hanghong Animal Husbandry Co. Ltd, Changchun, China). The nutritional requirement was followed according to the recommendations of the genetic line manual [11].

Laying performance

Eggs from all replicated cages were collected and recorded daily. Egg production percentage was calculated by dividing the total number of eggs by hen-day and was expressed in percent. Egg weight was presented as the average egg weight per hen divided by the number of days during the experimental period. Egg mass was calculated as laying percentage multiplied by egg weight. Total feed intake was determined as the differences between feed offered and residual feed in trough feeders on weekly basis. Feed conversion ratio (FCR) was then calculated as feed intake divided by egg mass respectively. Unmarketable eggs were calculated on number of curled eggs (abnormal size, shape, color, deformities, broken eggs, etc) divided by the total number of eggs laid per pen and expressed in percent during the total experimental period.

Chemical analysis of feed sample

Mushroom stem waste (FVW) sample and diet sample were prepared (0.01 mm) for proximate component analysis. The analyzed nutritional composition of the experimental diet and FVW are presented in Table 1. The dry matter (DM) (method number 934.01), ether extract (EE) (method number 920.39), crude fiber (Method number 962.09), and total ash (method number 942.05) were analyzed according to the procedures of AOAC [12]. Nitrogen was determined using an FP-528 nitrogen determinator (LECO Corporation, Joseph, MI, USA). The calcium and phosphorus content were determined following the method of Talapatra et al [13].

Calcium utilization

Samples of feed, excreta, and egg shell (3 eggs from each replicate, n = 105) from each replicated treatment groups were collected every 24 hour during the last three consecutive days of the experiment. Samples were dried in oven at 105°C for 24 hour, ground by laboratory high speed universal sample grinder (Huanghua xinxing electric Appliance Co, Hebei, China) and then ashed in a muffle furnace at 650°C for 6 hour. The calcium retention was calculated by deduction of calcium found in excreta from calcium intake in feed. The calcium balance was estimated by deduction of calcium in egg shell from calcium retention. Apparent calcium retention and mass calcium balance were determined according to the procedure described by Abdelqader et al [14].

Vaccination and immunological analyses

All birds were immunized, with combined vaccine of Newcastle diseases (ND); infectious bronchitis (IB) and egg drop syndrome vaccine, inactivated (strain La Sota+Strain M41+

Table 1. Ingredients and nutrient composition of the diets for layers (g/kg)

Items	Control	Antibiotic	2% FVW[1)]	4% FVW[1)]	6% FVW[1)]
Ingredients					
Maize corn	557.0	556.5	544.0	525.0	510.0
Soyabean meal (44% CP)	282.0	282.0	277.0	274.0	272.0
Soyabean oil	28.0	28.0	26.0	28.0	25.0
FVW[2)]	-	-	20.0	40.0	60.0
Lysine	2.0	2.0	2.0	2.0	2.0
Methionine	2.5	2.5	2.5	2.5	2.5
Dicalcium	36.0	36.0	36.0	36.0	36.0
Limestone	88.0	88.0	88.0	88.0	88.0
Common salt	2.5	2.5	2.5	2.5	2.5
Vit–mineral premix[3)]	2.0	2.0	2.0	2.0	2.0
Antibiotics	-	0.5	-	-	-
Total	1,000	1,000	1,000	1,000	1,000
Chemical analysis					
DM	912.2	912.2	913.3	913.55	913.50
CP	170.0	170.0	169.60	169.60	170.03
Calcium	41.10	41.09	41.11	41.10	41.2
Phosphorus	7.20	7.18	7.22	7.21	7.20
EE	52.3	52.3	50.40	52.20	49.30
CF	25.6	25.5	29.60	33.7	37.90
Calculated analysis (g/kg)					
ME (MJ/kg)	11.70	11.69	11.70	11.71	11.71
Lysine	10.51	10.51	10.40	10.45	10.43
Methionine	5.0	5.0	5.0	4.9	4.9
Linoleic acid	11.1	11.1	11.2	11.15	11.21
Arginine	10.5	10.5	10.7	10.6	10.6
Threonine	5.52	5.5	5.6	5.7	5.65
Cystine	2.8	2.8	2.8	2.7	2.75
Tryptophan	1.71	1.7	1.7	1.71	1.72

FVW, *Flammulina velutipes* mushroom stem waste; DM, dry matter; CP, crude protein; EE, ether extract; CF, crude fiber; ME, metabolizable energy.
[1)] FVW, *Flammulina velutipes* stem waste at 2%, 4%, and 6%.
[2)] FVW, analyzed composition of *F. velutipes* mushroom stem waste (g/kg) DM 889.0 ± 0.85; CP 12.75 ± 0.49; CF 21.05 ± 0.106; EE 2.7 ± 0.014; total minerals (ash) 11.5 ± 0.085; calcium 4.0 ± 0.1; phosphorus 6.2 ± 0.28; value are expressed as mean ± standard deviation (n = 6).
[3)] Provided per kg of the complete diet: retinyl acetate, 4,500 IU; cholecalciferol, 1,200 IU; DL-α-tocopheryl acetate, 25,000 IU; thiamin, 5,000 mg; riboflavin, 20,000 mg; phylloquinone,10,000 mg; niacin, 45,000 mg; pantothenic acid, 35,000 mg; biotin, 1,500 mg; folic acid, 3,000 mg; cyanocobalamin, 40 mg; zinc, 45 mg; manganese 50 mg; iron, 30 mg; copper, 4 mg; cobalt, 120 μg; iodine, 1 mg; selenium, 120 μg.

Strain HSH23, Beijing Ceva Huadu Biological Co, Ltd, Beijing, China) at day 140 via wing web (0.5 mL/bird). A blood sample was obtained from one bird of each replicated pen (7 birds per experimental group) via wing vein on day 147, day 154, day 161, and day 203. On the respective day of blood sample collection, serum was obtained by centrifuged at 3,000×g for 20 min at 4°C (Legend Micro 17R centrifuge, Thermo Fisher, Am Kalkberg, Germany) and was stored at –80°C until measuring antibody titers, serum immunoglobulin and serum cytokine concentration. Commercial enzyme-linked immunesorbent assay kits (Shanghai Jianglai industrial Ltd, Shanghai, China) were used to analyze ND and IB antibody titers. The serum immunoglobulin A (sIgA), immunoglobulin G (IgG), interleukin-2 (IL-2), IL-4, IL-6, and tumor necrotic factor-α (TNF-α) were measured using chicken specific sIgA, IgG, IL-2, IL-4, IL-6, TNF-α ELISA Quantitation Kits (Shang Hai Lengton Biosicences Co. Ltd, Shang Hai, China) according to the instructions of the manufacturer and absorbance were measured at 450 nm.

Statistical analysis

Data were subjected to one-way analysis of variance using SPSS (2006) software [15]. Significant effects of dietary treatments on experimental groups were evaluated with Duncan's test [16]. Statements of statistical significance are based on a probability of $p<0.05$.

RESULTS

Laying hen performance

There were no significant ($p>0.05$) differences on hen day egg production, egg weight, egg mass, feed intake, feed conversion efficiency (FCR) and live weight among experimental groups during the entire 70 days study period (Table 2). Unmarketable eggs were lower (p<0.05) in 4% FVW and 6% FVW fed groups than control. However, egg weight, egg mass were found higher with improved FCR in FVW fed groups compared with control and antibiotic fed groups although the results were not significant (Table 2).

Calcium utilization in layer

The calcium retention was improved (p<0.05) in FVW fed groups compared with control and antibiotic group although there were no differences on total calcium intake among the treatment groups. The calcium excretion in excreta was lower (p<0.05) in FVW fed group than control and antibiotic groups. The calcium in egg shell deposition was found higher (p<0.05) in FVW inclusion groups than control and antibiotic groups. There was no significant (p>0.05) differences in calcium balance among experimental groups (Table 3).

Immune response on vaccines

Feeding layers on diets with FVW resulted in a significant higher (p<0.05) in the antibody titers against ND in 6% FVW fed group (except combine with 4% FVW at day 147) than control and antibiotic fed groups. On the other hand, antibody titers against IB virus vaccines were significantly higher (p<0.05) in FVW fed groups (except 2% FVW and 4% FVW at day 161) in comparison to those fed on the control diets and antibiotic diets. The best responses for antibody titers were found in layer fed on diets containing 6% FVW. Interestingly negative antibody titers were observed for IB with antibiotic fed group at day 161 whereas mushroom fed groups showed highly positive in the defined period (Table 4).

Serum immunoglobulin and serum cytokines concentration

The sIgA and IgG were increased significantly (p<0.05) in FVW fed groups compared with control and antibiotic groups in this study (Table 5). The highest concentration for sIgA and IgG was observed in 4% FVW fed group among the treatments. Serum cytokine concentration (IL-2) was significantly higher (p<0.05) in 6% FVW; IL-6 and TNF-α were significantly higher (p<0.05) both in 4% FVW and 6% FVW than control and antibiotic fed groups. Moreover, IL-4 was found significantly higher (p<0.05) in antibiotic, 2% FVW and 4% FVW fed groups when compared with control (Table 5).

DISCUSSION

Demand for applying medicinal and functional natural herbs in chicken rations is increasing to improve health status as well as production performance after the ban of antimicrobial growth promoters (antibiotics) in different countries [17]. The estimated value of DM, crude protein (CP), crude fiber (CF) in FVW were mostly close to previously reported values [18,19]

Table 2. Effect of *Flammulina velutipes* mushroom stem waste (FVW) on performance in laying hens[1)]

Parameters	Control	Antibiotic	2% FVW	4% FVW	6% FVW	SEM	p-value
Hen day egg production (%)	84.74	84.45	83.95	89.92	85.89	1.345	0.654
Egg weight (g/egg)	57.41	57.50	58.22	58.14	58.79	0.433	0.865
Egg mass (g/d/hen)	48.81	48.57	48.95	52.36	50.53	0.988	0.740
Feed intake (g/d/hen)	111.25	111.06	110.20	111.17	111.13	0.490	0.966
FCR (g/g)	2.39	2.32	2.31	2.15	2.23	0.048	0.619
Unmarketable eggs (%)	1.21^{a}	0.89^{ab}	0.89^{ab}	0.41^{b}	0.39^{b}	0.098	0.025
Initial live weight (gm)	1,709.86	1,715.58	1,707.14	1,713.57	1,712.43	2.987	0.918
Final live weight (gm)	1,843.29	1,849.71	1,839.29	1,845.57	1,844.14	5.917	0.981

SEM, pooled standard error of the means; FCR, feed conversion ratio.
[1)] Data represent the mean value of 7 replicates with 6 hens each treatment.
[a,b] Means in the same row with different letters are significantly different (p < 0.05).

Table 3. Effect of *Flammulina velutipes* mushroom stem waste (FVW) on calcium utilization in layer[1)]

Parameters	Control	Antibiotic	2% FVW	4% FVW	6% FVW	SEM	p-value
Ca intake in feed (g/d)	4.59	4.57	4.53	4.57	4.57	0.015	0.718
Ca in excreta (g/d)	2.72^{a}	2.69^{a}	2.32^{b}	2.16^{c}	2.20^{bc}	0.045	0.001
Ca retention (g/d)	1.88^{c}	1.88^{c}	2.20^{b}	2.41^{a}	2.37^{ab}	0.048	0.001
Ca in egg shell (g)	1.79^{b}	1.80^{b}	2.12^{a}	2.24^{a}	2.19^{a}	0.040	0.001
Ca balance (g/d)	0.08	0.08	0.08	0.17	0.18	0.016	0.061

SEM, pooled standard error of the means.
[1)] Data represent the mean value of 7 replicates each treatment.
[a,b,c] Means in the same row with different letters are significantly different (p < 0.05).

Table 4. Effect of *Flammulina velutipes* mushroom stem waste (FVW) on antibody titers in layers[1)]

Parameters	Control	Antibiotic	2% FVW	4% FVW	6% FVW	SEM	p-value
Newcastle disease (ND) (ng/L)							
Day 147	497.67[ab]	328.50[c]	396.00[bc]	574.33[a]	620.17[a]	33.341	0.004
Day 154	331.83[c]	289.83[c]	295.17[c]	399.33[b]	559.33[a]	27.544	0.001
Day 161	271.00[b]	207.17[b]	251.17[b]	372.67[ab]	458.50[a]	31.218	0.037
Infectious bronchitis (IB)[2)]							
Day 147	0.63[bc]	0.52[c]	0.87[ab]	1.00[a]	1.12[a]	0.070	0.006
Day 154	0.46[b]	0.37[b]	0.76[a]	0.75[a]	0.82[a]	0.055	0.002
Day 161	0.39[b]	0.17[c]	0.54[ab]	0.56[ab]	0.68[a]	0.053	0.001

SEM, pooled standard error of the means.
[1)] Data represented the mean value of 7 hens per treatment.
[2)] Data represented more than cut off value (>0.228) indicates positive (+) for IB antibody as per kits instruction manual.
[a,b,c] Means in the same row with different letters are significantly different ($p<0.05$).

Table 5. Effect of *Flammulina velutipes* mushroom stem waste (FVW) on serum immunoglobulin and serum cytokine concentration in layers[1)]

Parameters	Control	Antibiotic	2% FVW	4% FVW	6% FVW	SEM	p-value
sIgA (μg/mL)	16.51[b]	18.35[b]	23.13[a]	24.79[a]	22.43[a]	0.790	0.001
IgG (mg/mL)	6.96[b]	7.82[b]	7.85[b]	10.82[a]	8.44[b]	0.307	0.000
IL-2 (ng/L)	12.68[d]	23.92[b]	16.67[c]	21.79[b]	28.16[a]	1.057	0.000
IL-4 (ng/L)	205.83[b]	257.64[a]	249.34[a]	258.21[a]	233.55[ab]	5.952	0.017
IL-6 (ng/L)	105.71[b]	124.80[b]	102.64[b]	223.30[a]	184.55[a]	11.820	0.001
TNF-α (ng/L)	607.79[b]	693.43[b]	581.14[b]	831.14[a]	748.86[a]	36.067	0.001

SEM, pooled standard error of the means; sIgA, serum immunoglobulin A; IgG, immunoglobulin G; IL, interleukin; TNF-α, tumor necrotic factor-α.
[1)] Data represented the mean value of 7 hens per treatment.
[a,b,c,d] Means in the same row with different letters are significantly different ($p<0.05$).

but EE, calcium and phosphorus were lower than the published values (EE, 18.4 to 73.3 g/kg; calcium 4.6 to 11.8 g/kg; and phosphorus 8.8 to 9.4 g/kg) [19,20]. These differences were associated with effects of soil, cultivation methods and environmental factors.

During the entire study period, this study did not find any significant differences in the measured laying parameters; hen day egg production, average egg weight, egg mass, FCR, among experimental groups. However, better laying performance was observed in layer fed on diets with 4% FVW and 6% FVW respectively. The present study was similar with the past study by Hong-Gu et al [21] who reported that fermented *F. velutipes* mycelium had no significant effects on egg production, egg mass, feed intake and FCR in laying hens. In addition Na et al [22] reported that inclusion of *F. velutipes* media at 5% to 10% in layer diets had no significant effects on egg production, egg weight, egg mass, and FCR. Lee et al [18] reported that higher levels up to 5% *F. velutipes* mycelium did not improve feed intake in broiler which was similar with the current study for feed intake. Unmarketable eggs were significantly lower in 4% FVW and 6% FVW fed groups, which was associated with higher calcium deposition in egg shell with dietary inclusion of FVW in the experimental groups. Enhancing calcium availability and absorption in gut of hens could be potential strategy for improving egg shell quality as well as reducing unmarketable eggs from aged hens [14]. This study confirmed the beneficial effects of dietary inclusion with FVW on calcium utilization. This study speculated that dietary calcium source from FVW fed groups may improve calcium absorption, resulted higher calcium retention and calcium deposition in egg shell. *F. velutipes* is excellent source of vitamin and minerals especially calcium and phosphorus [23]. Lower calcium retention in control and antibiotic groups were found in the present study, which was because of higher fecal calcium excretion in experimental hens.

Antibody titers against ND and IB were found higher in 6% FVW fed group in this study. The combination of garlic, oyster mushroom and propolis extract to the diets improved antibody response to ND when compared to control and antibiotic diets in broilers [24]. In contrast, Fard et al [10] found no significant differences for ND antibody titers in broilers fed with 1% oyster mushroom waste and suggested a reinvestigation of the antibody titer with mushroom supplement. These differences might be correlated with mushroom type and inclusion levels with experimental birds.

This study also confirmed the significant positive effects of FVW on serum immunoglobulin and cytokine production in layer. The appropriate level of immune modulatory materials such as polysaccharides, protein, and some flavonoids are reported to maintain the immune response in host [25] and cytokines are known to be regulators of the immune status [26]. The activity of IL-2 is mainly manifested as activation of B and T lymphocytes and NK cells [27]. Stimulation of the Th1 response is characterized by secretion of IL-2, TNF-α along with other cytokines which leads to the promotion of cellular immune mechanisms. Further stimulation of the Th2 response is characterized by secretion of IL-4, with other cytokines which promotes the humoral mechanisms of the immune response [28]. The polysaccharides in *F. velutipes* mushroom have strong immune modulatory activity and possess antioxidant activity that could enhance non-specific and specific immune responses *in vitro* [5]. Supplementation of β–glucan from edible mushroom had significant immune stimulatory effects in broiler birds that were reflected in the *in vitro* immune effector activities of the mucosal cells [29]. In addition, Badalyan and Hambardzumyan [30] stated that *F. velutipes* has immune modulatory effects via induction of cytokines. However, published data on the response of serum immunity and cytokine production with dietary FVW in layer are very limited to compare the current study.

CONCLUSION

In conclusion, present study highlighted the beneficial effects of FVW on immune response, serum immunity, calcium utilization, marketable eggs in laying hens without hampering egg production percent, egg weight and FCR. With a focus on organic egg production and sound health; golden needle mushroom (*F. velutipes*) stem waste can be used as a natural potential substitute for antibiotic in layer production.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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