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# Effects of Dietary Fermented *Flammulina velutipes* Mycelium on Performance and Egg Quality in Laying Hens

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**Abstract:** This study was conducted to evaluate the effect of dietary *Flammulina velutipes* mycelium (FFVM) fermented with *Bacillus subtilis* A8-8 and *Klebsiella* sp. *Sc* on the performance of laying hens including the egg quality, the pathogenic bacterial (*Escherichia coli* and *Salmonella* spp.) counts in caecal contents and the $NH_3$ production in excreta. One hundred eighty Hy-Line Brown hens were fed one of six diets, including a corn-soybean meal based control diet and control diets supplemented with FFVM at 1, 2, 3, 4, or 5% (n = 30 per experimental group). During the 5 week experimental period, laying hen performance, egg quality, pathogenic bacteria number in caecum and fecal $NH_3$ gas production were estimated. Although there were no significant differences in egg production, feed intake and feed conversion among experimental groups, egg weight was significantly increased in the 1 and 3% FFVM groups compared with the control diet group ($p<0.05$). Interestingly, the 4% FFVM experimental group improved egg quality including albumen height, Haugh unit, egg shell weight and shell thickness, but not yolk color ($p<0.05$). On the other hand, the number of pathogenic bacteria such as *Salmonella* spp. and *E. coli* in caecum was significantly decreased by high levels of dietary FFVM supplementation (3-5%) ($p<0.05$). Early stage fecal $NH_3$ gas production was also significantly suppressed by 3-5% dietary FFVM diets ($p<0.05$). Collectively, our results indicated that high level of dietary FFVM (4%) improves egg quality as well as suppressing pathogenic bacterial proliferation in the gastrointestinal tract and fecal $NH_3$ gas emission.

**Key words:** Fermented *Flammulina velutipes* mycelium, laying hen performance, egg quality, pathogenic bacteria, fecal $NH_3$ gas production

## INTRODUCTION

During the last several decades, antibiotics have been widely used in the animal industry to promote growth and feed efficiency as well as a prophylaxis to prevent animal diseases (Mee, 1984; Khachatourians, 1998). However, the extensive use of antibiotics has the possibility to generate antibiotic-resistant bacteria in animal products, which subsequently leads to treatment failure for bacterial disease after transferring to humans through the food chain (Zhang *et al*., 2011). Moreover, appearance of antibiotic-resistant bacteria, such as methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococci* (VRE) are growing this concern. For this reason, usage of antibiotics as an animal growth promoter in animal diets have been banned or limited in many countries (Wallinga and Burch, 2013) and research to search for alternative ways to promote growth has arisen.

The use of probiotics, products made from living microorganisms including bacteria, fungi and yeast, have been considered a strong alternative to antibiotics because probiotics do not lead to antibiotic-resistant bacteria in the gastrointestinal tract (Abe *et al*., 1995). In the poultry industry, accumulated studies reported that probiotics improved feed conversion rate (FCR) and body weight gain of broilers (Timmerman *et al*., 2006; Kalavathy *et al*., 2003; Zulkifli *et al*., 2000). Moreover, Davis and Anderson (2002) reported that dietary probiotics based on *Lactobacillus* spp. improved egg size and lowered feed consumption in laying hens. In accordance with this observation, improvement of egg production and egg quality by dietary probiotics was reported by several researchers (Kurtoglu *et al*., 2004; Panda *et al*., 2008). Although functional mechanisms of probiotics are not fully understood, it could be explained by the beneficial alteration of intestinal microflora (Fuller, 1989; Ryu and Park, 1998). In fact, dietary probiotics suppressed both intestinal *Escherichia coli* (Baba *et al*., 1991) and *Salmonella* spp. (Dunham *et al*., 1993) which subsequently lead to increases in beneficial intestinal

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microflora and therefore, increased retention and digestibility of nutrients (Hooper *et al*., 2002; Nahashon *et al*., 1994).

Among various kinds of microorganisms, *Bacillus subtilis* is widely used as a probiotic in the animal industry together with *Lactobacillus* spp. because this bacterium is easy to cultivate and maintain, as well as being highly stable under enzyme digestion, acidic conditions and high temperatures (Kim *et al*., 1997; Jang *et al*., 1999). Pelicano *et al*. (2004) reported that dietary probiotics based on *B. subtilis* improved FCR up to 21 days of age in broilers and Xu *et al*. (2006) observed improvement of feed consumption and FCR in laying hens. On the other hand, Santoso *et al*. (1999) demonstrated that dietary *B. subtilis* increased digestibility and availability of nutrients as well as decreased fecal $NH_3$ gas production in broilers.

The *Flammulina velutipes*, a fungus belonging to the *Tricholomataceae* family, currently produces a large amount of waste in the form of the discarded mycelium (root) during harvesting. Annually, about 23,000 tons of *F. velutipes* are harvested in Korea; however, only the fruiting body is used as food stuff and the mycelium is discarded leading to environmental pollution (Chung, 1999). However, numerous studies provide a possibility that *F. velutipes* mycelium can be used as a valuable animal feed resource. Meanwhile, Ko *et al*. (2007) reported that *F. velutipes* contains 27.5% protein, 7% fat, 58% carbohydrate and 7.4% ash, other studies have shown that *F. velutipes* also contains various kinds of bioactive substance such as β-glucan, hetero polysaccharides, mannofucogalactan and monoterpenes, which have anti-carcinogenic and immunomodulating effect (Smiderle *et al*., 2006; Hirai *et al*., 1998).

In this current study, we estimated the possibility of disposed *F. velutipes* mycelium as a feed ingredient as an alternative for antibiotics to improve animal productivity in laying hens. In order to increase availability, *F. velutipe* mycelium were fermented with *Bacillus subtilis* A8-8 and *Klebsiella* sp. *Sc* containing enzymes to digest the cell walls of the mycelium during fermentation and lyophilization.

## MATERIALS AND METHODS

**Preparation of fermented *Flammulina velutipes* mycelium (FFVM):** The *F. velutipes* mycelium was obtained from a domestic mushroom farm after the fruiting bodies were harvested. The *F. velutipes* mycelium was dried at room temperature then grinded using Feed mill (J-NCM, Jisico, Korea). The prepared *F. velutipes* mycelium powder was then mixed with the *B. subtilis* A8-8 and *Klebsiella* spp. *Sc* in a 1:1:1 ratio and fermented for 2 weeks at room temperature. The *B. subtilis* A8-8 and *Klebsiella* spp. *Sc* were prepared by cultivation for 2 days at 37°C in LB media (polypepton 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, glucose 1 g/L) and the supernatant from these cultivations were used for the fermentation of *F. velutipes* mycelium. Xylan has been considered a major component of plant cell walls and has the possibility to decrease digestibility of *F. velutipes* mycelium. Thus, *Klebsiella* spp. *Sc*, which contains high levels of β-xylosidase to digest xylan into xylose, was used for fermentation of FFVM to increase utilization of *F. velutipes* mycelium (Lee *et al*., 2010). Fermented *F. velutipes* mycelium (FFVM) was freeze-dried and grinded. The chemical composition of FFVM was analyzed by AOAC (1995) methods and presented in Table 1.

**Experimental design, birds and management:** A total of 180 Hy-line laying hens, approximately 60 weeks old, were randomly assigned to 6 dietary treatments. Each treatment consisted of 3 cages of 10 individually-cased hens (n = 30/treatment group) and all hens were raised in cages sized 0.36 m wide x 0.42 m deep x 0.40 m high equipped with nipple drinkers and trough feeders at a farm annexed to the School of Life Resources Sciences, Busan University. The animal protocols in the current study were approved by the Animal Ethics Committee of Busan University. According to the assigned experimental group, laying hens were fed with control diet or control diet containing 1, 2, 3, 4, or 5% FFVM for 5 weeks. Feed ingredients and nutrient composition of the control diet are shown in Table 2. During experimental periods, hens were provided light for 16 h per day and had free access to feed and water.

**Hen performance:** Laying percentage was calculated as the number of eggs per hen divided by the number of days during the experimental period. Egg weight was presented as the average egg weight per hen divided by the number of days during the experimental period and egg mass was calculated as laying percentage multiplied by egg weight. Total feed intake was determined as the difference between feed offered and residual feed in trough feeders and feed conversion ratio (FCR) was then calculated as feed intake divided by egg mass.

**Egg quality analysis:** Egg quality was examined every week using randomly selected 30 eggs per treatment group. The shell color was initially determined using egg shell color fan (Samyang Co, Korea) and eggs were broken to determine albumen height, yolk color, shell weight, shell thickness and Haugh unit. While egg albumen height was measured on the concentrated albumen using albumen height gauge (Technical Services Supplies, York, UK), yolk color was determined using egg yolk color fan (Roche Co, Switzerland). Shell thickness was measured using shell micrometers (Technical Services Supplies) on 3 replicates of shell chips from the equator of the egg. Haugh unit was

Table 1: Chemical composition of FFVM (%)

| Moisture[1] | Crude protein | Crude fat | Crude fiber | Crude ash | Ca | P |
|---|---|---|---|---|---|---|
| 10.52 | 23.04 | 2.56 | 7.69 | 6.82 | 0.02 | 0.76 |

[1]All values are expressed on a dry matter basis

Table 2: Ingredients and nutrient composition of the control diet

| Ingredients | Control diet (%) |
|---|---|
| **Ingredient (%)** | |
| Corn grain (CHI) | 60.34 |
| Soybean meal | 15.62 |
| Mixed grain (wheat) | 5.52 |
| Corn gluten | 2.24 |
| Animal fat | 1.00 |
| Salt dehydrated | 0.20 |
| Dicalcium phosphate (18.5/23.0) | 1.40 |
| Limestone (1 mm) | 8.68 |
| LIQ-methionine | 0.13 |
| LIQ-Choline chloride | 0.06 |
| Vitamin premix[1] | 0.15 |
| Trace mineral premix[2] | 0.15 |
| Avizyme-1500 | 0.03 |
| Smuos | 0.10 |
| Oxizory-D | 0.03 |
| Another | 4.35 |
| Total | 100 |
| **Nutrient composition (%)** | |
| ME (kcal/kg) | 2.764 |
| Crude protein | 15.70 |
| Crude fat | 3.73 |
| Crude ash | 12.88 |
| Crude fiber | 3.00 |
| Calcium | 3.82 |

[1]Vitamin premix provided/kg of diet: Vitamin A, 5,000 IU; Vitamin $D_3$, 1,000 IU; Vitamin E, 2 IU; Vitamin $K_3$, 3 mg; Vitamin $B_2$, 0.6 mg; Vitamin $B_6$, 1.5 mg; Vitamin $B_{12}$, 8 mg; Vitamin C, 15 mg; Nicotinic acid, 20 mg; Folic acid, 0.3 mg; Cal-pantothenate, 15 mg; Biotin, 0.25 mg; Lysin, 12 mg; Methionine, 12 mg; Citric acid, 10 mg; Copper sulphate, 12 mg; Magnesium sulphate, 12 mg; Zinc sulphate, 12 mg; Yeast Extraction, 10 mg

[2]Trace mineral premix provided per kg of diet: Magnesium, 60.5 mg; Zinc, 50 mg; Copper, 6 mg; Iron, 30.5 mg; Iodine, 0.35 mg; Selenium, 0.5 mg; Cobalt, 0.2 mg

calculated with the following equation using obtained egg weight and albumen height; Haugh unit = $100_{log}$ (albumen height-1.7 x egg weight$^{0.37}$+7.6).

**Identification of pathogenic microorganisms in the caecum:** Bacteriological examination in the caecum was conducted as described previously (Lee *et al*., 2012). Briefly, at the end of each experimental period, five hens, having similar body weight, were selected from each group and humanely sacrificed by cervical dislocation. The caecum was immediately removed from each bird and 3 g of caecal contents were serially diluted ($10^{-1}$ to $10^{-9}$) with phosphate buffered saline. Among various diluents, 0.1 mL of $10^{-4}$, $10^{-5}$ and $10^{-6}$ dilutions were plated on both Salmonella Shigella agar (Asan Pharmacy, Seoul, Korea) and MacConkey agar (Asan Pharmacy) and were cultivated under aerobic conditions at 37°C overnight. Based on the characteristics of each agar described in Table 3, the number of *E. coli* and *Salmonella* spp. colonies were measured using the digital colony counter (KT00-74A, Kartech, Seoul, Korea) and values were presented as $log_{10}$ colony forming unit (CFU) per g of caecum content.

**$NH_3$ gas measurement:** $NH_3$ gas production in excreta was determined as previously described (Lee *et al*., 2012). Briefly, fresh excreta were collected from three cages of each treatment group within 24 h after the last day of the 5 week treatment. After eliminating feathers or other foreign substances, 70 g of excreta samples from individual cages were stored in 1.6 L sealed plastic bottles and fermented at room temperature. At day 0, 1, 3, 6, 9 and 15 after collecting excreta, the gas samples were collected from headspace air about 2 cm above the excreta surface and $NH_3$ gas production was then measured using a gas sampling pump (GV-100S, Gastec Corp., Yokohama, Japan).

**Statistics analysis:** The values obtained from the test and analysis were variance analyzed by the SAS package (2008)'s ANOVA Procedure and the Duncan New Multiple Range Test was used to test for significance (SAS).

## RESULTS AND DISCUSSION

**Performance of laying hens:** The effects of dietary FFVM on the performance of laying hens including laying percentage, egg weight, egg mass, feed intake and FCR were summarized in Table 4. Although there were no significant differences in laying percentage and feed intake among experimental groups, dietary FFVM tended to increase feed intake. Interestingly, hens fed with 1% of FFVM had an increased average egg weight ($p<0.05$) and tended to improve egg mass and FCR compared with other experimental groups.

These results may be contributed by the effect of *B. subtilis* contained in FFVM rather than that of *F. velutipes* mycelium because our observation is similar with previous studies examining the effect of *B. subtilis* in poultry productivity. In accordance with our observation, when laying hens were fed a diet containing dried *B. subtilis* at 500 mg/kg, egg production, feed consumption and FCR were improved (Xu *et al*., 2006). Jin *et al*. (1996) also reported that dietary commercial *Lactobacilli* spp. or *B. subtilis* probiotics improved the weight gain of broilers and research by Santoso *et al*. (1995) showed that feed efficiency of broiler chicks fed with dietary dried *B. subtilis* culture was enhanced. Although the exact function of *B. subtilis* on the

Table 3: Characteristics of selective media

| Selective media | Micro organisms | ATCC | Reaction |
|---|---|---|---|
| S,S agar[1] | *Salmonella typhimurium* | 14028 | Colorless black centers colonies |
| | *Salmonella flexneri* | 12022 | Colorless colonies |
| | *Enterococcus faecalis* | 29212 | Inhibition (complete) |
| | *Escherichia coli* | 25922 | Inhibition (partial), colonies pink to rose-red |
| MacConkey agar[2] | *Escherichia coli* | 25922 | Pink color |
| | *Salmonella typhimurium* | 14028 | Colorless colony |
| | *Proteus mirabilis* | 12453 | Colorless colony, swarming inhibition |
| | *Enterococcus faecalis* | 29212 | Inhibition |

[1]*Salmonella*, *Shigella* agar: Selection discrimination separation
[2]MacConkey agar: Pathogenic intestinal bacillus, separation discrimination

Table 4: Effects of dietary FFVM on performance in laying hens

| Treatment[1] | Laying (%) | Egg weight (g/day) | Egg mass (g/day/hen) | Feed intake (g/day/hen) | Feed conversion rate |
|---|---|---|---|---|---|
| C | 89±2.7 | 65.3±0.66$^{cd}$ | 58.1±1.60$^{ab}$ | 138.1±0.52 | 2.38±0.057$^{ab}$ |
| T1 | 93±0.9 | 69.3±0.29$^{a}$ | 64.2±0.81$^{a}$ | 145.5±2.09 | 2.27±0.043$^{a}$ |
| T2 | 85±4.7 | 67.0±0.85$^{bc}$ | 57.0±3.00$^{ab}$ | 136.4±3.22 | 2.40±0.076$^{ab}$ |
| T3 | 87±4.1 | 67.3±0.46$^{b}$ | 58.9±3.08$^{ab}$ | 141.7±4.85 | 2.42±0.156$^{ab}$ |
| T4 | 83±4.0 | 64.8±0.45$^{d}$ | 53.6±2.67$^{b}$ | 143.9±4.10 | 2.69±0.124$^{b}$ |
| T5 | 89±1.7 | 66.0±0.25$^{bcd}$ | 59.1±1.21$^{ab}$ | 143.8±7.50 | 2.44±0.181$^{ab}$ |

$^{a,b}$Means with different superscripts within columns differ significantly ($p<0.05$)
Values are Mean±SE (n = 30). [1]C, none; T1, 1% FFVM; T2, 2% FFVM; T3, 3% FFVM; T4, 4% FFVM; T5, 5% FFVM

Table 5: Effects of dietary FFVM on egg quality in laying hens

| Treatment[1] | Albumin height (mm) | Haugh unit (HU) | Yolk color (R.C.F) | Shell color (S.C.F) | Shell weight (g) | Shell thickness (μm) |
|---|---|---|---|---|---|---|
| C | 7.5±0.22$^{b}$ | 84.7±1.28$^{b}$ | 7.8±0.11$^{ab}$ | 32.3±0.81$^{a}$ | 6.1±0.04$^{bc}$ | 376.7±2.81$^{b}$ |
| T1 | 8.3±0.35$^{b}$ | 88.2±2.08$^{b}$ | 7.6±0.18$^{ab}$ | 30.6±0.99$^{ab}$ | 6.1±0.03$^{bc}$ | 362.2±2.21$^{c}$ |
| T2 | 8.2±0.29$^{b}$ | 88.4±1.69$^{b}$ | 7.4±0.17$^{b}$ | 28.5±0.97$^{bc}$ | 5.9±0.12$^{c}$ | 358.8±4.97$^{c}$ |
| T3 | 8.0±0.29$^{b}$ | 87.4±1.74$^{b}$ | 7.6±0.07$^{ab}$ | 32.1±0.94$^{a}$ | 6.3±0.06$^{ab}$ | 380.6±1.94$^{b}$ |
| T4 | 9.2±0.21$^{a}$ | 94.3±1.12$^{a}$ | 7.9±0.19$^{a}$ | 25.1±1.29$^{d}$ | 6.4±0.08$^{a}$ | 391.4±4.93$^{a}$ |
| T5 | 8.2±0.20$^{b}$ | 88.1±1.21$^{b}$ | 7.5±0.07$^{ab}$ | 25.8±1.13$^{cd}$ | 6.2±0.04$^{ab}$ | 375.6±2.51$^{b}$ |

$^{a,b,c}$Means with different superscripts within columns differ significantly ($p<0.05$)
Values are Mean±SE (n = 30). [1]C, none; T1, 1% FFVM; T2, 2% FFVM; T3, 3% FFVM; T4, 4% FFVM; T5, 5% FFVM

Table 6: Effects of dietary FFVM on number of *Salmonella* spp. and *E. coli* in caecum of laying hens ($log_{10}$ CFU/g content)

| Treatment[1] | *Salmonella* spp. | *E. coli* |
|---|---|---|
| C | 4.73±0.036$^{a}$ | 5.90±0.020$^{a}$ |
| T1 | 4.49±0.006$^{bc}$ | 5.93±0.017$^{a}$ |
| T2 | 4.45±0.015$^{c}$ | 5.75±0.012$^{a}$ |
| T3 | 4.31±0.010$^{d}$ | 5.62±0.039$^{ab}$ |
| T4 | 4.56±0.032$^{b}$ | 5.32±0.306$^{bc}$ |
| T5 | 4.44±0.046$^{c}$ | 5.14±0.014$^{c}$ |

$^{a,b,c,d}$Means with different superscripts within columns differ significantly ($p<0.05$). Values are Mean±SE (n = 5)
[1]C, none; T1, 1% FFVM; T2, 2% FFVM; T3, 3% FFVM; T4, 4% FFVM; T5, 5% FFVM

improvement in laying hen performance is not fully understood, it can be explained by enhanced nutrient availability following *B. subtilis* feeding based on previous literature. The study by Sen *et al.* (2012) demonstrated that dietary *B. subtilis* enhanced growth performance of broilers as a result from increased nutrient retention and villus height in both the duodenum and ileum.

However, previous studies on dietary *F. velutipes* did not show any positive effects on both laying hen and broiler performance. In fact, when laying hens were fed with 5 or 10% of dietary *F. velutipes* media, there was no effect on egg production, egg weight, egg mass, or FCR (Na *et al.*, 2005). Moreover, our previous study also revealed that a broiler diet containing up to 5% of *F. velutipes* mycelium did not improve broiler performance such as feed intake and feed conversion (Lee *et al.*, 2012). These results imply that 1% of dietary FFVM is most effective to improve laying hen productivity.

**Egg quality:** The effects of dietary FFVM on egg quality were estimated and presented in Table 5. The inclusion of FFVM tended to enhance albumen height and Haugh unit compared with the control diet group, but there was no significant difference. However, 4% of dietary FFVM significantly increased Haugh unit compared with all other groups ($p<0.05$), which was caused by enhanced albumen height even though the egg weight was the lowest among the experimental groups. Next, we examined the effect of dietary FFVM on yolk and egg shell color. Although dietary FFVM did not affect yolk color, egg shell color was lowered by the inclusion of FFVM in the diet, especially at 4% of dietary FFVM ($p<0.05$). On the other hand, dietary FFVM tended to increase egg shell weight and thickness compared with the control diet group and 4% of dietary FFVM enhanced both significantly ($p<0.05$).

Table 7: Effects of dietary FFVM on fecal $NH_3$ gas production (ppm)

| | Days | | | | | | |
|---|---|---|---|---|---|---|---|
| Treatment[(1)] | 0 | 1 | 3 | 6 | 9 | 15 | AVG |
| C | 15±0.2[a] | 12±0.6[a] | 89±7.8 | 447±57.5 | 308±111.1 | 56±13.3 | 154±26.3 |
| T1 | 13±1.4[ab] | 8±0.4[bc] | 147±13.9 | 474±56.3 | 242±19.0 | 30±10.2 | 152±14.9 |
| T2 | 11±1.2[ab] | 8±1.4[bc] | 134±32.8 | 424±42.2 | 219±39.1 | 50±11.9 | 140±11.0 |
| T3 | 10±1.8[b] | 10±0.3[ab] | 154±23.8 | 436±89.5 | 242±61.4 | 37±10.4 | 148±22.5 |
| T4 | 10±0.6[b] | 6±0.9[cd] | 141±18.3 | 560±89.5 | 224±40.1 | 44±13.0 | 164±26.0 |
| T5 | 5±1.2[c] | 4±0.6[d] | 142±41.5 | 557±86.9 | 255±38.5 | 45±15.8 | 169±28.1 |

[a,b,c,d]Means with different superscripts within columns differ significantly ($p<0.05$)
Values are Mean±SE (n = 5)
[(1)]C, none; T1, 1% FFVM; T2, 2% FFVM; T3, 3% FFVM; T4, 4% FFVM; T5, 5% FFVM

Many previous studies showed a positive effect of probiotics on egg quality. A previous study by Nahashon *et al*. (1996) demonstrated that dietary probiotics significantly increased the color of the egg yolk and a study by Panda *et al*. (2003) also showed that inclusion of probiotics in diets of laying hens improved the egg shell weight and thickness. Moreover, the improvement of egg shell weight and thickness by dietary probiotics is further supported by previous studies reporting that dietary probiotics enhanced retention of calcium and phosphorus in the digestive tract of laying hens (Nahashon *et al*., 1994).

However, the effect of *B. subtilis* on egg quality is controversial. While one study by Kim *et al*. (2005) revealed that both 0.2 and 0.4% of *B. subtilis* did not affect egg quality such as Haugh unit, yolk color and egg shell thickness, Xu *et al*. (2006) reported that a diet containing dried *B. subtilis* at 500 mg/kg improved egg quality. However, Kim *et al*. (2005) may not have observed improvements in egg quality due to an insufficient concentration of *B. subtilis*. In the study by Xu *et al*. (2006), although they fed broilers with a diet containing 500 mg of *B. subtilis* per kg, the actual level of *B. subtilis* would be far greater compared with the study by Kim *et al*. (2005) because Xhu *et al*. (2006) utilized concentrated, dried *B. subtilis*. In the current study, since we also concentrated FFVM using the freezing-dry method after the fermentation step, the actual dietary level of *B. subtilis* will be greater compared with the research by Kim *et al*. (2005). Therefore, *B. subtilis* contained in FFVM may contribute to the improvement of egg quality. Our results imply that dietary FFVM tended to improve egg quality such as the albumen height, Haugh unit and egg shell thickness and that 4% of FFVM is most effective.

**Pathogenic microorganisms in the caecum:** The effects of dietary FFVM on the caecal pathogenic bacteria number were shown in Table 6 and the number of *Salmonella* spp. and *E. coli* in the appendix contents were presented as $log_{10}$ CFU ($p<0.05$). The number of *Salmonella* spp. was suppressed by all dietary levels of FFVM compared with the control diet group, with the 3% of FFVM being the most effective concentration ($p<0.05$). In case of *E. coli*, the number of bacteria significantly decreased in high levels of dietary FFVM groups (3-5%). Since *E. coli* and *Salmonella* spp. have been considered major pathogenic bacteria impairing productivity in laying hens and can be transferred to humans via egg consumption, these pathogenic bacteria should be controlled. Earlier, previous studies showed that dietary probiotics suppress proliferation of intestinal *E. coli* (Baba *et al*., 1991) and *Salmonella* spp. (Dunham *et al*., 1993) leading to an alteration to beneficial intestinal microflora (Fuller, 1989; Ryu and Park, 1998). Although there is limited information on the effect of *B. subtilis* on pathogenic bacteria proliferation in the gastrointestinal tract, dietary *B. subtilis* decreased intestinal *E. coli* in laying hens (Ryu *et al*., 1999) and caecal *Salmonella* spp. in broilers (Knap *et al*., 2011).

On the other hand, *F. velutipes* mycelium contained in FFVM also partially contributed to the inhibitory effect of FFVM on caecal pathogenic bacteria proliferation because dehydrated *F. velutipes* mycelium significantly decreased the number of caecal *Salmonella* spp. in our previous study (Lee *et al*., 2012). This is supported by an additional study that various kinds of mushroom mycelium culture medium's ethyl acetate extract has a high antibiotic effect against *S. typhimurium* spp. (Park *et al*., 2004). These results suggest that high levels of dietary FFVM (3-5%) are effective to control the number of pathogenic bacteria in caecum of laying hen.

**$NH_3$ production in the feces:** The effects of FFVM on the $NH_3$ gas emission from feces are shown in Table 7. The feces were gathered within 24 h of excretion and the $NH_3$ gas was measured at day 0, 1, 3, 6, 9 and 15 after excreta collection. At day 0 and 1, each FFVM group showed a decreased trend as compared to the control and the higher levels of 4 and 5% FFVM showed significant decreases ($p<0.05$). However, after 3 days, no experimental groups showed significant decreases as compared with the control.

Since high concentrations of $NH_3$ from manure impairs productivity, including growth rate (Reece *et al*., 1979) and egg production (Deaton *et al*., 1984), as well as damaging the respiratory tract (Nagaraja *et al*., 1983), previous studies have focused on suppressing $NH_3$ gas production by decreasing the protein level in the feed (Hobbs *et al*., 1996) and controlling the microorganisms in the intestines by adjusting the pH level (Van Heugten and Van kempen, 1999). On the other hand, dietary

*B. subtilis* could pose as a promising alternative for inhibiting $NH_3$ gas emissions via improving nutrient availability, especially for crude protein, followed by increased nutrient retention time in the gastrointestinal tract. In fact, Santoso *et al.* (1999) reported that dietary *B. subtilis* increased the digestion and nutrient availability and decreased the level of nitrogen in the feces and therefore decreased $NH_3$ emission. Moreover, a study by Sen *et al.* (2012) demonstrated that dietary *B. subtilis* increased intestinal retention time for nutrients including crude proteins and Kim *et al.* (2005) also reported that the inclusion of *B. subtilis* in broiler diet significantly decreased the $NH_3$ and nitrogen level in feces. However, the inhibitory effect of FFVM on $NH_3$ emission may result from a synergistic effect between *B. subtilis* and *F. velutipes* mycelium, but not only by *B. subtilis* because our previous study showed that $NH_3$ emission from broiler manure was suppressed by dietary dehydrated *F. velutipes* mycelium (Lee *et al.*, 2012). Collectively, our results suggest that the addition of FFVM in the laying hen's feed can decrease $NH_3$ gas emission, although by a small amount.

**Conclusion:** Although *F. velutipes* is a popular edible mushroom, the mycelium part has been discarded after harvesting. Therefore, we examined the possibility of *F. velutipes* mycelium as an animal feed resource after fermenting with *B. subtilis* A8-8 and *Klebsiella* sp. *Sc* (FFVM) to increase its availability in laying hens. One hundred eighty Hy-Line Brown laying hens were fed with a control diet or a control diet supplemented with 1, 2, 3, 4, or 5% of FFVM for 5 weeks and hen performance, egg quality, number of caecal pathogenic bacteria and fecal $NH_3$ gas production was estimated. For hen performance, 1% of dietary FFVM improved egg weight and egg mass. Dietary FFVM tended to improve egg quality including the albumen height, Haugh unit and egg shell thickness and 4% of FFVM significantly improved egg quality, including albumen height, Haugh unit, egg shell weight and shell thickness, compared with the control diet ($p<0.05$). Interestingly, the number of pathogenic bacteria and fecal $NH_3$ gas production during early periods were suppressed by high levels of dietary FFVM (3-5%). Taken together, the current study suggests that dietary FFVM can be used as a valuable feed resource, recycling agricultural byproduct.

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