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Evaluation of golden needle mushroom *(Flammulina velutipes)* stem waste on pullet performance and immune response

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Abstract

The driving force behind the interest in using natural herbs in poultry production is to eliminate the use of low-dose antibiotics. Therefore, this study was carried out to investigate the effect of *Flammulina velutipes* mushroom waste (FVW) on performance parameters, relative organ weight, apparent nutrient retention, excreta composition, immune response and serum immunity in pullets. A total of 360 x 10-week-old ISA Brown pullets were randomly assigned to five equal treatment groups, with nine replications of eight birds for each treatment. The dietary treatments included a standard basal diet as control; antibiotic (0.05% flavomycin); 2% FVW; 4% FVW; and 6% FVW. The total experimental duration was 42 days, from 10 weeks to 16 weeks old. Final live weight was higher in FVW groups than in the control and antibiotic groups. No differences were found for average daily feed intake, average daily weight gain and feed conversion ratio during the entire study period. Proventriculus weight and bursa weight were higher in FVW groups. No differences were observed for other inner relative organ weights (liver weight, gizzard weight spleen and abdominal fat weight) compared with the control and antibiotic groups. Dietary inclusion of FVW increased dry matter, crude protein and ether extract retention compared with control and antibiotic groups. Excreta dry matter content was higher and pH lower, in the FVW groups than in the control and antibiotic groups. Antibody titres against Newcastle disease, Infectious bronchitis and Avian influenza virus vaccines were higher in FVW groups. Serum immunoglobulin parameters (IgA, IgG, IgM) were higher in FVW than in the control and antibiotic groups. *Flammulina velutipes* mushroom waste can be used at inclusion levels up to 6% in pullet rations for better immune response and nutrient retention without hampering normal growth performance.

Keywords: Antibody titres, apparent nutrient retention, excreta dry matter, serum immunity
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Introduction

The popularity of mushrooms in human food and in the animal feed industry is increasing owing to their considerable health benefits and desirable functions. Recently, mushrooms have been recognized as a functional food and potential source of nutraceuticals (Tang *et al*., 2016). The fungus, *Flammulina velutipes* in the family physalacriaceae, is also known as the golden needle mushroom, winter mushroom, enoki mushroom, lily mushroom or velvet stem in different countries (Miles & Chang, 2004; Jing *et al*., 2014). *F. velutipes* has long been popular for its own flavour, texture, medicinal and functional properties (Yang *et al*., 2011, 2016; Tang *et al*., 2016). Several biological and therapeutic effects of *F. velutipes* have been reported in *in vitro* studies, such as the immune modulatory effect via stimulating the immune response, the production of cytokines and antibacterial, antiviral, antifungal, antioxidant, and anticancer activities (Badalyan & Hambardzumyan, 2001; Smiderle *et al*., 2006; Wang *et al*., 2012; Wu *et al.,* 2014). *F. velutipes* contains high levels of dietary fibre and protein, with all the essential amino acids, vitamins and minerals, and is low in

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energy and free from cholesterol (Karaman *et al*., 2010; Wu *et al*., 2010). In addition, *F. velutipes* is a good source of calcium (2.21%), phosphorus (1.68%) and vitamin D (Na *et al*., 2005; Ko *et al*., 2007).

Traditionally, antibiotics have been used in poultry feed as growth promoters and as treatment for infected chickens. Conventional synthetic feed additives, such as antibiotic growth promoters, antioxidants, anti-parasitic agents and anti-fungal agents have been used in poultry feed for decades (Ahmad *et al.*, 2017). But long-term and overuse of antibiotics in poultry diet may lead to animal and human health hazards. Zhang *et al.* (2011) reported that overuse of antibiotics in animal production created antibiotic-resistant bacteria in food and food products that originated from animals, and thus exposing humans to these bacteria. The inclusion of antibiotics as growth promoters in animal feed and poultry diet has been restricted in many countries (Wallinga & Burch, 2013; Neeraj, 2016). Currently, poultry researchers are committed to using unconventional natural feed supplements as replacements for antibiotics, which have been proved to be possible therapies to improve health status and production performance. Now animal researchers are searching for potential natural feed resources that can increase production performance and the health status of livestock and poultry (Pourhossein *et al.,* 2015; Mahfuz *et al*., 2017). Mushroom waste could be a proper substitute for antibiotics in the poultry industry (Mahfuz *et al.*, 2017). It is available from mushroom farms, and possesses prebiotic, antimicrobial, antifungal and antioxidant properties (Fard *et al*., 2014). In addition, unconventional natural pharmaceutical products that have originated from fungi or herbs have been used as feed supplements for centuries in veterinary medicine (Zhang *et al.*, 2015). *F. velutipes* stem waste (FVW) is abundantly available because of the increased cultivation of this mushroom. The stems are treated as agricultural waste, although they have medicinal and nutritive values. The popularity of mushrooms for human consumption has also lead to environmental pollution (Chung, 1999; Mahfuz *et al*., 2017). However, to date the uses of *F. velutipes* stem waste in farm animal production have been limited. An *in vitro* study of the effects of mushroom and herb polysaccharides on immune function may provide a theoretical basis for future use of these components to enhance production, health status and economic efficiency in farm animals (Guo *et al.,* 2003). The weight of bird inner organs is an indicator of good digestion and sound health. Al-Khalifa (2015) stated that immune responses in avian species can be measured by weighing lymphoid organs, such as the spleen, liver, thymus, bursa, and lymphoid tissues. The development of the gastro intestinal tract has a positive role in the immune status of the bird.

Taking these facts into consideration, this study examined the suitability of golden needle mushroom *(Flammulina velutipes)* stem waste as a phytogenic feed additive and substitute for antibiotics in pullet diets. The objective was achieved by assessing the effects on performance, apparent nutrient retention, excreta composition, immune response to vaccines and serum immune parameters of pullets.

Materials and Methods

A total of 360 x 10-week-old ISA Brown pullets was randomly assigned to five groups of eight birds, with nine replications. The pullet house was exposed to 15 hours light per day, up to 10 weeks, then the lighting was gradually reduced every week to 13 hours light per day at 14 weeks. It was then maintained constant according to the recommendations of the genetic line manual (ISA Brown commercial management guide, 2016). The pullet house temperature was maintained at approximately 21 °C. The experiment was carried out at the animal shed of Jilin Agricultural University. The handling with experimental pullets was approved by the Animal Care and Use Committee of Jilin Agricultural University.

The dietary treatments included i) control group (basal diet); ii) antibiotic (0.05% flavomycin) group; iii) 2% FVW group; iv) 4% FVW group; and v) 6% FVW group. The *F. velutipes* stems were collected from a domestic mushroom farm in Changchun. The stem sample was prepared (0.01 mm) by a laboratory high-speed universal sample grinder (Huanghua Xinxing Electric Appliance Co, Hebei, China) and the proximate components (dry matter (DM), crude protein (CP), ether extract (EE) and ash) including calcium and phosphorus were performed following the method of AOAC (2004). The mushroom stem was dried in sun light and then mixed into the pullet diet, formulated to the specifications of the National Research Council (NRC,1994) using a feed mixer in a feed mill (Jilin Hanghong Animal Husbandry Co. Ltd, China). The dietary source of vitamin D was only from the vitamin-mineral premix in the experimental diet. The nutritional composition of the experimental pullet diet and FVW are presented in Table 1. Feed and water were offered *ad libitum* during the whole experimental period from 10 weeks to 16 weeks.

Total feed intake was determined as the difference between feed offered and residual feed in trough feeders on a weekly basic. The feed conversion ratio (FCR) was then calculated as feed intake divided by body weight gain. Mortality per pen was recorded and calculated based on the number of dead bird during the experimental period.

Table 1 Chemical analysis (g/kg) of experimental diets and mushroom stem waste

Ingredients	Control	Antibiotic	2% FVW	4% FVW	6% FVW
Maize corn	675.0	674.5	670.0	645.0	615.0
Soybean meal (43% CP)	255.0	255.0	240.0	245.0	255.0
FVW [a]	-	-	20	40	60
Lysine	2	2	2	2	2
Methionine	2.5	2.5	2.5	2.5	2.5
Dicalcium	30	30	30	30	30
Limestone	31	31	31	31	31
Common salt	2.5	2.5	2.5	2.5	2.5
Vitamin–mineral premix[b]	2	2	2	2	2
Antibiotics	-	0.5	-	-	-
Total	1000	1000	1000	1000	1000
Chemical analysis[c]					
DM (g/kg)	913.4	915.4	913.5	916.5	915.0
CP (g/kg)	168.7	168.7	168.3	168.5	168.4
Ca (g/kg)	11.1	11.1	11.2	11.2	11.3
P (g/kg)	5.8	5.8	5.7	5.6	5.8
EE(g/kg)	28.5	28.5	28.64	28.49	28.10
CF (g/kg)	25.8	25.8	29.4	33.9	38.6
Calculated analysis					
ME (MJ/kg)	11.69	11.68	11.66	11.68	11.67
Lysine (g/kg)	10.0	10.0	9.7	9.9	10.2
Methionine (g/kg)	5.0	5.0	4.9	4.9	5.0
Cystine (g/kg)	2.9	2.9	2.8	2.8	2.8

[a] FVW: *F. velutipes* stem waste at 2%, 4% and 6% FVW. Analysed composition of *F. velutipes* mushroom stem waste (g/kg)[c]: DM 884.0 ± 0.85; CP 13.75 ± 0.49; CF 22.05 ± 0.106; EE 2.8 ± 0.014; ash 11.6 ± 0.085; Ca 4.0 ±0.1; P 6.3 ± 0.28; Value are expressed as mean ± standard deviation (n=6)

[b] Provided per kg of the complete diet: retinyl acetate, 4500 IU; cholecalciferol, 1200 IU; DL-α-tocopheryl acetate, 2500 IU; thiamin, 5000 mg; riboflavin, 20000 mg; phylloquinone, 10000 mg; niacin, 45000 mg; pantothenic acid, 35000 mg; biotin, 1500 mg; folic acid, 3000 mg; cyanocobalamin, 40 mg; zinc, 45 mg; manganese 50 mg; iron, 30 mg; copper, 4 mg; cobalt, 100 µg; iodine, 1 mg; selenium, 100 µg

[c] DM: dry matter; CP: crude protein; Ca: calcium; P: phosphorus; EE: crude fat; CF: crude fibre; ME: metabolizable energy

All birds were immunized with combined vaccines of Newcastle disease (ND), Infectious bronchitis (IB) and Avian influenza (AI) (H9 subtype) (strain La Sota + strain M41 + strain HN 106, Beijing Ceva Huadu Biological Co. Ltd) at day 77 via wing web (0.5 mL/bird). On days 84, 91 and 98 blood samples were obtained from one bird of each replicate pen (nine birds per experimental group) via wing vein, and at day 112 by cervical dislocation. On the day of blood sample collection, serum was obtained by centrifuge at 3000 × g for 20 min at 4 °C (Legend Micro 17R centrifuge, Thermo Fisher, Germany) and was stored at -80 °C until antibody titres and serum immunoglobulin concentration were measured. Commercial enzyme-linked immuno-sorbent assay kits (Shanghai Jianglai industrial Ltd, Shanghai, China) were used to analyse ND (Cat No JL 21698; JL 21699), IB (Cat no JL 18838; JL 18839) and AI (Catno JL-E-73473; Cat no JL-E-73477) antibody titres. The serum IgA, IgG, IgM were measured using chicken-specific IgA (Catno BPE 60017), IgG (Catno BPE 60033), IgM (Catno BPE 60023), ELISA quantitation kits (Shanghai Lexington Bio Sciences Co. Ltd, China) according to the instructions of the manufacturer. Absorbance was measured at 450 nm.

An apparent nutrient retention trial was conducted during the last five days of the experiment to determine DM, CP and EE retention in the experimental pullets. One bird from each replicate was transferred to an individual cage to facilitate the collection of excreta samples. Room temperature was maintained at 21 °C. Diets containing chromium oxide at 2 g/kg were used as indigestible markers. Feed intake and total

excreta output were measured daily. Sample excreta collections were pooled within a cage and sub-sampled following the method of total excreta collection by Sakomura & Rostagno (2007). The Cr_2O_3 content was determined by the method described by Shang *et al.* (2016). The DM and EE were analysed according to the procedures of the Association of Official Analytical Chemists (AOAC 2004). Nitrogen was determined using an FP-528 nitrogen determinator (LECO Corporation, USA). The apparent nutrient retention (ANR) was calculated using this formula, based on quantity of feed and faeces nutrients with appropriate corrections for differences in DM content (Shang *et al*., 2016).

ANR = 100 – 100 x [(Cr_2O_3 in diet x nutrient in excreta) / (Cr_2O_3 in excreta x nutrient in diet)]

Excreta pH was measured by diluting 1 g excreta sample with 9 mL distilled water by a pH meter (PHS-3C; Shanghai Peng Shun Scientific Instrument Co., Ltd., Shanghai, China) (Shang *et al*., 2016).

At the end of the trial (day 112), one bird from each pen was randomly selected (nine pullets from each group) and weighed before being euthanized. Inner organ samples, for example proventriculus, gizzard, liver, spleen, bursa and abdominal fat, were removed, weighed, and the relative organ weights were expressed as a percentage of live weight.

Data were subjected to one-way analysis of variance using SPSS (2006) software. Significant effects of dietary treatments on experimental groups were evaluated with Duncan's test (1995). Statements of statistical significance are based on a probability of P <0.05.

Results

There was no difference (P >0.05) in initial live weight, but the final live weight was higher (P <0.05) in both 4% and 6% FVW groups compared with control and antibiotic groups (Table 2). Daily weight gain was not affected by FVW feeding in this study (Table 2). No difference (P >0.05) in feed intake was observed in the current study on all the evaluating days including the 42-day study period (Table 2). Similarly, FCR was not affected (P >0.05) in FVW feeding at all the evaluation periods (Table 2). Mortality was nil in the antibiotic group and FVW groups throughout the trial period (Table 2).

Table 2 Effect of *Flammunila velutipes* mushroom stem waste on pullet performance[1]

Parameters	Control	Antibiotics	2%FVW	4%FVW	6%FVW	*P* -value
Average feed intake (g/day)						
Day 70–84	69.89 ± 0.57	69.70 ± 0.55	69.91 ± 0.54	70.07 ± 0.17	70.05 ± 0.35	0.867
Day 85–98	73.67 ± 0.76	73.33 ± 0.44	73.12 ± 0.28	72.91 ± 0.13	73.45 ± 0.62	0.437
Day 99–112	78.50 ± 0.58	78.32 ± 0.19	78.65 ± 0.80	78.58 ± 1.02	78.18 ± 0.73	0.923
Day 70–112	74.04 ± 4.31	73.78 ± 4.32	73.89 ± 4.42	73.85 ± 4.32	73.89 ± 4.08	1.00
Average body weight gain (g/day)						
Day 70–84	15.90 ± 3.15	13.40 ± 1.47	17.20 ± 4.30	19.60 ± 3.56	19.0 ± 1.75	0.380
Day 85–98	9.58 ± 1.86	11.43 ± 3.72	9.68 ± 4.85	9.73 ± 2.85	9.68 ± 1.27	0.935
Day 99–112	13.17 ± 0.58	14.01 ± 0.19	13.86 ± 0.80	13.74 ± 1.01	13.75 ± 0.75	0.200
Day 70–112	12.88 ± 3.16	12.95 ± 1.34	13.58 ± 3.76	14.36 ± 4.69	14.14 ± 4.67	0.982
Feed conversion ratio (g/g)						
Day 70–84	4.66 ± 1.26	5.27 ± 0.62	4.53 ± 1.09	3.66 ± 0.76	3.68 ± 0.16	0.389
Day 85–98	7.73 ± 0.72	6.81 ± 1.84	8.76 ± 1.53	7.88 ± 1.89	7.64 ± 0.63	0.839
Day 99–112	5.96 ± 0.06	5.59 ± 0.08	5.68 ± 0.12	5.73 ± 0.28	5.69 ± 0.23	0.210
Day 70–112	6.12 ± 1.54	5.89 ± 0.81	6.32 ± 2.18	5.76 ± 2.11	5.67 ± 1.98	0.991
ILW (g)	1018.75 ± 10.8	1016.33 ± 8.76	1018.42 ± 14.2	1017.50 ± 13.2	1016.17 ± 13.2	0.998
FLW(g)	1559.70^{c} ± 7.05	1560.23^{c} ± 6.09	1588.72^{b} ± 4.35	1620.07^{a} ± 18.74	1609.80^{a} ± 14.32	0.001
Mortality (%)	1.23	0	0	0	0	-

[1] Data represent the mean value ± standard deviation of 72 pullets per treatment
[a,b,c] means in the same row with different superscripts are significantly different at P <0.05
ILW: initial live weight; FLW: final live weight

Proventriculus weight was higher (P <0.05) in FVW groups than in the control and antibiotic groups. Bursa weight was higher (P <0.05) in antibiotic and FVW groups than in the control. Abdominal fat weight was lower in pullets on FVW compared with the control and antibiotic diets, but the result was not different (P >0.05). No difference (P >0.05*)* was observed in relative liver weight, spleen weight and gizzard weight, among the treatment groups (Table 3).

Table 3 Effect of *Flammunila velutipes* mushroom stem waste on inner organ weight (% live weight) of pullets at day 112[1]

Parameters	Control	Antibiotics	2%FVW	4%FVW	6%FVW	*P* -value
Proventriculus	$0.40^{c} \pm 0.05$	$0.44^{bc} \pm 0.48$	$0.50^{a} \pm 0.05$	$0.46^{abc} \pm 0.07$	$0.47^{ab} \pm 0.06$	0.009
Gizzard	2.46 ± 0.30	2.43 ± 0.40	2.48 ± 0.43	2.35 ± 0.36	2.08 ± 0.19	0.112
Liver	1.52 ± 0.13	1.53 ± 0.15	1.51 ± 0.07	1.54 ± 0.05	1.57 ± 0.29	0.960
Spleen	0.18 ± 0.04	0.20 ± 0.02	0.22 ± 0.05	0.23 ± 0.06	0.19 ± 0.06	0.248
Bursa	$0.18^{b} \pm 0.03$	$0.27^{a} \pm 0.05$	$0.26^{a} \pm 0.03$	$0.29^{a} \pm 0.07$	$0.31^{a} \pm 0.05$	0.001
Abdominal fat	1.64 ± 0.63	1.63 ± 0.72	1.46 ± 0.39	1.53 ± 0.50	1.55 ± 0.58	0.961

[1] Data represent the mean value ± standard deviation of nine pullets per treatment
[a,b,c] means in the same row with different superscripts are significantly different at P <0.05

Feeding experimental pullets on diets containing FVW resulted in higher (P <0.05) DM, CP and EE retention compared with those fed on the control and antibiotic diets. The best response for CP and DM retention was observed in pullets in the diet containing 6% FVW (Table 4). Excreta DM was higher (P <0.05) in the FVW groups than the control and antibiotic groups. The pH of excreta was lower (P <0.05) in pullets fed with FVW than the control group. However, excreta DM remained the same in both the control and antibiotic groups (Table 4).

Table 4 Effect of *Flammunila velutipes* mushroom stem waste on apparent nutrient retention (g/kg), excreta dry matter (%) and excreta pH in pullets[1]

Parameters	Control	Antibiotics	2%FVW	4%FVW	6%FVW	*P* -value
DM	$775.35^{b} \pm 8.97$	$784.82^{a} \pm 3.91$	$786.01^{a} \pm 5.95$	$789.69^{a} \pm 6.42$	$790.67^{a} \pm 3.41$	0.006
CP	$669.89^{d} \pm 4.75$	$688.94^{c} \pm 6.23$	$717.49^{b} \pm 3.91$	$717.26^{b} \pm 4.92$	$736.29^{a} \pm 4.25$	0.001
EE	$784.62^{cd} \pm 3.69$	$782.53^{d} \pm 2.37$	$795.70^{a} \pm 2.08$	$790.11^{b} \pm 1.98$	$789.56^{bc} \pm 5.20$	0.001
Excreta DM	$23.37^{b} \pm 0.85$	$23.80^{b} \pm 0.34$	$24.13^{b} \pm 0.66$	$25.77^{a} \pm 0.72$	$25.85^{a} \pm 0.09$	0.001
Excreta pH	$6.97^{ab} \pm 0.12$	$7.03^{a} \pm 0.10$	$6.91^{abc} \pm 0.16$	$6.83^{bc} \pm 0.17$	$6.77^{c} \pm 0.14$	0.005

[1] Data represented the mean value ± standard deviation of nine pens
[a,b,c,d] means in the same row with different superscripts are significantly different at P <0.05

Antibody titres against ND, IB and AI virus vaccines were higher (P <0.05) in FVW diets on all the evaluating days (except for AI at day 84) in comparison with those fed the control and antibiotic diets. The best responses for antibody titres were found in pullets fed 6% FVW. Interestingly, no antibody titres were observed for IB in the control group at day 112, whereas mushroom groups tested positive (Table 5).

Pullets in FVW groups showed higher (P <0.05) serum immunoglobulin (Ig A, Ig G, and Ig M) levels than the control and antibiotic groups (Table 6). The highest concentrations for immunoglobulin parameters were observed in both 4% and 6% FVW groups (except for serum IgG 2% FVW supplementation), whereas, IgA and IgM remained the same in the control and antibiotic feed groups.

Table 5 Effect of *Flammunila velutipes* mushroom stem waste on antibody titres (ng/L) in pullet[1]

Parameters	Control	Antibiotics	2%FVW	4%FVW	6%FVW	*P* -value
Newcastle disease (ND)						
Day 84	$747.17^{c} \pm 55.35$	$785.0^{bc} \pm 45.21$	$867.17^{abc} \pm 62.66$	$900.33^{ab} \pm 43.66$	$947.17^{a} \pm 45.12$	0.025
Day 91	$622.17^{bc} \pm 61.29$	$613.0^{c} \pm 49.93$	$704.67^{ab} \pm 45.17$	$731.33^{a} \pm 37.53$	$758.83^{a} \pm 42.35$	0.011
Day 98	$432.17^{b} \pm 36.92$	$420.5^{b} \pm 33.85$	$536.33^{ab} \pm 42.89$	$539.67^{ab} \pm 35.57$	$580.5^{a} \pm 44.11$	0.037
Day 112	$347.17^{c} \pm 27.16$	$384.67^{bc} \pm 28.12$	$498.83^{ab} \pm 18.38$	$483.0^{ab} \pm 16.98$	$528.83^{a} \pm 25.76$	0.025
Infectious bronchitis (IB)[2]						
Day 84	$0.473^{b} \pm 0.07$	$0.492^{b} \pm 0.05$	$1.022^{a} \pm 0.21$	$1.012^{a} \pm 0.24$	$1.050^{a} \pm 0.22$	0.040
Day 91	$0.465^{c} \pm 0.35$	$0.430^{c} \pm 0.16$	$0.692^{bc} \pm 0.12$	$1.112^{a} \pm 0.23$	$1.023^{ab} \pm 0.04$	0.007
Day 98	$0.190^{c} \pm 0.14$	$0.420^{b} \pm 0.06$	$0.473^{b} \pm 0.11$	$0.517^{b} \pm 0.01$	$0.722^{a} \pm 0.05$	0.002
Day 112	0.000	$0.082^{b} \pm 0.14$	$0.347^{ab} \pm 0.18$	$0.572^{a} \pm 0.09$	$0.645^{a} \pm 0.08$	0.019
Avian influenza (AI)						
Day 84	6.48 ± 1.20	7.87 ± 0.77	7.14 ± 0.87	8.42 ± 1.05	8.56 ± 1.15	0.386
Day 91	$5.44^{b} \pm 0.46$	$5.71^{b} \pm 0.36$	$6.40^{b} \pm 1.03$	$6.48^{b} \pm 0.71$	$7.87^{a} \pm 0.46$	0.012
Day 98	$4.82^{bc} \pm 0.50$	$4.62^{c} \pm 0.33$	$6.13^{a} \pm 1.0$	$5.89^{ab} \pm 0.09$	$6.68^{a} \pm 0.62$	0.010
Day 112	$4.41^{b} \pm 0.53$	$4.32^{b} \pm 0.17$	$5.67^{a} \pm 0.48$	$5.62^{a} \pm 0.47$	$6.43^{a} \pm 0.81$	0.004

[1] Data represent the mean value ± standard deviation of nine birds per treatment
[2] Data represent more than cut off value (> 0.250) indicate positive (+) for IB antibody as per kit instruction manual
[a,b,c] means in the same row with different superscripts are significantly different at $P < 0.05$

Table 6 Effect of *Flammunila velutipes* mushroom stem waste on serum immunoglobulin (mg/mL) in pullets at day 112[1]

Parameters	Control	Antibiotics	2%FVW	4%FVW	6%FVW	*P* -value
IgA	$3.89^{c} \pm 1.09$	$4.81^{c} \pm 0.93$	$5.10^{b} \pm 1.01$	$5.47^{a} \pm 1.24$	$5.57^{a} \pm 1.79$	0.021
IgG	$4.49^{c} \pm 0.69$	$5.50^{b} \pm 1.16$	$5.94^{ab} \pm 0.86$	$6.52^{a} \pm 1.18$	$7.03^{a} \pm 1.94$	0.001
IgM	$2.20^{c} \pm 0.36$	$2.45^{c} \pm 0.60$	$3.08^{b} \pm 0.81$	$3.73^{a} \pm 0.48$	$4.08^{a} \pm 1.08$	0.044

[1] Data represent the mean value ± standard deviation of nine pullets per treatment
[a,b,c] means in the same row with different superscripts are significantly different at $P < 0.05$

Discussion

The analysed values of *F. velutipes* for DM, CP, and CF in FVW were mostly close to those reported previously (Lee *et al.*, 2012; Reis *et al.*, 2012), but EE, total mineral (ash), Ca and P were lower than the published values. The nutritional composition of *F. velutipes* may differ with soil, harvesting methods and environmental factors (Miles & Chang, 2004).

Throughout the study period no significant differences were found in growth performance parameters, average daily feed intake, average daily weight gain and FCR among the experimental groups. However, the final live weight was significantly higher in the FVW groups than in control and antibiotic groups, which justified the improved nutrient retention in the FVW groups. This study speculated that the inclusion of FVW in diet might provide additional vitamins and minerals, especially vitamin D, calcium and phosphorus, which might lead to better nutrient retention and final live weight than non-supplemented groups. Hong-Gu *et al.* (2014) reported that inclusion of fermented *F. velutipes* mycelium had no positive effect on feed intake and FCR in laying hens. In addition, Lee *et al.* (2012) reported that levels up to 5% *F. velutipes* mycelium did not improve feed intake in broilers, which was similar to this study for feed intake. Dietary incorporation of the dried mushrooms in broiler chickens had no effect on feed intake but improved body weight and feed efficiency compared with the non-supplemented treatment (Giannenas *et al.*, 2010; Shang *et al.*, 2016). In contrast, Daneshmand *et al.* (2012) reported that inclusion of oyster mushrooms, garlic and propolis extract decreased birds' body weight and weight gain. This difference might be associated with mushroom type,

inclusion level and experimental bird types. Mortality rate was nil in the FVW and antibiotic groups, which resulted in higher pullet survival than in the control group. Mortality caused by infection is a major problem in the poultry industry. Antibiotics are commonly used as the main tools to prevent infection and mortality in poultry. This study found the feasibility of FVW as a substitute for antibiotic to prevent infection caused mortality. It was speculated that β-glucan, the active component of *F. velutipes* mushroom, may generate immunity to birds in FVW groups. No incidence of disease during the study period ensured that pullet immunity had been gained from dietary sources of mushroom waste. Wu *et al*. (2014) found that the polysaccharides in *Flammunila velutipes* had immune modulatory activity.

The experimental diets contained higher levels of crude fibre, which may serve to increase relative proventriculus and bursa weight. Yokhana *et al.* (2016) reported that digestive organ weights can be higher with the addition of insoluble fibre to the diet of growing pullets. The higher bursa weight resulted in better health, and a lower immune suppression effect on pullet physiology was observed in the current study. The higher bursa weight is an indicator of better health status and sound physiological response to body immune system (Willis *et al*., 2013). Additional abdominal fat in pullets is undesirable, which may result in poor egg production and metabolic diseases such as fatty liver syndrome. The current study speculated that FVW could reduce abdominal fat in pullets and the incorporation of FVW could prevent such metabolic disorders and improve performance in pullets. The weights of the other relative organs were not affected by FVW inclusion, which ensured that the normal health of pullets consuming FVW was not impaired during the experimental period and that greater immunity was gained. In some studies, it was reported that mushroom had no significant effect on lymphoid organ weight (Toghyani *et al*., 2012; Kavyani *et al*., 2012; Daneshmand *et al*., 2012; Fard *et al*., 2014).

The higher DM, CP and EE retention in pullets with FVW might be associated with significantly higher final live weight in FVW groups in this study. A previous study by Shang *et al.* (2016) reported that inclusion of the dried mushroom, *Hericium caput-medusae*, in diets improved (P <0.05) DM and EE digestibility in broiler chickens. In contrast with the current study, Daneshmand *et al.* (2012) reported that inclusion of oyster mushroom and antibiotic treatments could not affect protein and organic matter retention in experimental broilers. These different findings might be associated with mushroom type, mushroom inclusion level and types of experimental birds compared with the current study. Excreta DM content was higher with the dietary inclusion of FVW in this study, which suggests that the incorporation of FVW reduced excreta moisture, which could prevent the problems caused by wet litter in poultry houses, increase the absorption of nutrients and reduce ammonia gas production in excreta. Excreta pH was lower in the FVW group, which may have a positive effect in reducing excreta ammonia in pullet houses. There was a strong relationship between excreta pH and moisture with ammonia gas production. Factors such as relatively high moisture content, high temperatures and high pH can facilitate the production of ammonia from excreta (Teye *et al.*, 2008).

Daneshmand *et al.* (2012) found higher antibody response to ND with a combination of garlic, oyster mushroom and propolis extract than the control and antibiotic diets in broilers; which supports the current findings for ND, IB, and AI antibody titres with the inclusion of FVW in this study. In addition, the slightly higher antibody titres against ND and AI virus were reported by Toghyani *et al*. (2012). In contrast with this study, Fard *et al.* (2014) found 2% mushroom extract significantly decreased (P <0.05) antibody titre against ND in chickens, but slightly increased AI antibody titres, and suggested reinvestigating antibody titres with mushroom supplement.

Serum immunoglobulin concentrations can generate humoral immune response in animals owing to their important roles in the immune function in fighting against infection (Bai *et al.*, 2017). Fard *et al.* (2014) reported that inclusion of oyster mushroom waste improved certain immune parameters in broilers. Supplementation of β-glucan from edible mushrooms had a significant immune stimulatory effect in broilers (Muthusamy *et al*., 2013). The polysaccharides in *F. velutipes* mushroom have strong immune modulatory activity and possess antioxidant activity, which could enhance non-specific and specific immune responses *in vitro* (Wu *et al*., 2014; Tang *et al*., 2016; Mahfuz *et al*., 2017). However, no similar reports were found in the literature about the effects of the inclusion of FVW in pullet diets on serum immunity to compare with this study.

Conclusions

This study found positive effects of mushroom waste on apparent nutrient retention, excreta DM, excreta pH, humoral immune response to disease vaccines, and serum immunity. Collectively, this study suggested that FVW can be used at inclusion levels up to 6% as potential phytogenic feed additives in layer pullet diets with a view to improving health status via increasing the immune response without affecting normal growth.

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Authors' contributions

SUM as a lead author conducted this experiment as a part of his PhD research work under the supervision of ZL and HS. SM prepared the manuscript. MC, SW, JW helped in mushroom preparation, experimental diet and participated in the animal experiment. SUM, MC, JW, JSZ worked in vaccination and the ELISA test. SW, JSZ helped in statistical analysis and formatting the manuscript. ZL, HS and SUM managed the entire experiment and revised the manuscript. All authors read and approved the final manuscript for publication.

Conflicts of interest Declaration

There are no conflicts of interest that are relevant to this publication.

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