ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

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International Journal of Poultry Science 12 (4): 206-211, 2013
ISSN 1682-8356

Effect of Incubation Time and Level of Fungus Myceliated Grain Supplemented Diet on the Growth and Health of Broiler Chickens

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Abstract: A study was conducted to evaluate the effects of different incubation time and level of inclusion of Fungus Myceliated Grain (FMG) in diets of broiler chickens. The nine different dietary treatments were as follows: (1) Control-No-FMG (2) 5% FMG-14 d (3) 10% FMG-14 d (4) 5% FMG-28 d (5) 10% FMG-28 d (6) 5% FMG-42 d (7) 10% FMG-42 d (8) 5% FMG-56 d and (9) 10% FMG-56 d. Each diet was fed to 3 replicate pens of 10 chicks each in floor pens for 49 days. The 270 day-of-hatch straight-run chicks were fed FMG that included *Lentinula edodes*, *Cordyceps* sp. *Ganoderam* sp. and *Pleurotus ostreatus* incubated in grain for 14, 28, 42, or 56 days. FMG protein percentage, live weights, *Eimeria* oocyst, bifidobacteria counts, mortality, feed consumption, blood differential and immunoglobulins (IgA, IgG) were evaluated in this experiment. Percent protein between mushroom incubation periods differed significantly. Body weight results showed that all experimental treatments were significantly ($P \leq 0.05$) different from treatment 1 (Control) for male broilers but did not differ with the females, while the overall feed intake was significantly higher for the control broilers. Fecal oocyst count showed only one treatment (2) differed significantly ($P > 0.05$) from treatment 1 (Control). Bifidobacteria counts were highest in treatment 4 ($8.07_{\log 10}$) when compared to the treatment 1 control ($7.69_{\log 10}$) at week 5. Some hematological analysis values showed some elevation and decreased protein levels as the incubation time increased along with the antibody IgA and IgG titers. The results from this study suggest that the incubation time, mixture and level of FMG do not adversely affect growth and higher antibody levels in broiler chickens could enhance health as percent protein decline with increased beta-glucan and incubation time of the FMG.

Key words: Broiler chickens, mushrooms, performance, incubation time, health

INTRODUCTION

The removal of many feed additives/antibiotics in poultry rearing has negatively impacted health, production performance and product safety (Montagne *et al*., 2003). Therefore, new strategies and alternatives feed additives, including medicinal mushroom, are attracting heightened interest (Ohimain and Ofongo, 2012). Research on medicinal mushrooms for animals has focused on compounds that can modulate the biologic response of immune cells. Those compounds that appear to stimulate the animal's immune response are of interest so that drugs/antibiotics usage in poultry production can be reduced or eliminated. Recent research has paved the way for utilizing and furthering the potential for mushroom applications in poultry production. For instance, Guo *et al*. (2004) studied the effects of mushroom and herb polysaccharides on cellular and humoral immune responses of *Eimeria tenella*-infected chickens and concluded that these supplementations resulted in an enhancement of both cellular and humoral immune responses.

In recent decades, significant gains have been made with utilizing mushrooms for promoting animal health (Borchers *et al*., 2004). Dalloul *et al*. (2006) pioneered some studies on the immune-potentiating effect of lectin extracted from *Fomitella fraxinea* in poultry during coccidiosis. Their results demonstrated that the lectin extraction given to chickens was effective in promoting growth and enhancing immune-modulatory activity during coccidiosis. More recently, Selegean *et al*. (2009) studied the effect of a polysaccharide extract from the edible mushroom *Pleurotus ostreatus* against infectious bursal disease virus and found a positive synergistic relationship between the extracts and bursa vaccines in stimulating the production of antibodies. In a similar vein, a water extract method has been utilized to further

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study mushrooms in promoting poultry health (Willis *et al*., 2007, 2009). Other usages of this product, pioneered by Willis *et al.* (2009), utilized the mushroom extract to molt aging laying hens for additional eggs production cycles.

As these positive findings have evolved using mushroom extracts and their product in enhancing poultry health, some other issues and concerns have surfaced. These include: what are the most effective type and dosage and what is the cost of producing the mushrooms and the time and labor needed to extract the polysaccharides from them? These concerns and others led to studies by Willis *et al.* (2009) that utilized Fungus Myceliated Grain (FMG) instead of extracts in research with broiler chickens that cost less and was easy to administer. These investigations resulted in a FMG product composes of cultures of mushroom spawn incubated in sorghum grain. The product derived from the methodology employed is more economical to produce and administer to chickens via the feed rather than extracts added to water and other delivery methods. Their most recent studies have utilized FMG inclusion in the feed to molt laying hens and investigate coccidiosis infection protection, *Salmonella* population reduction bifidobacteria growth and immune enhancements in broiler chickens (Willis *et al*., 2012, 2011, 2010).

The present study relates to issues surrounding methods, type of mushrooms, level of supplementation, duration of incubation time, feeding regimes and performance. Accordingly, this specific experiment was undertaken to determine if specific antibody levels, body weights and other performance traits of broiler chickens were positively or negatively affected by FMG incubation time and inclusion level in their diet.

MATERIALS AND METHODS

Experimental design and husbandry: Two hundred seventy (n = 270) day-old-male and female broiler chickens (Ross x Ross) were obtained from a commercial hatchery. The chicks were weighed and randomly distributed into nine different treatment groups replicated three times with 10 chicks (5 males + 5 females) that were fed a control or a 5 or 10 percent FMG mixture, each made from four different mushrooms and with four different incubation periods as follows: (1) Control-No FMG (2) 5% FMG-14 d (3) 10% FMG-14 d (4) 5% FMG-28 d (5) 10% FMG-28 d (6) 5% FMG-42 d (7) 10% FMG- 42 d (8) 5% FMG-56 d and (9) FMG 10%-56 d. The mushroom mixture utilized in this study consisted of *Lentinus edodes (*Shiitake), *Ganoderman lucidum* (Reishi), *Pleurotus ostreatus* (Oyster) and *Cordyceps sp*. All FMG types were evenly mixed and fed at the 5 and 10 percent inclusion level of a balanced basal meal ration (Table 1). All chicks were vaccinated against Marek's disease, infectious bronchitis and Newcastle disease prior to leaving the hatchery. Each treatment group was housed and maintained within a 27 floor pen system containing unused wood shaving in each (1.2 m^2) pen with a drinker and one hanging feeder. Body weights of males and females, mortality, blood, fecal samples for oocyst and bifidobacteria counts were collected and evaluated at 49 days of age.

Table 1: Composition of basal diets

	Amount		
Ingredients	Starter	Grower	Finisher
Corn	1167	1324	1410
Soybean meal	716	563	478
Corn micro-flush	19.94	20.73	20.30
Limestone fine	19.42	20.40	21.37
Dicalcium phosphate (18.5%)	41.77	36.92	31.47
Lysine (78.5%)	0.01	1.26	4.27
Methionine (99%)	3.80	2.67	2.01
Threonine	1.06	0.02	1.58
Salt	10.00	10.00	10.00
PX NCSU Br Mineral (TM90)	4.00	4.00	4.00
Choline chloride (60)	4.00	4.00	4.00
PX NSCU Br Vitamin (NCSU90)	1.00	1.00	1.00
Selenium Premix NCSU (0.02%)	2.00	2.00	2.00
Poultry fat (Miter)	10.00	10.00	10.00
Total batch weight	2000	2000	2000

Mushroom cultivation, protein determination and basal diet: Shiitake, cordyceps, reishi and oyster were cultivated at the Mushroom Biology and Fungal Biotechnology Laboratory at North Carolina A&T State University. Sterile sorghum grain was separately inoculated with selected fungi, at 25°C for 2, 4, 6 and 8 weeks before use (Willis *et al*., 2010). The resulting myceliated grain was processed by air-drying at about 25°C for approximately 6 hours and ground into a powder that was used for supplementing the basal ration in the experimental trials. Samples were collected from the different mushrooms at different incubation times and sent to a commercial laboratory for protein assessment. The basal diet composition is shown in Table 1. The four different fungi were weighed and put into the basal diet at 5 and 10 percent levels, mixed thoroughly and placed into tube feeders in respective treatment pens. The basal meal ration was free of any medication. The chickens were fed *ad libitum* FMG which had been inoculated with a combination of four species of mushroom spawn that have documented health attributes.

Blood differential cell count: For determination of blood differential cell counts, peripheral blood samples were collected via the broilers' jugular veins in vacuum tubes containing EDTA to prevent clotting. Blood smeared slides were prepared, allowed to dry and then fixed and stained with HEMA3 Stain. A total of one hundred cells were counted and the results were expressed as percentages of lymphocytes, heterophils, eosinophils and basophils. Whole blood samples for ELISA IgA and IgG were obtained from broilers per treatment group at

d 49. The collected blood was put on ice and transported to a laboratory for processing. Serum was separated from whole blood by centrifugation, decanted and stored in a minus 80° laboratory freezer until analyzed. Chicken IgA and IgG ELISA kits were used to quantify the amount of IgA and IgG antibody levels present within the collected serum samples. Both standards and samples were analyzed according to the Chicken IgA (Cat. No. E33-103) and Chicken IgG (Cat. No. E33-104).

***Eimeria* fecal analysis:** Fecal samples were collected at day 49 from four birds in each treatment that had been placed in cages prior to collection. The samples were transported to the laboratory where two grams of feces per tube were measured out onto a small scale and then placed into a clear, sterile container in which 30 ml sodium nitrate was conjugated. The mixture of solution and fecal matter was strained through cheese cloth and the mixture was pipetted and transferred to the chambers of McMaster's slide. A total of 5 minutes elapsed in order for the eggs to reach the surface of the chambers of the slide. A microscope using 10x lens and 10x eye piece was used to read the McMaster's slide. The total number of eggs in the two chambers was multiplied by 50, thus indicating the eggs-per-gram for each sample (Hodgson, 1970).

Bifidobacteria fecal analysis: The population of *Bifidobacteria* in fecal samples was determined using the standard laboratory method (Ibrahim and Salameh, 2001; Brown *et al*., 2005). Fecal samples were collected from floor droppings at four and five weeks of the experimental trial and transported to the laboratory for analysis. The samples (11 g each) were diluted with 99 ml sterilized 0.1% peptone water and homogenized using a stomacher 400 lab system 4 for 2 min and 100 µL of appropriate dilution was plated onto modified BIM 24 agar. Plates were incubated at 35°C for at least 3 d to allow for bifidobacteria cell growth. In addition, the Gram Stain techniques were used to facilitate microscopic examination of morphological characteristics of bifidobacteria. Fructose 6-phospnloketalase activity was measured to confirm the identity of the bifidobacteria.

Statistical analysis: Data analysis was carried out using SPSS version 17.0 (SPSS Inc., Chicago, IL). Analysis of Variance (ANOVA) was performed to detect any significant differences between the treatment groups. The Duncan multiple range test was subsequently used to detect the source of difference in the ANOVA output. Mean values were considered significantly different at ($P<0.05$). Data are expressed as mean values±SEM. The critical level for the null hypothesis rejection in all the statistical test was 5% (a = 0.05).

RESULTS AND DISCUSSION

Protein percent of mushrooms: The effect of incubation growth time on the percent protein is shown in Table 2. There were significant ($P<0.05$) differences in protein levels of treatments. The data for the mushroom species showed a decline or decrease as incubation time increased for most species, except for Reishi. As such, the feeding at various levels of inclusion was expected to significantly decrease body weights. However, this was not a clear cut case, as body weights were somewhat constant. The mixture of different types is believed to balance the protein decline overall of the supplemented product into the basal ration at the levels of inclusion.

Body weights: The effects of dietary supplementation with FMG on body weights of male and female broiler chickens are presented in Table 3. Body weights of male broilers differed significantly ($P\leq0.05$) in all experimental treatments when compared to the Control-No FMG; whereas the females did not differ. The mixtures incubated for different periods of time did not show any decline in body weights as was shown in protein percent decline over extended periods of incubation (Table 2). Even at the 10% level of inclusion, no significant differences ($P>0.05$) were observed with the four-way mixture which is in contrast to previous research showing significant decline in weight using a single fungus at the 10% inclusion level. Moreover, as demonstrated in previous work by Willis *et al*. (2010), individual mushroom inclusion at the 5% level was comparable to the control groups. The fact that the females in each treatment did not differ from each other

Table 2: Protein level of mushroom for different incubation times

Mushroom	Incubation days	Protein (%)
Shiitake	0	7.477±0.234de
	14	7.540±0.130de
	28	5.543±0.227ab
	42	4.637±0.400a
	56	4.690±0.338a
Cordyceps	0	7.600±0.344de
	14	7.223±0.391de
	28	7.990±0.266e
	42	5.507±0.146ab
	56	5.143±0.121ab
Oyster	0	7.423±0.377de
	14	7.723±0.292de
	28	6.677±0.283cd
	42	5.877±0.551bc
	56	5.853±0.373bc
Reishi	0	7.260±0.372de
	14	7.627±0.363de
	28	8.130±0.188e
	42	7.143±0.757de
	56	7.050±0.367de

Each value is expressed as mean±standard error (n = 3) Different letters in each column indicate significant differences at $P<0.05$ according to the Duncan Statistics

Table 3: Body weight, feed intake and bifidobacteria population

Trts	Body weight, kg		Feed	Bifidobacteria population, $1og^{10}$ cfu's	
	Male	Female	intake (kg)	Week 4	Week 5
1	3.227±0.115b	2.552±0.081a	3.834±0.139b	7.403±0.003c	7.690±0.083ab
2	2.571±0.080a	2.261±0.252a	3.184±0.093a	7.643±0.066d	7.487±0.054a
3	2.632±0.052a	2.286±0.114a	3.547±0.135ab	6.723±0.137	7.883±0.032bc
4	2.726±0.152a	2.403±0.062a	3.261±0.005a	8.040±0.023e	8.070±0.015c
5	2.696±0.086a	2.384±0.057a	3.274±0.002a	6.390±0.000a	7.620±0.083a
6	2.728±0.095a	2.417±0.024a	3.271±0.003a	6.790±0.137b	7.857±0.085bc
7	2.702±0.026a	2.386±0.089a	3.281±0.005a	6.657±0.024b	7.960±0.035c
8	2.768±0.097a	2.437±0.136a	3.551±0.271ab	6.430±0.030a	8.010±0.114c
9	2.545±0.233a	2.307±0.045a	3.531±0.132ab	6.563±0.042ab	7.560±0.055a

Each value is expressed as mean ± standard error (n = 3).
Different letters in each column indicate significant differences at P<0.05 according to the Duncan Statistics

presents a challenge as to why this occurred. At this time, no explanation is offered; however, Willis *et al.* (2007) demonstrated that male and female chicken administered mushroom extracts responded differently with regards to body weight gain.

Feed intake: The average feed intake per bird is shown in Table 3. There were some significant ($P \leq 0.05$) differences observed amongst treatments. The birds receiving feed from treatments 2, 4, 5, 6, 7 consumed less feed compared to the control (treatment 1). As mentioned previously, the average protein for the control was 7.42% which declined as the incubation period increased. Therefore, less feed intake meant less protein intake, thereby reducing weights.

Bifidobacteria population: Bifidobacteria population results are presented in Table 3. There were significant ($P \leq 0.05$) differences for weeks 4 and 5 amongst treatments. Treatment 4 yielded the highest bifidobacteria population for both weeks. As noted in previous reports by Willis *et al.* (2009), bifidobacteria in chicken feces will not rise far above 8.00 cfu's at log_{10} and indeed, the level approached 8.00 cfu's in most treatment groups. This beneficial bacteria in the gut generally supports good gut health by competitive exclusion of harmful bacteria from the gut of chickens (Ohimain and Ofungo, 2012).

Oocyst counts: The oocyst counts are shown in Table 3. The egg count was low and consistent in all treatments except 2 which was significantly ($P \leq 0.05$) higher than the control and all others. It was also observed that the lowest numerical IgA values (data not shown) and lymphocyte percent was associated with treatment 2. The reason why this treatment has the highest and lowest values for oocyst count and IgA level is not known at this time and requires further investigations. In a recent experiment conducted by Willis *et al.* (2013), there was a very low level of natural oocyst shed from broilers that were fed different types and levels of mushrooms which is in agreement with the findings in this paper.

Table 4: Oocyst count, immunoglobulin (IgG) and percent mortality

Trts	Oocyst counts, eggs/g	IgG, ng/ml $1og10^6$	Mortality y (%)
1	1050±1050^{a}	3.5349±0.636434^{a}	0
2	20733±9057^{b}	4.5860±0.17626^{ab}	0
3	5233±3491^{a}	4.63216±0.19592^{ab}	3.33
4	783±613^{a}	5.0325±0.86969^{abc}	0
5	933±148^{a}	6.3111±0.31962^{cd}	0
6	2633±2533^{a}	7.0464±0.37223^{d}	0
7	4250±2157^{a}	4.7126±0.16348^{ab}	0
8	5633±4613^{a}	5.1509±0.33213^{bc}	6.67
9	4817±4058^{a}	5.9705±0.56389$_{bcd}$	3.33

Each value is expressed as mean±standard error (n = 3) Different letters in each column indicate significant differences at P<0.05 according to the Duncan Statistics

Blood differential percentages: Blood differential percents are shown in Table 4. There were significant differences in all components of blood evaluation. The most notably was seen with the heterophiles of treatment 6, 7, 8 and 9 which were significantly higher than the control. Differences were also observed in the monocytes. Recently, research results from Willis *et al.* (2010) observed no dose response influence by feeding different levels of shiitake FMG on heterophils and lymphocytes percentages. According to published work by Campbell (2007), healthy birds are expected to have more lymphocytes than heterophils in circulation which would influence the H:L ratio. However, Maxwell and Robertson (1995) noted that the numbers of heterophils increase during stressful conditions and because of that, the H:L ratio can be used to detect the presence of physiological stress. Although, no experimental challenge was involved here, birds appeared to be healthy based on performance traits. So these results are inconclusive, because the variations in hematological values could be dependent on many factors that influence the physiological state of the broiler chickens.

Mortality and serum IgA and IgG levels: The percent mortality for each treatment is shown in Table 3. There was no significant mortality related to treatments. The serum IgA levels were not significantly (P<0.05) influenced by the FMG supplementation (data not

Table 5: Blood differential percent of seven week old broilers

Trts. #	Heterophiles (%)	Monocytes (%)	Lymphocytes (%)	Basonophils (%)	Eosinophils (%)
1	32.740±1.822a	11.683±1.928a	36.707±1.465a	2.297±0.898a	16.890±0.376b
2	33.800±0.607ab	16.040±1.151abcd	38.897±2.349a	1.787±0.494a	9.470±1.859a
3	34.860±0.140ab	18.543±0.543bcd	34.440±2.125a	1.290±0.290a	10.803±1.197a
4	37.620±2.014abc	16.347±1.245abcd	36.740±2.433a	1.600±0.360a	7.997±2.087a
5	35.037±1.342ab	14.910±2.001abc	42.613±6.460a	1.260±0.306a	7.090±2.690a
6	40.160±2.939abc	12.770±1.498ab	36.780±3.841a	2.897±0.573a	8.053±1.759a
7	41.133±3.196bc	12.367±2.490ab	42.010±2.518a	1.823±0.644a	6.187±2.958a
8	44.653±4.367c	21.800±2.754d	35.083±5.960a	1.630±0.331a	8.833±1.612a
9	38.790±2.589abc	19.963±2.216cd	37.103±1.053a	1.837±0.082a	7.863±1.819a

Each value is expressed as mean ± standard error (n = 3) Different letters in each column indicate significant differences at P<0.05 according to the Duncan Statistics

shown). However, there was a trend toward higher elevation trend compared to the control group. Therefore, some health and performance enhancements were realized with the higher immunoglobulin levels. There were significant ($P \leq 0.05$) IgG concentration differences amongst treatments groups (Table 4). Treatments 5 and 6 produced higher IgG concentrations than all other treatments with the highest. The recovery of oocyst demonstrated a low challenge in all treatments; thus, the level of specific IgG in the sera of chickens may be attributed to the increase in beta-glucans at the 42 d incubation period. Lee *et al*. (2009) demonstrated that feeding chicks with a hyper immune egg yolk, containing IgY (IgG) against *E. tenella* and *E. maxima*, provided them with a significant protection against coccidiosis. Guo *et al*. (2004) saw a higher production of IgG in infected and non-infected *E. tenella* chicks fed polysaccharide extracts. The effect of FMG on immune response does not appear to be related to the inclusion levels of the FMG. Since body weights and oocyst counts did not differ greatly, further experiments are needed with experimental coccidial challenged chicks to determine an immune response to coccidiosis.

Conclusion: It is concluded that mushroom incubation time, with declining protein percent, does not impart declining body weights at the 5 and 10% inclusion levels. Additionally, no impairment of health and well-being were cited in association with level and incubation time during the preparation time of the different FMG. From this body of work and the average protein, 7.85% and beta-glucan level of 202 mg/g at d 28; this team of scientists recommend the usage of the 28 d incubated FMG of the combined mixture at the 5% inclusion level.

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