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Effect of Dietary Fungus Myceliated Grain on Broiler Performance and Enteric Colonization with *Bifidobacteria* and *Salmonella*

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Abstract: An experiment was conducted to investigate the effects of Fungus *(Lentinula edodes)* Myceliated Grain (FMG) supplementation on production performance, leukocyte levels and growth of *Bifidobacteria* and *Salmonella* populations in broiler chickens. A total of 240 broiler chickens were kept in floor pens and fed a corn and soybean meal based diet with added fungus myceliated grain at 0% (control), 1%, 5% and 10%. The four experimental feeding conditions were replicated six times with ten chicks per replicate. Parameters measured were male and female live body weight, carcass yield, bursa and spleen weights, *Salmonella sp*. and *Bifidobacteria* fecal populations and blood differential cell counts. The results from this study revealed no significant differences between treatments in male or female body weight, carcass yield, spleen or bursa weight. Significantly ($p \leq 0.05$) higher *Bifidobacteria* populations were observed in the 5 and 10% (FMG) treatments. In contrast, a lower *Salmonella* fecal population (log value) was observed in the 10% (FMG) treatment. The heterophil percent was significantly ($p \leq 0.05$) higher utilizing the 1% (FMG) supplements with lower lymphocyte percentages compared with other treatments. The results from this study indicate that fungus myceliated grain tested at all levels of inclusion did not adversely affect production performance and at higher levels led to increased *Bifidobacteria* and reduction in *Salmonella*. It is concluded that this feedstuff having beneficial properties from the fungal species is suitable as a broiler chicken food supplement.

Key words: Broilers, fungus myceliated grain, *Bifidobacteria*, growth performance

INTRODUCTION

The administration of therapeutic and subtherapeutic antimicrobials to animals has become a serious problem worldwide. This is due primarily to the emergence and spread of multiple antibiotic-resistant zoonotic bacterial pathogens. In some countries of the world, especially Europe, antimicrobial agents and drugs that promote growth have been eliminated partially or entirely from poultry feeding programs. This action has occurred due either to poultry companies voluntarily eliminating these agents to address consumer concerns or through legislative action. As a result, there is increasing interest in evaluating non-medical alternatives in terms of their ability to improve disease resistance, and enhance overall animal health and production performance in poultry.

Because non-therapeutic use of antibiotics in animal production is being phased out, immunological intervention and nutritional immunomodulators to enhance the immune system of broiler chickens during grow out is greatly desired. One alternative approach for controlling pathogens and enhancing immunity and health in poultry is the use of medicinal mushrooms. Thus, there is a need to research and develop new methods and forms of mushroom feeding applications to poultry.

There is a long history of using natural medicinal products such as mushrooms for feed supplements as growth and health promoters in farm animals in China (Li, 1998). *Lentinula edodes* (Shiitake) has many documented medicinal properties. Mushrooms, including Shiitake, are rich sources of polysaccharide compounds called beta-glucans that are natural antibiotics found in the cell wall as well as in extracellular secretions produced by the mycelium. They are known to combat bacteria and have immune modulating properties (Benedict and Brady, 1972; Kupra *et al*., 1979). Beta-glucans dock onto receptors on the outer cell walls of macrophages and activate them, so that they can assist in the fight against infectious disease (Battle *et al*., 1998; Mueller *et al*., 2000). However, those receptors in poultry have not yet been identified.

Recently, in the Western Hemisphere, interest has heightened regarding the potential of mushrooms to enhance health in animal production. This is reflected in several recent studies which have highlighted medicinal mushrooms as a promising alternative to antibiotic

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growth promoters (Guo *et al*., 2004; Willis *et al*., 2007). Kogut (2009), for example, reported that dietary bioactive food components that interact with the immune response may offer the potential to reduce susceptibility to infectious disease. He found that chickens fed beta-glucan diets had significantly enhanced heterophil efficacy to phagocytize and kill invading *Salmonella enteritidis*. Since Shiitake mushrooms contain beta-glucan in their cell walls, the potential for influencing health attributes in chickens is possible. Information regarding the use of myceliated grain and other myceliated agricultural materials as a poultry food supplement, however, is very limited. Therefore, the applicability of this material warrants more investigation. Finding effective non-therapeutic alternatives to antibiotics is of great importance. Although the use of mushrooms as feed supplements to enhance health in animal production has been demonstrated, there is a need for research to develop methods of successfully feeding mushrooms to poultry. The purpose of this study was to investigate the use of a digestible animal feed on which a fungal species has been grown and its potential when fed to young broiler chickens to promote desirable growth performance characteristics, enhance immunity and control pathogenic infections.

MATERIALS AND METHODS

A total of 240 day-of- hatch Ross x Ross straight-run broiler chicks were obtained from a local commercial hatchery. The chicks were weighed and distributed randomly into four treatment groups, replicated six times, with 10 chicks per pen. The floor pens measuring 1.5 m x 3.6 m contained wood shavings on a concrete pad and each included two hanging tube feeders and one suspended drinker. The experimental basal diets utilized with different levels of FMG supplementation were as follows: 1) 0% (control); 2) 1%; 3) 5% and 4) 10%. The chicks were fed a starter diet for two weeks, a grower for four weeks, and then a finisher for one week along with the supplement FMG as required for each treatment condition. The nutrient concentrations met or exceeded minimum requirements according to the National Research Council (1994). The basal mash feeds (North Carolina State University Feed Mill, Raleigh, NC) were free of drugs or medication (Table 1). The chicks were initially started at 35°C; the temperature was gradually decreased by 5°C each week to 25°C by the end of week 3. During the experiment, continuous lighting was provided and at week 2, lighting intensity was lowered for the duration of the 49 d experimental period. Feed and water were provided *ad libitum*. All broilers were vaccinated at the hatchery for Infectious Bronchitis, Newcastle and Marek's disease. All animal procedures were reviewed and approved by the University's (Institutional Animal Care and Use Committee) IACUC.

Table 1: Composition of the 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 NCSU 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

Fungus myceliated grain preparation: To make myceliated grain, sterilized sorghum grain (white milo) substrate was inoculated with mycelia of Shiitake *(Lentinula edodes)* and incubated in PPB75/SEH61x38-57 (from SAC02-Microsac, Eke, Belgium) at 25°C for two weeks before use. The resulting myceliated grain was processed by air drying at about 25°C for approximately six hours, ground into powder and added into the basal ration for the experiment.

Body and spleen weights and carcass yield: Birds were weighed by gender in replicated pens at the end of the 49 d experiment. Mortality was recorded daily per pen replicate and calculated for the 49 d period by treatment condition. Carcass yield and organ weights were obtained from sample male and female broilers who were weighed, stunned, and then killed by severing the carotid artery and jugular vein. After bleed-out, the broilers were defeathered in a rotary drum picker, eviscerated and spleen and bursa removed, and weighed, followed by reweighing of the broilers to determine carcass yield percentages.

***Bifidobacteria* and *Salmonella* culture methods and differential blood counts:** 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 the end of the experimental trial and transported to the laboratory for analysis. The samples (11 g) were diluted with 99ml sterilized 0.1% peptone water and homogenized using stomacher 400 lab system 4 for 2 min and 100 µL of appropriate dilution (10^4) was plated onto modified BIM 25 agar. Plates were incubated at 37°C for at least 3 d to allow for *Bifidobacteria* cell growth. In addition, the Gram stain technique was used to facilitate microscopic examination of morphological characteristics of *Bifidobacteria*. Fructose 6-phosphate phosphoketalase activity was measured to confirm the identity of the

Bifidobacteria. The population of *Salmonella* in fecal samples was determined by blending in a stomacher 400 lab system 4 for serial dilution examination.
Samples were serially diluted (1:10) in a 0.1% peptone solution. One hundred microliters from each dilution tube was placed onto Xylose Lysine Deoxycholate Agar (XLD) and spread evenly on the agar. All plates were then incubated for 24 h at 37°C. The number of colony forming units of *Salmonella* was expressed exponentially as Log_{10} *Salmonella* per gram of feces. Confirmation of *Salmonella* was conducted using microscopic observation and biochemical screening of *Salmonella* colonies from selective agar onto TSI agar slants. For determination of differential cell counts, blood samples were collected via the broiler's jugular veins in vacuum tubes containing EDTA to prevent clotting. Blood-smeared slides were prepared, allowed to air 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 macrophages, lymphocytes, heterophils and eosinophils.

Statistical analysis: Data were statistically analyzed using the GLM procedure of SAS (SAS Institute, 2001). The means were compared by Duncan's multiple range test and Fisher's protected least-significant difference (Steel and Torrie, 1980). Statements of significance were based on P values of 0.05 unless otherwise indicated.

RESULTS AND DISCUSSION

Final body weights, carcass dress weights, bursa and spleen weight: There were no differences in the final live weight of the chickens among the four different treatments ($p>0.05$). However, there were differences in terms of gender with males being significantly heavier than the females in all the four treatments (Table 2). Among the male chickens, the heaviest weights were recorded for treatments 1 and 2; however, there was no significant difference with the other treatments. On the other hand, among the female chickens, treatment 2 was significantly different from treatment 3 with no significant differences between the other treatments. The dress weight exhibited the same pattern with the live weight, with both parameters being directly correlated. The regression analysis exhibited a linear relationship, which is highly significant, showing that the dress weight is approximately 0.77 for the males and 0.76 for the females ($p<0.0001$). The significance of live body weight not being different among treatments is noteworthy in this experiment because the administering of extracts from mushroom fruiting bodies continually via the drinking water, in a previous study, had been reported to cause significant weight reduction in broiler chickens (Willis *et al*., 2007). Since the broilers were not adversely affected by this form of supplementation, we believe that it is more practical and economical to utilize the FMG vs. the use of extracts from the Shiitake mushroom fruiting bodies. The absolute bursa weight among the four treatments was not significantly different ($p>0.05$), hence relative percentage body weight/total weight was close in the order of 0.15-0.17 (Table 2). A similar pattern was observed in the spleen weights, where there were no significant differences among the four treatments and between males and females ($p>0.05$). The percentage spleen/total weight was close in the order of 0.11-0.13. The lymphoid organ weights and organ to body weight ratios data in this experiment are more consistent than data previously obtained by (Willis *et al*., 2007). These results imply that the addition of FMG did not exert stress and adversely affect body weight of the broilers.

***Bifidobacteria* and *Salmonella* fecal populations:** Demonstrated ($p<0.05$) differences were observed between treatments with regard to *Bifidobacteria* and *Salmonella* populations (Table 3). The 5% and 10% FMG treatments had significantly higher fecal *Bifidobacteria* populations when compared with levels in the 0% and 1% FMG. *Salmonella* fecal populations were significantly lower for broilers receiving the 10% FMG, when compared to the 0, 1 and 5% FMG. Although the reduction was less than 1 log, the trend follows previous findings in studies conducted by Willis *et al*. (2007). The higher level appeared to provide some protection against the extraintestinal natural *Salmonella* infections because the birds were not experimentally infected. These current findings are supported by a previous study conducted by Willis *et al*. (2008) which found a significant reduction of *Salmonella* populations in the ceca and crop of molted hens given alfalfa plus mushroom extracts. Another published report by Willis *et al*. (2009a) obtained similar *Salmonella* reduction results utilizing FMG for molt induction in aging laying hens. Similarly, Willis *et al*. (2009b) obtained data that showed increased *Bifidobacteria* population in broilers receiving mushroom extracts and a reduction in *Salmonella* population. Taken together, these data suggest that mushroom extracts support the outgrowth of beneficial bacteria and protect against colonization of pathogenic bacteria such as *Salmonella* and, therefore, have potential to control pathogenic infections and positively affect the health of poultry.

Blood differential percentages: There were significant ($p>0.05$) differences among treatment conditions regarding the levels of heterophils, macrophages, lymphocytes and eosinophils in the blood (Table 4); however, the responses were not dose dependent. The percentages of heterophils in the blood were increased only at the 1% FMG inclusion rate. In contrast, the all-inclusion rates lead to a decrease in the percentage of macrophages as compared to the control (0%

Table 2: Effect of levels of dietary supplemented fungus myceliated grain on final body weight and lymphoid organ measurements of 7 week broiler chickens

	Live weight (kg)			Dress weight (kg)			Bursa weight (g)			BLW %	Spleen weight (g)			SLW %
Trts	Total (n=12)	Male (n=6)	Female (n=6)	Total (n=12)	Male (n=6)	Female (n=6)	Total (n=12)	Male (n=6)	Female (n=6)	Total (n=12)	Total (n=12)	Male (n=6)	Female (n=6)	Total (n=12)
1	2.93± 0.11a	3.26± 0.07e	2.59± 0.07ab	2.26± 0.08a	2.50± 0.07e	2.02± 0.06ab	4.42± 0.38a	4.12± 0.40ab	4.67± 0.67ab	0.15	3.33± 0.26a	3.67± 0.42a	3.00± 0.26a	0.11
2	2.90± 0.0a	3.11± 0.07de	2.69± 0.07bc	2.21± 0.06a	2.35± 0.05de	2.08± 0.06bc	4.83± 0.41a	5.50± 0.56b	4.167± 0.48ab	0.17	3.67± 0.19a	3.83± 0.31a	3.50± 0.22a	0.13
3	2.67± 0.11a	2.98± 0.10d	2.36± 0.08a	2.05± 0.09a	2.28± 0.09cde	1.81± 0.08a	3.92± 0.23a	3.67± 0.33a	4.17± 0.31ab	0.15	3.58± 0.19a	3.67± 0.33a	3.50± 0.22a	0.13
4	2.72± 0.10a	2.90± 0.09cd	2.54± 0.14ab	2.09± 0.08a	2.18± 0.11bcd	1.99± 0.11ab	4.42± 0.40a	5.50± 0.43b	3.33± 0.21a	0.16	3.35± 0.33a	3.50± 0.56a	3.00± 0.366a	0.12

*Mean values down the column block having the same alphabets are not significantly different at p<0.05, according to the Duncan multiple range tests. BLW = Bursa/live weight %; SLW = Spleen/live weight %

Table 3: Mean log values for *Salmonella* and *bifidobacteria* fecal population of broiler chickens subjected to fungus myceliated grain on day 49

Treatments	*Salmonella*	*Bifidobacteria*
Control (0% FMG)	6.47^{a}±0.0[1]	7.44^{b}±0.13
1) Fungus myceliated grain (1% FMG)	6.47^{a}±0.0	7.65^{b}±0.09
2) Fungus myceliated grain (5% FMG)	6.47^{a}±0.0	8.03^{a}±0.03
3) Fungus myceliated grain (10% FMG)	5.77^{b}±0.09	8.00^{a}±0.07

^{abc}Mean values within the same column with no common superscripts differ significantly ($p \leq 0.05$).
[1]Means±SE. [2]FMG-fungal myceliated grain

Table 4: Blood differential percentages of broiler chickens subjected to fungus myceliated grain

	Percentage			
Treatments	Macrophages	Lymphocytes	Eosinophils	Heterophils
Control fungus myceliated grain (0%)	25.57^{a}±0.869[1]	41.67^{a}±5.48	2.0^{b}±1.53	30.63^{b}±4.94
Fungus myceliated grain (1%)	18.15^{b}±1.87	36.23^{b}±8.17	2.43^{b}±0.617	43.20^{a}±6.90
Fungus myceliated grain (5%)	18.98^{ab}±1.68	44.55^{a}±3.97	4.68^{a}±1.66	31.75^{b}±5.26
Fungus myceliated grain (10%)	19.48^{ab}±3.08	43.53^{a}±5.30	2.65^{b}±0.46	34.38^{b}±3.87

^{abc}Mean values within the same column with no common superscripts differ significantly ($p \leq 0.05$). [1]Means±SE

inclusion), with a significant decrease being observed at the 1% inclusion level, most likely due to the significant rise in the percentage of heterophils at that same rate of inclusion. Lymphocytes were also affected at the 1% inclusion rate. Heterophils have been shown to play a major role in the defense against *Salmonella*. Research by Kogut (2009) demonstrated heterophil-specific activation with corresponding protection against extraintestinal *Salmonella* infections in young chickens by adding highly purified beta-glucans. There are also reports that heterophils control both initial *Salmonella* infections and subsequent disease pathogenesis (Kogut *et al*., 1994). Because we observed a decrease in *Salmonella* and as mushrooms contain beta-glucans, we sought to analyze the effect of the treatments on the levels of heterophils and other leukocytes within the blood. Based on these data we cannot reasonably conclude that an increase in heterophils after FMG supplementation produces effective antimicrobial responses and is involved in the reduction of *Salmonella*. However, because there is limited published research with FMG and chickens and because the data suggest that FMG may affect leukocyte levels particularly that of heterophils, we therefore intend to further investigate the effect of FMG on leukocyte populations as well as other immune parameters.

Conclusion: we have demonstrated the potential value of FMG as a supplement in broiler rations for production performance, *Bifidobacteria* enhancement, and *Salmonella* reduction. In doing so, we have contributed to the small but growing body of information on the use of (fungal species) mushrooms and other digestible or indigestible materials to promote the health and well-being of animals raised without antibiotics.

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