Effect of dietary supplementation on improvement of growth and immune function of broilers

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Abstract: The purpose of this investigation was to evaluate the effect of a dietary supplement (Mirigen™), a fungal cell wall derivative product, as a new generation alternative to antibiotics, on the growth and the innate and adaptive immune functions in broilers from birth to 45 d of age. Newborn chicken were randomly assigned to one of three groups: G1 (n=150) controls no supplement fed; G2 (n=150) is fed with dietary supplement at a designed regular dose (0.5 %, weight of additive to food); G3 (n=150) is fed with dietary supplement at double doses (1 %). All three groups were housed in the same conditions. Body weight and blood were taken on day 1, 14, 28 and 45. Medications used and costs/treatment were recorded for each group. The whole blood was used to purify heterophils for reactive oxygen species (ROS) generation and E coli killing abilities examination assays, and the serum samples were preserved in freezer for enzyme linked immunosorbent assays (ELISAs) to determine concentration of macrophage inflammatory protein-1β (MIP-1β), CD4/CD8, interferon-γ (IFN-γ), and titers of antibody against Newcastle disease virus (NDV). Group differences were analyzed using analysis of variance (ANOVA) algorithm (S-Plus). There was no significant birth weight difference in three groups. After 45 d growth, the dietary supplement treated groups had significantly higher body weight gain (BWG) with lower mortality rate if compared to the untreated control group (P<0.05). Their BWG and mortality rate were 2.23 kg and 10 % in G1 (control group), 2.89 kg and 2 % in G2 (experimental group, 0.5 % dose), and 2.77 kg and 1 % in G3 (experimental group, 1 % dose), respectively. Heterophil ROS generation in treated groups were markedly improved through the addition of dietary supplement in both regular and double doses to the diet (P<0.05). The ability of heterophil to kill E coli was also significantly improved in dietary supplement treated groups (P<0.01). Comparing to control group, there was significantly higher serum IFN-γ concentration in treated groups (P<0.05) on day 45. The CD4/CD8 was also improved in treated groups (P<0.05). Newcastle di-sease is the most prevalent avian disease, and vaccination is an effective method to protect the animals from the virus infection. In our study, it is found G2 and G3 that fed with dietary supplement had higher antibody titers against NDV after vaccination (P<0.01) and the antibodies lasted longer. Results from this study demonstrated dietary supplement to broilers improved the immune capabilities of immune cells, which are vital to the establishment of immune response against pathogens, thereby, to improve chicken’s health and growth and reduce medication cost in chicken farming.

Key words: broilers; growth; health; immunity; dietary supplement

1 Introduction

Antimicrobials are medicines vital for the treatment of animal infections, but their effectiveness is threatened by over and inappropriate use that contributes to the growing resistance of bacteria. Mounting concern regarding antibiotics used in food animals, the natural products, such as yeast and fungal derivate products, can give the promising ways to solve the problem. In this study, we used dietary supplement Mirigen™ as alternatives to some antibiotics for the purpose to limit their consumption in the farm.
Mirigen™ is formulated to include active dried *Saccharomyces cerevisiae* fermentation product, niacin, vitamin B12, diatomaceous earth, calcium carbonate and potassium sorbate. One of its ingredients, yeast cell wall (YCW) component, has been used in animal feeding since the last decades [1]. Their inclusion in broiler diets has significantly improved animal productivity, which was attributed to the physiological effects on intestinal digestive mucosa [2,3]. In the digestive tract of animals, mannann-oligosaccharides (MOS) present in YCW could act as high-affinity ligands, with the potential benefit of offering a competitive binding site for pathogenic bacteria mannose-specific type-1 fimbriae. The MOS are indigestible to non-ruminant animals and can provide competitive binding sites for pathogenic digestive bacteria, decreasing their intestinal attachment and colonization [4,5]. The β-1, 3/1, 6-glucan also presents in YCW and is recognized as an immune modulator substance in animals and humans [6,7].

The mode of action (MOA) of Mirigen™ in chicken has been studied, and the hypothesis is that it can stimulate heterophils and other immune cells’ function in the broilers to kill pathogens. The YCW components might stimulate the gut-associated immune system by acting as a nonpathogenic microbial antigen, giving an adjuvant-like effect [8].

Heterophils in broilers are effective in eliminating most bacterial species via phagocytosis and intracellular killing [9]. Once heterophils reach the site of infection, their primary function is to phagocytize and kill bacteria. During phagocytosis, various processes take place to kill bacteria, such as the respiratory burst process in which the free radicals are released into infected tissue and the degranulation process in which various enzymes are released from neutrophils to degrade bacteria [10]. The respiratory burst and degranulation processes produce reactive oxygen species (ROS) that are instrumental in preventing bacterial growth and colonization [11].

Interleukins (ILs) are especially important to heterophil function, and include IL-1, IL-2 and IL-8 [2], IL-8, produced by leukocytes and epithelial cells, mediates neutrophil migration and activation, and also induces inflammation, which is vital to the activation of immune processes to fight against pathogens. Cytokine production during the initial stages of infections leads to an influx of heterophils into the sties, and includes ILs, colony-stimulating factors, interferon (IFN), and tumor necrosis factor (TNF). IFN-γ, or type II IFN, is a cytokine which is critical for innate and adaptive immunity against viral and intracellular bacterial infections and for tumor control. IFN-γ is an important activator of macrophages. The importance of IFN-γ in the immune system stems in part from its ability to inhibit viral replication directly and most importantly from its immunostimulatory and immunomodulatory effects. Activated macrophages are also important as Ag-presenting cells for T-cell interactions initiation and the development and proliferation in CD4/CD8, and play an important role in increasing titration of antibodies, such as antibody titers against Newcastle disease virus (NDV). Higher antibody titration after routine vaccination indicates a better protection in animals. Macrophage inflammatory protein (MIP) in microphages is crucial for immune responses towards infection and inflammation. They activate different types of granulocyte such as heterophils, eosinophils and basophils. There are two major forms; MIP-1α and MIP-1β. Both are produced by macrophages after they are stimulated with bacterial endotoxins [12]. And MIP-1β is a chemoattractant for natural killer cells, monocytes and a variety of other immune cells [13].

There are two main types of T-cells. T4 cells, also called CD4⁺ (CD4) cells, are “helper” cells. They lead the attack against infections. T8 cells [CD8⁺ (CD8) cells] are “suppressor” cells which end the immune response. CD8⁺ cells can also be “killer” cells which kill cancer cells and cells infected by virus. The ratio of CD4 cells to CD8 cells is often an indicator of immune response status. In chickens, the CD4/CD8 has been shown to be positively related to the amount of antibodies produced in response stimuli. Low CD4/CD8 has been associated with lower antibody responses. The enhanced CD4/CD8 in the blood was associated with increases in the ability of lymphocytes to respond to T-cell mitogen and in the antibody response to a T-independent antigen [14].

Pathogens are coated with complement factors (C3b and C3bi) or antibody, which is called opsonization, and they can as such be recognized by activated heterophils and other cells, and then be phagocytosed by these cells. Commercially available feed supplements containing antioxidants and various vitamins have been formulated and found to be effective in stimulating the immune system when challenged with stressors, for example, shipping stress or heat stress [15].

Immunosuppression is common in avian species...
and accounted for the high incidence of disease. High-producing avian species have been selected to manufacture high levels of meat or egg and, as a result, are always close to metabolic disease. Hence, it is believed that high-producing poultry are “stressed”. A stressed and immunocompromised animal is susceptible to pathogenic infection. Thus, improving immune function through feed supplementation with immunostimulants may aid in preventing infections and decreasing treatment and culling costs due to diseases. The purpose of this study was to evaluate the effect of a dietary immunostimulant on blood phagocyte activity and immune marker expression in broilers vaccinated with NDV vaccine as a measure of enhanced innate and adaptive immunity.

2 Materials and methods

2.1 Animal housing

The experiments were conducted using 450 commercial 1-day-old Ross broiler chicks, and these chicks were randomly assigned to either a control group (G1) or two treatment groups (G2 and G3) (150 chicks per group). The G1 was fed on a commercial starter, grower and finisher feeds formulated by the grower’s nutritionist. The G2 and G3 were fed on the same starter, grower and finisher, with the addition of 0.5% or 1% dietary supplement admixed into the feeds, respectively. The experimental house was provided with forced ventilation and lamb heating. The temperature inside the house on arrival was 26–28 °C and was increased by 1 °C each week until 33–35 °C. The lighting program was 23 h of light for the first 4 d, 20 h until 10 d, and 18 h afterwards. Feed and water were provided for ad libitum consumption throughout the 45 d of experimentation.

2.2 Data collect

Grower collected data on daily morbidity and mortality throughout the duration of the trial. And body weight gain (BWG) was recorded by 20 chicks each group on day 1, 14, 28 and 45.

2.3 Blood collection

In the experiment, prior to beginning the treatment diet, 8 chicks per group were sacrificed for blood sample. And on day 14, 28 and 45, blood samples of 8 chicks per group were collected using a 4.5 mL BD vacutainer® (Becton, Dickenson and Company, Franklin Lakes, NJ) blood tube containing buffered sodium citrate. After collection, blood samples were placed in a cooler containing ice packs, and samples were used for heterophil purification, and were analyzed for phagocytic activity and ROS production by blood heterophils as described below.

2.4 Heterophils purification and determination of phagocytic activity and ROS production

Heterophils were isolated from fresh blood samples to determine phagocytic activity and ROS production. For isolation of heterophils, a 6 mL aliquot of buffy coat from the blood samples was layered over 10 mL lymphocyte separation medium (LSM) and centrifuged. The phosphate-buffered saline (PBS) layer was removed and the monocyte layer was collected at the top of the tube using a 5 mL pipette. All LSM was removed and PBS was added to suspend pellet. After centrifugation, PBS was removed and red blood cells were lysed by adding 10 mL sterile water, vortexing, and adding 10 mL double strength PBS followed by 50 mL PBS. After the red blood cell membrane fraction was removed by centrifugation, heterophils were washed by centrifugation and suspended in 10 mL PBS. A viable cell count was performed using trypan blue.

To determine phagocytic activity, heterophils were incubated with E. coli, and after 2 h incubation, bacteria were quantified by reading the absorbance of dye solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)] at 570 nm according to techniques described, and ROS production was measured according to techniques described by Wiggins et al. Stimulants of ROS production included phorbol myristate acetate (PMA; Sigma-Aldrich, St. Louis, MO) at 10⁻⁷ M. Differences in mean ROS production measured by arbitrary fluorescence units (AFU) among heterophils were compared between treated and control broilers using two-sample t-tests.

2.5 Chicken CD4/CD8, IFN-γ, IL-6 enzyme linked immunosorbertent assay (ELISA)

Chicken CD4/CD8, IFN-γ, IL-6 ELISA and NDV titration kits (Cusarbio Biotech Co., Shanghai, China) were used to determine their concentrations in serum samples according to manufacturer’s recommendations.

2.6 Statistical analyses

Differences between treatments were assessed using analysis of variance (ANOVA) with S-Plus (Lucent Technologies Inc., Murray Hill, NJ). Where differences among treatment means were detected, and a Fisher multiple range test was used to examine differences among individual treatment means. A two-sided alternative hypothesis was assumed; \( P < 0.05 \) was considered statistically significant.
3 Results and discussion

3.1 Flock performance

Statistical analysis of data indicated that the average weight of control chicks was 2.23 kg (G1) on 45 d of age, and the average weights of dietary supplement fed chicks were 2.89 kg (G2) and 2.77 kg (G3) on 45 d of age (P<0.05), respectively, using house and age as covariates. Survival rates of control and treatment fed groups was significantly different (P=0.017) with 90% (G1), 98% (G2) and 99% (G3), respectively (Fig.1 and Fig.2).

3.2 Blood parameters

Heterophil function was evaluated using serum samples from broilers, including MIP, CD4/CD8, IFN-γ and NDV titers. The results that there is significantly higher MIP-1β expression level in treatment groups than in control (Fig.3, P<0.05), represent there is better innate immune response in the feed additive groups in the molecular level. Fig.4 showed there is significantly higher IFN-γ in treatment group (P<0.05) than in control. For CD4/CD8 (Fig.5), there is significantly higher CD4/CD8 in treatment group (P<0.05) than in control.

The Newcastle disease is the most prevalent avian disease, and NDV vaccination is an effective method to protect broilers. Fig.6 shows that supple-
ment resulted in a numerical increase in titer of NDV antibody on day 28 and 45, and there were significantly higher titration and longer lasting NDV antibodies in G2 and G3 than in G1 (P<0.01). The NDV vaccine was given on day 7, 14 and 27.

![Graph showing NDV antibody titer](image)

**Fig. 6** Effects of feeding 0.5% and 1% Mirigen™ on development of NDV titer

The enhancements in MIP-1β, CD4/CD8 and other markers’ expressions reflect that the Mirigen™ supplemented diet was provided in sufficient amounts to result in immune response in animals. MIP is believed to be instrumental in the host response to bacterial invasion. MIP, expressed by microphages on their cell surfaces, is instrumental in killing of the invading bacteria. While the mechanism by which the feed supplement enhances MIP expression in broilers is unknown yet, it is hypothesized that yeast and fungal cell wall components in the feed supplement contain molecules that interact with the innate immune system to prime leukocyte antimicrobial processes, specifically heterophils [10]. Alternatively, the interaction of supplement components (yeast and fungal cell walls) with immunoreactive lymphoid tissues in the gut may initiate the innate immune response leading to a cascade of events associated with supplement as well as increased proinflammatory cytokine. Significant increases in MIP, IFN-γ and CD4/CD8 enhanced by nutritional stimulation signify a direct relationship between nutrition and innate immune response.

### 3.3 Phagocytic activity and ROS production

Twenty-four broilers (16 from G2 and G3, 8 from G1) were used in the analysis of phagocytic activity and ROS production in blood heterophils to determine treatment differences, as shown in Fig.7. It demonstrated that there are significantly higher *E. coli* killing ability in phagocytosis and level of ROS expression in G2 and G3 (P<0.05) than G1, which showed heterophils from treatment groups have stronger killing ability than that from control group.

![Graph showing heterophil phagocytosis](image)

**Fig. 7** Effects of feeding 0.5% and 1% Mirigen™ on heterophil phagocytosis

The results of the present trial show that blood neutrophils from animals receiving the feed supplement may be primed and more readily available to begin producing ROS for bacterial killing. On the 45th day, ROS production was numerically higher in dietary supplement groups than in control group (Fig.8).

![Graph showing ROS concentration](image)

**Fig. 8** Effects of feeding 0.5% and 1% of Mirigen™ on ROS concentration

### 3.4 Antibiotics and broilers’ health

Health practices in animal husbandry reduce the need for antibiotics, and antibiotics should never be used as substitutes for inadequate hygiene. A decrease in use of antibiotics as growth promoters does not need to entail reduced productivity and economic losses to the food producers or increased prices for consumer [19, 20]. Research on alternative methods to improve animal growth and feed efficiency was on the way [21].
There is no significantly different mortality and BWG between those two treatment groups. However, we noticed that the BWG is higher in the lower dose (2.89 kg) than the higher dose (2.77 kg), indicating that the high dose of supplement might have caused more energy consumption for immune response, and this innate immune response diverts energy from growth and performance. Considering the historical data that high dose additive could prevent more death events in flock, it might be a necessary approach to increase the dose to prevent pathogens infection if animals are fed in compromising environment or conditions.

3.5 Mechanism of action

A stronger immune response invariably leads to a healthier animal, resulting in less antibiotic use, only novel alternatives will pave the new ways to enhance an animal’s immune response. This investigation sheds light on the effects of dietary supplementation on immunity, and demonstrates the resolve of animal scientists to appeal to the public and offers an alternative management tool to improve animal health without increasing antibiotic use and jeopardizing both consumer well-being and public perception of the agricultural industry. Mechanisms by which the additive may reduce bacterial and viral infections in animals were studied in this and in other studies.

In this study, the results demonstrated that chicken’s CD4/CD8, the concentrations of IFN-γ and IL-6, and antibody titers against NDV increased after we fed them dietary supplement. We believe that supplement not only can block the colonization of intestinal pathogens, such as Salmonella spp. and E coli, which contain type I fimbriae with mannose-binding lectins. The supplement has also been linked with improved gut health, and stimulated intestinal mucosal immunity, by acting as a non-pathogenic microbial antigen. Hence, the expression of multiple genes involved in immune processes was changed by supplement. Enhancement of chicken natural defense mechanisms, particularly during times of stress, may prevent new cases of infectious diseases thereby decreasing or possibly eliminating the need for antimicrobials. Broilers would appear to be less immunocompetent in treatment groups than in control group. The enhanced CD4/CD8 in blood was associated with increases in the ability of lymphocytes to respond to T-cell mitogen and in the antibody response to a T-independent antigen.

4 Conclusions

This trial demonstrated a positive advantage to bird performance in both average daily weight gain and average live weight due to the addition of MirigenTM at 0.5 % and 1 % of the diet. Heterophil killing ability and apparent immune function were markedly improved through the addition of daily supplement to the diet.

The exact mechanism by which the feed supplement may induce more active leukocyte antimicrobial activity is largely unknown. However, results demonstrated that blood phagocytes from treated animals may be more capable of traveling to a site of inflammation, binding to and phagocytizing bacteria for intracellular killing than untreated animals. Significantly increased CD4/CD8, MIP and IFN-γ suggested that the feed supplement may simulate the immune system in an effort to protect against future bacterial challenges. Production of ROS and phagocytes from treated animals did display an overall trend towards increased surface-binding of bacteria, phagocytic activity and ROS production.

Antigens in infected tissues may be phagocytosed by macrophages and dendritic cells and presented on the surfaces of antigen-presenting cells in association with major histocompatibility complex (MHC). Enhanced expression of MHC in blood of Mirigen™-fed animals implied that the additive increased either the total numbers of antigen-presenting cells in blood or increased the abilities of individual antigen-presenting cells to present antigens on their surfaces.

References

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