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# Immunopotentiating effect of *Inonotus obliquus* fermentation products administered at vaccination in chickens

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ABSTRACT

Vaccination is an important approach for the control of avian viral diseases. The aim of this study was to evaluate the immune-potentiating effect of oral administration of *Inonotus obliquus* fermentation products (IOFP) at vaccination in chickens. In total, 120 one-day-old specific-pathogen-free chickens were randomly assigned to six groups: groups 1 to 3 were vaccinated with Newcastle disease virus (NDV) LaSota live vaccine via intranasal and eye-dropped route at seven days of age, and boosted two weeks later. Before each immunization, chickens in groups 1 and 2 were orally administered 0.8% IOFP and 0.2% astragalus polysaccharide (APS) in their diets, respectively, for seven consecutive days and group 3 was fed with commercial diet. At the same time, group 4, 5 and 6 were inoculated in the same manner with PBS and fed with commercial diet, containing 0.8% IOFP and 0.2% APS diet, respectively, as negative controls. At 0, 7, 14, 21, 28, and 35 days post-inoculation (dpi) firstly, the temporal changes in serum Newcastle disease hemagglutination inhibition (HI) and neutralizing antibody titers were determined. Meanwhile, proliferations of peripheral blood mononuclear cells (PBMCs) isolated from each group in response to concanavalin A stimulation and the expression levels of Th1-type (IFN-γ) and Th2-type (IL-4) cytokines were determined by 3-(4,5-Dimethylthiazol-2-yl) −2,5-diphenyltetrazolium bromide, and ELISA methods. On days 0, 14 and 28 after the first vaccination, the percentages of $CD3^+$, $CD3^+CD8^+$, and $CD3^+CD4^+$ T lymphocytes were detected by flow cytometry. At 35 dpi, a challenge test was carried out and protective efficacy was determined. Results showed that oral administration of IOFP could significantly enhance ND HI and neutralizing antibody titers, proliferation of PBMCs, proportions of $CD3^+$, $CD3^+CD8^+$, and $CD3^+CD4^+$ T lymphocytes, as well as the ratio of Th1/Th2, and all of these values were superior to those seen with APS as a positive control, and other groups. Therefore, IOFP possesses significant immune-potentiating properties in chickens and may be a more economical and convenient oral adjuvant to improve vaccination in avian species.

## 1. Introduction

With the development of intensive culture, the breeding of poultry of superior genetic stock with short production cycle and high production has expanded both in number and scale. But due to relatively few breeds and high production pressure, chickens become more susceptible to many diseases, especially viral infectious disease. Avian influenza, chicken infectious bronchitis, avian leucosis [1–3] are always prevalent, and fowl Adenovirus, Newcastle disease virus and many other diseases emerge as variants of existing viruses or the development of new syndromes [4,5]. As a result, the emergence of such diseases seriously threatens the poultry production economy. Chickens, once infected, are increasingly susceptible to secondary infections and suboptimal response to vaccinations. Moreover, since 2013, several outbreaks of the H7N9 avian influenza epidemic, in which poultry are the intermediate hosts transmitting the virus to other birds and mammalian hosts, have jeopardized human health [6,7]. Therefore, effective control of avian viral infectious diseases not only could decrease economic losses, but also diminishes the interspecies transmission of avian zoonosis, which safeguards human health.

Vaccine is one of the most successful tools for the control of viral diseases. It is believed that the simultaneous application of a vaccine

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with an immunopotentiator could improve the efficacy of vaccination. However, some adjuvants commonly used in animal vaccines, such as oil emulsions and aluminum, ordinarily result in side effects or fail to increase the immunogenicity of weak antigens [8]. Therefore, development of novel immunopotentiators with high efficiency, low toxicity, and extensive availability is an urgent priority. Many Chinese herbal medicines and medicinal fungi possess various pharmacological activities, such as antitumor, antioxidant, antivirus, immune modulation, with no toxicity and side effects. Such adjuvants are considered a potent alternative to conventional immunopotentiators for a variety of vaccines [8–13].

*Inonotus obliquus* (Pers.:Fr.) Pil. (*Fuscoporia obliqua* (Pers.:Fr.) Aoshima) is a white rot fungus belonging to the family Hymenochaetaceae; Basidiomycetes. Since the sixteenth century, *I. obliquus* has been used to treat gastric cancer, intestinal cancer, heart disease and diabetes as a folk remedy in many countries [14–16]. Sporophores of *I. obliquus* are isolated from silver birch at high market cost resulting from its rarity and a 10–15 year requirement to maturity. Thus, related research is limited to human and mouse studies only, with no reports in avian species. However, with the development of fermentation technology, mycelium and other active compounds of *I. obliquus* could be commercially produced by submerged mycelium culture [16,17]. Owing to the productivity of submerged mycelium culture, the breadth and depth of research with *I. obliquus* could be expanded.

In last decades, many components of *I. obliquus* were successfully extracted, and then the characterization and biological activity were studied [16,18–22]. Polysaccharides, as one of the active ingredients of *I. obliquus*, are proved possessing immunostimulating, immunomodulatory, antiviral and antitumor activity in human and mice [16,20,23,24], but the mechanism are not fully understood, especially at immunopotentiation aspect [16,19]; apart from human and mice, it is also unclear whether *I. obliquus* or it's extracts could enhance growth and immune parameters of avian. In our previous study, *I. obliquus* fermentation products (**IOFP**) was administrated in diet of chicken, and the results demonstrated that growth performance, intestinal morphology, immune organ index and partial non-specific immune response were significantly enhanced [25,26], showing that IOFP was an excellent feed additive for chickens. However, there is little research on the effect of IOFP in promoting specific immune response in avian up to now. In the present study, we investigated the immune-potentiating effects of oral administration of IOFP at vaccination on humoral and cellular immune responses and protection from virulent NDV in chickens. APS was used as positive control.

## 2. Materials and methods

### 2.1. Birds

Ten-day-old specific-pathogen-free embryonated chicken eggs and healthy one-day-old specific-pathogen-free chickens (Female, leghorn) were obtained from Shandong Healthtec Laboratory Animal Breeding Co., Ltd (Shandong, China). The birds were reared in separate units and fed with different diets (details shown in experimental design).

### 2.2. Reagents

*I. obliquus* strain YU was isolated from sterile conks on *Betula platyphylla* collected from Heilongjiang province of China and conserved by the Shandong Key Laboratory of Disease Control and Breeding. The strain was maintained on YMA (no. A507034, Sangon Biotech, Shanghai, China) slants and sub-cultured every month. After the slants were incubated at 27 °C for 7 days, it was stored at 4 °C for stock. APS was produced by Beijing CENTRE Technology Co., Ltd (Beijing, China), and recommended intake was 0.2% (w/w). Roswell Park Memorial Institute-1640 medium (no. 11875093, Gibco, Grand Island, Nebraska, USA) was supplemented with 100 IU/mL of benzylpenicillin, 100 IU/mL of streptomycin, and 10% fetal bovine serum (no. 04-001-1A-US, Biological Industries, Cromwell, Oklahoma, USA) and was used for cell culture in vitro, washing and resuspending the cells, and mitogen dilution. Concanavalin A (**Con A**; no.C2272, Sigma, Saint Louis Missouri, USA) was dissolved to a concentration of 0.022 mg/mL 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (no. 0793-1G, Amresco, purchased from BIOSCIENCE Biotechnology Co. Ltd., Shanghai, China) was dissolved with calcium- and magnesium-free PBS (pH 7.4) to 10 mg/mL. Mouse anti-chicken CD3-FITC (no. GTX43629, GeneTex, San Antonio, Texas, USA), CD4-PE (no. ab25420, Abcam, Cambridge, UK) and CD8a-Cy5 (no. ab81990, Abcam, Cambridge, UK) were purchased from Hetuo biotechnology Co. Ltd. Lymphocyte separation medium and Ficoll-Hypaque were purchased from TBD Science (Tianjin, China). Chicken IL-4 (no. C972458) and interferon gamma (**IFN-**γ; no. C939647) ELISA kit were products of Break Innovation, purchased from Jinan jianbang biotechnology Co. Ltd. (Jinan, China).

### 2.3. Cultivation methods of IOFP

A modified YM broth used for both a seed and fermentation medium was prepared as described [16]. After sterilization, 0.5 mL of mineral solution was added to 1 L of the liquid medium. The mineral solution consisted of 13.20 g of $CaCl_2{\cdot}2H_2O$, 8.40 g of $FeSO_4{\cdot}7H_2O$, 2.40 g of $MnSO_4{\cdot}4H_2O$, 2.40 g of $ZnSO_4{\cdot}7H_2O$, 0.48 g of $CuSO_4{\cdot}5H_2O$, 0.48 g of $CoCl_2{\cdot}6H_2O$, 0.24 g of $Na_2MoO_4{\cdot}2H_2O$, and 0.06 g of $K_2B_4O_7{\cdot}H_2O$ per liter of 1 N HCl.

For flask cultures, the strain was grown on YMA medium in a petri dish for 10 days. Then 10 mL homogenized cell suspension was inoculated into 500 mL baffled flasks containing 200 mL of YM medium followed by cultivation for 5 days on a shaking incubator at 27 °C and 180 rpm.

For 100 L jar (no. 100SJ-S, Bailun Bio, Shanghai, China) cultures, seed cultivations were firstly carried out in 500 mL baffled flasks for 4 days on a shaking incubator at 27 °C and 180 rpm. When the cultures reached the mid log phase, they were stopped and mixed together. Then, 700 mL mixed cultivations were added to 10 L fermenter (no. 10SJ, Bailun Bio, Shanghai, China) containing 6.3 L of YM medium. The pH was initially set at 6.0 with 1 N HCl or $NH_4OH$, then not controlled thereafter. The dissolved oxygen tension (DOT) was initially set at 100% saturation and was maintained at 20% or higher during cultivation by controlling the air flow rate (0.1–0.5 vvm). After 6 days, 7 L of culture broth at mid log phase was added as an inoculum to a 100 L jar (stirred type, Kobio Tech., Seoul, Korea) containing 63 L of the modified YM medium. Cultivations were then performed for 10 days. The temperature, pH, and DOT levels in the jar were controlled in the same way as for the 10 L fermenter. The internal pressure was maintained in the range of 0.1–0.25 $kg/cm^3$. The fermentation end-products, biomass (dry weight) and crude polysaccharides, were measured as described [16,27], which were 20 g/L and 1.2 g/L, respectively.

### 2.4. Vaccine and virus

Newcastle disease virus (**NDV**, Lasota strain) live vaccine (no.1602006–1) was provided by Qilu Animal Health Products Co. Ltd (Jinan, China). NDV strain LaSota and standard virulent strain F48E8 were purchased from China Institute of Veterinary Drug Control and conserved in our lab.

### 2.5. Experimental design

A total of 120 one-day-old birds were randomly divided into six groups of 20 chickens each. All chickens were vaccinated through intranasal and eye-dropped route at 7-days-of-age (0 day after post-inoculation (**dpi**)) and booster immunization was performed interval 14 days (14 dpi). Group 1 (IOFP + Vaccine) was orally administered IOFP