



## Research Article

### $\beta$ -Glucan from Edible Mushroom (*Pleurotus florida*) Enhances Mucosal Immunity in Poultry

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#### ARTICLE HISTORY

Received: 2013-08-06  
Revised: 2013-08-17  
Accepted: 2013-08-18

**Key Words:** Broiler,  $\beta$ -glucan; intestinal intraepithelial leucocytes; mucosal immunity; mushroom

#### ABSTRACT

The study was undertaken to assess the effect of orally administered  $\beta$ -glucan on intestinal intra epithelial leukocytes (iIEL) of broiler to establish its role in mucosal immunity. After acclimatization for 7 days with control diet, two groups of broiler birds each containing 20 chicks, with replica of 10 chicks in each pen, were fed with experimental feed containing  $\beta$ -glucan @15mg/kg (group A) and 30 mg / kg (group B) for a period of 20 days and, then given normal diet. Birds kept with normal feeding regime (n=10) were considered as control (group C). Intestinal intraepithelial leucocytes (iIEL) of the experimental birds were isolated and *in-vitro* immune-effector activities were observed. Significant enhancement ( $P \leq 0.05$ ) of *in vitro* phagocytic activity, nitrite production, proliferative activity and cytokine production of iIEL were observed in birds of group B than C. The results showed that supplementation of  $\beta$ -glucan from edible mushroom in feed (30mg/ kg) for 20 days had significant immunostimulatory effect in broiler birds that was reflected in the *in vitro* immune-effector activities of the mucosal cells (iIEL).

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**ARTICLE CITATION:** Muthusamy G, Joardar SN, Samanta I, Isore DP, Roy B and Maiti KT (2013).  $\beta$ -Glucan from edible mushroom (*pleurotus florida*) enhances mucosal immunity in poultry. *Adv. Anim. Vet. Sci.* 1 (4): 116 – 119.

#### INTRODUCTION

Infectious diseases are the major challenge in commercial poultry industry. Preventive health management through vaccination is universally recommended practice to meet this challenge, although, cases of vaccine failure and disease outbreak are also common (Dhama et al., 2013).

Immunostimulants comprise of a group of biological and synthetic compounds that enhance the non-specific cellular and humoral defense mechanisms in animals (Chan et al, 2009). It also increases resistance to infectious diseases, and use of these immunostimulants is an effective means of increasing the immunocompetence and disease resistance of animals, birds and fishes. Generally, immunostimulants are promoting a greater and more effective sustained immune response to the infectious agent without the risks of toxicity, carcinogenicity or tissue residues (Weickert and Pfeiffer, 2008; Firenzouli et al., 2008). Beta glucans from the yeast cell wall and mushroom have been shown to stimulate both specific and non-specific immune responses and improve the growth performance also (Vetvicka et al., 2008). However, it has been observed that glucans from various sources appear to elicit diverse immunomodulatory effects in numerous animal tissues, including the blood, gastrointestinal tract and spleen (Ramberg et al., 2010; Samulsen et al., 2011). The use of immunostimulants that is  $\beta$ -1, 3/1-6-glucan can protect *Staphylococcus aureus*, *Listeria monocytogens*, *Bacillus anthracis*, *Venezuelan equine encephalomyelitis* infection in mice, *Salmonella enterica* serovar Enteritidis infection in chicken and *Staphylococcus aureus* infection in cattle (Li et al., 2004; Lowry et al., 2005; Tominac et al., 2010).

Immunostimulants should be active by oral route and should be stable both in its native state and after incorporation into food and water. Oral administrations of immunostimulants have already been reported for glucans, lactoferrin, levamisole and chitosan (Kamilya et al., 2008). Oral administration is the most practical method for delivery of immunostimulants and results in enhancement of leucocyte function and protection against infectious diseases (Tominac et al., 2010).

Studies on immunomodulatory role of edible mushroom *Pleurotus florida* in fish has produced encouraging results. It was observed that extracted proteoglycon/glucan from *Pleurotus florida* when used in feed enhanced the activities of immune effector cells of fish and the protection against pathogen (*Aeromonas hydrophila*) challenge in experimental condition (Kamilya et al., 2008). *In vitro* immunostimulatory effects as also adjuvancity of mushroom glucan was tested in fish (Kamilya et al., 2006). Earlier, we have shown that orally administered  $\beta$ -glucan of edible mushroom (*Pleurotus florida*) up-regulates innate immune response in broiler (Paul et al., 2012). Modulation of immune-effector activities of haematopoietic cells viz. neutrophil and blood mononuclear cells were reported in that study. Here we report, the effect of orally administered  $\beta$ -glucan of edible mushroom (*Pleurotus florida*) on intestinal intra epithelial leukocytes (iIEL) of broiler to establish its role in mucosal immunity.

#### MATERIALS AND METHODS

##### *Immunostimulant*

Purified Mushroom Glucan (MG) was prepared from the source of *Ostrea* Mushroom by alkali extraction method (Maiti et al., 2008). The purified product was obtained from the

Department of Biotechnology, Indian Institute of Technology, Kharagpur, India.

#### Experimental Bird

The experimental design and procedure adopted was approved by the Animal Ethics Committee of the University. Fifty day-old chicks were procured from a commercial hatchery and vaccinated at 5<sup>th</sup> day with RD vaccine (F strain) (Institute of Animal Health and Veterinary Biologicals, Govt. of West Bengal). On 7<sup>th</sup> day, they were divided into three diet groups (Group-A, B, C). A and B group contained 20 chick with replica of 10 chicks in each pen. Control group (C) had 10 birds with replica of 5 chicks in each pen.

#### Experimental Design

Birds were fed (normal) feed (Amrit Feed, India) for 20 days. On 7<sup>th</sup> day onward, the experimental birds of group A were given 15 mg MG / kg whereas, birds in the Group B were given 30 mg / kg in feed. Group C birds (Control) were given normal feed. After 20 days of feeding, birds of all three groups (A, B, C) were given normal feed for further 14 days. Birds were allowed to take feed and water *ad libitum* during the whole period of experiment. The period of observation was started from the last MG administration (0-day) with 7 days interval, viz. 0 day, 7<sup>th</sup> day and 14<sup>th</sup> day.

#### Isolation of intestinal Intra Epithelial leukocyte cells (iIEL)

Three birds of each group (viz. Group A, Group B and Group C) were sacrificed under anesthesia (following the standard guidelines of Institutional Ethical Committee) to collect the intestine in sterile PBS on each experimental day. The iIEL were isolated from chicken intestine with slight modification as adapted by Grachia et al. (1997). In brief, 12 to 15 cm of duodenal C loops, jejunum, ileum and caeca were removed from chicken intestine, washed with ice cold PBS buffer with antibiotics solution (containing streptopenicillin @400 IU/mL, gentamycin @4mg/mL) extensively. The gut tissues were taken into a beaker treated with pre warmed (41°C) 5mM dithiothreitol (DTT) and 0.1 mM Ethylene Diamine Tetra Acetic acid (EDTA) solution for 40 minutes in water bath (temperature 41°C) with occasional gentle shaking. After extensive washing, the treated intestinal tissues were placed in a beaker containing 30 ml of washing medium having 300 IU of collagenase per mL and kept in a shaking water bath (41°C). After 30 min, supernatants containing single cells were collected and replaced with fresh washing medium containing collagenase and incubated for an additional 30 min at 41°C. The viable cells (iIEL) were collected by centrifugation, washed twice in washing medium and separated from debris and dead cells by differential centrifugation using Histopaque<sup>R</sup> (Sigma, USA). Viability was assessed by trypan blue dye exclusion method (Daly et al., 1995). Then the iIEL cells were used for the functional assays.

#### Stock solutions for LPS and ConA

Stock solution of lipopolysaccharide (Sigma, USA) was prepared at a concentration of 20µg/mL of the growth medium RPMI-1640 (Sigma, USA), filtered through a sterile membrane filter (0.2µ) and stored at -20°C until use. Stock solution of concanavalin A (Sigma, USA) was prepared at the concentration of 20µg/mL of the growth medium RPMI-1640 (Sigma, USA), filtered through a sterile membrane filter (0.2µ) and stored at -20°C until use.

#### In vitro nitrite production assay

The production of reactive nitrogen intermediates was assessed following the method described by Tafalla and Novoa (2000). This method was based on the Griess reaction that quantified the nitrite content of the macrophage supernatants, as nitric oxide is an unstable molecule and degrades to nitrite and nitrate (Green et al., 1982).

#### Lymphoproliferation Assay (LPA)

The colorimetric - [4, 5- dimethylthiazol-2-yl]-2, 5- diphenyl tetrazolium bromide (MTT) assay described by Daly et al. (1995) was used to determine the proliferation of iIEL cells.

#### Macrophage Functional Assay

Phagocytosis was examined as described by Yoshida et al. (1993).

#### cDNA preparation

After separation from intestine, iIEL cells ( $2 \times 10^6$  cells / mL) were suspended in RPMI-1640 and 100 µL of cell suspension was dispensed into wells of 96-well tissue culture plates. The final volume of the wells made up to 200 µL with Con A at a concentration of 10µg/mL in positive control wells (in triplicate) and with RPMI-1640 growth medium in negative control wells (in triplicate). Finally, it was incubated at 37°C for 3 hr containing 5% CO<sub>2</sub> tension.

For cell harvesting, cells were thoroughly mixed with the culture medium and the cell suspension was transferred to a 15 mL polypropylene tube. The tube was centrifuged at 400g for 5 min to pellet the cells. The cells (pellet) were washed twice with 10 mL of PBS. Finally, the pellet was suspended in 1mL of culture medium. The cell density was determined and it was adjusted using ice-cold PBS so that it falls within the range of 5000 cell/µL. Now 10µL of cells were transferred to PCR tube and cDNA was prepared following the protocol mentioned in cDNA kit (GeNei™).

#### Detection of cytokine mRNA by RT-PCR

The presence of mRNA for chicken IFN-γ was determined by RT-PCR as described earlier following the touch-down PCR protocol for amplification of cDNA (Xing and Schat, 2000). The PCR product (485 bp) was analyzed by agarose gel electrophoresis and documented in the gel documentation system (UVP). As well as the image was analyzed by UVP software (Doc ILT) for semi quantification of the cDNA present in the product.

#### Statistical Analysis

The results of each experiment was expressed as the mean ± standard error of mean and analyzed by one-way analysis of variance (ANOVA) to test the significance between control and experimental groups.

## RESULTS

#### In vitro Nitrite Production Assay

Upon LPS stimulation the *in vitro* nitrite production of intestinal intra-epithelial leukocytes (iIEL) of broiler birds of group A, B, C are shown in Figure- 1. *In vitro* nitrite production was increased significantly ( $P \leq 0.05$ ) in treated birds of both the groups subsequently through 0 day, 7<sup>th</sup> day and 14<sup>th</sup> day than control group. The average values of Group B birds were more than Group A in all the post treatment periods.

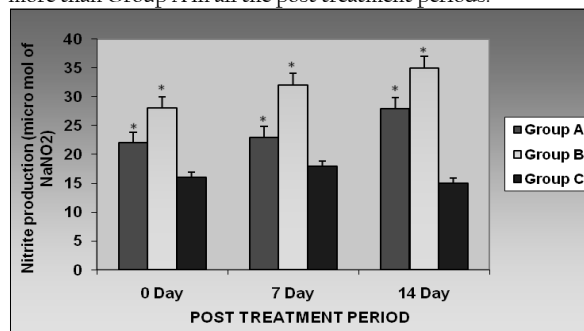


Figure 1: *In vitro* NO<sub>2</sub><sup>-</sup> production by intestinal intra-epithelial leukocytes of Mushroom Glucan fed and control broiler birds at different days post treatment

### Lympho-proliferation assay

Upon ConA stimulation, the *in vitro* iIEL of broiler birds, in group A, B and C were expressed as stimulation index (S.I.). The S.I. values of treated birds of both the groups enhanced in post treatment period, viz. 0-day, 7<sup>th</sup> day and 14<sup>th</sup> day. The average S.I. values were gradually increased from  $0.157 \pm 0.003$  to  $0.302 \pm 0.001$  in case of group A and  $0.174 \pm 0.005$  to  $0.331 \pm 0.056$  in case of group B from 0-day to 14<sup>th</sup> day. In all the cases values were more in case of Group B than Group A. These values were significantly ( $P \leq 0.05$ ) higher than that of group C in all the cases. The results are shown in the Figure 2.

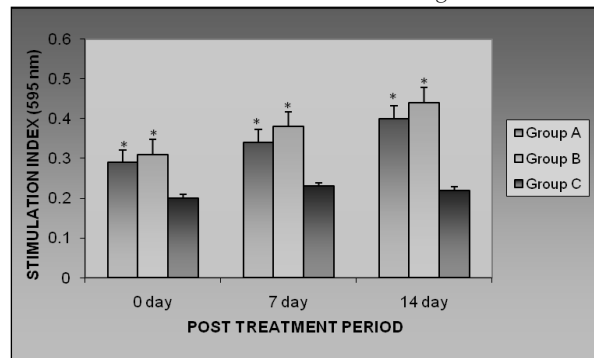


Figure 2: *In vitro* lymphoproliferation assay of intestinal intra-epithelial leukocytes of Mushroom Glucan fed and control birds at 0, 7<sup>th</sup> and 14<sup>th</sup>

### Macrophage functional assay

The Phagocytic Activity (PA) value of iIEL cells of control and MG fed birds are presented in Figure 3. The PA values of treated birds of both the groups enhanced significantly ( $P \leq 0.05$ ) in post treatment period, viz. 0-day, 7<sup>th</sup> day and 14<sup>th</sup> day. The average values of Group B birds were more than Group A in all the post treatment periods.

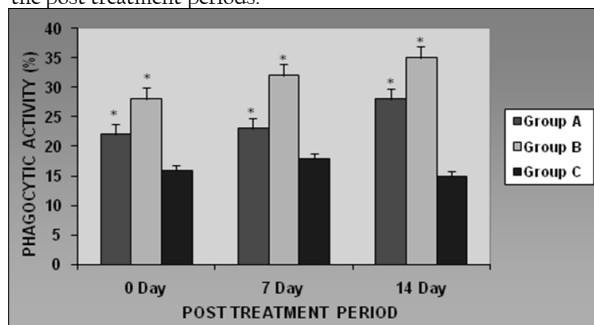


Figure 3: Phagocytic activity of intestinal intra-epithelial leukocytes of control and Mushroom Glucan fed broiler birds at 0, 7<sup>th</sup> and 14<sup>th</sup> day post treatment period

### Cytokine assay

iIEL from control and treated birds (group A and group B) were separated and stimulated by ConA *in vitro*. In culture, mRNA produced was reverse transcribed and amplified by RT-PCR to assess cytokines produced viz. IFN- $\gamma$  using specific primers. The RT-PCR showed mRNA production of IFN- $\gamma$  *in vitro* by iIEL of both control and treated group birds. Birds of control groups produced less IFN- $\gamma$ -cDNA in compared to birds of treated groups as per the results observed in the UVP software analysis.

### DISCUSSION

This work illustrates the influence of dietary supplementation of  $\beta$ -glucan from edible mushroom (*Pleurotus florida*) on the activities of immune effector cells of broiler birds. As the

transmission of various infectious diseases to animals is taken place through the alimentary tract route, understanding the nature and functions of various cellular components of gut associated lymphoid tissue (GALT) gives us a panoramic view about the type of immunological responses operated in gastrointestinal tract (GIT). Lamina propria and intestinal intra epithelial leukocytes (iIEL) are the major components of gut immunity. The iIEL are located at basal and epical part of the intestinal epithelium, which makes them virtually most important in immunosurveillance mechanism (Lee et al., 2010). Reactive nitrogen intermediates have been proved to be important molecules in regulating immune functions, as well as having direct anti-microbial effect (Liew, 1995).

In the present study, the iIEL of MG fed broiler chicks induced by LPS *in vitro* was assessed for nitrite production. In all the post treatment period, treated birds showed higher *in vitro* nitrite production than the control birds. It is known that LPS are capable of stimulating macrophage nitrite production in mice (Stuehr and Marletta, 1985) and in fish (Tafalla and Novoa, 2000). Our finding is supported by earlier works in mammals and birds where MG augmented nitric oxide production (Mucksova et al., 2001; Cox et al., 2010). Earlier workers also showed immunostimulatory effect of  $\beta$ -glucan of edible mushroom origin in other animal species like Indian major carp, *Catla catla* (Kamilya et al., 2006). In an experiment, broiler birds fed with 20 mg  $\beta$ -glucan gave higher *in vitro* nitrite production by blood leukocytes than control birds (Paul, 2008).

There was a significant enhancing effect of dietary MG on the proliferative response of iIEL, induced by Con A. *In vitro* lymphoproliferation was enhanced on group A and group B birds and the stimulation index (SI) was significantly ( $P \leq 0.05$ ) more than control group in all the post treatment periods. It was found to be that administration of oat  $\beta$ -glucan increased lymphocyte and neutrophil stimulation in beef steers (Estrada et al., 1999). L-Arginine was observed to stimulate *in vitro* lymphoproliferation of peripheral blood lymphocytes, iIEL and increase the systemic immune response in chickens that gave 100% protection in IBD virus challenge (Tayade et al., 2006).  $\beta$ -glucan of yeast cell wall origin (*Saccharomyces cerevisiae*) was found to have immunostimulatory effect in fish. Enhancing effect of dietary glucan on the response of lymphocytes of fish induced by ConA has been observed previously (Verlhac et al., 1998). Similarly, increased spleenocyte proliferation in response to LPS has been observed following oral administration of direct fed microbials in broiler birds (Lee et al., 2010).

An enhanced phagocytic activity (PA) of iIEL was observed in MG fed birds. Group A and group B birds showed high PA value than the control group in all the post experimental periods. Earlier workers reported similar observation in other species (mice). Sakurai et al. (1992) showed enhanced murine alveolar macrophage activity due to  $\beta$ -glucan feeding. Suzuki et al. (1990) reported that orally administered  $\beta$ -glucan enhanced the phagocytic activity of macrophage in mice. Similar results were observed in chicken treated with purified  $\beta$ -glucan. The PA activity increased in immature chickens against *Salmonella enterica* serovar Enteritidis (Lowry et al., 2005).

In the present study, semi quantitative RT-PCR for mRNA was conducted to assess the *in vitro* production of IFN- $\gamma$  from iIEL of treated and control birds stimulated by ConA. Birds of control groups produced *in vitro* cytokines viz. IFN- $\gamma$  less as compared to birds of treated groups. This shows that leukocytes of birds from treated groups where highly sensitized than the leukocytes of control birds. That means MG had positive response in treated birds in terms of cytokine production by iIEL. Earlier worker (Rice et al., 2005) also

obtained similar positive correlation between immunostimulation and cytokine production. In that study glucan as immunostimulatory carbohydrates has increased systemic levels of IL-12. The orally administered yeast  $\beta$ -1, 3 glucan to mice stimulated the production of cytokines (Rice et al., 2005). However, in contrast, Cox et al. (2010) observed reduced expression of IFN- $\gamma$  in broiler birds fed with beta glucan.

In the mammalian system, action of  $\beta$ -glucan is mediated through toll-like receptors (TLR) and dectin-1 (Brown and Gordon, 2003). It is known that after binding with dectin-1,  $\beta$ -glucan activates the transcription factor NF- $\kappa$ B that plays a critical role in immune responses (Tada et al., 2009; Samuelson et al., 2011). The authors hypothesize such type of molecular interactions at cellular level in poultry gut that substantiates the present findings.

## CONCLUSION

The results showed that supplementation of  $\beta$ -glucan from edible mushroom in feed (30mg/kg) for 20 days had significant immunostimulatory effect in broiler birds as reflected in the immune-effector activities of mucosal cells (iIEL).

## ACKNOWLEDGEMENT

Authors are thankful to the Vice Chancellor, WBUAFS for providing necessary research facilities.

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