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Dietary administered purified β-glucan of edible mushroom (*Pleurotus florida*) provides immunostimulation and protection in broiler experimentally challenged with virulent Newcastle disease virus

Gopi Muthusamy[1], Siddhartha Narayan Joardar[1*], Indranil Samanta[1], Devi Prasad Isore[1], Barun Roy[2] and Tapas Kumar Maiti[3]

Abstract

Background: To study the immunomodulatory and protective role of dietary administered purified β-glucan obtained from edible mushroom (*Pleurotus florida*) in commercial broiler chicken, experimentally challenged with virulent Newcastle disease virus (NDV) on 7th day post treatment. Mushroom glucan (MG) at 15 mg/kg feed (group A) and MG at 30 mg/kg feed (group B) was administered to broiler birds for 20 days keeping control birds (group C) with a normal diet throughout. After 7 days post treatment, three groups of birds ($n = 4$, in each case) were challenged with virulent NDV. The immunological parameters were assessed to observe the protective efficacy of MG.

Results: When compared to the treatment regime, it was observed that in all the cases, group B birds showed higher immune-cellular and humoral responses in terms of enhanced immune-effector activities of blood leucocytes and intestinal intra-epithelial leucocytes and antibody production besides protection against NDV challenge than the others. After NDV challenge, 100% mortality was observed in control birds within 4 days, whereas in treated birds 50% and 75% protection of challenged birds was observed in group A and group B birds, respectively. The superoxide anion production by blood leucocytes of group A (0.641 ± 0.01) and group B (0.721 ± 0.01) birds were significantly higher than the control birds (0.283 ± 0.04) when assessed on 4th day post challenge. Group A (27.33 ± 1.20 μl and 25.33 ± 2.02 μl) and group B (33.66 ± 0.33 μl and 32.66 ± 0.33 μl) birds showed higher in vitro nitrite production by peripheral blood mononuclear cells (PBMC) and intestinal intra-epithelial leucocytes (iIEL), respectively, than the control (14.00 ± 0.57 μl and11 ± 0.57 μl) after challenge with virulent ND virus. In vitro lymphoproliferation (expressed as stimulation index) was significantly high in PBMC and iIEL of group A (0.371 ± 0.02 and 0.295 ± 0.02) and group B (0.428 ± 0.01 and 0.314 ± 0.01), respectively, than control (0.203 ± 0.01 and 0.135 ± 0.01) on 4th day of NDV challenge.
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* Correspondence: joardar69@gmail.com
[1]Department of Veterinary Microbiology, Faculty of Veterinary and Animal Sciences, West Bengal University of Animal and Fishery Sciences, Kolkata 700037, India
Full list of author information is available at the end of the article

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The phagocytic activity of iIEL of the treated group birds showed higher values (24% and 32%) than the control group (14%). The haemagglutination inhibition (HI) titre was also observed higher in treated groups (group A, average HI titre 256, and group B, average HI titre 512) than control (HI titre, 32). Both groups (A&B) of birds were produced in vitro IFN-γ by PBMC and iIEL.

Conclusion: It is advisable to use 30 mg MG/kg feed in broiler birds to provide immunostimulation and for better output in terms of disease protection at least against ND virus.

Keywords: Broiler, Haemagglutination inhibition, Immunomodulation, Intestinal intra-epithelial leucocytes, Mushroom β-glucan, Newcastle disease virus

Background

Immunostimulants enhance the body's resistance against various infections through increasing the oxidative activity of neutrophils, engulfment capacity of phagocytic cells and stimulating cytotoxic cells as necessary defence mechanisms. Many disorders could be treated using immunostimulants such as autoimmune diseases, viral infections and cancer (Shahbazi and Bolhassani, 2016). Several types of stimulants such as bacterial products, complex carbohydrates (e.g. glucans, schizophyllan, scleroglucan, lentinan, statolon, bestatin, acemannan), vaccines, immunoenhancing drugs (e.g. levamisole, isoprinosine, fluoro-quindone, avridine, polyribonucleotides), nutritional factors (e.g. vitamins, carotenoids, lipids, trace elements like selenium), animal extracts (e.g. chitosan from shrimp), cytokines (e.g. macrophage activating factor, interferon, interleukin-2, tumour necrosis factor) and plant extracts (e.g. Lectins, mitogens such as phytohaemagglutinin, concanavalin A) which have different mechanisms and functions (Galeotti, 1998). Immunostimulants activate different elements of the immune system in human and animals. They develop the non-specific immunotherapy and immunoprevention by stimulating the basic factors of the immune system including phagocytosis, properdin and complement systems, protective secretory IgA antibodies, α- and γ-interferon release, T- and B-lymphocytes and synthesis of specific antibodies and cytokines. This also helps in the synthesis of pulmonary surfactant (Labh and Shakya, 2014).

Earlier, wide varieties of microbial products were employed as immunostimulants owing to their potentiality of non-specific activation of marcrophages, dendritic cells and NK cells, thereby releasing cytokines (Barakat et al., 1981; Vetskova et al., 2013). Muramyl dipeptide (MDP) obtained from cell wall of mycobacteria possess numerous biological activities including non-specific resistance against infectious challenges (Souvannavong et al., 1988). Heat-killed or formaldehyde-treated *Propionibacterium acnes* activates macrophages causing release of cytokines through toll-like receptor (TLR) stimulation. It enhances humoral and cell-mediated immune responses to protect from various bacterial, viral and protozoal infections (Becker et al., 1989; Isenberg et al., 1995). Un-methylated cytosine-guanosine nucleotide derived from bacteria; complex carbohydrates like zymosan, glucans, aminated polyglucose and lentinans derived from yeasts; and acemannan obtained from *Aloe vera* plant have potent cytokine inducing ability from macrophages (Scaringi et al., 1988; Wang et al., 2006).

The infections of various aetiologies are considered the greatest challenge in the profit-making process of the modern poultry industry. At this backdrop, immunostimulants can play quite significant role in enhancing the body defence mechanisms of poultry birds leading to improved growth and production performance with reduced mortality. Both specific and non-specific immune responses of poultry have been shown to be stimulated by β-glucans obtained from yeast cell wall and mushrooms (Vetvicka et al., 2002). Earlier, oral applications of various stimulants, viz. glucans, lactoferrin, levamisole, and chitosanhave been reported (Kamilya et al., 2008). Immunostimulatory role of edible mushroom (*Pleurotus florida*) was established in fish. It was observed that proteoglycan/glucan extracted from *Pleurotus florida* when used in feed, enhanced the activities of immune-effector cells in fish and the protection against pathogen (*Aeromonas hydrophila*) upon experimental challenge (Kamilya et al., 2008). In vitro immunostimulatory effect as well as adjuvanticity of mushroom glucan was reported in fish (Kamilya et al., 2008).

With this background, the objective of the present work was to study the immunomodulatory and protective role of dietary administered purified glucan obtained from edible mushroom (*Pleurotus florida*) in commercial broiler chicken against pathogenic infections, taking Newcastle disease virus (NDV) as a representative pathogen.

Methods

Mushroom glucan

Glucan was isolated from the edible oyster mushroom (*Pleurotus florida*) by ethanol precipitation and ion exchange chromatography. The neutral concentrated polysaccharides were subjected to Sephadex G-100 gel permeation chromatography. The gel permeation produced three fractions. The second yielded the most polysaccharide, with

a protein ratio of 24.6. It was frozen in a conical flask at − 20 °C, lyophilized and stored in desiccators at − 20 °C (Wasser, 2002; Kamilya et al., 2006).

Experimental bird

Broiler birds were used as experimental animals in the present study. The experimental design and procedure adopted was as per the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Ministry of Fisheries, Animal Husbandry and Dairying, Govt. of India, and was approved by the Animal Ethics Committee of the West Bengal University of Animal and Fishery Sciences (WBUAFS). Fifty (50)-day-old chicks were procured from a commercial hatchery of Kolkata (India). All chicks were vaccinated at the 5th day with Newcastle disease vaccine (F strain) (Institute of Animal Health and Veterinary Biologicals, West Bengal, India). On 7th day, they were divided into three groups (group A, B, C) on the basis of diet. A and B groups contained 20 chicks 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 offered feed containing maize, soya bean, groundnut cake, vitamin and mineral mixture for 20 days. On the 7th day onward, the experimental birds of group A were given mushroom glucan (MG) at 15 mg/kg feed and to group B birds at 30 mg/kg feed (Gopi et al., 2013). 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 up to the end of the experiment. Birds were allowed to take feed and water ad libitum. After 7 day of post treatment, three groups of birds (n = 4, in each case) were challenged intranasally with laboratory (Department of Veterinary Microbiology) isolated field strain of NDV (10^6 EID_{50}/ml dose). The birds were kept in observation for 7 days. Then, the immunological parameters were assessed to observe the protective efficacy of MG.

Neutrophil function assay (nitro blue tetrazolium reduction assay)

As a measure of non-specific immune response, superoxide anion production by blood neutrophils was determined by the nitro blue tetrazolium (NBT) reduction assay (Siwicki et al., 1998). Briefly, 100 μl of the blood from the treated and control (4th day post challenged) birds were placed into a micro-titre plate well. Equal amount of 0.2% filtered NBT in phosphate buffer saline (PBS) solution was added into it and incubated for 30 min, in room temperature. Then, 50 μl were taken from this mixture and 1 ml N, N-dimethylformamide was added, centrifuged for 5 min at 3000×g and read in a spectrophotometer at 540 nm.

Isolation of peripheral blood mononuclear cells (PBMC)

Isolation of Peripheral blood mononuclear cells was carried out from treated and control birds on 4th day post challenge (Chung and Secombes, 1988). The diluted blood samples were layered onto HiSep® (Himedia) at the ratio of 1:3 (1 part of HiSep and 3 parts of cell suspension) and centrifuged for 30 min at 1200 rpm. Then, the white blood cell interface layer was collected, transferred into clean sterile test tube and washed thrice. The cell viability of the isolated cells was enumerated by the Trypan blue exclusion method. In most of the cases, > 90% of the cells were viable.

Isolation of intestinal intra-epithelial leucocyte cells (iIEL)

Three birds of each group (group A, group B and group C) were sacrificed under anaesthesia (following the standard guidelines of Institutional Ethical Committee) on 4th day post challenge to collect the intestine in sterile PBS on each experimental day. The iIEL were isolated from chicken intestine (Chai and Lillehoj, 1988). 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 at 400 IU/ml and gentamycin at 4 mg/ml) extensively. The gut tissues were taken into a beaker and treated with pre-warmed (41 °C) 5 mM dithiothreitol (DTT) and 0.1 mM ethylene diamine tetra acetic acid (EDTA) solution for 40 min in a 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 two times in washing medium and separated from debris and dead cells by differential centrifugation using Histopaque^R (Sigma, 1.077). Viability was assessed by trypan blue dye exclusion method. Then, the iIEL cells were used for the functional assays.

Stock solutions for lipopolysaccharide (LPS) and concanavalin-A (ConA)

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

In vitro nitrite production assay

The production of reactive nitrogen intermediates was assessed in PBMC and iIEL of treated and control birds after virus challenge (Tafalla and Novoa, 2000). This method was based on the Griess reaction that quantified the nitrite content of the macrophage supernatants, as nitric oxide was an unstable molecule and degraded to nitrite and nitrate (Green et al., 1982).

Lymphoproliferation assay (LPA)

The colorimetric-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay (Daly et al., 1995) was used to determine the proliferation of PBMC and iIEL cells upon in vitro ConA treatment in glucan-fed and control birds on the 4th day post challenge.

Estimation of phagocytic activity

The adherent cells (macrophages) were separated from iIEL suspension of treated and control birds (on 4th day post challenge) on glass cover slips, methanol fixed and stained by Giemsa. The phagocytic activity (PA) of the cells was determined (Yoshida et al., 1993).

cDNA preparation

The PBMC and iIEL cells (2×10^6 cells/ml) were suspended in RPMI-1640 and 100 μl of cell suspension was dispended into wells of 96-well tissue culture plates. The final volume of the wells made up to 200 μl with ConA at a concentration of 10 μg/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 h with 5% CO_2 tension.

The 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 400×*g* for 5 min to pellet the cells. The cells pellet was washed twice with 10 ml of PBS and was suspended in 1 ml 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. The cDNA was prepared following the protocol (GeNei™, India).

Detection of cytokine mRNA by reverse transcription-polymerase chain reaction (RT-PCR)

The presence of mRNA for chicken interferon-gamma (IFN-γ) was determined in PBMC and iIEL of treated (group A and group B) and control birds by RT-PCR as described (Xing and Schat, 2000). The PCR product was observed by agarose gel electrophoresis and documented in the gel documentation system (UVP, UK).

Haemagglutination inhibition test

Haemagglutination followed by haemagglutination inhibition (HI) test was performed (Allan and Gough, 1974). Positive serum control, negative serum control, RBC control, and 4HA haemagglutinin control were kept. The end point was taken as the well in which haemagglutination is inhibited which was seen as clear button.

Statistical analysis

Data were analysed with SPSS 17.0 for Windows software and one-way analysis of variance (ANOVA). Means were compared by LSD post hoc test and a probability level of 0.05 was used.

Results

Effect of virulent virus challenge

All birds (four) of control group died showing clinical symptoms within 4 day after challenge with a virulent field isolate of ND virus. Two birds of group A died within 7 days after challenge. Two birds were protected in this group. Three birds were protected in group B after virus challenge. One bird died showing clinical symptoms after 7 days of viral challenge. All birds of control group showed characteristic post-mortem lesions as petechial haemorrhages in proventriculus and haemorrhages in ileo-coecal junction (Fig. 1). After NDV challenge, 100% mortality was observed in control birds within 4 days, whereas in treated birds 50% and 75% protection of challenged birds was observed in group A and group B birds, respectively (Table 1).

Neutrophil function assay

The super oxide anion production by blood leucocytes of group A (0.641 ± 0.01) and group B (0.721 ± 0.01) birds were significantly higher than the control birds (0.283 ± 0.04) when assessed on the 4th day of post challenge period (Fig. 2a).

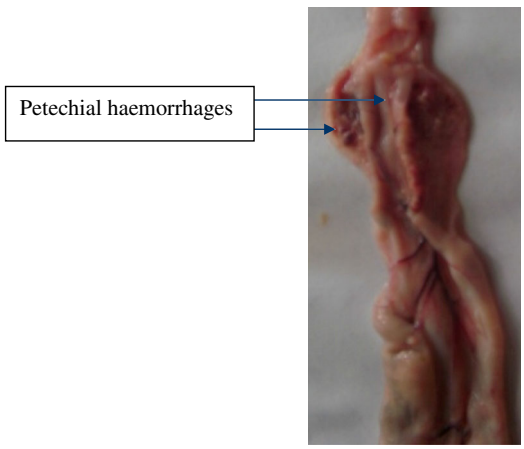

Fig. 1 Post-mortem lesion of NDV challenged control bird

Table 1 Percent protection in control and treated broiler birds after virulent ND virus challenge

Treatment group	No. of birds challenged	No. of birds died	No. of birds protected	Protection (%)
Group A (15 mg/kg feed)	4	2	2	50
Group B (30 mg/kg feed)	4	1	3	75
Group C (control diet)	4	4	0	0

In vitro nitrite production assay

The in vitro nitrite production of PBMC and iIEL of treated broiler birds was higher than control birds after challenge with virulent ND virus. Group A (27.33 ± 1.20 μl and 25.33 ± 2.02) and group B (33.66 ± 0.33 μl and 32.66 ± 0.33) birds showed higher production than control birds (14.00 ± 0.57 μl and11 ± 0.57) after challenge with virulent ND virus (Figs. 2b and 3).

Lymphoproliferation assay (LPA)

The results of lymphocyte proliferation of PBMC and iIEL upon in vitro ConA treatment was presented in terms of stimulation index (S.I.). The S.I. values of control and

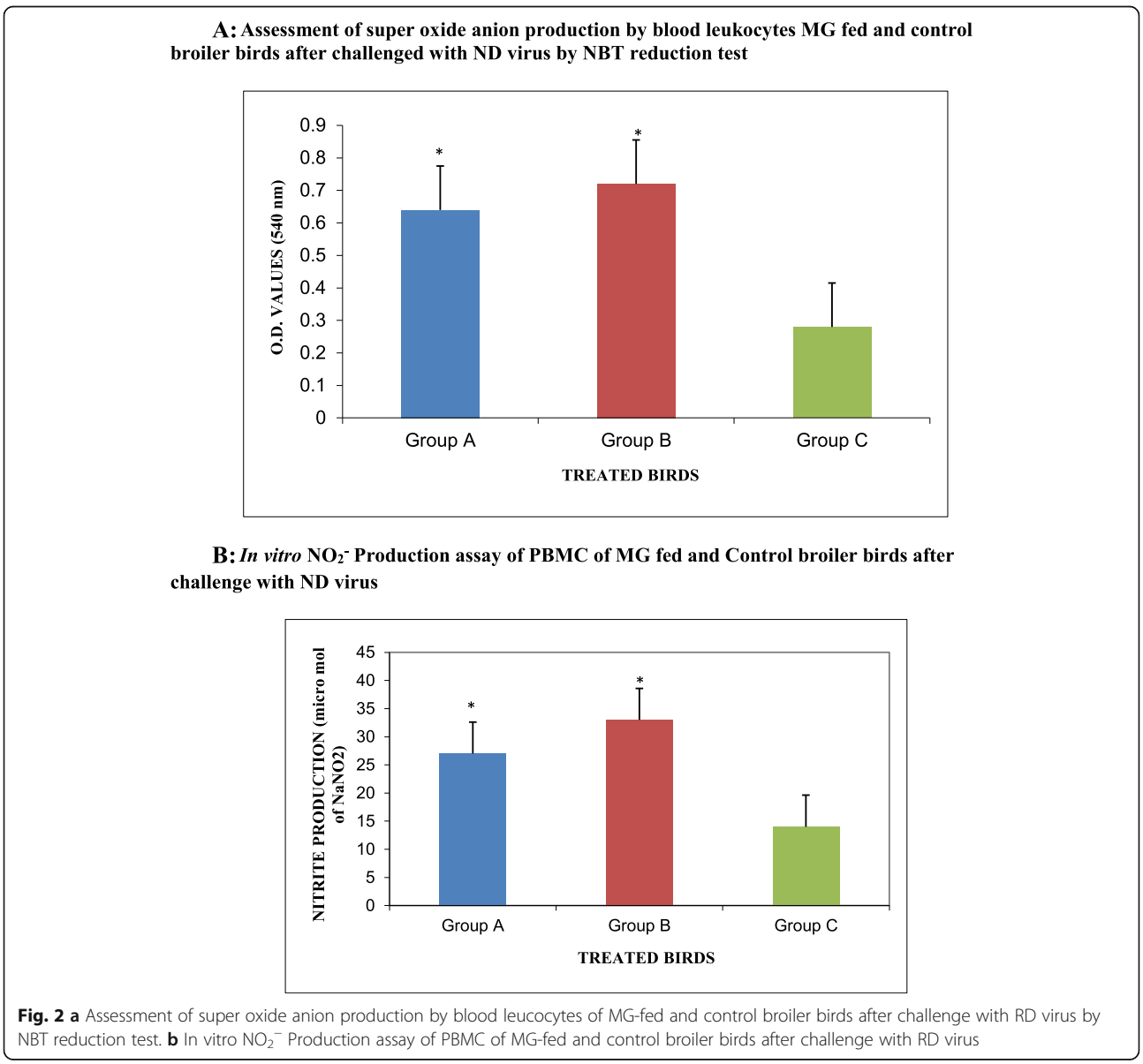

Fig. 2 a Assessment of super oxide anion production by blood leucocytes of MG-fed and control broiler birds after challenge with RD virus by NBT reduction test. **b** In vitro NO_2^- Production assay of PBMC of MG-fed and control broiler birds after challenge with RD virus

MG-fed broiler birds after virus challenge are presented in Figs. 4 and 5. MG-fed groups showed significantly higher ($P < 0.05$) stimulation indices in both the cases, i.e. group A 0.371 ± 0.02 and 0.295 ± 0.02, and group B 0.428 ± 0.01 and 0.314 ± 0.01, respectively, than control birds (0.203 ± 0.01 and 0.135 ± 0.01) after the 4th day of virus challenge.

Estimation of phagocytic activity

The phagocytic activity (PA) value of iIEL after virus challenge is presented in Fig. 6. The treated groups showed higher values (24% and 32%) than the control group (14%).

Detection of cytokine mRNA by RT-PCR

PBMC and iIEL of both treated group birds showed bands in gel indicating mRNA production of IFN-γ in vitro. However, no such band was observed in control birds. The band profile of cytokine IFN-γ is shown in Fig. 7.

Haemagglutination inhibition test

The haemagglutination inhibition (HI) titre was also observed higher in the treated groups (group A, average HI titre 256, and group B, average HI titre 512) than control (HI titre 32).

Discussion

Immunostimulants increase resistance to infectious diseases generally by enhancing non-specific defence mechanism. Use of immunostimulants is an effective means of increasing the immunocompetence and disease resistance in fish (Anderson, 1992; Siwicki et al., 1998; Sakai, 1999; Kamilya et al., 2008). Many immunostimulants from different sources have been studied in poultry (Yin et al., 1997; Hosamani et al., 2004; Ma et al., 2006). β-glucan is a potent immunostimulant having branched polysaccharide and its effect was tested on fish (Pal et al., 2007a; Pal et al., 2007b; Kamilya et al., 2006), poultry (Ali et al., 2019) and other animals (Williams and Di Luzio, 1979; Li et al., 1996; Waller and Colditz, 1999; Hiss and Sauerwein, 2003). β-glucans in oregano essential oils activate the immune cells, macrophages, dendritic cells neutrophils, B cells, T cells and natural killer cells (Kim et al., 2011).

In the present study, β-glucan was obtained from edible mushroom *Pleurotus florida* (Kamilya et al., 2008) and administered to broiler chicks in feed at 15 mg/kg of feed (group A) and 30 mg/kg of feed (group B) for 20 days. The protective role of orally fed β-glucan was evaluated after NDV challenge.

In the present study, broiler chicks of the MG-fed groups and control group were challenged with virulent field isolate of Newcastle disease virus at 10^6 EID_{50} dose. Moderately higher protection (75%) was observed in group B birds than group A (50%), whereas 100% mortality was observed in the control group. The protection in treated groups (groups A and B) correlated with enhanced cellular and humoral immune response detected by neutrophil and macrophage functional assay, lymphocyte proliferation assay, and HI.

In the similar way, earlier workers also reported protective ability of various immunostimulants that provided high humoral and cellular response in treated animals/birds. The ND vaccine adjuvinated with CpG ODN offered better protection against the lethal dosage of ND challenge associated with enhanced antibody production and lymphocyte proliferation (Linghua et al., 2007).

MOS (Mannan oligosaccharides) significantly improved the antibody against ND virus and lymphocyte proliferation of broiler chicks (Ma et al., 2006). Hosamani et al. (2004) studied the effect of *Mycobacterium phlei* as immunomodulator. *Mycobacterium*

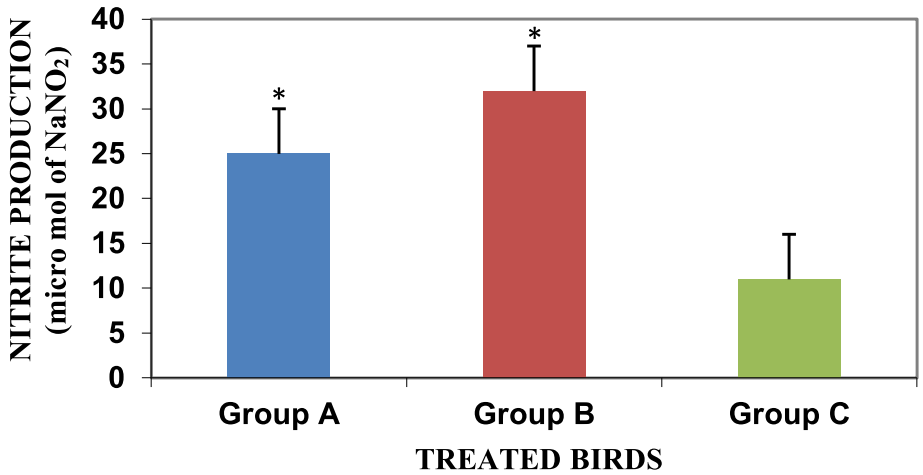

Fig. 3 In vitro NO_2^- production assay of intestinal intra-epithelial leukocytes (iIEL) of MG-fed and control broiler birds after challenge with ND virus

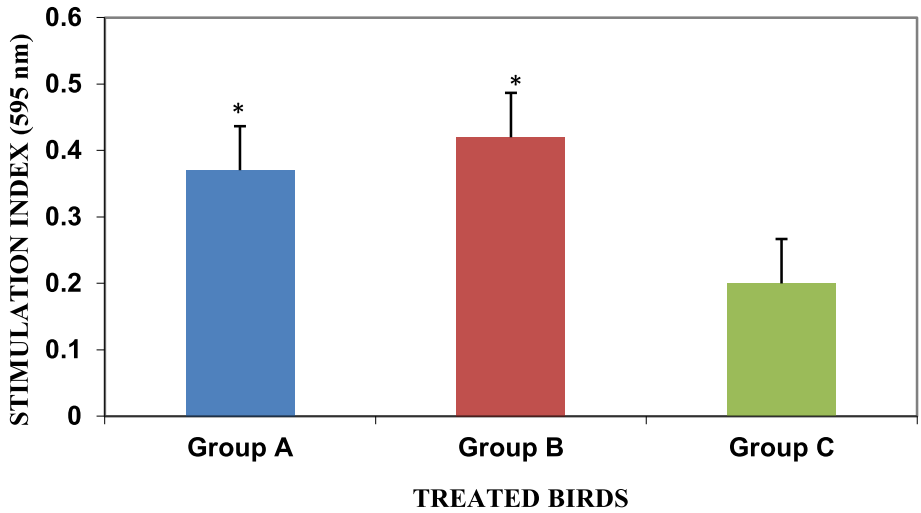

Fig. 4 In vitro lymphoproliferation assay of PBMC of MG-fed and control broiler birds after challenge with RD virus

phlei was administered orally to poultry and the protective efficacy against Newcastle disease was observed. In the present study, enhanced neutrophil, macrophage and lymphocyte functional activity in terms of superoxide production, phagocytic activity and cytokine (IFN-γ) production were observed in the MG-treated broiler birds that justified the protective ability of the treated birds against virulent ND virus as opposed to the control birds that showed 100% mortality.

In the present study, RT-PCR for mRNA was conducted to assess the in vitro production of IFN-γ from PBMC and iIEL of treated and control birds challenged with NDV. The MG-treated birds produced higher cytokines (IFN-γ) than the control group due to the presence of more sensitized leukocytes. Earlier, similar positive correlation between immunostimulantion and cytokine production was reported by Rice et al. (2005). They reported systemic increase of IL-12 in mice upon yeast β-1, 3 glucan treatment. However, in contrast, reduced expression of IFN-γ in broiler birds fed with beta glucan was reported in *Eimeria* challenge experiment (Cox et al., 2010). The action of β-glucan is mediated through pathogen recognition receptors like multiple toll-like receptors (TLR) and C-type lectin receptors, viz. Dectin-1, Dectin-2, mannose receptor, and DC-SIGN (Brown and Gordon, 2003, Sukhithasri et al., 2013; Hopke et al., 2018). The β-glucan after binding with dectin-1 activates the transcription factor NF-κB, thereby, enhancing lymphoproliferation and initiation of immune reactions (Tada et al., 2009; Samuelsen et al., 2011). In the present experiment, similar molecular interaction at the cellular level in poultry gut was anticipated.

When compared to the treatment regime, it was observed that in all the cases group B birds showed higher immune responses and protection than group A birds. In other words, broiler birds fed with 30 mg MG/kg feed

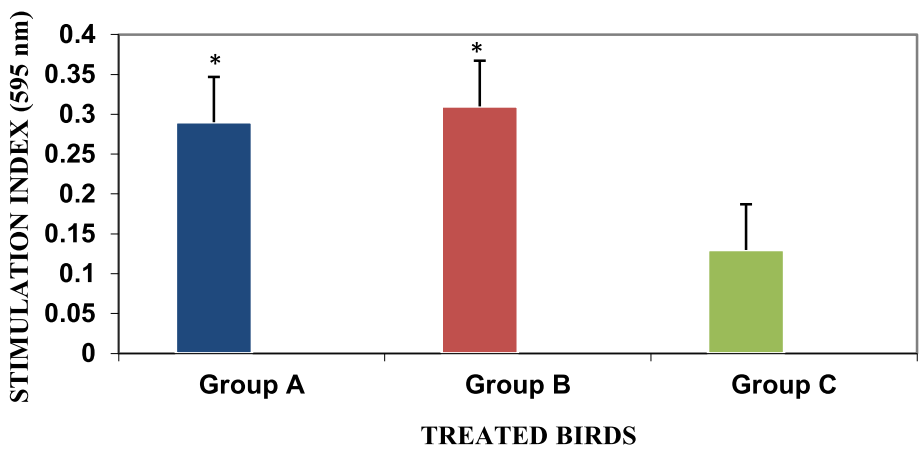

Fig. 5 In vitro lymphoproliferation assay of intestinal intra-epithelial leucocytes (iIEL) of MG-fed and control broiler birds after challenge with ND virus

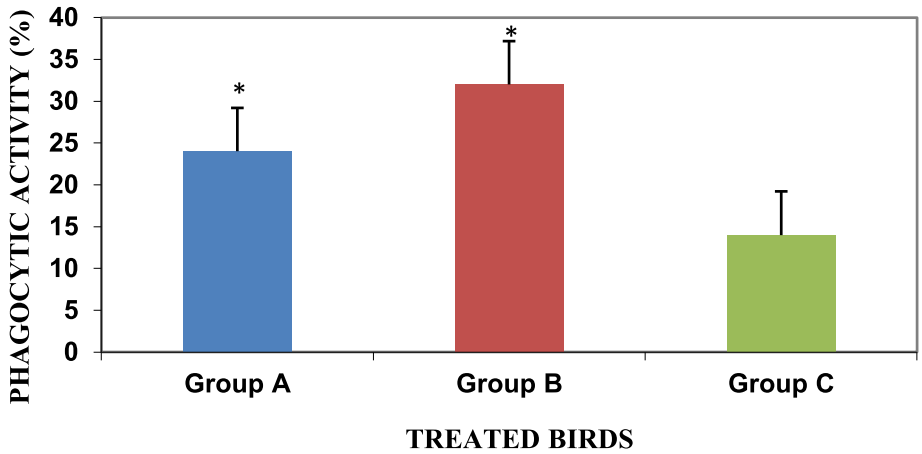

Fig. 6 Phagocytic activity of intestinal intra-epithelial leucocytes (iIEL) of control and MG-fed broiler birds after challenge with ND virus

showed better results. Therefore, it is advisable to use 30 mg MG/kg feed in broiler birds for better output in terms of protection from infectious pathogen, viz. NDV.

In short, supplementation of mushroom glucan with feed (preferably by 30 mg MG/kg) having feeding regime of 20 days, showed a considerable immunostimulatory effect on broiler birds. These could be considered useful in broiler birds to prevent highly infectious diseases like ND as immunostimulants have great importance in improving the immune system and minimizing the effect of infectious diseases in poultry flocks (Ali et al., 2019), thus allowing maximum performance in poultry production (Abdel-Hafez and Mohamed, 2016).

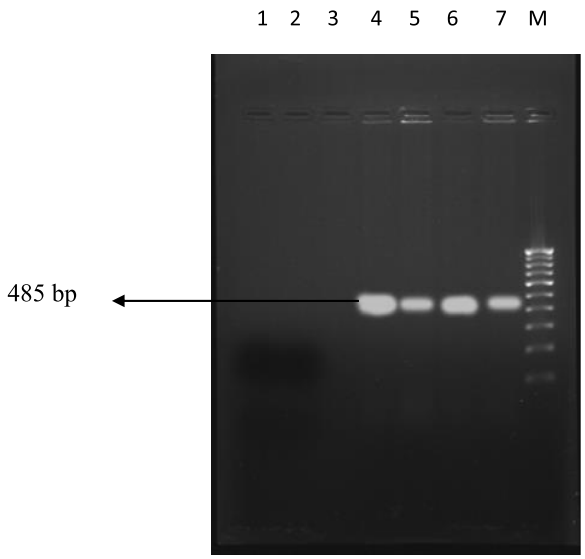

Fig. 7 PCR result showing production of IFN-γ in MG-fed birds and control group. Lane 1, negative control; lane 2 and 3, PBMC and iIEL of control group of birds; lane 4 and 5, PBMC and iIEL of group A bird; lane 6 and 7, PBMC and iIEL of group B bird; and lane M, 100 bp DNA ladder

Conclusion

It is observed from the experiment that protective immunity against Newcastle disease was comparatively more in mushroom glucan-fed broiler chicken. When compared to the treatment regime, it was observed that in all the cases, broiler chicken fed with 30 mg mushroom glucan/kg feed showed better immunostimulatory (and protective) activities. Therefore, it is concluded that the use of 30 mg mushroom glucan/kg feed may be used for better output in terms of disease protection against Newcastle disease (ND) virus in broiler chicken.

Abbreviations

cDNA: Complementary deoxyribonucleic acid; ConA: Concanavalin-A; CPCSEA: Committee for the Purpose of Control and Supervision of Experiments on Animals; DTT: Dithiothreitol; EDTA: Ethylene diamine tetra acetic acid; HI: Haemagglutination inhibition; iIEL: Intestinal intra-epithelial leucocytes; LPA: Lymphoproliferation assay; LPS: Lipopolysaccharide; MG: Mushroom glucan; MTT: [4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; NBT: Nitro blue tetrazolium; NDV: Newcastle disease virus; PA: Phagocytic activity; PBMC: Peripheral blood mononuclear cells; RPMI: Roswell Park Memorial Institute; RT-PCR: Reverse transcription-polymerase chain reaction; WBUAFS: West Bengal University of Animal and Fishery Sciences

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Authors' contributions

All authors suggested the study, participated in its design and coordination, interpreted the results and approved the final manuscript.

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Availability of data and materials

All the data and materials used to reach the conclusion of this study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the Institutional Ethical Committee of the Faculty of Veterinary and Animal Sciences, West Bengal University of Animal and Fishery Sciences, Kolkata.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]Department of Veterinary Microbiology, Faculty of Veterinary and Animal Sciences, West Bengal University of Animal and Fishery Sciences, Kolkata 700037, India. [2]Department of Animal Nutrition, Faculty of Veterinary and Animal Sciences, West Bengal University of Animal and Fishery Sciences, Kolkata 700037, India. [3]Department of Biotechnology, Indian Institute of Technology, Kharagpur 721302, India.

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