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Research Article
Phytogenic Feed Additive Enhance Innate and Humoral Immune Response to Newcastle Disease Virus Vaccination in Broiler Chickens

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Abstract

Objective: The objective of this study was to evaluate the effects of a phytogenic feed additive (Biostrong 510), which contains Saponins to modulate the immune system of poultry and to improve vaccination efficacy. **Materials and Methods:** In this study, four groups of broiler chickens (40 each) were reared for 10 days under same conditions. Groups 1 and 2 were fed diets containing phytogenic feed additives (PFA) at 150 mg kg^{-1} (Biostrong 510, Delacon Biotechnik GmbH, Austria) whereas groups 3 and 4 continued to be fed without any PFA as control groups. Chickens in groups 1 and 3 were vaccinated with Newcastle disease vaccine (NDV-Lasota strain) at 14 days of age whereas groups 2 and 4 were left unvaccinated as controls. Immune organ/body weight ratio; phagocytic activity; nitric oxide, lysozyme and total antioxidant activity were measured. In addition, mean titers of heamagglutinating antibodies to NDV were determined. **Results:** This study showed that in comparison to the control groups, chickens fed Biostrong 510 demonstrated a significant increase in bursa/body weight ratio, significant increase in total antioxidant activity and phagocytic indexes. However, no significant effects were determined in nitric oxide and lysozyme concentration. **Conclusion:** Biostrong 510 has the potential to stimulate the innate immune response and enhance antibody production against NDV.

Key words: Broiler chicken, immunity, NDV, phytogenic feed additive, vaccination

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Immunity failure in chickens occurs quite often because of biological or chemically induced immunosuppression, variation of avian pathogens and irregular use of vaccines[1]. Immunosuppressed flocks may have high susceptibility to secondary infections, poor feed conversion ratio and low response to commonly used vaccines[2]. Immunostimulants are substances which stimulate the immune system by inducing or increasing activity of any of its components. In poultry production, immunostimulants are applied to improve immunity. Herbal modulators can be administered along with the vaccine to elicit a faster and stronger immune response. Herbal immunomodulators are paving their way as safe to be used[3-5]. The PFA may add to the set of non-antibiotic growth promoters for use in livestock, such as organic acids and probiotics. Phytogenic Feed Additives (PFA, often also called phytobiotics or botanicals) are commonly defined as plant-derived compounds used in poultry nutrition to improve performance and health of the birds.

PFA contain a vast variety of different compounds like herbs and spices and products derived thereof, mainly essential oils but also saponins, pungent substances and bitter substance. Different effects of compounds of PFA like antioxidative properties (e.g. monoterpenes thymol and carvacrol, flavonoides, anthocyanes), antimicrobial actions (phenolic compounds being the principal active components) and growth promoting efficacy (e.g. by affecting the ecosystem of gastrointestinal microbes, improving digestibility by enhancing the activity of digestive enzymes) are reported for poultry.

Saponins are a diverse group of natural compounds occurring in a wide variety of plants, food and a few marine animals. They are classified into two categories (steroidal saponins and triterpenoid saponins) according to the nature of their aglycone skeleton[6]. Many plant based drugs and Traditional medicines contain saponins that have several kinds of bioactivities, such as antiviral, anti-inflammatory, anti-parasitic, immune-enhancing, anti-cancer and antimicrobial activities[7]. They derive their name from their ability to form stable, soap-like foams in aqueous solutions. Saponins are a class of high molecular weight secondary metabolites widely distributed in plants. They are important because of their use in industrial as well as potential pharmacological activity as immune-adjuvant[8,9]. There is significant demand for Saponins, particularly due to their presence in phytomedicines and as modern immune-adjuvants in commercial vaccines.

Medical pressure and public interest resulted in the systematic removal of antibiotics from poultry diet. Consequently, the poultry industry has attempted to obtain substances that could replace antibiotics as growth promoters.

The objective of this study was to evaluate the effects of a phytogenic feed additive (Biostrong 510), which contains Saponins to modulate the immune system of poultry and to improve vaccination efficacy.

MATERIALS AND METHODS

Ethical approval: This study was carried out in accordance with the regulations of the Public and Ethics Health committee in Egypt. The use of animals and protocols was approved by the Animal Care and Use Committee in Egypt.

Experimental design: A total of 200 one-day old chicks were obtained from a commercial poultry production company in Egypt. At one-day old the birds were vaccinated at the hatchery with vaccines against Newcastle disease, Gumboro, Influenza (H9N2) and Infectious Bronchitis viruses. Chicks were floor reared, fed a balanced commercial poultry ration and maintained under optimal hygienic conditions for 10 days. The birds were then divided into 4 groups. Two groups were fed a control diet and the other two groups were fed a diet containing Biostrong 510 (Delacon Biotechnik GmbH, Austria) at 150 g per ton. Biostrong 510 contains saponins, micro-encapsulated essential oils, bitter substances and pungent substances. The four different experimental groups included:

- **Group 1:** Chicks fed a ration supplemented with Biostrong 510 product and vaccinated with NDV vaccine at 14 days of age
- **Group 2:** Chicks fed a ration supplemented with Biostrong 510 product and not vaccinated with NDV vaccine
- **Group 3:** Chicks fed a basal ration without feed additives and vaccinated with NDV vaccine at 14 days of age
- **Group 4:** Chicks fed a basal ration and not vaccinated (control group)

Two blood samples were taken from four birds in each group weekly for 5 successive weeks via heart puncture. One sample was collected in sterilized plastic centrifuge tubes containing heparin for separation of mononuclear cells for use in phagocytic activity assays. The other sample was collected in tubes lacking anticoagulant to separate serum for use in hemagglutination inhibition (HI), lysozyme activity, nitric

oxide and total antioxidant capacity assays. Body weight was measured weekly. At the end of the experimental period, lymphoid organs (bursa of fabricius, thymus and spleen) were removed, cleared of fat and tissue debris and then weighed[10]. The weight was expressed as a percentage and calculated as:

$$\frac{\text{Weight of organs (g)}}{\text{Total body weight (g)}} \times 100$$

Measurement of phagocytic activity of peripheral blood monocytes using *Candida albicans*: Separation of peripheral blood mononuclear cells was carried out as previously described[11] using Ficoll-Hypaque. Mononuclear cell layers were collected, washed and re-suspended in RPMI media containing 10% fetal calf serum[12] and cell viability was measured as previously described by Cheung *et al.*[13] and Chu and Dietert[11]. Phagocytic percentage was calculated by dividing the number of macrophages that ingested *Candida* by the total number of macrophages×100. The phagocytic index was calculated by dividing the number of macrophages that ingested more than 3 blastospores by the total number of macrophages that had ingested blastospores.

Hemagglutination inhibition test (HI): Assays of hemagglutination inhibition were carried out as described by Beard[14]. Briefly, two-fold diluted serum samples were incubated with 4 HA unit of NDV antigen for 30 min at room temperature before 1% prepared chicken RBCs were added. The plates were left at room temperature for 30 min. Results were expressed as positive in when hemagglutination was inhibited. Positive and negative control sera were also assessed.

Measurement of lysozyme activity: Lysozyme activity was determined as previous described by Schultz[15]. Lysoplates were prepared by dissolving 1% agarose in 0.06 M PBS (pH 6.3) with 500 mg L^{-1} *Micrococcus lysodeikticus* added to 1 liter of agarose. The agarose mixture was distributed into 6 plates each on is 4:5 nm in diameter and 25 µL of serum samples and standard lysozyme were added to each well. After 18 h, the diameter of the cleared zones was measured and the lysozyme concentration was estimated.

Determination of nitric oxide (NO): Nitric oxide levels were determined according to the method described by Rajaraman *et al.*[16]. Serum samples (100 µL) were incubated at 25°C for 10 min with an equal volume of Griess reagent in flat bottom 96-well ELISA plates. The absorbance was measured at 550 nm using an ELISA plate reader and the NO concentration was calculated from a standard curve generated using $NaNO_2$.

Total antioxidant activity: Total antioxidant activity was determined according to the method described by Koracevic *et al.*[17]. In brief, the capacity of sera to inhibit production of thio-barbituric reactive substances from sodium benzoate under the influence of free oxygen radicals derived from Fenton's reaction was measured. A 1 mmol/liter uric acid solution was used as a standard.

Statistical analysis: Data obtained were statistically analyzed using one way analysis of variance and comparison between groups was performed using least significant difference (LSD) at $p = 0.05$ according to Petrie and Watson[18]. All analyses were conducted using SPSS and graphs were created using GraphPad Prism 7.0 software.

RESULTS

Effect of Biostrong 510 on phagocytic percentage and phagocytic index: Measurement of phagocytic percentage and phagocytic index revealed that among chickens fed a diet supplemented with Biostrong 510, only Group 2 (fed Biostrong, not vaccinated) showed a significant increase in phagocytic percentage at 2 and 4 weeks (Fig. 1). A significant increase was also observed in phagocytic index at 3 and 4 weeks compared with negative control birds (Group 4). Meanwhile, birds in Group 1 (fed Biostrong, vaccinated against NDV) showed a notable but not significant increase in phagocytic percentage and index relative to the other groups.

Effect of Biostrong 510 on lysozyme concentration (ug mL^{-1}): Measurement of lysozyme levels showed that Biostrong 510 supplementation did not affect lysozyme concentration at any point during the experimental period (Fig. 2).

Effect of Biostrong 510 on nitric oxide (NO) concentration (µmol mL^{-1}): The nitric oxide levels in serum samples showed no significant differences across all four experimental groups (Fig. 3).

Effect of Biostrong 510 on total antioxidants: Birds fed on a diet supplemented with Biostrong 510 and vaccinated with NDV vaccine (group 1) showed a significant increase in the concentration of total antioxidant compared to birds fed on

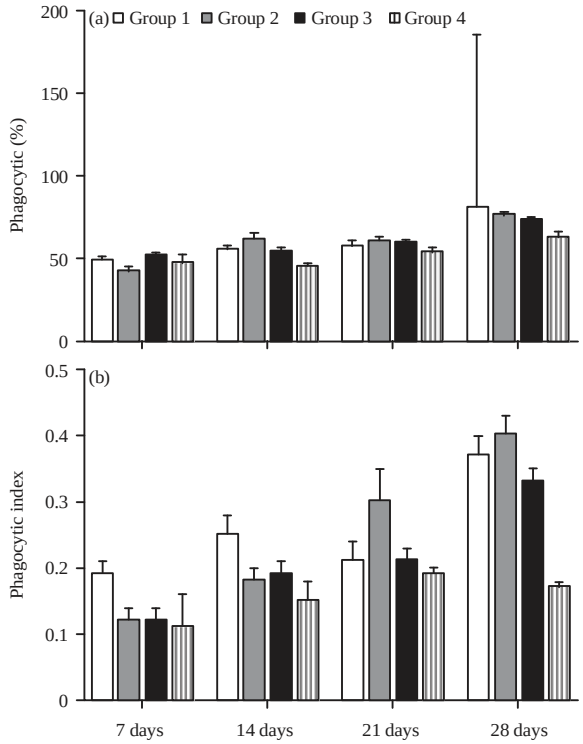

Fig. 1(a-b): Effect of Biostrong 510 on phagocytic percentage and phagocytic index of chicken macrophages isolated from the four experimental groups between day 7 and 28 of the experimental period

Results are expressed as mean phagocytic percentage and phagocytic index±standard error of mean (SEM). Error bars represent the SEM. Group 1 and 2 included chicks fed a diet containing Biostrong 510 that were and were not vaccinated with NDV vaccine at 14 days of age, respectively. Groups 3 and 4 included chicks fed on basal ration without feed additives that were and were not vaccinated with NDV vaccine at 14 days of age, respectively. Group 4 served as the control group

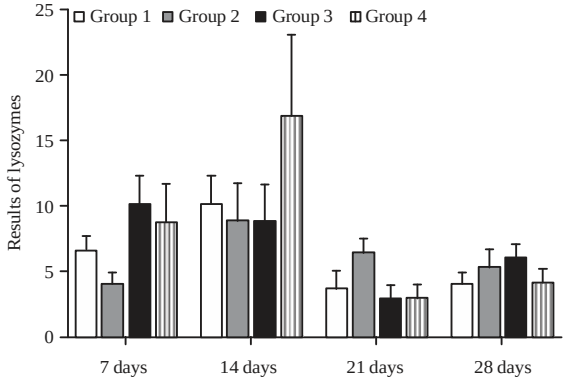

Fig. 2: Effect of Biostrong 510 on lysozyme concentration (μg mL^{-1}) in experimental groups between day 7 and 28

Results were expressed as the mean lysozyme concentration (μg mL^{-1}) ±SEM. Error bars represent the SEM. Groups are as defined in Fig. 1

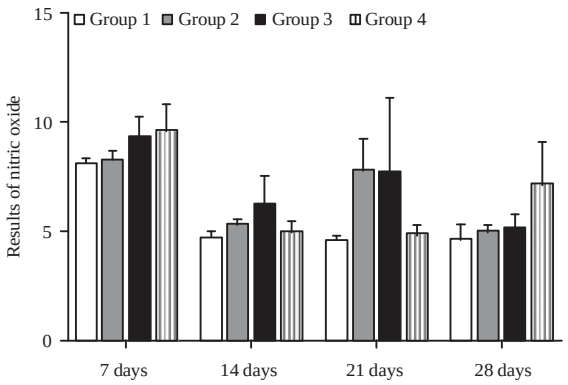

Fig. 3: Effect of Biostrong 510 on nitric oxide (NO) concentration (μmol mL^{-1}) in experimental groups between day 7 and 28

Results were expressed as mean nitric oxide (NO) concentration (μmol mL^{-1}) ± SEM. Error bars represent the SEm. Experimental groups are as described for Fig. 1

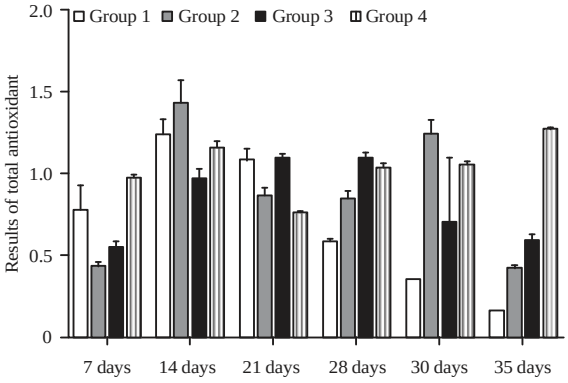

Fig. 4: Effect of Biostrong 510 on total antioxidants Mm L^{-1} in experimental groups (group 1-4), from 7-35 days

Results were expressed as mean total antioxidants Mm L^{-1} ±SEm. Error bars represent the SEm. Group 1: Chicks fed a ration with Biostrong 510 product and vaccinated with NDV vaccine at 14 days of age. Group 2: Chicks fed a ration with Biostrong 510 product and not vaccinated with NDV vaccine. Group 3: Chicks fed on basal ration without feed additives and vaccinated with NDV vaccine at 14 days of age. Group 4: Chicks fed on basal ration and not vaccinated (control group). SEm: Standard error of mean, NDV: New Castle disease virus

Biostrong 510 but not vaccinated with NDV vaccine (group 2) at 21 days' post vaccination. Meanwhile, at 30 days' post vaccination, birds in group 2 showed a significant increase in the level of total antioxidant compared with the negative control birds (group 4) (Fig. 4).

HI titers of the collected sera samples: The serological titers (Fig. 5) showed that control birds (group 4) demonstrated rapid and gradual decrease in antibodies reaching 3.2, while those of group 2 revealed slow decrease in antibodies. The

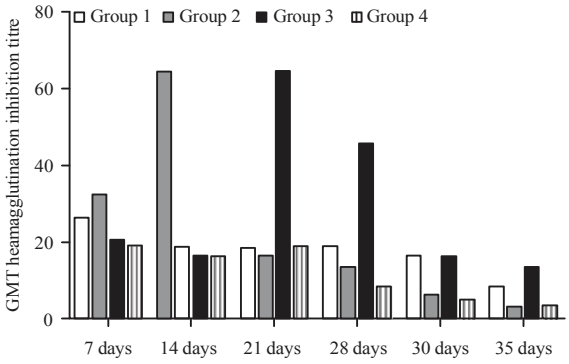

Fig. 5: Geometric mean titer (G.M.T) of chickens supplemented with Biostrong 510 and/or vaccinated with NDV vaccine in experimental groups (group1-4), from 7-35 days

Results were expressed as mean total antioxidants Mm L^{-1} ± SEm. Error bars represent the SEm. Group 1: Chicks fed a ration with Biostrong 510 product and vaccinated with NDV vaccine at 14 days of age. Group 2: Chicks fed a ration with Biostrong 510 product and not vaccinated with NDV vaccine. Group 3: Chicks fed on basal ration without feed additives and vaccinated with NDV vaccine at 14 days of age. Group 4: Chicks fed on basal ration and not vaccinated (control group). SEm: Standard error of mean, NDV: New Castle disease virus

level of HI antibody titer reached its maximum level in birds of group 1 at 14 days of age (GMT64). While, birds in group 3 showed the same result later at 21 days post vaccination.

Effect of Biostrong 510 on Bursa/body weight, spleen/body weight and thymus/body weight ratios: There was a significant increase in spleen/body weight ratio at 14 days of age in the groups fed on Biostrong 510 compared to the control groups. However, a significant decrease at such age was observed in the thymus/body weight ratio (Fig. 6) and there was no significant difference in bursa/body weight ratio.

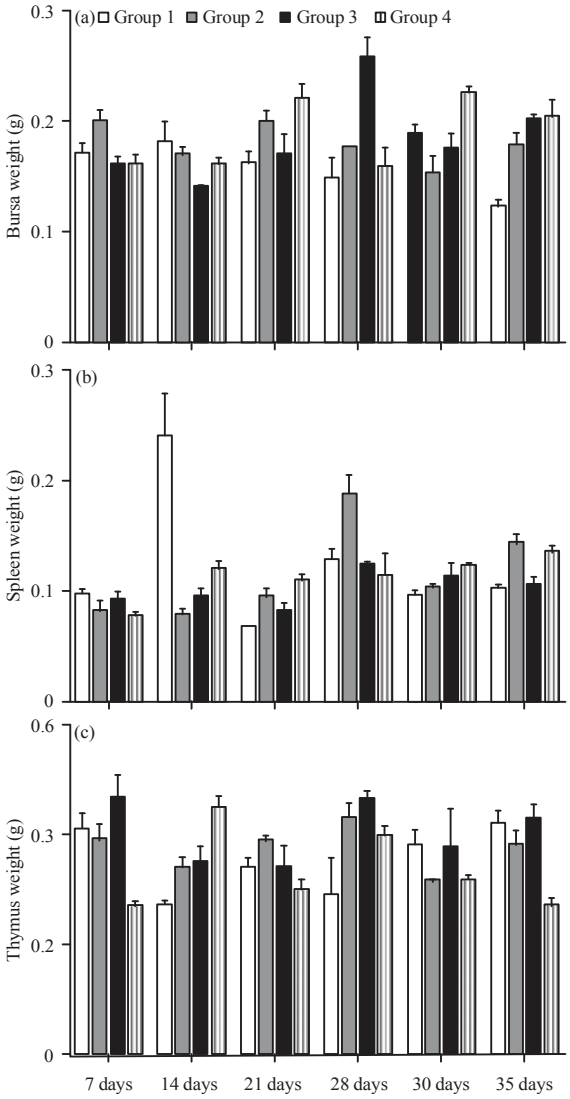

Fig. 6: The effect of Biostrong 510 on Bursa/body weight, spleen/body weight and thymus/body weight ratios in experimental groups (group 1-4), from 7-35 days

Results were expressed as mean grams ± SEm. Error bars represent the SEm Group 1: Chicks fed a ration with Biostrong 510 product and vaccinated with NDV vaccine at 14 days of age. Group 2: Chicks fed a ration with Biostrong 510 product and not vaccinated with NDV vaccine. Group 3: Chicks fed on basal ration without feed additives and vaccinated with NDV vaccine at 14 days of age. Group 4: Chicks fed on basal ration and not vaccinated (control group). SEm: Standard error of mean, NDV: New Castle disease virus

DISCUSSION

Macrophages play a crucial role in immunity against microbes by rapidly recognizing and phagocytosing pathogens. Moreover, macrophages activate antimicrobial effectors including NADPH oxidase (NOX), inducible nitric-oxide synthase (iNOS) and cationic antimicrobial peptides (AMPs) to contain and clear pathogens[19]. Increases in phagocytic activity can be elicited by saponins, as suggested by a previous study showing that saponin from *Ophiopogon japonicus* had marked macrophage-modulating activity represented by promotion of phagocytic capacity as well as increased macrophage viability rate, NO production and interleukin-1 release[20]. In humans and mice, orally administered Quillaja saponin induced significant increases in chemotactic and phagocytic activity of peritoneal macrophages and this effect continued for four days post-administration[21]. Mice fed rations containing saponins prior to intra-cerebral challenge with rabies virus showed enhanced production of rabies-neutralizing antibodies in serum.

Here, poultry given rations supplemented with saponin containing Biostrong 510 showed both enhanced

macrophage activity and adaptive immune responses. This result is consistent with a study by Jang *et al.*[22] who reported that ginseng extract containing saponin had immunomodulatory effects on phagocytic cells, lymphocytes and antibody production in humans and animals. The mechanism of saponins as an immunostimulants might be related to its ability to enhance the antigen presentation by macrophages through promotion of MHC class antigen expression[23] and by stimulation of the production of cytokines such as I-L1α by macrophages[22].

Saponins have natural antioxidant and immunostimulant properties. Naknukool *et al.*[21] reported that oral administration of Quillaja saponins enhanced the immune response through stimulation of macrophages. Saponins could also exert an immunostimulatory effect by modulating the activity of specific receptors such as toll-like receptor 4, which in turn affects production of cytokines and interferons that can activate macrophages.

Lysozymes perform many physiological and functional roles, including in host defense and innate immunity pathways. Lysozyme not only has antibacterial activity but also antiviral, anti-inflammatory, anticancer and immune-modulatory activities[24]. Whereas Helal and Melzig[25] found that exposure of human monocytes and epithelial cell lines to saponins initially increased lysozyme release. However, further incubation for up to 72 h decreased lysozyme secretion and activity. Here we saw no effect of Biostrong 510 supplementation on lysozyme levels and thus whether the saponins in the feed indeed contribute to enhanced anti-viral activity that was previously reported requires further investigation.

Nitric oxide is produced in large quantities by inducible nitric oxide synthases (iNOS) in activated macrophages and neutrophils during defense and immunological reactions[26]. Kim *et al.*[27] showed that total saponin of heat-processed ginseng stimulated the production of NO in IFN-g-primed macrophages and increased expression of iNOS protein. However, our results suggested that saponins might not affect nitric oxide production, as we saw no differences in nitric oxide levels in birds given Biostrong 510 supplementation.

Antioxidants inhibit oxidation and prevent production of free radicals to reduce oxidative stress that arises from an imbalance between reactive oxygen (free radicals) and/or antioxidants. Such imbalances stimulate the oxidation of macromolecules, such as proteins, enzymes, lipids and DNA[28]. Smith and Adanlawo[29] found that saponin extracts derived from *Garcinia kola* roots could be a source of natural antioxidants because it increases the free radicals scavenging ability as well as significant inhibition of Malondialdehyde (MDA) production and elevation in levels of free radical scavenging enzymes such as SOD and catalase. Furthermore, Akinpelu *et al.*[30] showed that saponins isolated from *E. suaveolens* could be used to prevent damage caused by free radicals and infections. Here we saw an initial increase in antioxidant levels for both vaccinated and unvaccinated birds fed Biostrong 510 that declined over time.

The results for HI were similar to those of Johansson and Lovgren-Bengtsson[31], who reported that Quillaja saponins enhance antibody production and can shift IgG subclass patterns to favor IgG2a in immunized mice. Also, immune-stimulating complexes formulated with Quillaja saponins induced antibody responses and/or protective immunity in guinea pigs, turkeys, cats, rabbits, dogs, seals, sheep, pigs, cows, horses and monkeys[32]. Herbal products containing saponins and essential oils enhanced the level of anti-NDV-HI antibodies in treated broilers[33,34]. Meanwhile, addition of plant extracts (*Radix astragali*, *Radix codonopis*, *Herba epimedii*, *Radix glycyrrhizae*) to drinking water or the use of vaccines containing ginseng stem-leaf saponins had higher antibody titers compared to untreated birds[35]. Birds inoculated with bovine serum albumin (BSA) in addition to saponin had a higher amount of BSA-specific IgA antibodies in the saliva and BSA IgY antibodies in the yolks of eggs produced by hens receiving lower doses of the glycoside[36].

Various lymphoid organs e.g., bursa of fabricius, thymus and spleen from birds in the four experimental groups were weighed. We saw a significant increase in the ratio of thymus weight to total body weight in birds supplemented with Biostrong 510, which could reflect increased production of plasma cells[35,36]. On the other hand, there was a significant increase in thymus/body weight ratios at 7 and 35 days post-vaccination in the groups fed Biostrong 510 compared to the control groups that may be due to increases in the number of activated macrophages that could prime the T cells to proliferate and produce cytokines that enhance antibody production[31].

Taken together, the results of this study demonstrate the potential of Biostrong 510 to stimulate innate immune responses and enhance antibody production against NDV following vaccination. The observed effects of Biostrong 510 could be due to the presence of saponins, which are known to have immunomodulatory activity.

CONCLUSION

The present study show the immune stimulatory effect of Biostrong 510 as one of PPA products especially on the innate immune system as well as the increased antibodies

production on the groups fed the PFA. The anti-inflammatory, anti-oxidative and cardiovascular activity due to saponin contents in the product may have contributed largely the main immune-stimulatory effects. However, the contribution of other components of Biostrong 510 like pungent substances and essential oils cannot be ruled out. Further characterization of modes of action of these substances on the cytokine profile in the chicken fed with PFA need to be addressed.

SIGNIFICANT STATEMENT

The present study documents the immune stimulatory effect of Biostrong 510 as one of PPA products especially on the innate immune system as well as the increased antibodies production in the groups fed on the PFA. The anti-inflammatory, anti-oxidative and cardiovascular activity due to saponin contents in the product may have contributed largely the main immune-stimulatory effects. The results of this study demonstrate the potential of Biostrong 510 to stimulate innate immune responses and enhance antibody production against NDV following vaccination. The observed effects of Biostrong 510 could be due to the presence of saponins, which are known to have immunomodulatory activity.

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