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INFLUENCE OF "AVIGEN DUCK" IMMUNOMODULATOR ON SOME HUMORAL FACTORS OF NON-SPECIFIC PROTECTION IN WHITE PEKIN DUCKS PARENTS

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Abstract

Field trials were carried out to investigate the impact of "AVIGEN DUCK" immunomodulator on the IFN-γ concentration, IL-2, IL-6, lysozyme, and beta-lysine activity in the blood serum in White Pekin ducks' parents. The IFN-γ, IL-2, and IL-6 were analyzed by immunoenzyme methods, while the lysozyme and beta-lysine activities were determined using conventional techniques. The measured values among experimental birds for most parameters were significantly higher than those in controls during the observed period. An exception to this trend were the concentrations of lysozyme and beta-lysine, in which an inverse relationship was noticed. During the experiments, the challenged flocks were bred without the use of antibiotics. Based on collected data we consider the immunomodulator "AVIGEN DUCK" a successful alternative tool in the fight against microbial resistance associated with the use of antibiotics in poultry farming, as well as a means of enhancing the natural immunity in aquatic birds.

Key words: immunomodulators, lysozyme, IFN, IL-2, IL-6, beta-lysine

Introduction. In the first days of life, congenital immune mechanisms predominate, and adaptive ones develop gradually, over the course of the first few

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weeks after hatching [1,2]. During this period the mucous surfaces of the respiratory and digestive tracts are largely unprotected, which is used as an entrance door for most pathogens. Intensive formation of IFN-γ in serum usually begins at the end of the second week of birds' life [3]. According to some authors [4,5], the lymphoid tissue of the bronchi and intestines is functionally mature even after hatching. Therefore, there are physiological foundations for the early activation of various immune factors. TEIJARO et al. [6], SONG et al. [5], and NETEA et al. [7] consider that this can be achieved by direct stimulation of the mucus, since parenteral administration of the antigen does not provide innate protection. The issue is also relevant to the economic losses caused by H5N1 influenza A viruses in breeding stock ducks.

In recent years, the problem of microbial antibiotic resistance has become a global threat [8,9]. WHO [8] indicates the mucous immunomodulators as one of the alternatives to the use of antibiotics in poultry farming.

The current field trials evaluate the impact of the immunomodulator "AVIGEN DUCK" on the degree of humoral non-specific protection and the possibilities of limiting the use of antibiotics in the industrial breeding of ducks.

Material and methods. A day-old White Pekin ducks were divided into two flocks – experimental and controlled and were grown under the same production conditions. Both flocks were treated with the preparations "ASPIVIT C" and "BIOXAN", which play an auxiliary role. The first has an anti-stress effect, while the second increases the permeability of the mucous intestinal surfaces. Polybacterial immunomodulator "AVIGEN DUCK" is a concentrated lipopolysaccharide extracted from the Enterobacteriaceae family. The immunomodulator was administered in liquid form, containing 6000 doses (ten days) in 1000 mL. It is used orally through drinking water, using the available farm dispensers. The experimental birds were treated twice from the 1st to the 10th day and then from the 110th to the 120th day of their life. The total number of birds in each group equals 12 000. Blood samples for analysis were obtained on the 160th and 210th day of life from 45 randomly selected birds from each group. The blood was collected aseptically from the axillary vein with disposable needles in plain vacuum tubes without anticoagulant. Serum was extracted via centrifugation at 3000 rpm for 10 min and stored at 4 °C until processed. Serum testing was done within 24 h after sampling.

Determination of interferon-γ, interleukin-2 and interleukin-6. All three parameters were analyzed by enzyme-linked immunosorbent assays via commercially available kits (DUCK Interferon (IFN-γ) and DUCK Interleukin ELISA (MYBIOSOURCE). Analyses followed the manufacturer's protocol and include: Addition of standards to the wells of the plate with levels: 0, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 pg/mL for IFN-γ; for IL-2 we prepared standards of 0, 6.25, 12.5, 25, 50, 100, 200 and 400 pg/mL; and for IL-6 – 6.25, 12.5, 25, 50, 100, 200 pg/mL, respectively. The optical density of the samples was determined spec-

trophotometrically at a wavelength of 450 nm. The calculation of the indicator levels was carried out on the basis of a standard curve.

Determination of lysozyme concentrations. Serum lysozyme levels were determined according to the method of Sotirov and Koynarski [10]. In this method, 20 mL of 2% agarose in phosphate buffer (0.07 M $NaHPO_4$ and NaH_2PO_4) and 20 mL suspension of *Micrococcus lysodeicticus* cultured for 24 h at 67 °C were prepared. Before it cools, the mixture is poured into a sterile petri dish. After the agar has solidified, 5 mm wells are drilled. Fifty µl of undiluted serum was added to the prepared wells. Eight standard dilutions of lysozyme (from 0.025 to 3.125 µg/mL) were made in advance and added dropwise to separate agar wells. The method proceeds with incubation for 20 h at 37 °C. Final lysozyme concentrations are calculated by special software developed at Trakia University (Bulgaria) by correlating the measured lytic zone for each serum with standard dilutions of lysozyme.

Determination of beta-lysine activity. The beta-lysine activity of blood serum was determined by a spectrophotometric method of Bucharin et al. [11], modified by Karakolev and Nikolov [12]. The analysis was performed using flat-bottomed plates. The method utilizes a pre-prepared spore suspension of Bacillus subtilis ATCC 6633. The controls are added with an automatic pipette – 80 µl of saline + 80 µl of spore suspension in each of the first 4 wells. Experimental sera are instilled with spore suspension in equal volumes (80 µl serum + 80 µl spore suspension) in subsequent wells, according to the number of samples tested. Samples are then homogenized via plate shaker and optical density is measured using a BioTek L80 spectrophotometer at a wavelength of 630 nm, before incubation. The technique proceeds with plate incubation at 37 °C for 2 h and second optical density measurement at the same wavelength. Since the optical densities of the controls did not change for 2 h in the incubator, we performed the calculations by taking the changes in the optical densities of the samples, for each well separately, according to the formula

$$\text{(1)} \qquad \%\ \text{of lysis} = \text{OD1} - \text{OD2}/\text{OD1} \times 100,$$

where OD1 is the sample's extinction before incubation and OD2 is the sample's extinction after incubation.

Statistical analysis. Descriptive statistics for each variable were calculated and presented as "Mean ± Standard Deviation". Data was processed by independent t-test with the fixed effect using the Data analysis tool pack, Microsoft Excel 2016, Microsoft Corporation Ltd., at a level of significance $P < 0.001$.

Results and discussion. The values of IFN-γ, IL-2, and IL-6 in the birds receiving "AVIGEN DUCK" (Table 1) differed significantly from the values of the same indicators for the control flock. The results for IFN-γ for the experimental herd at 160 days of age were significantly higher than the levels witnessed

T a b l e 1

Influence of "AVIGEN DUCK" immunomodulator on interferon-γ, interleukin-2 and interleukin-6 concentrations in blood serum

Groups	Interferon-γ, pg/mL	Interleukin-2, pg/mL	Interleukin-6, pg/mL
160th day of life			
Experimental	534.12±12.60*	212.38±15.10*	95.49±6.42*
Control	132.40±8.25	52.90±6.30	25.85±4.80
210th day of life			
Experimental	506.35±14.10*	186.14±16.22*	129.14±12.54*
Control	94.84±7.55	55.28±10.18	28.60±6.18

$^*P < 0.001$

among the control birds – 534.12 ± 12.60 pg/mL vs. 132.40 ± 8.25 pg/mL, respectively. The measured average value of interferon for the experimental birds had a value four times higher compared to the controls. At the next blood collection at 210 days of age, we obtained mean values of interferon 506.35 ± 14.10 pg/mL for the experimental flock, while the interferon concentrations among the controls remained significantly lower at 94.84 ± 7.55 pg/mL.

The birds receiving the immunomodulator, demonstrated high levels of cytokines at the first measurement on day 160 – 212.38 ± 15.10 pg/mL for IL-2 against only 52.90 ± 6.30 pg/mL for the control flock. As seen from the data in Table 1, a similar trend was also observed at 210 days of age. IL-6 values in birds receiving immunomodulator were 95.49 ± 6.42 pg/mL on day 160 and increased to 129.14 ± 12.54 pg/mL on day 210. In contrast, IL-6 concentrations in control birds remained at low levels.

The effects of the tested immunomodulator were witnessed for the blood serum lysozyme and beta-lysine concentrations as well. On the 160th day and on the 210th day of the life of the birds receiving an immunomodulator, lysozyme values were 7.85 ± 0.46 mg/mL and 8.64 ± 0.86 mg/mL, respectively (Table 2).

T a b l e 2

Serum lysozyme concentrations and beta-lysine activity

Groups	Lysozyme, mg/mL	Beta-lysine activity, %
160th day of life		
Experimental	7.85±0.46*	11.49±1.18
Control	2.50±0.45	36.45±1.82
210th day of life		
Experimental	8.64±0.86*	9.89±0.95*
Control	3.18±0.44	35.48±1.84

$*P < 0.001$

Suddenly, beta-lysine activity demonstrated an insignificant decrease between the two test periods – $11.49 \pm 1.18\%$ on the 160th and $9.89 \pm 0.95\%$ on the 210th day. Low lysozyme values below 4.0 mg/mL were witnessed in control birds that did not receive immunomodulator. In contrast, beta-lysine activity was high – $36.45 \pm 1.82\%$ on the 160th day and $35.48 \pm 1.84\%$ on the 210th day. These data testify to an inverse proportional dependence in the dynamics of the two factors studied.

During the entire period of rearing, no antibiotics in any form were administered to birds treated with the immunomodulator.

Several studies on germ-free animals show that symbiotic bacteria and/or bacterial molecules (lipopolysaccharides, β-glucan and peptidoglycans) can completely trigger a non-specific immune response [1, 2, 13, 14], as the intestinal mucosa has its leading meaning in the initial activation of the innate immunity and influence its regulation and maturation.

The nucleus of the polysaccharide represents the intrinsic, common to most bacteria areas which is not associated with the somatic antigen and makes possible the rapid recognition of many pathogens. These signals from the LPS nuclei are received by Toll-like receptors, which resemble a "start button" of innate immunity [15].

It has already been proven that in this case, the immune system recognizes not the unique region of the antigen, but group markers characteristic of a wide range of pathogens. In this way, the mucosal immune system conducts an "express analysis" for the presence of a dangerous marker, and the signal from the LPS nuclei activates the immune response. Thus, the immunomodulator prepares the bird's immune system for a rapid reaction against multiple pathogens simultaneously. This effect is associated with the stimulation of macrophages, interferon production, lysozyme, and other factors of nonspecific immunity. Several authors [16–18] highlight the crucial importance of interferon and cytokines for nonspecific antiviral protection in Pekin ducks. Some authors [18, 19] point to differences in the innate immune response of chickens and ducks against the low-pathogenic avian influenza virus. We found that after stimulation of mucosa with LPS by Enterobacteriaceae, ducks activate IFN-γ synthesis, similar to broiler chickens. Similar data were obtained regarding the concentrations of lysozyme in the blood serum, which marked a reliable increase in the ducks receiving the immunomodulator according to the respective schedule. In our experiments, we also applied two auxiliary preparations: "ASPIVIT C", with its anti-stress action and "BIOXAN", which improves the resorptive ability of mucosal surfaces. Since these preparations were applied in parallel in the control and experimental flock, it can be confidently concluded that the increased activity of nonspecific protection of the experimental birds is due to the action of the immunomodulator. In the period of the proliferation, we measured increased beta-lysine activity in both herds. This confirms the thesis expressed earlier by us that beta-lysine values rise

in stress states [20], and this indicator can be studied not only as a factor of innate immunity but also as a stress indicator.

According to the global report of the World Health Organization to combat microbial resistance to antibiotics, the solution to the problem goes through the creation and application of innovative tools and technologies to limit the use of antibiotics in poultry and livestock breeding. As evidenced by our large-scale field trials, it is possible to exclude antibiotic prophylaxis and therapy in raising parents for Pekin ducks.

In this way, several objectives are achieved: an essential contribution to the fight against the formation of microbial resistance to antibiotics; elimination of the risk of antibiotic residues in poultry meat, and reduction of the cost of production. WHO recommended the use of mucosal immunomodulators as an alternative to the uncontrolled use of antibiotics in animal husbandry. The trend in all EU countries is the complete exclusion of antibiotics for prophylactic purposes in poultry breeding.

Conclusion. The immunomodulator "AVIGEN DUCK" induces high values of IFN-γ, interleukin-2 and interleukin-6 in the blood serum of parents for Pekin ducks, thus providing an effective humoral protection of a non-specific nature. Beta-lysine activity and lysozyme concentration in the blood serum of birds demonstrated inversely proportional dynamics in our trials. Immunomodulator-treated birds may be reared without antibiotics throughout the laying period.

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