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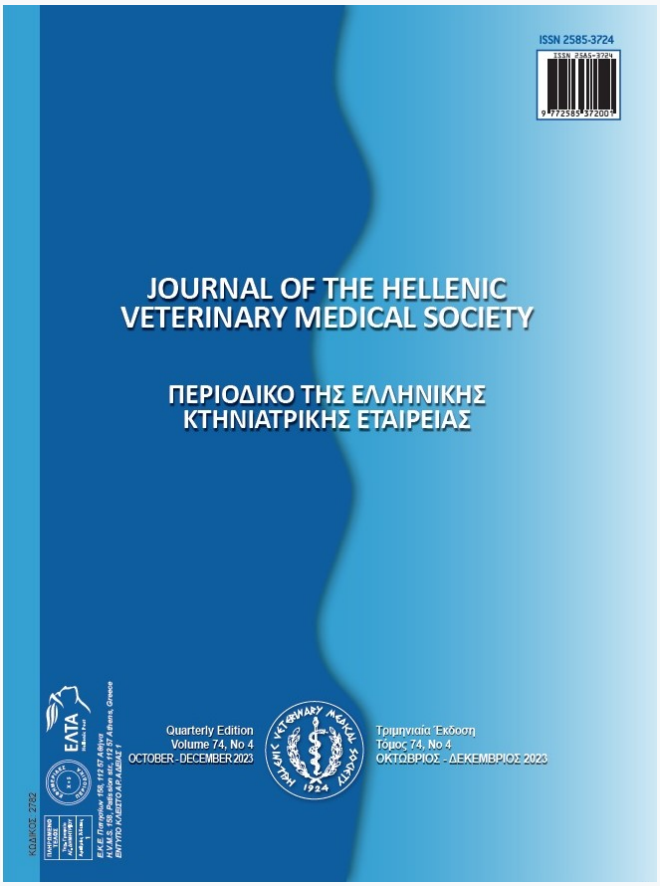


# Opportunities to improve the immune status of broiler chickens

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Research article
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# Opportunities to improve the immune status of broiler chickens

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**ABSTRACT:** Since 2006, the European Community has banned the use of nutritional antibiotics as growth promoters in livestock husbandry. The utilization of modern poultry production systems with a high degree of concentration and automation has resulted in the health status of sensitive poultry strains and hybrids. Some humoral indicators of natural immunity in broiler chickens from the ROSS 308 hybrid and growing broiler breeders were studied. The experiment was conducted in control and experimental herds raised under the same production conditions. The experimental herds were reared without the use of antibiotics but were treated with the immunomodulator Avigen. The values of serum lysozyme concentrations, the alternative pathway of complement activity (APCA), and concentrations of IFN γ, IL-2, and IL-6 of broilers treated with Avigen significantly exceeded the values in the control birds. The same tendency is observed among the growing broiler breeders. Serum IgY concentrations show a significant difference in growing broiler breeders but not in broilers. Based on the obtained data, the treatment with the immunomodulator Avigen increases the natural resistance of birds, which allows them to be raised without the use of antibiotics in production conditions.

***Keywords***: Complement; IFN γ; IL-2; IL-6;Lysozyme;Chicken.

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## INTRODUCTION

Modern poultry farming requires fast-growing periods, high food conversion ratios, and maintenance of health status at the lowest possible cost. One of the biggest challenges is the high bird population density, raised in relatively small spaces, which increases the risk of spontaneous disease outbreaks (Linares and Martin, 2010). The first two necessities are influenced by the nutritional properties of the food provided. The third requirement has a far more complex nature where animal selection and the introduction of specific food supplements play a crucial role. Currently, marker-assisted selection is a common approach in livestock breeding (Abasht et al., 2009) However, targeting a high immune response against a specific pathogen is not always the best option. Farmers face subclinical infections from a well-known cocktail of locally presented infectious agents in several cases. The unpredictable nature of such processes requires prominent levels of non-specific immune response factors among all animals (Al-Mansour et al., 2011). Lysozyme is a major factor in the humoral non-specific protection of birds and the natural resistance of the embryo (Xia et al., 2019). The serum lysozyme protects the host organism against a variety of Gram-negative bacteria and some large viruses, such as the Avipoxvirus (Gadde et al., 2017; Gong et al., 2017; Ma et al., 2017; Abdel-Latif et al., 2017). It is well-known that serum lysozyme is secreted by macrophages (Tagashira et al., 2018) and its concentrations depend on various factors. EL-Deepetal. (2020) reported that rabbit supplemented with dietary egglysozyme increased body live weight and decreased FCR. The following year (2021), the same authors repeated th eexperiment and confirmed the results obtained in 2020.Ferraboschietal. (2021) published an interesting review in which they investigated the application of lysozymeas a natural immuneprotective factor and alternative to antibiotics in human and veterinary medicine and as a food additive. The alternative pathway of complement activation (APCA) is an important factor of innate immunity. It is active against Gram-negative bacteria, viruses, virus-infected cells, neoplastic cells, agarose, lipopolysaccharides, contrast media used in radiology, etc. (Tirado et al., 2021; Idowu et al., 2021). In the body of birds, the complement system is inactive, but after activation, its action is cascading and includes a series of proteolytic reactions aimed at enhancing humoral and cellular interactions (Sotirov and Koynarski, 2003). Bainetal. (2020) reported that people with severe infections survived more successfully if they had a higheractivity of the alternative pathway of complementactivation compared to people who had an average or lower activity of this indicator.IgY is found in birds' blood serum and egg yolk and is formed by plasma cells in response to antigenic effects (Tizard, 2002). Its effect by affecting the intestinal mucosa may be a guideline for the relationship between mucosal and systemic immunity. Type II interferons (IFN γ) are products of T lymphocytes and natural killer cells in birds. IFN α/β is mainly involved in the formation of antiviral immunity, and IFN γ is a pleiotropic molecule that to one degree or another affects all stages of the immune response (Seo et al., 2002; Wigley and Kaiser, 2003). According to Rosenberger et al. (2000), and Sato and Iwasaki (2005),bacterial endotoxins can induce interferon production in the body. The rationale for using lipopolysaccharides (LPS) from gram-negative bacteria as an alternative to antibiotics to modulate the broiler immune response is the investigations done before (Bonovska et al., 2014; Karakolev et al., 2015; Fan et al., 2020; Karakolev et al., 2023). The results obtained are very good evidence to suggest that LPS is effective in improving the natural immunity of chickens. In the present experiment, the effect of the polybacterial immunomodulator Avigen on some indicators of natural humoral immunity in broiler chickens and their parents reared under production conditions was studied.

## MATERIALS AND METHODS

### Broiler chickens

The experiment was performed with broiler chicken's hybrid ROSS 308 reared in two halls. The experimental broiler chickens received immunomodulator Avigen from days 1st to 10$^{th}$ day and were reared without antibiotics. The control chickens received only antibiotics (Lincospectin and Lincomycin).

### Growing broiler breeders

The growing broiler breeder's herds used in this experiment were also reared in two different halls. The control herd did not receive immunomodulator Avigen and the experimental herd was treated with Avigen.

Polybacterial immunomodulator "Avigen" - contains lipopolysaccharide components in concentrated form, extracted from Gram-negative bacteria from the family *Enterobacteriaceae* and is produced as suspension.

**Method of treatment**

Each chick needs from the 1st to 10th day of the chick's life 700 ml water. The experimental broiler herd was treated orally with drinking water plus immunomodulator Avigen from day 1st to day 10th (1085 l water + 5,115 l Avigen for 1550 chickens). At age 120th to 130th day one chick drank 2250 ml water for 10 days. The experimental growing broiler breeder's flock was also treated orally from the 1st to the 10th day and from the 120th to the 130th day (3255 l water + 15,555 l Avigen for 1446 chicken).

**Samples**

Forty-five blood samples were randomly taken from broilers (on the 36th day) and from the growing broiler breeders (on the 36th day and the 146th day). The blood was taken from *v. subcutaneous ulnaris*. The samples were left to clot for 30 minutes, centrifuged at 2000 rpm, and the sera were harvested.

**Determination of lysozyme concentration in blood serum**

Serum lysozyme concentrations were tested by the method of Lie (1985).Briefly, twenty ml of 2% agarose dissolved in phosphate buffer (0.07 M NaHPO4 and NaH2PO4) was mixed with 20 milliliters suspension of the 24-hour culture of *Micrococcus lysodeicticus* at 67°C. The mixture was poured out in a 14-cm Petri dish. After solidifying at room temperature, thirty-two 5-mm wells were made with a special device. Fifty microliters of undiluted sera were piped in each well. Eight standard lysozyme dilutions (from 0.025 to 3.125 μg/ml) were prepared and pipetted in weight wells. The samples were incubated for 20 hours at 37°C and lytic zone diameters were measured. The final lysozyme concentrations were calculated by special software developed at Trakia University.

**Determining the activity of the alternative pathway for complement activation**

The activity of APCA was tested by the method of Sotirov (1991). Each serum sample was first diluted by mixing 100μl serum with 350 μl veronal-veronal Na buffer (in final concentrations: 146 mM NaCl, 1,8 mM 5,5- diethylbarbituric acid sodium salt; 3,2 mM 5,5- diethylbarbituric acid; 1 mM EGTA and 0,8 mM MgCl2). In U-bottomed plates (Flow Laboratories, UK), 7 other dilutions from each diluted serum were again prepared in veronal veronal Na buffer: 80 μl diluted serum + 20 μl buffer, 70 μl diluted serum + 30 μl buffer, 60 μl diluted serum + 40 μl buffer, 50 μl diluted serum + 50 μl buffer, 40 μl diluted serum + 60 μl buffer, 30 μl diluted serum + 70 μl buffer and 20 μl diluted serum + 80 μl buffer. The final serum dilutions were, respectively, 8/45, 7/45, 6/45, 5/45, 4/45, 3/45 and 2/45. Then 50 μl buffer and 100 μl of 1% rabbit erythrocyte suspension were added to each well. After incubation for 1 hour at 37°C, samples were centrifuged at 150 g for 3 minutes at room temperature (23°C). Thereafter, 150 μl of each supernatant was removed and placed in flat-bottomed plates for measurement of optical density at 540 nm using a 'Sumal-PE2' ELISA reader (Karl Zeiss, Germany). The final APCA activity was calculated using special computer programs developed at Trakia University and expressed as CH50 units (CH50 units correspond to 50% of complement-induced hemolysis of applied erythrocytes).

**Determination of IFN-γ, IL-2, IL-6, and IgY**

The traits mentioned above were investigated by using different ELISA tests - Chicken interferon γ (IFN γ) ELISA kit, Chicken Interleukin 2 (IL-2) ELISA kit, Chicken Interleukin 6 (IL-6) ELISA kit, Chicken Immunoglobulin Y (IgY) ELISA kit (CUSABIO).

**Statistical analysis**

Data were processed by one-way analysis of variance (ANOVA) with the fixed effect model using the Data analysis tool pack, Microsoft Excel 2016, Microsoft Corporation Ltd. at a level of significance $P<0.05$.

**RESULTS**

As can be seen from the Table. 1 the concentration of serum lysozyme in experimental broiler chickens was higher thanin the control flock ($P <0.001$). In the experimental broiler herd, lysozyme concentrations varied from 2.21 mg/L to 8.83 mg/L (average of 6.17 ± 0.49 mg/L). In control birds, these levels were significantly lower and ranged from 1.10 mg/L to 4.41 mg/L (average of 2.99 ± 0.27 mg/L). In experimental chickens, significantly higher APCA activity (549.00 ± 19.69 CH 50) was found while in control chickens this value was lower(377.40 ± 9.58 CH50; $P <0.001$). Similar results were obtained in the growing broiler breeder's herds. The mean lysozyme concentrations (6.42 ± 0.76 mg/mL) and APCA (465.00 ± 15.08 CH50) in birds treated with immunomodulator Avigen were significantly higher than in controls ($P <0.001$). In broilers, the concentrations of IgY, were

**Table1.** Serum lysozyme concentrations, APCA, and IgY concentrations in broiler and broiler breeders.

| Groups | Lysozyme (mg/mL) | APCA (CH50) | IgY (mg/mL) |
|---|---|---|---|
| | Broilers | | |
| Experimental | 6.17 ± 0.49*** | 549.00 ± 19.69*** | 4.74 ± 0.62 |
| Control | 2.99 ± 0.27 | 377.40 ± 9.58 | 4.68 ± 0.58 |
| | Growing broiler breeders | | |
| Experimental | 6.42 ± 0.76*** | 465.00 ± 15.08*** | 18.10 ± 1.45** |
| Control | 2.64 ± 0.25 | 312.00 ± 10.52 | 12.26 ± 1.28 |

*** P < 0.001; ** P < 0.01

**Table 2.** Concentrations of IFN γ, IL-2, and IL-6 in the blood serum of broilers and broiler breeders.

| Groups | IFN γ (pg/mL) | IL-2 (pg/mL) | IL-6 (pg/mL) |
|---|---|---|---|
| | Broilers | | |
| Experimental | 548.23 ± 2.18*** | 6.28 ± 0.76*** | 612.55 ± 2.54*** |
| Control | 127.04 ± 1.09 | 1.87 ± 0.61 | 204.18 ± 1.32 |
| | Growing broiler breeders | | |
| Experimental | 635.18 ± 2.56*** | 6.89 ± 0.64*** | 541.16 ± 2.74*** |
| Control | 109.32 ± 2.01 | 1.45 ± 0.90 | 165.14 ± 1.65 |

*** P < 0.001

not significant between the experimental and control flocks, while in the growing broiler breeders flocks this indicator was significantly higher (P <0.01) in experimental chickens. Interferon γ and interleukin concentrations in broilers and broiler breeders are presented in Table 2. In chickens treated with Avigen, significantly higher concentrations of IFN γ, IL-2, and IL-6 were found compared to the control flock (P <0.001). Similar data were also found for broiler breeders (P <0.001).

## DISCUSSION

There is no doubt that AGPs (antibiotic growth promoters) since the late 1940s have had a significant impact on the progress of the livestock industry. There are currently new methods and tools for the prevention and treatment of bacterial, viral, and parasitic infections in the production of food of animal origin. There are many substances, such as prebiotics, probiotics, phytonutrients (herbs and essential oils), hyperimmune antibodies, bacteriophages, antimicrobial peptides, and toll-like receptor (TLR) agonists, used in the animal industry, but it is generally known that neither one of these alternatives is not as effective as AGP. However, the combination of these substances has shown some efficacy in reducing production losses when not using AGP. Furthermore, the exact mechanisms of action of widely used AGPs are unknown. Niewold (2007) proposed a hypothesis that AGP interacts directly with the complex intestinal ecosystem, especially innate immune cells that mediate the inflammatory response. According to this hypothesis, AGP enhances growth by acting directly on inflammatory immune cells, which leads to an anti-inflammatory intestinal environment. However, the exact mechanisms of different types of AGP need to be better studied and identified. Recently, more and more scientific evidence supports the idea that non-antibiotic alternatives to AGP can be developed that can stimulate innate immune responses. As an alternative to AGP, several immunomodulators have been proposed. “Immunomodulation” according to the Merriam-Webster Dictionary (www.merriamwebster.com) is defined as “modulation of the immune response or the functioning of the immune system through the action of an immunomodulator”. Immunomodulators include antimicrobial peptides (AMP), TLR ligands and agonists, prebiotics, probiotics, hyperimmune antibodies, herbs, essential oils, and more. Studies in mice and humans have provided scientific evidence that many of these alternatives to AGP have immunomodulated host immunity by interacting directly with innate sensory molecules present in innate immune cells. When the host’s innate immune cells encounter foreign antigens, the cells of the innate immune system recognize various innate immune receptors of the

host, called pathogen recognition receptors (PRRs). Initial binding of PRR to pathogens triggers a series of complex and complex intracellular and intercellular signaling pathways that lead to definitive activation of the NFκβ and inflammatory response. Natural immune responses are determined by the different types of PRR that are activated. As a result, a specific set of adapter molecules containing the Toll/IL-1 receptor domain, such as MyD88 and TRIF, are secreted to initiate downstream events that lead to the secretion of inflammatory cytokines, type 1 interferons, chemokines, and antimicrobial peptides. In addition, activation of TLR leads to the maturation of dendritic cells, which contributes to the initiation of adaptive immunity and subsequent differentiation of natural T-helper cells into mature effector cell types with different functions, such as Th1, Th2, Th17, and Treg, which secrete different cytokine types, including IFN-γ, TNF, IL-10, TGF-β, IL-4, and IL-5. Activation of innate immune cells also leads to the development of an antigen-specific, long-lasting response, which is a secondary line of defense of the host, called adaptive immunity. These studies have shown close communication between innate and adaptive components of the host immune system through activated receptors and secreted soluble effector molecules (Lillehoj and Lee, 2012). Thus, the initial immune response elicited by an immunomodulator has a profound effect on the quality of the host's secondary line of defense. The high density of the bird population in modern-day commercial farming results in a dramatic rise in the spontaneous spread of various pathogens. The potential development and distribution of antibiotic resistance from animals to humans makes the traditional treatment of livestock with different antibiotics unacceptable (Millet and Maertens, 2011). These circumstances navigate the attention of science into two major directions - a marker-assisted selection of animals with strong immune responses and research for different food additives, probiotics, prebiotics, symbiotics, or immunostimulants with immunomodulating potential. Although very promising, so far animal selection has not provided the desired results. At the heart of this is the discrepancy between the living conditions in experimental and commercial farms. Moreover, market requirements do not always match the productive potential of animals selected for strong natural immune responses. This encourages the research of different substances with an immune-stimulating effect (Fathi et al., 2012). In recent years, there has been a great interest in the search for alternatives to the use of antibiotics to avoid the harmful effects of their use. It is well known that serum lysozyme is an important humoral factor of natural immunity in birds (Besarabov, 2013). Stimulation of the intestinal mucosa with lipopolysaccharides contained in the used immunomodulator leads to increased levels of lysozyme and APCA in broilers and broiler breeders. Besarabov(2013) and Bonovska et al. (2014)reported that lysozyme content below 3.5 mg/L in blood serum and egg white, shows low natural resistance in birds. According to Wigley et al. (2003), interferons and interleukins play a major role as mediators of immune responses. The application of polybacterial immunomodulators in production conditions can increase the natural resistance and productivity of birds and this is of great interest to poultry farming. In this regard, our experiments show that the immunomodulator Avigen may be a good alternative to antibiotics in broiler farming. In a similar experiment, Bozakova et al. (2020) explored the possibilities for increasing natural immunity via the application of immunomodulator Immunobetain turkeys and hens. The core components of that product are beta-glucans and mannan oligosaccharides extracted from several yeast strains. Compared with our experiment, the authors report far more moderate benefits of the applied additive. Both traits were almost indistinguishable among experimental and control turkeys. The same result was observed for the lysozyme concentration among hens. A small increase was observed for the APCA activity in both species but with a smaller value. Based on the similarities of both products we could assume the stimulating effect on the complement system. Lalev et al. (2015) tested the influence of the preparation Natstim on both parameters of natural immunity among White Plymouth Rock hens. The product has been applied in feed and water, where the results were opposed. The application of the product was much more effective in drinking water than fodder. Despite this fact, the increase in both traits is quite modest and on average about 20% higher than in control hens. The application of these substances has some benefits, but the Avigen immunomodulator, used in our experiment, has a higher potential to modulate the factors of innate immunity. Denev et al. (2020) studied the effect of Silymarin on the natural immunity in chickens. Despite the applied product's hepatoprotective and antioxidant properties, the authors detected that lysozyme concentration was almost 2.5 times lower than controls. Such experiments prove the need for detailed analyses of each food supplement before com-

mercial use for specific animal species. At present, due to the constant increase in the number of people in the world, it is necessary to keep short-lived animals (such as birds and pigs) to provide enough food for humanity. This is due to the use of modern technologies for their breeding, but at the same time, it leads to severe stress in animals and therefore increases their morbidity. This is the reason why farmers use antibiotics both for treatment and to stimulate the growth of animals to obtain greater production. As a result, however, antibiotic-resistant microorganisms are created, which is extremely dangerous to humans. This necessitates the search for alternative means of treatment and prevention aimed at reducing the use of antibiotics and improving the welfare of poultry, with an emphasis on natural immunity. All these circumstances are in the background of increasing demands for antibiotic-free human treatment and poultry welfare issues driven by global social pressure (Ordinance 44/20.04.2006; Ordinance 25/State Gazette 42/23.05.2006). Bedrania et al. (2013) consider that the antimicrobial protection of eggs is carried out by immunoglobulins presented in the yolk and antimicrobial proteins/peptides localized in the egg white. The authors suggest that it is possible to stimulate the synthesis of specific immunoglobulins by using genetically foreign antigens. To test this hypothesis, they treated chickens with intravenous injections of lipopolysaccharide derived from *Salmonella enterica Enteritidis* (LPS) at 24-hour intervals. Eggs from the control and experimental groups were collected for 21 days after the first LPS injection and the antimicrobial activity of the egg white against *Staphylococcus aureus* and *Escherichia coli* was evaluated. Increased antimicrobial activity of the protein in the eggs of treated hens against *Staphylococcus aureus* was found, which was 20.9% and 23.4% higher than in control birds on the 5th and 6th day after the first injection of LPS. Antimicrobial activity against *E. coli* was moderately increased only on the 9th and 15th days after LPS treatment. The results of this study could be used to increase embryo protection by using antimicrobial agents in addition to specific antibodies derived from hens.

## CONCLUSION

The use of the immunomodulator Avigen has been shown to increase APCA activity, lysozyme, interferon-gamma, and serum interleukins concentrations in broilers, which increases their natural resistance and allows birds that receive Avigen to be reared in production conditions without the use of antibiotics.

## CONFLICT OF INTEREST

All authors declare that there is no conflict of interest and disclose that we do not have any financial and personal relationships with other people or organizations that might inappropriately influence or bias our work.

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