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Original article

Influence of the Immunomodulator AVIGEN to Broiler Chicken Humoral Immune Factors

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Abstract

The nonspecific immune response plays an important role in organism's defense against a variety of pathogens. Two major factors in this process are blood serum lysozyme and alternative pathway of complement activation (APCA). Over the past few decades, the application of various substances, targeting improved levels of natural immunity have become part of the mainstream trends in livestock rearing programs. The current study examined the influence of the polybacterial immunomodulator AVIGEN on the performance of the aforementioned immune factors among broiler chicken hybrids. The experimental group demonstrated better overall performance for both parameters of interest. APCA activity for the treated group (549.10±19.69 CH50) was significantly higher compared to the result obtained for the controls (377.40±9.58 CH50), (P<0.001). Results for the other parameter were even more indicative. The measured concentration of the serum lysozyme for the treated group was twice as high compared to the control birds - 6.17±0.49 mg/L vs. 2.99±0.27 mg/L, respectively. Therefore, the introduction of the AVIGEN immunomodulator to animals' diet has strong potential to improve natural humoral immunity in poultry farming.

Keywords: lysozyme, complement system, immunomodulatory, biotechnology.

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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 density of the bird population, raised in relatively small spaces, which increases the risk of spontaneous disease outbreaks (Linares and Martin, 2010). If the first two necessities are influenced by the nutrition properties of the food provided. The third requirement has 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. In several cases, farmers face subclinical infections from a well-known cocktail of locally presented infectious agents. This category of diseases has a massive impact on animals' productivity and well-being. The unpredictable nature of such processes requires prominent levels of non-specific immune response factors among all animals (Al-Mansour et al. 2011).

Two major factors of natural humoral immunity are the activity of the alternative pathway of complement activation and the lysozyme concentration in the blood serum. The complement system and the two major pathways of its activation is one of the oldest mechanisms for protection against pathogenic bacteria and plays a crucial role in both specific and nonspecific immune response. The system consists of several proteins, produced in an inactive form (Sotirov and Koinarsky, 2003). Its activation results in a cascade of consecutive reactions aimed at eliminating a specific infectious agent. Although the classical pathway of complement activation is much more effective, its alternative variant does not require the formation of antigen-antibody complex. This makes it one of the very first defense mechanisms against wide range of pathogens. Its key role remains further evidenced by the fact that this mechanism was the first to evolve in evolution (Zhu et al. 2005). Once activated, the alternative pathway of complement activation (APCA) is quite effective against a variety of Gram-negative bacteria, viruses, neoplastic cells, etc. (Mueller-Ortiz et al. 2004). Lysozyme is an antimicrobial enzyme that has essential functions in the innate immune response (Gilbert, 1971). The muramidase is found in egg white, saliva, blood serum, etc. The serum lysozyme is predominantly produced by the macrophages (Eshbailat and Ibrahim, 2004; Bazlamit et al, 2009) and is effective against Gram-negative bacteria as well as some large viruses, such as *Avipoxvirus* (Zhang et al. 2017). Breed, sex, age, and species variations in both parameters have been observed (Koynarski et al. 2018; Koynaski and Sotirov 2013; Semerdjiev et al. 2011; Sotirov et al. 2011; Sotirov, et al. 2011). Given the large number of genes encoding different protein fractions for both parameters of interest, marker-assisted selection does not provide a useful solution. Moreover, poultry farming has always focused more on productive parameters than health and welfare (Bahmanimehr 2012). In the past, fighting pathogens favored using antibiotics, including some with nutritional effects. Nowadays trends for production of antibiotic-free animal products motivate the

use of different probiotics, symbiotics, immunostimulants, etc. Many of these substances have pathogen inhibition, growth performance, and welfare properties (Huyghebaert et al. 2011; Chen et al. 2017). At present, the market offers several immunomodulators of herbal origin, but their effect is limited (Georgieva et al. 2013). Given that triggering molecules for APCA and lysozyme actions are lipopolysaccharides found in bacterial cell membrane and viral envelopes, using an immunomodulator based on the same concept seems promising.

The major focus of this study was given to the impact of the polybacterial immunomodulator AVIGEN on the base levels of the aforementioned two innate immune factors among broiler chicken hybrids. Since the product is based on lipopolysaccharide components of the thermostable endotoxin of Gram-negative bacteria from *Enterobacteriaceae* we could assume its stimulating effect on both parameters of the innate immune response.

MATERIALS and METHODS

The effect of the immunomodulator AVIGEN on the blood serum lysozyme concentration and APCA activity was analyzed among the popular broiler chicken hybrids ROSS 308. The trial was done with two equal groups of chicken (n=45) – one experimental and one control. Birds have been raised from day-old under the same conditions for 42 days growing period in the commercial farm Planeta 98 Ltd. Veliko Tarnovo, Bulgaria. The administration of the immunomodulator AVIGEN for the experimental group was done via the drinking water for the first 10 days of birds' life. The product contains lipopolysaccharide components of the thermostable endotoxin of Gram-negative bacteria from *Enterobacteriaceae* family. The supplement was provided in liquid form, where 1 liter of the product contains 3000 daily doses.

Blood samples for analyses were collected on day 35 (25 days after treatment) aseptically from *v. ulnaris*. Blood was transported in cool bags at 6˚C. Serum was extracted via centrifugation at 3000 rpm (1,000 g) for 10 minutes.

The alternative pathway of complement activation was evaluated by the method of (Sotirov et al. 2005). One hundred microliters of each serum sample were diluted with 350 µl veronal-veronal Na buffer (in final concentrations: 146 mM NaCI, 1,8 mM 5,5- diethylbarbituric acid sodium salt; 3,2 mM 5,5- diethylbarbituric acid; 1 mM EGTA and 0,8 mM MgCl2). Using U bottomed plates, 7 other dilutions from each diluted serum were again prepared in veronal- veronal Na buffer, so the final serum dilutions were 8/45, 7/45, 6/45, 5/45, 4/45, 3/45, and 2/45, respectively. Subsequently, each well was supplemented with 100 µl of 1% rabbit erythrocyte suspension. Samples were incubated for 1 hour at 37°C statically and then centrifuged at 150 g for 3 minutes at room temperature. Thereafter, 150 µl of each supernatant was placed into a flat-bottomed plate for measurement of optical density at 540 nm using 'Sumal-PE2' ELISA reader (Karl Zeiss, Germany). The final APCA activity was calculated using

a dedicated software developed at Trakia University and expressed as CH50 units (correspond to 50% of complement-induced hemolysis of applied erythrocytes).

Blood serum lysozyme concentrations were analyzed by the method of (Sotirov et al. 2007). The method consists of mixing 20 ml of 2% agarose dissolved in phosphate buffer (0.07 M NaHPO4 and NaH2PO4) with 20 ml suspension of a 24-hour culture of *Micrococcus lysodeicticus* at 67°C. While still warm the mixture is poured into a 14-cm Petri dish. After solidifying at room temperature, 5 mm wells are made. Each well is filled with 50 µl of undiluted serum. Eight standard lysozyme dilutions (from 0.025 to 3.125 µg/ml) are prepared and pipetted into eight wells. The plate is then incubated for 20 hours at 37°C. The final lysozyme concentration is calculated by dedicated software developed at Trakia University, comparing the lytic zone of each sample with the standard lysozyme dilutions.

Obtained data have been processed by one-way analysis of variance (ANOVA) with the fixed-effect model using Data analysis tool pack, Microsoft Excel 2016, Microsoft Corporation Ltd.

RESULTS

The trial was conducted with broiler chicken where experimental and control groups were raised at equal conditions, including housing, nutrition, and vaccination programs, etc. Results represent mean values obtained for both parameters toward the end of the growing period (day 35), which is about 25 days after treatment with the immunomodulator. The results about the influence of the immunomodulator AVIGEN on broiler chicken serum lysozyme concentrations and APCA activity are presented in Figures 1 and 2. As seen from the graphs, under the influence of the applied immunomodulator the levels of both parameters exhibit significantly higher results in favor of the treated group. The alternative pathway of complement activation (fig.1) for the treated group was more than 170 units (CH50) higher compared to the controls - 549.10±19.69 vs. 377.40±9.58, respectively ($P<0.001$). A similar tendency was observed for the second parameter of interest. The concentration of blood serum lysozyme (fig. 2) was twice as high for the treated chicken (6.17±0.49 mg/L) compared to the control group (2.99±0.27 mg/L) ($P<0.001$).

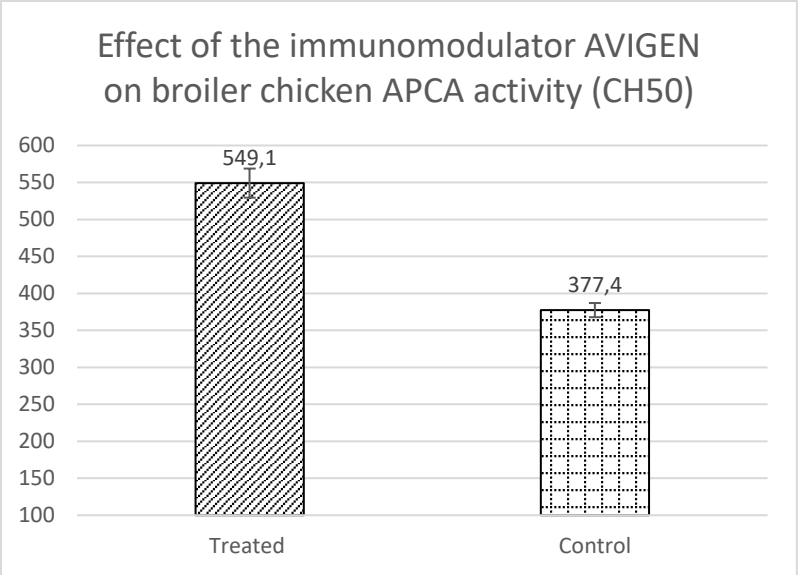

Figure 1. Effect of the immunomodulator AVIGEN on broiler chicken APCA activity (CH50).

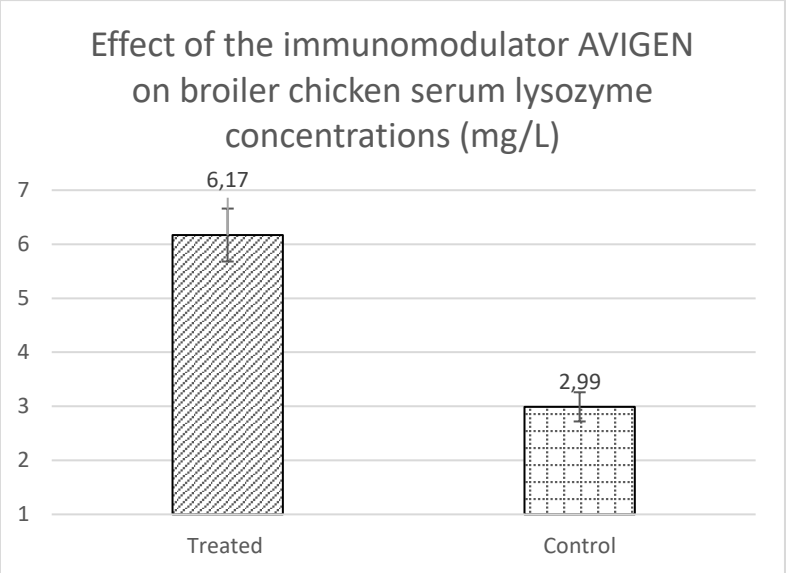

Figure 2. Effect of the immunomodulator AVIGEN on broiler chicken serum lysozyme concentrations (mg/L).

Discussion

The high density of bird population in modern-day commercial farming results in a dramatic risk of spontaneous spread of various pathogens. Potential development and distribution of antibiotic resistance from animals to humans makes 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 response 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 response. This encourages the research of different substances with an immune-stimulating effect (Fathi et al. 2012).

Serum lysozyme is a major factor of the nonspecific immune response (Eshbailat et al. 2004, Besarabov, 2013). The obtained results demonstrate that the stimulation of intestinal mucosa with lipopolysaccharides obtained from *Enterobacteriaceae*, the main component of the applied immunomodulator, leads to a significant increase in the blood serum lysozyme concentration of the treated birds. According to Besarabov (2013), serum lysozyme concentrations below 3.5 mg/L demonstrate a low natural immune response and harms the overall development during the growing period of chicken. The control group of our experiment exhibits twice as low serum lysozyme concentration compared to the treated group, which undoubtedly demonstrates the immune simulative capability of the tested product. Based on previous experiment of ours, we can consider that serum lysozyme concentration between 1.5 and 3 mg/L is the baseline of chicken capacity (Koynaski and Sotirov 2013). The current research endorses the possibility of a strong increase in the muramidase concentration and modulation of animals' immune response to various infectious agents.

The comparison of APCA activity between the control and treated group exhibited a similar trend. Considering the nature of the applied product and the triggering mechanism of this pathway for activation of the complement cascade, such results were expected. Indisputably, the consumption of the AVIGEN immunomodulator had facilitated the witnessed increase among the treated birds. High values for this trait are associated with a strong innate immune response to a variety of pathogens (Zhu et al. 2005). The tested supplement provides a precise tool for keeping the nonspecific immune response in its highest state, which has the potential to resist plurality of challenges of the enclosed environment.

In a similar experiment, Bozakova et al. (2020) explored the possibilities for improvement of humoral immunity via the application of Immunobeta® immunomodulator among turkeys and hens. The core components of that product are betaglucans and mannanoligosaccharides extracted from several yeast strains. Compared with our experiment, the authors report far more moderate benefits of the applied additive. Both parameters of interest were almost indistinguishable among treated and control turkeys. The same outcome was witnessed for the lysozyme concentration among hens. A slight increase was observed for the APCA activity in that species, but with a much smaller margin. Based on the similarities of both products we could assume the stimulating effect on the complement system. Lalev et al. (2015) tested the effect of the immunomodulator Natstim® on both parameters of innate immunity among White Plymouth Rock hens. The product has been applied in feed and water, where

the results were diametrically opposed. The application of the product was much more effective in drinking water than fodder. Despite this fact, the increase of both parameters was rather modest and averaged at about 20% higher compared to the control birds. Clearly, the application of such substances with drinking water has some benefits, but the nature of the AVIGEN immunomodulator, used in our experiment, has much higher potential to modulate the state of the studied factors of innate immunity.

Denev et al. 2020 explored the effect of silymarin on the natural immunity in broiler chickens. Despite the applied product's hepatoprotective and antioxidant properties, the authors detected far more inferior results among treated animals, where lysozyme concentration was almost 2.5 times lower than controls. Such experiments prove the need for detailed analyses of each food supplement before commercial use for specific animal species.

CONCLUSION

The application of polybacterial immunomodulators has definite benefits for the increased innate immune response in birds, reared under commercial growing technologies. Based on the current experiment, we consider the used AVIGEN immunomodulator a strong candidate for a mainstream supplement of chicken diet with a definite innate immunity boost effect.

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