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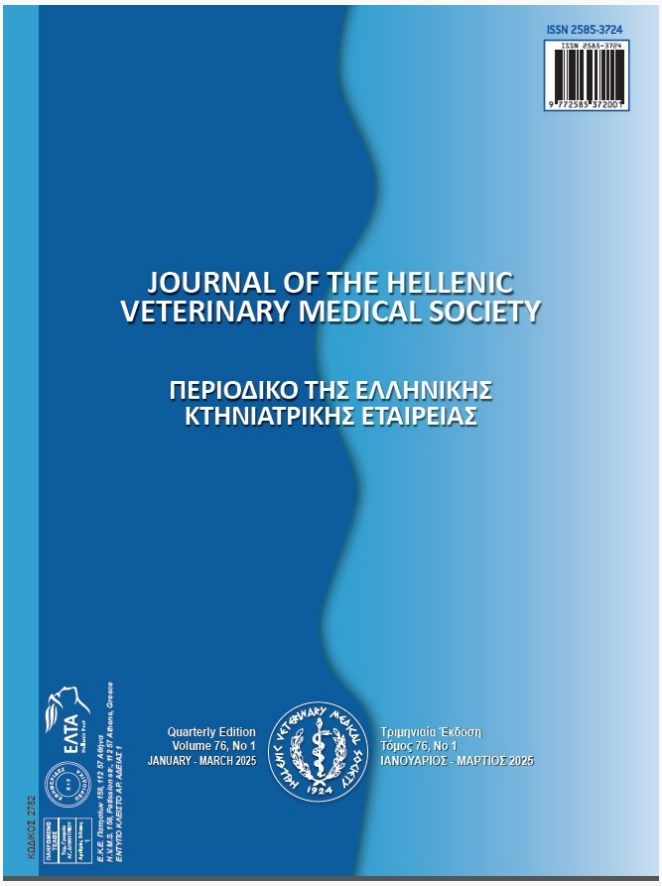

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Research article
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Influence of immunomodulator Immunobeta on the humoral immunity of lambs during the thermoneutral, hot, and cold season

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ABSTRACT: Infectious diseases cause devastating economic losses in the sheep industry. The current study aimed to evaluate the influence of immunomodulator Immunobeta on the humoral immunity of lambs, whose rations were supplemented with 4g /kg immunomodulator Immunobeta for 60 days during thermoneutral, hot summer and, cold winter periods. The lambs were divided into two groups - control and experimental. To the experimental animals, after 15 days of age, the immunomodulator Immunobeta was added to the standard concentrated feed, at a dose of 4g/kg of feed. It was found that the immunomodulator Immunobeta reliably increased the values of the investigated indicators in all three annual seasons. It can be concluded that the studied immunomodulator Immunobeta possesses an important immunomodulating potential in lambs, which could improve their health.

Keywords: Lambs; Immunobeta; Immunity; Seasons

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INTRODUCTION

Antimicrobial resistance continues to increase, and after just eight decades of antibiotic use, bacterial infections that were once easily treatable have become incurable. Successful outcomes of surgical procedures and immunosuppressive treatments are highly dependent on antibiotic prophylaxis. Antibiotic resistance is, therefore, a serious threat to much of health care, both in humans and animals. Antibiotic resistance correlates with the use of antibiotics, so the use of alternative preparations to antibiotics will limit the use of antibiotics and reduce the risk of the emergence of antibiotic-resistant bacteria. Significant global action and investment are needed to develop new anti-infectives to keep pace with increasing antibiotic resistance (MacGowan and Macnaughton, 2017). Probiotics and prebiotics are promising alternative therapies for animal prevention and treatment. Applying probiotics and prebiotics in livestock production is a very good opportunity to reduce the need for antibiotic use in agriculture and the negative impacts down the chain (Leistikow et al., 2022). For the last few decades, the ban on using antibiotics in livestock husbandry has increased interest in biotechnological and natural products to improve lambs' productivity and health (Liu et al*.,* 2011). Immunomodulators constitute a new class of growth promoters that recently gained importance in the food industry to produce functional foods. Research has focused mainly on the effect of immunomodulators on mortality, stress hormones, blood, muscle metabolism, and even the immune system function of domestic animals. The alternative pathway of complement activation (APCA) is known to be an important factor in innate immunity. It is active against Gram-negative bacteria, viruses, virus-infected cells, neoplastic cells, agarose, lipopolysaccharides, contrast media used in radiology, etc. (Sotirov et al., 2005). Grewal and Babiuk (1980) have studied the cytotoxic effect of neutrophils against herpes virus-infected cells and reported that it was dependent not only on the classical pathway of complement activation but on APCA as well. The antiviral activity of APCA was confirmed by the experiment of Ohta et al. (1983). They treated cell cultures of chicken embryos infected by the fowlpox virus with normal chicken serum and observed the lack of cytopathogenic effect, viral growth, and plaque formation (Sotirov et al., 2005). Lysozyme is an enzyme that breaks down bacterial cell walls by attacking peptidoglycan. The increased serum concentration of lysozyme serves as an indirect marker of inflammation and provides information on the activity of granulocytes and the functionality of the monocyte-macrophage system. In addition, it is a potential indicator of the number of pathogens in the environment (Miglio et al., 2018). Zhang et al. (2014) reported that the infusion of oligosaccharides into the gastrointestinal tract of sheep increases the percentage of CD4+ T-lymphocyte cell populations and the serum concentrations of IgA and IgG. Milewski et al. (2010) also found that lambs fed a diet supplemented with *Saccharomyces cerevisiae* yeast with increased β-1,3/1,6-D-glucan and mannan-oligosaccharide showed increased lysozyme activity, ceruloplasmin activity, and γ-globulin content. Khalkhane et al. (2013) reported that oral administration of beta-glucan increases total IgG and interferon-gamma levels in lambs.

The current study aimed to evaluate blood serum concentrations of lysozyme, gamma interferon, immunoglobulin G, immunoglobulin M, the activity of the alternative pathway of complement activation (APCA), betalysin activity, cortisol, and serotonin in lambs whose rations were supplemented with 4g/kg of the immunomodulator Immunobeta.

MATERIALS AND METHODS

Experimental Design

The experiment was conducted in 2022, with 12 female lambs of the French Lacaune breed, during manure-pasture rearing on a private sheep farm. The area is located in the transitional Mediterranean climate region, characterized by warm and dry summers and mild winters. The lambs were divided into two groups: control and experimental. The experimental animals, after 15 days of age, were fed with standard concentrated feed (corn, wheat, barley, sunflower meal, soybean meal, bran, chalk, salt, monocalcium phosphate, vitamin-mineral premix; guaranteed analysis (in %): crude protein - 15.50, raw ash - 5.00, crude fibers - 6.20, calcium - 0.67, phosphorus - 0.57), supplemented with the immunomodulator Immunobeta, at a dose of 4g/kg feed. In addition to the concentrated feed, the lambs were also fed good quality meadow hay. The lambs were reared in group pens, with an area of 2 m^2 provided per animal, with a norm of 0.7-1 m^2 (Regulation No. 44/2006). Lambs had *ad libitum* access to feed and water, and lighting was provided continuously. The experiments were conducted with standard ethical norms and no lambs were subjected to undue stress. The minimum

requirements for the protection and welfare of experimental animals and the requirements for facilities for their use, keeping, and supply are set out in Ordinance № 20 of 1.11.2012 on the minimum requirements for protection and welfare of experimental animals and the requirements for sites for use (8.1.2018), breeding and/or delivery, which transposes Directive 2010/63/EU.

The Italian company for veterinary pharmaceuticals, Chemifarma manufactures the immunomodulator Immunobeta. It is obtained from selected strains of yeast (*Saccharomyces cerevisiae*) by enzymatic autolysis and a process of natural extraction of the components from the yeast cells. The immunomodulator has the following composition: 1,3-1,6 Beta-Glucans 30%, Mannan oligosaccharides 25%, Nucleotides 7%, Vitamin B1, Vitamin B2, Vitamin B6, Niacin, Pantothenic Acid, Folic Acid, Choline, Iron, Zinc, Manganese, and Copper. The study animals didn't receive other preventive treatment.

Samples

Blood samples were taken during a thermoneutral period (May 21, 2022), a hot summer period (July 23, 2022), and a cold winter period (January 3, 2023) from the jugular vein (*v. jugularis*) of six lambs from each group. Blood for analysis was in Vacutests KIMA - 11020 for a serum with clot activator - 6 ml, red cap, Vacutests KIMA 135400 - for hematology with K2 EDTA 6 ml, purple cap, Needles for Vacutests KIMA - 21G x 1, ½ - sterile KIMA 1526504, Holders for Vacutests KIMA 169018 (Distributor CHIMTEX LTD). The blood was allowed to clot for one hour at room temperature (25°C), the samples were centrifuged at 2000 g for 10 min, and the extracted blood serum was stored at 4°C. The sera were analyzed not later than 1 hour after the samples were taken.

Determination of lysozyme concentration in blood serum

Serum lysozyme concentrations were tested by the method of Lie et al. (1985). Briefly, 20 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 into 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 pipetted in each well. Eight standard lysozyme dilutions (from 0.025 to 3.125 μg/ml) were prepared and pipetted in separate wells. The samples were incubated for 20 hours at 37°C and lytic zone diameters were measured. The final lysozyme concentrations were calculated using special software developed at Trakia University.

Determining the activity of the alternative pathway for complement activation (APCA)

The activity of APCA was tested by Sotirov (1991). Each serum sample was first diluted by mixing 100 μl 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 a 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 the betalysin activity

Betalysin activity was assessed by the method of Buharin et al. (1977).

Determination of gamma interferon (IFN-γ), immunoglobulin G (IgG), immunoglobulin M (IgM), cortisol, and serotonin

Concentrations of gamma interferon (IFN-γ), immunoglobulin G (IgG), immunoglobulin M (IgM), cortisol, and serotonin in lamb's samples were determined by ELISA tests mentioned below:

1. Sheep IFNA / Interferon Alpha ELISA Kit - LS-F74396 (LSBio, USA)

2. Sheep IFN Gamma / Interferon Gamma ELISA Kit - LS-F50569 (LSBio, USA)

3. Sheep IgG (Sandwich ELISA) ELISA Kit - LS-F32254 (LSBio, USA)

4. Sheep IgM (Sandwich ELISA) ELISA Kit - LS-F44932 (LSBio, USA)

Statistical analysis:

Data was 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

The results presented in Table 1 show no significant differences in lysozyme concentrations between tested seasons in the experimental and control groups. Lysozyme concentrations in the group treated with Immunobeta are significantly higher than the control group ($P < 0,001$). Looking at the results for the alternative pathway for complement activation in the experimental group, a significant increase in values was observed consistently from the thermoneutral to the cold winter period ($P < 0.001$). The same was observed in the control group but with lower values ($P < 0.001$). For betalysin, no significant differences were found between the studied seasons, but the group treated with Immunobeta showed significantly higher values compared to the control group. In winter, the control group showed a significant increase in IgG blood serum concentration ($P < 0.001$). The same was observed in the experimental group but with significantly higher values ($P < 0.001$). The dynamics in the concentration of IgM was similar to that of IgG, but for this indicator, the highest values in both groups were observed during the hot summer season ($P < 0.001$). If we compare the control with the experimental group, again the Immunobeta® treated group has significantly higher values ($P < 0.001$). Similar to IgM are the changes in IFN-γ dynamics. Here again, the highest values were observed during the hot summer season, with the concentration of IFN-γ in the experimental group being significantly higher than that in the control group. For cortisol, again the highest values were found in the cold winter season in both groups ($P < 0.001$). Unlike the other indicators, the concentration of cortisol was highest in the control group during the cold winter season ($P < 0.001$). Serotonin, in contrast to the indicators described so far, had the highest values during the thermoneutral period in both groups ($P < 0.001$). As with the other indicators, the Immunobeta-treated group showed significantly higher values compared to the control group ($P < 0.001$).

DISCUSSION

As can be seen from the results presented above, the immunomodulator Immunobeta reliably increases the values of the investigated indicators. It is known

Table 1. Influence of the immunomodulator Immunobeta on some humoral factors of immunity in lambs (S ± Sx)

Seasons	Groups	n	Thermoneutral period	Hot summer period	Cold winter period
Lysozyme (mg/L)	Immunobeta	6	0.68 ± 0.13	0.71 ± 0.11***	0.61 ± 0.07
	Control	6	0.07 ± 0.01	0.08 ± 0.01	0.07±0.01
APCA (CH50)	Immunobeta	6	225.59 ± 6.20	232.70 ± 5.72	253.97 ± 5.21***
	Control	6	178.60 ± 6.80	188.94 ± 4.98	206.04 ± 6.35
Betalysine (%)	Immunobeta	6	24.37 ± 1.52	26.28 ± 1.26	27.57 ± 1.73***
	Control	6	17.20 ± 0.83***	7.01 ± 0.34	13.37 ± 1.11
IgG (mg/ml)	Immunobeta	6	24.08 ± 0.16	23.45 ±0.20	30.68 ± 0.31***
	Control	6	20.94 ± 0.70	19.52 ±0.76	24.44 ± 0.72***
IgM (mg/ml)	Immunobeta	6	66.68 ± 1.46	84.06 ± 2.35***	80.85 ± 2.07
	Control	6	59.09 ± 1.05	68.15 ± 1.53***	62.94 ± 1.63
IFN-γ (pg/ml)	Immunobeta	6	14.95 ± 2.47	44.30 ± 0.84***	28.18 ± 0.58
	Control	6	10.23 ± 2.81	33.39 ± 1.60***	20.65 ± 1.01
Cortisol (ng/ml)	Immunobeta	6	20.70 ± 0.33	25.45 ± 0.74	26.15 ± 0.33***
	Control	6	26.75 ± 0.42	32.97 ± 0.56	39.37 ± 0.31***
Serotonin (ng/ml)	Immunobeta	6	107.63 ± 1.54***	99.01 ± 1.46	94.85 ± 1.56
	Control	6	99.08 ± 1.96***	71.73 ± 2.03	62.44 ± 2.27

*** - $P < 0.001$

that these indicators play a decisive role in the protection of animals and humans from infectious diseases. Primo et al. (2018) reported that lysozymes are enzymes that break down the bacterial cell wall and disrupt the bacterial life cycle by cleaving the linkage between the N-acetylglucosamine and N-acetylmuramyl pentapeptide carbohydrates. Wojcik (2010) reported that lambs supplemented with yeast extract significantly increased γ-globulin levels, lysozyme, and ceruloplasmin activity in the blood serum of experimental animals. Similar results are reported by Milewski et al. (2010), Małaczewska and Milewski (2010), Bozakova et al. (2017) and Bozakova et al. (2020). According to Inngjerdingen et al. (2005, 2007a, b) immunostimulatory polysaccharides can also directly activate other immune cells, such as NK cells and B lymphocytes. In addition to inducing macrophage responses, complement-fixing activity has been attributed to immunostimulatory polysaccharides, including mixed (β1→4)(β1→3)-D88 glucans (Samuelsen et al., 2011). Torrecillas et al. (2007) reported increased resistance of *European sea bass* (*Dicentrarchus labrax*) fed mannan oligosaccharides against artificial infection with *Vibrio alginolyticus*. The authors explain this resistance with the increased values of some factors of the immune system. Oblakova et al. (2022) reported that some medicinal herbs have a positive effect on natural humoral immunity in turkeys. Research on betalysin is scarce, and therefore we consider our research on this indication to be useful. Karakolev et al. (2023) reported that ducks treated with immunomodulator Avigen (created based on lipopolysaccharides extracted from the Enterobacteriaceae family) demonstrated lower values of betalysin than control birds. The authors explain this fact by suggesting that betalysin most likely recognizes the foreign antigen and fights against it. Gökçe and Atakişi (2019) proposed interesting data indicating that the first week of neonatal life is critical for a lamb's health and the passive transfer of immunity is of paramount importance for the maintenance of health. The study also revealed the critical cut-off point of serum IgG concentration and serum total protein concentrations at 24 h after birth for increased risk of disease and death in both periods. Investigations by other authors support these findings (Gokcea et al., 2014; Alves et al., 2015). Youssef et al. (2023) reported that hens given MOS at various doses significantly raised serum levels of immunoglobulin Y (IgY), immunoglobulin M (IgM), and avian influenza (AI) antibodies compared to control birds. Sun et al. (2009) found that galactic-mannan-oligosaccharides and chitosan supplemented in weaned piglets' diet led to higher serum concentrations of IgG, IgA, IgM, IL - 6, IL -2, and IL - lβ in experimental groups compared to the control group. Attia et al. (2020) provided information that *Oestrus ovis* increased the levels of IFN-γ and TNF-α significantly in infested sheep compared to non-infested ones. This is strange because it implies that IFN-γ and TNF-α are effective against this parasite. Mo et al. (2001) reported the in vivo and in vitro effects of chicken interferon alfa on Infectious Bursal Disease Virus and Newcastle Disease Virus infection. According to Delgado et al. (2011), IFN-γ levels are increased in ewes infected with *Mycobacterium avium (Map)*. These authors suggest that the peripheral immune response induced by *Map* infection in adult ewes is more efficient in controlling the progression of the infection than in lambs. This could likely be due to the existence of previous contact with *Map* or other mycobacteria in the adult sheep compared to young lambs. Some authors found a positive effect against ovine lentivirus in sheep treated with recombinant ovine interferon-tau (roIFN-τ) (Singh et al., 1998; Juste et al., 2000).

Cortisol is a catabolic steroid hormone secreted by the adrenal glands. It is sometimes called stress hormone because its levels rise significantly after physical or emotional stress. Cortisol's main roles are to regulate blood pressure and the functions of the cardiovascular system. It also regulates the body's use of protein, carbohydrates, and fat. Lester et al. (1991) compared the effects of different surgical methods on cortisol responses. Cut lambs (tailing only, castration only, castration plus tailing) experienced more distress than other groups. Additionally, the distress response (indicated by elevated plasma cortisol concentrations) lasted longer than 4 hours in cut lambs, unlike in all other groups. Coulon et al. (2013) studied the relationship between humans and lambs and found that the lambs showed a friendly attitude towards their caregiver, evidenced by a lack of a cortisol response. Pajor et al. (2013) studied the influence of temperament on cortisol concentration and found that at the end of fattening, calmer lambs had lower cortisol concentrations (2.60 nmol/l) compared to more nervous lambs (8.07 nmol/l).

Serotonin or 5-hydroxytryptamine (5-HT) is a monoamine neurotransmitter that is synthesized in serotonergic neurons of the central nervous system (about 10%) and in enterochromaffin cells of the gas-

trointestinal tract (about 90%). Serotonin plays an important role in regulating various aspects of human and animal behavior such as aggression, mood, vomiting, body temperature, sexuality, and sleep. It is also related to aging, learning, and memory. Low levels of serotonin can contribute to aggressive behavior, clinical depression, migraines, irritable stomach, fibromyalgia, and various nervous disorders. On the other hand, high levels of serotonin in the blood can be associated with conditions such as osteoporosis (Mohammad-Zadeh et al., 2008). Bacqué-Cazenave et al. (2020) presented an interesting review of the role of serotonin in animal cognition and behavior.

Within this experiment, the influence of Immunobeta on the development of the live weight of lambs was investigated and it was found that Immunobeta increased the live weight of lambs by 12%, which is a good economic indicator and contributes to higher incomes of the farmer (Bozakova et al., in press). The positive action of yeast can be considered in several directions - stimulation of immunity, improvement of the rumen microbiome, and due to the antiparasitic action against the gastrointestinal parasite *Haemonchus contortus* in sheep (Pérez-Ruchel et al., 2012; Hooper et al., 2014).

CONCLUSION

Based on the obtained results, it can be concluded that the studied immunomodulator Immunobeta has significant immunomodulating potential in lambs, which could improve their health and, consequently, their productive performance.

CONFLICT OF INTEREST

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

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