

# Possibilities for Improvement of Humoral Innate Immunity in Turkeys and Hens in Conditions of Thermal Stress by the Immunomodulator Immunobeta®

Research Article

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## ABSTRACT

The experiments for evaluation of the effect of Immunobeta® were performed with turkeys and organically reared layer hens. Turkeys received Immunobeta® at a dose of 4 g/kg feed during the beginning of lay (cold period), thermoneutral period and hot summer period. In hens, two dietary levels of Immunobeta® were tested: 2 g/kg and 4 g/kg. Blood serum and egg white lysozyme levels, alternative pathway of complement activation (APCA) and beta-lysins concentrations were determined. It was found out that Immunobeta® had a beneficial effect on serum lysozyme, APCA activity and beta-lysins in turkeys and hens. The lysozyme concentration in egg white was significantly higher in eggs produced by birds treated with both doses of the immunomodulator. Immunobeta®, offered at a dose of 4.0 g/kg feed had a beneficial effect on natural humoral immunity factors. Applied at a dose of 4.0 g/kg, the tested immunomodulator increased significantly egg white lysozyme concentrations in hens, presuming a higher shelf life of eggs and possibly, better protection of chick embryos.

**KEY WORDS** beta-lysins, complement, hens, immunobeta, lysozyme, turkeys.

## INTRODUCTION

In modern society, the welfare aspects of birds' prophylaxis and treatment become especially important. During the last two decade, there are a number of potential non-therapeutic alternatives of antibiotic growth promoters (AGPs) in animal nutrition and production of safety (antibiotic-free) products, including probiotics, prebiotics, symbiotics, immunostimulants, direct feed microbials and etc. (Denev, 1996; Denev, 2006; Denev, 2006a; Denev, 2008; Denev *et al.* 2000; Denev *et al.* 2006b; Denev *et al.* 2009; Staykov *et al.* 2007; Huyghebaert *et al.* 2011). A lot of them demonstrated positive results, equivalent to the AGPs, but without the stigma of increasing antimicrobial-resistant bacteria.

They stimulated probiotic intestinal flora, ecological balance, inhibition of pathogens, humoral immunity, gut health, animal welfare, growth performance, quality and safety of animal products (Denev, 2008; Zhang and Kim, 2014; Chen *et al.* 2017).

There are no reports about the effects of the immunomodulator Immunobeta® on the innate immunity of turkeys and layer hens at the time of beginning of lay as well as in birds under heat stress. The purpose of these experiments were to investigate the effects of the immunomodulator Immunobeta® on serum lysozyme concentrations, alternative pathway of complement activation and beta-lysins in turkeys and organically reared layers in conditions of environmental thermal stress.

## MATERIALS AND METHODS

### Experimental design

The effects of Immunobeta® were evaluated on turkeys and hens in a free-range system during 2015-2016. The birds were treated with Immunobeta® as per recommendations of the manufacturer Chemifarma, Italy. The immunostimulatory preparation was produced by enzymatic autolysis of selected yeast strains (*Saccharomyces cerevisiae*) followed by natural extraction of components of yeast cells. The immunomodulator contains three important components: beta-glucans (30%), mannanoligosaccharides (25%) and nucleotides (5%). The experiments for evaluation of immunomodulator's effects in turkey and organically fed hens were performed during different periods of the year: beginning of lay (cold period), thermoneutral period and hot summer period.

The effect of the immunomodulator was tested on turkey egg productive light line (LL) selected in Agricultural Institute, Stara Zagora. The experiment was performed with 30 turkeys divided in two groups (15 experimental and 15 control birds). The influence of the preparation was tested from February to August 2015. The duration of the experiment was 7 months. Treated birds received the immunomodulator Immunobeta® at a dose of 4.0 g/kg feed during the beginning of egg lay (February-March 2015, cold period), the thermoneutral period (April-May 2015) and the hot summer period (June-July 2015). During these periods, the blood serum and egg white were collected three times and lysozyme, alternative pathway of complement activation (APCA) and beta-lysins were determined.

The study on the effect of Immunobeta® on humoral immunity of hens reared in a free-range system was performed in the Experimental farm of the Agrarian University - Plovdiv from November 2015 (19 weeks of age) to July 2016 (54 weeks of age). The tests were carried out on 75 dual-purpose rural hens Tetra Super Harco (originating in Babolna Tetra Kft, Hungary), housed in sleeping houses and walking yards. The effects of two dietary Immunobeta® levels were followed out: 2.0 g/kg and 4.0 g/kg. The birds were divided in three groups: group I (control), group II (supplemented with 2.0 g/kg) and group III (supplemented with 4.0 g/kg). The effect of the immunomodulator was monitored during three subperiods: beginning of egg lay (January-March 2016, cold period), thermoneutral period (April-May 2016) and hot summer period (June-July 2016). During these periods, the blood serum and egg white lysozyme, alternative pathway of complement activation (APCA) and beta-lysins were determined.

The blood samples from turkeys and hens were collected aseptically from vena ulnaris with disposable needles in vacuum tubes without anticoagulant.

Blood was transported in cool bags at 6 °C and left for 1 hour to clot in room temperature and centrifuged for 10 min at 2000 g. Present investigation have been conducted according to animal welfare documents in Bulgaria (regulation 25/2006; Regulation 44/2006).

### Assay methods

Serum lysozyme concentrations were determined by method of [Lie et al. \(1985\)](#). Briefly twenty ml of 2% agarose dissolved in phosphate buffer (0.07 M NaHPO<sub>4</sub> and NaH<sub>2</sub>PO<sub>4</sub>) was mixed with 20 mL suspension of 24-hour culture of *Micrococcus lysodeicticus* at 67 °C. The mixture was poured out in 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 mg/L) were prepared and pipetted in eight 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 the Trakia university.

Alternative pathway of complement activation (APCA) was evaluated by method of [Sotirov \(1986\)](#). 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 MgCl<sub>2</sub>). 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.

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 (25 °C). Thereafter, 150 µL of each supernatant was removed and placed in flat bottomed plates for measurement of optical density at 540 nm using 'Sumal-PE2' ELISA reader (Karl Zeiss, Germany). The final APCA activity was calculated using special computer programs developed in the Trakia university, and expressed as CH50 units (CH50 units correspond to 50% of complement induced haemolysis of applied erythrocytes). Beta lysins were assessed by method of [Buharin et al. \(1977\)](#). Briefly in flat bottomed plate (Flow Laboratories, UK) were drop 80 µL serum + 80 µL suspension of *Bacillus subtilis* (Merck, cat. № 1.10649) and optical density were measured at 600 nm using 'Sumal-PE2' ELISA reader (Karl Zeiss, Germany).

After 2 hours incubation at 37 °C optical density was measured again and bera lysin activity was calculated by following formula:

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

Where:

OD1: optical density before incubation.

OD2: optical density after incubation.

### Statistical analysis

Data were processed by one-way analysis of variance (ANOVA) with the fixed effect model using data analysis tool pack, Microsoft Excel 2010, Microsoft Corporation Ltd. at a level of significance  $P < 0.05$ .

## RESULTS AND DISCUSSION

The results about the effect of Immunobeta® supplementation on serum lysozyme, APCA activity and beta-lysins in turkeys are presented in Table 1. Under the influence of Immunobeta® lysozyme concentrations were increased during all three subperiods. The highest lysozyme concentration was observed during the cold period ( $P < 0.001$ ). A similar tendency occurred in the time course of APCA after supplementation with the immunomodulator. Although not statistically significant, the differences between control and experimental birds marked a tendency for higher serum APCA in supplemented birds at the time of beginning of lay. It is remarkable that high temperatures in the hot period suppressed significantly the APCA activity. The beta-lysins percentages were significantly higher during the hot period. As egg white lysozyme was concerned, there was positive significant effect of Immunobeta® on this parameter in experimental turkeys (Table 2).

The results summarizing the effects of Immunobeta® on serum lysozyme, APCA and beta-lysins in organic fed layer hens are shown in Table 3. Lysozyme concentrations decreased parallelly to increase in Immunobeta® dose and during the hot period, the reduction is statistically significant ( $P < 0.05$ ). This dose-dependent change confirmed the effects of Immunobeta® on the innate humoral immunity of hens. There was a significant increase in APCA activity during the three subperiods after supplementation with 4.0 g/kg immunomodulator ( $P < 0.05$ ). A similar trend for higher APCA activity was also observed in hens treated with 2.0 g/kg Immunobeta® as a dietary supplement although the differences *vs.* controls were not significant.

Table 4 presents the results for egg white lysozyme concentrations in organic fed layer hens under the influence of Immunobeta®. They were significantly higher in eggs produced by hens treated with 4.0 g/kg Immunobeta® ( $P < 0.05$ ). A similar change was noted in eggs produced by

hens treated at 2.0 g/kg immunomodulator but the differences *vs.* controls was insignificant ( $P < 0.05$ ).

The results about the effect of Immunobeta® on parameters of natural humoral immunity in studied birds affirmed categorically the positive effect of the immunomodulator Immunobeta®. It is well known that lysozymes are endo-acting enzymes that catalyze the hydrolysis of (1→4)-β-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan and between N-acetyl-D-glucosamine residues in chitooligosaccharides (Iacono *et al.* 1980; Leitch and Willcox, 1999; Zhang *et al.* 2017). Improvements of gastrointestinal morphology, physiology, microbiology and growth performance were reported by Denev *et al.* (2006b), Denev (2008), Yin *et al.* (2008), Al-Mansour *et al.* (2011), Silva *et al.* (2009), Fathi *et al.* (2012) and Bozakova *et al.* (2016) in broiler chickens, and by Huff *et al.* (2013) in turkeys. Other studies have evidence enhanced humoral immunity, resistance to diseases and lower death rates in birds after dietary supplementation of yeasts (Gao *et al.* 2008; Yin *et al.* 2008; Fathi *et al.* 2012). Vahabi-Asil *et al.* (2017) provide evidence that supplementing prebiotics in turkey's diet with different protein levels did not affect growth performance, blood biochemistry and haemagglutination-inhibition. Pournazari *et al.* (2017) and Tayeri *et al.* (2018) reported similar results when antibiotics, prebiotics and probiotics were supplemented to chicken broiler's diet. Bozakova *et al.* (2012) demonstrated that the welfare of hens during the hot summer period has been substantially improved through supplementation of zinc (100 mg/kg Zinteral 35, Lohmann Animal Health, Germany), containing 35 mg zinc/kg as zinc oxide) and zinc + 250 mg/kg vitamin C (100 mg/kg Zinteral 35 together with 250 mg/kg vitamin C). Bozakova *et al.* (2013a) confirmed the beneficial effects of these dietary supplements on the welfare of New Hampshire hens reared during the cold winter period. Similar positive influence was reported after supplementation of 1% L-arginine, zinteral 35, 250 mg/kg vitamin C and 1% arginine and 250 mg/kg vitamin C to the feed of turkeys and hens both during the hot and cold periods of the year (Bozakova *et al.* 2009; Bozakova *et al.* 2013b; Bozakova and Gerzilov, 2014). Sotirov *et al.* (2000) and Denev (2008) observed positive effects of the probiotic Lacto-Sacc® and at a lesser extent of lactose (as prebiotic) on the serum lysozyme concentrations and the alternative pathway of complement activation in broiler chickens. In turkeys treated with the above feed supplements, Sotirov *et al.* (2001) obtained similar results for the same factors of non-specific resistance. Sotirov *et al.* (2007) established also the stimulating effect of organic selenium (Sel-Plex®) in sows and their progeny, on blood serum lysozyme concentrations and complement levels.

**Table 1** Effect of immunobeta on serum lysozyme concentrations, APCA and beta lysines in turkeys (Means±SE)

Periods	Lysozyme (mg/L)		APCA (CH50)		Beta lysines (%)	
	Controls	Immunobeta 4 g/kg	Controls	Immunobeta 4 g/kg	Controls	Immunobeta 4 g/kg
Cold	0.96±0.29	1.13±0.27 <sup>a**</sup>	692.78±24.54 <sup>β***</sup>	695.90±9.84	34.7±2.9 <sup>γ</sup>	47.8±5.5
Termoneutral	0.32±0.05 <sup>a</sup>	0.72±0.11 <sup>a**</sup>	619.45±29.34	632.54±24.83	47.6±5.01	57.9±6.8
Hot	0.31±0.03	0.35±0.04 <sup>a</sup>	468.55±11.06 <sup>β</sup>	488.66±9.36	56.9±1.6 <sup>γ***</sup>	56.5±1.9

The means within the same row with at least one common letter, do not have significant difference (P>0.05).

\* (P<0.05); \*\* (P<0.01) and \*\*\* (P<0.001).

**Table 2** Effect of immunobeta on egg white lysozyme concentrations in turkeys (mg/L) (Means±SE)

Periods	Controls	Immunobeta
Cold	499.33±37.19	511.04±17.98 <sup>a***</sup>
Termoneutral	532.67±59.55	475.09±10.01
Hot	587.83±51.83	306.51±21.59 <sup>a</sup>

The means within the same row with at least one common letter, do not have significant difference (P>0.05).

\*\*\* (P<0.001).

**Table 3** Effect of immunobeta on serum lysozyme concentrations, APCA and beta lysines in hens (Means±SE)

Groups	Lysozyme (mg/L)	APCA (CH50)	Beta lysines (%)
Controls	1.71±0.22	543.77±28.15 <sup>a</sup>	40.56±1.29 <sup>b</sup>
0.2% immunobeta	1.50±0.25	567.12±25.96	48.99±5.62
0.4% immunobeta	1.53±0.15	613.74±26.39 <sup>a*</sup>	53.17±3.23 <sup>b**</sup>
<b>Termoneutral period</b>			
Controls	1.88±0.35	493.77±32.67 <sup>d</sup>	40.56±2.22 <sup>c</sup>
0.2% immunobeta	1.50±0.41	533.79±24.84	52.32±5.82 <sup>c*</sup>
0.4% immunobeta	1.53±0.49	563.74±14.48 <sup>d*</sup>	50.34±3.90
<b>Hot period</b>			
Controls	2.05±0.50 <sup>k</sup>	443.77±18.99 <sup>m</sup>	38.90±4.35
0.2% immunobeta	1.33±0.39	464.45±31.19	30.65±3.51
0.4% immunobeta	0.87±0.29 <sup>*</sup>	530.41±10.13 <sup>m*</sup>	33.67±4.01

The means within the same row with at least one common letter, do not have significant difference (P>0.05).

\* (P<0.05); \*\* (P<0.01) and \*\*\* (P<0.001).

**Table 4** Effect of immunobeta on egg white lysozyme concentrations in hens (Means±SE)

Groups	Lysozyme (mg/L)		
	Cold period	Termoneutral period	Hot period
Controls	482.66±22.60 <sup>n</sup>	494.73±30.82 <sup>u</sup>	521.16±30.12 <sup>v</sup>
0.2% immunobeta	527.71±19.01	525.09±22.36	539.84±17.05
0.4% immunobeta	561.04±27.76 <sup>n*</sup>	575.09±25.82 <sup>u*</sup>	589.84±11.13 <sup>v*</sup>

The means within the same row with at least one common letter, do not have significant difference (P>0.05).

\* (P<0.05).

Karakolev *et al.* (2013a), Karakolev *et al.* (2013b) and Gospodinova *et al.* (2013) provided proofs for significant stimulating effect of the preparation Helpankar on serum lysozyme, gamma interferon and alternative pathway of complement activation in layer hens. Immunological mechanism of alternative pathway of complement activation is very well described by Pangburn and Müller-Eberhard (1984).

Zhang *et al.* (2012) established that a dietary supplement containing yeast cellular wall products boosted the immune system of broiler chickens treated with the immunosuppressing agent cyclosporine A. Furthermore, Sadeghi *et al.* (2013) reported about a positive effect of a dietary prebiotic based on mannan-oligosaccharides and beta-glucans on the immune response of infected chickens.

Comparable data about favourable influence of mannan-oligosaccharides and beta-glucans on immune performance of chickens were also reported by Huff *et al.* (2013), Shanmugasundaram *et al.* (2013), Shao *et al.* (2013). Similar data have been published by Valtchev *et al.* (2015) in ducks. Kovitvadhi *et al.* (2019) let us know that prebiotic based on mannan-oligosaccharides and beta-glucans increase of phagocytic activity, relative weight of thymus and bursa of fabricius. According to Czech *et al.* (2014) the dietary supplementation with 6% *Yarrowia lipolytica* yeast increased lysozyme levels in turkeys. Results about increased egg white lysozyme concentrations were reported in Lohmann Brown hens, treated with 0.4% (4.0 g/kg feed) Immunobeta® in previous studies of ours (Bozakova *et al.* 2017).



Bozakova et al. (2016) reported that immunomodulator Immunobeta®, applied as dietary supplement at doses of 3.0 and 4.0 g/kg feed, stimulated the intestinal villi height and outer diameter of glandular crypts in the small intestine of broiler chickens. The application of the preparation at doses of 2.0 and 4.0 g/kg had beneficial effects on the growth of epithelium lining the glandular crypts and adjacent intestinal villi.

## CONCLUSION

Immunobeta®, offered at a dose of 4.0 g/kg feed had a beneficial effect on natural humoral immunity factors – it increased significantly lysozyme levels in turkeys, enhanced APCA activity and beta-lysins in hens. Applied at a dose of 4.0 g/kg, the tested immunomodulator increased significantly egg white lysozyme concentrations in hens, presuming a higher shelf life of eggs and possibly, better protection of chick embryos.

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