

## Effect of silymarin and ochratoxin A on humoral natural immunity of broiler chickens

### Влияние на силимарин и охратоксин А върху хуморалния естествен имунитет при пилета бройлери

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#### ABSTRACT

The aim of this work was to investigate the effect of Ochratoxin A (OTA) and Silymarin on serum lysozyme concentrations, complement and betalysin activity in broiler chickens. In this experiment 144 one-day-old Ross 308 male broiler chicks were used. All chicks were divided in four groups of 36 birds each: Group 1: Basal diet (BD) with no supplementation of Ochratoxin A (OTA) and Silymarin; Group 2: BD with 1.0% Silymarin; Group 3: BD with 3.0 mg/kg OTA; Group 4: BD with 3.0 mg/kg OTA plus 1.0% Silymarin. It was found that lysozyme concentration in the 2nd group there is a significant difference to groups 3 and 4. The immunosuppressive effect of Ochratoxin A is underlined but no protective effect of Silymarin in the group 4 was found. The alternative pathway of complement activation (APCA) is affected in the group 4. Betalysine there is a significant decreasing in the group 3 but slightly increasing of Betalysine in 4th group. Based on these results it can be concluded that OTA there is an immunosuppressive effect on the studied traits and there is a positive effect of Silymarin only on serum betalysine.

**Keywords:** betalysine, chicken, complement, lysozyme, ochratoxin A, silymarin

#### АБСТРАКТ

Целта на това изследване е да се проучи влиянието на Охратоксин А (ОТА) и Силимарин върху концентрациите на серумния лизоцим, активността на комплемента и бетализина при пилета бройлери. За този експеримент бяха използвани 144 едnodневни мъжки пилета бройлери от хибрида Ross 308. Пилетата бяха разпределени в четири групи по 36 пилета: Първа група (1): Основна диета (ОД) без добавяне на ОТА и Силимарин; Втора група (2): ОД с добавяне на 1.0% Силимарин; Трета група (3): ОД с добавяне на 3.0 mg/kg ОТА; Четвърта група (4): ОД с добавяне на 3.0 mg/kg ОТА и 1.0% Силимарин. Беше намерено, че лизоцимната концентрация при втора група е достоверно по-висока от тази при групи 3 и 4. Имуносупресивният ефект на Охратоксин А е подчертан, но не се установява протекция от Силимарина при 4-та група. Активността на алтернативния път за активиране на

комплемента е понижена при 4-та група. Активността на бетализина е достоверно по-ниска при 3-та група, но е достоверно увеличена при 4-та група. Въз основа на получените резултати може да се заключи, че ОТА има подчертан имуносупресивен ефект върху изследваните показатели при пилетата, но Силимарина има известен положителен ефект само върху активността на бетализина.

**Ключови думи:** бетализин, комплемент, лизоцим, охратоксин А, пилета силимарин

## INTRODUCTION

It is well known that mycotoxins are produced by a variety of molds (Frisvad et al., 2004; El Khoury and Atoui, 2010; Grenier and Oswald, 2011). Ochratoxin A (OTA) is the important member of this toxin family (van der Merwe et al., 1965). Ochratoxin A represents a serious health issue and has been associated with several human and animal diseases including poultry ochratoxicosis, porcine nephropathy, human endemic nephropathies, and urinary tract tumors in humans. In rodents, OTA was shown to be carcinogenic. OTA is the most toxic family member but other ochratoxins or their metabolites may cause serious problems to human and animal health. In vivo experiments in chicken showed that OTA undergoes extensive metabolism after oral administration resulting in different metabolites in chicken (Dietrich et al., 2005). The toxicological profile of OTA has been investigated in a lot of studies (Frisvad et al., 2004; Gekle et al., 2005; Grenier and Oswald, 2011; Haighton et al., 2012; Klarić et al., 2013; Limonciel and Jennings, 2014). These studies showed that OTA has nephrotoxic, hepatotoxic, neurotoxic, teratogenic and immunotoxic effects in various animals. The genotoxic mechanism involving OTA activation and DNA complex formation is discussed by some authors (Sarandan et al., 2012). They attempt to explain the mechanism of in vivo gene expression (Pfohl-Leskowicz and Manderville, 2012). According to Xiao et al. (1996), all animals died within 72 h supplementing the diet with OEOTA (DC-OTA, OM-OTA or M-OT $\alpha$ ) at a concentration of 500 mg/kg b.w. The same authors showed that there was no lethality at 200 mg/kg b.w. In comparison, OTA showed 30% and 90% lethality at 20 and 50 mg/kg b.w. The structure-activity analysis shows that the toxicity of OTA is associated with its isocoumarin moiety and most likely with the lactone carbonyl group, but not with the phenyl hydroxyl group nor the iron-

chelating properties (Mally and Dekant, 2009). Müller et al. (2004) compared the effects of different ochratoxins on extracellular radical formation in porcine blood monocytes and granulocytes and presented the following ranking: OTA (89.1) > OTC (154.2) > OTA methylester (285.5) > OTB (>1000) = OT $\alpha$  (>1000) (IC<sub>50</sub> in ng/mL).

In conclusion, based on the above-mentioned studies, no clear general toxicity ranking can be drawn; however, OTA seems has overall the most toxic effect, followed by OTC, OTB, and OT $\alpha$ . In nature, often is observed co-occurrence of different mycotoxins. One mold species may produce many different mycotoxins, and the same mycotoxin may be produced by several species (Speijers and Speijers 2004; Mally et al., 2005; Tozlovanu et al., 2006; Mally and Dekant, 2009; El Khoury and Atoui, 2010; Han et al., 2013; Soares et al., 2013; Sorrenti et al., 2013).

Considering this finding, it is very likely that humans and animals are always affected by mixtures rather than by individual compounds. In general, the combined effect of OTA with other mycotoxins showed mostly additive or synergistic interactions.

Silymarin is a natural compound derived from the species *Silybum marianum* (known also as Milk thistle). This plant contains at least seven flavoligands and the flavonoid taxifolin. The hepatoprotective and antioxidant activity of Silymarin is caused by its possibility to inhibit the free radicals' production in the metabolism of toxic substances (Dietrich D.R., et al. 2005). Silymarin increases hepatic glutathione and may boost the antioxidant defense of the liver. It has also been shown that Silymarin increases protein synthesis in hepatocytes by stimulating RNA polymerase I activity (Tedesco et al., 2004; Ahmad et al., 2012; Khatoun et al., 2013; Vargas-Mendoza et

al., 2014; Yu et al., 2018). Pozzo et al. (2013) reported that feeding broiler chickens, with a food contaminated with molds the maximum level admitted by the European Commission Recommendation (0,1 mg OTA/kg), did not affect the animal performance, slaughter traits, organ weights, haematological parameters, liver enzyme or renal function. However, in this concentration it has an overall immunosuppressant effect, with reduction in the thymus weight and the total serum protein, albumin, alpha, beta and gamma globulins concentrations. Sarandan et al. (2012) investigated the effect of Deoxynivalenol (DON) and Ochratoxin A and found that the inclusion of mycotoxin inhibitor reduced the severity of histological lesions and had a significant effect on body weight, feed conversion ratio, serum lysozyme, properdin, and phagocytic activity. Lea et al. (1989) established that the Ochratoxin A causes immunosuppression through interference with essential processes in cell metabolism irrespective of lymphocyte population or subpopulation. According to Corrier (1991), Ochratoxin A immunosuppression may be manifested as depressed T or B lymphocyte activity, suppressed immunoglobulin and antibody production, reduced complement, or interferon activity. Stewart et al. (1985) showed that Ochratoxin A in dose of 2.5 µg/g decreases significantly complement activity in chicken. Similar results reported Valtchev et al. (2015) in ducks treated with different doses of Aflatoxin B<sub>1</sub>. Generally, information about the influence of Ochratoxin A and Silymarin on humoral natural immunity in chicken broilers is very limited. That is why the aim of this work was to investigate the immunotoxic effect of Ochratoxin A and possible preventive effect of Silymarin on serum lysozyme concentrations, complement and betalysin activity in broiler chickens.

## MATERIAL AND METHODS

### *Experimental Design, Birds, Housing and Diets*

The study was conducted in the Poultry Unit, Faculty of Agriculture, Trakia University, Stara Zagora, Bulgaria, during April-May 2019. The experimental poultry house and equipments were cleaned and disinfected before starting the experiment. The completely randomised

experimental design included 144 one-day-old Ross 308 male broiler chicks that were obtained from a local commercial hatchery. Upon arrival all chicks were individually weighted, wing-banded, and assigned randomly in four groups of 36 birds each, with six subgroups (replicates) of 6 birds each. They were housed in separate pens into wire type experimental cages that were placed in an environmentally controlled experimental poultry house. Pens were equipped with plastic feeders and drinkers. All broilers were kept under the same managerial, hygienic and environmental conditions. The rearing environment complied according to the Ross breeder's recommendations (Aviagen, 2009). Water and feed were provided *ad libitum* throughout the experimental period. The trial was terminated when the broiler chicks were 42 days of age. A three-phase feeding program was used with commercial complete standard, no medicated type corn-wheat-soybean based diets in mash form, produced by Melchran Ltd., Stara Zagora, Bulgaria: Starter (from 1<sup>st</sup> to 21<sup>st</sup> day); Grower (from 22<sup>nd</sup> to 35<sup>th</sup> day) and Finisher (from 36<sup>st</sup> to 42<sup>nd</sup> day). All experimental basal diets were formulated to meet broiler chick's nutritional requirements, according to the NRC (1984) and Aviagen (2009) nutrient recommendations. The basal diets were the same for all groups (Table 1).

The commercially used standard feeds did not contain detectable amounts of mycotoxins as aflatoxins, deoxynivalenol, fumonisin B<sub>1</sub>, OTA, T-2 toxin and zearalenone. Basal diets were mixed as a single batch to reduce diet variability, after which the feed supplement was added to create the different dietary treatments. The Silymarin and OTA were periodically homogenized with the chick ration (each week) in order to give the required levels. The complete feeds per experimental treatment were stored in sacks that had been appropriately and clearly labelled. During the study a total of four dietary treatments were applied:

- Group 1: Basal diet (BD) with no supplementation (Negative control, **NC**);
- Group 2: BD with 1.0% Silymarin (Positive control, **PC**)
- Group 3: BD with 3.0 mg/kg OTA
- Group 4: BD with 3.0 mg/kg OTA plus 1.0% Silymarin.

**Table 1.** Composition and nutrient content of basal diets

Dietary ingredient, % (as-is)	Diets		
	Starter	Grower	Finisher
Corn	30.00	33.00	33,34
Wheat	29.00	30,40	30.00
Soybean meal (47% CP*)	30.00	23.00	19,00
Sunflower meal (37% CP)	3.00	5.00	8.00
Sunflower oil	3,50	4,70	6.00
L-Lysine HCL (56%)	0,30	0,28	0,35
DL-Methionine (free base)	0,25	0,18	0,17
L-Threonine (free base)	0,08	0,08	0,08
Phytase®	0,01	0,01	0,01
Calcium carbonate	0,61	0,5	0,28
Dicalcium phosphate	2,20	1,85	1,80
Salt (NaCl <sub>2</sub> )	0,25	0,20	0,15
Sodium bicarbonate	0,30	0,30	0,32
Vitamin-Mineral Premix**	0,50	0,50	0,50
<i>Calculated nutrient composition</i>			
Metabolizable energy, Kcal/kg	3003	3121	3195
Crude Protein (%)	22.00	19,70	19,00
Calcium (%)	1,00	0,90	0,76
Available phosphorus (%)	0,46	0,40	0,39
Methionine (%)	0,58	0,49	0,48
Methionine + Cysteine (%)	0,94	0,82	0,80
Lysine (%)	1,32	1,14	1,12
Threonine (%)	0,86	0,77	0,74
Tryptophan (%)	0,25	0,22	0,21
Sodium (%)	0,20	0,18	0,16

\* CP - Crude Protein.

\*\* The vitamin and mineral premix contained vitamins and trace elements to meet the requirements specified by the NRC (1994). The premix provided (units/kg diet): Retinol, 3,600 µg; Cholecalciferol, 125 µg; α-tocopherol, 34 mg; Menadione, 3 mg; Thiamine, 2 mg; Riboflavin, 7 mg; Pyridoxine, 5 mg; Cobalamin, 15 µg; Nicotinic acid, 50 mg; Pantothenic acid, 15 mg; Folic acid, 1 mg; Biotin, 200 µg; Iron, 80 mg; Copper, 10 mg; Manganese, 100 mg; Cobalt, 0.5 mg; Zinc, 80 mg; Iodine, 1 mg; Selenium, 0.2 mg; Molybdenum, 0.5 mg

### Silymarin

Silymarin with molecular weight 482.44 g/mol and purity (UV 60%) used in this study was produced by Samwon International LTD., Nanjing, China.

### Ochratoxin A Production

The OTA used in this study was produced by *Aspergillus ochraceus* (isolate D2306), as used by Tapia and Seawright (1984) and Stoev et al. (2000, 2000a, 2002, 2019). The methodology for OTA production, investigation and using are described by Stoev et al. (2019).

### Ethics approval and consent to participate

The Animal care Ethics Committees approved the study protocol and ethical clearance (N° 111 from 20.11.2014) was issued for the study by the Bulgarian Agency for Food Safety. The experiments were conducted within standard ethical norms and no birds were subjected to undue stress. The chicks were housed and maintained in accordance with the relevant international rules and recommendations.

### Assay methods

At 42 day of age blood samples were collected from wing vein (*v. subcutanea ulnaris*) of six birds per group and were left to clot for 1.0 h. Then blood sera were centrifuged at 2000g for 10 min, then collected in different sterile tubes, and stored at - 25 C° until the time of investigation.

Serum lysozyme concentrations were determined by method of Lie (1985); Alternative pathway of complement activation (APCA) by method of Sotirov (1986); β-lysine by method of Buharin et al. (1977).

### Statistical Analyses

All data were analysed by one-way ANOVA with fixed effects of the factor, using Statistica 6.0 (StatSoft Inc.). Results were presented as mean ± SEM (pooled). Differences among groups were considered significant at P<0.05.

## RESULTS AND DISCUSSION

The results obtained are presented in the attached Table 2. As it is seen in the table, highest lysozyme concentrations are found in the Control group (group I) but there are no significant differences to other groups of the experiment. The group treated with Silymarin (group II) is in second place with respect to lysozyme concentrations and there is a significant difference to Ochratoxin A (group III) and Silymarin + Ochratoxin groups (group IV) ( $P < 0,05$  and  $P < 0,001$ ). The immunosuppressive effect of Ochratoxin A is underlined. It is also seen that no protective effect of Silymarin in the group treated with Silymarin + Ochratoxin (group IV).

Sarandan et al. (2012) and Pozzo et al. (2013) also reported about the immunosuppressive effect of Ochratoxin A in serum lysozyme concentrations. Khatoon et al. (2013) reported for a dose-dependent protective effect of Silymarin to immunotoxicity of Ochratoxin in chicks. According to these authors, Silymarin and Vitamin E alone or in combination improved the immunotoxic effects induced only by mg OTA/kg but could not influence the effect when 2.0 mg OTA/kg is ingested. In our experiment, a dose of Ochratoxin A 3 mg/kg was used and according to the data of Khatoon et al. (2013), it is impossible to expect a protective effect of Silymarin. According the results reported in this work these data are very important for veterinary and human medical experts in their clinical practice. The alternative pathway of complement activation (APCA) is affected in the highest degree in the group treated with Silymarin and Ochratoxin A (group IV,  $P < 0,001$ ). Here is not established any protective effect of Silymarin on APCA

as well. Stewart et al. (1985) showed that Ochratoxin A in dose 2,5  $\mu\text{g/g}$  decreases significantly complement activity in chicken. Similar results reported Valtchev et al. (2015) in ducks treated with different doses of Aflatoxin B1. Generally, it was not found more information about the effect of Ochratoxin A on complement activity.

Regarding the results for Betalysine it can be seen that there is a significant decreasing of the activity of this trait in the group treated only with OTA (group III,  $P < 0,05$ ) but in the same time there is slightly increasing of Betalysine activity in group treated with Silymarin and Ochratoxin A (group IV). This fact shows that in this case there is positive effect of Silymarin on immunosuppressive effect of OTA on Betalysine.

Dwivedi and Burns (1984), Muller et al. (1999) reported that Ochratoxin A manifests immunomodulator effects. According to them a reduction in the number of lymphoid cells was observed after OTA ingestion, especially in the thymus, bursa Fabricii and spleen of poultry. This indicates a potential suppression of cell-mediated immunity. According to them a reduction in serum immunoglobulins and phagocyte capacity of leucocytes and neutrophil also occur, resulting in reduced resistance to viral and bacterial infections. In the same year, both authors found that total immunoglobulin levels were also reduced in the sera from OTA fed birds.

Fuchs and Hult (1992) described two mechanisms for the toxic effect of OTA. First one is probably connected with the influence of OTA on enzymes participating in the phenylalanine metabolism (phenylalanine-transferase, phenylalanine-hydroxylase, phenylalanine-lipoperoxide) and the functions of mitochondria.

**Table 2.** Effect of Silymarin and Ochratoxin A on some humoral factors of innate immunity in chicken broilers

Groups/Traits	Lysozyme	APCA	Betalysine
Group I Control	1,73 $\pm$ 0,91	616,13 $\pm$ 14,9 <sup>a***</sup>	30,72 $\pm$ 9,6 <sup>a*</sup>
Group II Silymarin	0,67 $\pm$ 0,08 <sup>a***</sup>	607,14 $\pm$ 13,8	26,15 $\pm$ 7,8
Group III Ochratoxin	0,45 $\pm$ 0,08 <sup>a</sup>	599,32 $\pm$ 32,9	13,46 $\pm$ 4,9 <sup>a</sup>
Group IV Silymarin+ Ochratoxin	0,30 $\pm$ 0,09 <sup>b</sup>	524,25 $\pm$ 27,1 <sup>a</sup>	17,45 $\pm$ 5,9

\*-  $P < 0,05$ ; \*\*\*-  $P < 0,001$  (APCA – alternative pathway of complement activation)

The secondary mechanism of the toxic effect is based on increased lipid peroxidation in liver and kidney microsomes. The third mechanism of OTA's toxic effect is based on the inhibition of respiration in mitochondria, where it acts as a competitive inhibitor of the carrier's proteins, localized on the inner membrane of mitochondria (Uraguchi and Yamazaki, 1978).

## CONCLUSION

On the bases of the results obtained in this experiment it can be concluded that OTA has an important immunosuppressive effect on chicken treated with a dose of 3 mg/kg food and there is a positive effect of Silymarin only on serum Betalysine.

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