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Original Article

Effect of Modified Clinoptilolite to Counteract the Deleterious Effects of Ochratoxin A on Egg Production and Quality

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ABSTRACT

The objective of this study was to evaluate the efficacy of modified clinoptilolite as mycotoxins adsorbent in preventing negative effects of ochratoxin A (OTA) on egg production and egg quality of laying hens exposed to this mycotoxin. Forty-eight (n=48) laying hens (27 weeks old) were used in this study. The hens were randomly divided into six equal groups and were fed for 7 weeks with a standard diet in addition to: E-I group - 1 mg/kg OTA; E-II group 0.25 mg/kg OTA; E-III group 1mg/kg OTA + 0.2% of MZ; E-IV group 0.25 mg/kg OTA + 0.2% of MZ. MZ group of hens was fed with standard diets containing 0.2% of the adsorbent (MZ). The control group of hens was fed with standard diet, without any addition of OTA or MZ. The present study showed that laying hens fed with 1 mg/kg of OTA (E-I), had a significant decrease (p<0.05) of all performance parameters during the trial, while group fed with 0.25 mg/kg OTA have shown no adverse effects on egg production and egg quality. Addition of modified clinoptilolite (0.2%) to the diet containing the OTA, minimized these effects bringing values not significantly different from the control diet for most of the parameters. These findings clearly indicate the protective potential of modified clinoptilolite against the toxic effects of OTA in laying hens.

INTRODUCTION

Eggs are an important source of high-quality proteins, lipids, vitamins and trace minerals for humans (Cimrin *et al.,* 2020). The egg belongs to the limited category of foods containing the nine amino acids that human cannot synthesize. It was thus chosen by World Health Organization (WHO) as the reference protein source for children (Moula *et al.,* 2013). Furthermore, due to the increasing human populations and incomes, poultry meat and eggs demand is continuously growing across the world, making a significant and increasing contribution to the national economy of most countries (FAOSTAT, 2020).

Thus, the poultry production in EU and worldwide has undergone significant changes, and clear criteria have been established to improve the quality of the products (meat and eggs), to guarantee the safety of the food, and to protect the environment in line with the animal welfare (Mitrović *et al.*, 2018). According to the latest available information, global production of table eggs has increased by 150 % over the last three decades, bringing production to 77 million tonnes in 2018 (FAO, 2020), which is expected to increase further because of the high demand for animal-originated protein. Asia is the largest egg-producing region, with more than 64 percent of global output, where production increased almost fourfold (FAO, 2020). In Serbia general, poultry production has fluctuated in the recent years. Egg production in 2020 was approximately 1.706 billion eggs, with average of around 200 eggs/laying hen (Statistical Office of the Republic of Serbia, 2021).

Feed intake (g feed/egg) represents 70-80% of the total costs of poultry production, thus significantly affect the economic efficiency of egg production (Carvalho *et al.*, 2015). The poultry feed industry suffers the greatest economic losses as compared to other animal species due to mycotoxins contamination (Zhai *et al.*, 2021). Mycotoxins are defined as a chemical hazard of microbiological origin, structurally diverse group of secondary metabolites, produced mainly by filamentous fungi during their secondary metabolism, that can contaminate feedstuffs commonly used in poultry feeds (Milićević *et al.*, 2011). Because of co-occurrence usually at low concentrations, mycotoxins in farm animals can cause subclinical losses in production and increase the risk and incidence of infectious diseases mainly due to their immunosuppressive effects (Pleadin, 2015). Ochratoxin A (OTA) is one of the most relevant mycotoxins with great public health and agroeconomic significance, due to the confirmed nephrotoxic, genotoxic, neurotoxic, imunotoxic, embriotoxic, teratogenic effects and its suspected carcinogenicity (Nedeljković-Trailović, *et al.*, 2015, Pleadin *et al.*, 2016). In commercial birds, consumption of OTA-contaminated diets may cause significant losses to the poultry industry due to reduced performance, weight gain, impaired feed efficiency, reduced egg production and quality, and also impaired health by reduced resistance to infectious diseases. Unlike the other monitored mycotoxins, OTA has the potential to bioaccumulate in the organism. Thus, feeding birds with contaminated feed containing OTA could lead to the presence of OTA residues in tissues and eggs, which represent a public health risk due to human consumption of contaminated animal products (Milicevic *et al.*, 2016). Predisposing factors like extreme weather events in Serbia, pre-harvest and harvest conditions, poor storage conditions, etc., are one of the greatest risks for contamination of cereals such as wheat, maize, barley and oat by OTA (Milićević *et al.*, 2020).

Several treatments and dietary strategies have been developed for the detoxification of OTA in contaminated feeds. In intensive poultry production the most promising and economical approach for reducing harmful effects of mycotoxins is the use of feed additives known as adsorbents. Therefore, in order to protect human health, animal health and welfare, a new functional group was added in the EU in the category of technological feed additives defined as 'substances that can suppress or reduce the absorption, promote the excretion of mycotoxins or modify their mode of action' (EC Commission Regulation, 386/2009). Moreower, the use of adsorbents is considered to be more of a preventative approach than therapeutic (Nedeljković Trailovic *et al.,* 2015). In the last decade, several studies have suggested that alumino-silicates (Al_2SiO_5), particularly clinoptilolite $(Na,K,Ca)_4(Al_6Si_{30}O_{72})\cdot 24H_2O$ have been effectively used as adsorbent for organic and inorganic substances. A characteristic of their honeycomb structure is a system of open microchannels in the crystal structure, renders them ideal as ion-exchangers, catalysts, and binding agents. Hence, they have been used for decades in animal feeds to diminish the adverse effects of mycotoxins to livestock and the carry-over of toxin compounds to animal products (Banaszak *et al.,* 2020). Over the last decade, there has been increasing interest of researchers in the adsorption properties of Serbian clinoptilolite as adsorbent in preventing negative effects of mycotoxins. The aim of this study was to investigate the effectiveness of locally available, low-cost, patented and modified clinoptilolitein reducing the adversely effect on the growth performance and egg quality parameters of laying hens fed with OTA-contaminated feed.

MATERIAL AND METHODS

Ethical approval

All procedures were carried out in accordance with the permit of the Ethics Committee of the Ministry of Agriculture, Forestry and Water Management as well as the Veterinary Directorate Republic of Serbia no 323-07-00241/2019-05-01. The permit of Ethics Committee is according with Directive 2010/63/EU.

Adsorbent

Tested adsorbent - modified clinoptilolite (Minazel Plus®) used in the current study, was provided from the Patent Co, Misicevo, Serbia. Minazel Plus® is a preparation made of organically modified natural clinoptilolite consisting of a mineral and an organic component, which form an organo-complex in the defined technological process as a result of ion exchange between inorganic cations on the mineral surface and long chain organic cations added during the production process. It is chemically stable in the digestive tract of the animals (in the pH range between 2 and 9), and it is non-resorptive. By the adsorption of long-chain organic cations, the physicochemical characteristics of the outer mineral surface are profoundly altered, reflecting in the creation of a double layer of organic ligand on which nonpolar organic molecules are adsorbed. Organo-complex as a boundary phase has a hydrophobic surface, making it compatible with the nonpolar organic molecules of mycotoxins (zearalenone, ochratoxins, T-2 toxin and others). The latest developments include the enhancement of the adsorptive characteristics by addition of new active centers on the surface, making Minazel Plus® effectively adsorb mycotoxins, with the insignificant adsorption of vitamins, amino acids, and microelements. Chemical composition of the modified clinoptilolite, which was added in the diet are shown in Table 1.

Table 1 – Chemical composition of the modified clinoptilolite, which was added in the diet.

Component	Content
Moisture	6.54%
Clinoptilolite content	>85.00%
SiO_2	64.19%
Al_2O_3	7.64%
Fe_2O_3	0.70%
CaO	3.71%
Arsenium (As)	0.38±0.08 mg/kg
Lead (Pb)	12.03±2.85 mg/kg
Cadmium (Cd)	<0.04 mg/kg
Mercury (Hg)	<0.02 mg/kg
Total capacity of cationic exchange	215.3 mmolM^+/100g

Experimental Design

The experiment was conducted at the reaserch farm (unit for poultry) of the Department of equine, small animal, poultry and wild animal desease Faculty of Veterinary Medicine University of Belgrade, Belgrade. Forty-eight (n=48) laying Lohmann Brown hens (27 weeks old) were used in this study. The average body weight of the birds was 1520±87 g, while the study lasted 49 days. The birds were placed in a light-controlled (16 h Light:8 h Dark) and temperature-controlled (22°C) room in wire cages having the following dimensions (length × depth × height): 47 cm × 32 cm × 45 cm ×35 cm, with unlimited access to drinking water. The percentage relative humidity in the cages was maintained at between 60 and 65. The hens were randomly divided into six equal groups, and fed 7 weeks by standard diet in addition to: E-I group - 1 mg/kg OTA; E-II group 0.25 mg/kg OTA; E-III group – 1 mg/kg OTA + 0.2% of MZ; E-IV group 0.25 mg/kg OTA + 0.2% of MZ; E-V group 0.2% MZ. The control group of hens (C) was fed only with standard diet, without any addition of OTA or MZ. The contaminated diet was prepared with OTA obtained by contamination of corn with *Aspergillus ochraceus.* OTA was produced using the culture of *Aspergillus ochraceus* Wilhelm NRRL 263.67 from the Dutch collection.

Hens were fed once a day, while the standard diet contained complete mixtures to meet the nutrient requirements of laying hens according to the NRC recommendations (National Research Council, 1994). All used feed was from the same source (company) and had the same production date. Diet composition is shown in Table 2. Throughout the study (7 wk) feed and water were provided *ad libitum*, and each hen was clinically observed.

Table 2 – Composition of basal diets (%).

Ingredient	%
Maize	44.48
Wheat	10.00
Wheat meal	5.00
Soybean meal (440 g crude protein/kg)	17.60
Sunflower meal (330 g crude protein/kg)	10.00
Soybean oil	3.05
MCP	1.10
Limestone	8.35
Iodiezed Salt	0.10
dl-methionine 99%	0.09
Phytasa	0.003
Xylanase Ron WX	0.02
Calculated nutrient and energy	%
Crude protein	16.5
Metabolic energy MJ/kg	11.5
Crude fat	5.6
Crude fiber	5.09
Calcium	3.65
Total phosphorus	0.66
Phosphorus available	0.38
Total sodium	0.16
Chloride	0.16
Kalium	0.70
Lysine	0.78
Methionine	0.65

Analysis of mycotoxins

Before artificial contamination with OTA, the feed was tested for the presence of other mycotoxins (aflatoxin B1, deoxynivalenol, trichothecenes, fumonisins and zearalenone) in order to avoid synergistic toxic effects on broilers.

The analyses of mycotoxins were carried out ~~OTA~~ as previously published by Nedeljković-Trailović *et al.*, (2015) using ELISA kits for aflatoxin B1 (Celer® Afla B1, Tecna, Trieste, Italy, limit of detection LoD = 0.5 µg/kg; limit of quantification LoQ = 1 µg/kg), deoxynivalenol (Celer® DON v3, Tecna, Trieste, Italy, LoD = 40 µg/kg; LoQ = 125 µg/kg), trichothecenes (Celer® T2, Tecna, Trieste, Italy, LoD = 15 µg/kg; LoQ = 25 µg/kg), fumonisins (Celer® Fumo, Tecna, Trieste, Italy, LoD = 750 µg/kg; LoQ = 1,000 µg/kg) and zearalenone (MaxSignal® zearalenone ELISA test kit, Bioo Scientific Corp, Austin, TX, USA, LoD = 0.7 µg/kg; LoQ = 1 µg/kg).

Parameters of productivity and egg quality

The feed intake rate was recorded weekly per pen and the average daily feed intakes (ADFI) and feed conversion ratios (FCR) were determined for the entire period for each treatment. The Feed Conversion Efficiency (FCE) was calculated by dividing mass egg by feed intake.

Egg production and egg quality were evaluated by the daily collection of eggs (n = 3/day/per group) and summarized data for each during 7 wks (n = 588). The following parameters were assessed:

Egg weight (EW) and yolk weight was determined in a digital scale (0.0001g accuracy, Chyo, model JL 200, Japan).

After the eggs were broken the shell with membranes were washed under running water to remove adhering albumen. The wet eggshell was left for 24 h at room temperature for drying and then eggshell thickness without the membrane was measured using a commercially available micrometer (Digimatic micrometer, Series 293-330, Mitutoyo, Japan). The measurements were taken at three random locations at the equator, blunt and pointed edges of each eggshell without membrane. The average of the three measurements represented the shell thickness (Crosara *et al.,* 2018).

Eggshell color was measured at the large end of the eggs using a colorimeter (Minolta, CR-600, Osaka, Japan) by determination of L^*, a^*, b^*. As shown below, L* indicates lightness, a* is the red/green color space, and b* is the yellow/blue color space (Eleroğlu *et al.,* 2016).

The pigmentation of the egg yolk was measured visually using the Roche Yolk Color Fan (F. Hoffmann-La Roche Ltd., Basel, Switzerland), which is a scale of colours ranging from 1 (light pale) to 15 (dark orange). Sensory evaluation of egg yolk colour was performed by well-trained panel of experts consisting of 5 people experienced in sensory egg analysis.

Haugh units - After the eggs were weighed, they were broken on a flat glass surface. The height of the albumen was registered using a tripod micrometer (AMES S-6428). Egg weight (g) and albumen height (mm) were used to calculate the Haugh units according to Pardi (1977): $HU = 100_{log}(h + 7.57 - 1.7\,W^{0.37})$, where: h = albumen height (mm) and W = egg weight (g).

The diameter of a yolk portion was measured using a vernier caliper.

The yolk and albumen were separated from each egg and weighed separately. The yolk ratio (participation of yolk in the egg mass) was calculated using a mathematical formula in which %Y=yolk weight/egg weight×100.

Statistical analysis

Descriptive statistics and effects of dietary clinoptilolite and OTA on certain egg parameters were separately analyzed. Data were further subjected to analysis of variance (ANOVA), and when significant differences ($p<0.05$) were obtained, Tukey's test was used. The results were analysed by the use of Graph Pad Prism® 5.0 software (Graph Pad Software Inc., San Diego, California, USA). All values are expressed as the mean ± SE.

RESULTS AND DISCUSSION

Parameters of productivity

Results of feed conversion ratio (FCR) is shown in Table 3. The FCR in laying hens represents the ratio of the consumed feed and the mass of eggs. After 7 wks of treatment average FCR for the E-I, E-II, E-III, E-IV, Mz and C was 2.23, 1.90, 1.95, 1.91, 1.87 and 1.98 kg feed/mass egg, respectively, with the lowest (better). When compared between groups, the best FCR was observed in the group where MZ (0.2%), added in feed (1.87) while group that received 1 mg of OTA/kg of feed, had the lowest FCR (2.23) indicating poor performance an impaired parameters of productivity (all data not presented). FCR was improved in the group of laying hens fed the diet supplemented with MZ by 14%, when compared with the group that consumed feeds contaminated with 1 mg of OTA/kg of feed. The assessment of the differences in FCR between groups observed in our study, support the results of a previous study where the inclusion of MZ in laying hen feed could increase feed efficiency (Vasiljević *et al.*, 2021).

Table 3 – Feed conversion ratio (kg feed/mass egg).

Group	Week		
	1 -3 st	4-6 st	1-7 st
E-I	2.13^{a}	2.22^{a}	2.23^{a}
E-II	1.90^{b}	1.89^{b}	1.90^{b}
E-III	1.96^{c}	1.91^{b}	1.95^{c}
E-IV	1.91^{b}	1.92^{b}	1.91^{b}
Mz	1.92^{b}	1.90^{b}	1.87^{d}
K	$2.02^{a,c}$	1.95^{c}	1.98^{c}

[a,b,c] Means followed by the same letter within a column are not different by the test (*p*-value<0.05). Experimental groups: E-I group - 1 mg/kg OTA; E-II group 0,25 mg/kg OTA; E-III group 1 mg/kg OTA + 0,2% of MZ; E-IV group 0,25 mg/kg OTA + 0,2% of MZ; Control group (C) - standard diet without any addition; MZ - Minazel Plus® - modified adsorbent; OTA – ochratoxin A.

Due to feed costs constitute the greatest share of total costs in intensive poultry production, FCR is a remarkable parameter contributed to the economic efficiency and profitability of poultry production. As the diets of all treatment groups were the same, from a practical point of view these results suggest that feed efficiency (feed consumed per egg produced) and egg quality is adversely affected only when the hens are exposed to higher concentrations of OTA. Dietary OTA at a level of 1 mg/kg (E-I), which seldom occur naturally, resulted in a significantly poorer feed efficiency shortly after the start of feeding, with a tendency toward the impairment of nutritient utilization for the egg production. Conversely, group fed with 0.25 mg/kg OTA showed no adverse effects on feed efficiency, as well on egg productivity and egg quality. The data reported in this study were consistent with the results of earlier experiments (Nedeljković-Trailović *et al.,* 2001, 2004; Duarte *et al.,* 2011) which confirm that exposure to OTA contaminated feed without supplementation of mineral adsorbent for a longer period causes a significant decrease in poultry productivity in terms of weight gain, poor feed conversion, reduced egg production, poor egg shell quality and nephrotoxicity. Based on its polar composition, OTA is quickly absorbed from the digestive tract of the hens, thus compared with other tissues gut is exposed to OTA at higher concentrations, tended to OTA first interact with the intestinal epithelium. Several researchers suggested that OTA in the ingested feed negatively affected the microvilli of the intestine, thereby affect gut permeability, reduced digestibility, energy and nutrient absorption, including water with consequently gut and other organ injury (Constantinescu & Chou, 2016; Pietro *et al.,*2017; Zhai *et al.,* 2021). Anyhow, the gut is the first defense barrier that prevent harmful substances, particularly bacteria and toxins from feed to enter the body, and also is closely connected to immune responses. Taken together, gastrointestinal dysfunction can lead to decreased growth performance followed by increasing susceptibility of animals to various diseases (Zhang, 2018; Ruan *et al.,* 2019; Wang *et al.,* 2019).

External quality traits of egg

Results of effects of dietary MZ and OTA on external quality traits of egg during the experimental period, shown with significant differences between the mean values, are summarized in Table 4. The present study showed that laying hens exposed to 1 mg/kg of OTA

Table 4 – External quality traits of egg.

Traits	Group	Week						
		1st	2nd	3rd	4th	5th	6th	7th
Number of egg	E-I	52.0±0.14^{a}	40.0±0.42^{a,A}	39.0±0.20^{a,A}	47.0±1.38^{a,A}	46.0±0.53^{a,A}	45.0±0.97^{a,A}	38.0±0.53^{a,A}
	E-II	52.0±0.37^{a}	53.0±0.53^{b,B}	55.0±0.14^{b,c,B}	55.0±0.38^{c,d,B}	55.0±0.14^{c,B}	54.0±0.48^{b,B}	54.0±0.18^{b,B}
	E-III	54.0±0.28^{b}	54.0±0.48^{b,c,B}	55.0±0.14^{b,c,B}	53.0±0.53^{b,B}	53.0±0.30^{b,B}	53.0±0.78^{b,B}	53.0±0.20^{b,B}
	E-IV	54.0±0.18^{b}	55.0±0.38^{c,d,B}	54.0±0.18^{b,B}	54.0±0.48^{b,c,B}	55.0±0.14^{c,B}	56.0 ± 0.00^{c,B}	53.0±0.30^{b,B}
	Mz	52.0±0.00^{a}	56.0±0.00^{d,B}	56.0±0.00^{c,B}	56.0±0.00^{d,B}	56.0±0.00^{c,B}	56.0 ± 0.00^{c,B}	56.0±0.00^{c,B}
	C	51.0±0.30^{a}	52.0±0.20^{b,B}	55.0±0.14^{b,c,B}	56.0±0.00^{d,B}	55.0±0.14^{c,B}	54.0±0.48^{b,B}	50.0±0.14^{d,B}
Egg weight (g)	E-I	60.96±5.34^{a}	57.07±3.82^{a}	58.59±5.97^{a}	60.78±4.05^{a}	60.91±4.88^{a}	61.74±8.83^{a}	61.33±9.31^{a}
	E-II	64.36±2.31^{b,c}	63.11±3.26^{b}	63.98±4.30^{b,d}	63.11±3.05^{c,d}	63.64±3.65^{b,c}	63.10±3.37^{b,c}	64.67±2.42^{b}
	E-III	61.57±5.22^{a}	62.38±4.22^{b}	61.71±5.68^{c}	61.83±4.27^{a,b}	61.32±2.62^{a,d}	61.69±5.21^{a}	60.33±6.11^{a}
	E-IV	64.39±4.48^{b}	63.65±2.86^{b}	64.04±4.08^{b}	64.37±3.96^{d}	62.86±2.81^{b,d}	63.95±4.22^{c}	65.43±5.16^{b,c}
	Mz	61.55±4.72^{a}	63.90±3.84^{b}	61.74±3.93^{c}	62.27±2.73^{b,c}	62.15±4.31^{b,d}	62.88±5.22^{b}	66.50±4.81^{c}
	C	62.36±2.66^{a}	63.08±5.04^{b}	62.45±4.03^{c,d}	63.43±1.96^{d}	64.28±2.90^{c}	63.93±2.85^{c}	68.17±3.86^{d}
Color of eggs shell	E-I	4.00±0.00^{a,b}	4.00±0.00^{a,b}	4.11±0.33^{a}	4.00±0.00	3.83±0.41^{a}	3.66±0.51	3.66±0.51^{a}
	E-II	3.75±0.46^{a}	4.00±0.00^{a,b}	4.12±0.64^{a}	4.00±0.00	4.00±0.00^{a,b}	3.87±0.35	4.00±0.00^{b}
	E-III	4.00±0.57^{a,b}	4.13±0.35^{b}	4.00±0.00^{a,b}	4.17±0.98	4.25±0.46^{b}	4.12±0.35	4.12±0.64^{c}
	E-IV	4.12±0.35^{b}	3.75±0.46^{a,c}	3.83±0.41^{b}	4.00±0.00	4.12±0.35^{b}	3.85±0.37	4.00±0.57^{b}
	Mz	3.87±0.35^{a}	3.86±0.38^{a,c}	4.00±0.00^{a}	4.00±0.00	3.87±0.35^{a}	3.62±0.74	3.87±0.35^{a}
	C	3.75±0.46^{a}	3.57±1.13^{c}	3.86±0.38^{b}	4.00±0.00	3.75±0.46^{a}	3.75±0.46	3.83±0.41^{a}
Thickness of the egg shell (mm)	E-I	0.339±2.64^{a}	0.316±4.50^{a,A}	0.332±4.79^{a}	0.320±4.51^{a,A}	0.348±1.83^{a}	0.325±1.04^{a,A}	0.342±2.31^{a,A}
	E-II	0.389±2.38^{b,c}	0.377±1.60^{b,B}	0.381±2.23^{b}	0.375±2.14^{b,c,B}	0.380±2.39^{b}	0.377±1.97^{b,B}	0.390±3.68^{b,B}
	E-III	0.387±1.21^{b,c}	0.384±2.13^{b,B}	0.381±1.77^{b}	0.353±0.81^{d,B}	0.384±1.13^{b}	0.36.5±2.20^{b,c,B}	0.388±1.60^{b,c,B}
	E-IV	0.351±2.47^{d}	0.384±2.77^{b,B}	0.379±3.06^{b,c}	0.380±2.60^{b,B}	0.367±2.06^{c}	0.373±3.20^{b,B}	0.404±1.71^{b,B}
	Mz	0.377±1.66^{b}	0.381±2.23^{b,B}	0.380±2.38^{b}	0.387±2.69^{b,B}	0.385±2.13^{b}	0.379±3.48^{b,B}	0.390±2.14^{b,B}
	C	0.395±2.87^{c}	0.363±3.35^{c,B}	0.367±3.30^{c}	0.361±1.34^{c,d,B}	0.366±2.66^{c}	0.381±1.72^{c,B}	0.378±2.40^{c,B}

$^{a.b.c,\ A,B}$ Means followed by the same small or capital letter within a column are not different by the test (p-value<0.05, *p*-value <0.01, respectively). Experimental groups: E-I group - 1 mg/kg OTA; E-II group 0,25 mg/kg OTA; E-III group 1 mg/kg OTA + 0,2% of MZ; E-IV group 0,25 mg/kg OTA + 0,2% of MZ; Control group (C) - standard diet without any addition; MZ - Minazel Plus® - modified adsorbent; OTA – ochratoxin A.

(E-I) in their diets had a significant decrease on egg production as well as egg quality parameters that were considered, as compared to the other groups (p<0.05). Changes in quality parameters were observed mainly by the end of the trial (week 7). The results showed that the addition of 0.2% MZ in OTA contaminated feed (E-III and E-IV) for laying hens had a positive impact on increasing laying capacity and mitigated the negative effects of ochratoxicosis in terms of number of eggs and thickness of the eggshell. Thus, no differences were found between experimental (E-II to E-IV and MZ) and control groups during the 7 wk period of investigation (p>0.05). On the other hand, the addition of MZ (0.2%) to the diet containing 1 mg/kg of OTA (E-III) did not minimize adversely effect of OTA on egg weight, whereas, there were no effects (p>0.05) of treatments (E-III and E-IV) on the color of the eggshells. The current findings suggest the decrease observed in egg production (number of egg and egg weight), as also thickness of the eggshell, could probably be associated with gastrointestinal and kidney dysfunction, which has been related with disturbances in nutritient metabolism, particularly calcium and phosphorous absorption (Khatoon & Abidin, 2018). It is known that OTA also disturbs calcium homeostasis, inhibiting ATP production in the mitochondria, suggesting that protein synthesis inhibition is also a consequence of bird exposed to OTA (Murugesan *et al.,* 2015).

Among many quality characteristics of table eggs, external factors including cleanliness, egg weight, color of eggs shell and shell quality are important in consumer's acceptability of eggs. Moreover, eggshell quality has always been a critical parameter in the egg industry, because eggs with poor eggshell quality cause losses both to the farmers and the egg-processing industries. Nearly eight percent of all losses in egg production is obtained due to the poor eggshell quality (Gheisari *et al.*, 2011). It is a major concern for consumers, as strong resistance to breaking and lack of shell defects are essential for protection against the penetration of pathogenic bacteria into the eggs. Thus, eggshell quality has a huge impact on the profitability of egg production. The use of MZ in OTA contaminated feed showed to have a positive impact on the eggshell quality throughout this study. However, this external quality parameter can also be influenced by environmental factors, e.g., the age of the flock, molting, nutrition, general stress, heat stress, diseases, production systems, and food supplements (Samiullah & Roberts, 2014; Kang *et al.,* 2020). Poultry are vulnerable to heat stress which markedly reduces feed intake, metabolism of nutrient, causing deterioration in production performance, accompanied with increased mortality which compromise economy of the poultry industry (Eleroğlu *et al.,* 2016).

Internal quality traits of egg

Effects of dietary MZ and OTA on internal quality traits of egg during the experimental period with significant differences between the mean values according to weeks of the study are shown in Table 5. Regarding effect of OTA (E-I and E-II) and adsorbent added in the diet (E-III and E-IV) on internal quality traits of egg, in group (E-I) significantly (p<0.05) reduced the values of all investigated parameters. Moreover, diet containing 0.25 mg/kg OTA (E-II), had a negative impact (p<0.05) on the diameter of yolks, Haugh's units and yolk ratio, while the addition of MZ (0.2%) to the diet (E-III and E-IV) did not ameliorated the adverse effect on diameter of yolks and Haugh's units (p>0.05). Average color of egg yolks and yolk weight were similar (p>0.05) in all experimental groups throughout the study (exception of E-I group).

Data related to the color of egg yolks, diameter of yolks, participation of yolk in the egg mass, Haugh's units and yolk weight might be connected to the OTA interference with lipid metabolism, carotenoid absorption, or deposition in yolk (Osborne *et al.,* 1982; Verma *et al.,* 2003). Egg yolk is a liquid mixture which contains phospholipids, fatty acids, and cholesterol Many *in vitro* and *in vivo* experiments suggest that these components possess a positive health effect, with regard to antioxidant, anti-inflammatory, and cardiovascular protection (Xiao *et al.,* 2020). Therefore, from an economical point of view, the yolk color is important because it is a quality criterion for consumers which are willing to pay higher prices for eggs of high and controlled quality (Popova *et al.,* 2020). Color of egg yolk strongly depends on the diet of laying hens, thus producers can tune the right yolk color by adding natural raw materials or additives to the layer feed when developing new types of eggs according to market strategies. Carotenoids (lutein and zeaxanthin) are natural, highly active yolk colorants that provide yellow, while canthaxanthin provides red yolk color (Loetscher *et al.,* 2013). Grass meal will give a darker yolk color and feeding most other grains will actually make the yolk lighter. However, transfer efficacy of carotenoids to egg yolk and their influence on yolk coloration differ greatly depending upon the type of carotenoids present and the chemical form of the

Table 5 – Internal quality traits of egg.

Traits	Group	Week						
		1st	2nd	3rd	4th	5th	6th	7th
Color of egg yolk	E-I	11.00±0.92	8.85±2.26^{a}	6.33±0.98^{a}	7.17±2.04^{a}	7.33±2.87^{a}	9.5±1.76^{a}	9.0±1.90^{a}
	E-II	11.00±1.07	11.43±0.53^{b,c}	11.75±0.46^{c}	11.63±0.51^{b}	11.63±0.51^{b}	11.63±0.74^{b}	11.67±0.81^{b}
	E-III	11.43±0.78	10.75±1.58^{c}	9.75±2.86^{b}	10.00±2.44^{c}	10.25±1.28^{c}	10.50±0.75^{c}	11.38±0.51^{b}
	E-IV	11.13±0.64	11.25±0.46^{c}	11.56±0.52^{c}	11.33±0.51^{b}	11.13±0.64^{b}	11.43±0.53^{b}	11.43±0.53^{b}
	Mz	11.38±1.06	11.63±0.51^{b}	11.71±0.48^{c}	11.43±0.53^{b}	11.50±0.53^{b}	11.38±0.51^{b}	11.25±0.46^{b}
	C	11.63±0.74	11.57±0.53^{b}	11.71±0.48^{c}	11.33±0.51^{b}	11.50±0.53^{b}	11.0±0.00^{b}	11.17±0.40^{b}
Yolk width (mm).	E-I	37.75±1.75^{a}	36.75±1.19^{a}	37.73±2.04^{a}	38.44±0.77^{a}	37.67±1.36^{a}	36.00±2.10^{a}	37.17±2.56^{a}
	E-II	37.88±1.13^{a}	38.78±1.20^{b}	39.29±0.77^{c}	40.21±1.60^{b}	39.25±0.88^{b}	39.00±1.19^{b,c}	39.50±1.05^{b}
	E-III	38.90±2.00^{b}	39.26±0.97^{b,c}	38.16±1.68^{b}	39.63±1.55^{b}	38.56±1.17^{c}	39.50±1.41^{c}	38.88±0.99^{c}
	E-IV	37.38±1.06^{a}	39.52±1.24^{c}	38.27±4.12^{b}	41.25±1.33^{c}	39.00±1.30^{b}	38.86±1.34^{b}	39.43±0.97^{b}
	Mz	38.25±1.16^{b}	38.14±1.07^{b}	39.48±1.66^{c}	40.61±1.50^{b}	40.56±1.39^{d}	39.25±1.83^{c}	39.50±2.07^{b}
	C	38.63±1.30^{b}	38.43±0.79^{b}	38.28±3.33^{b}	39.99±2.28^{b}	46.54±9.20^{e}	39.88±1.55^{c}	40.33±1.36^{d}
Yolk ratio (%).	E-I	25.26±1.44^{a}	22.75±1.34^{a}	22.97±1.33^{a}	22.91±1.57^{a}	23.54±2.05^{a}	21.33±1.24^{a}	22.11±1.59^{a}
	E-II	23.70±1.38^{b}	23.88±1.44^{b}	23.37±1.67^{b}	23.55±1.92^{b}	24.53±1.15^{b}	24.04±1.33^{b}	24.49±1.10^{b}
	E-III	24.35±1.52^{c}	24.45±1.30^{c}	24.21±2.05^{c}	25.24±1.97^{c}	25.57±2.11^{c}	25.39±1.51^{c}	24.93±1.71^{b}
	E-IV	23.92±2.11^{b}	24.44±1.58^{c}	24.00±1.64^{c}	25.37±1.52^{c}	25.35±1.98^{c}	24.75±1.24^{b}	24.82±1.34^{b}
	Mz	23.34±1.39^{b}	23.91±1.31^{b}	24.72±1.28^{d}	25.38±1.16^{c}	26.06±1.41^{d}	25.49±1.30^{c}	25.92±1.70^{c}
	C	24.56±1.99^{c}	24.70±1.36^{c}	24.93±1.87^{d}	25.79±2.18^{c}	26.05±2.27^{d}	25.66±2.07^{c}	26.39±1.70^{c}
Haugh's units	E-I	83.00±5.29^{a}	83.86±9.24^{a}	84.50±7.34^{a,A}	80.00±7.15^{a}	83.33±7.31^{a}	82.83±6.73^{a,A}	84.83±9.57^{a}
	E-II	87.13±5.84^{b,A}	87.29±8.26^{b}	90.25±6.36^{b}	85.25±3.40^{b}	80.75±7.61^{b}	88.50±6.09^{b}	88.00±4.98^{b,c}
	E-III	81.86±4.63^{c,B}	86.43±4.42^{c}	91.13±4.61^{c}	81.67±7.91^{c}	79.00±8.22^{c}	87.63±6.97^{c}	81.75±5.47^{c}
	E-IV	88.5±10.45^{d}	86.75±9.30^{c}	89.25±6.31^{d}	87.33±7.63^{d}	82.50±7.29^{d}	92.29±5.64^{d,B}	85.43±5.96^{d}
	Mz	88.88±5.64^{d}	90.88±5.08^{d}	95.0±3.26^{e,B}	88.43±5.35^{e}	85.38±7.23^{e}	89.38±3.50^{e}	87.38±4.34^{e}
	C	86.88±6.17^{d}	86.71±5.12^{c}	89.29±5.99^{d}	84.67±3.77^{b}	86.36±3.81^{f}	86.88±4.85^{f}	87.67±3.72^{e,c}
Yolk weight (g)	E-I	15.36±1.24^{a}	13.49±1.07^{a}	13.45±1.45^{a}	13.88±0.74^{a}	14.31±1.40^{a}	13.10±1.37^{a}	13.52±1.90^{a}
	E-II	15.50±0.62^{a}	15.05±0.79^{b}	14.90±0.68^{b}	14.84±1.20^{b}	15.78±0.90^{b}	15.40±1.15^{b}	15.83±0.75^{b}
	E-III	15.00±1.62^{a,b}	15.26±1.36^{b}	14.89±1.27^{b}	15.56±0.94^{c}	16.02±1.78^{c}	15.66±1.60^{b}	15.46±1.33^{b}
	E-IV	15.36±1.23^{a}	15.65±1.33^{b}	15.72±1.70^{c,d}	16.34±1.53^{d}	15.93±1.40^{b}	15.81±1.00^{b}	16.20±0.93^{c}
	Mz	14.96±1.12^{b}	15.06±1.11^{b}	15.05±1.32^{c}	15.79±0.81^{c}	16.16±0.89^{c}	16.48±1.05^{c}	17.25±1.83^{d}
	C	15.31±1.34^{a}	15.80±1.42^{b}	16.06±1.64^{d}	16.61±1.85^{d}	16.75±1.74^{c}	16.41±1.62^{c}	18.00±1.67^{d}

$^{a.b.c,\ A,B}$Means followed by the same small or capital letter within a column are not different by the test (*p*-value<0.05, *p*-value <0.01, respectively). Experimental groups: E-I group - 1 mg/kg OTA; E-II group 0,25 mg/kg OTA; E-III group 1 mg/kg OTA + 0,2% of MZ; E-IV group 0,25 mg/kg OTA + 0,2% of MZ; Control group (C) - standard diet without any addition; MZ - Minazel Plus® - modified adsorbent; OTA – ochratoxin A.

molecules. The lower yolk proportion may be due to a deficiency of some nutrients, primarily methionine given the fact that methionine is the first limiting amino acid in diets for laying hens (Nassiri *et al.,* 2012). It is not surprising therefore that mycotoxins are the cause of impairing animal productivity (Bryden, 2012), which could also be observed in this study.

Despite other studies which indicate that many of adsorbents used in animal feed have limited efficiency to prevent ochratoxicosis, the present study showed that modified clinoptilolite seems to be a promising strategy to reduce the adverse effects of OTA on animal's health and productivity, or its entering into the food chain. Moreover, results of this study, may be considered as more acceptable as MZ inclusion (0.2%) resulted in increased laying capacity and lowered negative effects of OTA exposure in terms of number of eggs, egg weight and thickness of the eggshell, reaching the levels not significantly different for most of the parameters obtained from hens on control diet. Considering that average color of egg yolks and yolk weight were similar in all groups throughout this study, with exception only of group exposed to 1 mg/kg OTA in the diet (without MZ), results of color of egg yolk, yolk weight and relative participation of yolk in the egg mass pointed out that MZ is able to eliminate negative impact of OTA on these internal quality traits. These results pointed towards significant beneficial effects of MZ as adsorbent on egg production and food efficiency in OTA exposed hens, as it was also concluded earlier for pullets by Karović *et al.* (2013). Results of this study show that egg production could be adversely affected only when the hens are exposed to higher concentrations of OTA than maximum permitted limit for poultry feed (0.20 mg/kg). Nevertheless, under climate conditions recordered in the last decade in Serbia, the occurrence of various species of toxigenic fungi and consequent co-occurrence of mycotoxins in feeds is a considerable threat inducing major economic losses for farmers, industry, international trade and society.

The present study showed that laying hens fed with 1 mg/kg of OTA in their diet had a significant decrease in egg production and egg quality. Considering effects of consumption of the contaminated diet with 0.25 mg/kg of OTA, it can be concluded that the level encountered minimal effect on egg production. The addition of MZ in the diets of laying hens show that it is a potent adsorbent, which can reduce harmful effects of OTA on production parameters in laying hens without any adverse effect on performance and nutrient utilization. Moreover, this adsorbent can help to increase egg production in laying hens, improve feed conversion and to prevent the occurrence of various negative changes which may be the result of hen's exposure to OTA contaminated diet. The results obtained pointed out that the use of mineral adsorbents (MZ) in feeds and premixes could provide a solution to prevent mycotoxicosis, showing better productive performance and economic returns to growers. In addition, specific benefits related to use of mycotoxins adsorbents could be assessed not only on animal health and productivity, but also in an indirect way for human consumption due to the improvement of safety of animal products.

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