The effect of feed supplementation with Transcarpathian zeolite (clinoptilolite) on the concentrations of acute phase proteins and cytokines in the serum and hepatic tissue of chickens

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ABSTRACT The aim of the this study was to determine the effect of different levels of Transcarpathian zeolite (clinoptilolite) on selected indicators of the immune response in chickens by assessing the concentrations of the acute phase proteins haptoglobin, C-reactive protein (**CRP**), serum amyloid A, transferrin, and alpha-1-acid glycoprotein and the cytokines tumor necrosis factor-α (**TNF-α**), interferon-γ (**IFN-γ**), IL-2, and IL-10 in the serum and hepatic tissues of chickens. The study was conducted on 450 one-day-old male Ross 308 chickens. The total rearing period was 42 D. The samples of blood and liver were collected before the start of the study (day 0) and on day 42, after rearing was completed. ELISA kits specific for chicken CRP, haptoglobin, alpha-1-acid glycoprotein, serum amyloid A, transferrin, TNF-α, IFN-γ, IL-2, and IL-10 were used to determine the levels of acute phase proteins and cytokines in the serum and liver homogenates. The results of immunological tests suggest that for long-term maintenance of homeostasis in chickens, the addition of 2% zeolite as a feed additive is most beneficial. The results indicate that 3% clinoptilolite induce production of Th1 pro-inflammatory cytokines, increasing the synthesis of IL-2, IFN-γ, and TNF-α. The high concentration of IL-10 after the use of zeolite in conjunction with the high concentration of IL-2, TNF-α, and IFN-γ indicates a reduction in the intensity of inflammatory processes, the enhancement of the humoral immune response, and the simultaneous inhibition of the production of Th1-type cytokines. The increase of CRP concentration in conjunction with high concentrations of pro- and anti-inflammatory cytokines in the birds from the group receiving 3% clinoptilolite demonstrates indicates that it can influence the development of local inflammatory processes and enhance immune regulation in birds. Our research has shown that clinoptilolite influences on an increase in birds' resistance to infection, as confirmed by clinical observations and anatomopathological examination and by the increase in the synthesis of acute phase proteins with immunoregulatory properties.

Key words: Transcarpathian zeolite (clinoptilolite), chickens, acute phase protein, cytokine concentration

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INTRODUCTION

The ban on the use of antibiotics as feed additives introduced in 2006 in the European Union, combined with growing antibiotic resistance and accumulation of antibiotic residues in animal products and the environment, requires poultry producers to look for alternative substances that positively affect the health of birds (Commission Regulation [EC] No 1831/2003). The various feed additives used in poultry feeding, such as aluminosilicates (Al_2SiO_5), increase weight gains, stabilize the gut microbiota, prevent the proliferation of specific intestinal pathogens and the development of intestinal inflammation, and also exert an immunostimulatory effect (Pavelic et al., 2002; Yadav and Jha, 2019). These substances have no toxic effects on chickens, do not pollute the farm environment, and are inexpensive to produce (Gilani et al., 2016). Among commercially available aluminosilicates, poultry producers are showing increasing interest in Transcarpathian zeolite (clinoptilolite).

Owing to its structure, composed of a microporous arrangement of silica and alumina tetrahedra, and complex formula, (Na,K,Ca)2-3Al3(Al,Si)2Si13O36·12H2O,

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Transcarpathian zeolite (clinoptilolite) is used in various branches of the economy as an adsorbent, a catalyst, or an ion exchanger (Laurino and Palmieri, 2015). However, its biological properties predispose it to use in livestock feeding, to reduce the occurrence of diarrhoeal diseases, and improve nutrient digestibility (Cabuk et al., 2004; Nassiri-Moghaddam et al., 2008). Numerous studies indicate a positive effect of zeolite on performance parameters and egg shell quality (Olver, 1989), as well as the bioavailability of minerals contained in feed (Mumpton, 1999; Christaki et al., 2001, 2006), ion exchange in the intestines (Elliot and Edwards, 1991), intestinal enzyme activity, stabilization of the gut pH, enterocyte morphology, and gut microflora composition (Khambualai et al., 2009). This effect improves digestion and intestinal absorption (Khambualai et al., 2009), contributing to increased weight gain and feed utilization, as well as reduced production costs (Eleroğlu and Yalçin, 2005; Shariatmadari, 2008). Transcarpathian zeolite (clinoptilolite) is also used in human medicine as an anti-diarrhoeal, bactericidal, and antifungal medicine, as well as for the treatment of wounds (Neidrauer et al., 2014). Antibacterial activity of zeolite in poultry has been demonstrated against *Salmonella* spp. and *Escherichia coli* (Ricke et al., 1995; Tang et al., 2014) and consists in the selective adsorption of pathogenic bacteria in the broiler gut (Afaf et al., 2011; Wu et al., 2013c).

The antiviral and antibacterial effects of Transcarpathian zeolite (clinoptilolite) have been shown to correlate with its immunomodulatory properties (Ivkovic et al., 2004). Ueki et al. (1994) and Aikoh et al. (1998) indicate that zeolites act as nonspecific immunostimulants similar to superantigens, which are T-cell mitogens with high affinity for class II MHC molecules. Other authors (Holian et al., 1997; Martin et al., 1997) have demonstrated that zeolites stimulate phagocytic cells, mainly macrophages, enhancing phagocytosis and reactive oxygen species production. Stimulation of phagocytic cells by zeolites contributes to increased synthesis of pro-inflammatory cytokines, such as IL-1a, IL-6, and tumor necrosis factor-α (**TNF-α**) (Simeonova et al., 1997). Production of proinflammatory cytokines and consequently the body's general immune response (Pavelic et al., 2002; Chen et al., 2018) are conditioned by direct stimulation of enterocytes promoting the adhesion of leukocytes to the epithelium and their migration and activation in the peripheral blood. Zeolites may also have an indirect influence through their effect on the intestinal ecosystem and interference with the maturation and development of the local mucosa-associated lymphoid tissue immune response (Kraljević Pavelić et al., 2018). Stimulation of intestinal epithelial M cells by zeolites facilitates the transfer of aluminosilicate molecules from the intestinal lumen to the deeper cells of the lamina propria, mainly macrophages and dendritic cells, responsible for antigen presentation to T cells, resulting in stimulation of the general immune response. In a study by Jarosz et al. (2017), the use of zeolite as a feed additive for broiler chickens resulted in increased antigen presentation and stimulation of lymphocyte proliferation, especially $CD4^+CD25^+$, $CD8^+CD25^+$, and MHC Class II T cells. The simultaneous stimulation of expression of BU-1^+ and MHC class II on B lymphocytes indicates the modulatory effect of zeolites on the Th1 and Th2 response, which determines the body's multifaceted response to antigens.

The available literature lacks information on the effect of Transcarpathian zeolite (clinoptilolite) used as a feed supplement on the concentrations of acute phase proteins and cytokines in chickens. Acute phase proteins and cytokines are secreted by specialized immunocompetent cells in the acute phase response, which accompanies local or systemic inflammation. This reaction may be caused by numerous endogenous and exogenous factors constituting antigens that stimulate the acute phase response. The resulting proteins play a profound role in defense mechanisms, tissue repair, and restoration of health (Murata et al., 2004). Determination of acute phase protein (**APP**) concentrations in broilers can thus be used to diagnose and predict the intensity of inflammatory processes in various disease states (Chamanza et al., 1999; Cray et al., 2009). Understanding the mechanisms of immune system function in chickens whose feed is supplemented with Transcarpathian zeolite (clinoptilolite) will make it possible to determine the effect of this additive on the immune response at the cellular level. The research will also help to explain the mechanisms of the clinically confirmed beneficial effect of the preparation in the course of inflammatory disease in chickens. The aim of the study was to determine the effect of Transcarpathian zeolite (clinoptilolite) on selected indicators of the immune response in chickens by assessing the concentrations of acute phase proteins haptoglobin (**Hp**), C-reactive protein (**CRP**), serum amyloid A (**SAA**), transferrin (**TRF**), and alpha-1-acid glycoprotein (**α-1-AGP**) and cytokines TNF-α, interferon-γ (**IFN-γ**), IL-2, and IL-10 in the serum and hepatic tissue.

MATERIAL AND METHODS

Experimental Animals and Feeding Principles

The study complied with European Directive 2010/63/EU. All procedures used in the research were approved by the Local Ethics Committee for Animal Testing at the University of Life Sciences in Lublin, Poland (resolution no.: 37/2011 of 17 May 2011).

The study was conducted on 450 one-day-old male Ross 308 chickens owned by the Small Animals Teaching and Research Station of the University of Life Sciences in Lublin, Poland. The experimental chickens were kept in cages in a room with controlled temperature and humidity. The chickens were weighed and randomly placed in battery cages (1 m^2) with 5 chickens per cage. The metal cages had grates that were replaced as the chickens

grew. The cages were equipped with nipple drinkers and feeders whose height was continually adjusted to the age of the chickens. All cages were in the same room, with electric lighting 24 h/D until the 10th D of the experiment and 16 h/D from days 10 to 42 of the experiment, according to the lighting scheme for chickens raised on the farm at the Small Animals Teaching and Research Station of the University of Life Sciences in Lublin, Poland. Three days before the chickens were placed in the cages, the floor was heated to 29°C and the air to 33°C, with relative humidity of 63%. During the first week of the experiment, the temperature was kept at 33°C and thereafter was reduced weekly by 2°C to 3°C until reaching a final temperature of 20°C to 22°C. Humidity during successive periods of the experiment was as follows: days 1 to 21: 55 to 60%; days 22 to 35: 60 to 65%; and days 36 to 42: 65 to 70%.

The chickens were fed *ad libitum* with compound feeds appropriate for each period of rearing, that is, starter, S (days 1–21), grower, G (days 22–35), and finisher, F (days 36–42) (Agropol-Motycz, Poland) and had unlimited access to water. The starter feed was provided to the chickens in crushed form, while the grower and finisher feeds were pelleted. The starter, grower, and finisher feeds were prepared from corn meal, wheat meal, and soybean extraction meal. No coccidiostats or antibiotics were used in the feed during the entire experiment. The composition and nutritional values of the basal diets are presented in Table 1.

The total rearing period was 42 D. Ninety chickens were used in one experiment, and the chickens were randomly divided into 3 groups (30 chickens per one treatment): 1 control group (I) and 2 experimental groups (II and III). The same basal diet was used in all groups, but groups II and III received a feed additive in the form of 2 and 3% Transcarpathian zeolite (clinoptilolite), respectively (Andalusia sp. z o.o., Poznań, Poland). The preparation contained at least 87% clinoptilolite as the active substance, with the following composition: 67.07% SiO_2, 12.4% Al_2O_3, 2.09% CaO, 2.8% K_2O, 0.9% Fe_2O_3, 0.72% MgO, 2.05% Na_2O, 0.19% TiO_2, 0.04% MnO, and 0.014% P_2O_5. The moisture content did not exceed 6%.

On the first day of the experiment, 10 one-day-old chicks from each group were killed by decapitation to collect blood samples and liver tissue. The remaining 20 chickens in each group were used for further stages of the experiment. On day 42, the remaining 20 chickens from all groups were killed to collect blood and liver tissue. The experiment was replicated 5 times. A total of 450 Ross 308 broiler chickens were used in the 5 replications of the experiment.

Table 1. Raw material composition (%) and nutrition value of experimental mixtures.

Components (%)	Starter (day 1–21)	Grower (day 22–35)	Finisher (day 36–42)
Corn	24.44	40.00	40.00
Wheat	42.99	27.84	28.84
Soybean extraction meal[6]	25.0	24.97	22.87
Soy oil	2.50	3.69	3.98
1-Ca phosphate	0.90	0.90	0.81
Feed lime	1.40	1.13	1.09
Acidic sodium carbonate	0.08	0.08	0.08
NaCl	0.29	0.25	0.26
Vit.-min. prefix (no Fe)	0.50[1]	0.50[2]	0.50[3]
Protein and fat concentrate[7]	1.00	-	1.00
DL-methionine 99%	0.30	0.23	0.23
L-lysine HCl	0.42	0.28	0.27
L-threonine 99%	0.18	0.13	0.07
Nutrient value of 1 kg of mixture			
ME, MJ kg^{-1}	12.7	13.1	13.2
BO, %[4]	21.7	20.2	19.6
WS, %[4]	2.41	2.32	2.31
TS, %[4]	4.52	5.28	5.64
Lysine, %[4]	1.28	1.14	1.10
Meth + Cys, %[4]	0.94	0.84	0.83
Ca total, %[4]	0.87	0.79	0.76
P total, %[4]	0.67	0.66	0.64
P assimilable, %[5]	0.43	0.40	0.41
Ca total/P assimilable[5]	2.11	1.91	1.90

Abbreviations: BO, total protein; ME, metabolic energy; TS, raw fat; WS, raw fiber.

[1]Content of vitamins and minerals in 1 kg of starter mixture: Mn 100 mg, J 1 mg, Se 0.15 mg, vit. A 15,000 UI, vit. D_3 5,000 UI, vit. E 75 mg, vit. K_3 4 mg, vit. B_1 3 mg, vit. B_2 8 mg, vit. B_6 5 mg, vit. B_{12} 0.016 mg, biotin 0.2 mg, folic acid 2 mg, nicotinic acid 60 mg, pantothenic acid 18 mg, choline 1,800 mg.

[2]Content of vitamins and minerals in 1 kg of grower mixture: Mn 100 mg, J 1 mg, Se 0.15 mg, vit. A 12,000 UI, vit. D_3 5,000 UI, vit. E 50 mg, vit. K_3 3 mg, vit. B_1 2 mg, vit. B_2 6 mg, vit. B_6 4 mg, vit. B_{12} 0.016 µg, biotin 0.2 mg, folic acid 1.75 mg, nicotinic acid 60 mg, pantothenic acid 18 mg, choline 1,600 mg.

[3]Content of vitamins and minerals in 1 kg of finisher mixture: Mn 100 mg, J 1 mg, Se 0.15 mg, vit. A 12,000 UI, vit. D_3 5,000 UI, vit. E 50 mg, vit. K_3 2 mg, vit. B_1 2 mg, vit. B_2 5 mg, vit. B_6 3 mg, vit. B_{12} 0.011 µg, biotin 0.05 mg, folic acid 1.5 mg, nicotinic acid 35 mg, pantothenic acid 18 mg, choline 1,600 mg.

[4]Analyzed values.

[5]Calculated values.

[6]Forty-six percentage general protein in dry matter.

[7]One-kilogram protein and fat concentrate contains: 2% raw fat, 39% raw protein, 10.8 MJ EM.

Clinical Signs and Growth Performance in the Animals

Throughout the experiment, the chickens were under clinical observation, with special attention paid to the activity of the birds, their appetite, respiratory symptoms, and the occurrence of digestive disorders in the form of diarrhoea. In all groups, feces were examined on days 20 and 42 for the presence of *Eimeria* spp. oocysts by the flotation method, using a saturated NaCl solution described by Ryley and Ryley (1978). The health status of the chickens was evaluated by determining clinical parameters, anatomopathological changes in dead chickens, and the mortality rate (Table 2).

During the experiment, all chicks were weighed, and their initial and final weights were calculated.

Blood Samples

The tested material consisted of 2 mL of peripheral blood samples taken from the wing vein on day 42 of the experiment or from one-day-old chickens killed by decapitation (day 0). The blood samples were collected into sterile vacuum tubes containing a clot activator and serum separator (Vacuette, Medlab Products, Raszyn, Poland). Blood samples were collected before the start of the study (day 0), and on day 42, after rearing was completed. The samples were transported to the laboratory at +4°C to +8°C within 1 h. Serum was obtained by centrifuging the blood at room temperature (20°C–22°C) for 15 min at 4,000 × *g*. The serum was apportioned and stored at −80°C for further analysis.

Assays of CRP, Hp, α-1-AGP, SAA, TRF and TNF-α, IFN-γ, IL-2, and IL-10 in Chicken Serum

ELISA kits specific for chicken CRP, Hp, α-1-AGP, SAA, and TRF (Wuhan Fine Biotech Co., Ltd., East Lake High-tech Development District, Wuhan, Hubei Province, China) were used to determine the levels of acute phase proteins in the serum, and ELISA kits specific for chicken TNF-α, IFN-γ, IL-2, and IL-10 (Wuhan Fine Biotech Co., Ltd., East Lake High-tech Development District, Wuhan, Hubei Province, China) were used to determine cytokine levels in the serum. Optical densities of kit standards and test samples were read at 450 nm using an ELISA plate reader (Benchmark Plus Microplate Spectrophotometer System with Incubator, Bio-Rad Laboratories, CA). All procedures were performed according to the manufacturer's instructions.

Assays of CRP, Hp, α-1-AGP, SAA, TRF and TNF-α, IFN-γ, IL-2, and IL-10 in Liver Homogenates

Birds were euthanized on days 1 and 42 of the experiment. On the first day of the experiment, 10 one-day-old chicks from each group were euthanized by decapitation to collect blood and liver tissue. The remaining 20 chickens in each group were euthanized on the 42nd D of the experiment. The livers (from all birds in each group) were immediately excised, rinsed of blood, and homogenized using a mechanical homogenizer in PBS containing 0.05% sodium azide and 0.5% Triton X-100 (pH 7.4). Liver samples were then sonicated for 10 min. All solid particles were removed by centrifugation of the homogenates at 12,000 × *g* for 10 min. Dichloromethane (0.4 mL) was added to 1 mL of supernatant to eliminate all lipid contaminants. This was followed by a second centrifugation for 10 min at 12,000 × *g*, after which the supernatant was removed and immediately assayed. ELISA kits for the determination of chicken CRP, Hp, α-1-AGP, SAA, TRF and TNF-α, IFN-γ, IL-2, and IL-10 were obtained from Wuhan Fine Biotech Co., Ltd. (East Lake High-tech Development District, Wuhan, Hubei Province, China). The ELISA experiments were performed according to the manufacturer's specifications. APP and cytokine content were expressed as ng/mL total protein.

Statistical Analysis

The results were analyzed statistically using Statistica 10.0 PL (StatSoft, Krakow, Poland). The analysis included the arithmetic mean and standard deviation

Table 2. Evaluation of health and production parameters.

Item	Group I, control, 0% clinoptilolite	Group II, 2% clinoptilolite	Group III, 3% clinoptilolite
Body weight gain (g)	1949.34 ± 5.35	2315.70 ± 8.08*	2317.60 ± 6.57*
Mortality rate (%)	8% (12 birds)	0.66% (1 bird)	5.33% (8 bird)
Gastrointestinal symptoms	Diarrhoea lasting 4–5 D, remitting spontaneously (between 10th and 20th D of life)	Diarrhoea lasting 2 D, remitting spontaneously (between 10th and 15th D of life)	None
Respiratory symptoms	Cough n = 6 (between 32nd and 42nd D of life) Sneezing n = 17 (between 20th and 42nd D of life) Conjunctivitis n = 9 (between 20th and 42nd D of life)	Sneezing n = 11 (between 15th and 20th D of life) Conjunctivitis n = 7 (between 15th and 20th D of life)	Sneezing n = 4 (between 15th and 20th D of life) Conjunctivitis n = 5 (between 15th and 20th D of life)
Anatomopathological changes in dead birds	Intestinal hyperemia, petechiae in the mucosa of the small intestine at 42nd D of life	Intestinal hyperemia at 42nd D of life	Intestinal hyperemia, isolated pinpoint petechiae in the mucosa of the small intestine at 42nd D of life

Data represent means from 5 replications of the experiment.
*Asterisks indicates significant differences ($P < 0.05$) between the experimental groups and the control group.

(α ± SD). The significance of differences between means for the control and experimental groups of animals and between sampling times was assessed by the one-way ANOVA with post-hoc Tukey HSD test and the median test, with P values of less than 0.05 considered to indicate statistical significance (Tables 2–5). The statistically significant difference between the group of birds receiving 2% zeolite in their feed and the group receiving 3% zeolite was assessed by the one-way ANOVA with post-hoc Tukey HSD test and the median test. P values for statistically significant differences are shown in Tables 2–5.

The experimental data on growth performance were analyzed with the general linear model ANOVA procedure in Statistica 10.0 PL. Statistically significant effects were further analyzed, and means were compared using Tukey's honestly significant difference (**HSD**) multiple comparison procedure. Statistical significance was determined at $P < 0.05$.

RESULTS

Results of Clinical Observations

In the experimental group, which received the 3% clinoptilolite supplement, only respiratory symptoms (sneezing and conjunctivitis) were observed. In the group supplemented with 2% clinoptilolite, apart from respiratory symptoms, there was also diarrhoea, lasting 2 D and remitting spontaneously. Both respiratory symptoms and diarrhoea were also observed in the control group. The lowest mortality rate (0.66%) was noted in the group of chickens supplemented with 2% clinoptilolite. There were also 2 deaths in the control group; anatomopathological examination of these chickens revealed catarrhal inflammation of the mucosa of the small intestine. Similar but more severe inflammatory changes were observed in the intestines of one dead chicken from experimental group III. Parasitological examination of feces samples did not show the presence of *Eimeria* oocysts in any of the groups of chickens. Body weight gain in both experimental groups was statistically significantly higher ($P < 0.05$) than that in the control. Detailed data are presented in Table 2.

Assay of CRP, Hp, α-1-AGP, SAA, and TRF in Chicken Serum

Compared with the control group, a statistically significant decrease ($P < 0.05$) in the serum concentration of CRP was observed at day 42 in the group supplemented with 2% clinoptilolite. Interestingly, in the group receiving 3% clinoptilolite, the CRP concentration was higher than that in the control group on day 42 of the study.

In both the control group and the group supplemented with 3% clinoptilolite, a statistically significant ($P < 0.05$) increase in the CRP level was observed on day 42 of the study compared with that on day 0. In the group where the chickens received 2% clinoptilolite, a statistically significant decrease in CRP was observed on day 42 of the study compared with day 0.

Comparison of the serum concentration of CRP between groups showed a statistically significantly higher ($P = 0.0001$) concentration of this protein in the group of chickens receiving 3% clinoptilolite on day 42 of the study than that in the group supplemented with 2% clinoptilolite. Detailed data are presented in Table 3.

The data in Table 3 present concentration of Hp in the chicken serum. In comparison to the control group, no statistically significant differences in the concentration of this protein were found in the groups receiving 2 and 3% clinoptilolite. In the control group as well as in the groups supplemented with 2 and 3% clinoptilolite,

Table 3. Comparison of the concentration of acute phase proteins in chicken serum.

		Control group	2% Clinoptilolite	3% Clinoptilolite
Parameter	Day	n = 150	n = 150	n = 150
SAA	0	51.79 ± 9.11	49.17 ± 5.36	49.40 ± 3.50
	42	82.44 ± 9.26, $^{a}P = 0.0002$	84.65 ± 7.54, $^{a}P = 0.0001$	182.94 ± 10.92*, $^{a}P = 0.0001$,$^{A}P = 0.03$
CRP	0	0.88 ± 0.06	0.89 ± 0.04	0.88 ± 0.07
	42	1.63 ± 0.30, $^{a}P = 0.0001$	0.64 ± 0.07*, $^{a}P = 0.0001$	2.60 ± 0.82*, $^{a}P = 0.0003$,$^{A}P = 0.0001$
α-1-AGP	0	10.59 ± 0.63	10.60 ± 0.89	10.67 ± 0.36
	42	23.97 ± 7.11, $^{a}P = 0.0004$	12.62 ± 2.42*	9.46 ± 1.02*, $^{a}P = 0.01$
TRF	0	0.81 ± 0.11	0.76 ± 0.24	0.79 ± 0.18
	42	2.15 ± 0.21, $^{a}P = 0.0001$	0.65 ± 0.31*	3.49 ± 0.56*, $^{a}P = 0.0001$,$^{A}P = 0.0001$
Hp	0	0.10 ± 0.01	0.09 ± 0.01	0.09 ± 0.007
	42	0.55 ± 0.05, $^{a}P = 0.0001$	0.49 ± 0.04, $^{a}P = 0.0001$	0.58 ± 0.05, $^{a}P = 0.0001$, $^{A}P = 0.008$

Values are expressed as mean and standard deviation (α ± SD). n, number of chickens used in 5 replications of the experiment. Data represent means from 5 replications of the experiment.

^{A}Statistically significant differences between the group of birds receiving 2% zeolite in their feed and the group receiving 3% zeolite, assessed by a one-way ANOVA with the post-hoc Tukey HSD and median tests.

^{a}Statistically significant differences ($P < 0.05$) within groups between day 0 and day 42.

Abbreviations: α-1-AGP, alpha-1-acid glycoprotein; CRP, C-reactive protein; Hp, haptoglobin; SAA, serum amyloid A; TRF, transferrin.

Asterisks indicate a significant increase in the parameter between experimental groups and the control on each testing day ($P < 0.05$).

Table 4. Comparison of the concentration of acute phase proteins in chicken liver tissue.

		Control group	2% Clinoptilolite	3% Clinoptilolite
Parameter	Day	n = 150	n = 150	n = 150
SAA	0	95.54 ± 4.06	93.70 ± 5.85	95.36 ± 3.79
	42	123.44 ± 18.76, $^{a}P = 0.002$	141.23 ± 19.61, $^{a}P = 0.0002$	204.16 ± 9.54*, $^{a}P = 0.0001$,$^{A}P = 0.0001$
CRP	0	0.71 ± 0.09	0.78 ± 0.14	0.77 ± 0.10
	42	1.85 ± 0.23, $^{a}P = 0.0001$	1.18 ± 0.08*, $^{a}P = 0.0002$	2.91 ± 0.63*, $^{a}P = 0.0001$,$^{A}P = 0.0001$
α-1-AGP	0	134.66 ± 12.15	135.55 ± 20.70	134.65 ± 6.38
	42	313.47 ± 9.02, $^{a}P = 0.0001$	180.98 ± 12.93*, $^{a}P = 0.0005$	275.16 ± 18.53*, $^{a}P = 0.0001$,$^{A}P = 0.0001$
TRF	0	1.03 ± 0.10	0.98 ± 0.08	0.99 ± 0.09
	42	1.82 ± 0.25, $^{a}P = 0.0001$	1.28 ± 0.24*, $^{a}P = 0.01$	3.69 ± 0.42*, $^{a}P = 0.0001$,$^{A}P = 0.0001$
Hp	0	9.43 ± 0.53	9.31 ± 0.63	9.54 ± 0.59
	42	13.55 ± 1.92, $^{a}P = 0.0003$	17.25 ± 1.72*, $^{a}P = 0.0001$	41.09 ± 3.37*, $^{a}P = 0.0001$, $^{A}P = 0.0001$

Values are expressed as mean and standard deviation (α ± SD). n, number of chickens used in 5 replications of the experiment. Data represent means from 5 replications of the experiment.

[A]Statistically significant differences between the group of birds receiving 2% zeolite in their feed and the group receiving 3% zeolite, assessed by a one-way ANOVA with the post-hoc Tukey HSD and median tests.

[a]Statistically significant differences ($P < 0.05$) within groups between day 0 and day 42.

Abbreviations: α-1-AGP, alpha-1-acid glycoprotein; CRP, C-reactive protein; Hp, haptoglobin; SAA, serum amyloid A; TRF, transferrin.

*Asterisks indicate a significant increase in the parameter between experimental groups and the control on each testing day ($*P < 0.05$).

a statistically significant increase in the Hp level was observed on day 42 of the study compared to day 0.

Comparison of the serum concentration of Hp between groups showed a statistically significantly higher ($P = 0.008$) concentration of this protein in the group of chickens receiving 3% clinoptilolite on day 42 of the study than in the group supplemented with 2% clinoptilolite. Detailed data are presented in Table 3.

The assays of serum concentrations of α-1-AGP revealed statistically significantly lower ($P < 0.05$) levels of this protein in both experimental groups at day 42 of the study than those in the control group. Compared with day 0, a statistically significant increase in the serum concentration of α-1-AGP on day 42 was observed in the control group. However, in the group supplemented with 3% clinoptilolite, on day 42 of the study, a statistically significant decrease in α-1-AGP concentration was observed relative to day 0. Comparison of the α-1-AGP concentration in chicken serum between groups supplemented with 2 and 3% clinoptilolite showed no statistically significant differences.

The concentration of SAA in the serum of chickens is shown in Table 3. Compared with the control group, the serum concentration of SAA on day 42 was statistically significantly higher in the group supplemented with 3% clinoptilolite. Compared with day 0 of the study, a significant increase in the serum concentration of SAA was observed at day 42 in both the experimental groups and the control. Comparison of the serum concentration of SAA between the supplemented groups showed a statistically significantly higher ($P = 0.03$) concentration of this protein in the group of chickens receiving 3% clinoptilolite on day 42 of the study than in the group supplemented with 2% clinoptilolite.

The concentration of TRF in the chicken sera is shown in Table 3. Compared with the control group, a statistically significant increase ($P < 0.05$) in TRF concentration was observed at day 42 in the group supplemented

Table 5. Comparison of the concentrations of interleukins in chicken serum.

		Control group	2% Clinoptilolite	3% Clinoptilolite
Parameter	Day	n = 150	n = 150	n = 150
TNF-α	0	9.26 ± 0.46	9.14 ± 0.83	9.37 ± 0.36
	42	19.82 ± 4.74, $^{a}P = 0.0001$	12.03 ± 1.60*, $^{a}P = 0.0001$	16.19 ± 3.01, $^{a}P = 0.0001$, $^{A}P = 0.02$
IFN-γ	0	4.33 ± 1.03	4.45 ± 0.53	4.76 ± 0.71
	42	16.61 ± 2.45, $^{a}P = 0.0001$	14.93 ± 2.78, $^{a}P = 0.0001$	26.33 ± 3.25*, $^{a}P = 0.0001$, $^{A}P = 0.0001$
IL-2	0	32.96 ± 0.90	33.45 ± 0.71	33.28 ± 0.65
	42	68.80 ± 6.22, $^{a}P = 0.0001$	32.90 ± 1.61*	131.33 ± 4.57*, $^{a}P = 0.0001$, $^{A}P = 0.0001$
IL-10	0	6.31 ± 0.64	7.05 ± 0.62	7.34 ± 0.43
	42	18.44 ± 1.49, $^{a}P = 0.0001$	9.88 ± 1.15*, $^{a}P = 0.0002$	13.38 ± 2.80*, $^{a}P = 0.0002$, $^{A}P = 0.009$

Values are expressed as mean and standard deviation (α±SD). n, number of chickens used in 5 replications of the experiment. Data represent means from 5 replications of the experiment.

[A]Statistically significant differences between the group of birds receiving 2% zeolite in their feed and the group receiving 3% zeolite, assessed by a one-way ANOVA with the post-hoc Tukey HSD and median tests.5

[a]Statistically significant differences ($P < 0.05$) within groups between day 0 and day 42.

Abbreviations: IFN-γ, interferon-γ; IL-2, interleukin-2; IL-10, interleukin-10; TNF-α, tumor necrosis factor-α.

*Asterisks indicate a significant increase in the parameter between experimental groups and the control on each testing day ($*P < 0.05$).

with 3% clinoptilolite. Conversely, in the group supplemented with 2% clinoptilolite, the TRF level on day 42 of the study was statistically significantly lower than that in the control group. Compared with day 0 of the study, a statistically significant increase in TRF concentration was noted on day 42 in the group supplemented with 3% clinoptilolite and in the control group. Comparison of the serum TRF concentration between the supplemented groups showed a statistically significantly higher ($P = 0.0001$) concentration of this protein in the group of chickens receiving 3% clinoptilolite on day 42 of the study than in the group supplemented with 2% clinoptilolite. Detailed data are presented in Table 3.

Assay of CRP, Hp, α-1-AGP, SAA, and TRF in Chicken Liver Tissue

The level of CRP in the liver homogenates is shown in Table 4. Compared with the control group, a statistically significant increase in the concentration of this protein was observed on day 42 in the group supplemented with 3% clinoptilolite. Conversely, in the group supplemented with 2% clinoptilolite, the CRP level in the liver homogenates on day 42 of the study was statistically significantly lower than that in the control group. In all, in the control group and the 2 and 3% clinoptilolite groups, the CRP level in the liver homogenates on day 42 of the study was statistically significantly higher than that on day 0 of the study. Comparative analysis of the CRP concentration in the chicken liver tissues between the supplemented groups (2 and 3% clinoptilolite) showed a statistically significant ($P = 0.0001$) increase in the concentration of the protein on day 42 of the study in the group of chickens supplemented with 3% clinoptilolite.

Compared to the control group, the concentration of Hp in the liver homogenates was statistically significantly higher ($P < 0.05$) at day 42 in both experimental groups. Compared with day 0 of the experiment, a significantly higher concentration of Hp at day 42 was observed in the groups supplemented with 2 and 3% clinoptilolite and in the control group. Comparison of the Hp concentration of in the chicken liver tissues between the supplemented groups showed a statistically significantly higher concentration of the protein in the group of chickens receiving 3% clinoptilolite on day 42 of the study than in the group supplemented with 2% clinoptilolite (Table 4).

Table 4 shows the concentration of α-1-AGP in the liver homogenates. Compared with the control group, a decrease in the concentration of this protein was observed on day 42 in the groups supplemented with clinoptilolite. Compared with day 0 of the study, a significant increase in α-1-AGP concentration in the liver homogenates was observed at day 42 in both experimental groups and the control. Comparison of the α-1-AGP concentration in the chicken liver tissues between the supplemented groups showed a statistically significantly ($P = 0.0001$) higher concentration of the protein in the group of chickens receiving 3% clinoptilolite on day 42 of the study than in the group supplemented with 2% clinoptilolite.

Compared with the control group, SAA levels in the liver homogenates showed statistically significant differences on day 42 of the study only in the group supplemented with 3% clinoptilolite. Compared with day 0 of the experiment, a significantly higher concentration of SAA at day 42 was observed in the groups supplemented with 2 and 3% clinoptilolite and in the control group. In both supplemented groups, there was an increase in the concentration of this protein on day 42 of the study. Detailed data are shown in Table 4. Comparison of the SAA concentration in chicken liver tissues between the supplemented groups showed a statistically significantly higher ($P = 0.0001$) concentration of the protein in the group of birds receiving 3% clinoptilolite on day 42 of the study.

Table 4 shows the concentration of TRF in the chicken liver homogenates. Compared with the control group, a statistically significant increase ($P < 0.05$) in TRF concentrations was observed on day 42 of the study in the group supplemented with 3% clinoptilolite. In the group supplemented with 2% clinoptilolite, the level of TRF in the liver homogenates on day 42 of the study was statistically significantly lower than that in the control group. Compared with day 0 of the study, a statistically significant increase in TRF concentration was observed in both supplemented groups and the control at day 42. Comparison of the TRF concentration in the chicken liver homogenates between the supplemented groups showed a statistically significantly higher ($P = 0.0001$) concentration of the protein in the group of birds receiving 3% clinoptilolite than in the group supplemented with 2% clinoptilolite (Table 4).

Expression of Cytokines and Interleukins in Chicken Serum

The data presented in Table 5 indicate that compared with the control group, the concentration of TNF-α on day 42 of the study in the chicken serum was statistically significantly lower in the group of birds supplemented with 2% clinoptilolite. Compared with day 0 of the experiment, significantly higher expression of TNF-α at day 42 was observed in groups supplemented with 2 and 3% clinoptilolite and in the control group. Comparison of TNF-α expression in the chicken serum between the supplemented groups showed a statistically significantly higher ($P = 0.02$) concentration of this cytokine in the group of chickens receiving 3% clinoptilolite than in the group supplemented with 2% clinoptilolite.

Compared with the control group, a statistically significant increase ($P < 0.05$) in IFN-γ expression in the chicken serum was observed at day 42 in the group supplemented with 3% clinoptilolite. Compared with day 0 of the experiment, significantly higher expression of IFN-γ at day 42 was observed in the groups supplemented with 2 and 3% clinoptilolite and in the control

group. Comparison of the expression of IFN-γ in the chicken serum between the supplemented groups showed a statistically significantly higher ($P = 0.0001$) concentration of this cytokine in the group of chickens receiving 3% clinoptilolite than in the group supplemented with 2% clinoptilolite.

The data in Table 5 present IL-2 expression in the chicken sera in the study groups. Statistically significantly higher relative concentration of IL-2 was observed for the group supplemented with 3% clinoptilolite on day 42 than for the control group. In contrast, in the group supplemented with 2% clinoptilolite, relative expression of IL-2 in the serum of chickens on day 42 of the study was significantly statistically lower than that in the control group. Furthermore, compared with day 0 of the experiment, a statistically significant increase in IL-2 expression was observed in the control group and in the group supplemented with 3% clinoptilolite. Comparison of the relative expression of IL-2 between the supplemented groups showed a statistically significant increase in the expression of this interleukin on day 42 in the group supplemented with 3% clinoptilolite compared with the group supplemented with 2% clinoptilolite.

Statistically significantly lower relative expression of IL-10 was observed for both groups supplemented with clinoptilolite on day 42 than for the control group. Compared to day 0 of the experiment, significantly higher expression of IL-10 at day 42 was observed in the groups supplemented with 2 and 3% clinoptilolite and in the control group. Comparison of the relative expression of IL-10 between the supplemented groups showed a statistically significant ($P = 0.009$) increase in the expression of this interleukin on day 42 in the group supplemented with 3% clinoptilolite compared with the group supplemented with 2% clinoptilolite.

Expression of Cytokines and Interleukins in Chicken Liver Tissue

The data presented in Table 6 indicate that compared with the control group, the expression of TNF-α in the chicken liver tissues on day 42 of the study was statistically significantly lower in the group of chickens supplemented with 2% clinoptilolite. Compared with day 0 of the experiment, significantly higher expression of TNF-α at day 42 was observed in the groups supplemented with 2 and 3% clinoptilolite and in the control group. Comparison of TNF-α expression in the chicken liver tissue between the supplemented groups showed a statistically significantly higher ($P = 0.024$) concentration of this cytokine in the group of chickens receiving 3% clinoptilolite than in the group supplemented with 2% clinoptilolite.

Compared with the control group, statistically significantly lower ($P < 0.05$) expression of IFN-γ in the chicken liver tissue was observed at day 42 in the group supplemented with 2% clinoptilolite. Compared with day 0 of the experiment, significantly higher expression of IFN-γ at day 42 was observed in the groups supplemented with 2 and 3% clinoptilolite and in the control group. Comparison of the expression of IFN-γ in the chicken liver tissue between the supplemented groups showed a statistically significantly higher ($P = 0.0002$) concentration of the cytokine in the group of chickens receiving 3% clinoptilolite than that in the group supplemented with 2% clinoptilolite.

Statistically significantly lower relative expression of IL-2 in the chicken liver tissue was observed for both groups supplemented with clinoptilolite on day 42 than for the control group. Furthermore, compared with day 0 of the experiment, a statistically significant increase in IL-2 expression was observed in the control group and in both groups supplemented with clinoptilolite. Comparison of the relative expression of IL-2 between the supplemented groups showed a statistically significant increase in the expression of the interleukin on day 42 in the group supplemented with 3% clinoptilolite compared with the group supplemented with 2% clinoptilolite.

Statistically significantly lower relative expression of IL-10 was observed for the group supplemented with 2% clinoptilolite on day 42 than for the control group. Compared with day 0 of the experiment, significantly higher expression of IL-10 at day 42 was observed in the groups supplemented with 2 and 3% clinoptilolite and in the control group. Comparison of the relative expression of IL-10 between the supplemented groups showed a statistically significant ($P = 0.001$) increase in the expression of the interleukin on day 42 in the group supplemented with 3% clinoptilolite compared with the group supplemented with 2% clinoptilolite (Table 6).

DISCUSSION

Aluminosilicates, which include clinoptilolite, positively influence the physiological functions of animals as well as the quality of feed in terms of shelf life, consistency, smell, appearance, and taste (Mumpton, 1999). They also have a beneficial effect on protein and energy metabolism in animals by influencing nutrient absorption from feed (Zhou et al., 2014). Studies have shown that zeolite used as a dietary supplement for poultry increases weight gains and reduces the incidence of gastrointestinal disorders (Papaioannou et al., 2002). These observations are confirmed by the results of our research, in which the administration of clinoptilolite to experimental broilers chickens increased weight gains (Table 2). Moreover, the mortality rate was significantly lower in the groups of chickens that received feed with clinoptilolite than in the control group. Our observations also demonstrate that the administration of clinoptilolite as a feed additive improves the health of chickens by reducing the severity of respiratory and gastrointestinal symptoms. The beneficial effects of zeolite in poultry demonstrated in the study may be associated with improved nutrient digestibility, which has also been shown in studies by other authors (Ouhida

Table 6. Comparison of concentrations of interleukins in chicken liver tissue.

		Control group	2% Clinoptilolite	3% Clinoptilolite
Parameter	Day	n = 150	n = 150	n = 150
TNF-α	0	25.32 ± 3.46	26.31 ± 2.26	23.52 ± 2.39
	42	44.27 ± 4.03, $^{a}P = 0.0001$	35.92 ± 2.66*, $^{a}P = 0.0001$	40.44 ± 1.43, $^{a}P = 0.0001$, $^{A}P = 0.024$
IFN-γ	0	25.45 ± 2.92	21.67 ± 1.04	21.55 ± 0.97
	42	52.27 ± 2.99, $^{a}P = 0.0001$	44.45 ± 2.96*, $^{a}P = 0.0001$	52.32 ± 2.11, $^{a}P = 0.0001$, $^{A}P = 0.0002$
IL-2	0	35.27 ± 1.61	34.64 ± 2.19	33.61 ± 3.15
	42	82.62 ± 3.24, $^{a}P = 0.0001$	58.85 ± 1.77*, $^{a}P = 0.0001$	66.88 ± 4.19*, $^{a}P = 0.0001$, $^{A}P = 0.0006$
IL-10	0	31.58 ± 1.70	31.19 ± 1.14	30.74 ± 0.61
	42	64.97 ± 2.70, $^{a}P = 0.0001$	51.33 ± 2.24*, $^{a}P = 0.0001$	60.57 ± 1.54, $^{a}P = 0.0001$, $^{A}P = 0.0001$

Values are expressed as mean and standard deviation (α±SD). n, number of chickens used in 5 replications of the experiment. Data represent means from 5 replications of the experiment.

^{A}Statistically significant differences between the group of birds receiving 2% zeolite in their feed and the group receiving 3% zeolite, assessed by a one-way ANOVA with the post-hoc Tukey HSD and median tests.

^{a}Statistically significant differences ($P < 0.05$) within groups between day 0 and day 42.

Abbreviations: IFN-γ, interferon-γ; IL-2, interleukin-2; IL-10, interleukin-10; TNF-α, tumor necrosis factor-α.

Asterisks indicate a significant increase in the parameter between experimental groups and the control on each testing day ($P < 0.05$).

et al., 2000; Wu et al., 2013c). Moreover, clinoptilolite has been found to reduce toxic ammonia compounds and hydrogen sulfide in the lumen of the digestive tract, which burden the liver and other internal organs, and owing to the absorption of microorganisms, it reduces the concentration of pathogenic bacteria within the intestines (McCrory and Hobbs, 2001; Wu et al., 2013b). The use of zeolites in broiler chicken feed plays an important role in the final phase of digestion and in nutrient absorption, increasing body weight, influencing small intestine morphology, and improving the feed conversion rate (Mumpton, 1999; Christaki et al., 2001, 2006; Lamprecht et al., 2015). However, the lower mortality rate in the experimental group demonstrated in our research may be linked to the better environmental conditions in which these chickens were kept than the conditions on commercial farms. The effect of clinoptilolite on animals may vary depending on the composition of the feed additive, its purity and concentration, and the decomposition rate of active compounds contained in it (Wu et al., 2013a). The mechanism of action of zeolites at the molecular level and their impact on animal growth are not fully understood. The results of our research indicate the need for further research on clinoptilolite as a feed additive in terms of its impact on production parameters and the health of poultry of various use types and raised in various environmental conditions.

The available literature contains little information on the modulatory or suppressive effects of zeolites on the defense mechanisms of the body. The results of previously published studies on the impact of various feed additives on the immune system of poultry indicate that they stimulate proliferation of Th1/Th2 cells and the release of proinflammatory and anti-inflammatory cytokines (Holian et al., 1997; Pavelić et al., 2001). A similar effect is exerted by clinoptilolite, which stimulates the immune system by activating T cells, resulting in stimulation of other immunocompetent cells, release of cytokines, and thus a protective effect on the body (Simeonova et al., 1997). Clinoptilolite has been shown to induce production of Th1 proinflammatory cytokines, increasing synthesis of IL-1, IL-6, and TNF-α (Simeonova et al., 1997). This effect is confirmed by the results obtained in the experimental groups in the present study, in which the TNF-α concentration in the serum and hepatic tissue on day 42 of the experiment was higher in the group of chickens receiving 3% zeolite than in the chickens receiving 2% zeolite. However, the highest TNF-α concentration was obtained in the chickens of the control group. Our observations are consistent with those of Wu et al. (2013c), who showed that natural or modified clinoptilolites reduce the serum concentration of proinflammatory cytokines including TNF-α and IL-1β in poultry. These compounds also affect the permeability of the intestinal mucosa and exert a protective effect by stimulating the intestinal barrier and preventing infections in chickens (Wu et al., 2013a,b,c).

Analysis of the results for IFN-γ in the present study showed a higher concentration of this cytokine in the serum and hepatic tissue of chickens receiving feed with 3% zeolite than that in chickens receiving 2% zeolite. The serum concentration of this cytokine in the control chickens was statistically significantly lower than that in the experimental groups, while no statistically significant differences were found in the liver tissue.

Research on a *Salmonella* sp. infection model in poultry has shown that one of the mechanisms of antiinfective host defense is activation of the phagocytic system, which can be achieved by administering dietary supplements that exert an immunomodulatory effect (Kogut et al., 2001a). Activated phagocytes with nonspecific activity protect against infection by presenting antigens to T cells and stimulating cellular defense mechanisms. Phagocytic cells, for example, macrophages, have a direct bactericidal effect by activating TNF-α and IFN-γ or act indirectly by activating them in the peripheral blood (Kogut et al., 2001b). These observations are confirmed by the results of the present study, in which high serum concentrations of TNF-α

and IFN-γ were demonstrated in the group of chickens receiving 3% zeolite. Similarly, Jung et al., (2010) have shown higher mRNA expression for IFN-γ, IL-4, and TNF-α produced by spleen T cells and macrophages of mice whose feed was supplemented with zeolites. Similarly, Martin et al. (1997) have shown that zeolite molecules absorbed by phagocytic cells, such as respiratory epithelial macrophages, stimulate them to release large amounts of TNF-α. Research results by Jung et al. (2010) and our own results indicate that zeolites stimulate the mitogenicity and immune activity of T cells and macrophages. Other authors (Ueki et al., 1994; Aikoh et al., 1998) have also shown that zeolites act as nonspecific immunostimulators of general host immune mechanisms, which determines effective protection of the body against infection. A similar effect was found in our experiment, manifested by a lack of clinical respiratory and gastrointestinal symptoms in chickens.

IL-2, a proinflammatory cytokine, also has a stimulating effect on the defense mechanisms of the body. Its pleiotropic effects are manifested by activation of B, T, and natural killer cells and innate immune mechanisms associated with the function of phagocytic cells, such as macrophages and heterophils (Rochman et al., 2009). The present study showed a statistically significantly higher serum concentration of IL-2 in the poultry receiving 3% zeolite in their feed relative to the group of chickens receiving 2% zeolite and the control group. IL-2 produced in poultry under the influence of zeolite stimulates recognition of zeolite as an antigen, as well as the cytotoxicity of Tc lymphocytes, thereby promoting cellular immune response mechanisms. This phenomenon is confirmed in a study by Jarosz et al. (2017) in poultry, which demonstrated a statistically significant increase in the percentage of $CD4^+$ T and $CD8^+$ T cells, indicating activation of cells involved in the cellular immune response, mainly involving cytotoxic $CD8^+$ T lymphocytes. That study also showed that zeolite, which irritates the gastrointestinal tract of chickens, triggers a cascade of reactions promoting the proinflammatory Th1CD4+ cell phenotype. Our study also found that IFN-γ, whose concentration was high in the serum of poultry receiving 3% zeolite in their feed, together with IL-2 increases the cytotoxicity of Tc lymphocytes. Different results were obtained for the IL-2 concentration in the liver tissue. The highest concentration of this cytokine was demonstrated in the chickens from the control group. This may indicate a developing response to infection, hepatocyte activation, or a cascade inflammatory reaction, which could cause a clinical form of disease if it persisted longer than the 42 D of the experiment. A high concentration of IL-2, which activates regulatory T lymphocytes, indicates suppression of the immune response after recognition and elimination of an antigen, which may be zeolite for poultry. Both hypotheses can be confirmed by the statistically significantly higher concentrations of IL-10 in the group of chickens receiving a 3% zeolite supplement in the feed relative to the chickens receiving 2% zeolite. The activity of this cytokine reduces the intensity of the inflammatory process and supports the humoral immune response while inhibiting the production of Th1-type cytokines.

Pavelic et al. (2002) have shown that natural clinoptilolite, as an endotoxin adsorbent, can induce a local response in the gastrointestinal tract and a systemic response. A systemic response is indicated in part by the increase shown in the present study in the concentrations of cytokines TNF-α, IL-2, IFN-γ, and IL-10 and acute phase proteins SAA, CRP, Hp, and TRF in the serum and hepatic tissue of chickens receiving 3% zeolite compared with the group of chickens receiving 2% zeolite. Comparison of these data with the results of research by Jarosz et al. (2017) indicates that the local inflammation induced by zeolite promotes the proliferation of Th1CD4+ cells. At the same time, activated lymphocytes releasing IL-10 limit cytokine production by Th1 lymphocytes and inhibit the cellular response. In the long term, the use of 3% zeolite in feed could lead to breakdown of the immunoregulatory barrier, resulting in damage to the body through the dominance of the inflammatory reaction, leading to the development of the active form of disease and increased mortality. The results of immunological tests suggest that for long-term maintenance of homeostasis in poultry, the addition of 2% zeolite as a feed additive is most beneficial. The lower concentration of cytokines in the liver tissue and serum of poultry from the experimental groups relative to the control group may be linked to the anti-inflammatory potential of clinoptilolite, which regulates the concentration of cytokines secreted by Th1/Th2 cells or repression of T-cell responses, which is also confirmed by the results of our previous research (Jarosz et al., 2017).

One of the most important cytokine markers of the immune response profile is IL-10, released by many cell types, but primarily by B lymphocytes (Rothwell et al., 2004). A significant role is attributed to IL-10, released locally by immunocompetent cells, which may inhibit the inflammatory response in the gut (Kuhn et al., 1993). IL-10 on the one hand suppresses the immune response and on the other hand modulates cells with a Th profile. In vitro studies have shown that IL-10 inhibits the synthesis and release of proinflammatory cytokines, including IL-1, TNFα, IL-6, and IL-12, by antigen-presenting cells and inhibits T-cell proliferation in response to antigen or superantigen (Rothwell et al., 2004). In the present study, the concentration of IL-10 in the serum and hepatic tissue of poultry receiving 3% zeolite was higher than that in the group receiving 2% zeolite. The high concentration of IL-10 following the use of zeolite in conjunction with the high concentration of IL-2, TNF-α, and IFN-γ indicates a reduction in the intensity of inflammatory processes, enhancement of the humoral immune response, and simultaneous inhibition of the production of Th1-type cytokines. The high concentration of IL-10 in the liver tissue indicates its regulatory effect, resulting in the maintenance of homeostasis and suppression of the inflammatory response, which is the response to feed supplementation with zeolite, leading to enterocyte damage. Similarly, Lamprecht et al. (2015) showed a higher concentration of IL-10 and pro-

inflammatory TNF-α in humans given zeolites in their diet, which should be associated with the immunoregulatory function of these cytokines in the gut. The health-promoting properties of zeolite are also evidenced by the mitigation or resolution of gastrointestinal symptoms in poultry receiving the preparation as a feed supplement in the amount of 2 and 3%.

The highest concentrations of IL-10 were found in the serum and liver tissue of chickens from the control group. These results together with the high concentration of TNF-α in the serum and liver tissue and of IFN-g and IL-2 in the liver tissue of chickens in this group indicate slow development of generalized inflammation in chickens. The high concentration of IL-10 found in this group protects chickens against Th1/Th2 imbalances by reducing the concentration of proinflammatory cytokines, mainly TNF-α. The effect of these phenomena is to limit the severity of clinical respiratory and gastrointestinal symptoms, which could be exacerbated and lead to the development of systemic disease if the experiment were continued for a longer period.

The results of our own previously published research (Jarosz et al., 2017) indicate that zeolites used as a dietary supplement for poultry promote the humoral and cellular immune response, characterized by a high concentration of proinflammatory and anti-inflammatory cytokines in chicken serum (Jarosz et al., 2017). Cytokines released in excess initiate and modulate the acute phase response in the chickens, which is manifested in the synthesis of numerous proteins. Our observations indicate that the excess clinoptilolite in feed, in the amount of 3%, contributes to excessive synthesis of proinflammatory cytokines acting locally and systemically, which increases synthesis of liver proteins and leads to disturbances of homeostasis. (Hallquist and Klasing, 1994; Sevimli et al., 2013).

In most animal species, including poultry, one of the most important APPs is SAA, which exerts an immunomodulatory and inhibitory effect on proinflammatory tissue-damaging factors (Urieli-Shoval et al., 2000). Chamanza et al. (1999) and Sevimli et al. (2008) have shown that the SAA concentration increases as a result of the effect of proinflammatory cytokines on the body, particularly IL-1β, IL-6, and TNF-α. The results of the present study showed that the serum SAA concentration was higher in the chickens receiving 3% clinoptilolite than in the group of chickens receiving 2% clinoptilolite and the control group. Similarly, a statistically significantly higher concentration of this protein was demonstrated in the liver tissue of chickens receiving 3% clinoptilolite than in the other groups. The high TNF-α concentration in this group of chickens supports studies by Chamanza et al. (1999) and Sevimli et al. (2008) and is linked to developing local inflammation and the release of proinflammatory cytokines from immunocompetent cells. This is also confirmed by the high concentrations of other APPs found in this group, including CRP and TRF. The low serum concentration of SAA in the chickens receiving 2% clinoptilolite indicates the absence of inflammation and that the protein is not produced in the acute phase response but is only involved in immunomodulation and maintenance of homeostasis. The high concentrations of SAA and proinflammatory cytokines in the group of chickens receiving the 3% clinoptilolite supplement, in the absence of clinical disease symptoms, suggest a stimulatory effect of clinoptilolite on the synthesis of this protein in response to local intestinal inflammation. Confirmation of this hypothesis, however, requires precise immunohistopathological tests. The concentration of SAA and other APPs in the serum and hepatic tissue suggest that clinoptilolite stimulates the acute phase systemic response, but the synthesized APPs are involved in maintaining homeostasis of the body and participate in innate immune mechanisms.

In the course of many metabolic and inflammatory processes in the serum and liver of chickens, changes take place in the concentrations of other proteins, including TRF (Giansanti et al., 2012). In the present study, the highest TRF concentration in the serum and in the liver was demonstrated in chickens receiving 3% clinoptilolite. The increase in the concentration of this protein in conjunction with the high concentrations of other acute phase proteins suggests the possibility of development of local inflammatory changes, which occurs as a result of the irritating effect of zeolite on the gastrointestinal tract. TRF is a negative acute phase reactant, and an increase in its concentration usually accompanies processes reducing inflammation (Gabay and Kushner, 1999; Gruys et al., 2005). TRF also exhibits immunomodulatory activity, prevents the proliferation of bacteria, stimulates the activity of heterophils and macrophages, and facilitates tissue remodeling and angiogenesis, thus supporting tissue repair processes (Xie et al., 2002; Rath et al., 2009). The high concentration of TRF shown in the study in conjunction with high concentrations of TNF-α demonstrates that it is produced in response to stimulation of macrophages by the superantigens contained in clinoptilolite. The modulatory role of TRF, which has bactericidal, anti-inflammatory, and homeostatic effects, is confirmed by the results of clinical and anatomopathological tests in which no symptoms or pathological lesions were found in the chickens in experimental groups (Xie et al., 2002).

Hp is another acute phase protein that exhibits anti-inflammatory activity, modulates prostaglandin synthesis, and inhibits granulocyte chemotaxis and phagocytosis (Ceron et al., 2005). The statistically significant increase in Hp concentration in the liver of chickens receiving 3% clinoptilolite in the liver and the lack of differences between groups in the serum concentration of this protein indicate that clinoptilolite is involved in stimulating APP synthesis in the liver, which is also confirmed by the higher concentrations of other acute phase proteins. Excessive Hp synthesis, on the other hand, may be due to the anti-inflammatory effect of this protein on processes in the gastrointestinal tract.

In most animal species, an increased concentration of CRP is an early marker of inflammation in the body

(Eckersall and Bell, 2010). In the present study, CRP concentrations in the liver were higher in chickens from the experimental groups than in the control group, while the highest serum concentration of the protein was demonstrated in chickens from the groups receiving 3% clinoptilolite. The increase in the concentration of this protein in conjunction with high concentrations of proinflammatory and anti-inflammatory cytokines in the chickens from the group receiving 3% clinoptilolite demonstrates that it can influence the development of local inflammatory processes and enhance immune regulation in chickens. The low levels of CRP in the serum and the liver of chickens receiving 2% clinoptilolite rule out an inflammatory reaction.

A similar function in poultry is performed by α1-acid glycoprotein (**AGP**) (Murata et al., 2004; Ceron et al., 2005), an acute phase protein which has immunoregulatory properties and is involved in regulating the inflammatory reaction (Hochepied et al., 2003). In the present study, the concentration of AGP in the serum and in the liver was statistically significantly higher in the chickens from the control group than that in the experimental groups. In conjunction with the clinical picture and the anatopathological changes in the chickens in this group, this is indicative of an anti-inflammatory effect of AGP in response to the developing infection. The study did not show statistically significant differences in the serum concentration of this protein between the experimental groups, while a higher concentration of AGP in the liver was found in the group of chickens receiving 3% clinoptilolite. These data demonstrate the immunostimulatory effect of clinoptilolite on synthesis of liver proteins, including APPs. Our previous research (Jarosz et al., 2017) has shown that clinoptilolite influences regulation of the Th1/Th2 immune response and suppression of inflammatory changes (Jarosz et al., 2017). In conjunction with the concentration of AGP and other acute phase proteins and cytokines in the liver and serum, this suggests that AGP has an immunomodulatory effect that maintains homeostasis in the body. These processes are also associated with an increase in chickens' resistance to infection, as confirmed by clinical observations and anatomopathological examination, as well as the increase in the synthesis of APPs with immunoregulatory properties, such as AGP and SAA (Hochepied et al., 2003).

The research shows that the addition of 2% zeolite to feed is the most beneficial for poultry in terms of health, production, and immunity. This concentration of the preparation was shown to have an immunomodulatory effect in poultry, maintaining systemic homeostasis. These processes contribute to increased resistance to infection, which was confirmed by clinical observations, anatomopathological examination, and an increase in synthesis of APP with immunoregulatory properties, such as AGP and SAA. The increased concentrations of APP and proinflammatory and anti-inflammatory cytokines in chickens receiving 3% clinoptilolite indicate that it stimulates the development of local inflammation and enhances immunoregulation processes.

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