Effect of feed supplementation with zinc glycine chelate and zinc sulfate on cytokine and immunoglobulin gene expression profiles in chicken intestinal tissue

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ABSTRACT The aim of the study was to evaluate the effect of inorganic and organic forms of Zn on the expression of cytokines (IL-2, TNF-α, IFN-γ, IL-12, IL-17, IL-4, IL-10, and TGF-β) and immunoglobulins (IgA and IgG) in the tissues of the small intestine (jejunum and ileum) of broiler chickens. In the experiment, 90 broiler chickens were divided into 4 experimental groups and a control group, with 18 birds each. The birds received Zn supplements in inorganic form with and without phytase ($ZnSO_4$ and $ZnSO_4$ + F), and in organic form with glycine, with and without phytase (Zn–Gly and Zn–Gly + F). The total rearing period was 42 days. Quantitative real-time (RT)-PCR was used to measure the expression of the cytokines and immunoglobulins. The differences between the results obtained for the control and experimental groups, between the groups receiving $ZnSO_4$ and Zn-Gly, and between groups $ZnSO_4$-F and Zn-Gly-F were analyzed statistically. High relative expression of IL-2 was observed for the chickens in the groups receiving $ZnSO_4$-F, Zn-Gly, and Zn-Gly-F on d 42 in comparison to the control group. High relative expression of TNF-α, IL-12, and IL-17 was noted in the group that received $ZnSO_4$ + F. High expression of IgG, IgA, IL-4, TGF-β, and IL-10 was noted in the groups of chickens that received feed supplemented with Zn–Gly and Zn–Gly + F chelates on d 42 of the study in comparison to the control group. In conclusion, supplementation with Zn–Gly chelates can ensure Th1 and Th2 balance during the immune response in the gut-associated lymphoid tissue (GALT), and, by increasing IgA and IgG expression, also can stimulate potentiation of the immune response involved in passive protection of the body from infection. In contrast, the use of inorganic forms of Zn, in the form of sulfates, can induce local inflammatory processes in the intestines, which, in the case of long-term supplementation, lead to the development of infections.

Key words: zinc glycine chelate, zinc sulfate, cytokines, quantitative RT-PCR, chicken

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INTRODUCTION

Proper functioning of the immune system in poultry and other animal species maintains homeostasis and ensures protection against infection by pathogenic microbes (Fraker et al., 2000). Immune activity in poultry is significantly influenced by diet, particularly the use of feed supplements in the form of micro- and macronutrients (Park et al., 2002; Choct, 2009). Zn is a micronutrient that plays an important role in physiological and pathological processes, including modulation of the immune response (Mohanna and Nys, 1999; Feng et al., 2009). These effects of Zn are directly reflected in economic indicators in intensive poultry rearing and breeding (Feng et al., 2009). The main components of poultry feed do not contain sufficient Zn to ensure normal physiological processes (Oberleas and Harland, 2008). Furthermore, factors that limit absorption of this element (e.g., a high concentration of Ca in the raw diet and the presence of phytates) necessitate the use of various forms of Zn as feed additives (Önnerdal, 2000;

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Huang et al., 2007, 2009). The zinc phytate complex forms an insoluble and unabsorbable compound, which is one mechanism for reducing zinc availability to animals. Phytase is an enzyme that acts specifically on phytate, breaking it down to release trace elements in a form available to the animal. Phytase is present in low concentrations in the gastrointestinal tract of poultry, but can be added to the diet of broilers to hydrolyze phytate in the gastrointestinal tract, allowing the bound minerals to become available to the animal (Selle and Ravindran, 2008).

Zinc deficiencies in feed are usually corrected using inorganic forms of this element—sulfates or oxides. Unfortunately, these compounds have low bioavailability, may irritate the gastrointestinal mucosa, decrease the digestibility of other nutrients, and have negative effects on the environment by increasing the excretion of Zn in the feces (Cao et al., 2002). Various studies have indicated that the use of organic trace minerals in broiler diets can enhance mineral uptake, improve body weight gain, and reduce mineral excretion (Burrell et al., 2004; Ao et al., 2007). Organic forms of Zn are increasingly used in poultry—mainly Zn chelates or complexes of organic forms of Zn with methionine (Önnerdal, 2000; Huang et al., 2009; Ma et al., 2011). Many authors point to the obvious advantages of supplementing poultry feed with organic forms of zinc (Cao et al., 2000). For example, a study by Ao et al. (2007), indicates that the tibias of chicks fed a diet containing organic Zn had significantly higher Zn content than those of chicks fed a diet supplemented with inorganic Zn (Ao et al., 2007). However, there are continuing efforts to identify new organic Zn complexes with better bioavailability. Such criteria are met by a glycine complex, which is characterized by greater stability and by chemical and physical homogeneity (Ma et al., 2011). Moreover, this formula ensures better absorption of Zn through the intestinal wall, which makes it possible to reduce the weight content of the glycine complex in the feed and thus production costs (Sahin et al., 2005; Feng et al., 2007; Huang et al., 2009). The use of Zn–Gly chelates has been shown to have a beneficial effect on the growth of birds and on certain immune parameters (Sahin et al., 2005; Huang et al., 2009). These compounds contribute to increased concentrations of IgA, IgG, and IgM in the peripheral blood, increased activity of cytoplasmic Zn-dependent superoxide Cu/ZnSOD-1 and glutathione peroxidase, and a decreased amount of malondialdehyde in the liver (Powell, 2000; Sahin et al., 2005).

The literature has reported numerous biological functions of zinc as a feed additive for poultry, including an anti-inflammatory effect and maintenance of intestinal epithelium integrity (Roselli et al., 2003; Wapnir, 2004; Bonaventura et al., 2015). Information is lacking, however, on the effect of Zn–Gly chelates and $ZnSO_4$ on the immune phenomena taking place in the gut-associated lymphoid tissue (**GALT**) in poultry. Structures that form intestinal lymphoid tissue (e.g., Peyer's patches) are the sites at which intraepithelial B and T lymphocytes mediate a local immune response. These lymphocytes, by secreting cytokines such as tumor necrosis factor-α (**TNF-α**), interferon-γ (**IFN-γ**), IL-2, IL-4, and IL-5, control the immune responses to antigens, eliminate pathogenic microorganisms, regulate the intensity of inflammation, induce synthesis of IgA and enhance its activity, and take part in repair processes in the body, ensuring maintenance of homeostasis (Beagley and Elson, 1992). Our previous study (Jarosz et al., 2017) showed that feed supplementation with Zn chelates activates systemic cellular and humoral immune responses and ensures that a balance is maintained between Th1 and Th2 immune responses. The use of $ZnSO_4$ as feed additives, in contrast, may contribute to the development of local inflammatory processes in the gut, as indicated by high serum concentrations of IL-2 and TNF-α (Jarosz et al., 2017). The available literature lacks data on the effect of Zn–Gly chelates and $ZnSO_4$ on local immune processes in the intestines. The aim of the study was to determine the effect of inorganic and organic forms of Zn on the expression of cytokines (IL-2, TNF-α, IFN-γ, IL-12, IL-17, IL-4, and IL-10, and transforming growth factor-β [TGF-β]) and immunoglobulins (IgA and IgG) in the tissues of the small intestine of broiler chickens.

MATERIAL AND METHODS

Experimental Animals

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 experiments were conducted at the Small Animal Teaching and Research Station of the University of Life Sciences in Lublin, Poland. The study was conducted on 270 one-day-old Ross 308 roosters (Lublin, Poland).

The experimental birds were kept in cages in a room with controlled temperature and humidity. The broilers were weighed and randomly placed in battery cages (1 m × 1 m), with 5 birds per cage. The cages were equipped with nipple drinkers and feeders whose heights were continually adjusted to the age of the birds. All cages were in the same room, and electric lighting was used throughout the rearing period (24 h/d). Three d before the chickens were placed in the cages, the floor was heated to 29°C and the air to 33°C, with a relative humidity of 63%. The temperature was kept at 33°C during the first wk of the experiment and reduced weekly thereafter by 2°C to 3°C, until a final temperature of 20°C to 22°C was reached. The humidity during the experiment was as follows: d 1 to 21, 55 to 60%; d 22 to 35, 60 to 65%; and d 36 to 42, 65 to 70%.

The birds were fed ad libidum mixtures appropriate for each period of rearing, i.e., starter - S (d 1 to 21), grower - G (d 22 to 35), and finisher - F (d 36 to 42),

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

Components (%)	Starter (day 1 to 21)	Grower (day 22 to 35)	Finisher (day 36 to 42)
Maize	24.44	40.00	40.00
Wheat	42.99	27.84	28.84
Soybean extraction meal*	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[a]	0.50[b]	0.50[c]
Protein and fat concentrate**	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
[d] BO, %	21.7	20.2	19.6
[d] WS, %	2.41	2.32	2.31
[d] TS, %	4.52	5.28	5.64
[d] Lysine, %	1.28	1.14	1.10
[d] Meth + Cys, %	0.94	0.84	0.83
[d] Ca total, %	0.87	0.79	0.76
[d] P total, %	0.67	0.66	0.64
[e] Available P, %	0.43	0.40	0.41
[e] Ca total/available P	2.11	1.91	1.90
[e] Fe, mg	40, 22	39, 92	39, 68
[e] Cu, mg	14, 54	14, 72	13, 59

[a]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 5000 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 1800 mg.

[b]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 5000 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 1600 mg.

[c]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 5000 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 1600 mg.

[d]Analyzed values.

[e]Calculated values.

*46% general protein in dry matter.

**1 kg protein and fat concentrate contains: 2% raw fat, 39% raw protein, 10.8 MJ EM.

with unlimited access to water. The starter, grower, and finisher feeds were prepared from maize meal, wheat meal, and soybean extract meal. The composition and nutritional value of the basal diets are presented in Table 1. The total rearing period was 42 days.

Ninety Ross 308 broiler chickens were used in the experiment. The birds were divided into 5 groups: 4 experimental groups and a control group, with 18 birds each. The birds in the control group (group I) received a balanced feed mixture in accordance with the requirements for Ross 308 chickens and a mineral and vitamin premix without Zn. The birds in the experimental groups received Zn in inorganic form ($ZnSO_4$; group II), in inorganic form with a phytase supplement ($ZnSO_4$ + F; group III), in organic form in combination with glycine (Zn–Gly; group IV), and in organic form in combination with glycine and a phytase supplement (Zn–Gly + F; group V).

The Zn requirement in the feed mixtures, based on the dietary recommendations for Ross 308 broiler chickens (Aviagen, Broiler Ross Nutrition Supplement, 2013, www.aviagen.com), was 100 mg/kg, which did not include the content of the element in the feed components. Thus, 100 mg/kg Zn were added to the feed of all experimental groups. The concentration of Zn in the water provided to the chicks during the experiment was 0.299 mg l^{-1}. The content of Zn in the experimental diets is presented in Table 2. The Zn content in the feed samples was determined using the AAS flame technique in a Unicam 939 (AA Spectrometer Unicam, Shimadzu Corp., Tokyo, Japan) apparatus, after ashing at 550°C, according to the methods adopted by AOAC (2000).

In addition, 500 phytase activity units (FTU)/kg (RONOZYME HiPhos (DSM Nutritional Products Sp. z o.o., Mszczonów, Poland) were added to the mixtures. The GLYSTAR FORTE chelate by ARKOP Sp. z o.o. (Bukowno, Poland), which contained 15% Zn, was used in the experiment.

Each group, i.e., the control and the groups whose diets were supplemented with $ZnSO_4$, $ZnSO_4$ + F, Zn–Gly, or Zn–Gly + F, comprised 18 birds. On the first d of the experiment, 6 one-day-old chicks from each group were killed by decapitation to collect intestinal samples. The remaining 12 birds in each group were used for further stages of the experiment. On the 20th

Table 2. Analyzed Zn concentrations in diets for broilers (as-fed basis).*

Zn source	Added Zn, mg kg^{-1}	Analyzed Zn, mg kg^{-1}		
		Starter	Grower	Finisher
Control[a]	0	22, 10	22, 33	20, 95
$ZnSO_4$[b]	100	123, 90	123, 10	125, 90
Zn-Gly[c]	100	125, 90	124, 80	124, 10

*Values based on triplicate determinations of diet samples.
[a]Control negative (corn-wheat and soybean meal control diet with no supplementation of Zn).
[b]Experimental diet with 100 mg of Zn as zinc sulfate and zinc sulfate with phytase supplement/kg of diet.
[c]Experimental diet with 100 mg of Zn as glycine chelate and glycine chelate with phytase supplement/kg of diet.

d, the next 6 birds were killed, and on the 42nd d, the remaining 6 birds were killed. The experiment was replicated 3 times.

Intestinal Sample Collection

The chicks were euthanized (6 chicks from each group on experiment d 0, 20, and 42), and the intestines were removed and flushed with cold PBS. Cross-sections from the jejunum (midsection between Meckel's diverticulum and the duodenum) and the ileum (midsection between Meckel's diverticulum and the ileocecal junction) were collected from all birds in all experiments and snap-frozen in liquid nitrogen. Slices were stored at −80°C until analysis. The intestinal samples for further analysis were not pooled. In the 3 replications of the experiment, 18 samples were analyzed on each test day.

Quantitative Real-time (RT)-PCR for Cytokine and Immunoglobulin Expression

Total RNA was prepared from the mixed (jejunum and ileum) homogenized snap-frozen samples of each bird using an RNeasy mini kit (QIAGEN, Crawley, United Kingdom) following the manufacturer's instructions. Purified RNA was eluted in 50 μl RNase-free water and stored at −80°C until use. The RNA quantity was determined using an ND-1000 spectrophotometer at 260 nm/280 nm (NanoDrop Technologies, Silverside, Wilmington). cDNA for quantitative reverse transcription-PCR (**qRT-PCR**) was synthesized from 1 μg of purified RNA using 50 ng of random hexamers and the SuperScript II first-strand cDNA synthesis kit according to the manufacturer's instructions (Invitrogen, Chorzów, Poland). The expression of cytokines IL-2, TNF-α, IFN-γ, IL-12, IL-17, IL-4, IL-10, and TGF-β and immunoglobulins IgA and IgG was determined by qRT-PCR using the Applied BioSystems 7500 RT-PCR system (Applied Biosystems, Warrington, United Kingdom) as previously described (Lammers et al., 2010). The primer sequences used for qRT-PCR are listed in Table 3. Each sample was subjected to qRT-PCR in triplicate, and the mean values were used for analysis. The threshold cycle ($\mathbf{C_T}$) values for the genes of interest were normalized to an average C_T value of the housekeeping genes, and the relative expression of each replicate was calculated as $2^{-\Delta\Delta Ct}$ (Applied Biosystems user Bulletin #2; AI prism 7700 detection system, 2001). The reference gene used was 28S. The C_T values of each gene were normalized against that of 28S RNA (housekeeping gene).

Statistical Analysis

The results were analyzed statistically using Statistica 10.0 PL (StatSoft, Krakow, Poland). The analysis included the arithmetic mean and standard deviation ($\alpha \pm$ SD). The significance of differences in the means between the results obtained for the control and experimental groups of animals and between sampling times was assessed by the Kruskal–Wallis and median tests, and P-values of less than 0.05 were considered to indicate statistical significance (Figure 1). The differences in the means between the results obtained for $ZnSO_4$ and Zn-Gly, between $ZnSO_4$-F and Zn-Gly-F, and between sampling times were assessed by the Kruskal–Wallis and median tests. P-values for statistically significant differences are shown in Table 4.

RESULTS

qRT-PCR for Cytokine and Immunoglobulin Expression

The data presented in Figure 1.I represent IL-2 expression in the different animal groups. The data indicate that IL-2 expression in groups III, IV, and V on d 42 was higher ($P < 0.05$) than in the control group. IL-2 expression was also higher in all study groups on d 20 and 42 than on d 0 ($P < 0.05$). Furthermore, an increase in the expression of this cytokine ($P < 0.05$) was observed on d 42 compared to d 20 for groups III, IV, and V. A statistically significant increase ($P = 0.06$) in the relative expression of IL-2 was observed in group ZnGly as compared to the $ZnSO_4$ group on d 42 of the study. Detailed data are presented in Table 4.

An increase in IFN-γ expression was observed on d 20 and 42 for groups III ($ZnSO_4$ + F) and V (Zn–Gly + F) compared to the control group ($P < 0.05$). In addition, significant increases in the expression of this cytokine

Table 3. List of primers used for the chicken cytokine mRNA quantification.

RNA target	Primer	Sequence (5'→3')	Source of reference	Accession no.
Il-2	For	5′- TTCAAAATATCGAAAAGAACCTCAAG-3′	Lammers et al., 2010	AF033563
	Rev	5′-CGGTGTGATTTAGACCCGTAAGAC-3′		
IFN-γ	For	5′-GTG AAG AAG GTG AAA GAT ATA TCA TGG A-3′	Kaiser et al., 2003	Y07922
	Rev	5′-GCT TTG CGC TGG ATT CTC A-3′		
TNF-α	For	5′-AAT TTG CAG GCT GTT TCT GC-3′	Crhanova et al., 2011	Designed by Rychlik et al., 2009
	Rev	5′-TAT GAA GGT GGT GCA GAT GG-3′		
Il-12β (p40)	For	5′-TGG TCC ACG CTT TGC AGC T-3′	Crhanova et al., 2011	AJ564201
	Rev	5′-AAG GTT AAG GCG TGG CTT CTT A-3′		
Il-17	For	5′-TAT CAG CAA ACG CTC ACT GG-3′	Crhanova et al., 2011	Designed by Crhanova et al., 2011
	Rev	5′-AGT TCA CGC ACC TGG AAT G-3′		
Il-10	For	5′-CAT GCT GCT GGG CCT GAA -3′	Rothwell et al., 2004; Basaraddi et al., 2013	AJ621735
	Rev	5′-CGT CTC CTT GAT CTG CTT GAT G-3′		
Il-4	For	5′-GTG CCC ACG CTG TGC TTA C-3′	Lammers et al., 2010	AJ621249
	Rev	5′-AGG AAA CCT CTC CCT GGA TGT C-3′		
TGF-β_4	For	5′-ACC TCG ACA CCG ACT ACT GCT T-3′	Lammers et al., 2010	M31160
	Rev	5′-ATC CTT GCG GAA GTC GAT GT-3′		
IgA H(alpha heavy chain)	For	5′-GTC ACC GTC ACC TGG ACT ACA-3′	Lammers et al., 2010	S40610
	Rev	5′-ACC GAT GGT CTC CTT CAC ATC-3′		
IgY H-chain (IgG)	For	5′-ATC ACG TCA AGG GAT GCC CG-3′	Lammers et al., 2010	X07174.1
	Rev	5′-ACC AGG CAC CTC AGT TTG G-3′		
28S	For	5′-GGCGAAGCCAGCCAGAGGAAACT-3′	Kaiser et al., 2003	X59733
	Rev	5′-GACGACCGATTGCACGTC-3′		

compared to the control group were demonstrated for group III on d 20 and 42 and for groups IV and V on d 42. Furthermore, for group III birds treated with $ZnSO_4$ + F, IFN-γ expression was increased ($P < 0.05$) on d 42 compared to d 20. A comparison of relative expression of IFN-γ between the supplemented groups showed a statistically significant increase in this parameter in the $ZnSO_4$-F group as compared to the Zn-Gly-F group on d 20 ($P = 0.01$) and 42 ($P = 0.05$) of the study. Detailed data are presented in Figure 1.III and in Table 4.

Expression of TNF-α was increased compared to the control group on d 20 for groups III and V and on d 42 in all examined groups ($P < 0.05$). The expression of this cytokine also was increased for all experimental groups on d 20 and 42, and for the control group on d 20, compared to d 0 ($P < 0.05$). The highest relative value (12) was recorded for group V on d 42, which was greater than that obtained on d 20 for this group ($P < 0.05$). A statistically significant increase in TNF-α expression between d 20 and 42 also was observed in group II for birds that received $ZnSO_4$. Comparison of the relative expression of TNF-α between the supplemented groups showed a statistically significant increase in this parameter in the $ZnSO_4$-F group as compared to the Zn-Gly-F group on d 20 ($P = 0.03$). Detailed data are presented in Figure 1.II and in Table 4.

The data presented in Figure 1.V represent IL-12 expression in the different study groups. A greater level of relative IL-12 expression was observed for group III on d 20 and 42 and for group V on d 42 compared to the control group ($P < 0.05$). Increases in IL-12 expression also were observed on d 42 in all groups (including the control) and on d 20 for groups III and V compared to d 0 ($P < 0.05$). Furthermore, a statistically significant increase in IL-12 expression was observed between d 20 and d 42 for groups III and V. Comparison of the relative expression of IL-12 between the supplemented groups showed a statistically significant increase in this parameter in the $ZnSO_4$-F group compared to the Zn-Gly-F group on d 20 ($P = 0.004$) and 42 ($P = 0.005$) of the study. Detailed data are presented in Table 4.

Figure 1.VI shows the relative expression of IL-17 in response to Zn supplementation in the study groups. The highest value (0.04) was observed on d 42 in the group that received $ZnSO_4$ + F (group III). An increase in this parameter also was exhibited on d 42 for groups III, IV, and V, and on d 20 for group III in comparison to the control group ($P < 0.05$). Moreover, a statistically significant increase in IL-17 expression occurred on d 20 for group III and on d 42 for groups III, IV, and V compared to d 0. In groups III and V, there were also increases in IL-17 expression between d 20 and 42. Comparison of the relative expression of IL-17 between the supplemented groups showed a statistically significant increase in this parameter in the Zn-Gly group as compared to the Zn-SO_4 group on d 20 ($P = 0.03$) and 42 ($P = 0.03$) of the study. Also, statistically significantly higher expression of IL-17 was observed in the $ZnSO_4$-F group than in the Zn-Gly-F group on d 20 ($P = 0.03$) and 42 ($P = 0.04$). Detailed data are presented in Table 4.

Compared to the control group, the expression of IL-10 was greater on d 20 and 42 for groups III and V and on d 42 for group IV ($P < 0.05$). The highest value (3) for IL-10 was obtained for group III on d 42. An

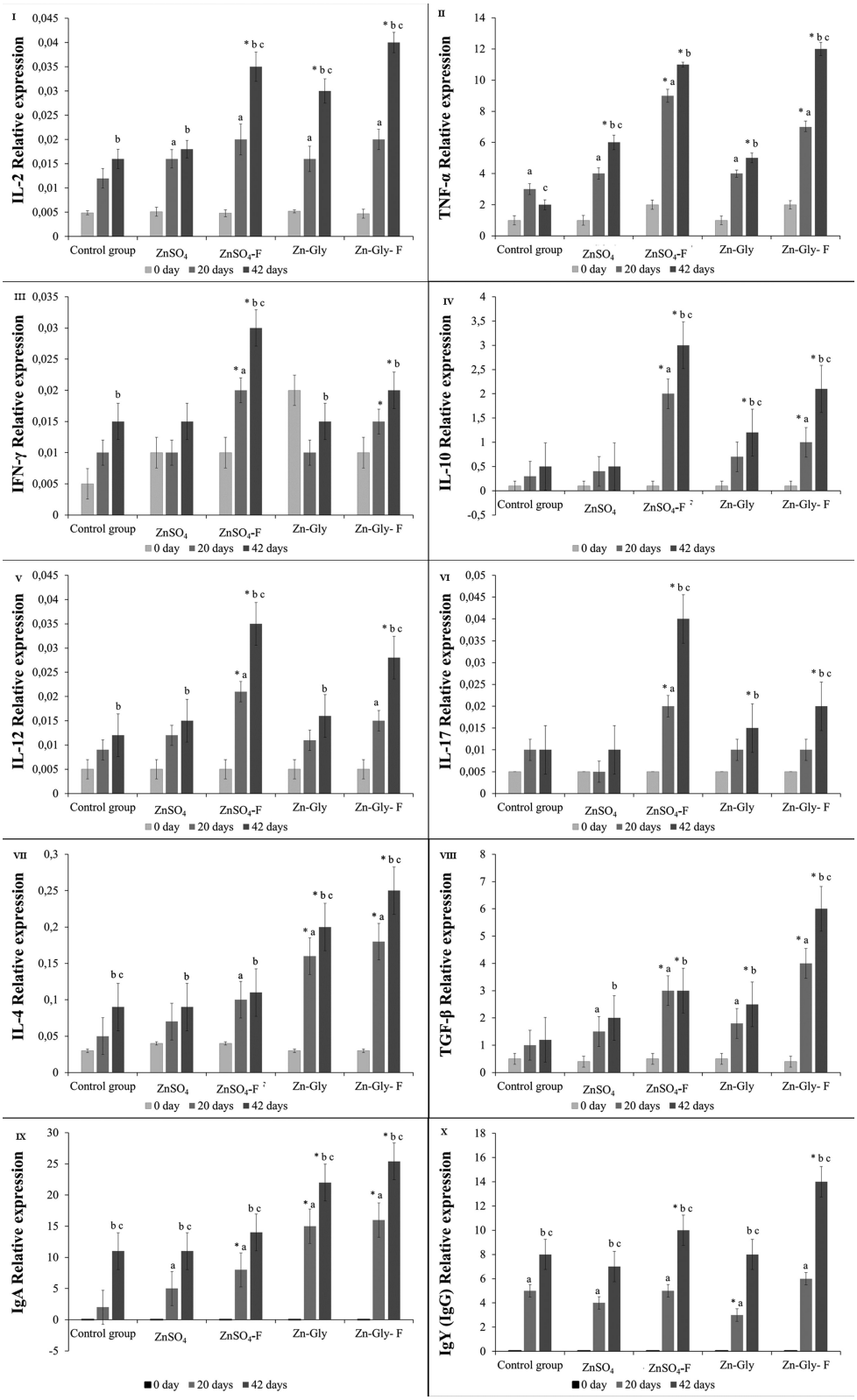

Figure 1. Gene expression in chicken intestine (jejunum and ileum): I—IL-2, II—TNF-α, III—IFN-γ, IV—IL-10, V—IL-12, VI—IL-17, VII—IL-4, VIII—TGF-β, IX—IgA, and X—IgY (IgG). Values are expressed as the mean and standard deviation ($\alpha \pm$ SD). Asterisks indicate a significant increase in the parameter between experimental groups and the control on each testing d ($^*P < 0.05$). a—statistically significant differences ($P < 0.05$) within groups between d 0 and d 20, b—statistically significant differences ($P < 0.05$) within groups between d 0 and d 42, and c—statistically significant differences ($P < 0.05$) within groups between d 20 and d 42 assessed by the Kruskal–Wallis and median tests.

Table 4. Comparison of the relative expression of cytokines between groups $ZnSO_4$ and ZnGly and groups $ZnSO_4$-F and ZnGyl-F, taking into account individual d of the treatment.

Parameter (relative expression)	Day	$ZnSO_4$	$ZnSO_4$-F	Zn-Gly	Zn-Gly-F
	0	0, 0051	0, 0048	0, 0052	0, 0047
Il-2	20	0,016	0, 02	0,016	0, 02
	42	0,018	0,035	0, 03[A]	0, 04
				$P = 0,006$	
	0	1	2	1	2
TNF-α	20	4	9	4	7[B]
					$P = 0, 03$
	42	6	11	5	12
	0	0, 01	0, 01	0, 02	0, 01
IFN-γ	20	0, 01	0, 02	0, 01	0.015[B]
					$P = 0, 01$
	42	0,015	0, 03	0,015	0, 02[B]
					$P = 0, 05$
	0	0, 1	0, 1	0, 1	0, 1
Il-10	20	0, 4	2	0, 7[A]	1[B]
				$P = 0,004$	$P = 0,005$
	42	0, 5	3	1, 2[A]	2, 1[B]
				$P = 0,02$	$P = 0,007$
	0	0,005	0,005	0,005	0,005
Il-17	20	0,005	0, 02	0, 01[A]	0, 01[B]
				$P = 0,03$	$P = 0,003$
	42	0, 01	0, 04	0,015[A]	0, 02[B]
				$P = 0,03$	$P = 0,004$
	0	0,005	0,005	0,005	0,005
Il-12β (p40)	20	0,012	0,021	0,011	0,015[B]
					$P = 0,004$
	42	0,015	0,035	0,016	0,028[B]
					$P = 0,005$
	0	0, 04	0, 04	0, 03	0, 03
Il-4	20	0, 07	0, 1	0,16[A]	0,18[B]
				$P = 0,005$	$P = 0,004$
	42	0, 09	0, 11	0,2[A]	0,25[B]
				$P = 0,004$	$P = 0,005$
	0	0, 4	0, 5	0, 5	0, 4
TGF-β	20	1,5	3	1,8[A]	4[B]
				$P = 0,03$	$P = 0,02$
	42	2	3	2,5[A]	6[B]
				$P = 0,005$	$P = 0,005$
	0	0, 03	0, 03	0, 03	0, 03
IgA	20	5	8	15[A]	16[B]
				$P = 0,005$	$P = 0,005$
	42	11	14	22[A]	25, 4[B]
				$P = 0,005$	$P = 0,005$
	0	0,03	0,03	0,03	0,03
IgY (IgG)	20	4	5	3[A]	6[B]
				$P = 0,005$	$P = 0,005$
	42	7	10	8[A]	14[B]
				$P = 0,03$	$P = 0,005$

[A]Statistically significant difference between the group of birds receiving Zn-Gly in their feed and the group receiving $ZnSO_4$.

[B]Statistically significant difference between the group of birds receiving Zn-Gly-F in their feed and the group receiving $ZnSO_4$-F.

increase in IL-10 expression also was observed on d 20 for groups III and V and on d 42 for groups III, IV, and V, compared to d 0. Furthermore, differences in the expression level of this parameter between d 20 and 42 were recorded for groups III, IV, and V ($P < 0.05$). Comparison of the relative expression of IL-10 between the supplemented groups showed a statistically significant increase in this parameter in the Zn-Gly group as compared to the Zn-SO_4 group on d 20 ($P = 0.004$) and 42 ($P = 0.02$) of the study. Also, higher expression of IL-10 was observed in the $ZnSO_4$-F group as compared to the Zn-Gly-F group on d 20 ($P = 0.005$) and 42 ($P = 0.007$). Detailed data are presented in Figure 1.IV and in Table 4.

The data presented in Figure 1.VII represent the IL-4 expression of the study groups. Compared to the control group, an increase in the relative expression of IL-4 was observed for groups IV and V on d 20 and 42 ($P < 0.05$). For all study groups including the control, the IL-4 expression was significantly higher on d 42 compared to d 0. In addition, expression of this cytokine was statistically higher for groups III, IV, and V on d 20 compared to d 0. An increase in IL-4 expression also was observed for 3 groups (I, IV, and V) between d 20

and 42 ($P < 0.05$). Comparison of relative expression of IL-4 between the supplemented groups showed an increase in this parameter in the Zn-Gly group as compared to the Zn-SO_4 group on d 20 ($P = 0.005$) and 42 ($P = 0.004$) of the study. Also, higher expression of IL-4 was observed in the Zn-Gly-F group than in group $ZnSO_4$-F on d 20 ($P = 0.004$) and 42 ($P = 0.005$). Detailed data are presented in Figure and in Table 4.

Figure 1.VIII shows the expression of TGF-β for the study groups. The highest TGF-β value (6) was obtained on d 42 for birds that received organic Zn–Gly + F. In comparison to the control group, an increase in the relative TGF-β expression was observed on d 20 for groups III and V and on d 42 for groups III, IV, and V ($P < 0.05$). For group III, the expression of TGF-β was maintained at the same level (3) on both d 20 and 42, but these values were higher than on d 0 ($P < 0.05$). The relative expression of TGF-β on d 42 was only significantly higher than that of d 20 for group V. Comparison of the relative expression of TGF-β between the supplemented groups showed an increase in this parameter in the Zn-Gly group as compared to the Zn-SO_4 group on d 20 ($P = 0.03$) and 42 ($P = 0.005$) of the study. Also, higher expression of TGF-β was observed in the Zn-Gly-F group than in group ZnSO4-F on d 20 ($P = 0.02$) and 42 ($P = 0.005$). Detailed data are presented in Table 4.

The analysis of IgA expression showed an increase on d 42 for all experimental groups (II-V) compared to the control group ($P < 0.05$). Furthermore, on d 20, a statistically significant increase in IgA expression was observed for groups III, IV, and V compared to the control group. IgA expression was also higher on both d 20 and 42 for all groups compared to d 0 ($P < 0.05$). The highest IgA value (25.4) was obtained for group V on d 42. In all groups (including the control), an increase in IgA expression between d 20 and 42 was observed ($P < 0.05$). Comparison of the relative expression of IgA between the supplemented groups showed a statistically significant increase in this parameter in the Zn-Gly group as compared to the Zn-SO_4 group on d 20 ($P = 0.005$) and 42 ($P = 0.005$) of the study. Also, higher expression of IgA was observed in the Zn-Gly-F group than in group $ZnSO_4$-F on d 20 ($P = 0.005$) and 42 ($P = 0.005$). Detailed data are presented in Figure 1.IX and in Table 4.

The data presented in Figure 1.X illustrate IgY (IgG) expression for all experimental groups. An increase in the relative IgY (IgG) expression in comparison to the control group ($P < 0.05$) was observed only on d 42 for groups III and V. Compared to d 0, an increase in IgY (IgG) expression was observed on both experimental d for both the experimental and control groups ($P < 0.05$). In addition, statistically significant increases in IgY (IgG) expression between d 20 and 42 were observed in all groups. Comparison of the relative expression of IgY (IgG) between the supplemented groups showed a statistically significant increase in this parameter in the Zn-Gly group as compared to the Zn-SO_4 group on d 42 ($P = 0.03$). However, on d 20 of the study, the expression of IgY (IgG) was higher ($P = 0.005$) in the group receiving $ZnSO_4$ than in the Zn-Gly group. Statistically significantly higher expression of IgY (IgG) also was observed in the Zn-Gly-F group than in group $ZnSO_4$-F on d 20 ($P = 0.005$) and 42 ($P = 0.005$). Detailed data are presented in Table 4.

DISCUSSION

The immune system of the intestinal mucosa in poultry (GALT) consists of various immunocompetent cells, including B and T lymphocytes, NK cells, dendritic cells, macrophages, and heterophils, which, through the induction of local and systemic immune responses, provide protection against infections (Lillehoj and Chung, 1992). In the initial stage of a bird's life, the GALT system is not yet fully developed and has no active B or T lymphocytes (Keen and Gershwin, 1990); it reaches full functional maturity only upon contact with the microflora that colonize the intestines and stimulation by environmental antigens (Lowenthal et al., 1994). In adult chickens, the dominant role among active intraepithelial lymphocytes (**IEL**) is played by T lymphocytes and a subpopulation of $CD4^+$ Th cells, which are differentiated into Th1 or Th2 lymphocytes, depending on the cytokines they secrete. The phenotype of lymphocytes forming the GALT, in addition to the gut microflora, is influenced by genetic determinants, exposure to pathogenic microbes, and feed components or various dietary supplements (Bar-Shira et al., 2003). The immunological effect of feed additives is affected by the form of organic and inorganic Zn used in the preparations. Too little or too much Zn in chelate complexes with amino acids, which are characterized by good bioavailability, has been shown to impair the function of T and B lymphocytes (Zhao et al., 2015).

Stimulation of the Th1 response is characterized by secretion of IL-2, IL-12, and IFN-γ and leads to the promotion of cellular immune mechanisms, which ensures what is known as the functional response. Zinc is known to play an important role in the development of the Th1 response, which is characterized by a cascade of secretion of cytokines that stimulate immunocompetent effector cells (Amitava et al., 2014). This is confirmed by the results of our study, in which high IL-2 mRNA expression was observed for the chickens in the groups receiving $ZnSO_4$-F, Zn-Gly, and Zn-Gly-F on d 42 as compared to the group of birds receiving no supplement (Figure 1). The activity of IL-2 is mainly manifested as activation of B and T lymphocytes and NK cells (Chang et al., 2006). Increased mRNA expression of this cytokine in intestinal tissues after ingestion of Zn can be linked to its modulating effect on immune processes through the activation of innate immune response mechanisms. The statistically high IL-2 mRNA expression induced by the Zn–Gly chelates (Table 4) as

compared to the group of birds receiving $ZnSO_4$ shows that this cytokine may stimulate proliferation and differentiation of T lymphocytes, which promote their cytotoxic functions. It should be emphasized that comparison of the groups of birds receiving $ZnSO_4$-F and Zn-Gly-F revealed no statistical differences in the expression of IL-2 mRNA (Table 4), which indicates that the addition of phytase did not significantly affect the availability of the organic forms of zinc, and the effects are not associated with phytase as a supplement improving digestibility and availability, but only with the effect of Zn itself on the intestinal mucosa.

IL-2 also stimulates the synthesis and release of other pro-inflammatory cytokines, mainly IFN-γ and TNF-α (Lillehoj et al., 2001). This is consistent with the results of this study, in which the highest IFN-γ mRNA expression on d 20 and 42 was noted for the groups of chickens that received $ZnSO_4$ + F as compared to the groups receiving Zn–Gly + F chelates (Table 4). The stimulation of IFN-γ production in T and NK cells increases their cytotoxic potential and promotes the cellular phenotype of the Th1 response. Excessive IFN-γ synthesis in intestinal tissues, as noted in the group of chickens that received $ZnSO_4$ + F, may lead to compromised integrity of the intestinal epithelial barrier and the development of inflammation, which facilitates infection.

Similarly, statistically high expression of TNF-α mRNA was noted in the group of chickens that received $ZnSO_4$ + F on d 20 as compared to the groups receiving Zn–Gly + F chelate (Table 4). TNF-α is mainly secreted by activated macrophages and NK cells stimulated by bacterial antigens or IL-2 (Withanage et al., 2004). The increase in TNF-α mRNA expression, together with very high expression of IFN-γ mRNA, in the intestinal tissues in the group receiving $ZnSO_4$ + F as compared to the group receiving Zn–Gly + F chelate (Table 4), indicates the stimulation of defense mechanisms associated with inflammation in the intestines and promotion of the Th1 cellular response. High expression of TNF-α mRNA also was observed for the group of chickens that received Zn–Gly + F chelate as compared to the control (Figure 1). It is likely that in this group, TNF-α not only induced the cellular phenotype of the Th1 response, but also took part in regulating the immune response. Clinical examination of the birds did not reveal inflammation, which suggests that the pro-inflammatory effect of this cytokine was subject to modification and that it may be involved in the stimulation of apoptosis. This possibility was confirmed by the low expression of IFN-γ mRNA in the chickens that received Zn–Gly + F chelate (Table 4).

Promotion of the pro-inflammatory profile of the Th1 immune response in the group receiving $ZnSO_4$ + F as compared to the group receiving Zn–Gly + F chelate (Table 4) is also evidenced by the high expression of mRNA of IL-12, which stimulates production of T lymphocytes and NK cells. The results obtained for this group demonstrate that this cytokine also stimulated production of IFN-γ and TNF-α, which can increase the cytotoxicity of T lymphocytes involved in inflammatory processes.

A pro-inflammatory effect is also exerted by interleukin IL-17, which plays an important role in chronic inflammation and in allergic and autoimmune diseases. Damage to the intestinal mucosa by various factors, including dietary supplements (e.g., irritating $ZnSO_4$), may lead to an increase in the concentration of IL-17 released from macrophages migrating to the site of inflammation. This hypothesis was confirmed by our study, in which the highest expression of IL-17 mRNA in the intestinal tissues was obtained for the groups receiving $ZnSO_4$ and $ZnSO_4$ + F, as compared to the groups receiving Zn-Gly and Zn–Gly + F chelate (Table 4). Administration of Zn as a dietary supplement may lead to a toxic effect, particularly in the enterocytes. $ZnSO_4$ used in feed has good solubility in water, which is conducive to the formation of a large quantity of Zn ions, which damage the enterocytes. The high expression of pro-inflammatory cytokines shown in our study may thus indicate a local inflammatory reaction within the intestines. It should also be emphasized that an excess of Zn in feed may lead to suppression of Th17 cells that secrete IL-17, thereby impairing the inflammatory response (Kitabayashi et al., 2010; Crhanova et al., 2011).

Stimulation of the Th2 response is characterized by secretion of IL-4, IL-5, and IL-10, which promote the humoral mechanisms of the immune response (Degen et al., 2005). The Th2 response in chickens is mainly involved in combating infection by parasites (Avery et al., 2004), but excessive stimulation of this response may lead to the development of allergic diseases (Hwang et al., 2005). IL-10 plays an important role in immune phenomena. It modifies the immune response through a direct effect on T and B lymphocytes, inhibition of synthesis of pro-inflammatory cytokines, and reduction of the Th1 response (Rothwell et al., 2004; Prasad et al., 2011). The statistically high expression of IL-10 mRNA and the pro-inflammatory cytokines TNF-α, and IFN-γ noted in the study in the tissues of the group receiving $ZnSO_4$ + F as compared to the group receiving Zn–Gly + F chelate (Table 4) suggest the presence of inflammatory processes induced by the $ZnSO_4$ used in the diet in combination with phytase. Haritova and Stanilova (2012) also reported high IL-10 expression in the intestinal tissue of chickens that developed inflammation induced by infection with *Eimeria*. High expression of IL-10 mRNA in correlation with low expression of other pro-inflammatory cytokines, shown in the groups of birds receiving the Zn–Gly chelate as compared to the group receiving $ZnSO_4$, may indicate that this compound has an immunoregulatory effect on the immune response. Statistically high expression of both IL-10 mRNA and IL-2 mRNA on d 42 of the study in the birds receiving Zn–Gly chelate as compared to the group receiving $ZnSO_4$ is indicative of the activation and differentiation of $CD4^+$ T lymphocytes and the

formation of subpopulations of regulatory Treg cells, which suppress excessive immune responses. Due to its regulatory functions, IL-10 also determines immune tolerance for a wide spectrum of bacterial flora, including pathogenic intestinal flora that colonize the gut of chickens during their lives. Because of the potential allergenic effect of Zn preparations, the appearance of this cytokine prevents the development of allergic reactions. The high concentration of IL-10 mRNA in correlation with higher expression of IL-4 mRNA in the chickens that received the Zn–Gly and Zn–Gly + F chelate as compared to the groups receiving $ZnSO_4$ and $ZnSO_4$ + F may lead to increased B lymphocyte proliferation and antibody synthesis and the stimulation of humoral immune mechanisms in the GALT. This hypothesis is confirmed by the high expression of IgG and IgA mRNA noted in the intestinal tissue in the groups of chickens that received feed supplemented with Zn–Gly and Zn–Gly + F chelate on d 20 and 42 of the study as compared to the groups receiving $ZnSO_4$ and $ZnSO_4$ + F (Table 4). Similar observations were made by Lammers et al. (2010), who showed that enhanced levels of IL-10 mRNA in the intestinal tissue of poultry are linked to an increase in IgA mRNA levels.

IL-10 also has increased IgA production in the intestines by promoting differentiation of B cells into plasma cells that take part in the Th2 response (Deng et al., 2012). Analysis of the literature shows that TNF-α and IFN-γ also have a mobilizing effect on the differentiation of B cells and synthesis of IgA antibodies (Sato et al., 2003; Strober et al., 2005). The high expression of IgA mRNA, observed in this study in the intestinal tissues of the chickens that received Zn–Gly and Zn–Gly + F chelate on d 20 and 42 as compared to the groups receiving $ZnSO_4$ and $ZnSO_4$ + F (Table 4) suggests that this compound stimulates humoral immune response mechanisms through activation and differentiation of B lymphocytes into IgA-producing plasma cells in the GALT. The high mRNA expression of this immunoglobulin is evidence of active protection of the intestinal mucosa against antigens, which affects overall potential immunity. Lammers et al. (2010) also showed a positive correlation between IgA protein production and mRNA expression; hence the increased expression of IgA mRNA in the groups receiving the zinc glycine chelate suggests active protection of the intestinal mucosa against penetration by intestinal microbiota and helps to maintain mucosal homeostasis. In contrast with IgA, statistically higher mRNA expression of IgG was shown on d 20 and 42 of the study in the group that received Zn–Gly + F as compared to the groups receiving $ZnSO_4$ + F and on d 42 of the study in the group receiving Zn–Gly as compared to the group receiving $ZnSO_4$ (Table 4), which appears in the intestinal epithelium in response to stimulation by bacterial antigens (Folwaczny, 1997).

Another of the most important immunoregulatory and anti-inflammatory cytokines is TGF-β. The high expression of TGF-β mRNA noted in the group of chickens that received the Zn–Gly and Zn–Gly + F chelate on d 20 and 42 of the study as compared to the groups receiving $ZnSO_4$ and $ZnSO_4$ + F (Table 4) is indicative of maintenance of homeostasis in the organism through suppression of the inflammatory immune response, inhibition of the Th1 response, and promotion of the Th2 response. TGF-β also stimulates B lymphocytes to synthesize antibodies—mainly of class IgA—that protect the intestinal mucosa against damage (Deng et al., 2012), which was confirmed in the experiment.

In conclusion, the balance between the Th1 and Th2 response in the GALT in poultry ensures homeostasis of the organism and protects it against an excessive systemic immune response. The use of Zn–Gly chelates as feed additives ensures that this balance is maintained through the activation of a cascade of cytokines released in the GALT by stimulated T and B lymphocytes. The use of Zn-Gly and Zn-Gly-F as feed supplements enhances the humoral immune response in poultry by promoting synthesis of Th2 cytokines, mainly IL-4 and TGF-β, inhibiting synthesis of Th1 cytokines, and reducing the severity of inflammatory processes. The increased mRNA expression of immunoglobulins IgA and IgY (IgG) following the use of Zn–Gly and Zn–Gly + F chelate is also indicative of potentiation of the immune response involved in passive protection against infections. Furthermore, organic Zn preparations effectively protect the body against infections and improve the overall health of the birds. In contrast, the use of inorganic forms of Zn, in the form of sulfates, induces local inflammatory processes in the intestines, which in the case of long-term supplementation may lead to the development of infections. Further research is needed to determine the cluster differentiation phenotype of immunocompetent cells in the GALT.

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Conflict of interest

The authors declare that they have no competing interests. The authors certify that they have no affiliation with or financial involvement in any organization or entity with a direct financial interest in the subject matter or materials discussed in the paper.

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