Inorganic or organic zinc and MUC-2, IgA, IL-17, TGF-β4 gene expression and sIgA secretion in broiler chickens

Martin Levkutá, Eva Husákováa, Katarina Bobíkováa, Viera Karaffováa, Mikuláš Levkutováa, Okasana Ivanšinováb, Lubomira Grešákováb, Klaudia Čobanováb, Katarína Reiterováč and Mikuláš Levkutad

aDepartment of Pathological Anatomy and Pathological Physiology, University of Veterinary Medicine and Pharmacy, Košice, Slovak Republic; bDepartment of Pathological Anatomy and Pathological Physiology, Institute of Animal Physiology, Slovak Academy of Sciences, Košice, Slovak Republic; cDepartment of Pathological Anatomy and Pathological Physiology, Institute of Parasitology, Slovak Academy of Sciences, Košice, Slovak Republic; dDepartment of Pathological Anatomy and Pathological Physiology, Neuroimmunological Institute, Slovak Academy of Sciences, Bratislava, Slovak Republic

ABSTRACT
The study compared the effect of dietary supplementation with an inorganic or organic source of zinc (Zn) on mucin 2 (MUC-2) and IgA gene expression, the cytokines IL-17 and TGF-β4 and the secretory IgA content (sIgA) in broiler jejunum. One-day-old chickens were fed an unsupplemented basal diet (BD) or the same BD supplemented with 30 or 70 mg/kg of added Zn from ZnSO4·H2O or Zn chelate of glycine hydrate for 40 days. The highly expressed MUC-2 and IgA genes were observed in both groups supplemented with the low-dose Zn sources (30 mg/kg). A higher sIgA concentration was observed in both the ZnSO4 groups and the glycine-zinc/30 mg group. Our data indicate that the organic Zn chelate has better availability than the inorganic Zn source, and the low-dose Zn diets proved to be more beneficial to the maintenance of intestinal immune homeostasis.

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1. Introduction
The nutritional importance of Zinc (Zn) has been known for a long time, but in recent decades its importance in immune modulation has been recognized. Zn can come from organic and inorganic sources. Organic forms of Zn sources include amino acid chelates, bioplexes and proteins as well as lactates and acetates. Zinc from amino acid complexes has been reported to be more bioavailable than Zn from inorganic sources (Bao, Choct, Iji, & Bruerton, 2010; Yu et al., 2010). The zinc-glycine (Zn-Gly) complex has a slightly higher stability constant for Zn than methionine, which may be important for better availability for absorption. Zn-Gly diet can improve intestinal morphology by increasing the villus height, thickness of intestinal wall at the jejunum mucosa and reducing the crypt depth in animals (Payne, Bidner, Fakker, & Southern, 2006).

Intestinal mucosal tissue represents the site of infection or route of access for the majority of viruses, bacteria, yeast, protozoa and multicellular parasites in animals. The interaction of sIgA and mucins is very important in direct or indirect antimicrobial
activity. Not only do mucin oligosaccharides bind microbes, but in some cases, they also have either direct antimicrobial activity or carry other antimicrobial molecules. MUC2 has been shown to be important in the establishment of the mucus layer (Forder, Nattrass, Geier, Hughes, & Hynd, 2012). Secretory IgA is secreted via mucosal epithelial cells and needs to be retained in the immediate mucosal environment (Herich, 2016; Linden, Sutton, Karlsson, Korolik, & McGuckin, 2008). Follicular inductive sites in the intestine are rich in cytokines with IgA-inducing functions, including transforming growth factor β (TGF-β) and interleukin 17 (IL-17) (Cerutti & Rescigno, 2008). On the other hand, zinc is known to modulate some immunological parameters in vitro (Zhao, Li, Chen, Hu, & Zhao, 2016) and in broilers has a positive effect on the immunological capacity by improving the levels of immunoglobulins IgA, IgM, and IgG (Vieira et al., 2013). Zinc supplementation decreases oxidative stress markers and increases phagocytosis. Moreover, Zn promotes monocyte adhesion to endothelial cells in vitro and is important for the production of pro-inflammatory cytokines (Bonaventura, Benedetti, Albarède, & Miossec, 2015).

National Research Council (1994) recommended 40 ppm for broiler chickens, which appeared to be based on the results that considered growth performance as the only criterion (Burrell et al., 2004; Sunder et al., 2008). However, there are several reports that demonstrate that higher Zn levels (60–180 ppm) produce better immune status in broiler chickens (Feng et al., 2010; Tang, Wen, Li, Wang, & Zhou, 2014).

However, there is a lack of information on the mucosal immune protection in the intestine. For these reasons, the goal of this paper was to compare the effect of organic and inorganic zinc sources with different supplementation levels of zinc on the expression of IgA and the MUC-2 gene and the secretion of IgA into intestinal lumen. Moreover, the cytokines inducing IgA functions (TGF-β, IL-17) were followed up.

2. Materials and methods

2.1. Animals

A total of 210 one-day-old ROSS 308 hybrid broilers (MACH Hydina Budmerice, Slovak Republic) of both sexes were randomly allotted into 5 treatment groups consisting of 6 replicate pens with 7 chickens in each pen. During the 40-day feeding trial birds were offered dietary treatments included an unsupplemented basal diet (BD, control – Table 1) and the same BD supplemented with 30 or 70 mg/kg of added Zn from ZnSO₄·H₂O (Sigma-Aldrich, USA) or zinc chelate of glycine hydrate (Glycinoplex-Zn 26, Phytobiotics, Germany). Commercial broiler starter (1–19 days) and grower (20–39 days) diets were formulated as a BD with no supplemental zinc for the control treatment. The mean analyzed values of the Zn content in the starter and grower BD were 31.6 and 31.9 mg Zn/kg, respectively.

The birds were housed in large pens with wood shavings. The environmental temperature was kept at about 35°C during the first week and then was gradually reduced to reach a final temperature of about 24°C. Broilers were maintained on a 23-h constant light schedule and 1 h of darkness and had free access to experimental diets and tap water during the entire experiment.
All procedures were in accordance with European Community guidelines (Directive 2010/63/EU) for animal experiments, and the experimental protocol was approved by the Ethics Committee of the Institute of Animal Physiology SASci and by the State Veterinary and Food Office (Ro-4160/13–221).

### 2.2. Sample collection

After the 40-day feeding period, two birds from each replicate (12 birds/group) with average body weight 2108 ± 78.4 g were slaughtered for sample collection. The tissue samples from the terminal section of the jejunum were collected for the determination of sIgA content and the mRNA levels of MUC-2, IgA, IL-17 and TGF-β4.

### 2.3. Homogenization of tissue and isolation of total RNA of cytokines, IgA and MUC-2

Tissue samples (caudal part of jejunum) were cut into 20 mg pieces, immediately placed into RNA Later solution (Qiagen, UK) and stored at -70°C prior to RNA purification. A single tissue fragment was transferred into 1 ml of TRI reagent (Molecular Research Center, USA), and homogenized using zirconium silica beads (BioSpec Products, USA) in a vortex mixer (Labnet, USA). To separate the phases, 50 µl of 4-bromanisole (Molecular Research Center, USA) was added. The whole content of the tube was centrifuged and upper aqueous phase was collected for total RNA purification using the RNAeasy mini kit.

### Table 1. Composition of the BD.

| Item                              | Starter diet (days 1–19) | Grower diet (days 20–39) |
|-----------------------------------|--------------------------|---------------------------|
| Ingredient (%)                    |                          |                           |
| Wheat, ground                     | 30.96                    | 26.56                     |
| Maize, ground                     | 35.00                    | 45.00                     |
| Soybean meal, extracted           | 28.20                    | 25.00                     |
| Fish meal                         | 2.50                     | –                         |
| Monocalcium phosphate             | 0.90                     | 0.95                      |
| Limestone                         | 1.70                     | 1.70                      |
| Feed salt                         | 0.35                     | 0.36                      |
| Coccidiostat                      | 0.05                     | 0.05                      |
| Lysine                            | 0.10                     | 0.10                      |
| Methionine                        | 0.16                     | 0.20                      |
| Vitamin premixa<sup>a</sup>       | 0.04                     | 0.04                      |
| Mineral premixa<sup>b</sup>       | 0.04                     | 0.04                      |
| Nutrient composition              |                          |                           |
| Dry matter (g/kg)                 | 883.02                   | 881.55                    |
| Crude protein (g/kg)              | 210.75                   | 182.19                    |
| Crude fat (g/kg)                  | 28.27                    | 27.44                     |
| Crude fiber (g/kg)                | 30.85                    | 29.97                     |
| Lysine (g/kg)                     | 12.10                    | 9.90                      |
| Methionine (g/kg)                 | 5.09                     | 4.89                      |
| Zinc (mg/kg)                      | 31.64                    | 31.86                     |
| Manganese (mg/kg)                 | 90.99                    | 88.48                     |
| Copper (mg/kg)                    | 14.23                    | 13.58                     |
| ME (MJ/kg)                        | 12.08                    | 12.24                     |

<sup>a</sup>The vitamin premix provided per kg of diet: vitamin A 12,000 IU, vitamin D₃ 4000.0 IU, vitamin K 3.0 mg, vitamin E 45.5 mg, vitamin B₁ 2.0 mg, vitamin B₂ 6.0 mg, vitamin B₆ 4.0 mg, vitamin B₁₂ 0.02 mg, niacin 40.0 mg, pantothenic acid 12.0 mg, biotin 0.2 mg, folic acid 1.5 mg, choline 440 mg.

<sup>b</sup>The mineral premix provided per kg of diet: I 0.64, Mn 64.0, Cu 6.4, Se 0.1, Fe 84.0 mg.
Following the manufacturer’s instructions, Turbo DNA-free kit (Ambion, USA) was used for the treatment of RNA samples to remove genomic DNA. Both the purity and concentration of RNA were determined spectrophotometrically on NanoDrop 200c (Thermo Scientific, USA) and 1 μg of the total RNA was immediately reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, USA). Resulting cDNA was 10× diluted in UltraPure™ DNase/RNase-Free distilled water (Invitrogen, USA) and used as a template in real-time PCR, or stored at −20°C until used.

### 2.4. Real-time PCR analysis

The amplification, melting analysis and detection of specific products were performed using the CFX96 RT-PCR system (Bio-Rad, USA), with the temperature–time profile as initial denaturation at 95°C for 15 s, and annealing at 59–95°C with a reading every 0.5°C was performed for each individual RT-PCR plate. After each cycle the intensity of the fluorescence was read. Melting curve analysis was carried out after the final elongation at 72°C for 5 min. The melting analysis started at 55°C followed by heating to 95°C by 0.5°C increments and continuous plate read. The primer sequences used for qPCR are listed in Table 2.

Each sample was subjected to real-time PCR in duplicate, and the mean values of the duplicates were used for subsequent analysis. We also confirmed that the efficiency of amplification of each target gene (including GAPDH) was essentially 100% in the exponential phase of the reaction, where the quantification cycle (Cq) was calculated. The Cq of the genes studied were normalized to an average Cq value of the reference genes (ΔCq), and the relative expression of each gene was calculated as $2^{-\Delta Cq}$. These expression levels were then used for comparative data analysis. The relative expression of IgA, MUC-2, IL-17 and TGF-β4 in the jejunum was determined in 12 chickens.

### 2.5. ELISA

The terminal section of the jejunum was excised, and 10 ml of warm flush solution (1 M tris/glycine buffer with 0.25% Tween 20, pH 7, Sigma-Aldrich, USA) was injected into the intestine lumen. The solution was aspirated and injected several times to flush secretions from the intestine wall, and then the contents were collected into the syringe and dispelled into a tube. The intestine flushes were centrifuged for 5 min at 12,000 × g (Hettich Rotina 75 420R Centrifuge, DJB Labcare, UK), and the supernatants from each sample were used

### Table 2. List of primers used in qRT-PCR for cytokines and IgA, Muc 2 mRNA detection in chicks.

| Primer   | Sequence 5′–3′ | References                        |
|----------|----------------|-----------------------------------|
| IgA For  | GTACACGTCTACCTGGAGACTACA | Lammers et al. (2010)             |
| IgA Rev  | ACCGTGCTTGCCTTCACATC    |                                   |
| Muc 2 For| GCTGATTCATCCTGACGCTCTT | Smirnov, Tako, Ferket, and Uni (2006) |
| Muc 2 Rev| ATCTGCCTGAAATCAAGTGTCGC |                                   |
| TGF-β4 For| AGGATCTGAGTGGAAAGTGATGG | Swaggerty et al. (2004)           |
| TGF-β4 Rev| CCCGCGTTGTTGTTGTTGTTG |                                   |
| IL-17 For| TATACGCCAAAGCTGACGCTG | Chránova et al. (2011)            |
| IL-17 Rev| AGTTCCAGCGACCTGGAATTG |                                   |
| GAPDH For| CCTGCATCTGCCCATTT | De Boever, Vangestel, De Backer, Croubels, and Sys (2008) |
| GAPDH Rev| GCCAGCGCATCCTATC |                                   |
for enzyme-linked immunosorbent assay (Husáková, Bobíková, & Stašová, 2015). The chicken IgA ELISA kit (Kamiya Biomedical Company, USA) was used for the detection of total sIgA content. The samples were diluted at 1:5 in 1 × diluent solution, and 100 μl was added into pre-designated wells in duplicates. The 96-well plates were coated with affinity-purified anti-chicken IgA antibody. After the incubation of the plates (22°C for 20 min), the contents of the wells were aspirated and washed three times with wash solution. Then, 100 μl of diluted enzyme–antibody conjugate binding with horseradish peroxidase in stabilizing buffer AQ8 was applied into the plate wells and incubated at 22°C for 20 min. One-hundred microliters of substrate solution (3,3′,5,5′-tetramethylbenzidine) was added into each well, followed by the addition of stop solution at 100 μl per well. Absorbance was measured spectroscopically at 450 nm on a microplate reader (Revelation Quicklink, Opsys MR, Dynex technologies, USA).

2.6. Statistical analysis

Statistical analysis of the data was done by one-way analysis of variance (ANOVA) with the post hoc Tukey multiple comparison tests using GraphPad Software (USA). Differences between the mean values for the different treatment groups were considered statistically significant at \( P < .05 \), \( P < .01 \), \( P < .001 \). The values are expressed as means ± standard deviation mean (±SD).

3. Results

3.1. Expression of MUC-2 IgA, IL-17 and TGF-β4 gene

The expression of the MUC-2 (Figure 1) gene was increased in the ZnSO4/30 and Gly-Zn/30 mg groups compared with the control broilers (\( P < .001 \)). On the other hand, changes

![Figure 1](image_url)

Figure 1. Effect of Zn supplementation on the relative expression of the MUC-2 gene in jejunum by RT-PCR. Means with different superscripts above the columns are significantly different (\( ^{ac}P < .01 \); \( ^{ad}P < .001 \)).
in the expression of MUC-2 gene were not found in the birds fed diet supplemented with a higher dose of organic and anorganic Zn source as compared to control birds.

The expression of the IgA gene (Figure 2) in the ZnSO₄/30 and Gly-Zn/30 mg groups followed a similar pattern compared with both groups supplemented with higher doses of Zn sources \((P < .01; P < .001)\). Similarly, the expression of IgA was found to be elevated in the ZnSO₄/30 and Gly-Zn/30 mg groups compared with the control \((P < .001)\).

The relative expression level of IL-17 (Figure 3) was upregulated in the Gly-Zn/30 mg group compared with the control broilers \((P < .001)\) and the birds fed diets supplemented with inorganic Zn sources \((\text{ZnSO₄/30 mg} – P < .001; \text{ZnSO₄/70 mg} – P < .01)\). Similar tendency in the expression of IL-17 but with lower significance was detected in the Gly-Zn/70 mg group compared with other groups \((P < .05)\).

**Figure 2.** Effect of Zn supplementation on the expression of IgA in jejunum by RT-PCR. Means with different superscripts above the columns are significantly different \((^{ab}P < .05; ^{ad}P < .001)\).

**Figure 3.** Effect of Zn supplementation on the relative expression level of the IL-17 gene in jejunum by RT-PCR. Means with different superscripts above the columns are significantly different \((^{ab}P < .05; ^{ac}P < .01; ^{ad}P < .001)\).
Relative mRNA expression of TGF-β4 (Figure 4) was upregulated in the Gly-Zn/30 mg group comparing with chickens fed the BD (P < .01) and the both diets supplemented with the inorganic form of zinc (P < .001; P < .01). No significant differences were determined in the expression of TGF-β4 between the Gly-Zn/30 and Gly-Zn/70 mg groups.

3.2. Evaluation of sIgA

Excluding the Gly-Zn/70 mg group, the concentration of sIgA in the intestine flush was upregulated in all Zn-supplemented groups (Figure 5) compared with the control.
group, regardless of the Zn source: Gly-Zn/30 and ZnSO₄/70 mg (P < .001) and ZnSO₄/30 mg (P < .01). We found no significant decrease in the sIgA concentration in broilers fed the diet enriched with the high-dose organic Zn supplement compared with the animals supplemented with the same dose of the inorganic Zn source.

4. Discussion

Our current results demonstrate the higher expression of the MUC-2 gene and IgA gene in the jejunum of broilers given diets enriched with both low-dose Zn supplements (30 mg/kg), regardless of the Zn dietary source. Similarly, Zn supplementation resulted in an increased level of sIgA in the intestinal flush. Sunder et al. (2008) observed the highest antibody titer (diet supplemented with Zn at 10, 20, 40, 80, 160 and 320 ppm) after inoculation of non-pathogenic antigen in chicken fed 80 ppm of Zn. The authors in the evaluation of cellular immune response to inoculation of nonpathogenic antigen did not find statistically different response from 20 to 160 ppm Zn levels in the feed. Similarly, Shao et al. (2014) observed an increased number of beneficial Lactobacillus bacteria and a reduced number of Salmonella typhymurium after the zinc supplementation with 120 mg Zn/kg in the broiler diet. Additionally, the same Zn content (120 mg Zn/kg) in the feed of broiler chickens reduced the expression of the inflammatory cytokine TNF-α in the hypothalamus of Salmonella-challenged broilers (Hu et al., 2015).

In contrast, the broiler feed supplemented with 70 mg/kg of both zinc sources did not modulate the expression of the MUC-2 and IgA genes in our trial. Therefore, our data indicate that Zn accumulation in the healthy and stable intestinal microbial community of non-infected broilers seems to be tolerant in the investigated parameters of immune response. Our results suggest that increased level of supplemented Zn is needed in the case of elevated stress.

In our study, the elevated IgA expression and sIgA content in the jejunum of the supplemented broilers showed no differences between the Zn dietary sources upon low-dose Zn supplementation. These data also revealed that a low-dose Zn supplementation is sufficient to stimulate mucosal immunity of jejunum.

Among IgA-inducing cytokines, transforming growth factor β (Gonella et al., 1998) and interleukin-17 (Jaffar, Ferrini, Herrit, & Roberts, 2009) were chosen for study. TGF-β drives naive B cells to differentiate into IgA-producing cells. IL-17 increases polymeric Ig receptor (pIgR) expression and thus increases the rate of sIgA secretion into the lumen (Cao, Suxia, Gong, Elson, & Cong, 2012). Our current experiment demonstrated the promotion of TGF-β and IL-17 in the intestine of chickens fed a diet supplemented with an organic form of zinc in feed. The obtained values for TGF-β and IL-17 in the current experiment showed better effectiveness of the organic form of zinc on these cytokines. The higher expression of examined cytokines in chickens fed only diet supplemented with organic zinc is not clear. Both followed cytokines influence different functions in the host immune response (Kim et al., 2012). On the other hand, glycine appears to exert several protective effects, including anti-inflammatory, immunomodulatory and direct cytoprotective actions (Zhong et al., 2003).

Due to the lack of promotion of immune response parameters in broilers fed a diet supplemented with higher-dose Zn (70 mg Zn/kg), our findings support the suggestion about
the occurrence of an immune response tolerance to a high dose of zinc supplementation in a healthy organism.

In conclusion, low-dose dietary supplementation with either the inorganic or organic zinc source increased the expression of the MUC-2 and IgA genes and sIgA in the intestine of the broilers. On the other hand, the expression of TGF-β and IL-17 cytokines suggested better influence of low-dose supplemented organic zinc source in the diet for clinically healthy chickens. Collectively, our data indicate that the organic Zn chelate had better effect than inorganic Zn source, and the low-dose Zn diets appeared to be more beneficial to the maintenance of intestinal immune homeostasis.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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**Notes on contributors**

**Martin Levkut**, DVM, PhD, actually is young assistant at the departement of Pathology at University of Veterinary medicine and Pharmacy. He interest is focused on interaction between infection agents caused infection diseases and defense barrier of mucosal immunity. In his research seeking to find correlations between mucin composition/degradation and influencing factors of it, then immune mucosal response and also morphometrical analysis of certain microstructures of GIT in poultry and other farm animals. In his dissertation thesis has dedicated to factor’s influencing production of mucin production in the intestine. During his professional works also has written a few chapter’s about beneficial effects of essential oils extracted from organic plants.

**Eva Husáková**, DVM, PhD, is a doctorand from Slovakia, University of Veterinary Medicine in Kosice, Department of pathological anatomy. Her research interest is enzyme-linked immunosorbent assay for detection of immunoglobulins.

**Katarina Bobikova**, DVM, PhD, is a postdoctoral researcher from Slovakia, University of Veterinary Medicine in Kosice, Department of pathological anatomy. She deals with the field of infectious and bacterial diseases of food producing animals in relation to foodborne diseases in humans.

**Viera Karaffová**, DVM, PhD, is a researcher from Slovakia, University of veterinary medicine and pharmacyin Košice, Department of Pathological anatomy. Her research interest are molecular immunology, is a specialist for the PCR methods and bacteriology.

**Mikuláš Levkut** is a professor and doctor of science. He is pathologist and interested in immunopathology of intestinal tract and toxicopathology.

**Okasana Ivanišinová** is a PhD student at the IAP SAS in Kosice. The topic of her PhD thesis is focused on the organic and inorganic zinc sources used in animals nutrition.

**Lubomíra Grešíková** is a researcher of the Department of digestive tract physiology at the IAP SAS in Kosice. Her research area is focused on the metabolism and utilisation of trace elements from their different dietary sources used in animal nutrition.

**Klaudia Cobanová** is a senior researcher of the Department of digestive tract physiology at the Institute of Animal Physiology Slovak Academy of Sciences (IAP SAS) in Kosice. She studies the metabolism of trace elements (Se, Mn, Zn) in farm animals and compares their impact on parameters of antioxidant status.
Dr. Katarína Reiterová, D.Sc. – Research professor; Institute of Parasitology of the Slovak Academy of Sciences (SAS), Košice, Slovakia. Her scientific interests are Immunology, serological and molecular diagnostics of medical and veterinary important parasites, their epidemiology and control; Determination of the occurrence and spreading of intracellular protozoan parasites in Slovakia, above all *Toxoplasma gondii* (Protist, Apicomplexa) – parasite of all warm-blood animals with zoonotic potential and *Neospora caninum* (Protist, Apicomplexa) – serious parasite of dogs and canine carnivores as definitive hosts; as well as domestic and wildlife ruminants, omnivores, small rodents and birds as intermediate host; *In vitro* cultivation of alive isolates from seropositive animals as source for own antigen’s preparation; Preparation and standardization of commercially unavailable specific antigens for diagnostic of helminthozoonoses (*Echinococcus* spp., *Trichinella* spp., *Toxocara canis*).

Mikuláš Levkut is a professor and doctor of science. He is pathologist and interested in immunopathology of intestinal tract and toxicopathology.

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