BIOAVAILABILITY AND RESPONSE OF BROILER CHICKENS IN HEALTH AND DISEASE-CHALLENGE STATES TO DIFFERENT SOURCES AND LEVELS OF ZINC SUPPLEMENTATION

BY

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(Under the Direction of OLUYINKA A OLUKOSI)

Abstract

Zinc is involved in many biochemical functions essential for the birds' health and growth. The relative bioavailability values were 107%, 76%, and 127% for zinc hydroxychloride (ZH), zinc-glycinate (ZG), and zinc-lysine-glutamate (ZL) sources, respectively, relative to zinc sulfate (ZS). There were no Zn sources or levels effects on the growth performance of the broiler. However, there was a significant effect of Zn sources on *in vitro* nitric oxide production ($P < 0.05$), wherein ZL increases its production compared to ZH diets. Birds receiving ZS diets had lower ($P < 0.01$) P digestibility than those fed ZH, ZG, and ZL-supplemented diets, and birds fed the ZL diet had higher Cu digestibility ($P < 0.05$). An interaction was observed for GPX3, in which the expression was upregulated by increasing the Zn level from 25 to 100 mg/kg in ZS, ZH, and ZL-fed diets and downregulated in ZG-fed diet. In our last study, *Eimeria* challenge significantly ($P < 0.05$) impaired all performance traits assessed in different ages. Zn source affected weight gain at the end of the grower phase and showed an increase in ZL diet compared to the birds fed ZS diets ($P < 0.05$). Tibia weight, ash weight, and tibia Zn concentration were significantly ($P < 0.05$) decreased in challenged birds at 6 days post inoculation (6-dpi) than in non-challenge. *Eimeria*

inoculation decreased the expression of ZnT1 in duodenum, jejunum, and kidney, while upward expression of ZnT5 in duodenum, jejunum and ileum. mRNA expression of JAM2 and CLDN1 in jejunum and CLDN1 in ileum upregulated during Coccidiosis. Metallothionines (MTs) was upregulated by *Eimeria* challenge in both kidney and pancreas ($P < 0.05$). Birds displayed lower expressions of ALP1 and GPX1 in the kidney and ALP1 in the liver at 6-dpi.

Our studies showed that organic trace minerals of ZL can be beneficial to physiological responses like growth, bone, immunology, and nutrient utilization in broiler chickens. Also, ZS increases VH in the ileum, SCFA, mcC of Cu, CAT1, and GLUT1 mRNA expression. More research needs to be done to justify the higher cost of organic mineral supplementation compared to inorganic one in poultry feed.

(Keywords: Zinc, bioavailability, inorganic, organic, Zn transporters)

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A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2024

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December 2024

DEDICATION

To my beloved parents, whose unwavering support and love have been my pillars for hard work. I will forever be grateful for your encouragement to embrace my full potential. I dedicate this dissertation to my wife, Neda, for her patience, compassion, and most importantly, love. To my son, Behrad, remember that all things are possible. Never be afraid to pursue your dreams and stand up for people. I love you without measure.

ACKNOWLEDGMENT

I would like to start off by saying if it was not for my upbringing in Khuda, I would have never gotten to the point I am today. Through the thick and thin my faith has always brought me to a better place no matter how long it takes.

Thank you, Dr. Oluyinka Olukosi, for giving me the opportunity to join your research team. The trust, independence, support, and guidance you have provided had a major impact on my scientific and moral advancement. He patiently mentored me through this program.

Thank you, Dr. Robert Dove, for providing me with the opportunity to measure minerals in your Lab. I would also like to thank my committee members Dr. Rami Dalloul and Andrew Benson for their mentorship throughout my Ph.D.

I would like to thank Lindsey Rackett, Jesse Hanks, and Christopher McKenzie for their help in the farm trials.

Thank you, Dr. Lin Yang, Dr. Shravani Veluri, Dr. Ajao Adeleye Mathew, Dr. Gabriel Akerele, this work would not have been done without your help.

A special thank you to our lab group, Iyabo Oluseyifunmi, Bhargavi Kasireddy, Rama Devi Kolli, Sujitha Veeraganti, Olanrewaju Ayinde, June Hyeok Yoon and Asghar Sedaghat.

Lastly, I would like to thank our lab technician in the Poultry Science Department, Trevor Ard, Derrel Hardman, Brett Marshal, and Jonah Sta Monica.

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CHAPTER 1

1. INTRODUCTION

Zinc is an important mineral due to its many vital functions in the body, like immune response, intestinal ion transfer, catalytic activity, and support for overall optimal growth and health. In practical diets, Zn is supplemented since it is marginally deficient, and supplementation has shown many beneficial effects in poultry. Poultry breeders' recommendations of Zn level supplementation are around 100 mg/kg Zn, whereas NRC recommends 40 mg/kg Zn supplementation for a practical diet for broiler chickens.

Traditionally, Zn has been supplemented as inorganic salts in the feed for economic reasons, but organic sources of Zn have received more attention recently since their bioavailability is higher than inorganic Zn. The use of organic Zn as a mineral supplement in poultry diets was shown to be more stable throughout the changes in pH in the digestion process, protected from chemical interactions with nutrients to form insoluble substances, less excretion, and environmental pollution. Organic Zn can be formed by bonding amino acids (AA) with Zn atom(s). Amino acids are usually absorbed in the small intestine more efficiently than minerals, and it is assumed that a portion of Zn-AA can be absorbed via AA transporters, such that intestinal absorption of organic Zn occurs more effectively than inorganic Zn sources.

Studies have shown that organic Zn sources are not always superior to inorganic ones. These differences in observation are likely due to different chelation strengths, manufacturing processes, or different inorganic brands. Although laboratory analysis can give some information about the Zn contents and possibly hydrolyzability of Zn from organic and inorganic sources, animal trials (bioavailability, growth performance, nutrient utilization or response during a disease

challenge) can be considered the ultimate test for suitability of these different Zn sources for the broiler chickens.

Therefore, it was hypothesized that Zn complexed with organic compounds (organic Zn sources) compared to inorganic Zn sources in broiler chicken diets will have greater bioavailability and promote superior growth performance and improvement in response to disease. To test the hypothesis, three animal studies with the following aims were developed:

Aim1: Evaluate the relative bioavailability of Zn from hydroxychloride (inorganic Zn source) and glycine or lysine chelated-Zn as organic sources, using zinc sulphate hepta-hydrate ($ZnSO_4.7H_2O$) as the reference Zn source.

Aim 2: To evaluate the influence of adding phytase in low or high-Zn diets from different sources (organic and inorganic) on growth performance, gene expression (Zn transporters, AA transporters, and antioxidant enzymes), in vitro immune response, and intestinal histomorphology of broiler chickens.

Aim 3: To characterize the effect of organic and inorganic Zn on growth performance, gut permeability, nutrient transporter, and intestinal morphology of broiler chickens challenged with *Eimeria*.

CHAPTER 2

2. LITERATURE REVIEW

The first deficiency of Zn was reported in 1958, when a 21-year-old male patient in the Iranian city of Shiraz presented with mental lethargy, dry skin, and dwarfism, and it turns out he only ate unleavened bread and no animal protein (Prasad et al., 1961). World Health Organization (WHO) showing 2 billion people may have Zn deficiency (Kumssa et al., 2015). This is mainly due to organic phosphate compounds in cereal proteins, which combine with Zn, making it unavailable for absorption. Zn deficiency may result in other symptoms, such as increased oxidative stress (Bao et al., 2010), decreased cell-mediated immunity (Bao et al., 2011), and upregulation of inflammatory cytokines (Prasad et al., 2007). Zinc supplements or animal-protein foods improve growth and support immune function (Wessels et al., 2017), DNA and protein synthesis, and insulin and thyroid function (Severo et al., 2019). Growth is the first limiting effect of Zn deficiency in animals. Both pituitary growth hormone and insulin-like growth factor-I decreased in response to a Zn-deficient diet. The mechanism can explain how Zn affects membrane signaling and intracellular second messengers, thereby directing cell proliferation in response to Insulin-like Growth Factor-1(MacDonald, 2000).

Zinc in the Poultry Industry

Zinc is the second most abundant mineral in body composition and a critical trace mineral with many functions like growth, immunity, and antioxidant defense in poultry nutrition (Salim et al., 2008). Zinc is a key element of many molecule enzymes involved in essential metabolic reactions, including digestion, energy metabolism, and protein synthesis. Zinc exert its antioxidant activities by synthesizing metallothionein (Naz et al., 2016). Enough Zn intake and digestion are

needed for functions like immunity against pathogens, development, egg production, growth, and reproductive performance (Chand et al., 2014). The role of Zn in nutritional biology was first reported by Raulin (1869) when he observed that Zn was essential for the evolution of *Aspergillus niger*.

The level of Zn needed for broilers differs across reports, and some studies report that the required dose is greater than the NRC recommendation. Over past decades, the Zn requirement for broilers recommended by the major breeding companies has far exceeded the NRC recommendations, at up to 110 mg/kg added Zn for a Ross 308 broiler (Aviagen, 2014) or 100 mg/kg supplemental Zn for a Cobb 500 broiler (Cobb, 2018). In a commercial production setting, it is common practice to formulate diets that contain 100 to 120 mg/kg of supplemental Zn (Feng et al., 2007).

Organic and Inorganic Zinc

In poultry diets, Zn may be supplemented into the diet either as organic Zn (e.g., Zn protein, Zn amino acid, or Zn picolinate) (Hu et al., 2023a) or inorganic Zn (e.g., $ZnSO_4$, Zn Hydoxychloride, or ZnO) (Qudsieh et al., 2018). The recommended Zn level in broiler diets by the National Research Council (NRC) (Council, 1994) is 40 mg/kg of poultry diet, which inorganic or organic Zn sources can supplement. The chemical bond between metals (cations: Zn, Ca, Mn, etc.) and non-metal (anions: S, O, etc.) named ionic bonds, which form when oppositely charged ions (anions and cations) attract to each other. The weak ionic bonds in Zn sulfate and Zn oxide allow the connection between the salt and metal molecule to fully dissociate in an aqueous gut solution (Solutions, 1980). The disassociated Zn molecule can bond to many nutrients in the intestinal lumen, like other minerals, fat, vitamins, and phytates (Kornegay, 2001), leading to lower mineral absorption and nutrient absorption (Batal et al., 2001). However, oxide salts of trace minerals, such

as Zn, are less reactive and comprise up to twice the cation concentration as sulfate salts, requiring lower supplementation in trace-mineral premixes. Zinc oxide has lower bioavailability than ZS (Edwards III & Baker, 1999). Another chemical interaction, called a covalent bond, happens when one or more pairs of electrons exchange between atoms. Zn hydroxychloride (ZH) crystallizes in a layered structure where Zn ions are coordinated in geometry with three hydroxide (OH) groups and one chloride (Cl) ion, creating a Zn salt with low water solubility due to its crystalline structure. This structure gives ZH less reaction with other nutrients in the intestine lumen (Cromwell et al., 1998). Generally, inorganic mineral sources are widely used in the poultry industry since they are cheaper. However, less absorption leads to a high concentration of Zn in poultry litter and adverse environmental effects (Leeson & Caston, 2008)—organic minerals formed by chelation of minerals with an organic molecule of amino acids, peptide, etc. (Zhao et al., 2010) found that chelated trace minerals can be an excellent alternative mineral source for favorable broiler growth needs and reduce dietary mineral inclusion.

Zinc Bioavailability

The quantity of a nutrient that reaches the body and is involved in metabolism after ingestion and absorption is called bioavailability (Ammerman et al., 1995). Many researchers have discussed that Zn bioavailability differs when comparing organic and inorganic supplements (N. F. Suttle, 2022). Zinc methionine, Zn lysine, and Zn threonine are Zn amino acid complexes that improve digestion and availability by dropping antagonism compared to the inorganic form, which may react with no absorbable compounds in the gastrointestinal tract (Neto et al., 2020). Organic sources of trace minerals arriving at the brush border of enterocytes can be present with intact structure (Ashmead, 1993), thus increasing bioavailability compared to inorganic mineral sources and potentially reducing Zn supplementation levels in poultry diet (Gheisari et al., 2011; Kumar

et al., 2021). Many researchers have shown that chelation strength can affect organic mineral bioavailability (Huang et al., 2009; Li et al., 2005). Organic mineral sources with moderate or high chelation strength showed lower interference in high dietary phytate and higher relative bioavailability (RBV) than inorganic sources. Chelated minerals are chemically well-defined and promote laying hen performance. In addition, it has been suggested that organically generated Zn has been prioritized in cases in which the dietary levels of trace elements are pushed to the margins (Sun et al., 2012). Organic Zn (a complex of metal amino acids) has effectively improved the FCR, broken egg percentage, and Haugh units in young laying hens (Abedini et al., 2018). Bakhshalinejad et al. (2024) reported that the concentration of Zn in eggs of laying hens fed dietary organic Zn was higher than that fed Zn hydroxychloride.

Zinc and Bone development

Due to rapid growth, there is a higher rate of skeletal disorders in broiler chickens, like lameness and tibial disorder. Research shows that these issues are partly due to deficiencies of some trace minerals (Lilburn, 1994). Some of these problems are reported even with enough minerals in the diet, likely due to poor bioavailability and nutrient-antagonistic interactions. It has been documented that supplemental Zn mitigates leg disorders in broilers (Akhavan-Salamat & Ghasemi, 2019). Seo et al. (2010) have shown that Zn administration positively affects bone formation, mainly with the help of direct impacts on protein synthesis (Scrimgeour et al., 2007). Zn can act as hormonal growth mediator, such as influencing insulin-like growth factor I on osteoblasts (Wang et al., 2002). The effect of Zn when feeding to a laying hen could be different when the origin of supplementation is different.

Tibia and eggshell thickness can be affected by Zn when inorganic Zn is replaced with Zn amino acid complexes (Gheisari et al., 2011). Also, the dietary addition of organic Zn could ease

the adverse effects of age on egg quality parameters in laying hens (Behjatian Esfahani et al., 2021). An increase in tibia ash in birds fed Zn proteinate compared to those fed Zn sulfate has been observed (Shelton & Southern, 2006). Organic Zn caused higher Zn concentration in chicks' tibia than those fed inorganic Zn. The authors concluded that the observation might be due to antagonism between Zn and other minerals while using inorganic sources but not organic sources. Ten-day-old chicks fed a basal diet of 15 ppm Zn require 40 ppm Zn supplementation for average growth and tibia length, but ten ppm Zn is necessary for maximal tibial ash (Young et al., 1958). Meanwhile, 25 to 30 ppm Zn supplementation to a basal diet is needed for maximal growth on day 21. One of the prevalent results in research on the effect of Zn on bone is little or no effect of Zn supplementation on bone ash (Davies & Motzok, 1971). Consistent decreases in ALP have been reported to be linked with the minimum alterations in bone ash due to variations in the enzyme distribution within different parts of the growing bone (Westmoreland & Hoekstra, 1969).

Zn and Reproduction

Zinc deficiencies in animals as well as humans are known to lead to hypogonadism. This condition is associated with decreased spermatozoa, spermatogenic size, and reduced testicular weight and size (McClain et al., 1984). Zn deficiency can also depress development of the pituitary gland (Morley et al., 1980) and testis weight (Hamdi et al., 1997). Testes have a strict Zn requirement, and severe deficiency compromises spermatogenesis and motility, resulting in low fertility. Low Zn nutrition is an essential risk factor for low sperm quality and male infertility(Colagar et al., 2009). Zn deficiency in mice can reduce the mRNA expression and protein abundance of Zip6 and Zip10 (influx transporter) in Sertoli cells (Croxford et al., 2011). The authors also observed a lower density of seminiferous tubules in mice fed a Zn-deficient diet compared with mice fed a control diet in tissue stained with hematoxylin and eosin. The tubes also

featured an irregular germinal epithelium in Zn-deficient diet mice. They demonstrate how Zn transfers from Sertoli cells to the developing gametes during spermatogenesis. Low Zn diets in male studies (2.7–5 mg Zn/d) resulted in poor Leydig cell function and lower testosterone, which can be reversed by Zn supplementation (Abbasi et al., 1979). Song et al. (2010) showed that reproductive function in male rats is sensitive to a more marginal Zn deficiency (~6 μg Zn/g diet), leading to low prostate Zn concentration and increased ROS production. Zip6, Zip8, and Zip10 are all expressed in round spermatids and play critical roles in Zn import.

Zn is an important factor in the quality of eggshells and regulates it by affecting the structure of the epithelium in the isthmus and influencing HCO_3^- secretion (Zhang et al., 2022). Most of the Zn in the egg is deposited in the yolk, and about 85% of the Zn can be transferred to the progeny (Richards, 1997), which is required for the rapid growth and development of the embryos. Breeder birds fed a Zn-deficient diet had irregular embryonic development, lower hatchability, and poor-performing offspring (Zhu et al., 2017), whereas *in-ovo* Zn injections in the yolk can reduce these adverse effects (Sun et al., 2018). These studies provided new evidence that Zn deficiency harms embryonic development and is related to epigenetic status alterations (Sun et al., 2018).

Work from various labs has uncovered many Zn-dependent processes that regulate mammalian female reproduction like oocyte development, maturation, and ovulation in rats (Bernhardt et al., 2012), swine (Jeon et al., 2015), and bovine (Pascua et al., 2020). Follicle activation involves a complex interaction between germ cells and somatic factors to ensure steady maintenance of the growing follicle pool. The Role of Zn is described as a regulator of early meiosis in germ cells and impairment of gametogenesis due to Zn deficiency (Hester et al., 2017).

It was reported that the presence of Zn salt in hypophysial extract injection increased the ovarian weight of young female rats (Maxwell, 1934).

Zn and Enzyme Cofactors

Zn participates in enzymes and other proteins as a basis of its catalytic roles. Zn contributes to 300 enzymes and 2000 transcription factors required for their functions and the stability of their structures. Zn is a cofactor in all six classes of enzymes, including oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases (Vallee & Falchuk, 1993). Zn functions in biology have been categorized into catalytic or structural functions within an enzyme (Cousins et al., 1996), and sometimes both, like alcohol dehydrogenase in the liver (Vallee & Hoch, 1957). Zn has better stability than Fe, Co, or Cu, making it a perfect cofactor since redox-stable enzyme ions are required (Butler, 1998). The role of Zn as a cofactor in the two key enzymes of carbonic anhydrase and alkaline phosphatase is critically important for egg quality and skeletal development, respectively.

The biochemistry of Zn in enzyme was first discovered in 1939 in erythrocyte carbonic anhydrase (Baranauskienė & Matulis, 2019). Carbonic anhydrase (CA) is a Zn-containing enzyme that controls the reversible hydration of carbon dioxide to bicarbonate and acid protons. Metal Zn is located in a cavity linked to three histidyl residues and a solvent molecule. The catalytic mechanism of CA involves an attack of Zn-bound OH on a CO_2 molecule loosely associated with a hydrophobic compartment. The resulting Zn-coordinated HCO_3 ion is shifted from the metal ion by H_2O (Lindskog, 1997). The Carbonic anhydrase research in poultry is historically well-documented (Davenport, 1940; Keilin & Mann, 1940; Mann & Keilin, 1940; Meldrum & Roughton, 1933). In laying hens, Zn supplementation can change the construction of eggshells by changing the formation of calcite crystals (Creech et al., 2004; Yildiz et al., 2010). It has also been

proven that Zn deficiency disrupts egg production (Tabatabaie et al., 2007). There are some discrepancies in the required supplementation level of Zn for laying hens. Some higher levels of 100 or 150 mg/kg Zn were reported to significantly increase eggshell thickness (Zamani et al., 2005), while others reported lower levels, as little as 30 or 60 mg/kg Zn, to improve eggshell thickness (Mabe et al., 2003). It has been well documented that chelated Zn has a better effect on egg quality (Neto et al., 2011) and ameliorates the age effect of egg quality (Mabe et al., 2003) than inorganic counterpart sources. Treating animals with organic minerals can significantly increase CA concentration in the liver (Sun et al., 2012).

Alkaline phosphatases (ALPs) are another enzyme that requires Zn in its structure to function. In humans, Zn deficiency accompanies low ALP activity in serum, liver, kidney, and small intestine (Cho et al., 2007). ALPs are more effective in an alkaline pH (pH 10), high concentrations in growing bones, and remove phosphate groups from molecules like nucleotides, proteins, and alkaloids. ALP is a crucial enzyme for the formation and calcification of bone tissues. Severe Zn deficiency in chicks depleted Zn more in the tibia than in the liver or intestine. ALP activity in the tibia was 70%, and the intestine was 34% lower, while the liver was 66% more, comparing Zn deficient birds to a control diet (Davies & Motzok, 1971). The very first research (Forrai, 1923) mentioned that the hydrolysis activity of ALP facilitates the utilization of phosphate esters in intestine. ALPs also play a crucial role in eggshell quality. The studies by (Yair & Uni, 2011) highlighted a significant decline in Zn levels during egg incubation, with approximately 94% of total Zn disappearing by the hatch, and noted that noted that 37% of the egg's Zn was associated with the albumen at the beginning of incubation. Moreover, the patterns of embryonic Zn uptake observed by both Dewar et al. (1974) and Yair and Uni (2011) reflect a significant decline in Zn-dependent alkaline phosphatase activity noted by Li et al. (2014) during the later

stages of incubation. This correlation suggests that Zn availability may influence enzymatic activity during embryonic development. It was observed that, in 12 days of incubation, ALP activity in bone was three times higher than in 1-day-old chicks (Kay, 1926).

In a very early study, researchers (Hove et al., 1940) observed that intestinal phosphatase activity significantly decreased by Zn deficient diet in rats and no changes in bone phosphatase activity. Forty years later, a follow-up study similarly showed a reduction in ALP activity in femur, kidney, serum, and small intestine of young rats fed 2.8 ppm Zn compared to 98 ppm Zn of control diet (Adeniyi & Heaton, 1980). Zn is involved in the regulation of the activity of glutathione peroxidase (GPX) (Scheideler et al., 2010), and supplementation of organic Zn has more advantages than their inorganic forms in improving antioxidant status and lipid metabolism (Akhavan-Salamat & Ghasemi, 2019). Results from Yang et al. (2024) showed that Zn supplementation in laying hen diet can improve antioxidant parameters in both liver and blood.

Eimeria and Zn responses

Eimeria is a protozoa that invades the intestinal tract and causes coccidiosis in poultry. Scholars have estimated that the damage in productivity induced by clinical coccidiosis in poultry, along with expenses in prevention and treatment, would be about US$ 14 billion annually (Blake et al., 2020). The enteric disease result can range from subclinical disease to bloody enteritis with high mortality. Subclinical infection influencing food conversion ratio and daily gain. The most prevalent *Eimeria* spp in poultry are *E. tenella, E. acervulina, and E. maxima* with invasion of specific intestine sites (Tewari & Maharana, 2011). *Eimeria*'s life cycle in poultry involves oocysts being excreted into the environment (Exogenous) and parasite development in the host intestine (Endogenous). During the latter, parasites complete their life cycle within the enterocytes, disrupting the gastrointestinal tract's epithelial integrity (Castro et al., 2020). Coccidiosis damage

to the intestine is accompanied by increased mortality and morbidity (Hafeez et al., 2020; M. Khan et al., 2023). Anticoccidial drugs are the main tactics used to manage *Eimeria* infection, although excessive use raises concerns regarding coccidiosis resistance capacity and drug residues in poultry meat (Khan et al., 2022). Subsequently, it has been demanding alternative exploration like feed supplements to boost immune response and performance when encountering *Eimeria* spp. (Adhikari et al., 2020; Ali et al., 2019; Chand et al., 2016; Tanweer et al., 2014). Organic Zn supplementation of diet reduced lesion score and oocyst shedding in broiler inoculated with *Eimeria* Spp (Bortoluzzi, Vieira, et al., 2019; Chand et al., 2021).

Coccidiosis can also lead to changes in the structure of the intestinal microbiota. A decrease in the cecal microbial diversity and changes in short-chain fatty acids produced in the ceca of chickens (Stanley et al., 2014). The distribution of minerals within the animal's body may change during enteric infections. When the absorptive capacity of the intestine is weakened due to enteric infections such as coccidiosis, it is safe to say that sources of minerals of greater availability may be needed. Dietary Zn concentrations higher than the 40 mg/kg recommended by NRC can help to lower the impact of coccidiosis in broilers. Gut health is vital in broiler performance from hatch to the point of harvest (Shannon & Hill, 2019). Many studies have investigated the importance of Zn in gastrointestinal functionality and health. Zinc deficiency adversely affects gut integrity by compromising intestinal permeability (Li et al., 2015) and the function of intestinal barriers (Lambert et al., 2004). Zn status in the body has been shown to influence the intestinal microbiota community (Shannon & Hill, 2019) and mRNA expression levels of several genes and proteins in the gut (Finamore et al., 2008). Zn supplementation changed cecum microorganisms, enhancing the population of beneficial probiotics such as Lactobacillus and Prevotella and increasing villus height in duodenum and ileum (Yang et al., 2024).

The crypts constantly renew the intestinal lumen's epithelial cells by migrating new cells from the crypts to the villus tip. During this migration, cells mature and become more efficient in nutrient absorption. The villous epithelial cells are directly in contact with the lumen content and are therefore susceptible to damage, which often leads to an increase in the loss of villous epithelial cells in cases of intestinal *Eimeria* infection. Supplementation with chelated Zn can increase villus length and villus length to crypt depth ratio (Højberg et al., 2005). An increased villus length is associated with increased digestion and absorption of nutrients and an increase of brush border enzymes and nutrient transport systems (Awad et al., 2017). The intestinal surface is directly proportional to digestive and absorptive efficiency and, thus, also to feed conversion efficiency. It has been discovered recently in Japanese quails that Zn-glycine in diet can improve growth performance in response to *Eimeria* infection (Saeeda et al., 2023). S. Khan et al. (2023) concluded that chelated Zn in combination with methionine ameliorates the negative effect of coccidia challenge and conserved the growth of broilers. Oxidative stress resulting from *Eimeria* infection in broilers is well-published (Abbas et al., 2011; Liu et al., 2024). Infection with *Eimeria* can increase Malondialdehyde (MDA), a biomarker for radical-induced damage, accompanied by a decrease in superoxide dismutase (SOD) in chicken (Georgieva et al., 2006; Liu et al., 2024), goat (Razavi et al., 2024), and rabbit (Zhou et al., 2022)

Zinc and Immunity

Zinc supports a robust immune system, aiding in the prevention and management of diseases. It contributes to the assembly and activation of immune cells. Zinc is a nonpharmacologic booster of the immune system in broiler chicks (Sajadifar et al., 2013). Also, Zn is a cofactor of thymulin, causes proliferation, and modulates cytokine release (Maggini et al., 2007). Zinc can enhance cellular and humoral immune systems (N. F. Suttle, 2022). As a limitation on antibiotics

in the poultry diet imposed by many countries, Zn is necessary as an enhancer of broiler immunity. Organic Zn induced higher anti-inflammatory regulator (A20) expression, downregulated the mRNA expression of inflammatory cytokines, including NF-kB, and promoted MUC2 and IgA production compared to its inorganic counterpart (Prasad et al., 2011). It has been proposed that a chelated Zn source can increase IgA in the small intestine and delay parasite attachment to the gut epithelium (Saeeda et al., 2023).

Low intracellular levels of Zn cause abnormal development of T-lymphocytes and cause lower weights of the thymus and spleen (Beach et al., 1982). Inadequate Zn in the diet causes damage to the lymphocyte activity that is responsible for the activation of T- and B-cell proliferation (Vruwink et al., 2020). Zinc deficiency can decrease the DNA synthesis or cell division required for regular organ growth since Zn is an essential component of many metalloenzymes, including those involved in gene replication, such as DNA and RNA polymerases (Prasad, 2020). The immune system can be affected indirectly by Zn through the interaction of growth and infection from pathogens introduced to animals. Zn shortage in animals is associated with infections with microorganisms like increased bacterial populations in the liver and kidneys (Srinivas et al., 1989). Chickens showed hypozincemia when being infected with the Newcastle disease virus, and Zn concentrations in the liver increased (Squibb et al., 1971). Increased liver size by infection could result from interleukin (IL-1) stimulation of acute-phase reactant protein synthesis, such as metallothionein. Zn concentrations in serum initially decrease when birds are infected—the effectiveness of Zn inhibition of bacterial growth results from changing the active transport system. Zinc involves defense mechanisms such as phagocytosis, leukocytosis, and cell-mediated immunity, as well as indirectly inhibiting the proliferation of pathogens (Hill, 1989). It

has been shown that dietary Zn nanoparticles alleviate inflammatory necrosis in hepatocytes of laying hens (Yang et al., 2024).

Zinc and Transporters

Zinc is a widely used biological metal in all living organisms. Zinc transporters regulate cellular Zn and extracellular distributions. Two different families of Zn transporters (ZnTs and ZIPs) were discovered in the 1990s. ZnT and ZIP transporters are encoded by *SLC30A1-10* and *SLC39A1-14* genes, respectively. ZnT are the efflux transporters on the basolateral surface of cells, which remove Zn from cells or take Zn into cellular organelles (Palmiter & Huang, 2004).

On the other hand, ZIPs are the influx transporters that replenish cytosolic Zn by importing Zn from extracellular or cell compartments into the cytoplasm (Jeong & Eide, 2013). They transport Zn in opposite directions to keep Zn's cellular homeostasis balanced. Most ZnT transporters are located in intracellular compartments, while ZnT1 is the only ZnT transporters located on the plasma membrane. Instead, ZIP is positioned chiefly on the plasma membrane (Hara et al., 2017). ZnT1 is important regarding cell toxicity by transferring cytosolic Zn into the extracellular space, and researchers observed early embryonic death in ZnT1 knockout mice (Andrews et al., 2004). ZnT1 is on the basolateral side of enterocytes, exporting absorbed Zn into the blood (Hennigar & McClung, 2016). At a high Zn level, the affinity of metal-response element-binding transcription factor-1 (MTF-1) binding to metal response elements (MREs) in the promoter of the ZnT1 gene increased and resulted in upregulation of transcription of ZnT1 (Hardyman et al., 2016). ZIP4 expression in the apical plasma membrane of the small intestine is upregulated during Zn deficiency and removes more dietary Zn from the lumen of the gut to enterocytes (Cousins, 2010). This upregulation has been proposed due to the abundance of Krüppel-like factor 4 (KLF4) transcription factor in the intestine. It binds to the ZIP4 promoter in

a restricted Zn mouse diet (Liuzzi et al., 2009). ZIP4 mRNA degradation and endocytosis by plasma membrane happen after Zn repletion or in high Zn concentration (Weaver et al., 2007). It was concluded in pigs that high dietary Zn concentration significantly increased ZnT1 and decreased ZIP4 in jejunal tissue (Martin et al., 2013). Some research has shown that protein expression of ZnT1, ZnT10, ZIP3, and ZIP5 was higher in the jejunum of broilers fed organic Zn in comparison to inorganic sources of Zn. The possibility could be that chelated Zn shows more resistance to phytate or fiber in the gut or low pH in the stomach. In that case, more intact Zn will arrive at the absorptive site of the brush border and then be transferred into the cells to uphold the mRNA and protein expression of Zn transporters (Hu, Huang et al.,). In a Zn-deficient diet, urinary Zn responded more rapidly than plasma Zn, and endogenous losses were reduced to conserve Zn (Baer & King, 1984), and then mobilization of Zn increased from rapidly exchangeable pools (Liver and serum) (Miller et al., 1994) and less from bone (Zhou et al., 1993).

The small intestine is where most of the digestion and absorption of nutrients (lipids, amino acids, minerals, and sugars) happen. The process is accomplished with digestive enzymes or nutrient transporters at the brush border of gut epithelial cells. The expression of these transporters can be changed drastically in *Eimeria*-infected chicken (Fetterer et al., 2014) or solely by selected diets (Gilbert et al., 2007). Amino acid transporters L-type amino transporter 1 (LAT1) and B0-type amino acid transporter 1 (B0AT1) help in the absorption of Zn as Zn amino acid chelates in the jejunum of broilers (Lu et al., 2018). Wen et al. (2022) reported that peptide-transporter 1 (PepT1) affects the transport of organic copper in the jejunum of pigs. Increased absorption in organic Zn as the Zn-Protein in the small intestine of broilers can be associated with the improved mRNA and protein expression of y+L-type amino transporter 1 (y+LAT1) and b0,+- type amino

acid transporter (rBAT) in the duodenum, suggesting the fact that the amino acids transporters can also help in the absorption of the organic Zn-Protein (Hu et al., 2022b).

Metallothionein

Metallothioneins (MTs) are intracellular metal-binding proteins that were first isolated from horse kidneys over 60 years ago (Margoshes & Vallee, 1957). It is a 61 amino acids protein with 30 percent cysteine and metal content. It can bind up to 7 Zn atoms with solid affinity and serve as a reservoir of Zn in different cells (King, 2011). A review of MT showed that MT in white blood cells was a more reliable biomarker in response to low Zn intake than plasma Zn (Hennigar et al., 2016). MT bound to Zn is the prevalent shape of MT in tissue, and its expression increases and decreases by Zn supplementation and exclusion in diet, respectively (Allan et al., 2000; Sharif et al., 2015). Metallothionein expression is induced in response to heavy metals and oxidative stress or stress hormones (glucocorticoids). It starts with a connection between Zn and metal transcription factor 1 (MTF1) in the cytoplasm; then it shuttles to the nucleus, where it bonds to specific DNA sequence named metal-responsive elements (MREs) in the promoter of MT gene (Heuchel et al., 1994). Some Zn transporters like ZnT1, ZnT2, and ZIP10 have the same MREs in their promoters.

Metallothionein binding to metal ions is one of the pathways for metal detoxification in cells. Particular cadmium detoxification is one of the vital roles of MT. High cadmium in living cells can damage the kidney and liver, triggering osteoporosis and metabolic disorders (Divya et al., 2018). MT can also play a role as an antioxidant agent. When the production of free radicals is high, and the antioxidant defense system cannot handle it, oxidative stress happens. MT is a significant source of thiols for cells, which contain cysteine to reduce oxidative damage by blocking the generation of ROS or function as a redox unit (Patankar et al., 2019).

Additionally, MT3 is mainly expressed in the central nervous system (CNS). It can protect neuron cells from toxicity with CU, thus playing a role in preventing Alzheimer's and Parkinson's diseases (Zaręba & Kepinska, 2020). Another role of MT is its anti-inflammatory effect. This role comes from its capacity to scavenge reactive oxygen species. Inflammation can activate MT expression through MREs (Choo et al., 2022). Increased MT expression parallels the rise in cellular nitric oxide (inflammation response). Nitric oxide promotes dissociation of Zn from MT, which may repress inducible nitric oxide synthase (iNOS) (Zangger et al., 2001). Higher expression of iNOS in a deficient diet of mice concluded that MT-bound Zn is unavailable (Cui et al., 2003).

Findings with intestinal epithelial cell-J2 of pigs confirmed the dose-dependent upregulation of MT with increasing Zn concentration. They also confirmed higher expression and protein abundance for MT in the jejunal tissue of pigs fed high compared with low amounts of dietary Zn (Martin et al., 2013). In both egg yolk sac membrane and embryonic liver tissue of duck, Zn mobilization and MT1 mRNA expression increased from day 17 to day 32 during duck embryonic development (Bai et al., 2022). Meanwhile, Zn concentration in egg yolk sac membrane and duck embryonic liver tissue decreased, respectively. MT's mRNA and serum protein levels were significantly higher in patients with chronic inflammatory disease (Ma et al., 2023). In birds infected with *Eimeria*, MT was considerably higher in response to the challenge in the liver, along with a high concentration of Zn (Richards & Augustine, 1988).

Studies evaluating Zn sources and *Eimeria* infection always assessed one of each organic or inorganic Zn source along the disease. In addition, the response in multiple tissues simultaneously for transporters, mineral utilization, and histomorphology has never been studied before. Therefore, this dissertation evaluates RBV of different Zn sources and their in vivo

intervention in low and high-diet supplementation. Moreover, studies in this dissertation focused on characterizing the Zn and other transporters in corresponding tissues in response to broilers' coccidiosis.

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CHAPTER 3

3. THE RELATIVE BIOAVAILABILITY OF INORGANIC AND ORGANIC ZINC SOURCES FOR WEIGHT GAIN IN BROILER CHICKENS[1]

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Abstract

A 21-day study was conducted to determine the relative bioavailability of zinc (Zn) from hydroxychloride as an inorganic Zn source and glycine and lysine chelated-Zn as organic sources, using zinc sulfate heptahydrate ($ZnSO_4.7H_2O$) as the reference Zn source (ZS). A total of 288 male broiler chicks (Cobb 500) were allocated into nine treatments, each with eight replicates and four birds per replicate in a Randomized Complete Block Design. The chicks received a corn-soybean meal diet on days 0 to 3, followed by a Zn-free cornstarch-soybean protein concentrate purified diet from days 3 to 7 to deplete the bird's Zn stores. The purified diet was supplemented with two levels of each of the 4 Zn sources: Zn Sulfate (ZS), hydroxychloride Zn (ZH), Zn-Glycinate (ZG), and Zn-Lysine-glutamate (ZL) to provide 50 mg/kg or 100 mg/kg Zn in experimental diets which were fed from day 7 to 21. The basal diet was Zn-free and used as a control diet. The relative bioavailability of Zn, based on weight gain, was assessed using slope-ratio assay and ZS as the standard Zn source. Body weight and feed intake data were collected on days 7 and 21 of the experiment. On day 21, two birds per cage were selected and euthanized for tibia and toe sampling. Body weight gain was regressed against Zn intake, and the ratio of the slopes was used to calculate the relative bioavailability of Zn for weight gain. The relative bioavailability values were 107%, 76%, and 127% for Zn-hydroxychloride, Zn-Glycinate, and Zn-Lysine sources, respectively, relative to Zn sulfate. There was a linear increase ($P < 0.05$) in % tibia and toe ash of chicks fed Zn-Lysine. Additional investigations are being conducted to elucidate the modes of action for the differences in response observed with the different Zn sources.

(Keywords: Zinc, inorganic, organic, bioavailability, chicks)

Introduction

Trace minerals like zinc (Zn) are involved in many biochemical functions, making them essential for overall birds' health and growth. Zinc is a cofactor of more than 300 enzymes which are required for cell replication, improving digestive enzyme activity, intestinal barrier function (Xie et al., 2021), synthesis of structural proteins (Underwood, 1999), and collagen content of the skin (Salim et al., 2010).

The very first Zn requirement for broilers determines to be 30 mg/kg (Roberson & Schaible, 1958). After that, other research set new requirements for broiler diets to be supplemented with 40 mg/kg of Zn from the hatch to the finisher phase (NRC, 1994). Other producers recommend a supplementation level of Zn at 100 mg/kg (Cobb-Vantress, 2018) or 110 mg/kg (Aviagen, 2019) for broilers in the diet. Zinc can be obtained from organic and inorganic sources. The primary difference between these sources is that inorganic sources do not contain carbon. However, organic sources include carbon. Zinc sulfate, Zinc oxide, and hydroxychloride are the primary sources of inorganic Zn. An ionic bond with Zn atoms forms sulfate and oxide sources of Zn, while the hydroxychloride Zn has a crystalized structure formed by covalent bonds between metal, multiple hydroxyl groups, and chloride ions (Cemin et al., 2017). These covalent bonds can reduce chelation with other nutrients in the diet and intestinal lumen (Cromwell et al., 1998), increasing bioavailability and reducing mineral excretion.

Measuring trace elements in specific tissues during dietary supplementation is an excellent way to estimate the relative bioavailability (RBV) of Mn (Henry et al., 1989; Wedekind & Baker, 1990), Cu (Kim et al., 2016), Zn (Brooks et al., 2013; Cao, Henry, Ammerman, et al., 2000; Star et al., 2012). However, other factors like weight gain have also shown high responsiveness to different Zn sources and levels in diets (Batal et al., 2001), while others see no difference in

bioavailability among sources and levels of Zn supplementation (Pimentel et al., 1991). In RBV measurement, a slope-ratio method using multiple regression can be used to calculate the bioavailability of Zn based on the tibia ash or weight gain relative to standard Zn sources.

Organic minerals are chelated to amino acids, proteins, or carbohydrates and has been suggested to be supplemented in poultry diet instead of inorganic sources since they have higher bioavailability for manganese (Mn) (Brooks et al., 2012; Saldanha et al., 2020), iron (Fe) (Ma et al., 2014), Zn (Brooks et al., 2013). Organic minerals can compete with antagonistic nutrients like Ca, P, or phytic acids for their Zn-biding capacity (Kratzer, 2018) since they have a stronger bond compared to inorganic mineral sources. Another possibility for higher bioavailability in organic minerals can be a result of peptide or AA transport systems (Nitrayova et al., 2012).

Knowing the RBV of Zn in different sources is essential for nutritionists to formulate diets accurately to meet the animal's Zn requirement. Recent research has focused on organic minerals as a potential feed ingredient for poultry diets. This study aimed to determine the RBV of Zn from hydroxychloride as an inorganic Zn source and glycine and lysine chelated-Zn as organic sources, using zinc sulfate as the reference Zn source.

Material and Methods

Birds and housing

A total of 288 male broiler Cobb 500 chicks were used in a dose-response experiment in cages to evaluate the bioavailability of 4 different zinc (Zn) sources, including zinc sulfate (ZS) and zinc hydroxychloride (ZH) as inorganic sources, zinc-glycinate (ZG) and zinc-lysine-glutamate (ZL) as organic sources. A total of 9 treatments were employed in this study consisting of a basal diet without supplemented Zn (Table 3.1). All the birds received a corn-soybean diet

supplemented with Zn for the first three days. On days 3 to 7, all the chicks received a Zn-free corn-starch-soybean protein concentrate diet to deplete the chick Zn storage. On day 7, chicks were individually weighed and allocated into nine treatments. Each treatment had eight replicate cages of 4 birds until day 21. The nine treatment were: 1) Zn-free purified diet (CON); 2) CON plus Zn sulfate to provide 50 mg/kg Zn (ZS-50); 3) CON plus Zn sulfate to provide 100 mg/kg Zn (ZS-100); 4) CON plus zinc hydroxychloride to provide 50 mg/kg Zn (ZH-50), 5) CON plus zinc hydroxychloride to provide 100 mg/kg Zn (ZH-100), 6) CON plus zinc-glycinate to provide 50 mg/kg Zn (ZG-50), 7) CON plus zinc-glycinate to provide 100 mg/kg Zn (ZG-100), 8) CON plus zinc-lysine-glutamate to provide 50 mg/kg Zn (ZL-50), 9) CON plus zinc-lysine-glutamate to provide 100 mg/kg Zn (ZL-100). The ingredient composition of the basal corn-soybean and the Zn-free purified diets are shown in Table 3.2. Broilers were housed in batteries with stainless-steel feeders and nipple drinkers. Feed and water were available ad libitum.

Experimental procedures

Feed consumption and body weights were recorded on a pen basis on days 7 and 21. Mortality was checked twice daily, and the weight of the dead birds was used to adjust the feed intake and gain to feed. At the end of the feeding trial, two birds from each replicate (16 birds per treatment) were chosen for bone parameters. The relative bioavailability of Zn, based on weight gain, was assessed using slope-ratio assay using Zn sulfate as the standard Zn source.

Chemical analysis

The left tibia and toes were collected from two birds per cage, placed in sealed plastic bags, and frozen at -20° C for analysis. The tibias were de-fleshed after autoclaving at 121° C for 1 minute. Then dry at 105° C for 24 h. The middle finger from each toe was removed and then dried

at 105° C for 24 h. Both dried tibia, and toes were ashed at 550° C in a muffle furnace for 24 h. The mineral profile in the tibia and diet were measured by inductively coupled plasma (ICP) emission spectroscopic method , as mentioned by (Leske & Coon, 2002).

Statistical Analysis

The data were analyzed using the one-way PROC ANOVA procedure of SAS with a cage as the experimental unit. Differences among treatment means were assessed using the Tukey test. The CON diet and the two diets of each source were compared using contrast. P value less than 0.05 was taken to be significant, and p-values between 0.05 and 0.1 were taken as a tendency to be significant. Relative bioavailability values were determined using Zn sulfate as the reference source by multiple linear regression and slope ratio assay. Multiple linear regression was conducted by regressing weight gain as a dependent variable (g/chick) on supplemental Zn intake from ZH, ZG, or ZL as independent variables. The bioavailability of Zn from Zn sulfate was assumed to be 100%. The calculated ratios of the slope of ZH, ZG, or ZL response lines to the ZS as the standard line resulted in the relative bioavailability estimate for Zn.

Results

Weight gain (WG), feed intake (FI), and gain-feed ratio were not affected ($P > 0.05$) by Zn source or supplementation levels of inclusion (Table 3.2). However, there was a tendency ($P < 0.1$) for both WG and FI to be increased in the ZL source compared to the other group. Both WG and FI were quadratically increased as the level of ZL increased ($P < 0.05$) compared to the control diet and peaked in the ZL-100 group. On the other hand, only WG was linearly increased as the diet was supplemented with Zn-Glycinate.

Results on tibia and toe parameters are given in Table 3.3. Tibia ash content ranged between 43 to 46 percent. In the present study, the percentage of tibia ash was significantly increased ($P = 0.022$) either by dietary inclusions of ZS-100, ZL-50, or ZL-100 diets compared to other dietary treatments. Toe ash percentage and tibia Zn content were not affected ($P > 0.05$) by Zn source or supplement level. Toe ash percentage was linearly increased ($P = 0.048$) by dietary supplementation of Zn-Glycinate compared to the control diet. The weight gain variable (figure 1) was more sensitive to changes in Zn in diet and was used to perform slope-ratio assay, comparing relative bioavailability (RBV) of 4 sources of supplementation, with Zn-sulfate being considered the standard source of Zn (100%). Based on ratios of slopes from multiple linear regression analysis of WG on Zn intake from various sources, the RBV of ZH, ZG, and ZL relative to ZS (100%) was 107%, 76%, and 127%, respectively, in 21-day-old chicks.

Discussion

The result from the present study has demonstrated that supplementation of either organic or inorganic Zn sources did not alter performance results. In agreement with our result, Han et al. (2020) Azad et al. (2020), and Vieira et al. (2013) reported no changes in the performance of broilers when comparing organic sources of Zn to inorganic sources, Contrary to this study, many researchers have reported that organic Zn can improve performance or digestibility in the broiler (Arbabi-Motlagh et al., 2022; Lu et al., 2020). Zinc is among the most essential mineral in bone homeostasis (Star et al., 2012). Supporting this study, Liu et al. (2013) demonstrated that organic Zn supplementation can increase ($P < 0.05$) tibia ash when compared to inorganic Zn sources. It's well reported that different Zn source supplementation may increase Zn concentration in bone, resulting in higher tibia strength (Min et al., 2019; Star et al., 2012). Although organic Zn

supplementation did not change growth performance, an apparent decrease in FI in the control diet was observed. This suggests that a Zn-deficient diet can affect the feed intake.

In this study, ZH and ZL have higher RBV of 107% and 127 %, respectively, at 21 days, considering ZS (100%) as a standard Zn supplementation source. The higher bioavailability of organic Zn sources was also reported by body weight gain and Zn content in tibia ash (ChangSha, 2001). Wedekind et al. (1992) found bioavailability ranging from 177 to 206 % for organic Zn compared (100%) using 45 mg/kg Zn in the diet. Cao, Henry, Guo, et al. (2000) reported smaller RBV values for Zn-AA (83%) and Zn-pro (108%). Organic sources of trace minerals arriving at the brush border (low pH) of enterocytes can be present with intact structure (Ashmead, 1993), thus increasing bioavailability compared to inorganic mineral sources and potentially reducing Zn supplementation levels in poultry (Gheisari et al., 2011; Kumar et al., 2021).

The higher RBV value indicates that more Zn is being absorbed and digested for growth and bone deposition from each source. This discrepancy could result from different organic Zn sources, chelation strength, or measurement phases.

In conclusion, the organic source of ZL had a higher bioavailability than ZS. Therefore, the present study might give evidence for further application of ZL as a novel Zn source in poultry feed.

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Table 3.1: Dietary composition and nutrients level of diets

	Day 0 to 3	Day 3 to 21
Corn	539	0
Corn-DDGS	75	0
Soybean meal	320	0
HP 300	0	360
Dextrose	0	408
Corn starch	0	190
Soybean oil	22	5
Dicalcium phosphate	17	17
Limestone	9	4.2
Sodium bicarbonate	2	2
Vitamin premix [1]	5	5
Trace minerals premix [2]	5	0
Trace minerals premix (Zn free) [3]	0	2.5
Methionine	1.2	2
Lysine	2	1.5
Threonine	0.5	0.5
Salt NaCl	2.4	2.4
Calculated Nutrients		
Protein, g/kg	220	202
ME, kcal/kg	2980	3134
Ca, g/kg	9	8
P, g/kg	7	6
Available P, g/kg	4	4
Cu, g/kg	85	72
Zn, g/kg	302	216

[1] Supplied per kg of diet: vitamin A, 13,233 IU; vitamin D3, 6,636 IU; vitamin E, 44.1 IU; vitamin K, 4.5 mg; thiamine, 2.21 mg; riboflavin, 6.6 mg; pantothenic acid, 24.3 mg; niacin, 88.2 mg; pyridoxine, 3.31 mg; folic acid, 1.10 mg; biotin, 0.33 mg; vitamin B12, 24.8 µg; choline, 669.8 mg;

[2] Supplied per kilogram of diet: Mn, 107.2 mg; Zn, 85.6 mg; Mg, 21.44 mg; Fe, 21.04; Cu, 3.2 mg; I, 0.8 mg; Se, 0.32 mg.

[3] Supplied per kilogram of diet: Mn, 80 mg; Fe, 33.3; Cu, 6.7 mg; I, 1.3 mg; Se, 0.2 mg; Mg, 20.9 mg.

Table 3.2: Growth performance responses to different sources and levels of Zn (d7-d21)

Diets	Weight Gain, g	FI, g/bird	G: F
No Zn	451.0	704.7	640.6
ZS[1] 50 mg	461.3	725.7	635.3
ZS 100 mg	466.6	749.6	622.6
ZH 50 mg	452.2	732.2	618.1
ZH 100 mg	469.2	732.8	642.2
ZG 50 mg	486.7	734.3	665.0
ZG 100 mg	458.1	718.6	637.7
ZL 50 mg	460.9	718.1	643.6
ZL 100 mg	485.1	782.8	625.0
P value	0.089	0.090	0.573
Pooled SEM	9.56	16.61	15.30
P values for contrasts			
Linear			
1,2,3	0.429	0.253	0.620
1,4,5	0.939	0.226	0.158
1,6,7	0.014	0.083	0.287
1,8,9	0.391	0.658	0.876
Quadratic			
1,2,3	0.445	0.063	0.137
1,4,5	0.188	0.458	0.343
1,6,7	0.346	0.951	0.444
1,8,9	0.009	0.015	0.310

[1] ZS: Zn Sulfate (22.7 % Zn); ZH: Zn Hydroxychloride (55% Zn); ZG: Zn Glycinate (26% Zn); ZL: Zn Lysine-Glutamate (17% Zn)

Table 3.3: Effect of different dietary Zn sources and levels on bone mineralization (d 21)

Diets	Tibia ash[1] (%)	Toe ash (%)	Tibia Zn (ppm)
No Zn	43.9[abc]	11.8	225.0
ZS[2] 50 mg	45.2[ab]	12.2	232.3
ZS 100 mg	45.6[a]	12.3	232.6
ZH 50 mg	43.2[c]	11.7	230.8
ZH 100 mg	44.8[ab]	12.2	236.9
ZG 50 mg	44.4[ab]	12.3	235.5
ZG 100 mg	44.4[ab]	12.0	237.3
ZL 50 mg	45.7[a]	12.1	234.4
ZL 100 mg	45.7[a]	12.2	230.3
P value	0.022	0.457	0.687
Pooled SEM	0.57	0.22	4.59
P values for contrasts			
Linear			
1,2,3	0.118	0.156	0.119
1,4,5	0.479	0.817	0.364
1,6,7	0.093	0.048	0.175
1,8,9	0.083	0.202	0.143
Quadratic			
1,2,3	0.139	0.295	0.309
1,4,5	0.136	0.191	0.112
1,6,7	0.761	0.780	0.219
1,8,9	0.337	0.325	0.916

[1] Means represent = 6 cages with 2 bones per cage

[2] ZS: Zn Sulfate (22.7 % Zn); ZH: Zn Hydroxychloride (55% Zn); ZG: Zn Glycinate (26% Zn); ZL: Zn Lysine-Glutamate (17% Zn)

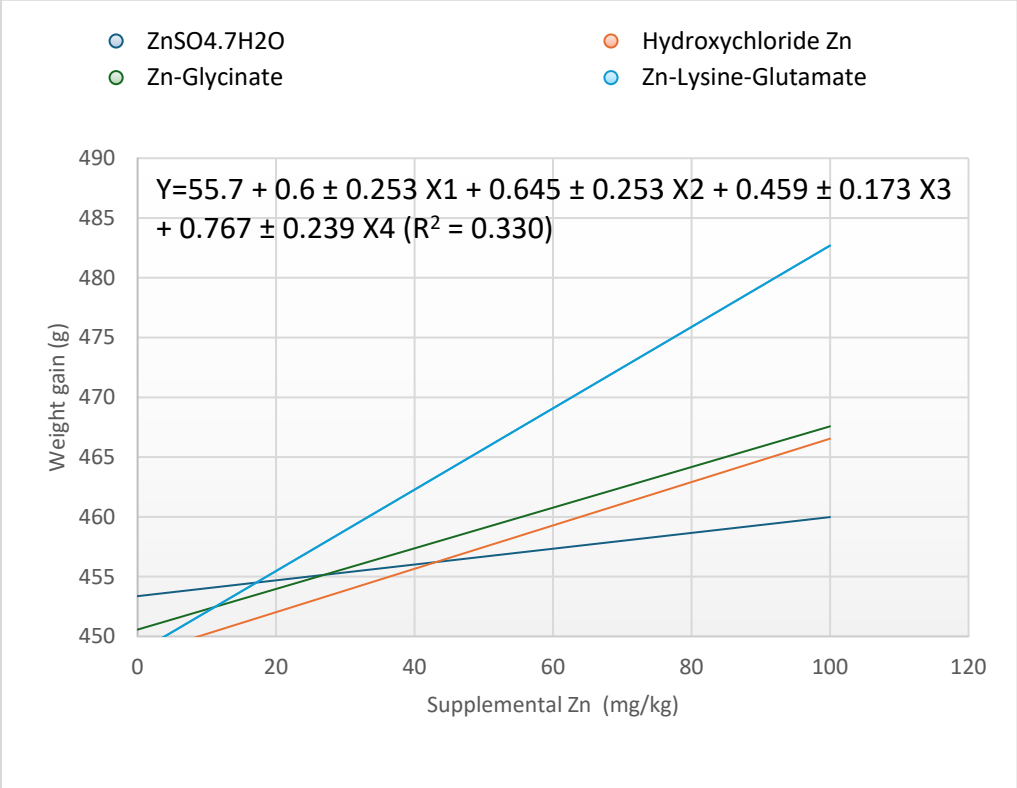

Figure 3.1: Relative bioavailability (RB), by the slope-ratio assay, ZS as standard supplementation (100%), and ZH, ZG, ZL, for the variables of weight gain

CHAPTER 4

4. EFFECTS OF DIFFERENT DIETARY SOURCES AND LEVELS OF ZINC ON GROWTH PERFORMANCE, BONE MINERALIZATION, MINERAL DIGESTIBILITY, INTESTINAL MORPHOLOGY AND NUTRIENTS TRANSPORTERS IN BROILER CHICKENS[2]

[2] M. Pilevar, and O. A. Olukosi. To be submitted to a peer-reviewed journal.

Abstract

Zinc is an essential mineral due to its catalytic and structural function. An experiment was conducted to determine the effect of different sources and levels of Zn in broilers on growth performance and physiology. The basal diet was a corn-soybean meal diet without supplemental Zn (analyzed Zn was 60 mg/kg). The diets were corn-soybean meal based and supplemented with 500 FTU/kg phytase. On day zero, chicks with the same body weights (± 4g) were allocated into nine treatments, each with seven replicates and six birds/ replicate cages. The birds and feed were weighed on days 0, 10, and 20 to measure weight gain and feed intake. There were no treatment effects on any growth performance responses at any point during the experiment. No significant source × level interaction existed for toe ash, tibia ash, and tibia Zn concentration. Toe ash was greater ($P < 0.05$) for birds receiving 100 ppm (12.7%) compared to 25 ppm (12.3%). ZL significantly increased nitric oxide (NO) production ($P = 0.036$) compared to ZH diets. Zn sources significantly affected the digestibility of P, Cu, and marker-corrected concentration of Cu. Birds receiving ZS diets had lower ($P < 0.01$) P digestibility than those fed ZH, ZG, and ZL-supplemented diets. Birds fed the ZL diet had higher Cu digestibility ($P < 0.05$). A significant main effect of Zn level ($P < 0.01$) was observed for marker-corrected concentration of Zn, with the ileal Zn concentration being higher ($P < 0.05$) in birds receiving a 100 ppm diet. mRNA expression of CAT1, CAT2, ZIP10, and ZIP8 was downregulated for ZL-fed diets and upregulated for ZG-fed diets in response to increasing Zn supplementation. An interaction was observed for GPX3, in which the expression was upregulated by increasing the Zn level from 25 to 100 mg/kg in ZS, ZH, and ZL-fed diets and downregulated in ZG-fed diet. In conclusion, Zn source, rather than level, significantly influenced responses reported herein regarding mineral digestibility. It has been

shown that Zn transporters are being downregulated at the brush border in response to high lumen Zn concentration to decrease the Zn influx into the cytosol and maintain homeostasis.

(Keywords: inorganic, organic, minerals digestibility, broiler chickens, gene expression)

Introduction

Zinc is essential for digestive enzyme activity (Lee et al., 2022). It acts as a catalytic and structural cofactor in a few hundred enzymes that help the metabolism of nutrients (Maret, 2013). In recent years, different organic Zn sources have emerged as a replacement for inorganic sources, including Zn-Methionine (Star et al., 2012), Zn-Glycinate (Yaqoob et al., 2020), Zn-Lysine-Glutamate (Sun et al., 2020), Zn-protein (Hu et al., 2023b). Studies have shown that organic Zn sources have more bioavailability (Star et al., 2012), improve growth performance (Mwangi et al., 2017), and lowered mineral excretion (Burrell et al., 2004). Traditionally, inorganic trace minerals are being used in feed more than the amount recommended (Council, 1994) to avoid trace mineral deficiencies (Zhao et al., 2010), which can cause soil contamination and decrease crop yield (Giordano et al., 1975; Tucker, 1997). The inorganic Zn is readily dissociated from its salt due to acidic pH in the upper section of the intestine. It subsequently forms bonds with other nutrients and reduces their availability for absorption (Yan & Waldroup, 2006). Zinc doesn't have a particular storage system, so a daily intake is necessary to maintain homeostasis and ensure its various functions. Data has shown that organic Zn supplementation can improve intestine morphology by increasing the villus height or crypt depth (De Grande et al., 2020; Payne et al., 2006) and improve digestibility. Several studies have reported enhancement of the immune system in response to Zn supplementation in diet (Bonaventura et al., 2015; Stahl et al., 1989).

Zinc is a hydrophilic ion and requires cellular uptake since it cannot cross the cell membrane by diffusion. A Zn transporter is required to move Zn along the brush border and

basolateral side. There are two types of Zn transporter, including the SLC30 (ZnT) family and the SLC39 (ZIP) family, to control cellular Zn homeostasis (Kambe et al., 2017). Some reported that Zn transporters can be affected by Zn sources (He et al., 2019) or ages (Han et al., 2020). Some research has shown that protein expression of ZnT1, ZnT10, ZIP3, and ZIP5 was higher in the jejunum of broilers fed organic Zn in comparison to inorganic sources of Zn. The possibility could be that Zn chelated shows more resistance to phytate or fiber in the gut or low pH in the stomach (Cousins, 2010). Interestingly, some Zn transporters are associated with some other physiological relevance. For example, ZnT8 can regulate glucose homeostasis in pancreatic islet β-cells (Kambe et al., 2017), ZIP14 transport is increased by IL-6 in an acute-phase response to LPS (Liuzzi et al., 2005).

This study aims to investigate how different sources of organic or inorganic Zn at various levels can affect performance, mineral digestibility, and intestine histomorphology using a practical diet. Moreover, this study examines the mRNA expressions of Zn and AA transporters in jejunum, antioxidant enzymes in the liver, and *in vitro* immune responses of chickens to better understand their mode of action.

Materials and Methods

Experimental Design and Diets

The experiment was conducted in the Poultry Research Center at the University of Georgia. Three hundred seventy-eight one-day-old Cobb 500 broiler chicks were allocated to 9 dietary treatments in a randomized design with a 2×4 factorial arrangement. Each treatment consists of 7 replicates and six birds per cage. The birds were placed in battery cages on day 0. The factors were four sources (ZS, ZH, ZG, ZL) and two levels of Zn (25 mg/kg and 100 mg/kg). The control diet

has 0 mg /Kg Zn supplementation. Each Zn source has a different Zn concentration, as reported by the manufacturer. All diets were fed in two phases: starter (d 1-10) and grower (d 10-20) (Table 4.1). The calculated and analyzed Zn concentrations of 9 experimental diets are shown in Table 4.2. All broilers were allowed ad libitum access to the mesh feed and water. The experimental diets are a corn and soybean meal-based diet (basal), basal diet supplemented with 25 mg/kg Zn sulfate (ZS-25), a basal diet supplemented with 100 mg/kg Zn sulfate (ZS-100), a basal diet supplemented with 25 mg/kg Zn hydroxychloride (ZH-25), a basal diet supplemented with 100 mg/kg Zn hydroxychloride (ZH-100), a basal diet supplemented with 25 mg/kg Zn-glycinate (ZG-25), a basal diet supplemented with 100 mg/kg Zn-Glycinate (ZG-100), a basal diet supplemented with 25 mg/kg Zn-lysine-glutamate (ZL-25) and a basal diet supplemented with 100 mg/kg Zn-lysine-glutamate (ZL-100). Each basal diet was supplemented with vitamin and trace mineral premix Zn-free. The basal diet contained 32 mg/Kg Zn and was formulated to meet all broiler requirements except for Zn. All the experimental diets in both phases were made from a large basal diet by adding different Zn sources to minimize mixing errors.

On days 10 and 20, growth performance parameters like weight gain, feed intake, and weight gain to feed intake ratio were measured. In the first three days of the study, 24 hours of light were provided, which gradually decreased to 16 hours of light and 8 hours of darkness. A digital thermometer measured the minimum and maximum temperature and relative humidity values every morning.

Diet Proximate composition

One representative composite sample from all the grower diet phases was collected and milled (Retsch ZM 200, Retsch GmbH and Co., K.G., Haan, Germany) through a 0.5 mm sieve before chemical analysis. Each diet analysis for dry matter (DM), nitrogen, Zn, Cu, and P. Dry

matter was measured by drying samples in an oven at 105 C for 24 hours (AOAC Method 934.01). Nitrogen was measured using a combustion method using a nitrogen analyzer (Leco FP 828-MC analyzer). To analyze the mineral composition, each sample was ash in duplicate in a furnace (12 hours, 600°C) and then underwent digestion in concentrated HNO3 and HCl (AOAC, 1990). Digested samples were analyzed for Zn and Cu concentration using an atomic flam spectrophotometer. Phosphorous concentration was measured in digested samples via colorimetric assay, determining molybdate complexes' reaction with phosphate in the presence of Fiske-Subbarow solution acting as a reducing agent. Each sample was pipetted in triplicate in 96-well plates and then read on a plate reader at 630 nm (AOAC, 1990).

***In vitro* immune responses**

Cell viability was measured using methyl thiazolyl tetrazolium (MTT) assay described by (Moon et al., 2013). On day 21, one bird per replicate was randomly chosen per treatment to collect heparinized blood from the wing vein. The blood was layered on 1077 Histopaque (1077-1, Sigma-Aldrich, St. Louis, MO) in a 15 ml tube and centrifuged at 1200 × *g* for 10 minutes. Then, the buffy coat layer (white blood cells) was collected from a gradient interface and washed two times with 5 ml antibiotic-free RPMI 1640 (R0883, Sigma-Aldrich) to thoroughly remove the Histopaque by centrifuging at 500 × *g* for 8 min. Resuspended in 1 ml of complete RPMI and continued to cell counting. Hundred µl of counted cells were transferred to a 96-well plate at 2 × 10^6 cells/ml/well density and treated with Concanavalin A (ConA) for 72 hours. During the final 4 hours, 20 µl of MTT (catalog no. M2128, Hyclone; 5 mg/mL) was added to each well and incubated for 3.5 hours at 37°C in the 5% CO_2. The MTT assay is based on the intracellular reduction of MTT to purple formazan granules in living cells. So, the assay can be used as an indicator of cell viability as well as cell proliferation. After incubation, plates were

centrifuged at 241 × g for 5 min, and the supernatant was removed. Then 100 µl of dimethyl sulfoxide (catalog no. 27,043-1, Hyclone) was added to each well and incubated in the dark for 15 minutes at room temperature. Dimethyl sulfoxide was used to lyse the cells and dissolve the formazan crystals formed by MTT. The absorbance was read in a plate reader at 570 nm wavelength.

The same cell concentration from the MTT assay (2×10^6 cells/ml/well) was incubated in 96-well plats for 4 hours. Then, plates were incubated again with lipopolysaccharide (LPS) (10 ng/ml) for 24 h. In aerobic conditions, nitric oxide (NO) reacts with oxygen to produce nitrite, and the quantity was measured by adding 100 µL of the Griess reagent (0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride in distilled water and 1% sulfanilamide in 5% phosphoric acid) to 100 µL of supernatant for 15 min at room temperature. The absorbance was measured at 540 nm with a microplate reader, and the concentrations of NO were calculated using the absorbance at 540 nm of standard sodium nitrate solutions prepared in the culture medium.

Bone Parameters

On day 20, the left tibia and middle toe of the left foot were excised from one bird per replicate (7 birds/diet) and then individually put into a zip-lock bag at -20 °C. Tibias then autoclaved at 121 ℃ for 5 minutes. After autoclaving, tissues were removed, and the remaining tissue was washed away with water and a brush. All the tibia were numbered with tape, and the toes were kept in a small aluminum container before putting into an oven for 24 hours at 105 ℃. Bones were then put in a desiccator to let them cool, and dry weights were obtained after this period. All the bones and toes were placed in a crucible in an ash oven overnight at 600 ℃. Tibia, and toes ash was weighted after cooling. The tibia ash sample was digested on the hot plate, and Zn concentration was measured using atomic spectrophotometry, as described in another chapter.

Intestinal Morphology and Gene Expression

On day 20, two birds per replicate were necropsied to test the intestinal morphology, according to (Liu et al. 2022). The ileum samples (about a 2 cm portion in the mid-ileum) were collected for histological analyses. Samples were washed using 0.1 M PBS and fixed in 10% formaldehyde. The fixed section was dehydrated, embedded in paraffin wax, cut in 5 μm, and stained with hematoxylin-eosin. At least five villi per section were chosen to measure crypt depth (CD), villus height (VH), and villus width (VW) using a digital camera microscope (BZ-X800, Keyence Inc., Itasca, IL) and digital image software (Image J). The ratio of VH: CD was also calculated.

One bird per replicate was used to analyze the mRNA expression level of antioxidant enzyme and transporter genes in liver and jejunum tissue, respectively. Briefly, small tissue samples were put into a 1.5 ml centrifuge tube and snap-frozen in liquid nitrogen to be transported to the lab for storage at -80 °C. Total RNA was extracted using TRIzol methods. Briefly, 20 to 30 mg of liver or jejunum were mixed with 1 ml of TRIzol and homogenized with homogenizer. Two hundred microliters of chloroform were added to each homogenized tissue and then centrifuged to separate total RNA from other reagents in the tube. Total aqueous RNA content was precipitated in propanol and then washed two times with ethanol 70%. The pellet then resolved in warm nuclease-free water. The RNA purity and concentration were determined using a nanodrop spectrophotometer (BioTek Synergy HTX, Agilent, Santa Clara, CA) with an optical density at 260 nm wavelengths. Values of 1.7 to 2.0 and 2.0 to 2.2 for 260/230 (RNA: Ethanol Contamination) and 260/280 (RNA: protein contamination), respectively, were considered good-quality RNA (Dash, 2013). Otherwise, RNA extraction should be repeated. A thousand nanograms of total RNA from each sample were reverse transcribed to cDNA in a 20 ul volume by PrimeScript

RT Reagent Kit (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's guidelines. All cDNA is then stored at -20 C.

The PRIMER BLAST algorithm checked gene-specific primers for *Gallus gallus* mRNA databases to ensure a unique amplicon. Primer sequences used are described in Table 4.3. and each reaction included 12.5 ng RNA equivalents along with 200 nmol/L of forward and reversed primers for each gene. Each reaction volume was 10 ul containing 0.5 ul of each primer (4 umol/L), 2.5 ul DEPC-treated water, 5 ul of iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA), and 2 ul of cDNA sample diluted 1:10. Real-time quantitative PCR (qPCR**)** was accomplished in 96-well microplates sealed with optically clear sealing film (Axygen Scientific, Inc., Union City, CA). Samples were incubated at 95°C for 3 min, accompanied by 40 cycles of 95°C for 5 s, 59.5 to 62°C (depending on the target gene) for 20 s, and 72°C for 33 s. Each sample was measured in duplicate using the Bio-Rad iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The reference gene of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to calculate ΔCt. Then ΔCt used as a calibrator for calculating ΔΔCt for treatments, and fold change relative to control was calculated using the $2^{-\Delta\Delta CT}$ method (Livak, 2001).

Mineral Digestibility

Apparent ileal trace mineral digestibility was measured using titanium dioxide (TiO_2) as a marker. Ileal digesta samples were collected on day 20 from 4 birds per replicate, pooled together in a container, and kept on ice. Then, they were dried at 60 C for 48 hours—nitrogen concentration was measured by a nitrogen analyzer machine. The dried digesta were placed in a furnace at 600 C overnight. The ash samples were then subject to HNO_3 (100 ul) and 4N HCl (20 ml) digestion. Zinc and Cu were analyzed using atomic absorption and P by plate reader. The titanium dioxide

concentration in grower diets and dried digesta was measured using a spectrophotometer (Short et al., 1996). The absorbance was measured at 410 nm.

Results

Table 4.1 shows the diet formula and analyzed composition of starter and grower diets. Analyzed Zn in both starter (61 g/kg) and grower (59 g/kg) basal diets had higher values than calculated Zn in starter (33.5 g/kg) and grower (31.6 g/kg) basal diets. These higher values are consistent in experimental diets of both phases (Table 4.2).

The effects of Zn supplementation levels and sources on growth performance are presented in Table 4.4 with 4×2 factorial analysis and simple analysis, including the basal diet. There was no interaction effect between the level and sources of Zn supplementation on growth performance of starter period. Also, this study did not observe the interaction between two factors for WG, FI, and G: F during d 10 to 20. The results obtained from this study showed no significant differences in performance among the groups fed different sources and levels of Zn from day 0 to 20. In addition, no significant effects comparing the basal diet to all other eight dietary treatments.

The proliferation of peripheral blood lymphocytes and nitric oxide production in response to different dietary Zn sources and levels are shown in Table 4.5. The stimulating index (SI) of blood lymphocytes in response to Con A was not affected by the levels or sources of Zn supplementation on day 21. There was no significant interaction between the main effect of level and sources of Zn on NO production of *in vitro* LPS-stimulated macrophages. On the other hand, Zn sources have a significant impact on NO ($P = 0.036$), wherein ZL diets increased their production compared to ZH diets.

Bone parameter data are presented in Table 4.6. While there was no interaction between the Zn source and level on tibia and toe measurements, Zn level significantly increased toe ash percentage when birds were fed 100 mg/kg Zn. Also, there was a significant increase in toe ash percentage when comparing chicks fed the basal diet with other supplemented groups (P = 0.011).

The effects of supplementing different dietary Zn levels and sources on ileum histomorphology are depicted in Table 4.7. No significant interaction between Zn source and level of Zn on Crypt depth and villus height was observed. However, there was a significant interaction for villus width, wherein 25 mg/kg Zn in ZS and ZG has increased width when compared to 100 mg/kg Zn supplementation, with no changes in other sources. Increasing the Zn level showed a trend (P = 0.08) toward increasing VH: CD in ZS, ZH, and ZL-fed diets and decreasing by increasing the Zn level in the ZG diet. Twenty-five mg/kg Zn fed birds had increased (P = 0.008) villus width compared to birds received 100 mg/kg Zn. Also, the same trend (P = 0.077) was observed for crypt depth.

The mRNA expression of 6 genes in the liver involved in antioxidant enzymes was investigated in response to different Zn sources and levels (Table 4.8). An interaction of Zn level by source was observed for GPX3, whose expression was upregulated by increasing the Zn level from 25 to 100 mg/kg in ZS, with no changes in other Zn-fed diets. In GPX1 expression, there was a trend (P = 0.096), and it was upregulated by increasing the Zn level in all four sources.

Gene expression data of Zn and amino acid transporters of jejunum are presented in Table 4.9. In the jejunum, there was an interaction of source by level of Zn for CAT1 (P = 0.051), CAT2 (P = 0.088), ZIP10 (P = 0.088), and ZIP8 (P = 0.044). mRNA expression of CAT1, CAT2, ZIP10, and ZIP8 were downregulated for ZL-fed diets and upregulated for ZG-fed diets when increasing Zn supplementation in the diet. There was an effect of Zn level (P = 0.06) on PEPT1, which 100

mg/kg Zn supplementation downregulated PEPT1 compared to 25 mg/kg Zn diet. Also, there was a tendency effect of Zn source in which ZS and ZH diets have the highest, and ZG and ZL show the lowest expression of ZnT10.

Data on the apparent ileal digestibility of DM, N, Zn, P, and Cu in 20 d old birds are presented in Table 4.10. Zn sources have a significant effect on P, Cu digestibility, and Cu DM intake. ZS-fed diet significantly decreased P digestibility (P = 0.007) compared to ZH and ZL diets. While ZG decreased Cu digestibility (P = 0.045) compared to ZL, there were no differences between ZS and ZH-fed diets. The Zn mg/kg intake was increased (P = 0.02) by the level of Zn supplementation and showed the highest value for a 100 mg/kg Zn-fed diet (178 vs. 76). Also, there was a tendency of decrease in Cu digestibility (P = 0.06) by increasing Zn level in diet.

Discussion

Trace minerals, specifically Zn, are one of the important nutrients for growth performance in poultry. This study examined the effect of 4 different sources of Zn in 2 different levels on performance, intestinal morphology, gene transporters, and digestibility in broilers. Different mineral organic sources, including Zn (Arbabi-Motlagh et al., 2022; Liu et al., 2013; Lu et al., 2020), Cu (Jegede et al., 2011), Mn (Attia et al., 2010) and Fe (Shinde et al., 2011) have been reported to improve performance in broilers. However, in this study, neither the inorganic Zn source diet (ZS&ZH) nor the organic Zn source (ZG&ZL) improved growth performance. In agreement with our findings, (Han et al., 2020; Mohanna & Nys, 1999; Vieira et al., 2013) found no differences in growth when using two sources of organic Zn compared to an inorganic source in their study. Also, (Kwiecień et al., 2017); Salim et al. (2011); (Star et al., 2012) reported that graded level of organic Zn in broiler diets did not have any effect on performance. Likewise, Bortoluzzi, Vieira, et al. (2019) observed no differences in growth of broilers fed either inorganic

Zn (97.4 mg/kg Zn) or proteinate Zn (104 mg/kg Zn). Liu et al. (2013) concluded that the lack of increase in feed intake or body weight gain in a supplemented Zn diet could be due to the adequate Zn in the unsupplemented diet for growth in the early phase of life as recommended 40 mg/kg Zn by (Council, 1994). The inconsistency effects of organic Zn on the performance of broilers among authors may result from the diet composition, breed of broiler, growth phase, presence of phytate that can bond to minerals and make them undigestible in the intestine, basal Zn level, amino acid type chelated to the Zn, or the chelation strength (Hu et al., 2022a).

Zn is important for the integrity of cells involved in the immune response and the highest antibody response (Bun et al., 2011; Park et al., 2004; Sunder et al., 2008). In the current study, the *in vitro* lymphocyte proliferation by mitogen ConA stimulating T cells was not affected by the sources and level of Zn supplementation. Bun et al. (2011) similarly reported that *in vivo* cell viability of PBMC in response to ConA was not affected by the Zn level in diet. In chicken, NO is mainly produced by macrophages or monocytes, which can be stimulated by pathogens like LPS, IFNγ, and killed bacteria (Crippen et al., 2003). In an inflammatory response, inducible nitric oxide synthase enzymes (iNOs) synthesized NO to diminish the cytotoxic effect of free radicals. The current study showed that Zn sources significantly increased NO production in response to LPS. This antigen is a stimulant for macrophages and antibody production in B cells. Bortoluzzi, Lumpkins, et al. (2019) find out similarly an upregulation in iNOS gen in cecal tonsils of broilers fed organic Zn compared to inorganic source. Nitric oxide synthase has three different forms: inducible NOS, neuronal NOS, and endothelial NOS (Dellamea et al., 2014). Other researchers (Croix et al., 2004) point out that Zn regulates iNOS activity and NO production in inflammation.

Zinc is crucial in bone homeostasis (Huang et al., 2007; Star et al., 2012). Contrary to our result, Liu et al. (2013) reported an increase in tibia ash in birds fed Zn proteinate compared to

those fed Zn sulfate. Also, in our study, tibia Zn concentration was not affected by Zn sources and levels conflicting with published data by (Ao et al., 2006); Shelton and Southern (2006), Ao et al. (2007). Similar to them, Ao et al. (2009) reported that organic Zn caused higher Zn concentration in the tibia of chicks in comparison to those fed inorganic Zn and concluded that it might be due to antagonism happen between Zn and other minerals while using the inorganic sources, but not when using organic sources. Liu et al. (2013) explained that higher feed intake, thus higher dietary Zn intake, can rationalize more Zn concentration in tibia of birds fed the diets supplemented with organic sources than those supplemented with Zn sulfate. Accordingly, our data doesn't show any feed intake increase by using organic Zn in our study, which can explain the lack of changes in Zn concentration in the tibia.

Zinc can change the histomorphology of small intestine, improve absorption and finally growth performance (Højberg et al., 2005; Katouli et al., 1999). Southon et al. (1986) reported that Zn deficiency in rats reduced the jejunum villus height and brought it back to normal after Zn supplementation. In the current study, neither organic nor inorganic Zn sources caused any changes in crypt depth, villus height, or villus height to crypt depth ratio of ileum of chicks at 21 d old. Organic Zn inclusion in the diet improved the villus length and the ratio of villus length to crypt dept in broilers on days 10 and 28 in the duodenal section (De Grande et al., 2020) and also villus height in broilers on 39 days of both duodenal and jejunum sections (Abuajamieh et al., 2020). Ninety mg/kg Zn glycine chelate vs, Zn sulfate did increase villus height and decrease crypt depth of both jejunum and ileum of chicks at 42 d old but not 21 d old (Ma et al., 2011). Likewise, in a *C. perfringens* study (Sun et al., 2020) partial replacement of organic Zn with Zn sulfate affected ($P < 0.05$) the VH and VH: CD ratio of 14-day-old broiler and did not have any effect on the 21-day old chicks. This is similar to the current study, in which the lower level of organic Zn

supplementation tended ($P = 0.07$) to improve the crypt depth and also increased ($P < 0.05$) width in the ileum at 21 days. Cell proliferation in crypts (base) and migration to villus tips are responsible for constant epithelium renewal and specialized epithelial cell types. Some well-evidenced experimental studies show that an increase in villus length or intestinal surface is directly proportional to digestion and growth (Awad et al., 2017; Collett, 2012). Considering all this, the ages of birds and Zn level should be considered, as no changes in VH were observed, along with no changes in growth performance data in the present study.

In our study, the organic source of ZL increased ($P < 0.05$) apparent digestibility of DM, P, and Cu compared to an inorganic source of ZS. Dietary Zn inclusion may influence the digestibility of other nutrients (Meyer et al., 2002). In accordance with this study, De Grande et al. (2020) reported a higher digestibility coefficient for ZnAA diet compared to ZS. Min et al. (2019) reported apparent retention of Cu and energy in the organic group was significantly higher than in the inorganic group. Zarghi et al. (2022) revealed a significant linear increase in the apparent digestibility of DM and N by increasing dietary Zn concentration. Contrary to our result, Zn-Glycine chelates, compared to ZS, were not able to make any significant changes in the digestibility of nutrients (Kwiecień et al., 2017). Zinc is a cofactors for many enzymes in GI tract (Hedemann et al., 2006; Szabó et al., 2004) and has been reported improvement in digestive enzyme activity by Zn supplementation in broiler diets (Xie et al., 2021). It can also be hypothesized that chelated amino acids protect Zn and help improve Zn's absorption and digestibility than in Zn sulfate.

Mammals regulate Zn homeostasis in different tissues by controlling influx and efflux of Zn (Jou et al., 2009; Kambe et al., 2021). Zip family transporters can influx Zn into the cytosol from lumen or intracellular compartments which increase cytoplasmic Zn. On the other hand, ZnT

transporters are Zn efflux proteins to decrease cytoplasmic Zn, by carrying the Zn from cytosol into extracellular and intracellular compartments (Myers et al., 2012). Measurement of the abundance of Zn related transporters can be used for assessment of Zn status (Gibson et al., 2008; Huang et al., 2007). Our present result showed that the mRNA expression of influx Zn transporter (ZIP8, 10) is affected mostly compared to efflux Zn transporters (ZnT 1, 10, 5) in jejunum. ZIP8 had the highest expression in 25 mg/kg of ZS compared to 100 mg/kg ZS, and no differences in other Zn sources by changing the Zn level. In general, ZIP8 and ZIP10 transporters are downregulated when compared to organic and inorganic Zn sources of ZS and ZH. These results conflict with previous results that dietary organic Zn supplementation upregulated both influx and efflux Zn transporters (Hu et al., 2023b; Hu et al., 2022a; Patrushev et al., 2012). In consistent to our result, Martin et al. (2013) reported that high dietary Zn supply downregulated ZIP4 (influx), upregulated ZnT2 (efflux) or no changes in ZnT1,5 expression in jejunum. As shown in marker-corrected DM intake data for Zn, a 100 mg/kg Zn-fed diet increased Zn mg/kg intake. It's also reported that in decreased dietary Zn intake, ZIP is upregulated, underlining its function in the homeostatic control of Zn levels (Jou et al., 2009; Pfaender et al., 2017). In conclusion, the lower expression of ZIP10, 8 in this study, is a response to high Zn level in lumen and more ZL source bioavailability (data not published) as an attempt to reduce influx to intestine.

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Table 4.1: Ingredient and chemical composition (g/kg) of experimental diets.

	Starter (0 to 10 d)	Grower (10 to 20 d)
Corn	572	616
Corn-DDGS	75	75
Soybean meal	295	245
Soybean oil	16	25
Dicalcium phosphate	9	7
Limestone (B)	9.5	8.5
Sodium bicarbonate	2	2
Vitamin premix[1]	5	5
Zn free Trace minerals premix[2]	5	5
Methionine	1.3	1.4
Lysine	2	2.4
Threonine	0.5	0
Salt NaCl	2.4	2.4
Titanium	5	5
Q-blue phytase	0.1	0.1
Calculated Nutrients		
Protein, g/kg	211	191
ME, kcal/kg	2981	3083
Ca, g/kg	7.20	6.16
P, g/kg	5.35	4.82
Available P, g/kg	3.03	2.60
Na	1.87	1.87
K	8.22	7.37
Cl	1.83	1.84
Cu	78.6	73.7
Zn	33.5	31.6
Mg	1.69	1.62
Analyzed Nutrients		
Protein, g/kg	210	191
Zn, ppm	61.0	59.0
P, g/kg	7.41	6.59
Cu, ppm	41.2	47.3

[1] Supplied per kilogram of diet: vitamin A, 5,511 IU; vitamin D3, 1,102 ICU; vitamin E, 11.02 IU; vitamin B12, 0.01 mg; biotin, 0.11 mg; menadione, 1.1 mg; thiamine, 2.21 mg; riboflavin, 4.41 mg; d-pantothenic acid, 11.02 mg; vitamin B6, 2.21 mg; niacin, 44.09 mg; folic acid, 0.55 mg; choline, 191.36 mg.

[2] Supplied per kilogram of diet: Mn, 80 mg; Fe, 33.3; Cu, 6.7 mg; I, 1.3 mg; Se, 0.2 mg.

Table 4.2: Calculated and Analyzed Zn composition of experimental diets

	Starter (0 - 10 d)			Grower (10 - 20 d)		
	Analyzed	Calculated	Supplemented Zn (mg/kg)	Analyzed	Calculated	Supplemented Zn (mg/kg)
Basal	61	33	0.000	59	32	0.000
ZS 25 mg 1	105	58	0.110	82	57	0.110
ZS 100 mg	142	133	0.441	131	132	0.441
ZH 25 mg	92	58	0.045	78	57	0.045
ZH 100 mg	150	133	0.182	153	132	0.182
ZG 25 mg	91	58	0.096	81	57	0.096
ZG 100 mg	135	133	0.385	142	132	0.385
ZL 25 mg	86	58	0.147	95	57	0.147
ZL 100 mg	147	133	0.588	140	132	0.588

1 ZS: Zn Sulfate (22.7 % Zn); ZH: Zn Hydroxychloride (55% Zn); ZG: Zn Glycinate (26% Zn); ZL: Zn Lysine-Glutamate (17% Zn)

Table 4.3: List of primers used for real-time qPCR of nutrient transporter genes

Gene name	Forward primer	Reverse primer
ZnT1	TCCGGGAGTAATGGAAATCTTC	AATCAGGAACAAACCTATGGGAAA
ZnT5	TGGAGGACCAGCCAAGACAA	TCCCTCAGGATGTTCTGCTATTTT
ZnT10	ATCAGATGGCACAAGGCAAACA	GAACAGACCTACGATCCCGACA
ZIP8	CTATCATTAACTTGGCATCGCTTCTG	CTACATGGGAATCAAATCCAAATGC
ZIP10	CCACCCTCATCCGCATCAT	TTCTTCTTCTGTTTCCGCTGTTTT
SOD1F	TGTGCATGAATTTGGAGACAAC	TTGCAGTCACATTZCCGAG
SOD2F	TGCACTGAAATTCAATGGT	GTTTCTCCTTGAAGTTTGCG
SOD3F	TTTTCTCCTAAAGATGGCAAG	CTTCCTGCTCATGGATCACAA
GPX1F	ACGGCGCATCTTCCAAAG	TGTTCCCCCAACCATTTCTC
GPX2F	ATCGCCAAGTCCTTCTACGA	ACGTTCTCGATGAGGACCAC
GPX3F	CCTGCAGTACCTCGAACTGA	CTTCAGTGCAGGGAGGATCT

Table 4.4: Effects of dietary Zn supplemental level and source on performance of broilers

		Starter (d 0 to 10)			Grower (d 10 to 20)			d 0 to 20		
		WG, g	FI, g	Gain:Feed g/kg	WG, g	FI, g	Gain:Feed g/kg	WG, g	FI, g	Gain:Feed g/kg
	Basal	185	307	604	524	985	533	709	1292	604
	ZS[1] 25 mg	180	315	571	563	1022	557	743	1337	571
	ZS 100 mg	192	329	584	547	1033	530	739	1362	584
	ZH 25 mg	194	309	646	530	979	548	724	1288	646
	ZH 100 mg	188	305	618	583	996	599	771	1301	618
	ZG 25 mg	194	320	607	561	1035	542	755	1355	607
	ZG 100 mg	195	316	618	573	1075	533	768	1391	618
	ZL 25 mg	182	305	597	537	994	542	719	1299	597
	ZL 100 mg	177	309	574	568	1006	567	745	1315	574
	Contrast Basal vs others	0.719	0.597	0.930	0.129	0.370	0.529	0.102	0.333	0.930
	SEM	7.47	11.3	25.9	20.8	34.7	29.5	20.9	38.3	25.9
	P value	0.600	0.850	0.551	0.471	0.656	0.794	0.441	0.548	0.551
Level	25 mg/kg Zn	188	312	606	548	1007	547	736	1319	606
	100 mg/kg Zn	188	315	599	568	1027	557	756	1342	599
	SEM	3.92	5.89	13.2	10.8	18.3	15.4	10.8	20.1	13.2
Source	ZS	186	322	578	555	1027	543	741	1350	578
	ZH	191	307	632	556	988	574	748	1295	632
	ZG	194	318	613	567	1055	537	761	1372	613
	ZL	180	307	587	553	999	555	733	1306	587
	SEM	5.55	8.33	18.6	15.2	25.9	21.8	15.3	28.4	18.6
						P value				
Level		0.960	0.789	0.725	0.205	0.437	0.651	0.200	0.431	0.725
Source		0.320	0.480	0.155	0.917	0.275	0.655	0.619	0.195	0.155
Level*Source		0.596	0.841	0.782	0.416	0.982	0.592	0.679	0.994	0.782

[1] ZS: Zn Sulfate (22.7 % Zn); ZH: Zn Hydroxychloride (55% Zn); ZG: Zn Glycinate (26% Zn); ZL: Zn Lysine-Glutamate (17% Zn)

Table 4.5: Effects of dietary Zn supplemental level and source on proliferation of peripheral blood lymphocytes stimulating index (SI) and nitric oxide (NO) production (µM)

		Cell Viability (SI)	NO (µM)
	Basal	1.19	1.43
	ZS[1] 25 mg	1.22	2.42
	ZS 100 mg	1.27	1.58
	ZH 25 mg	1.19	1.90
	ZH 100 mg	1.23	2.03
	ZG 25 mg	1.27	2.16
	ZG 100 mg	1.30	2.04
	ZL 25 mg	1.29	3.58
	ZL 100 mg	1.16	3.17
	Contrast Basal vs others	0.606	0.099
	SEM	0.134	0.526
	P value	0.969	0.101
Level	25 mg/kg Zn	1.16	2.52
	100 mg/kg Zn	1.19	2.20
	SEM	0.071	0.274
Source	ZS	1.23	1.99[ab]
	ZH	1.09	1.96[b]
	ZG	1.25	2.10[ab]
	ZL	1.13	3.37[a]
	SEM	0.099	0.378
		P value	
Level		0.815	0.414
Source		0.620	0.036
Level*Source		0.850	0.822

[a,b] Means in the same column without common superscripts differ significantly ($P < 0.05$).

[1] ZS: Zn Sulfate (22.7 % Zn); ZH: Zn Hydroxychloride (55% Zn); ZG: Zn Glycinate (26% Zn); ZL:Zn Lysine-Glutamate (17% Zn)

Table 4.6: Effects of Zn concentration and Zn sources on the bone characteristics of broiler chickens

		Tibia Ash%	Toes Ash%	Tibia Zn(ppm)	Tibia (g)
	Basal	46.6	13.2	332	3.49
	ZS[1] 25 mg	44.9	12.1	354	3.49
	ZS 100 mg	46.0	12.9	347	3.28
	ZH 25 mg	45.2	12.4	360	3.36
	ZH 100 mg	45.7	12.7	361	3.68
	ZG 25 mg	45.1	12.3	348	3.55
	ZG 100 mg	46.0	12.4	344	3.29
	ZL 25 mg	46.4	12.4	342	3.49
	ZL 100 mg	46.4	12.9	336	3.53
	Contrast Basal vs others	0.177	0.011	0.244	0.815
	SEM	0.617	0.237	13.6	0.142
	P value	0.415	0.053	0.759	0.581
Level	25 mg/kg Zn	45.3	12.3	351	3.47
	100 mg/kg Zn	46.0	12.7	347	3.44
	SEM	0.30	0.123	16.1	0.072
Source	ZS	45.4	12.5	351	3.38
	ZH	45.5	12.6	361	3.52
	ZG	45.6	12.3	346	3.42
	ZL	46.4	12.6	339	3.51
	SEM	0.432	0.175	9.85	0.102
			P Value		
Level		0.150	0.018	0.658	0.798
Source		0.328	0.710	0.408	0.738
Level*Source		0.820	0.667	0.991	0.190

[1] ZS: Zn Sulfate (22.7 % Zn); ZH: Zn Hydroxychloride (55% Zn); ZG: Zn Glycinate (26% Zn); ZL:Zn Lysine-Glutamate (17% Zn)

Table 4.7: Effects of dietary Zn supplemental level and source on performance of broilers

		Crypt depth	Villi height	Villi width	VH:CD
	Basal	115	950	112[cd]	8.43
	ZS 25[1] mg	130	902	134[ab]	7.06
	ZS 100 mg	115	926	102[cd]	8.34
	ZH 25 mg	126	851	127[abc]	7.31
	ZH 100 mg	116	881	127[abc]	8.21
	ZG 25 mg	116	938	140[a]	8.36
	ZG 100 mg	121	833	110[cd]	7.14
	ZL 25 mg	129	922	116[bcd]	7.26
	ZL 100 mg	110	848	121[abcd]	7.70
	Contrast Basal vs others	0.547	0.137	0.185	0.180
	SEM	7.71	39.5	7.65	0.52
	P value	0.550	0.314	0.015	0.306
Level	25 mg/kg Zn	125	903	130	7.50
	100 mg/kg Zn	115	872	116	7.85
	SEM	3.70	20.1	3.31	0.25
Source	ZS	122	914	119	7.70
	ZH	121	866	127	7.76
	ZG	118	886	126	7.75
	ZL	119	885	119	7.48
	SEM	5.23	27.8	4.79	0.36
			P Value		
Level		0.077	0.281	0.008	0.327
Source		0.943	0.703	0.477	0.937
Level*Source		0.394	0.237	0.013	0.083

[a,b,c,d] Means with different superscripts within the same column differ significantly ($P < 0.05$).

[1] ZS: Zn Sulfate (22.7 % Zn); ZH: Zn Hydroxychloride (55% Zn); ZG: Zn Glycinate (26% Zn); ZL: Zn Lysine-Glutamate (17% Zn)

Table 4.8: Effects of dietary Zn supplemental level and source on Zn and amino acids transporters gene expression in the liver of broilers

		GPX 1	GPX 2	GPX 3	SOD 1	SOD 2	SOD 3
	Basal	1.20	0.89	1.06[ab]	1.07	1.04	1.31
	ZS[1] 25 mg	0.87	0.97	1.06[ab]	1.05	1.00	1.52
	ZS 100 mg	0.97	1.05	1.50[a]	1.28	1.05	1.11
	ZH 25 mg	0.95	1.74	0.54[c]	0.86	0.92	1.58
	ZH 100 mg	1.14	0.68	1.49[ab]	0.84	0.84	0.93
	ZG 25 mg	0.66	1.40	1.71[a]	1.00	1.16	1.12
	ZG 100 mg	1.49	1.10	1.18[ab]	0.97	0.86	0.68
	ZL 25 mg	1.70	0.76	1.06[ab]	0.99	1.06	0.93
	ZL 100 mg	2.01	1.18	1.29[ab]	1.11	1.02	1.38
	Contrast Basal vs others	0.736	0.754	0.346	0.875	0.945	0.649
	SEM	0.533	0.320	0.201	0.149	0.127	0.323
	P value	0.757	0.717	0.046	0.802	0.568	0.504
Level	25 mg/kg Zn	1.11	1.24	1.06	0.98	1.03	1.30
	100 mg/kg Zn	1.45	1.05	1.35	1.06	0.95	0.99
	SEM	0.383	0.177	0.110	0.072	0.059	0.149
Source	ZS	0.91	1.01	1.34	1.18	1.03	1.32
	ZH	1.07	1.35	0.95	0.85	0.88	1.25
	ZG	1.21	1.24	1.36	0.98	0.98	0.86
	ZL	1.87	1.02	1.19	1.06	1.03	1.13
	SEM	0.516	0.248	0.157	0.119	0.082	0.212
				P Value			
Level		0.168	0.267	0.112	0.893	0.223	0.217
Source		0.172	0.974	0.358	0.682	0.770	0.373
Level*Source		0.096	0.462	0.044	0.912	0.533	0.418

[a,b,c] Means with different superscripts within the same column differ significantly ($P < 0.05$).

[1] ZS: Zn Sulfate (22.7 % Zn); ZH: Zn Hydroxychloride (55% Zn); ZG: Zn Glycinate (26% Zn); ZL:Zn Lysine-Glutamate (17% Zn)

Table 4.9: Effects of dietary Zn supplemental level and source on Zn and amino acids transporters gene expression in the liver of broilers

		b0+AT	B0AT	CAT1	CAT2	EAAT3	PEPT1	ZIP10	ZIP8	ZnT1	ZnT10	ZnT5
	Basal	1.05	0.90	1.12^{bc}	0.91	0.26	0.17	1.23	1.024^{abcd}	1.13	1.40	1.03
	ZS[1] 25 mg	0.68	0.91	1.10^{abc}	1.99	0.24	0.20	1.50	1.401^{a}	1.11	1.74	1.44
	ZS 100 mg	1.21	1.30	1.47^{abc}	1.98	0.27	0.17	1.57	1.00^{bcd}	1.11	2.36	1.13
	ZH 25 mg	1.32	0.98	1.88^{a}	1.47	0.25	0.19	1.73	1.19^{abc}	1.09	1.59	1.15
	ZH 100 mg	1.10	1.21	1.41^{abc}	2.00	0.30	0.14	1.39	1.27^{ab}	0.86	2.31	0.98
	ZG 25 mg	1.30	1.56	0.76^{c}	0.61	0.28	0.19	0.74	0.76^{bcd}	0.85	1.14	0.82
	ZG 100 mg	0.88	0.95	1.25^{abc}	1.95	0.24	0.10	1.60	1.23^{abc}	1.06	1.38	1.09
	ZL 25 mg	0.95	0.83	1.72^{ab}	2.27	0.18	0.14	1.34	1.03^{abcd}	0.93	1.36	1.10
	ZL 100 mg	0.93	0.97	0.68^{c}	0.61	0.25	0.14	0.97	0.83^{cd}	0.83	1.01	0.88
	Contrast Basal vs others	0.960	0.422	0.430	0.247	0.968	0.864	0.655	0.569	0.324	0.908	0.690
	SEM	0.18	0.18	0.25	0.49	0.03	0.04	0.28	0.14	0.10	0.37	0.13
	P value	0.226	0.136	0.071	0.255	0.379	0.506	0.185	0.044	0.194	0.146	0.108
Levels	25 mg/kg Zn	1.07	1.08	1.38	1.63	0.24	0.18	1.31	1.10	1.00	1.46	1.13
	100 mg/kg Zn	1.03	1.11	1.23	1.67	0.26	0.13	1.38	1.08	0.96	1.78	1.01
	SEM	0.096	0.098	0.129	0.239	0.015	0.018	0.144	0.074	0.055	0.194	0.066
Sources	ZS	0.97	1.12	1.30	1.98	0.25	0.19	1.53	1.20	1.11	2.05	1.29
	ZH	1.21	1.09	1.65	1.76	0.27	0.17	1.55	1.24	0.97	1.95	1.07
	ZG	1.09	1.25	1.01	1.33	0.26	0.15	1.17	0.99	0.96	1.25	0.94
	ZL	0.94	0.90	1.25	1.50	0.22	0.14	1.15	0.93	0.88	1.19	0.99
	SEM	0.133	0.134	0.186	0.343	0.022	0.025	0.213	0.099	0.076	0.272	0.097
							P Value					
Level		0.724	0.805	0.280	0.785	0.207	0.060	0.804	0.633	0.701	0.276	0.332
Source		0.419	0.325	0.150	0.621	0.296	0.566	0.236	0.126	0.169	0.051	0.164
Level*Source		0.081	0.068	0.051	0.088	0.415	0.706	0.088	0.044	0.250	0.478	0.154

[a,b,c,d] Means with different superscripts within the same column differ significantly ($P < 0.05$).

[1] ZS: Zn Sulfate (22.7 % Zn); ZH: Zn Hydroxychloride (55% Zn); ZG: Zn Glycinate (26% Zn); ZL:Zn Lysine-Glutamate (17% Zn)

Table 4.10: Effects of dietary Zn supplemental level and source on mineral digestibility of broilers

		DM Dig (%)	N Dig (%)	Zn Dig (%)	P Dig (%)	Cu Dig (%)	marker-corrected mg/100g DM intake	
							Cu	Zn
	Basal	0.668[b]	0.740	-0.089	0.51[abc]	0.449[ab]	28.9	71.4[b]
	ZS[1] 25 mg	0.671[b]	0.740	-0.162	0.483[c]	0.418[ab]	34.6	98.1[ab]
	ZS 100 mg	0.676[b]	0.741	0.236	0.504[bc]	0.455[ab]	30.6	141.7[ab]
	ZH 25 mg	0.7a[b]	0.760	0.089	0.597[abc]	0.455[ab]	29.3	81.4[b]
	ZH 100 mg	0.701[ab]	0.763	0.011	0.643[a]	0.395[b]	32.6	159.4[ab]
	ZG 25 mg	0.708[ab]	0.774	0.231	0.617[ab]	0.487[ab]	30.3	74.1[b]
	ZG 100 mg	0.672[b]	0.744	-0.015	0.525[abc]	0.447[ab]	34.6	147.1[ab]
	ZL 25 mg	0.683[ab]	0.750	-0.001	0.587[abc]	0.547[a]	25.6	92.01[ab]
	ZL 100 mg	0.720[a]	0.784	-0.511	0.617[ab]	0.467[ab]	27.0	263.2[a]
	Contrast Basal vs others	0.016	0.250	0.874	0.052	0.844	0.471	0.136
	SEM	0.009	0.014	0.222	0.026	0.031	2.200	37.823
	P value	0.0004	0.236	0.433	0.001	0.042	0.063	0.018
Level	25 mg/kg Zn	0.691	0.756	0.039	0.572	0.389	29.9	86.4
	100 mg/kg Zn	0.692	0.758	0.070	0.573	0.320	31.2	177.8
	SEM	0.004	0.005	0.117	0.015	0.026	1.1	20.0
Source	ZS	0.674[b]	0.739	0.037	0.495[b]	0.435[b]	32.5[a]	120
	ZH	0.7[a]	0.762	0.050	0.62[a]	0.426[b]	30.9[a]	120
	ZG	0.69[ab]	0.758	0.108	0.572[a]	0.468[b]	32.4[a]	111
	ZL	0.707[a]	0.767	0.256	0.602[a]	0.508[a]	26.2[b]	178
	SEM	0.006	0.010	0.165	0.019	0.023	1.59	28.3
					P Value			
Level		0.813	0.831	0.512	0.972	0.064	0.430	0.002
Source		0.011	0.211	0.411	0.0005	0.045	0.026	0.331
Level*Source		0.002	0.208	0.276	0.075	0.534	0.271	0.424

[a,b,c] Means with different superscripts within the same column differ significantly ($P < 0.05$).

[1] ZS: Zn Sulfate (22.7 % Zn); ZH: Zn Hydroxychloride (55% Zn); ZG: Zn Glycinate (26% Zn); ZL: Zn Lysine-Glutamate (17% Zn)

CHAPTER 5

5. EFFECT OF ORGANIC AND INORGANIC ZN SOURCES ON PERFORMANCE, TIBIA PARAMETERS, GUT PERMEABILITY, NUTRIENTS DIGESTIBILITY AND TRANSPORTERS OF BROILER CHICKENS TO *EIMERIA* CHALLENGE[3]

[3] M. Pilevar, and O. A. Olukosi. To be submitted to a peer-reviewed journal.

Abstract

Coccidiosis is an enteric disease of poultry that hinders growth performance and impairs gut health. In our previous study, Zn-lysine-glutamate (ZL) increased apparent digestibility and bioavailability compared to inorganic Zn sources. The current experiment investigated the effect of four Zn sources on broiler performance, nutrient transporters, intestine histology, short-chain fatty acids, bone parameters, and gut permeability under a coccidiosis challenge. Three hundred fifty one-day-old chicks were randomly allocated to treatments in a 5×2 factorial arrangement of 7 replicates each. Factors were five diets: 1) control without supplemental Zn (C); 2) C plus 80 mg/kg Zn sulfate (ZS); 3) C plus 80 mg/kg hydroxychloride Zn (ZH); 4) C plus 80 mg/kg Zn glycine (ZG); 5) C plus 80 mg/kg Zn lysine-glutamate (ZL) with or without challenge. On day 17, half of the birds were orally gavaged with a mixed *Eimeria* solution containing *E. maxima*, *E. tenella*, *E. acervuline.* Performance parameters were evaluated during the starter phase (0-10 days) and grower phase (11-23 days). Challenge significantly impaired all performance traits assessed from 12 to 23 or 0 to 23 days of age ($P < 0.05$). Zn source affected weight gain at the end of the grower phase and showed an increase in ZL diet compared to the birds fed ZS diets ($P = 0.045$). There was an interaction between Zn sources × challenge, whereas challenge increased crypt depth (CD) in jejunum for all Zn sources with ZL having the highest value. *Eimeria* challenge significantly increased CD and decreased VH: CD ratio and VH of all three sections of the intestine. The villi in the ZL group have the lowest height compared to the ZS-fed birds. There was an interaction of Zn sources by *Eimeria* challenge for cecal butyrate, valerate, and total SCFA. *Eimeria* inoculation decreased the expression of ZnT1 in duodenum, jejunum, and kidney. Our duodenum, jejunum, and ileum data revealed an upward expression of ZnT5 in response to the challenge. Downward expression of Ca and P-related genes was observed in jejunum. mRNA

expression of JAM2 and CLDN1 in jejunum and CLDN1 in ileum upregulated by Eimeria challenge. MT was upregulated by *Eimeria* challenge in both kidney and pancreas at 6 days post inoculation (6-dpi). An interaction between the *Eimeria* challenge and Zn sources was observed in the liver (P = 0.026). ZL showed the highest expression of ALP1 in liver in non-challenged birds compared to other Zn sources. As a result of the infection at 6-dpi, birds displayed lower expressions of ALP1 and GPX1 in the kidney and ALP1 in the liver at 6-dpi. In conclusion, ZL ameliorated coccidiosis-induced decrease in weight gain and ALP1 expression in liver. Also, ZS increases VH in the ileum, SCFA, mcC of Cu, CAT1, and GLUT1 mRNA expression.

(Keywords: inorganic, organic, minerals digestibility, broiler chickens, Zn transporters)

Introduction

Many dietary nutrients are required to promote good health and growth of animals. Trace minerals such as zinc and copper are essential for a variety of physiological processes like bone and skeletal development (Zeigler et al., 1961) (Philippi et al., 2023), catalytic function (Galdes & Vallee, 1983), and immune response to a disease challenge (Kidd et al., 1996).

Typically, inorganic salt minerals in the form of oxides or sulfates are supplemented in poultry diets. To compensate for the lower bioavailability of this mineral (Star et al., 2012), the industry is using them in higher concentrations in diets (Pacheco et al., 2017), leading to higher mineral excretion and cost. Additionally, to absorb inorganic minerals, they need to be solubilized in the gastrointestinal tract. This increases the interaction with other nutrients and lowers availability to the animal (Close, 2003). On the other hand, organic minerals in poultry diets resulted in better response, though smaller quantities of them were supplemented in the diet (Abdallah et al., 2009; Bao et al., 2007; Maciel et al., 2010). When minerals are chelated with

amino acids, their uptake by the gut will be facilitated by the amino acid transporter pathway, as shown in the human cell model (Gao et al., 2014).

The poultry industry has an increased rate of skeletal disorders in broiler chickens, particularly leg problems such as lameness and tibial dyschondroplasia. Research shows that these issues are partly due to deficiencies of some trace minerals (Lilburn, 1994). Some of these problems are reported even with enough minerals in the diet, likely due to poor bioavailability and nutrient-antagonistic interactions. It has been documented that supplemental Zn mitigates leg disorders in broilers (Akhavan-Salamat & Ghasemi, 2019). Seo et al. (2010) have shown that Zn administration positively affects bone formation, mainly with the help of direct impacts on protein synthesis (Scrimgeour et al., 2007). Zn can act as a hormonal growth mediator, such as influencing insulin-like growth factor I on osteoblasts (Wang et al., 2002). The effect of Zn when feeding to a laying hen could be different when the origin of supplementation is different. Tibia and eggshell thickness can be affected by Zn when replacing inorganic Zn with zinc amino acid complexes (Gheisari et al., 2011). Also, the dietary addition of organic Zn could ease the adverse effects of age on egg quality parameters in laying hens (Behjatian Esfahani et al., 2021). An increase in tibia ash in birds fed Zn proteinate compared to those fed Zn sulfate has been observed. Organic Zn causes higher Zn concentration in the tibia of chicks in comparison to those fed inorganic Zn, and researchers concluded that it might be due to antagonism occurring between Zn and other minerals while using the inorganic sources, but not when using organic sources (Shelton & Southern, 2006).

Zrt-irt-like proteins (ZIPs) and Zn transporters (ZnTs) are the primary transporters of Zn in intestinal cells (Ford, 2004). The ZIP transporters family mediate the influx of Zn from the intracellular organelles into the enterocyte. In contrast, the ZnT family helps the efflux of Zn from the cytosol into intracellular organelles (Wessels et al., 2017). Organic Zn significantly increased

ZnT1 mRNA expression in the jejunum of broilers challenged with Eimeria (He et al., 2019). Also, Zn-Prot significantly improved the Zn absorption in the intestine by upregulating the expression of the mRNA and/or protein expression of Zn transporter 7 (ZnT7), Zn transporter 9 (ZnT9), and Zrt-irt-like protein 3 (ZIP3), in the duodenum section of chicken (Hu et al., 2022b). Zn status in the body has been shown to influence the mRNA expression levels of several genes and proteins in Caco-2 cell line (Finamore et al., 2008).

Dietary Zn inclusion may influence the digestibility of other nutrients. Some reports show a higher digestibility coefficient for the chelated Zn diet than ZS (De Grande et al., 2020). It has been reported that the apparent retention of Zn in the organic group was significantly higher than in the inorganic group (Min et al., 2019). There is a significant linear increase in the apparent digestibility of dry matter (DM) and nitrogen (N) by increasing dietary Zn concentration (Zarghi et al., 2022).

Coccidiosis is an enteric disease in poultry caused by protozoa of the *Eimeria*. *Eimeria* life cycle in the epithelium causes enterocyte destruction and tissue injury, adversely affecting poultry health and economic status (Chapman, 2014). Coccidiosis can also lead to changes in the structure of the intestinal microbiota and a decrease in the cecal microbial diversity along with changes in short-chain fatty acids produced in the ceca of chickens (Stanley et al., 2014). The distribution of minerals within the animal's body may change during enteric infections. When the absorptive capacity of the intestine is weakened due to enteric infections such as Coccidiosis, it is safe to say that sources of minerals of greater availability may be needed. Organic Zn induced higher expression of an anti-inflammatory regulator (A20), downregulated the expression of pro-inflammatory cytokines, including NF-kB, and promoted MUC2 and IgA production compared to its inorganic counterpart (Prasad et al., 2011). Therefore, we hypothesized that organic Zn would

attenuate the severity of damage in broilers challenged with coccidia compared to inorganic Zn sources.

Materials and Methods

Broilers Management, Housing, and Diets

One-day-old male Cobb 500 broilers (n = 350; 70 cages) were used in a 23-day coccidiosis challenge experiment to study the response of broiler chickens receiving different sources of Zn and challenged with mixed *Eimeria* spp. Feed and water were provided ad libitum throughout the experiment. The room temperature gradually decreased from 34 °C to 23 °C. The light schedule was kept 24L: 0D for the first three days, then gradually reduced to 16L: 8D.

Diets were formulated to a 2-phase feeding program of starter (0-12 d) and grower (12-23 d) to meet the birds' nutritional requirements. The feed composition and calculated nutrients are presented in Table 5.1. Diets were based on corn and soybean meal. Titanium dioxide (TiO_2) was used as an indigestible marker for digestibility measurement with an inclusion level of 5 g/kg. Five experimental diets were formulated in a 5×2 factorial arrangement of treatments. Factors were Zn sources and *Eimeria* challenge (with or without). The five diets are the control diet without Zn supplementation (C), C plus 80 mg/kg Zn sulfate (ZS), C plus 80 mg/kg hydroxychloride Zn (ZH), control plus 80 mg/kg Zn glycine (ZG), and control plus 80 mg/kg Zn lysine-glutamate (ZL). One basal diet was prepared without Zn supplementation and then divided into five batches to be supplemented with different sources of Zn. For the C diet, no supplementation of Zn; for the ZS diet, 0.35 mg of Zn sulfate/kg; for the ZH diet, 0.14 mg of Zn hydroxychloride/kg; for the ZG diet, 0.30 mg of Zinc-Gly/kg and ZL diet 0.47 mg of Zn-Lys-Glu/kg added to the basal diets. All diets

were fed as mash. Birds and feed were recorded on days 0, 12, and 23 to measure weight gain and feed intake in both phases.

Eimeria Challenge, Intestinal Permeability, and Lesion Scoring

On day 17, birds in the challenge group were gavaged with a solution containing 12,500 sporulated oocysts of E. *maxima*, 12,500 sporulated oocysts of E. *tenella*, and 62,500 sporulated oocysts of E. *acervulina* suspended in 1 ml of distilled water. In contrast, the non-challenge groups were orally gavaged with 1 mL water. On day 22 (5-dpi), one bird from each cage was selected and orally administered 1 ml of fluorescein isothiocyanate dextran (FITC-d; 2.2 mg/mL, MW 4000; Sigma-Aldrich, St. Louis, MO) to measure intestinal permeability. Birds were then killed by CO_2 two hours after that, and immediately, blood was collected via cardiac puncture with a 5 ml syringe.

Blood was centrifuged at 1,500 × *g* for 10 min. Then, serum was collected, and the concentration of FITC-d was measured by a spectrophotometer plate reader (Spectramax M5, Molecular Devices, San Jose, CA) at 485 nm. At 6-dpi, duodenum, jejunum, ileum, and ceca were removed from 2 birds for intestinal lesion scoring. The tissue was scored by the four-scale guideline described by Johnson and Reid (1970).

Digestibility, Short Chain Fatty Acids, and Intestinal Morphology

At the end of the study, all birds were euthanized for ileum content collection. The ileal digesta were then oven-dried for two days at 75 C to measure ileal digestibility (DM, N, Zn, Cu, and P). Titanium dioxide analysis was done by wet digestion and optical density reading at 410 nm (Short et al., 1996). Diets, ileum, and excreta samples were ashed in duplicate and then digested in concentrated HNO_3 and HCl (AOAC, 1990). Phosphorous concentration was measured in

digested samples via colorimetric assay, measuring phosphate in the presence of a molybdate solution and acting as a reducing agent. Each sample was done in triplicates and read at 630 nm on a spectrophotometer. Zinc and copper were determined using a SHIMADZU AA-6800 atomic absorption spectrometer. Nitrogen content was determined by the combustion method using a Leco N analyzer (Leco FP 828-MC analyzer, Leco Corporation, St. Joseph, Michigan, USA). The analysis of volatile fatty acid (VFA) followed the protocol by Lourenco et al. (2020) using gas chromatography. One part of cecal samples was solubilized in 3 parts of water. Then, they were centrifuged at 10,000 x g for 10 minutes, and 1 ml of supernatant was added to 0.2 mL of a metaphosphoric acid solution (25% wt/vol), and samples were frozen overnight. Samples were then thawed and centrifuged at 10,000 x g for 10 minutes. The supernatant was transferred into polypropylene tubes and mixed with ethyl acetate in a 2:1 ratio of ethyl acetate to supernatant. Then, 0.5 mL of the top portion of the solution was transferred to a new tube for analysis of short-chain fatty acids in a Shimadzu GC-2010 Plus gas chromatograph (Shimadzu Corporation, Kyoto, Japan)

Samples from duodenum, jejunum, and ileum tissue (3 cm length) were collected (n = 7 per treatment) and after rinsed with phosphate-buffered saline; the tissues were then kept in 10% formalin bottle for about three days for tissue fixation and then embedded in Tissue Path (Fisher, Scientific, Pittsburgh, PA). Each sample was then gradually dehydrated by bathing in ethanol from 50% to 100%, diaphonized in dimethylbenzene, and fixed in paraffin. Then, a rotary microtome (model RM 2255, Leica, Wetzlar, Germany) was used to obtain a section thickness of 5 μm/sample on a glass slide stained with hematoxylin and eosin. Mucosal morphology was assessed according to the procedure by Luna (1968). Slides were viewed at 40× magnification using a Keyence microscope (BZ-X800, Keyence Inc., Itasca, IL). Villus height

(VH), crypt depth (CD), and villus height-to-crypt depth ratio (VH: CD) were measured for at least ten intact villi and crypts for each intestinal segment. VH was measured from the tip of the villus to the base of the intestinal mucosa, and CD was calculated as the length of the invagination between 2 adjacent villi.

Expression Analysis of Genes

On 6-dpi using one bird per cage, the duodenum, jejunum, ileum, liver, kidney, and pancreas were collected immediately, kept at –80°C, and used for gene expression analyses. Tissues (0.1 g) were homogenized with TRIzol reagent (QIAGEN, Hilden, Germany) using a bead beater. The concentration and quality of total RNA were determined using a nanodrop spectrophotometer (BioTek Synergy HTX, Agilent, Santa Clara, CA). RNA was dissolved in nuclease-free water. Then, cDNA was created using a cDNA reverse transcription kit (Thermo Fisher Scientific, Waltham, MA). For each target gene, cDNA was expressed in duplicate for real-time PCR analysis. Real-time PCR was done using Power SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA) on Real-Time PCR Systems (Applied Biosystems). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reference gene was used to normalize the expression of target genes and calculate ΔCt. Then ΔCt of C was used as a calibrator for calculating ΔΔCt for treatments, and fold change relative to control was calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). The primer sequence for the housekeeping gene and the target genes are presented in Table 5.3.

Bone Ash

Left tibia bones were sampled (6-dpi) from one bird per cage and autoclaved at 121 °C for 5 minutes to loosen tissue and caps in a modified method described by Cloft et al. (2018). Tibia was left to dry overnight before recording the weight and then ashed overnight at 600°C. Bone ash

was then weighed. To analyze the ash for mineral content, the same procedure was followed for ilea digesta and excreta samples.

Calculations and Statistical Analysis

The apparent ileal digestibility (**AID**) of DM, Zn, Cu, and P (%) were calculated in the experimental diets based on the following formula and using TiO2 as a marker:

$$AID\ \% = 100 \times (1 - ((Ci/Co) \times (No/Ni)))$$

Marker-corrected concentration (mcC) of Zn and Cu in ileum calculated using the following formula:

$$mcC\ (ppm) = No \times (Ci/Co)$$

Where Ci = Titanium concentration in diet (%)

Co = Titanium concentration in ileal digesta (%)

Ni = Nutrient concentration in diet (%)

No = Nutrient concentration in ileal digesta (%)

All data were analyzed using a 2-way ANOVA in a 5 × 2 grouping (Zn sources × Challenge) using the MIXED procedure of SAS OnDemand for Academics. In case of a significant interaction, means were separated using Tukey's HSD. Statistical significance was set at $P \leq 0.05$, and trends at $P \leq 0.10$.

Results

The calculated and analyzed composition of starter (0-12 d) and grower (12-23 d) diet formulas are shown in Table 5.1. The analyzed Zn levels were about 23 and 34 ppm more than calculated levels in starter (30 ppm) and grower (28 ppm) diets, respectively. Zn concentration for each experimental diet is shown in Table 5.2.

The growth performance response of broiler chickens receiving different sources of Zn and challenged with mixed *Eimeria* spp are presented in Table 5.4. There was no significant challenge × Zn sources interaction for the growth performance parameters (BW gain, FI, and FCR) in any periods of pre- or post-challenged. The dietary Zn source significantly affected weight gain at the end of the grower phase (day 23), wherein it was increased in ZL diet compared to the birds fed ZS diets ($P = 0.045$). Additionally, it was observed that the challenge significantly impaired all performance traits evaluated from 12 to 23 or 0 to 23 days of age ($P < 0.05$).

Bone, gut permeability and scoring

Bone responses of broiler chickens at 6-dpi receiving different sources of Zn and challenged with mixed *Eimeria* spp are depicted in Table 5.5. No interaction between challenge and Zn source supplementation was found for bone characterization ($P > 0.05$). Also, there was no main effect of Zn source on bone parameters, but ZL and ZG diets tend ($P = 0.116$) to increase Zn concentration in tibia compared to other Zn sources. On the other hand, the tibia weight, ash weight, and tibia Zn concentration were significantly decreased in challenged birds at day 23 ($P < 0.05$).

Figure 5.1 shows the effect of receiving different sources of Zn and challenged with mixed *Eimeria* spp on gastrointestinal permeability on day 22 (5-dpi). Intestinal permeability significantly increased in challenged birds ($P < 0.05$) compared to non-challenged birds, but there

were no differences among Zn sources. High FITC-d level shows intestinal leakage due to intestinal epithelium damage by *Eimeria*.

Eimeria challenge caused intestinal lesions in duodenum, jejunum, ileum, and ceca (Figure 5.2). While Zn supplementation had no significant effect on intestinal lesion scores (6-dpi).

Intestinal Histomorphometry, SCFA, and Digestibility

The results regarding intestinal morphology are depicted in Table 5.6. There was a significant interaction between dietary Zn sources and challenge for CD in jejunum at day 23 ($P = 0.016$). Briefly, Coccidiosis challenge increased CD values in jejunum in all Zn sources and has the highest value in ZL diet. *Eimeria* challenge significantly increased the CD of all three intestine sections compared to non-challenged birds ($P < 0.05$). On the other hand, VH and the ratio of VH to CD were significantly decreased in the challenge group in duodenum, jejunum, and ileum ($P < 0.05$). Dietary Zn sources significantly affected the ileum villus height at 6-dpi ($P<0.05$). The VH in the ZL group has the lowest height compared to the ZS fed birds, and there are no differences in VH between ZL and C diet.

Cecal SCFA composition at 6-dpi of broiler chickens receiving different sources of Zn and challenged with mixed *Eimeria* spp are shown in Table 5.7. An interaction was observed for Zn sources by *Eimeria* challenge for butyrate, valerate, and total SCFA. Zn supplementation changes *Eimeria*-induced effect in cecal butyrate, valerate, and total SCFA. Higher cecal valerate and butyrate ($P < 0.05$) were observed in ZS in challenged birds compared to challenged ZL and ZG. A trend ($0.05 < P \leq 0.10$) for the main effect of Zn source was observed for butyrate at 6-dpi, wherein birds fed ZS had the highest and ZL showed the lowest value. The concentration of

propionate, isobutyrate, isovalerate, valerate, and BCFA was significantly higher in challenged birds than in unchallenged birds ($P < 0.05$).

Mineral digestibility and marker-corrected concentration (mcC) for broilers at 6-dpi receiving different sources of Zn and challenged with mixed *Eimeria* spp are shown in Table 5.8. There was no significant interaction between Zn sources and challenges for any nutrient utilization factors. However, the main effect of Zn source was P digestibility and mcC of Zn and Cu, wherein all the Zn supplementation showed better digestibility compared to no Zn supplementation. Regarding the mcC, no Zn supplementation showed a significant decrease ($P < 0.05$) in Zn concentration compared to all other 4 Zn supplementation. On the other hand, ZS supplementation showed a substantial increase in mcC of Cu in ileum digesta compared to the other four diets.

Relative mRNA Expression of Intestinal Gene

Duodenal gene expression of 23-day-old broiler chicks (6-dpi) receiving different sources of Zn and challenged with mixed *Eimeria* spp are shown in Table 5.9. There was neither significant interaction nor significant main effect of Zn sources on any targeted gene in duodenum. However, challenge ($P < 0.05$) had a significant effect on ZnT1, ZnT5, and Ea-SaG mRNA expression in duodenum at 6-dpi. The *Eimeria* infection downregulated ZnT1 and upregulated ZnT5, and Ea-SaG mRNA expression of duodenum was found at 6-dpi. For both ZIP8 and ZIP10 expression, birds in the challenged group tended ($P = 0.1$) to have lower expression compared to the unchallenged diets.

The relative mRNA expression of mineral transporters, amino acids transporters, and tight junctions in the jejunum of 23-day-old broilers are shown in Table 5.10, 5.11, and 5.12, respectively. There was no challenge × Zn sources interaction among mineral transporters in

the jejunum. The *Eimeria* challenge caused a significant downregulation ($P < 0.05$) for mRNA expression of ZnT1, ZIP10, NPT2, CaSR, and CalbidinD28 while upregulating for ZnT5 and ZIP8 in Jejunum at 6-dpi. In Table 5.11, the significant challenge × Zn sources interaction ($P < 0.05$) for EAAT3 showed the lowest expression in ZS and highest expression in no Zn diet when not challenged. Also, a trend for interaction ($P=0.08$) was observed for CAT1, wherein the Zn sources decreased the CAT1 expression in all unchallenged groups but showed more expression for ZS compared to ZL. In Table 5.12, no interaction was found between challenge by Zn sources for tight junction expression gene. However, the *Eimeria* infection significantly ($P < 0.05$) increased the JAM2F and CLDN1 expression while tending to downregulate Occludin ($P = 0.093$) and had no effect on the ZO-1 gene.

Results for Zn transporters and intestinal barrier functions gene in the ileum are shown in Table 5.13. There was no Challenge × Zn sources interaction for targeted genes in ileum. Higher ($P < 0.05$) relative mRNA expression of ZnT1, ZnT5, CLDN1 and lower ($P < 0.05$) expression of ZIP10 and JAM2 in challenged birds were recorded. Also, there was a tendency ($P = 0.063$) for mRNA expression of Occludin to show downregulation in challenged birds at 6-dpi. As a main effect, ZL significantly ($P < 0.05$) upregulates the expression of ZIP8 compared to no Zn supplementation. No Zn diet showed upregulation ($P = 0.066$) for ZIP10 compared to ZG.

Relative mRNA expression in Liver, Kidney, and Pancreas

The gene expression of targeted genes in the liver of broiler chickens at 6-dpi receiving different sources of Zn and challenged with mixed *Eimeria* spp are shown in Table 5.14. The interaction between main effects was observed wherein ALP1 was upward in all unchallenged Zn sources while ZL diet showed the highest upregulation among them. The challenge with *Eimeria* significantly ($P < 0.05$) increased the expression of PEPT1 in liver. Another main effect of

challenge was observed for ALP1 being downregulated for challenged birds ($P < 0.05$). An effect of Zn sources was observed ($P < 0.05$) on the expression of ALP1 being upwardly express in ZL when compared to the birds receiving ZS or ZG supplementation. An additional significant ($P = 0.003$) effect of Zn sources was the expression of CAT1 in the liver, which was the highest expression among other Zn sources. ZL tended ($P = 0.124$) to upregulate the expression of SOD in comparison to other Zn sources.

No interaction between challenge by Zn source nor main effect of Zn source was noticed for mRNA expression in kidney (Table 5.15). Though, the *Eimeria* inoculation significantly ($P < 0.05$) upregulated MT expression and downregulated ZnT1, SOD, and ALP1 in kidney at 6-dpi. Among Zn sources, ZL was tended ($P = 0.187$) to increase the expression of MT in kidney.

Eimeria challenge significantly increases mRNA expression of MT in the pancreas, as shown in Table 5.16. Oppositely, challenge tended ($P=0.106$) to produce a downward expression of SOD in the pancreas. There was no interaction between main effects or Zn source on relative mRNA expression in pancreas at 6-dpi.

Discussion

Eimeria are intracellular protozoan parasites that cause Coccidiosis in livestock and a huge economic loss (15 billion dollars) in the poultry industry (Blake et al., 2020). The current study aimed to assess the potential effects of different Zn sources, *Eimeria* challenges, and their interactions on broiler chicks' growth performance and health responses. Sources of ZS/ZH and ZG/ZL as representatives of inorganic or organic Zn sources, respectively, were used. The result indicated that performance of challenged birds was negatively affected, which then was ameliorated by an organic Zn source of ZL at 6-dpi. These results are consistent with previous

studies indicating that *Eimeria* challenging impaired performance (Allen & Fetterer, 2002; Choi et al., 2023). Additionally, (El-Husseiny et al., 2012); Yuan et al. (2011) reported that replacing inorganic minerals with organic sources improved growth performance and carcass characteristics. Likewise, Feeding 80 mg/kg of organic Zn Vs Zn sulfate significantly increases weight gain in broilers (Gong et al., 2024). To maximize the growth performance, using higher levels of Zn more than requirement (NRC, 1994) can be beneficial in broiler chickens (Leeson, 2005). Contrary to our results, many studies found no changes in performance when using organic Zn compared to inorganic sources when birds are challenged with *Eimeria* (Bakhshalinejad et al., 2024; Bun et al., 2011). Both Bafundo et al. (1984) and Southern and Baker (1983) found a reduction in growth performance when challenged with *Eimeria* and no beneficial effects in response to Zn supplementation. In a pheasant study by Gugała et al. (2019), replacing 75% of the mineral salt with glycine chelates (90 mg/kg Zn) improves laying performance and hatchling survival. In a 51-day-old broiler study, 60 mg/kg of Chelated zinc methionine resulted in higher weight gain and FCR improvement compared to birds fed inorganic Zn source (Sirri et al., 2016). The discrepancies may result from multiple factors, such as the age, chelation strength (Hu et al., 2023a), sex (Zhao et al., 2010), genetics, amino acids type, *Eimeria* spp or level of organic Zn supplementation (Manangi et al., 2012).

Fast-growing broilers need higher levels or more bioavailable of minerals for proper bone development (Dibner et al., 2007). More bioavailability of organic minerals compared to inorganic sources has been proven in chapter 3 of this dissertation and other researchers (Cao, Henry, Guo, et al., 2000; Wang et al., 2007). The current study shows the negative effect of *Eimeria* inoculation on tibia and ash weight. In agreement with our findings, Shi et al. (2024) and Kakhki et al. (2019) reported reduced tibia ash weight in *Eimeria*-challenged birds. Sakkas et al. (2018) concluded that

lower mineralization in the bone of Eimeria-challenged birds causes lower bone growth. During *Eimeria* infection, impairment of bone growth could be due to malabsorption, nutrient deficiency, or imbalance of osteoblastic and osteoclastic cells (Sharma et al., 2023). Mireles et al. (2005) discussed that release of tumor necrosis factor (TNF-α) and IL-1 can increase bone resorption and have an adverse effect on bone quality in an inflammatory response of challenged birds. In an *Eimeria* challenge study, there were no differences in using 0, 80, or 100 mg/kg of ZS on tibia ash percentage (Santos et al., 2020). Like the current study, tibia Zn concentration increased when inorganic Zn sources were partially replaced with Zn proteinate (Riboty et al., 2024). Lower tibia Zn concentration could also be explained by lower Zn intake in inorganic sources (ZS/ZH) compared to organic sources (ZG/ZL) (Mwangi et al., 2017).

The current study evaluated the effects of different sources of Zn and challenged Eimeria on gut permeability by measuring the passage of FITC-d from the intestinal lumen to the blood at 5-dpi. Higher levels of FITC-d in the serum of challenged birds indicate the severity of damage to tight junction barrier (Liu et al., 2021). Zn is essential in gut health as it has antimicrobial and antioxidant properties (N. Suttle, 2022). Contrary to our result, Zn amino acids, compared to Zn sulfate, restored gut permeability in challenged birds (Troche, 2012). Likewise, Zn proteinate as an organic source of Zn decreased intestinal permeability compared to birds fed $ZnSO_4$ (Bortoluzzi, Lumpkins, et al., 2019). They concluded that the form of Zn along the brush border is essential for proper immunological and intra-cellular responses to *Eimeria*. The intestinal permeability of birds receiving 100 mg/kg of ZS was significantly improved compared to no Zn supplementation in an *Eimeria* study (Santos et al., 2020). Similar to our data, organic Zn (90 mg/kg) could not compensate for the increase in passage of FITC-d from the intestinal lumen to

the blood in *Eimeria* inoculation (Bortoluzzi, Vieira, et al., 2019). Damage to the integrity of epithelium increases antigen flow and consequently decreases nutrient absorption.

Gut absorption and digestion are directly related to the intestine's surface area. Higher villus height and lower crypt depth in the intestine are essential for better nutrient digestion and absorption (Jia et al., 2010). The crypt rapidly divides to produce new epithelial cells, and then the new cells migrate to the villus tip to replace the old cells. We observed the impairment in all three sections of the intestine due to the mixed *Eimeria* spp inoculation. *Eimeria* damage decreased VH and increased CD, indicating a higher epithelial turnover. In the jejunum, CD was significantly lower in challenged birds fed ZS/ZH compared to ZG/ZL. These results are in total agreement with Bortoluzzi, Vieira, et al. (2019) findings in which inorganic form of Zn decreased CD, not the organic Zn, in challenged birds. In a breeder study, supplementation of either inorganic or organic Zn exhibited higher VH or lower CD on days 14 and 35 of progeny compared with Zn-deficient diet (Li et al., 2015). They concluded that Zn supplementation increased enterocyte proliferation, detected by an increase in jejunum's proliferating cell nuclear antigen (PCNA). In current study, we observed increase in VH in ileum by ZS compared to ZL. Zn amino acids compared to ZS increased VH, which led to an increase in digestion/absorption more enzyme (Awad et al., 2017). On the contrary, VH was 16 % higher in broiler fed organic Zn compared to inorganic sources in *Eimeria* challenge study Bortoluzzi, Lumpkins, et al. (2019), explained by lower expression of cytokines involved in the inflammatory process (IL-8 or IL-10) in jejunum. Contrary to our result, a mixture of inorganic and organic Zn was able to increase VH in *C. perfringens*–challenged broilers compared to fed each source separately (Sun et al., 2020).

The negative effect of *Eimeria* challenge on short chain fatty acids are in agreement with previous studies (Lin et al., 2023). Isobutyrate and isovalerate are categorized into branched short-

chain fatty acids (BCFA) and produced in less quantity by the fermentation of protein or amino acids in the hindgut (Macfarlane & Macfarlane, 2003). Butyrate, propionate, and valerate are the end products of fermentation of nondigestible carbohydrates like fiber. SCFA is vital for intestinal health and enterocyte functions, upholding an epithelial cell barrier (JL, 1995). In a study of pigs, zinc supplementation decreased the production of SCFA. The authors concluded that Zn increases ammonia, which is required for fermentation, leading to less N incorporation into the bacterial mass and reduced SCFA production (Pardo et al., 2023). This is in line with the current study in which ZL supplementation in a challenge study significantly decreased SCFA when compared to the No Zn challenge diet.

Infection reduced N and DM digestibility in ileum of 23-day-old broilers. Similarly, Castro et al. (2020) reported that *Eimeria* challenge significantly decreased nutrient digestibility compared to unchallenged group. Similarly, ileal digestible energy and apparent ileal digestibility of Zn, Cu, P, Ca, and N were decreased at 6-dpi (Teng et al., 2020). When *Eimeria* sporozoites are in the gut, they damage the epithelium of the intestine and, leading do a decrease in VH (Rochell et al., 2016). The impairment of epithelium leads to lower digestion and absorption. In our data, ZL improved P digestibility compared to no Zn diet. This can be explained by the weight gain improvement in the ZL grower phase (Table 5.6). Min et al. (2019) apparent retention of Cu was significantly higher when comparing Zn AA to inorganic Zn source. Some reports have shown that organic Zn, compared to inorganic Zn, can improve the zinc digestibility coefficient. This can be explained by villus morphology improvement or oxidative stress alleviation (De Grande et al., 2020).

There are two complementary families of Zn transporter of ZIPs or ZnTs. ZIP transporters are responsible for the influx of Zn into the cytoplasm, and ZnT transporters are responsible for

the efflux of Zn from cells. In our study, *Eimeria* inoculation downregulated the expression of ZnT1 in duodenum, jejunum, and kidney. This was reported previously by Lin et al. (2023); (Paris & Wong, 2013) as the effect of challenge with *Eimeria*. ZnT1 is a Zn efflux transporter localized to the basolateral membrane of epithelial cells and can export cytosolic zinc to the extracellular space (Liuzzi & Cousins, 2004). ZnT1 expression is regulated by cellular Zn status, in which under zinc-sufficient conditions, ZnT1 is upward on the plasma membrane and efflux more Zn out of the cell.

On the other hand, under zinc-deficient conditions, ZnT1 molecules on the plasma membrane were degraded through lysosomal pathways (Nishito & Kambe, 2019) to maintain intracellular zinc homeostasis. In a comparison between ZG and ZS in a non-challenge experiment, ZnT1 was upregulated in duodenum. ZnT1 is considered as an efficient Zn carrier and a sensitive indicator of the intestinal response to dietary Zn (Tako et al., 2005). In our study, ZnT1 in liver was unaffected by challenge, and it could be due to the higher Zn concentration in liver after infection (Bortoluzzi, Vieira, et al., 2019) compared to low Zn concentration in ileum in our study. ZnT5 is an operative zinc transporter that transports Zn into the Golgi apparatus; only insignificant zinc-dependent changes were reported for ZnT5 expression (Sansuwan et al., 2023). Our data in duodenum, jejunum and ileum revealed upregulation in ZnT5 in response to challenge, which was observed by others in jejunum in *Eimeria* infection (He et al., 2019). In the present study, both ZIP 8 and 10 were numerically upregulated in duodenum in response to challenge and possibly to compensate for cell Zn deficiencies. It was explained by Kim et al. (2004) that ZIP4 (influx) expression increased at the plasma membrane, and Zn supplementation caused low ZIP4 expression by stimulating a rapid endocytosis of the transporter. Similarly, influx Zn transporter (ZIP13) upregulated and efflux Zn transporter (ZnT7) were downregulated in jejunum of *Eimeria*

challenge birds compared to non-challenge group (Bortoluzzi, Vieira, et al., 2019). Higher Zn concentration in diet resulted in higher Zn concentration in digesta and jejunum along with upregulation and downregulation of ZnT1 and ZIP4 transporters, respectively (Martin et al., 2013). They explained that high intracellular Zn due to high dietary Zn resulted in increased Zn export (ZnT1 upregulation) and decreased Zn uptake (ZIP4) by gut lumen to homeostasis Zn concentration.

In our study, *Eimeria* infection decreased Ca (CalbidinD28k and CaSR) related genes in jejunum. This is in agreement with Lin et al. (2023), who observed the downregulation of those genes following the *Eimeria* challenge. CaSR is key in regulating calcium homeostasis, absorption, and mobilization. The enterocyte expression of Calbidin D28K reflects the intestine's capacity to absorb Ca (Nys et al., 1992; Wasserman & Fullmer, 1983). NPT2 downregulation by *Eimeria* infection was also reported by Paris and Wong (2013).In the current study, it was not reflected in P digestibility (Table 5.8) but reflected on lower performance in challenge birds (Table 5.4).

Among all brush border transporters in jejunum (B0AT, PEPT1, EAAT3, and b0+AT), B0AT was the only one affected by *Eimeria* challenge. It's a Na-dependent neutral AA transporter and, contrary to our result, being reported in some *Eimeria* infection studies to be downregulated (Fetterer et al., 2014; Miska & Fetterer, 2017; Su et al., 2018; Teng et al., 2021) or unaffected (Paris & Wong, 2013). Similar to our result, B0AT was upregulated in ileum in response to *Eimeria tenella*, but downregulated in duodenum and unchanged in jejunum (Su et al., 2015). The discrepancies could result from different spp of *Eimeria*, doses of inoculation, or sections of the intestine. Expression of all basolateral nutrient transports in jejunum is being upregulated (CAT1 and GLUT1) or downregulated (Y+LAT2) by *Eimeria* challenge. Similarly, Teng et al. (2021) observed upward for CAT1 and GLUT1 in jejunum at 6-dpi. GLUT1 is responsible for transferring

carbohydrate molecules from enterocytes to the blood. While CAT1 is a cationic AA transporter, this upregulation resulted in more AA being exported out of the enterocyte at 6-dpi (Miska & Fetterer, 2017; Su et al., 2015). At the same time, in *Eimeria* life cycle, the parasite is changing from asexual to sexual reproduction. Our results in higher expression of CAT1 are in agreement with others (Fetterer et al., 2014; Gilbert et al., 2007; Teng et al., 2021). They suggested that upregulation in basolateral transporters depleted the AA in the infected cells and may cause apoptosis in intestinal epithelial. In our study, ZS significantly increased the expression of both CAT1 and GLUT1 in jejunum when compared to ZG fed diet. Like jejunum Cat1 expression, we observed that ZS in liver showed the highest expression compared to other Zn sources, but we observed no *Eimeria* effect on CAT1 in liver.

The intestinal epithelial barrier in poultry comprises a physical barrier that protects the bird from the luminal environment and has selective absorption of nutrients while avoiding the entry of pathogens. The barrier functions via tight junction proteins (Groschwitz & Hogan, 2009). These proteins help to regulate epithelial cell permeability via connecting cells to impede the transportation of large molecules across the intestinal border. The current finding shows that the expression of JAM2 and CLDN1 in jejunum and CLDN1 in ileum upregulated during Coccidiosis. Meanwhile, JAM2 gene expression in the ileum decreased in response to *Eimeria*. JAM2 and CLDN1 are responsible for tight junction formation and epithelial polarity. The CLD1 upward expression could result from high cytokine production in the intestines of E. maxima-infected chickens (Otani et al., 2019). Similar to our data, it was reported that *Eimeria* upregulated CLD1 (Poritz et al., 2011). They mentioned that TNFα, a key inflammatory cytokine, increased significantly. Another tight junction protein, Occludin, was marginally downregulated in both jejunum ($P = 0.093$) and ileum ($P = 0.063$) by *Eimeria* challenge. Parallel to this data, Bortoluzzi,

Vieira, et al. (2019) reported no effect of different Zn sources supplementation on the expression of tight junction protein-encoding genes.

Metallothioneins (MTs) are cysteine-rich intracellular metal-binding proteins in every organism. Zn is the primary inducer of MT (Bremner, 1991). Briefly, the binding of Zn to MT, initiates MT-gene transcription. It has been proposed that the free Zn concentration can regulate the transcription of ZnT1 (Langmade et al., 2000). Some reports show MT upregulated by cytokines, metals, and stress hormones (Bremner & Beattie, 1990; Hernández et al., 2000). In the current study, MT was upregulated by *Eimeria* challenge in both kidney and pancreas at 6-dpi. It has been reported that Zn concentration was increased in the liver both by *Eimeria* challenge (Richards & Augustine, 1988) or high Zn concentration fed diet (Martin et al., 2013). Also, high *MT* mRNA expression in pigs fed Zn diet (Martinez et al., 2005) has been shown. A positive correlation between MT expression and Zn concentration is believed to be regulated by a zinc-responsive transcription factor induced by cellular zinc or oxidative stress (Richards & Augustine, 1988).

Eimeria produces reactive oxidative species (ROS), which lead to damage the intestinal epithelial barrier, diarrhea, and disrupt the antioxidant systems of chickens. The challenged birds displayed lower expression of ALP1 and GPX1 in kidney and ALP1 in liver at 6-dpi. Also, non-challenge birds had the highest ALP1 expression in liver in ZL diet compared to other Zn sources. Reduction in total antioxidant capacity in response to *Eimeria* infection can result in high ROS, and induce programmed cell death (Ryter et al., 2007)

From the current study, it was concluded that ZL was able to ameliorate coccidiosis-induced decrease in weight gain and ALP1 expression in liver. Also, ZS increases VH in the ileum, SCFA, mcC of Cu, CAT1, and GLUT1 mRNA expression.

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Table 5.1: Ingredient and chemical composition (g/kg) of experimental diets.

	Starter (0 to 12 d)	Grower (12 to 23 d)
Corn	569.6	629.6
Corn-DDGS	70	75
Soybean meal	310	245
Soybean oil	10	10
Dicalcium phosphate	14.7	6.8
Limestone (B)	7.4	10.3
Sodium bicarbonate	2	2
Vitamin premix[1]	5	5
Zn free Trace minerals premix[2]	5	5
Methionine	1.3	1.4
Lysine	2	2.4
Threonine	0.5	0
Salt NaCl	2.4	2.4
Titanium	0	5
Q-blue phytase	0.1	0.1
Calculated Nutrients		
Protein, g/kg	216.5	191.7
ME, kcal/kg	2943.7	3001.9
Ca, g/kg	7.9	6.8
P, g/kg	6.6	4.9
Available P, g/kg	4.2	2.6
Na, g/kg	1.9	1.9
K, g/kg	8.5	7.4
Cl, g/kg	1.8	1.8
Cu, g/kg	80.7	74.5
Zn, g/kg	30	28
Mg, g/kg	1.7	1.6
Analyzed Nutrients		
Protein, g/kg	214	185
Zn, g/kg	52.9	64.7
P, g/kg	7.50	5.66
Cu, ppm	25.8	25.9

[1] Supplied per kilogram of diet: vitamin A, 5,511 IU; vitamin D3, 1,102 ICU; vitamin E, 11.02 IU; vitamin B12, 0.01 mg; biotin, 0.11 mg; menadione, 1.1 mg; thiamine, 2.21 mg; riboflavin, 4.41 mg; d-pantothenic acid, 11.02 mg; vitamin B6, 2.21 mg; niacin, 44.09 mg; folic acid, 0.55 mg; choline, 191.36 mg.

[2] Supplied per kilogram of diet: Mn, 80 mg; Fe, 33.3; Cu, 6.7 mg; I, 1.3 mg; Se, 0.2 mg.

Table 5.2: Calculated and Analyzed Zn composition of experimental diets

	Starter (0 - 12 d)		Grower (12 - 23 d)	
Diets	Analyzed (ppm)	Calculated (ppm)	Analyzed (ppm)	Calculated (ppm)
No Zn	52.9	30	64.7	28
ZS[1] 80 mg	121.7	110	126.1	108
ZH 80 mg	117.5	110	115.2	108
ZG 80 mg	134.5	110	146.3	108
ZL 80 mg	115.5	110	136.9	108

[1] ZS: Zn Sulfate (22.7 % Zn); ZH: Zn Hydroxychloride (55% Zn); ZG: Zn Glycinate (26% Zn); ZL: Zn Lysine-Glutamate (17% Zn)

Table 5.3: List of primers used for real-time qPCR of nutrient transporter genes and intestinal barrier functions

Gene name	Forward primer	Reverse primer	Accession number
GAPDH	GAGGGTAGTGAAGGCTGCTG	CCACAACACGGTTGCTGTAT	NM_204305.2
ZnT1	TCCGGGAGTAATGGAAATCTTC	AATCAGGAACAAACCTATGGGAAA	XM_040673965.1
ZnT 5	TGGAGGACCAGCCAAGACAA	TCCCTCAGGATGTTCTGCTATTTT	NM_001031419.2
ZIP8	CTATCATTAACTTGGCATCGCTTCTG	CTACATGGGAATCAAATCCAAATGC	XM_025145573.1
ZIP10	CCACCCTCATCCGCATCAT	TTCTTCTTCTGTTTCCGCTGTTTT	XM_004942587.2
MT	GCTCCTGTGCTGGGTCGTG	CCGGTTCCTTGCAGACACAG	NM_205275.1
NPT2	GGAAGCATTGCTGCGGATT	GCACCCTCCTGTTCTGCATT	AY389468.2
CaSR	GCCAATCTGCTGGGACTCTT	CTGATGCTCGTCATTGGGGA	XM_040661543.2
Calbidin D28k	TTTGATGCAAACAATGATGGA	TTTTGCACACATTTTGACACC	AH003256.2
ALPI	AGTCACTTCTCCCTGACTCTG	GCCTTCTGTGTCCATGAAGC	XM 0.152,9148
SOD1	TGGCTTCCATGTGCATGAAT	AGCACCTGCGCTGGTACAC	NM_205064
GPX1	TCCCCTGCAACCAATTCG	AGCGCAGGATCTCCTCGTT	NM_001277853
Ea-SAG	TGAGTTCCGCACGCAAGA	TCGATGTCTCGGCAACGAA	XM_013394133.1
Muc2	CTGATTGTCACTCACGCCTTAATC	GCCGGCCACCTGCAT	JX284122.1
Notch1	GAGGATCCATCGTCTACTTGGAA	ATCGGTTGCGCTCTGGAA	NM_001030295.1
AvBD10	CAGACCCACTTTTCCCTGACA	CCCAGCACGGCAGAAATT	NM_001001609.2
bo,+AT	CAGTAGTGAATTCTCTGAGTGTGAAGCT	GCAATGATTGCCACAACTACCA	NM_001199133.1
EAAT3	TGCTGCTTTGGATTCCAGTGT	AGCAATGACTGTAGTGCAGAAGTAATATATG	XM_021294088
y+LAT2	GCCCTGTCAGTAAATCAGACAAGA	TTCAGTTGCATTGTGTTTTGGTT	XM_040681086.1
PepT1	CCCCTGAGGAGGATCACTGTT	CAAAAGAGCAGCAGCAACGA	KF366603.1
b 0AT	GGTGAAAGTCAATGAAGAACTG	GCACACCAGCGATGATTA	XM_005509991
GLUT1	CTTTGTCAACCGCTTTGG	CAGAATACAGGCCGATGAT	NM_205209.1
CAT1	CCAAGCACGCTGATAAAG	TACTCACAATAGGAAGAAGGG	XM_005501421
CLDN1	TGGAGGATGACCAGGTGAAGA	CGAGCCACTCTGTTGCCATA	NM_001013611.2
JAM2	AGCCTCAAATGGGATTGGATT	CATCAACTTGCATTCGCTTCA	XM_025149444.1
Occludin	ACGGCAGCACCTACCTCAA	GGCGAAGAAGCAGATGAG	XM_026041453.1
ZO-1	CAACTGGTGTGGGTTTCTGAA	TCACTACCAGGAGCTGAGAGGTAA	XM_015278981.2

Table 5.4: Growth performance response of broiler chickens receiving different sources of Zn and challenged with mixed *Eimeria* spp

		Starter (0-12)			Grower (12-23)			0 to 23		
Challenge	Sources	Gain	FI, g/bird	FCR	Gain	FI, g/bird	FCR	Gain	FI, g/bird	FCR
-	No Zn	259	352	1.36	449	871	1.95	707	1223	1.73
+		271	363	1.34	360	821	2.29	631	1184	1.88
-	ZS[1] 80 mg	251	354	1.42	465	873	1.88	715	1227	1.73
+		255	350	1.37	345	867	2.53	600	1217	2.03
-	ZH 80 mg	282	377	1.34	462	902	1.95	743	1279	1.72
+		255	338	1.32	346	843	2.67	601	1181	2.00
-	ZG 80 mg	248	331	1.34	480	935	1.95	728	1266	1.74
+		231	318	1.38	383	809	2.13	614	1127	1.84
-	ZL 80 mg	267	373	1.40	501	954	1.91	768	1327	1.73
+		272	340	1.25	394	868	2.22	666	1208	1.80
	SEM									
Source	No Zn	265	358	1.35	404[ab]	846	2.12	669	1204	1.81
	ZS 80 mg	257	357	1.39	397[b]	858	2.21	655	1215	1.87
	ZH 80 mg	268	357	1.33	403[ab]	872	2.31	672	1230	1.86
	ZG 80 mg	239	325	1.36	431[ab]	872	2.04	671	1196	1.79
	ZL 80 mg	267	351	1.31	451[a]	910	2.04	718	1260	1.76
	SEM	10.0	14.5	0.027	15.4	24.5	0.111	18.6	29.3	0.045
Challenge	-	263	359	1.37	468	902	1.93	731	1262	1.73
	+	256	340	1.33	367	841	2.36	623	1181	1.91
	SEM	6.47	9.3	0.017	9.46	15.8	0.072	12.0	18.9	0.027
						P value				
	Source	0.246	0.437	0.355	0.045	0.474	0.3324	0.1171	0.495	0.342
	Challenge	0.424	0.142	0.115	<.0001	0.008	<.0001	<.0001	0.004	<.0001
	Source*challenge	0.701	0.683	0.107	0.975	0.355	0.3732	0.802	0.394	0.171

[a,b] Means with different superscripts within the same column differ significantly ($P < 0.05$).

[1] ZS: Zn Sulfate (22.7 % Zn); ZH: Zn Hydroxychloride (55% Zn); ZG: Zn Glycinate (26% Zn); ZL: Zn Lysine-Glutamate (17% Zn)

Table 5.5: Bone response of broiler chickens receiving different sources of Zn and challenged with mixed *Eimeria* spp

Challenge	Sources	Tibia (g)	Tibia ash (%)	Ash weight (g)	Tibia Zn (ppm)
-	No Zn	2.10	42	0.749	377.2
+		1.79	43	0.752	311.3
-	ZS[1] 80 mg	1.74	42.2	0.837	369.3
+		1.75	42.9	0.816	330.7
-	ZH 80 mg	1.99	42.3	0.855	366.8
+		1.85	43.3	0.779	334.4
-	ZG 80 mg	1.95	42.0	0.828	395.2
+		1.76	44.1	0.742	352.8
-	ZL 80 mg	2.01	42.9	0.878	432.3
+		1.85	42.7	0.797	328.0
	SEM	0.085	0.617	0.749	16.3
Source	No Zn	1.95	42.6	0.751	344.3
	ZS 80 mg	1.75	42.6	0.826	350.0
	ZH 80 mg	1.92	42.8	0.817	350.6
	ZG 80 mg	1.86	43.1	0.785	374.0
	ZL 80 mg	1.93	42.8	0.837	380.2
	SEM	0.060	0.436	0.026	11.5
Challenge	-	1.96	42.4	0.829	388.2
	+	1.80	43.1	0.777	331.4
	SEM	0.038	0.276	0.017	7.30
				P value	
	Source	0.131	0.930	0.143	0.116
	Challenge	0.006	0.059	0.031	<.0001
	Source*challenge	0.470	0.444	0.6749	0.180

[1] ZS: Zn Sulfate (22.7 % Zn); ZH: Zn Hydroxychloride (55% Zn); ZG: Zn Glycinate (26% Zn); ZL: Zn Lysine-Glutamate (17% Zn)

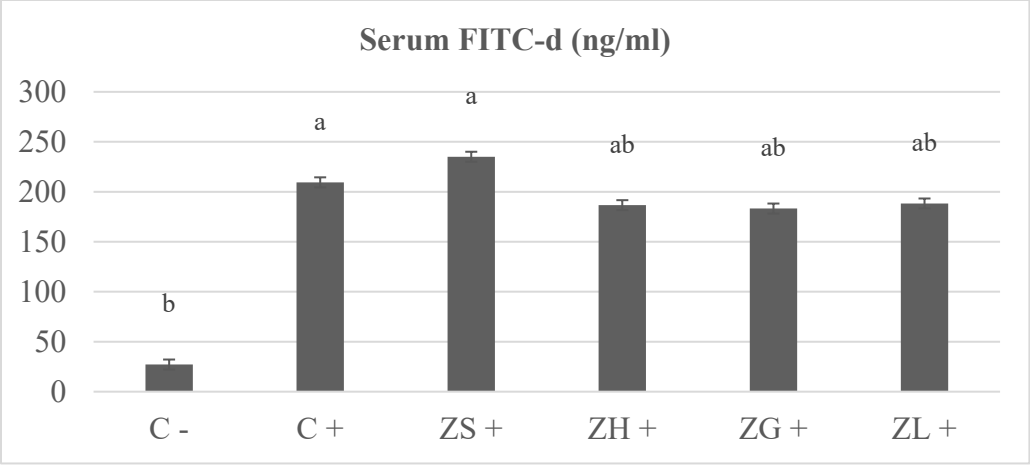
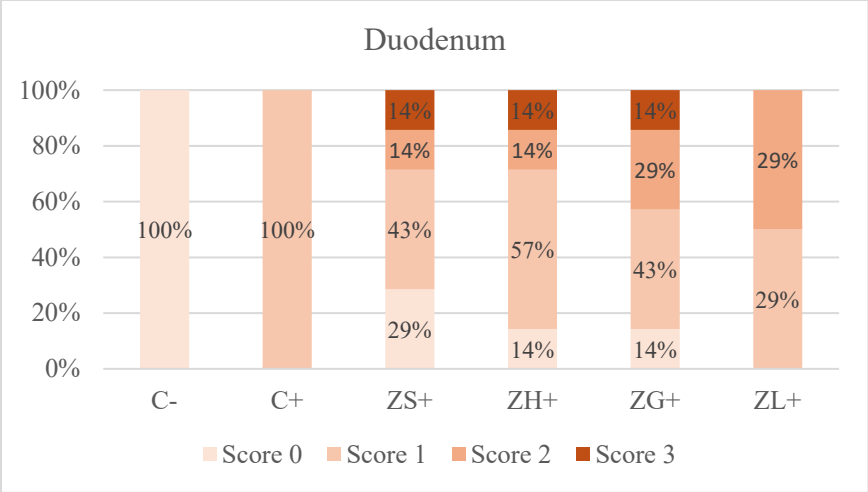


Figure 5.1: Fluorescein isothiocyanate dextran concentration (FITC-d, µg/mL) in serum of broiler chickens in response to *Eimeria* challenge and feeding with different sources of Zn. ZS: Zn Sulfate (80 mg/kg Zn); ZH: Zn Hydroxychloride (80 mg/kg Zn); ZG: Zn Glycinate (80 mg/kg Zn); ZL: Zn Lysine-Glutamate (80 mg/kg Zn)


Duodenum
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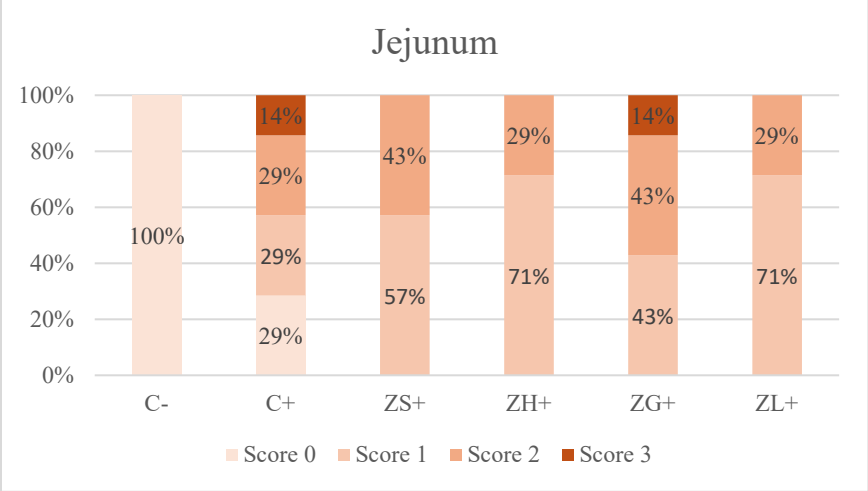
Jejunum
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ZH+
ZG+
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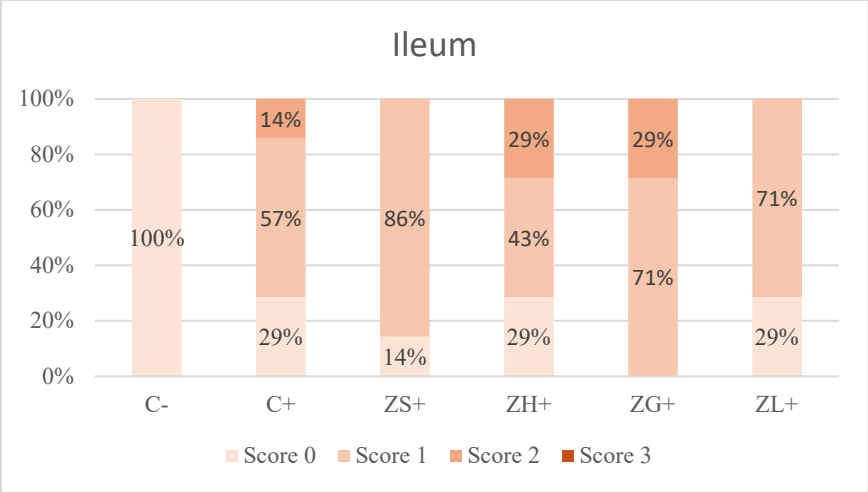
Score 0
Score 1
Score 2
Score 3



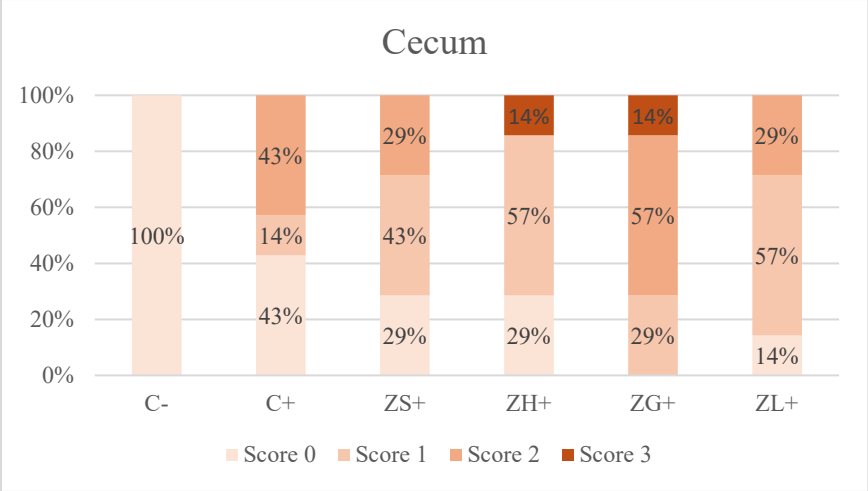


Figure 5.2: Lesion scores in the duodenum, jejunum, ileum, and ceca of *Eimeria*-challenged broiler chicken in response to different dietary Zn sources supplementation (6-dpi). ZS: Zn Sulfate (80 mg/kg Zn); ZH: Zn Hydroxychloride (80 mg/kg Zn); ZG: Zn Glycinate (80 mg/kg Zn); ZL: Zn Lysine-Glutamate (80 mg/kg Zn).

Table 5.6: Intestinal histology of broiler chickens receiving different sources of Zn and challenged with mixed *Eimeria* spp

		Duodenum			Jejunum			Ileum		
Challenge	Sources	CD	VH	VH:CD	CD	VH	VH:CD	CD	VH	VH:CD
-	No Zn	106.5	2080.4	19.7	121.3^{d}	1081.5	9.11	104.9	982.0	9.42
+		321.1	1396.0	4.57	170.9^{bc}	827.9	4.99	346.9	792.8	2.30
-	ZS[1] 80 mg	98.1	2128.0	22.0	112.1^{d}	964.6	8.67	114.1	1304.7	11.4
+		337.9	1369.1	4.16	154.6^{c}	898.8	5.87	338.1	788.1	2.43
-	ZH 80 mg	114.6	2043.0	19.4	115.5^{d}	1004.1	8.81	117.7	1059.0	8.97
+		373.9	1450.7	4.04	178.0^{bc}	887.0	5.04	350.7	696.3	1.99
-	ZG 80 mg	97.8	2016.3	21.0	111.7^{d}	1062.7	9.73	98.7	926.8	9.41
+		359.2	1445.9	4.32	196.6^{ab}	879.7	4.71	341.4	757.7	2.45
-	ZL 80 mg	95.8	2091.1	22.0	106.9^{d}	1086.0	10.5	105.8	900.8	8.73
+		356.1	1376.8	3.87	220.2^{a}	918.9	4.25	281.7	704.2	2.60
	SEM	18.3	76.1	0.99	11.1	68.1	0.602	17.8	78.5	0.633
Source	No Zn	213.8	1738.2	12.2	146.1	954.7	7.05	225.9	887^{ab}	5.86
	ZS 80 mg	218.0	1748.6	13.1	133.4	931.7	7.27	226.1	1046^{a}	6.91
	ZH 80 mg	244.3	1746.9	11.7	146.8	945.6	6.92	234.2	877^{ab}	5.48
	ZG 80 mg	228.5	1731.1	12.7	154.2	971.2	7.22	220.1	842^{ab}	5.93
	ZL 80 mg	226.0	1733.9	12.9	163.6	1002.4	7.36	193.7	802^{b}	5.66
	SEM	13.0	53.8	0.697	7.87	48.2	0.426	12.6	55.5	0.447
Challenge	-	102.6	2071.8	20.8	113.6	1039.8	9.36	108.2	1034.6	9.59
	+	349.6	1407.7	4.19	184.1	882.5	4.97	331.7	747.8	2.35
	SEM	8.19	34.0	0.441	4.97	30.5	0.269	7.94	35.1	0.283
						P value				
	Source	0.515	0.999	0.629	0.106	0.864	0.953	0.208	0.035	0.205
	Challenge	<.0001	<.0001	<.0001	<.0001	0.001	<.0001	<.0001	<.0001	<.0001
	Source*challenge	0.657	0.694	0.452	0.016	0.708	0.067	0.316	0.137	0.249

[a,b,c,d] Means with different superscripts within the same column differ significantly ($P < 0.05$).

[1] ZS: Zn Sulfate (22.7 % Zn); ZH: Zn Hydroxychloride (55% Zn); ZG: Zn Glycinate (26% Zn); ZL: Zn Lysine-Glutamate (17% Zn)

Table 5.7: Short chain fatty acids response of broiler chickens receiving different sources of Zn and challenged with mixed *Eimeria* spp

Challenge	Sources	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	SCFA	BCFA
-	No Zn	50.8	3.10	0.782	7.20^{b}	0.710	0.827^{b}	61.9^{ab}	1.49
+		66.9	7.91	1.47	16.07^{ab}	2.09	1.62^{a}	92.4^{a}	3.57
-	ZS[1] 80 mg	52.0	2.45	0.708	10.06^{b}	0.635	0.759^{b}	65.3^{ab}	1.34
+		64.5	5.15	1.36	21.4^{a}	1.98	1.61^{a}	92.7^{a}	3.34
-	ZH 80 mg	55.4	3.10	0.656	10.1^{b}	0.649	0.917^{b}	69.4^{ab}	1.31
+		44.5	4.98	0.643	7.55^{b}	1.02	0.762^{b}	57.8^{ab}	1.67
-	ZG 80 mg	67.0	2.72	0.703	15.2^{ab}	0.619	1.02^{ab}	85.1^{ab}	1.32
+		50.2	5.20	1.18	9.28^{b}	1.74	1.21^{ab}	65.8^{ab}	2.91
-	ZL 80 mg	54.5	3.20	0.902	8.34^{b}	0.687	0.763^{b}	66.8^{ab}	1.59
+		42.8	3.92	0.955	7.63^{b}	1.54	1.104^{ab}	55.4^{b}	2.50
	SEM	6.52	0.658	0.175	3.54	0.236	0.167	9.08	0.407
Source	No Zn	58.8	5.50	1.13	11.6	1.40	1.23	77.2	2.53
	ZS 80 mg	58.3	3.80	1.03	15.8	1.31	1.18	79.0	2.34
	ZH 80 mg	50.0	4.04	0.649	8.80	0.837	0.840	63.7	1.49
	ZG 80 mg	58.6	3.96	0.941	12.2	1.18	1.11	75.9	2.12
	ZL 80 mg	48.7	3.56	0.928	7.99	1.11	0.934	61.2	2.04
	SEM	5.13	0.558	0.149	2.193	0.201	0.141	7.70	0.346
Challenge	-	56.0	2.91	0.750	10.2	0.660	0.858	69.9	1.41
	+	53.8	5.43	1.121	12.4	1.675	1.262	72.9	2.80
	SEM	2.91	0.294	0.080	1.156	0.108	0.074	4.061	0.186
					P value				
	Source	0.514	0.112	0.254	0.096	0.254	0.204	0.326	0.199
	Challenge	0.268	0.0002	0.015	0.557	<.0001	0.020	0.768	<.0001
	Source*challenge	0.083	0.130	0.284	0.022	0.323	0.041	0.038	0.310

[a,b] Means with different superscripts within the same column differ significantly ($P < 0.05$).

[1] ZS: Zn Sulfate (22.7 % Zn); ZH: Zn Hydroxychloride (55% Zn); ZG: Zn Glycinate (26% Zn); ZL: Zn Lysine-Glutamate (17% Zn)

Table 5.8: Mineral digestibility response of broiler chickens receiving different sources of Zn and challenged with mixed *Eimeria* spp

Challenge	Sources	DM Dig (%)	N Dig (%)	Zn Dig (%)	P Dig (%)	Cu Dig (%)	marker-corrected mg/100g DM intake	
							Zn	Cu
-	No Zn	0.658	0.738	-0.284	0.343	-0.866	92.3	53.7
+		0.430	0.377	-0.042	0.293	-0.775	74.6	50.8
-	ZS 80 mg	0.713	0.785	-0.042	0.421	-0.563	145.5	45.8
+		0.368	0.388	-0.086	0.333	-0.802	151.3	52.6
-	ZH 80 mg	0.717	0.797	-0.103	0.482	-0.455	141.1	44.9
+		0.431	0.391	0.067	0.421	-0.392	118.8	42.8
-	ZG 80 mg	0.731	0.787	0.348	0.528	-0.398	128.8	40.6
+		0.486	0.423	0.458	0.388	-0.043	107.0	30.3
-	ZL 80 mg	0.721	0.788	0.091	0.510	-0.209	137.8	39.3
+		0.499	0.401	0.207	0.377	0.072	120.3	30.2
	SEM	0.039	0.038	0.094	0.088	4.966	13.3	5.66
Source	No Zn	0.544	0.557	-0.163	0.317[b]	-0.820	83.4[b]	52.2[ab]
	ZS 80 mg	0.541	0.586	-0.064	0.376[ab]	-0.682	148.4[a]	49.2[a]
	ZH 80 mg	0.574	0.594	-0.018	0.451[a]	-0.423	129.9[a]	43.8[ab]
	ZG 80 mg	0.609	0.605	0.403	0.458[a]	-0.220	117.8[ab]	35.4[b]
	ZL 80 mg	0.610	0.595	0.149	0.443[a]	-0.068	129.0[a]	34.7[b]
	SEM	0.028	0.026	0.066	0.060	3.370	9.84	4.18
Challenge	-	0.708	0.779	0.002	0.457	-0.498	129.1	44.9
	+	0.443	0.396	0.121	0.362	-0.388	114.4	41.3
	SEM	0.018	0.016	0.043	0.037	2.076	6.059	2.578
					P value			
	Source	0.250	0.704	<.0001	0.011	0.361	0.0002	0.049
	Challenge	<.0001	<.0001	0.052	0.395	0.272	0.121	0.737
	Source*challenge	0.514	0.956	0.624	0.230	0.315	0.830	0.404

[a,b] Means with different superscripts within the same column differ significantly ($P < 0.05$).

[1] ZS: Zn Sulfate (22.7 % Zn); ZH: Zn Hydroxychloride (55% Zn); ZG: Zn Glycinate (26% Zn); ZL: Zn Lysine-Glutamate (17% Zn)

Table 5.9: Gene expression in the duodenum of 23-day-old broiler chickens at 6-day post challenge receiving different sources of Zn and challenged with mixed *Eimeria* spp

Challenge	Sources	ZnT1	ZnT5	ZIP8	ZIP10	MUC2	AVBD10	Ea SaG	Notch1
-	No Zn	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
+		0.572	1.17	1.04	1.10	0.851	0.622	106.7	1.22
-	ZS[1] 80 mg	0.946	0.817	0.879	0.745	0.686	1.69	0.66	1.38
+		0.519	1.11	0.987	1.04	1.05	1.16	72.8	1.27
-	ZH 80 mg	0.835	0.841	0.831	1.03	0.915	0.914	0.42	1.03
+		0.496	1.18	1.36	1.27	0.923	1.18	106.4	1.33
-	ZG 80 mg	0.910	0.843	0.932	0.710	0.914	0.946	1.14	0.95
+		0.499	1.23	1.07	0.998	1.01	0.650	56.5	1.31
-	ZL 80 mg	1.06	1.02	1.32	1.07	0.900	1.16	0.59	1.06
+		0.600	1.26	1.32	0.970	0.849	0.865	103.0	1.01
	SEM	0.081	0.076	0.160	0.193	0.103	0.284	24.8	0.20
Source	No Zn	0.786	1.09	1.02	1.05	0.926	0.811	53.8	1.11
	ZS 80 mg	0.733	0.964	0.933	0.895	0.865	1.42	36.7	1.32
	ZH 80 mg	0.666	1.01	1.09	1.15	0.919	1.05	53.4	1.18
	ZG 80 mg	0.705	1.04	1.00	0.854	0.964	0.798	28.8	1.13
	ZL 80 mg	0.832	1.14	1.32	1.02	0.874	1.01	51.8	1.04
	SEM	0.058	0.054	0.118	0.137	0.073	0.200	17.6	0.150
Challenge	-	0.951	0.904	0.993	0.909	0.883	1.14	0.763	1.08
	+	0.537	1.19	1.16	1.08	0.936	0.896	89.1	1.23
	SEM	0.036	0.034	0.074	0.088	0.046	0.128	11.1	0.092
						P value			
	Source	0.277	0.192	0.160	0.556	0.873	0.175	0.847	0.689
	Challenge	<.0001	<.0002	0.108	0.173	0.416	0.163	<.0001	0.254
	Source*challenge	0.957	0.659	0.489	0.821	0.148	0.603	0.757	0.706

[1] ZS: Zn Sulfate (22.7 % Zn); ZH: Zn Hydroxychloride (55% Zn); ZG: Zn Glycinate (26% Zn); ZL:Zn Lysine-Glutamate (17% Zn)

Table 5.10: Gene expression in the jejunum of 23-day-old broiler chickens at 6-day post challenge receiving different sources of Zn and challenged with mixed *Eimeria* spp

Challenge	Sources	ZnT1	ZnT5	ZIP8	ZIP10	NPT2	CaSR	CalbidinD28
-	No Zn	1.00	1.00	1.00	1.00	0.70	1.00	1.00
+		0.353	1.14	1.66	0.558	0.400	0.437	0.128
-	ZS[1] 80 mg	0.975	0.748	0.857	0.849	1.08	0.753	0.704
+		0.353	1.29	2.17	0.634	0.330	0.436	0.129
-	ZH 80 mg	0.773	0.733	0.968	0.775	0.980	0.797	0.768
+		0.302	1.19	2.42	0.503	0.387	0.582	0.210
-	ZG 80 mg	0.809	0.743	0.692	0.703	1.12	1.09	0.953
+		0.354	1.14	1.65	0.515	0.301	0.338	0.068
-	ZL 80 mg	0.943	0.750	0.759	0.623	0.772	1.01	0.788
+		0.275	1.23	2.24	0.609	0.313	0.409	0.224
	SEM	0.092	1.00	0.214	0.116	0.195	0.139	0.192
Source	No Zn	0.677	1.07	1.33	0.779	0.550	0.719	0.564
	ZS 80 mg	0.664	1.02	1.51	0.741	0.704	0.595	0.417
	ZH 80 mg	0.538	0.962	1.70	0.639	0.684	0.689	0.489
	ZG 80 mg	0.581	0.941	1.17	0.609	0.708	0.716	0.511
	ZL 80 mg	0.609	0.991	1.50	0.616	0.543	0.708	0.506
	SEM	0.065	0.062	0.152	0.082	0.144	0.098	0.142
Challenge	-	0.900	0.795	0.855	0.790	0.929	0.930	0.843
	+	0.327	1.20	2.03	0.564	0.346	0.440	0.152
	SEM	0.042	0.040	0.096	0.052	0.095	0.063	0.089
					P value			
	Source	0.633	0.601	0.222	0.434	0.841	0.919	0.987
	Challenge	<.0001	<.0001	<.0001	0.003	<.0001	<.0001	<.0001
	Source*challenge	0.674	0.231	0.234	0.481	0.719	0.321	0.707

[1] ZS: Zn Sulfate (22.7 % Zn); ZH: Zn Hydroxychloride (55% Zn); ZG: Zn Glycinate (26% Zn); ZL: Zn Lysine-Glutamate (17% Zn)

Table 5.11: Gene expression in the jejunum of 23-day-old broiler chickens at 6-day post challenge receiving different sources of Zn and challenged with mixed *Eimeria* spp

Challenge	Sources	CAT1	B0AT	PEPT1	EAAT3	b0+AT	GLUT	Y+LAT2
-	No Zn	1.00	1.00	1.00	1.00[a]	1.00	1.00	1.00
+		4.08	0.959	0.510	0.448[ab]	0.914	3.13	0.429
-	ZS[1] 80 mg	1.06	0.575	0.527	0.355[b]	0.678	0.685	0.795
+		7.58	1.26	0.612	0.809[ab]	1.09	6.45	0.411
-	ZH 80 mg	1.16	0.638	0.734	0.645[ab]	0.758	0.570	0.749
+		4.79	1.17	0.635	0.725[ab]	0.869	3.72	0.567
-	ZG 80 mg	0.736	0.831	1.06	0.938[ab]	0.851	0.631	0.560
+		2.82	0.912	0.455	0.526[ab]	0.814	3.19	0.389
-	ZL 80 mg	0.913	0.570	0.629	0.619[ab]	0.636	1.051	0.454
+		4.42	1.35	0.781	0.862[ab]	0.904	4.76	0.406
	SEM	0.751	0.188	0.209	0.181	0.150	0.742	0.113
Source	No Zn	2.53[ab]	0.979	0.755	0.724	0.957	2.06[ab]	0.714
	ZS 80 mg	4.31[a]	0.917	0.569	0.583	0.883	3.56[a]	0.603
	ZH 80 mg	2.97[ab]	0.902	0.684	0.685	0.814	2.14[ab]	0.658
	ZG 80 mg	1.77[b]	0.872	0.758	0.733	0.832	1.90[b]	0.475
	ZL 80 mg	2.66[ab]	0.959	0.705	0.741	0.770	2.90[ab]	0.430
	SEM	0.556	0.139	0.155	0.123	0.106	0.550	0.087
Challenge	-	0.974	0.723	0.790	0.712	0.785	0.787	0.712
	+	4.74	1.13	0.598	0.674	0.918	4.25	0.441
	SEM	0.348	0.087	0.093	0.092	0.069	0.359	0.056
					P value			
	Source	0.026	0.974	0.929	0.946	0.749	0.038	0.134
	Challenge	<.0001	0.002	0.106	0.648	0.195	<.0001	0.003
	Source*challenge	0.099	0.144	0.284	0.050	0.520	0.215	0.195

[a,b] Means with different superscripts within the same column differ significantly ($P < 0.05$).

[1] ZS: Zn Sulfate (22.7 % Zn); ZH: Zn Hydroxychloride (55% Zn); ZG: Zn Glycinate (26% Zn); ZL: Zn Lysine-Glutamate (17% Zn)

Table 5.12: Gene expression in the jejunum of 23-day-old broiler chickens at 6-day post challenge receiving different sources of Zn and challenged with mixed *Eimeria* spp

Challenge	Sources	JAM2F	CLDN1	Occludin	ZO-1
-	No Zn	0.69	1.00	1.00	1.00
+		0.875	9.72	0.604	0.573
-	ZS[1] 80 mg	0.669	1.10	0.751	0.561
+		1.40	14.22	0.662	0.631
-	ZH 80 mg	0.805	1.21	0.745	0.582
+		1.33	7.00	0.756	0.528
-	ZG 80 mg	0.519	1.16	0.860	0.668
+		1.01	5.91	0.752	0.474
-	ZL 80 mg	0.734	0.861	0.750	0.509
+		1.14	8.98	0.685	0.652
	SEM	0.222	1.84	0.115	0.157
Source	No Zn	0.783	5.36	0.802	0.787
	ZS 80 mg	1.03	7.66	0.706	0.596
	ZH 80 mg	1.07	4.11	0.750	0.555
	ZG 80 mg	0.767	3.54	0.806	0.571
	ZL 80 mg	0.937	4.92	0.717	0.580
	SEM	0.150	1.36	0.085	0.116
Challenge	-	0.683	1.07	0.821	0.664
	+	1.15	9.17	0.692	0.572
	SEM	0.100	0.932	0.055	0.077
			P value		
	Source	0.619	0.307	0.919	0.626
	Challenge	0.001	<.0001	0.093	0.333
	Source*challenge	0.859	0.368	0.557	0.519

[1] ZS: Zn Sulfate (22.7 % Zn); ZH: Zn Hydroxychloride (55% Zn); ZG: Zn Glycinate (26% Zn); ZL: Zn Lysine-Glutamate (17% Zn)

Table 5.13: Gene expression in the ileum of 23-day-old broiler chickens at 6-day post challenge receiving different sources of Zn and challenged with mixed *Eimeria* spp

Challenge	Sources	ZnT1	ZnT5	ZIP8	ZIP10	JAM2F	CLDN1	Occludin	ZO-1
-	No Zn	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
+		0.968	0.963	0.808	0.795	0.751	2.15	0.748	0.739
-	ZS[1] 80 mg	0.708	0.885	1.28	0.903	0.846	0.989	0.831	0.786
+		1.03	1.13	0.921	0.549	0.618	1.97	0.771	0.830
-	ZH 80 mg	0.873	0.929	1.34	0.840	1.04	1.15	0.844	0.942
+		0.880	1.07	1.40	0.615	0.859	2.31	0.746	0.840
-	ZG 80 mg	0.807	0.899	1.16	0.558	1.03	1.26	0.896	0.877
+		1.28	1.18	1.13	0.527	0.891	1.49	0.856	0.843
-	ZL 80 mg	1.03	0.975	1.23	0.733	1.07	1.03	1.00	1.04
+		1.19	1.30	1.70	0.697	0.975	2.83	0.914	0.979
	SEM	0.148	0.101	0.185	0.159	0.134	0.645	0.089	0.096
Source	No Zn	0.984	0.982	0.903b	0.898	0.875	1.58	0.874	0.869
	ZS 80 mg	0.868	1.01	1.09ab	0.726	0.732	1.48	0.801	0.808
	ZH 80 mg	0.876	0.997	1.367ab	0.728	0.948	1.73	0.795	0.891
	ZG 80 mg	1.05	1.04	1.14ab	0.542	0.958	1.37	0.876	0.860
	ZL 80 mg	1.11	1.14	1.462a	0.715	1.02	1.93	0.957	1.01
	SEM	0.102	0.062	0.114	0.098	0.095	0.456	0.063	0.068
Challenge	-	0.883	0.938	1.20	0.807	0.996	1.09	0.914	0.929
	+	1.07	1.13	1.19	0.637	0.819	2.15	0.807	0.846
	SEM	0.078	0.033	0.060	0.072	0.060	0.289	0.040	0.043
						P value			
	Source	0.397	0.464	0.015	0.066	0.253	0.921	0.359	0.357
	Challenge	0.057	0.012	0.507	0.037	0.041	0.012	0.063	0.184
	Source*challenge	0.337	0.339	0.143	0.811	0.978	0.823	0.776	0.592

[a,b] Means with different superscripts within the same column differ significantly ($P < 0.05$).

[1] ZS: Zn Sulfate (22.7 % Zn); ZH: Zn Hydroxychloride (55% Zn); ZG: Zn Glycinate (26% Zn); ZL: Zn Lysine-Glutamate (17% Zn)

Table 5.14: Gene expression in the liver of 23-day-old broiler chickens at 6-day post challenge receiving different sources of Zn and challenged with mixed *Eimeria* spp

Challenge	Sources	SOD	ZnT1	ALP1	CAT1	EAAT3	PEPT1
-	No Zn	1.00	1.00	1^{b}	1.00	1.00	0.993
+		0.962	1.11	0.175^{c}	1.19	0.858	1.35
-	ZS[1] 80 mg	0.933	1.11	0.722^{b}	1.75	1.06	0.298
+		1.08	1.02	0.224^{c}	1.72	0.989	0.805
-	ZH 80 mg	0.925	1.19	0.824^{b}	1.41	0.936	0.324
+		1.18	1.22	0.291^{c}	0.972	0.949	1.67
-	ZG 80 mg	1.06	1.16	0.78^{b}	1.11	0.972	0.526
+		1.18	0.747	0.229^{c}	0.857	1.26	1.34
-	ZL 80 mg	1.61	0.804	1.40^{a}	0.677	0.767	0.563
+		1.15	0.989	0.262^{c}	0.774	0.859	1.91
	SEM	0.177	0.166	0.111	0.247	0.133	0.380
Source	No Zn	0.981	1.06	0.587^{ab}	1.09^{ab}	0.929	1.17
	ZS 80 mg	1.01	1.07	0.473^{b}	1.73^{a}	1.02	0.552
	ZH 80 mg	1.05	1.21	0.557^{ab}	1.19^{ab}	0.943	0.996
	ZG 80 mg	1.12	0.953	0.505^{b}	0.985^{b}	1.12	0.935
	ZL 80 mg	1.38	0.896	0.832^{ab}	0.725^{b}	0.813	1.24
	SEM	0.120	0.123	0.079	0.175	0.098	0.298
Challenge	-	1.11	1.05	0.946	1.19	0.946	0.541
	+	1.11	1.02	0.237	1.10	0.984	1.42
	SEM	0.073	0.076	0.050	0.113	0.059	0.17
					P value		
	Source	0.124	0.418	0.019	0.003	0.262	0.455
	Challenge	0.980	0.728	<.0001	0.650	0.887	0.003
	Source*challenge	0.219	0.460	0.026	0.678	0.581	0.674

b,c Means with different superscripts within the same column differ significantly ($P < 0.05$).

[1] ZS: Zn Sulfate (22.7 % Zn); ZH: Zn Hydroxychloride (55% Zn); ZG: Zn Glycinate (26% Zn); ZL: Zn Lysine-Glutamate (17% Zn)

Table 5.15: Gene expression in the kidney of 23-day-old broiler chickens at 6-day post challenge receiving different sources of Zn and challenged with mixed *Eimeria* spp

Challenge	Sources	MT	GPX	SOD	ZnT1	ALP1	Calbidin28
-	No Zn	1.00	1.00	1.00	1.00	1.00	1.00
+		1.45	1.22	0.694	0.650	0.437	1.72
-	ZS[1] 80 mg	1.03	1.53	1.25	1.09	1.14	1.62
+		2.37	1.17	0.614	0.633	0.489	1.21
-	ZH 80 mg	0.766	0.893	1.02	0.950	0.930	1.41
+		2.04	1.13	0.718	0.690	0.481	0.986
-	ZG 80 mg	0.844	0.978	0.927	1.00	0.884	1.32
+		1.91	1.26	0.837	0.663	0.560	2.00
-	ZL 80 mg	1.57	0.977	0.973	0.949	1.15	1.59
+		2.48	1.27	0.783	0.671	0.615	1.37
	SEM	0.313	0.167	0.101	0.067	0.119	0.297
Source	No Zn	1.22	1.11	0.85	0.82	0.72	1.36
	ZS 80 mg	1.70	1.35	0.93	0.86	0.82	1.41
	ZH 80 mg	1.40	1.01	0.87	0.82	0.71	1.20
	ZG 80 mg	1.38	1.12	0.88	0.83	0.72	1.66
	ZL 80 mg	2.02	1.13	0.88	0.81	0.88	1.48
	SEM	0.24	0.12	0.07	0.05	0.08	0.21
Challenge	-	1.04	1.07	1.03	0.998	1.02	1.39
	+	2.05	1.21	0.729	0.661	0.516	1.46
	SEM	0.149	0.076	0.046	0.031	0.054	0.136
					P value		
	Source	0.187	0.489	0.936	0.956	0.473	0.648
	Challenge	<.0001	0.153	<.0001	<.0001	<.0001	0.644
	Source*challenge	0.432	0.334	0.129	0.685	0.757	0.144

[1] ZS: Zn Sulfate (22.7 % Zn); ZH: Zn Hydroxychloride (55% Zn); ZG: Zn Glycinate (26% Zn); ZL: Zn Lysine-Glutamate (17% Zn)

Table 5.16: Gene expression in the pancreas of 23-day-old broiler chickens at 6-day post challenge receiving different sources of Zn and challenged with mixed *Eimeria* spp

Challenge	Sources	MT	GPX	SOD
-	No Zn	1.00	1.00	1.00
+		2.03	0.936	0.914
-	ZS[1] 80 mg	0.747	1.14	0.784
+		2.57	0.986	0.785
-	ZH 80 mg	1.39	1.26	1.21
+		3.13	0.936	0.717
-	ZG 80 mg	0.956	1.05	0.993
+		2.58	1.04	0.831
-	ZL 80 mg	0.841	1.24	0.933
+		3.24	1.09	0.612
	SEM	0.529	0.335	0.190
Source	No Zn	1.51	0.968	0.957
	ZS 80 mg	1.66	1.06	0.785
	ZH 80 mg	2.26	1.10	0.965
	ZG 80 mg	1.77	1.05	0.912
	ZL 80 mg	2.04	1.17	0.773
	SEM	0.391	0.241	0.134
Challenge	-	0.987	1.14	0.985
	+	2.71	0.999	0.772
	SEM	0.249	0.140	0.092
			P value	
	Source	0.684	0.988	0.775
	Challenge	<.0001	0.603	0.106
	Source*challenge	0.822	0.994	0.720

[1] ZS: Zn Sulfate (22.7 % Zn); ZH: Zn Hydroxychloride (55% Zn); ZG: Zn Glycinate (26% Zn); ZL: Zn Lysine-Glutamate (17% Zn)

CHAPTER 6

6. CONCLUSION

Much research exists regarding Zn and its importance to growth performance and disease mitigation. Coccidiosis is a frequent, costly, and potentially fatal disease in poultry farms caused by protozoan parasites of *Eimeria*. Mineral additives like Zn may help birds with coccidiosis by enhancing intestinal integrity and reducing inflammation. Therefore, this dissertation aims to assess the relative bioavailability of Zn, broiler response to different sources and levels of Zn, and the response of broiler chickens challenged with *Eimeria* to diets supplemented with Zn from various sources.

The first study evaluates the relative bioavailability of inorganic and organic zinc sources for weight gain in broiler chickens. In this study, body weight gain was regressed against Zn intake, and the ratio of the slopes was used to calculate the relative bioavailability of Zn for weight gain. The relative bioavailability values were 107%, 76%, and 127% for Zn-hydrochloride, Zn-Glycinate, and Zn-Lys-Glu sources, respectively, relative to Zn sulfate. There was also a quadratic increase ($P < 0.05$) in tibia and toe ash percentage of chicks fed Zn-Lysine. It was concluded that organic sources of ZL had a higher RBV than ZS.

The second study in this dissertation evaluated the effect of different organic or inorganic Zn sources at different levels on performance, mineral digestibility, or intestine histomorphology using a practical diet. Moreover, the mRNA expressions of Zn and AA transporters in jejunum, antioxidant enzymes in the liver, and *in-vitro* immune responses of chickens were investigated as possible modes of action . The data showed that Zn source, rather than level, had a greater influence on responses reported herein regarding mineral digestibility. It was shown that Zn transporters

were downwardly expressed at the brush border in response to high luminal Zn concentration possibly as a means to decrease the Zn influx into the cytosol and to help maintain Zn homeostasis.

In the final study, the effect of four Zn sources on broiler performance, nutrient transporters, intestine histomorphology, cecal short-chain fatty acids, bone mineralization, and gut permeability under a coccidiosis challenge were investigated. The data showed that ZL helped ameliorate coccidiosis-induced decrease in weight gain and ALP1 expression in the liver. In addition, ZS increases VH in the ileum, SCFA, and Marker-corrected concentration (mcC) of Cu, CAT1, and GLUT1 mRNA expression.

With the findings of this study and previous work, it is evident that organic trace minerals of ZL can benefit physiological responses like growth, bone, immunology, and nutrient utilization in broiler chickens. Due to the high cost of organic minerals as a replacement for inorganic sources, additional research is required to determine precise mineral levels that may aid in the poultry industry. Also, complementary research needs to be done to consider organic Zn supplementation in bird markets aged 35–42 days.