EFFECTS OF ZINC ON NECROTIC ENTERITIS IN BROILER CHICKENS

by

THEROS T. NG

(Under the Direction of Ramesh Selvaraj)

ABSTRACT

Three studies were conducted to determine the effects of zinc glycinate on the immune response and growth performance in broiler chickens. The objective of this thesis is to provide insight into the optimal zinc concentration in poultry diets with the focus on supporting the immunity of chickens against diseases. In the first study, broiler chickens were supplemented with two zinc sources, zinc sulfate (ZS) and zinc glycinate (ZG), and three concentrations of supplemental zinc at 40, 80, and 120 mg/kg in a 2 x 3 factorial arrangement. The results indicate that zinc concentration above 120 mg/kg improves the growth performance of broiler chickens and that the growth advantage is accompanied by improvements in intestinal morphology and modulation in immunity through cytokine production. In the second study, broiler chickens were supplemented with 40, 80, and 120 mg/kg of ZG in experiment 1 and supplemented with 100, 120, 140, and 160 mg/kg of ZG in experiment 2. The chickens were experimentally infected with necrotic enteritis by oral gavage of 5,000 oocysts of *Eimeria maxima* on day 14 and 10^8 CFU of *Clostridium perfringens* on day 19, 20, and 21. The results indicate that the alleviation of necrotic enteritis in chickens by ZG at 120 mg/kg was not mediated through the adaptive immunity. In the third study, the effects of zinc on *Clostridium perfringens*, chicken intestinal cells, and chicken macrophages (HD-11) were investigated *in vitro*. The results indicate that zinc concentration at

100 μM is beneficial to chicken intestinal cells and macrophages regardless of the zinc source. Taken together, zinc concentration in broiler chicken diets should be supplementaed at 120 mg/kg, and the inclusion of zinc at 100 μM is beneficial to ensure the viability of the cells.

INDEX WORDS: Zinc, immunology, infectious disease, necrotic enteritis, nutrition, broiler chickens

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DEDICATION

This thesis is dedicated to my loving parents, Sunny Ng and Quenevere Chan. They supported me since the beginning. I also dedicate this to the love of my life, Brandi Sparling, who challenged me to be a better scientist.

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CHAPTER 1

INTRODUCTION

Zinc is an essential nutrient for many biological processes in growth, reproduction, immune response, and molecular pathways. Although the NRC recommends total zinc in broiler chicken diets at 40 mg/kg (NRC, 1994), poultry producers recommend supplementing zinc at 100 mg/kg (Cobb-Vantress, 2018) or 110 mg/kg (Aviagen, 2019) for broiler chickens in the diets. There is currently a debate on increasing the zinc concentration in chicken diets in case of disease outbreaks. Furthermore, organic zinc, which is higher in bioavailability (Sridhar et al., 2015a), has been proposed to replace inorganic zinc.

The objective of this thesis is to provide insight into the optimal zinc concentration in broiler chicken diets with the focus on supporting the immunity of chickens against diseases. In this dissertation, the relevant research on the immune system, zinc metabolism, and necrotic enteritis infection are highlighted. Three studies were conducted to determine the effects of zinc. The first study is a non-infectious *in vivo* study that aimed to provide baseline effects of zinc on immunity in non-challenged chickens. The second study is an infectious *in vivo* study using a necrotic enteritis model in chickens. The third study is an *in vitro* study to determine the effects of zinc on *Clostridium perfringens* and the chicken independently.

CHAPTER 2

LITERATURE REVIEW

INTRODUCTION

The estimated yearly worldwide cost of necrotic enteritis (NE) in 2000 was US$2.5 billion (Van der Sluis, 2000a; Hofacre, 2001). The cost can be as high as US$6 billion, accounting for the cost associated with subclinical cases and increased prevalence due to the switch to antibiotic-free chicken production in the United States (Wade and Keyburn, 2015a). The nutritional requirements of chickens have not been updated since the latest publication by the National Research Council (NRC) in 1994, however the NRC has a working committee currently working on a revised edition. Table 2.1 provides the years of the most up to date publications on nutritional requirements by species as of 2020. The publication for chickens is one of the least up to date among other species. In addition to the changes in poultry production, the feed efficiency of modern broiler birds has been drastically improved (Applegate and Angel, 2014). Zinc is one of the essential micronutrients because of the broad reach in metabolic functions. For example, zinc is an essential co-factor for immune responses such as the production of zinc/copper superoxide dismutase to neutralize reactive oxygen species, and zinc also functions

Table 2.1.
Latest NRC publications by species.

Beef	2016
Swine	2012
Fish and Shrimp	2011
Horses	2007
Small Ruminants (Sheep, Goats, Cervids)	2007
Dogs and Cats	2006
Nonhuman primates	2003
Dairy	2001
Laboratory Animals	1995
Poultry	1994
Mink and Foxes	1982
Rabbits	1977

structurally in many zinc-finger transcription factors. Recently, organic minerals have been proposed to substitute inorganic minerals due to the increase in bioavailability. In this review, we highlighted some of the critical aspects of immunology, zinc metabolism, and necrotic enteritis that relate to the potential benefit of zinc to alleviate necrotic enteritis in chickens.

ZINC

Zinc is an essential component for many biological processes in growth, reproduction, immune response, and molecular pathways. Understanding the adequate amount of zinc intake poses a challenge due to the kinetics in absorption, endogenous excretion, retention, and utilization of zinc to maintain homeostasis. Other factors that affect zinc homeostasis include the species, life stage, sex, dietary form, and interactions with other nutrients. Elemental zinc was used the early animal production and later from zinc carbonate. Zinc sulfate and zinc oxide are currently most commonly used. Some “organic” forms of zinc, such as chelated zinc, have been proposed to improve bioavailability. Although the new zinc forms may have higher bioavailability, the physiological benefits remain unproven. There is a difference in expressing the amount of zinc across studies. For example, references for animals are often expressed in milligrams per kilograms of body weight or tissue in wet or dry matter basis, while references for humans are sometimes expressed in micromolar or milligrams per day basis. For humans, two recommendation systems were established: the recommended daily allowance (RDA) in the dietary reference intake (DRI) by the National Academy of Sciences and the daily value (DV) by the Food and Drug Administration (FDA); both are used in the United States. The criteria of the studies to evaluate the adequate zinc intake are not uniform. Therefore, these factors should be taken into account when establishing the requirement of zinc for animals.

Zinc Distribution in the Body

Zinc concentrations in the body are consistent between species. The integument contains a high concentration of zinc. Since some animals are covered with fur or hair while others are not, the distribution of zinc can vary slightly. High zinc diets increase zinc concentration in blood in many species (Lutz, 1926; Berfenstam, 1952b; Lewis Jr et al., 1956; Ott et al., 1966b; Perry et al., 1968), although only extreme zinc deficiency affects zinc concentration in blood (Hove et al., 1938; O'dell and Savage, 1957; Miller and Miller, 1960; Miller et al., 1966; Mills et al., 1967; Miller et al., 1968a).

In humans, the body contains about 20 to 33 mg/kg of zinc (Widdowson et al., 1951). The majority of zinc (85%) is found in the muscle and bone (Paik et al., 1999). Whole-body zinc remains at about 30 mg/kg in animals, regardless of zinc intake from 10 to 100 mg/kg (Kirchgessner, 1993). The average zinc concentrations of the tissue types (as wet basis) were reported as follow: prostate (102 mg/kg), kidney and liver (55 mg/kg), muscle (54 mg/kg), heart (33 mg/kg), pancreas (29 mg/kg), spleen (21 mg/kg), testes (17 mg/kg), lungs (15 mg/kg), brain (14 mg/kg), and the adrenal glands (12 mg/kg) (Tipton and Cook, 1963). Of the total zinc in the body, it was estimated that 20% (4 to 7 mg/kg) was found in the skin (Pories et al., 1967), an average of 151 mg/kg was found in the nails (Livingston et al., 1967), and an average of 173 mg/kg was found in the hair (Livingston et al., 1967). The blood only contains 0.1% of the total zinc in the body, but the concentration is maintained tightly at 10 to 15 μmol/l unless severe deficiency occurs (Cousins, 1989). In the blood, whole blood, plasma, erythrocytes, and leukocytes contain 8.8, 1.21, 14.4 mg/kg, 13 mg/kg of zinc, respectively (Smirov, 1948; Vallee and Gibson, 1949; Prasad et al., 1965). The majority of zinc in the blood (75 to 88%) is found in red blood cells, and the rest resides in the plasma (12 to 22%) and leukocytes (3%) (Vallee and Gibson, 1948). However, leukocytes contain a higher zinc

concentration than erythrocytes per cell basis due to the relative amounts of leukocytes and erythrocytes in the body. Plasma zinc in the blood is associated with albumin (33%), and the rest is bound to globulins (Wolff, 1956). Human platelets contain 0.071 μg/ml of zinc in the blood (Foley et al., 1968). It was demonstrated that serum (plasma without the clotting factors) contains higher zinc concentration from the release of zinc from the platelet clotting process and hemolysis (Foley et al., 1968). Zinc concentration in the hair is not different between males and females (Schroeder and Nason, 1969). In the human prostate, zinc concentration is about 891 mg/kg (as DM basis) (Mawson and Fischer, 1953). The human semen contains between 50 and 200 μg/ml of zinc depends on the subjects (Bertrand and Vladesco, 1921). In the milk, zinc concentration is between 3 and 5 μg/ml depends on the species (Archibald, 1944; Berfenstam, 1952a; Cavell and Widdowson, 1964); and is higher in colostrum (Berfenstam, 1952b).

Zinc is excreted into the feces from the pancreas, and to a lesser extent, the bile, cecum, or large intestine (Methfessel and Spencer, 1973). Endogenous loss is reduced during zinc depletion to 1.3 to 4.6 mg/day (Paik et al., 1999). In humans, zinc excretion in the urine is about 0.1 to 0.7 mg/day or 7.5 μg/day/kg of body weight (Copper, 2001). Zinc homeostasis is maintained in the first week after a depletion diet (Johnson et al., 1993a). Persistent low zinc intake increases absorption but decreases total absorption, which indicates that homeostasis is mainly maintained by decreasing endogenous fecal loss, rather than increasing zinc absorption (Lee et al., 1993). Zinc excretion in the urine is low but can decrease with low zinc intake within days (Lee et al., 1993). Zinc depletion occurs in the plasma, liver, bone, testes before the hair, skin, heart, and skeletal muscle (Jackson et al., 1982).

Similarly in nonhuman primates like the monkeys, the average zinc concentrations of the tissue types (as wet basis) were reported as follow: liver (51 mg/kg), muscle (54 mg/kg), pancreas (48

mg/kg), kidneys (29 mg/kg), muscle (24 mg/kg), heart (22 mg/kg), spleen (21 mg/kg), lungs (19 mg/kg), testes (17 mg/kg), and the adrenal glands (16 mg/kg) (Macapinlac et al., 1967).

In rats, the average zinc concentrations of the tissue types (as wet basis) were reported as follow: prostate (223 mg/kg), pancreas (33 mg/kg), liver (30 mg/kg), spleen (24 mg/kg), kidney (23 mg/kg), lungs and testes (22 mg/kg), heart (21 mg/kg), brain (18 mg/kg), and muscle (13 mg/kg); and 38% of total zinc in the body is found in the hair, skin, and bristles (Spray and Widdowson, 1950; Mawson and Fischer, 1953; Gilbert and Taylor, 1956). In rat testes, zinc concentration (as DM basis) is about 176 mg/kg (Pařízek et al., 1966). Zinc concentration in the testes (as DM basis) increases from 120 mg/kg in the first month of age to 200 mg/kg in the second month of age. In rat prostate, zinc concentration is about 891 mg/kg (as DM basis) (Mawson and Fischer, 1953); and is more concentrated in the luminal edge of the acinar cells (Millar et al., 1961). In the rat sperm (Millar et al., 1961) as well as in the ram (Garnica et al., 1993), zinc is more concentrated in the tail portion. Castration decreased the concentration of zinc in the dorsolateral prostate region and can be reversed by exogenous testosterone and gonadotropin (Gunn and Gould, 1956; Millar et al., 1957; Millar et al., 1958a). In the blood, rat leukocytes contain 10 mg/kg of zinc (Smirnov, 1948).

In pigs, the average zinc concentrations of the tissue types (as wet basis) were reported as follow: pancreas (45 mg/kg), liver and kidneys (40 mg/kg), adrenal glands (33 mg/kg), and spleen (28 mg/kg) (Hoekstra, 1964a). Boar semen contained 10 µg/ml in the semen (Westmoreland et al., 1967). In pig blood, zinc concentration was 0.6 µg/ml in plasma and 7 µg/ml in erythrocytes (Lewis Jr et al., 1956; Miller et al., 1968b).

Zinc concentration in other animals is not as studied extensively. In the ram, the testes contain 105 mg/kg of zinc (as DM basis) (Prasad et al., 1967b). In rabbits, the blood contains 2.5 µg/ml of zinc

in whole blood, 2.7 µg/ml of zinc in plasma, and 7 µg/ml or 9 µg/g of zinc in erythrocytes (Smirnov, 1948).

In chickens concentration 80 mg/kg zinc from zinc sulfate with 24 mg/kg in the basal feed, zinc concentrations in serum, liver, muscle, yolk, and albumin were 108.11 µmol/L, 76.84, 19.95, 51.83, and 8.37 mg/kg respectively (Zhang et al., 2017b). In the chicken eggs, zinc concentration is between 0.7 to 1.0 mg, mostly in the yolk (Birckner, 1919; Zhang et al., 2017a) bound to vitellin (Tupper et al., 1954). Plasma zinc concentration in broiler chickens fed a soybean meal diet that contains 45.1 mg/kg of zinc is about 2.25 µg/ml (Olkowski et al., 2005). Plasma zinc concentration is dependent on boron and vitamin D3 content of the diet (Kurtoğlu et al., 2005). Plasma zinc is 1.88 µg/ml in broiler chickens fed 5 mg/kg boron and 6.25 µg/kg vitamin D3. Plasma zinc concentration decreases to 1.27 µg/ml when boron in the diet is increased to 25 mg/kg. Plasma zinc concentration decreases to 1.20 µg/ml when vitamin D3 in the diet is increased to 50 µg/kg. Plasma zinc concentration is lowest in *Eimeria acervulina* infected birds on day seven and then peaks on day nine of infection (Turk, 1986). Plasma zinc concentration is lowest in *Eimeria necatrix* infected birds on day 7 of infection, but the plasma zinc concentration does not increase afterward. Plasma zinc concentration of *Eimeria brunetti* infected birds does not decrease on day seven but peaks on day nine to ten of infection. Plasma zinc concentration of *Eimeria tenella* birds peaks on day three and then drops to the lowest on day six of infection.

Zinc Transport

Table 2.2.
A list of zinc transporter proteins. Adapted from (Bonaventura et al., 2015).

Importers	Tissue expressed	Reference
ZIP1	All	(Costello et al., 1999)
ZIP2	Only in prostate and uterine epithelial cells	(Cao et al., 2001)
ZIP3	Bone marrow, spleen, small intestine, liver	(Liuzzi and Cousins, 2004)
ZIP4	Kidney, small intestine, stomach, colon, jejunum, duodenum	(Andrews, 2008)
ZIP5	Intestine, pancreas, liver, kidney	(Wang et al., 2004)
ZIP6	Prostate, placenta, mammary gland, HeLa cells	(Taylor and Nicholson, 2003)
ZIP7	Mammary gland	(Taylor et al., 2008)
ZIP8	Fibroblast and chondrocytes	(Thévenod, 2010; Liu et al., 2013; Song et al., 2013)
ZIP9	Human lymphocytes	(Taniguchi et al., 2013)
ZIP10	Mammary gland	(Kagara et al., 2007)
ZIP11	Testes, stomach, ileum, cecum	(Martin et al., 2013; Yu et al., 2013)
ZIP12	Brain, eye	(Bly, 2006)
ZIP13	Bone teeth, connective tissues	(Jeong et al., 2012)
ZIP14	Mammalian cells	(Taylor et al., 2005)
Exporters		
ZnT1	All	(Qin et al., 2009)
ZnT2	Mammary gland prostate, retina, pancreas, small intestine, kidney	(Huang and Tepaamorndech, 2013)
ZnT3	Brain, testes, pancreas	(Huang and Tepaamorndech, 2013)
ZnT4	All	(Huang and Tepaamorndech, 2013)
ZnT5, 6, 7	All	(Kambe et al., 2002)
ZnT9	All	(Chen et al., 2005)
Metallothionein		
MT1	All	(Gumulec et al., 2014)
MT2	All	(Gumulec et al., 2014)
MT3	CNS, small intestine	(Pountney et al., 2011)
MT4	Skin, gastrointestinal tract	(Aschner and West, 2005)

Cellular transports of zinc have been studied extensively in mammals (Cousins et al., 2006). Two zinc transporter families have been identified in mammals: the zinc transporter (ZnT) in the solute-

linked carrier family 30 (SLC30A) and the zinc importer and Irt-like protein (ZIP) in the solute-linked carrier family 39 (SLC39A). Table 2.2 (adapted from (Bonaventura et al., 2015)), provides a list of ZIP and ZnT and the tissues that the zinc transporters are expressed in.

Zinc absorption occurs throughout the small intestine (Lee et al., 1989). The rate of absorption of zinc is the fastest in the jejunum and the slowest in the ileum, while the duodenum is in the middle.

Zinc is transported via active or facilitated diffusion (MacDonald, 2000a; Bridges and Zalups, 2005). Zinc homeostasis is regulated by transporter proteins and binding proteins. Zinc import proteins (ZIP-1 to ZIP-14) increase cytosolic zinc by allowing entry from extracellular space or release from intracellular compartments (Cousins et al., 2006). Structurally, ZIP proteins contain nine transmembrane domains. Zinc export proteins (ZnT-1 to ZnT-10) decrease cytosolic zinc by allowing exit from the cytosol into the extracellular space or sequestration into intracellular compartments. The binding proteins, metallothionein (MT-1 to MT-4), sequester zinc in the cytosol; thereby, reducing free-flowing zinc.

Plasma membrane ZnT-1 regulates intracellular zinc concentration through zinc efflux with the L-type calcium channel (Segal et al., 2004). Znt-2 to 8 regulates zinc efflux into endosomal vesicles in different tissues. ZnT-2 regulates intracellular zinc concentration by sequestering zinc into endosomal vesicles (Palmiter et al., 1996a). ZnT-3 transporters are found abundantly in the synaptic vesicle neuro-tissues (Palmiter et al., 1996b; Cole et al., 1999). ZnT-4 regulates zinc influx into endosomal vesicles in the mammary gland (Kambe et al., 2002), liver (Cousins and McMahon, 2000a), lung, kidney, spleen, heart, brain, and intestine (Murgia et al., 1999). ZnT-4 is highly expressed in the brain. Mutation of the ZnT-4 gene results in cognitive impairment (Smith et al., 2006) and zinc deficiency in milk (Cousins and McMahon, 2000b). ZnT-5 is highly expressed in pancreatic ß cells (Murgia et al., 1999). ZnT-5 and 6 are expressed in the apical

membrane of the enterocytes to export zinc into the lumen of the intestine for excretion. ZnT-6 is highly expressed in brain tissues of Alzheimer Disease and Pick Disease patients (Lovell et al., 2006). ZnT-7 is localized in the Golgi apparatus (Kirschke and Huang, 2003) and retina (Wang et al., 2006). ZnT-8 is only expressed in pancreatic β cells (Chimienti et al., 2004; Chimienti et al., 2005). ZnT-5 and 8 transport zinc in pancreatic beta cells for insulin secretion. ZnT-9, also known as HUEL, was discovered in human embryonic lung cell lines, but the function of this transporter is not well understood (Sim and Chow, 1999). A novel ZnT transporter, ZnT-10, has been sequenced, but more studies are needed for this transporter (Sim and Chow, 1999). Increase in zinc intake upregulates ZnT-1 and ZnT-2 mRNA expressions (McMahon and Cousins, 1998) but not ZnT-4, 5, and 7 (Liuzzi et al., 2001); however, decrease in zinc intake upregulates ZnT-4 mRNA expression in the mammary glands (Kelleher and Lönnerdal, 2002) to prioritize nutrients to the neonate.

Zinc and ZIP-4 transporter expression is highly correlated. ZIP-4 imports zinc into the enterocytes from the intestinal lumen. ZIP-5, expressed in the intestinal cells, pancreas, and kidney, imports zinc from the extracellular space through the basolateral membrane into the enterocyte (Gropper and Smith, 2012). ZIP-14 imports zinc into hepatocytes, especially during acute phase response (Gropper and Smith, 2012). Additionally, ZIP-14 can also carry other ions, such as iron (Gropper and Smith, 2012).

Other proteins, such as divalent mineral transporter 1 (DMT-1) and protein transporters may contribute to zinc absorption, but the extent is thought to be minimal (Gropper and Smith, 2012).

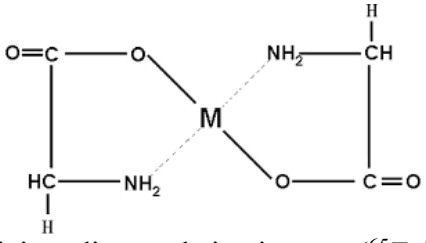
After zinc is absorbed, it may be used by the enterocytes or transported through the basolateral membrane by mainly ZnT-1 into the systemic blood circulation. In the blood, the majority (60%) is zinc is bound to albumin. Other proteins such as transferrin, α-2 macroglobulin, and

immunoglobulin (IgG) are thought to be responsible for the rest of zinc transport in the blood, while a small amount is bound to histidine and cysteine (Gropper and Smith, 2012).

Mineral Source and Bioavailability

Zinc glycinate contains a 2:1 glycine to zinc ratio and a higher concentration of zinc at 33.1% than zinc sulfate at 28.2%. The zinc molecules are connected to the carboxyl moieties of the glycine molecules (Yin et al., 2017).

Figure 2.1.
Structure of zinc glycinate.



The bioavailability of zinc glycinate was studied using zinc-deficient diets and zinc isotope (^{65}Zn) in rats (Schlegel and Windisch, 2006). In the control group with normal diets (55 mg/kg of zinc from zinc sulfate), blood plasma, femur, and whole-body zinc concentrations were 1.36 ug/ml, 156 mg/kg DM, and 26.5 mg/kg, respectively. Apparent absorption, true absorption, urinary, and retention of zinc as a percentage of intake of these control animals were 21.5, 30.8, 0.7, and 20.7, respectively. Rats fed zinc glycinate (10 mg/kg) had higher apparent and true zinc absorption as a percentage of intake than those fed with zinc sulfate (10 mg/kg). In addition, the bioavailability of zinc glycinate (48.6%) was higher than zinc sulfate (41.8%).

Inorganic zinc, such as zinc oxide and sulfate, is the primary sources in poultry feed (Wedekind and Baker, 1990). Organic sources such as B-TRAXIM®2C Zn-260 are glycinate sourcethat increases bioavailability, growth performance, and mineral retention (Sridhar et al., 2015b), reproductive performance (Zhang et al., 2017a), and immune responses (Kwiecień et al., 2017) in broiler chickens. Zinc status affects epithelial integrity (Zhang et al., 2017a) as well as reproductive performances (Zhang et al., 2017a) in chickens. Organic zinc supplemented at 30 mg/kg increases epithelial cell layer and collagen contents than zinc sulfate supplemented at 40 mg/kg in broiler chickens (Sridhar et al., 2015d). Average egg weight, broken egg rate, fertility, and hatchability

can be improved using zinc glycinate supplemented mg/kg (Zhang et al., 2017a). In this study, liver and serum Cu-Zn superoxide dismutase were higher in chickens fed zinc glycinate. Regardless of the concentration and sourceof zinc, zinc concentration was the highest in the liver and the lowest in the muscle. Serum and kidney zinc retention were higher at 30 mg/kg of supplemental zinc from zinc glycinate than supplemented at 40 mg/kg from the zinc sulfate (Sridhar et al., 2015a). An interaction between zinc and copper was observed in this study. Although liver zinc concentration was not different between zinc glycinate supplemented at 30 mg/kg and the zinc sulfate supplemented at 40 mg/kg, liver copper concentration was higher in the zinc glycinate treatment. No differences were observed in the bones, regardless of zinc treatments. Zinc glycinate increased liver and muscle zinc concentrations. Zinc supplemented at 100 mg/kg from zinc glycinate had no effects on body, liver weight, serum zinc concentration at 42 days of age compared to zinc oxide (Kwiecień et al., 2017). However, zinc concentration in the liver was increased from 55 to 61.9 mg/kg in zinc glycinate-fed chickens, and excretion of zinc was decreased from 48 to 43.9 mg/kg.

The feed conversion ratio was not affected regardless of the sourceof zinc and the concentration of zinc in multiple studies (Sridhar et al., 2014a; Zhang et al., 2017a). FCR of chickens improved when birds are fed 30 mg/kg of zinc from glycinate and 40 mg/kg of zinc from zinc sulfate from 10 mg/kg of zinc from zinc glycinate (Sridhar et al., 2014b). However, there was no difference in FCR between 30 mg/kg of zinc glycinate and 40 mg/kg of the sulfate form. In this study, breast yield, visceral organ size (% of body weight) (heart, liver, gizzard, bursa, spleen, thymus, kidney, and pancreas), abdominal fat percentage, and tibia percentage were not different regardless of the zinc sources and levels.

One study investigated the effects of zinc sources on immune response (Jarosz et al., 2017). The study measured $CD3^+/CD4^+$, $CD3^+/CD8^+$, $CD25^+$, and Bu-$1A^+$ lymphocyte percentage, MHC-II^+ cells, monocyte/heterophil phagocytosis ability, and proinflammatory cytokines (IL-2, IL-10, and TNF-α) on day 0, 20, and 24 of age in peripheral blood. However, differences between groups were unclear due to poor statistical analyses of the data.

Another organic source of zinc, zinc methionine, was studied (Mohanna and Nys, 1999). Weight gain, food intake, feed conversion, plasma zinc concentration, and tibia zinc concentration on 21 days of age were not different between chickens fed a basal diet, containing 20 mg/kg of zinc, and supplemented zinc sulfate and zinc methionine at 0, 10, 25, and 40 mg/kg. In chickens fed zinc sulfate, feather zinc ranged between 30 and 40 mg/kg, whole-body zinc ranged between 10 to 19 mg/kg, and zinc retention ranged between 20 and 19%. High zinc diets resulted in higher feather and whole-body zinc concentration but lower zinc retention. Immune responses of the chickens measured by anti-sheep red blood cell antibodies and alkaline phosphatase activities were not different between groups. In a separate experiment, chickens were fed a basal diet with 65 mg/kg of zinc and supplemented zinc sulfate at 40, 50, 65, 85, and 170 mg/kg. Weight gain ranged between 515 and 580 grams, food intake ranged between 783 and 877 grams, and feed conversion ranged between 1.51 and 1.53, but not statistically significant between groups. Plasma zinc ranged between 1.85 and 2.04 mg/l, tibia zinc ranged between 170 and 209 mg/kg, whole-body zinc ranged between 19 and 22 mg/kg, excreta zinc ranged between 40 and 170 mg/kg, and zinc retention ranged between 20 and 8 percent. High zinc diets resulted in higher plasma, tibia, whole body, and excreta zinc concentrations but lower zinc retention.

The organic source of other minerals, such as selenium, have been studied in broiler breeder chickens (Torres et al., 2009). The effect of organic selenium, B-TRAXIM®Se, was studied using

inorganic selenium, sodium selenite, as control. Selenium was supplemented to broiler diets at 0.3 mg/kg. Two concentrations, 0.1 and 0.3 mg/kg, of each of the selenium source, were tested. Selenium source did not affect egg production, egg weight, shell quality, and hatchability. Higher selenium concentrations increased egg production only. Selenium content in the yolk was higher in the egg yolk in chicken fed the organic zinc diet. Selenium content in the yolk, albumin, liver, and breast meat was higher in chickens fed higher selenium diet; but not in the serum. Glutathione peroxidase activity in the liver and plasma was decreased by the organic selenium diet than the inorganic selenium diet but was not affect by selenium concentration. This suggests that the increased bioavailability of minerals lowered oxidative stress in non-challenged chicken.

In ovo administration of organic zinc at 20 or 40 μg/egg (100 μl/egg) on day-18 of embryonic stage alleviated necrotic enteritis infection post-hatch (Lee et al., 2014b). Eggs were incubated and candled at 37.5°C with 55 to 60% relative humidity for 18 days. The organic selenium was injected into the amniotic cavity using the Intelliject system (Avitech, Easton, MD) to avoid negative pressure. PBS without selenium was used as control. The eggs were incubated at 36°C with 60 to 65% relative humidity until hatch. The hatched chickens were fed 0.1 mg/kg selenium. Necrotic enteritis was induced by a high crude protein diet (24%) between day 28 and 24, and oral gavage with 100,000 oocysts/bird of *E. maxima* strain 41A on day 15 and 10^9 CFU/bird of *C. perfringens* strain Del-1 (α-toxin and netB toxins positive) on day 14. Hatchability and pre-necrotic enteritis body weights of chickens were not affected. Body weights of organic zinc-treated and necrotic enteritis infected chickens on day 20 were higher than the chickens not treated with organic selenium (not different between selenium concentrations). Lesion scores, oocysts shedding, serum α-toxin and netB toxins, were lower and serum anti-α-toxin and netB toxins antibodies were higher in the selenium treated than the untreated chickens (not different between selenium

concentrations). Gene expressions of IL-1β, IL-6, and TNFSF15, were higher in selenium treated than untreated chickens (not different between selenium concentrations). Gene expressions of IL-8 and peroxiredoxin 6 were only higher in high selenium treated chickens. Gene expression of catalase were lower in selenium treated chickens than untreated chickens (not different between selenium concentrations). Gene expression of inducible nitric oxide synthase and paraoxonase 2 were higher in high selenium treated chickens but lower in low selenium treated chickens than non-treated chickens. Gene expression of glutathione peroxidase 7 was lower in low selenium treated chickens than non-treated chickens. Gene expression of superoxide dismutase was not affected by selenium treatment. Serum MDA, catalase, and SOD activities were decreased by selenium treatments.

Dietary organic selenium supplemented at 0.25, 0.5, and 1 mg/kg was studied in necrotic enteritis infected chickens, induced by high protein diet (24% crude protein), *E. maxima* Beltsville strain 41A (10^4 oocysts/bird) by oral gavage on 14 days of age, and *C. perfringens* (10^9 CFU) on 18 days of age (Xu et al., 2015). Organic selenium improved body weight gain (0.5 and 1 mg/kg) and decreased serum anti-netB antibodies (1 mg/kg), but selenium did not affect lesions, oocyst shedding, and serum anti-α-toxin antibodies. Selenium decreased IL-1β in the intestine (1 mg/kg) and the spleen (0.5 and 1 mg/kg) in infected chickens; decreased IL-8 in the intestine (0.5 mg/kg) and increased IL-8 in the spleen (0.5 mg/kg); decreased LITAF in the intestine (1 mg/kg) and increased LITAF in the spleen (1 mg/kg); decreased TNFSF15 in the intestine (1 mg/kg) and in the spleen (0.5 and 1 mg/kg); increased iNOS in the intestine (1 mg/kg) but not in the spleen; and did not affect IL-6 in the intestine and the spleen. β-defensin are antimicrobial peptides in innate immunity. β-defensin-6 was increased in the intestine (0.5 and 1 mg/kg) and in the spleen (1 mg/kg). β-defensin-8 was increased in the intestine (0.5 mg/kg) and decreased in the spleen (0.5

mg/kg). β-defensin-13 was increased in the intestine (0.5 and 1 mg/kg) and in the spleen (0.5 mg/kg).

Zinc requirements

The requirements of zinc can be estimated with several methods. First, the intake required to match the daily endogenous loss can be used. In this method, endogenous loss from the feces and urine is determined. Integument, semen, or menstrual losses of zinc are often negated due to the small quantity. The urinary loss remains constant from 4 to 25 mg/day of zinc intake (Baer and King, 1984). Endogenous excretion is estimated by tracer methods, mathematical modeling, or from ileal cannula method. Second, growth performance, often accompanied by mathematical modeling, is used to estimate zinc requirements. This is especially true in production animals. Other methods include measuring plasma, serum, erythrocyte, or hair zinc concentration, and enzyme activities. Plasma zinc remains stable for weeks during severe zinc deficiency (Wade et al., 1985; Johnson et al., 1993b); therefore, deviation from the normal range can be a valid indicator of zinc deficiency. Erythrocyte zinc concentration is decreased with less severe zinc deficiency (Thomas et al., 1992). The remaining methods can be used as a secondary marker, but those are not consistent among literature.

Zinc from zinc carbonate ($ZnCO_3$) was used in early studies of zinc in animals. Zinc concentration of 12 to 18 mg/kg from $ZnCO_3$ was adequate for growth in rats depends on the protein source (Forbes and Yohe, 1960b), but it was increased to 12 to 18 mg/kg due to the interaction of zinc and calcium. It was later suggested to increase zinc to 100 mg/kg based on reproductive performance (Swenerton and Hurley, 1968).

For pigs, total dietary zinc at 45 mg/kg were adequate for the female piglets but not the male (Liptrap et al., 1970). Parakeratosis was alleviated above 41 mg/kg of zinc in weanling pigs

(Liptrap et al., 1970). Taken together, the requirement of zinc was raised to 45 to 50 mg/kg of zinc for growing pigs. High copper (above 125 mg/kg) (O'hara et al., 1960; Hoekstra, 1964b) and calcium (above 1.6%) (Hoekstra et al., 1967) diets increase zinc requirement and should be taken into account.

For chickens, zinc requirement was 35 to 40 mg/kg with soybean-based diets with 1.6% calcium (O'dell and Savage, 1957); and the requirements can be lowered with lower calcium concentration (1.1%). Lower zinc concentration (25 to 30 mg/kg) can be used for casein or egg white-based diet (Moeller and Scott, 1958; Pensack et al., 1958). The recommended concentration of zinc for chickens was based on early studies with zinc-deficient diets. Chickens fed 15 mg/kg of had a slower growth rate, bone and feather abnormality, and lower immune cell counts (O'Dell et al., 1958b). From these growth abnormalities, the NRC of 1994 recommended 40 mg/kg of total dietary zinc in broiler diets from hatch to finish.

Meanwhile, the NRC recommended 40 mg/kg of total dietary zinc in the diet for white-egg laying chicks during the first six weeks and down to 35 mg/kg after the first six weeks (NRC, 1994). For the brown-egg laying chicks, the NRC recommended 38 mg/kg in the first six weeks and 33 mg/kg after that of total dietary zinc. High phytate and fiber diet decreased bioavailability of zinc and therefore increased the zinc requirement of chickens.

The Recommended Dietary Allowance (RDA) for adults 8 mg/day men, 11 mg/day for women, 12 mg/day for adolescent pregnant female, 11 mg/day for adult pregnant female, 13 mg/day for lactating adolescent, or 12 mg/day for lactating adults (Copper, 2001). RDA for children from birth to the first year of age is 3 mg/day; from the first year to 3 years of age is 5 mg/day, and from 5 to 8 years of age is 8 mg/day.

For humans, zinc intake from 5 to 22 mg/day was adequate in human studies (McCance and Widdowson, 1942); but variation occurs due to the range of dietary habits in humans. Typical human consumption ranged from 12 to 15 mg/kg of zinc (Sandstead et al., 1967; Schroeder et al., 1967). It was suggested that 6 mg/kg was adequate for preadolescence girls based on average consumption of 4.6 to 9.3 mg/kg (Engel et al., 1966). Breastfed infants were received from 0.2 to 1.2 mg/kg of zinc from breast milk (Cavell and Widdowson, 1964). Therefore, formula-fed infants may require a higher amount.

Zinc intake of 0.25% or 2,500 mg/kg from elemental zinc, zinc chloride, or zinc carbonated showed no adverse effects in rats, but decreased growth and increased mortality were observed at 5,000 mg/kg from zinc chloride and zinc oxide (Heller and Burke, 1927). Zinc intake at 0.2% or 2,000 mg/kg from zinc oxide did not affect the reproduction of female rats (Schlicker and Cox, 1968b). Decreased growth and anemia were observed with the intake of zinc above 5,000 mg/kg from zinc carbonate (Sutton and Nelson, 1937). Fetal development and high mortality were observed with an intake of zinc above 4,000 mg/kg (Schlicker and Cox, 1968a). Intake of 1000 mg/kg for several weeks from zinc sulfate or zinc carbonate showed no adverse effects in pigs (Lewis et al., 1957b; Brink et al., 1959); but high mortality, decreased growth, decreased intake, and internal hemorrhages were observed above 4000 mg/kg (Brink et al., 1959). Intake of zinc below 1400 mg/kg showed no adverse effects in broiler chickens, but decreased growth was observed above 3000 mg/kg. The adverse effects of high zinc diet can be explained by the unpalatability (Grant-Frost and Underwood, 1958; Ott et al., 1966a) or copper/iron deficiency induced by the increase of zinc, which can be resolved by copper/iron supplementations (Smith and Larson, 1946; Van Reen, 1953; Grant-Frost and Underwood, 1958; Cox and Harris, 1960; Magee and Matrone, 1960).

The Association of American Feed Control Officials recommended 120 mg/kg for dogs and 75 mg/kg for cats on a DM basis (Officials, 2013). High calcium in the diet, reduced zinc absorption (Heth and Hoekstra, 1965; Heth et al., 1966a; Heth et al., 1966b) and exacerbated zinc deficiency symptoms in pigs (Tupper et al., 1954; Stevenson and Earle, 1956; Lewis et al., 1957a; Luecke et al., 1957), dogs (Robertson and Burns, 1963) and birds (Kienholz et al., 1961a); but not in human (Spencer et al., 1965). Zinc absorption was 35.7% in humans using stable isotope ^{65}Zn. Copper reduced zinc absorption in rats, and vice versa (Van Campen and Scaife, 1967). Cadmium exacerbated zinc deficiency in chickens (Supplee, 1963), pigs, and calves (Powell et al., 1964). Other nutrients, such as iron (Solomons and Jacob, 1981; Valberg et al., 1984; O'Brien et al., 2000) or phytate (Oberleas et al., 1966), have been shown to decrease zinc availability.

Functions of Zinc in Animals

Growth and Development

Animal growth is the primary criterion used to evaluate nutritional requirements in animals. Zinc deficiency leads to growth hindrance in rats (Todd, 1980), lamb and calves (Mills et al., 1967); skin lesions in monkeys (Ott et al., 1966b; Barney et al., 1967a, b); alopecia in rats and mice (Follis et al., 1941b); crackling and scaling in the paws in rats (Forbes and Yohe, 1960a); parakeratosis in pigs (Miller et al., 1965a) and ruminants (Blackmon et al., 1967; Mills et al., 1967; Somers and Underwood, 1969a); disrupted horn growth in horned lamb (Mills et al., 1967); malformed hooves in lambs (Mills et al., 1967; Somers and Underwood, 1969b); poor feathering and dermatitis in birds (O'dell and Savage, 1957; Kratzer et al., 1958; Scott et al., 1959); bowing of the hind legs and stiff joints in calves (Miller and Miller, 1960); and bone malformation in rats (Hurley and Swenerton, 1966) and birds (O'dell and Savage, 1957; Kratzer et al., 1958; Young et al., 1958; Scott et al., 1959; Blamberg et al., 1960; Kienholz et al., 1961b; Zeigler et al., 1962; Fox and

Harrison, 1964). Bone malformation from zinc deficiency is due to the decrease in osteoblast activity in long bone, decrease in chondrogenesis, and increase in cartilage matrix formation (O'dell and Savage, 1957; Young et al., 1958).

Regulated apoptotic pathways are essential for cell development. Using zinc chelator, *N*, *N*, *N'*, *N*-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN), it was demonstrated that neuron apoptosis was mediated by zinc (Cho et al., 2010). Pro-apoptosis proteins caspase-9 and -3, and mitochondrial Bax were decreased in brain tissues of mice, while anti-apoptotic protein Bcl-2 was increased. In diabetes-induced testicular apoptosis, p38 MAPK and P53 proteins mediated apoptosis was increased by zinc deficiency induced by TPEN together with a decrease in antioxidant Nrf2 gene expression (Zhao et al., 2011).

Reproduction

Underdevelopment of the reproductive system is observed in zinc deficiency; such as seminiferous tubules atrophy in rats (Follis et al., 1941a), testes, epididymis, prostate, pituitary gland, testicular germinal epithelium deformity in rats (Mawson and Fischer, 1953; Millar et al., 1957), spermatogenesis disruption (Millar et al., 1958b; Wetterdal, 1958; Somers and Underwood, 1969b), hypogonadism in human (Miller et al., 1964; Prasad et al., 1983), in bull calves (Miller et al., 1966; Pitts et al., 1966), and ram lambs (Somers and Underwood, 1969b), estrous cycle disruption in rats (Hurley and Swenerton, 1966), and decrease egg hatchability and increase embryo mortality in chickens (Blamberg et al., 1960; Kienholz et al., 1961a).

Immunity

Zinc's beneficial role in wound healing and immunity has been demonstrated in humans (Pories et al., 1966; Pories et al., 1967), in calves (Miller et al., 1965b), and rats (Sandstead and Shepard,

1968). The risk of vascular inflammation (Shen et al., 2008), hypertension, hypertriglyceridemia (Singh et al., 1997; Singh et al., 1998), ischemic heart disease, and acute myocardial infarction (Vlad et al., 1994) have been correlated to zinc deficiency.

Immune cells are derived from hematopoietic stem cells in the bone marrow into two lineages: the lymphoid progenitor cell and the myeloid progenitor cell (Janeway et al., 2004). These progenitor cells mature in the primary lymphoid organs such as the bone marrow, the thymus, and the bursa in avian species. The mature immune cells survey antigens in peripheral lymphoid organs such as the lymphoid node, spleen, and the mucosa-associated lymphoid tissues or gut-associated lymphoid tissues where antigens are presented to the immune cells.

Immune Organ Weight and Cell Number

Immune organ size is one of the most common criteria to evaluate zinc on immunity in chickens in early studies (Pimentel et al., 1991). In this study, chickens were supplemented with zinc from zinc oxide at 8, 18, 25, 28, 38, 48, 58, 68, 68, 78, 88, and 125 mg/kg. The spleen weight was increased by the supplementation of zinc at 88 mg/kg compared to 58 mg/kg. Meanwhile, the bursa and thymus weights were increased by the supplementation of zinc at 18 mg/kg compared to 8 mg/kg. To adjust for the differences in body weight, the weight of the immune organs are normalized to the body weight. The relative spleen weight as the percentage of organ weight was increased by zinc from zinc sulfate supplementation at 60 mg/kg compared to the non-supplemented group (Akbari et al., 2008). Spleen weight and bursa weight in non-challenged chickens were increased by increasing dietary zinc from 20 to 40 mg/kg from zinc sulfate (Sunder et al., 2008). Inorganic and organic zinc did not result in the difference in bursa and spleen weight in non-challenged chickens between 40, 80, and 160 mg/kg of zinc (Gajula et al., 2011). Other zinc sources such as zinc-nanoparticle complexes did not result in differences in relative spleen

and bursa weight in non-challenged chickens compared to zinc sulfate or zinc methionine despite a reduction of growth performance by zinc-nanoparticle complexes (Mohammadi et al., 2015). Immune organ development is highly sensitive to zinc status and can be a reliable indirect method to evaluate immunity.

Another indirect method of evaluating zinc status on immunity is by the immune cell counts of WBC, heterophils, monocytes, lymphocytes, and heterophil to lymphocyte ratios. Dietary zinc sulfate supplementation at 60 mg/kg increased in WBC and lymphocytes, and decrease in heterophils, and heterophil to lymphocyte ratio in non-challenged chickens compared to the non-supplemented group (Akbari et al., 2008). The heterophil to lymphocyte ratio was decreased by increasing zinc from zinc sulfate from 20 to 40 mg/kg (Sunder et al., 2008). The zinc-nanoparticle complex supplemented at 80 mg/kg resulted in an increase in heterophils in non-challenged chickens compared to zinc sulfate (Mohammadi et al., 2015). Organic zinc from zinc-polysaccharide complex supplementation at 60 mg/kg decreased heterophils and basophils, and increased lymphocytes and monocytes in non-challenged chickens compared to zinc oxide (El-Katcha et al., 2017). In the same study, nano-zinc decreased heterophils and increased eosinophils, basophils, and lymphocytes compared to zinc oxide. Zinc status can be evaluated by immune cell counts and may show whether the effect of zinc is directed on innate immunity, adaptive immunity, or both.

Innate Immunity

The immune response is separated into innate immunity and adaptive immunity. The mucosal epithelium is protected by commensal bacteria, acid environment, mucous layer, enzymes (lysozyme), chemicals (bacteriocin, cathelicidin, and β-defensin), cilia movements, and physical cell barrier (Lee et al., 2010). The innate immune response is the first defense against pathogens

immediate upon exposure when the physical barrier is disrupted (Munyaka et al., 2012). The receptors are germline-encoded that recognizes common patterns of pathogens. Macrophages, neutrophils, and dendritic cells are phagocytic cells, while only macrophages and dendritic cells are antigen-presenting cells. Neutrophils, mast cells, eosinophils, and basophils circulate in the vessel before being activated. Mast cells are found mainly in the tissues that can activate other leukocytes.

In innate immunity, zinc upregulates chemotaxis of granulocytes (Ibs and Rink, 2003) and phagocytosis of macrophages and dendritic cells (Sheikh et al., 2010), and secretion of cytokines or chemokines, such as TNF-α, IL-1β, and IL-6, for recruitment of other immune cells (Mayer et al., 2014). The formation of phagolysosome is essential in phagocytic cells to eliminate ingested antigens by phagocytes. The process begins with the activation of the early endosome antigen-1 (EEA1) in the endosomes. Upon activation, EEA1 binds to the C-terminal of phosphatidylinositol 3-phosphate (PI3K), which then binds to the zinc finger domain of Rab5. Zinc functions as a catalytic cofactor in the zinc finger domain at the N-terminal of Rab5 protein of phagocytic cells (Simonsen et al., 1998; Merithew et al., 2003).

Acute Phase Response

The acute phase response is part of the innate immunity (Baumann and Gauldie, 1994). The hepatocytes are responsible for acute-phase proteins, such as C-reactive proteins (CRP) and mannose-binding lectin, the production that is initiated by TNF-α, IL-1, and IL-6. The c-reactive protein of the pentraxin protein family consists of five identical subunits. It binds to phosphocholine of pathogen LPS but not the on host LPS. CRP opsonizes the pathogen and activates complement by binding to C1q of the classical pathway. Mannose-binding lectin acts as an opsonin for monocytes, but not macrophages because macrophages lack mannose receptors.

Pulmonary surfactant proteins, SP-A and SP-D, in the collectin family, binds to pathogens with the globular domain to promote phagocytosis.

Zinc glycinate supplemented at 100 mg/kg decreased CRP and increased in alpha-1-acid glycoprotein (α-1-AGP) in the serum compared to zinc sulfate supplemented at 100 mg/kg in non-challenged chickens (Jarosz et al., 2017). In the same study, the inclusion of phytase decreased serum amyloid A (SAA) and increased α-1-AGP and haptoglobin in the serum compared to zinc sulfate. Zinc glycinate increased CRP, α-1-AGP, and haptoglobin in the liver compared to zinc sulfate. The inclusion of phytase decreased SAA, increased α-1-AGP, fibrinogen, and haptoglobin in the liver compared to zinc sulfate. The acute phase response is an integral part of immunity. However, few studies of zinc have been conducted on the acute phase response in chickens.

Adaptive Immunity

The adaptive immunity includes the humoral immunity by B lymphocytes and the cellular immunity by T lymphocytes (Zekarias et al., 2002). In humoral immunity, B-cell receptors (BCR) are immunoglobulins or antibodies that are membrane-bound (mIg) or are secreted (sIg) by plasma cells. Surface immunoglobulins of B cells and secreted immunoglobulins from plasma cells are receptors for antigens in humoral immunity. In thymus-dependent (TD) B-cell activation, B cells are activated by T helper cells that recognize protein antigens bound by immunoglobulins. The antigens are internalized by the T helper cells and repackaged in a peptide:MHC-II complex on the surface that is presented to BCR on B cells. B-cell co-receptor complex, CD19:CD21:CD81, amplifies B-cell activation. CD21 is also a complement receptor, CR2, that binds to complement protein, C3dg. TD activation requires the B cells and T helper cells to respond to the same antigen, which is referred to as linked recognition. In thymus-independent (TI) B-cell activation, antibody production is induced by two classes of antigens without T cells: TI-1 and TI-2. TI-1 antigens

activate B cells regardless of antigen specificity in polyclonal activation only in high concentration. In low antigen concentration, only the antigen-specific B cells are activated. LPS is one of the TI-1 antigens, also known as B-cell mitogens, that binds to LPS-binding protein and CD14. The LPS:LPS-binding protein:CD14 complex activates TLR-4 on B cells. TI-2 antigens such as bacterial capsular polysaccharides have repetitive structures that can only activate mature B cells, unlike TI-1 antigens, which can only activate immature and mature B cells. T1-2 antigens primarily activate B-1 and marginal zone B cells. Since TI-2 antigens only activate mature B cells and marginal zone B cells are not developed early in life, neonates do not receive response well with TI-2 antigens. B cells activation by TI-2 antigen cross-linking. Therefore, a high density of the isotopes is required, but density too high will cause mature B cells to become anergic.

In the chicken antibody response, zinc sulfate supplementation at 60 mg/kg increased the antibody response to sheep red blood cell (SRBC) challenge on 7 day post-injection but not 14 days post-infection compared to the non-supplemented group (Akbari et al., 2008). Antibody titer response to SRBC was increased when dietary zinc sulfate was increased from 20 to 80 mg/kg (Sunder et al., 2008). Antibody titers response to SRBC was not different in chickens that were fed zinc sulfate or zinc methionine supplemented at 40, 50, 65, or 170 mg/kg (Mohanna and Nys, 1999). Zinc sulfate, oxide, or propionate did not result in differences in hemagglutination (HA) titer (Yogesh et al., 2013). However, the HA titer was increased by increasing zinc concentration from 40 to 60 mg/kg. Antibody titer body titer response to Newcastle Disease Virus was not affected by zinc-nanoparticle complex compared to zinc sulfate or zinc methionate (Mohammadi et al., 2015). Organic zinc supplementation at 20 mg/kg increased secretory IgA (sIgA) on 7 days post-infection after challenged with *Eimeria maxima* compared to the non-supplemented group (Bun et al., 2011). In the same study, on 14 days post-infection, not only sIgA of the supplemented groups was higher

than thenon-supplemented group, the supplemented group at 40 mg/kg had lower sIgA than the supplemented group at 20 mg/kg. However, sIgA was increased unexpectedly in the supplemented group at 60 mg/kg. Zinc plays a major role in the humoral immunity. However, the role of zinc on the cellular immunity is not as well documented.

In cellular immunity, T cells target four types of antigens: defected host cells, pathogen-infected cells, phagocytosed pathogens, and extracellular pathogens or toxins (Sharma, 1997). T-cell receptors consist of TCRα and TCRβ chains. Some TCR consists of TCRγ and TCRδ chains. Unlike B-cell receptors that can recognize antigen surfaces, TCR recognizes intracellular peptides of processed antigens presented by MHC. T cells are separated into two classes: CD4 and CD8. CD4 T cells recognize MHC-I, and CD8 T cells recognize MHC-II. CD4 co-receptor can bind MHC-II by itself; however, CD4 co-receptor and TCR binding to MHC-II together increases activation by 100-fold. Zinc deficiency reduces thymus development; and subsequently reduces T-cell maturation and proliferation (King et al., 2005). CD8 to CD4 ratio is increased in zinc deficiency (Beck et al., 1997). T-cell proliferation is regulated by the Gfi1 (Karsunky et al., 2002) and ThPOK (Carpenter et al., 2012) using zinc in the zinc finger structural domain and STAT1 and STAT3 transcription factors that are regulated by the zinc interactions with metallothionein (Wu et al., 2013). T-cell activation is depending on the bind of the T-cell receptors. The binding of tyrosine kinase to CD4 and CD8 T-cell receptors requires zinc as a co-factor (Huse et al., 1998; Lin et al., 1998). Zinc deficiency decreases Th-1 (IFN-γ, IL-2, and TNF-α) cytokines without affecting TH-2 cytokines (IL-4, IL-6, and IL-10) (Rosenkranz et al., 2016); thus favoring TH-2 responses (Prasad, 2000). B cell maturation is regulated by zinc (DePasquale-Jardieu and Fraker, 1984a). Zinc functions in the zinc finger structural domains for Bcl-6 for expansion of B- cell germinal center (Phan et al., 2007), B cell maturation-induced protein (Blimb1) for B cell

differentiation (Xin et al., 2011). Antibody response by B cells is reduced from zinc deficiency (Fraker et al., 1978; Luecke et al., 1978; DePasquale-Jardieu and Fraker, 1984b; raker, 1984). TD B-cell response is more affected than TI B-cell response (Fraker et al., 1977; Fraker et al., 1978; Fraker and Zwickl, 1981; Fraker et al., 1984; Fraker et al., 1986). In addition, zinc is found in the zinc-finger motif of pro-myelocytic leukemia zinc finger (PLZF) for NKT cell development (Savage et al., 2008). Post-transcriptional epigenetic modification is regulated by gene methylation. Regulatory T cells are involved in this epigenetics process. In mixed lymphocyte culture, zinc increased T-cell activation marker CD69 and CD4+CD25+Foxp3+ inducible regulatory T cells populations (Rosenkranz et al., 2016). However, the CpG rich Foxp3 promoter region of PBMC is not affected by zinc. Acetylation of K31, K263, and K268 prevents ubiquitin-mediated proteasome degradation of Foxp3 (Kwon et al., 2012). Sirt-1 is a NAD-dependent deacetylase decrease Foxp3 activity in post-transcriptional modification. Zinc inhibits Sirt-1 activity and increases K31 acetylated Foxp3 (Rosenkranz et al., 2016).

Thymulin, secreted from thymic epithelial cells, promotes T-cells maturation and cytotoxicity (Prasad et al., 1988). Zinc is a thymulin co-factor (Iwata et al., 1979; Bendtzen, 1980; Iwata et al., 1981; Dardenne et al., 1982). Adrenal glucocorticoid was increased by zinc deficiency (DePasquale-Jardieu and Fraker, 1979; Quarterman and Humphries, 1979; Compton and Cidlowski, 1992; Concordet and Ferry, 1993; Garvy et al., 1993; Ozeki et al., 1997); which in part explained thymic dystrophy (DePasquale-Jardieu and Fraker, 1980a), B cell maturation (Fraker et al., 1995), and lymphocytes responses (DePasquale-Jardieu and Fraker, 1980b) caused by zinc deficiency. The cutaneous basophil hypersensitivity (CBH) response to phytohemagglutinin-P is an indication of the cell-mediated immune response. The CBH response was increased by increasing dietary zinc sulfate from 10 to 80 mg/kg (Sunder et al., 2008). Although

supplementation of zinc above 0 mg/kg to 30 mg/kg increased lymphocyte proliferation in the blood, it was not different when zinc was supplemented at 30, 60, or 120 mg/kg (Yang et al., 2011).

Immune Mediators

Cytokines (about 25 kDa in size) are immune mediators that are secreted by immune cells. The three families of cytokines are the hematopoietins, interferons, and TNF (Paul and Seder, 1994). The structural domain of the cysteine and histidine-rich zinc-finger, such as TNF-α converting enzyme (TACE) for signaling molecules such as cytokine and chemokines (Menghini et al., 2013), Calprotectin for chemotaxis (Nakatani et al., 2005), and MMP proteins for chemokines secretion (Dollery et al., 1995; McQuibban et al., 2002; Marikovsky et al., 2003; Zhang et al., 2003) require zinc as catalytic co-factors. The cytokine receptors have four categories: class I cytokine receptors (IL-2, -3, -4, -5, -7, -9, -13, -15, and CM-GSF), class II cytokine receptors (IFN- α, β, and γ), TNF-receptors (TNF, CD40, Fas, CD30, CD27), and chemokine receptors (CCR1 to 10, CXCR1 to 5, XCR1, and CX3CR1). **IL-1β**, produced by macrophages and keratinocytes, induces fever, activates T cells and macrophages, activates vascular endothelium, and increase acute-phase protein secretion in the liver. **CCL-5** promotes effector T cell migration (Janeway et al., 2004). **IL-6**, produced by macrophages and dendritic cells, activates lymphocytes, increases antibody production, and acute-phase protein secretion in the liver. **IL-8/CXCL-8**, produced by monocytes, macrophages, fibroblasts, keratinocytes, endothelial cells, and dendritic cells, mobilizes neutrophil, basophil, and naïve T cell. **CCL-2 and CXCL-8** are both involved in leukocytes recruitment. **IL-12**, produced by macrophages and dendritic cells, triggers the $CD4^+$ T cells differentiation into T_H-1 and activates NK cells. TNF-α, produced by macrophages and dendritic cells, increases vascular permeability and cell-adhesion molecules (E- and P-selectin) of the endothelium. **TNF-α**, produced by macrophages, activates vascular endothelium and increases

vascular permeability for IgG and complement entry. **CXCL-13** chemoattracts B cells to the respective region of the spleen, lymph nodes, and intestine. **TGF-β**, produced by chondrocytes, monocytes, and T cells, inhibits cell growth, activates anti-inflammatory responses, and induction of IgA production. **IFN-γ**, produced by T cells and NK cells, activates macrophages, increases MHC and antigen processing, immunoglobulin class switching, and suppresses T_H-2 response. The systemic response is induced by IL-1, IL-6, and TNF-α in the hypothalamus to raise body temperature and in the adipocyte and muscle cells to catabolize for energy. Interferons are a class of chemokines that target viruses by disrupting viral replication. dsRNA induces interferon production.

Zinc deficiency decreases cytokines, IL-1β (Bendtzen, 1980), IL-2 (Dowd et al., 1986; Moulder and Steward, 1989), IL-4 (Winchurch et al., 1987; Shi et al., 1994), IFN-α (Radhakrishnan et al., 1996), and IFN-γ (Salas and Kirchner, 1987) secretions, and decreases IL-2R expression (Dowd et al., 1986; Tanaka et al., 1990). Zinc supplementation increases cytokines, IL-1β, IL-6, IFN-α, and TNF-α (Winchurch et al., 1987; Scuderi, 1990; Driessen et al., 1994b, a). Monocyte adhesion to endothelial cells (Chavakis et al., 1999), dendritic cells maturation (Kitamura et al., 2006), and neutrophil ROS production (Singh et al., 1994) can be increased by zinc supplementation. Macrophage phagocytosis and elimination of intracellular parasites are weakened by zinc deficiency (Salvin et al., 1987; Wirth et al., 1989; Singh et al., 1992). In an *in vitro* study, zinc supplementation increased monocyte cytokines production (Winchurch et al., 1987; Scuderi, 1990; Driessen et al., 1994a). However, monocyte-mediated cytotoxicity (Allen et al., 1983) and macrophage phagocytosis (Wirth et al., 1984; Ercan and Bor, 1991) were enhanced by zinc deficiency.

In chickens, organic zinc supplementation at 90 mg/kg down-regulated IL-8 and upregulated IL-10 expressions compared to zinc sulfate supplemented at 90 mg/kg in coccidia and *Clostridium perfringens* co-infection but not in coccidia infection alone (Bortoluzzi et al., 2019a). In the same study, the expression of inducible nitric oxide synthesis (iNOS) was decreased by organic zinc compared to inorganic zinc by coccidia infection alone but was not different in the co-infection. In a similar study that supplemented chickens with zinc sulfate or organic zinc in the co-infection model, IFN-γ was decreased by the organic zinc while IL-8 and IL-10 were unaffected (Bortoluzzi et al., 2019b).

Although the cytokine response has been widely used to evaluate immunity in recent studies, interpreting up- and down-regulation of these immune mediators is complicated (Salvesen, 2002). Many of the cytokines have dual functions in pro-inflammatory and anti-inflammatory properties. While the reduction of IL-10 increases T cell IFN-γ and monocyte TNF-α, the increase of IL-10 T cell IFN-γ and decrease monocyte TNF-α. Therefore, cytokines should not be viewed solely in the lens of up- or down-regulation.

Antioxidant Status

Zinc is an essential component of an antioxidant enzyme, superoxide dismutase, and nuclear factor kappa B (NFκB) transcription factor activity. Zinc deficiency leads to an increase in oxidative stress, NFκB, and COX-2 activity; and a decrease in PPARα activity, which inhibits NFκB (Shen et al., 2008). In rat glioma cells, zinc deficiency increased nitrite production and p53 NFκB expression (Ho and Ames, 2002). Antioxidant enzymes are essential for macrophage survival following phagocytosis of pathogens. Zinc deficiency impairs macrophage phagocytosis, humoral and cellular immune responses against intracellular pathogens such as *Eimeria* species. NFkB consists of a zinc-finger binding domain. Activation of NFkB regulates inflammatory processes.

PPAR and RXR also consist of zinc-finger binding domains (Meerarani et al., 2003; Reiterer et al., 2004; Shen et al., 2007). Ligand binding with PPAR leads to heterodimerization with retinoid X receptor (RXR) and binding with peroxisome proliferator response elements (PPRE). PPAR activation regulates NFkB and AP-1 proinflammatory transcription factors gene expression. Intracellular zinc concentration is maintained by membrane zinc transporters and metallothionein (Maret, 2005; Cousins et al., 2006).

Enzyme activities can be used to evaluate the immune status of chickens (Rao et al., 2016). Lipid peroxidase measured by the product of polyunsaturated fatty acids peroxidation, malondialdehyde (MDA), was decreased by organic zinc supplemented at 40 mg/kg compared to diets with supplemented with inorganic zinc. Glutathione peroxidase (GPx) is an antioxidant enzyme that reduces reactive oxygen species (ROS). Supplementation of organic zinc from zinc-polysaccharide complex or nano-zinc at 60 mg/kg did not affect GPx or MDA compared to zinc oxide (El-Katcha et al., 2017). The copper/zinc superoxide dismutase (Cu/Zn-SOD) is an antioxidant enzyme that utilizes zinc as a co-factor (Marikovsky et al., 2003; West et al., 2011). In the liver of chickens challenged with *Eimeria tenella*, Cu/Zn-SOD was decreased by organic zinc supplementation at 60 mg/kg compared to the supplemented group at 40 mg/kg (Bun et al., 2011). Total-SOD, Cu/Zu-SOD, T-AOC, and MDA in the liver were increased by supplementing zinc sulfate at 80 mg/kg in non-challenged broiler chickens compared to the non-supplemented group (Zhang et al., 2017a). Furthermore, zinc glycinate supplementation at 80 mg/kg increased T-SOD and Cu/Zn-SOD in the liver compared to the zinc sulfate group. The same observations occurred in the serum in this study. However, there may be a difference between inorganic sources such as zinc sulfate and zinc oxide. Zinc glycinate supplementation at 100 mg/kg did not result in differences in Cu/Zn-SOD, catalase (CAT), or MDA in the chicken liver compared to zinc oxide (Kwiecień et al., 2017). The

interpretation of immune status based on antioxidant enzymes is similar to that for cytokine responses. While the increase of antioxidant enzyme by zinc may be interpreted as the improvement in immunity, it can also be interpreted as the increase of inflammation that leads to oxidative stress. Therefore, as with cytokine responses, the antioxidant status should not be viewed solely in the lens of up- or down-regulation.

Intestinal Gross Lesions

In *Eimeria brunetti* and *Clostridium perfringens* infection, intestinal gross lesions were increased by zinc sulfate supplemented at 1000 mg/kg compared to the non-supplemented group (Baba et al., 1992a). However, in another study of coccidia and *C. perfringens* infection, zinc from inorganic or organic sources did not result in differences in gross lesions (Bortoluzzi et al., 2019a).

Intestinal Epithelial Cells

Porcine intestinal epithelial cell line (IPEC-J2) from jejunum was used to study zinc on intestinal cell function using transepithelial electrical resistance (TEER) (Lodemann et al., 2013). IPEC-J2 cells were cultured in Dulbecco's modified eagle medium (DMEM)/F-12 medium (1:1) supplemented with 5% fetal bovine serum (FBS), 2.5 mmol/l L-glutamine, 5 µg/ml insulin, 5 µg/ml transferrin, and 5 ng/ml sodium selenite, 5 ng/ml epidermal growth factor (EGF), and Penicillin-Streptomycin at 37°C and 5% CO_2; and were trypsinized using 0.15 g/l porcine trypsin and 0.05 g/l EDTA. The medium was changed three times a week. Cells between 72 and 77 passages were used for the experiments. Alternatively, human colorectal adenocarcinoma, Caco-2, cells (ATCC HTB-37) were used as a comparison. Caco-2 cells were cultured in Eagle's Minimum Essential Medium with Earle's buffered saline solution (BSS) supplemented with 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 0.1 mmol/l nonessential amino acids, 1.5g/l

sodium bicarbonate, 20% FBS, and Penicillin-Streptomycin. The medium was changed every four to seven days. Cells between 36 and 41 passages were used for the experiments.

Zinc sulfate was diluted in sterile water at 4 mM. The zinc solution was added to the cells before the experiments. In the experiments, FBS at 10% was used. Cells were seeded on clear polyester membrane cell culture inserts (Snapwell®, 12 mm diameter, 1.12 cm^2 area, 0.4 µm pore size) at a density of 10^5 cells/1.12 cm^2 for 9 to 12 days for IPEC-J2 or 21 days for Caco-2 cells and TEER was measured using the Millicell-Electrical Resistance System (Millipore GmbH, Schwalback, Germany). Cells were seeded at 10^4 density and measured at 80% confluency and post-confluency (7 to 10 days for IPEC-J2 and 21 days for Caco-2 cells) using WST-1. LDH, and ATP assays.

WST-1 cell proliferation solution (10 µl) in 1:10 dilution was added to the cells after treated with zinc sulfate for one hour at 37°C. Absorbance was measured at 450/630 nm. Lactate dehydrogenase (LDH) as an indicator of loss in cell integrity was measured using the CytoTox-ONE™ Homogenous Membrane Integrity Assay (Promega GmbH, Mannheim, Germany). The fluorimetric reagent (100 µl) was incubated with the cells for 10 minutes, and the reaction was stopped using 50 µl stop solution with shaking for 10 seconds. Fluorescence was measured at 560/590 nm excitation/emission wavelengths. ATP assay as an indicator of viability was measured using CellTiter-GloLuminescent Cell Viability Assay. Cells seeded in 50 µl were plated in clear-bottom 96-well plates. The cell titer-Glo reagent (50 µl) was added to the cells and mixed for two minutes in a shaker. After 10 minutes of incubation, the luminescence was measured with one-second per well integration time using the Fluostar Optima (BMG Labtech GmbH, Offenburg, Germany). Heat shock protein-70 (Hsp70) mRNA gene expression of cells seeded at 10^5 density was measured in pre- and post-confluent cells (1 to 2 days for pre-confluent and 7 to 10 days for post-confluent IPEC-J2 cells; 3 to 4 days for pre-confluent and 21 days for post-confluent Caco-2

cells). Background absorbance without the cells was subtracted in the WST-1 and LDH assays. Zinc did not affect the TEER of post-confluent Caco-2 and IPEC-J2 cells. Zinc did not affect WST-1 conversion, LDH release, and ATP luminescence in Caco-2 cells. However, at 200 μM of zinc, WST conversion and ATP luminescence were decreased, and LDH release was increased in post-confluent IPEC-J2 cells, indicating a higher sensitivity of IPEC-J2 than Caco-2 cells and possible toxicity effect at this highest zinc concentration. In pre-confluent Caco-2 cells, only ATP luminescence was decreased at 200 μM, indicating possible toxicity at this concentration to the cells. In pre-confluent IPEC-J2 cells, WST-1 conversion and ATP luminescence were decreased, and LDH release was increased starting at 100 μM, indicating higher zinc sensitivity in pre-confluent than post-confluent IPEC-J2 cells. Similarly, Hsp70 gene expression was increased at 6 hours pre-incubation with 200 μM of zinc but not at 24 hours post-incubation and post-confluent cells. Hsp70 gene expression was increased at 6 and 24-hours post-incubation in post-confluent cells and 24-hours post-incubation in pre-confluent cells with 200 μM of zinc but not at 6 hours post-incubation in pre-confluent cells and other concentrations. The beneficial effects of zinc on intestinal cells is uncertain, but possible toxicity is possible at high concentrations.

Other Functions

Zinc is involved in all six of the metalloenzyme zinc classes as electron acceptors (Vallee and Galdes, 1984). These enzymes include RNA polymerases, alcohol dehydrogenase (ADH), carbonic anhydrase, and alkaline phosphatase. ADH contains four zinc atoms (two for enzymatic and two for structural function), converts alcohol to aldehydes using NADH. An example of the alcohol to aldehyde conversion includes retinol to retinal in vitamin A metabolism. Carbonic anhydrase in erythrocytes and renal tubule cells maintain pH balance. The enzyme catalyzes the reaction to remove carbon dioxide into carbonic acid. Oxyhemoglobin is the erythrocyte that

utilizes the protons from the carbonic acid to release oxygen to other tissues. Alkaline phosphatase, mostly found in bones and the liver, contains four zinc atoms (two for enzymatic and two for structural function), hydrolyzes monoester of phosphate groups of compounds. Protein digestion enzymes like carboxypeptidases A and B, exopeptidases, and aminopeptidases in protein digestion are structurally bound by zinc.

Several *in vitro* studies have demonstrated the involvement of zinc in molecular mechanisms, such as nucleic acid and protein synthesis (Nason et al., 1953; Winder and Denneny, 1959; Fujioka and Lieberman, 1964; Hsu et al., 1968; Sandstead and Rinaldi, 1969). *In vivo* studies have shown the role of zinc on ^{32}P and thymidine incorporation during nucleotide synthesis in rats (Sandstead and Rinaldi, 1969; Williams and Chesters, 1970), transcription and translation in pigs (Ku et al., 1970), glycine metabolism in glutathione synthesis (Hsu et al., 1968) and methionine metabolism in protein synthesis (Hsu et al., 1969), and cysteine metabolism in rats (Hsu and Anthony, 1970), and glucose metabolism (Quarterman et al., 1966). Zinc deficiency decreases lactate dehydrogenase (LDH), malic dehydrogenase (MDH), alcohol dehydrogenase (ADH), and NADH diaphorase in the testes; LDH, MDH, ADH, and alkaline phosphatase in the bones; MDH, AND, NADH diaphorase in the esophagus; and MDH and alkaline phosphatase in the kidney of the rat (Prasad et al., 1967a). Similar enzyme activities were observed in pigs (Prasad et al., 1969). Alkaline phosphatase activity in the intestine was decreased in zinc-deficient rats (Luecke et al., 1968). Δ-aminolevulinic acid dehydratase contains eight subunits with zinc in each one and can be condensed to form porphobilinogen for heme synthesis. Phospholipase C hydrolyzes glycerophosphate bond in phospholipid, and it contains three zinc atoms for the catalytic activity. Polyglutamate hydrolase (also known as γ-glutamyl hydrolase or pteroylglutamic hydrolase) removes the glutamic acids bound to the folate into monoglutamine folate for folate absorption

and contains zinc for the catalytic activity. Collagenase, gelatinase, matrilysins, and stromelysins, contains zinc in the catalytic site, degrade extracellular matrix for tissue repair.

Zinc in Necrotic Enteritis Infection in Chickens

The main cause of necrotic enteritis is netB toxin from *Clostridium perfringens* infection (Keyburn et al., 2008). Several factors, such as the dietary components and coccidiosis, predispose chickens to necrotic enteritis. Dietary components such as water-soluble non-starch polysaccharides (NSP) (β-glucans and arabinoxylans) high in cereals and high fish meal diets with high protein increase the chance of NE infection. NSP is not digested by birds because of the lack of β1-3 and β1-6 glycosidase, which acts as a substrate for microbiota growth and promotes *C. perfringens* proliferation in the gastrointestinal tract of the birds (Iji and Tivey, 1998). High protein diets increase intestinal pH by increasing metabolites such as ammonia and amine products from digestion (Juśkiewicz et al., 2004). High pH favors *C. perfringens* growth (Allison and Macfarlane, 1989) that leads to NE infection. Coccidiosis in chickens is caused by an apicomplexan (Barta et al., 1991) protozoa, *Eimeria*. The disease manifestation of coccidiosis in chickens differs between species (*E. acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix*, *E. mitis*, and *E. tenella*) and the number of oocysts that produces infection varies between 2×10^2 to 2×10^3 oocysts (Raman et al., 2011). While coccidia is present in most poultry flocks, coccidiosis is generally self-limiting depend on the immunity of the birds (Hong et al., 2006) and litter management. Particularly, the parasite is killed by ammonia in composting poultry litter; therefore, routine refreshing of poultry litter may exacerbate coccidiosis and related disease outbreak.

The *Eimeria* lifecycle has an exogenous and endogenous phase (Lal et al., 2009). In the exogenous phase, the oocysts are excreted by the chicken in the feces. The oocysts require a humid and oxygenated environment to sporulate. Each oocyst consists of four sporocysts with two sporozoites

in each sporocyst. Once sporulated, the oocysts infect the chicken through the oral-fecal route into the endogenous phrase. The mechanical digestion in the chicken's gizzard and enzymatic digestion in the intestine release sporozoites from the sporocysts into the intestine lumen. The sporozoites invade the intestine cells and undergo schizogony to produce merozoites inside the cells in the asexual developmental phase. The schizonts rupture and release merozoites that infect other cells. After two to four generations of asexual development or schizogony phase, the sexual phase occurs. Some merozoites undergo gametogenesis to produce micro- and macro-gametes that develop into unsporulated oocysts that are shed into the feces.

The oocyst shedding pattern varies between *Eimeria* species (Cha et al., 2018). The peak time of oocyst production for *E. acervuline* occurs six days post-infection, while for *E. maxima* and *E. tenella* occurs seven days post-infection. The oocysts shedding peak pattern influences the pathogenicity of the infection. The precise control of the timing is crucial to experimentally induce necrotic enteritis when inoculating coccidia with *Clostridium perfringens*. *E. acervuline*, *E. tenella*, *and E. maxima* are the most prevalent coccidiosis species in broiler chickens (Györke et al., 2013). *E. acervuline* oocysts are the smallest amongst the other two species, while *E. maxima* oocysts are the largest comparatively (Saif et al., 2011). The *Eimeria* species affect different regions of the intestinal tract. Particularly, *E. acervuline* infection manifests primarily in the foregut region near the duodenum; *E. maxima* infection manifests primarily in the midgut region near the jejunum and after the yolk sac diverticulum; and *E. tenella* infection manifests primarily in the ceca (Johnson and Reid, 1970). The pathogenicity of the three species of *Eimeria* is a distinguishing factor. While *E. acervulina* has the slowest sporulation time of 17 hours, *E. tenella* has a similar sporulation time of 18 hours compared to *E. acervuline*, and *E. maxima* has the longest sporulation time of 30 hours (Edgar, 1954; Johnson and Reid, 1970). *E. acervuline* lesions

are produced by vary lifestage of the parasite affecting the epithelium (Vetterling and Doran, 1966; Fernando, 1974). However, *E. maxima* lesions are generated mainly by the second-generation of schizonts that affect the deeper epithelium (Millard et al., 1971). *E. tenella* produces the most morbidity and mortality of the three. First, the short schizont stage of *E. tenella* allows the parasite to mature its first schizont stage by day 3 post-infection and propagate a large number of oocysts by the ruptured schizonts (Long, 1965; McDougald and Galloway, 1973; Del Cacho et al., 2004). Second, *E. tenella* lesions are generated by first and second-generation schizonts that result in hemorrhages and necrosis of the ceca. While the schizogony of *E. acervulina* is short as well, the first generation schizonts contain few merozoites (Norton and Joyner, 1981), which makes *E. tenella* particularly problematic.

The interrelationships of zinc and *Eimeria* have been studied for years. Excess dietary zinc above 1000 mg/kg exacerbates coccidiosis (Southern and Baker, 1983b; Baba et al., 1992a). Zinc utilization is increased by coccidiosis (Southern and Baker, 1983a). In addition, there is an antagonistic effect by copper and calcium on zinc utilization (Bafundo et al., 1984). Zinc supplementation at 50 mg/kg improves growth performance of birds after *E. acervulina* infection (Southern and Baker, 1983a). In addition, zinc supplementation reduces oocysts shedding after *E. tenella* infection (Bun et al., 2011).

Table 2.3.
C. perfringens isotypes and toxins produced by the bacteria.

	Alpha	Beta	Epsilon	Iota	β2 toxin (CPB2)	netB	TpeL	CPE
A	+	-	-	-	+	+	+	+
B	+	+	+	-				
C	+	+	-	-	+			
D	+	-	+	-				
E	+	-	-	+				

Clostridium perfringens is a spore-forming gram-positive bacterium that is the primary cause of necrotic enteritis infection in chickens. *C. perfringens* is categorized into five

types (A to E) based on the four major types of endotoxins that are produced (α, β, ε, and ι) (Table 2.3). More *C. perfringens* toxins have been discovered recently: β2 toxin (CPB2), netB, TpeL (Songer and Meer, 1996).

Other toxins are found in non-NE-associated *C. perfringens* strains. For example, CPE toxins (35 kDa) is responsible for *C. perfringens* type A food poisoning, which is the third most common foodborne illness in the US (Olsen et al., 2000). The *cpe* gene encodes the CPE toxin that can be located in the plasmid or the chromosome (Cornillot et al., 1995; Collie and McClane, 1998). *C. perfringens* type A food poisoning usually contains the *cpe* gene in the chromosome. CPE related *C. perfringens* type A that results in occasional diarrhea usually contains the *cpe* gene in the plasmid. Chromosomal *cpe* isolates are associated with food poisoning because they are more resistant to heat treatment in food processing than the plasmid *cpe* isolates. CPE binds to receptors (Wnek and McClane, 1986) and claudin-3 and -4 (Katahira et al., 1997a; Katahira et al., 1997b; Fujita et al., 2000) in the intestinal lumen. CPE activates the caspase-3 mediated apoptosis of CaCo-2 cells (Hockenbery, 1995; Majno and Joris, 1995; Steller, 1995). The CPE activated apoptosis involves calcium signaling (Caspar et al., 1987).

ε toxin of type B and D strains causes fatal enterotoxemia in lambs and goats (Songer, 1996). ε toxin is secreted in an inactive form and then converted the active form by cleavage of 13 amino acids from the N-terminus and 22 amino acids in the C-terminus by intestinal proteases, such as trypsin and α-chymotrypsin (Miyata et al., 2001; Smedley et al., 2004). The encoding gene of ε toxin, *etx*, is located in the plasmid (Katayama et al., 1996).

NE is caused by *C. perfringens* type A but rarely type C (Songer, 1996; Engström et al., 2003). *C. perfringens* grow between 15 and 50°C, and the spores can tolerate 100°C for two hours. Less than 10^2 to 10^4 CFU/g of *C. perfringens* is found in the healthy chicken intestine, and 10^7 to 10^9 CFU/g

of *C. perfringens* is found in NE infected chickens (Kondo, 1988). NE generally manifests between 14 and 42 days of age in broiler chickens (Songer, 1996; Cooper and Songer, 2010). The mortality of NE is between 10 and 40% (McDevitt et al., 2006). Clinical manifestation of NE includes lethargy, dehydration, diarrhea, ruffled feathers, low feed intake, and body weight loss. The subclinical manifestation of NE includes a decrease in weight gain and a reduction in feed conversion efficiency. *C. perfringens* contain 56 glycoside hydrolase enzymes that degrade glycoproteins (Ficko-Blean et al., 2011; Ficko-Blean et al., 2012). *C. perfringens* can be cultured anaerobically at 37°C using fluid thioglycollate medium (FTG) with dextrose (Long and Truscott, 1976; Cowen et al., 1987; Riddell and Kong, 1992b; McReynolds et al., 2004b; Olkowski et al., 2006; McReynolds et al., 2007a; McReynolds et al., 2009). The addition of peptone and starch can increase alpha-toxin production (Al-Sheikhly and Truscott, 1977b). Other media, such as cooked meat medium (CMM)(Baba et al., 1992a; Dahiya et al., 2007a) and brain heart infusion broth (BHI) (Kaldhusdal et al., 1999; Pedersen et al., 2003; Pedersen et al., 2008). The culture of *C. perfringens* using FTG less than 15 hours (log phase) produced more severe NE than using 24 hours culture (stationary phase) (Long and Truscott, 1976; Thompson et al., 2006).

The CPE toxin (35 kDa) of *C. perfringens* type A is responsible for the third most common foodborne illnesses in the US (Olsen et al., 2000). CPE binds to junction proteins Claudin-3 and -4 at the second extracellular loop domain but not Claudin-1 and -2 (Fujita et al., 2000). *C. perfringens* type A is the primary cause of NE in broiler chickens (Engström et al., 2003; Siragusa et al., 2006; Crespo et al., 2007b; Cooper and Songer, 2009). Alpha toxin is a zinc metalloenzyme with lecithinase, phospholipase, sphingomyelinase, and hemolytic activities that are produced in *C. perfringens* (Songer, 1996; Titball et al., 1999; Awad et al., 2001). Alpha toxins (Keyburn et al., 2006), netB (Keyburn et al., 2008; Van Immerseel et al., 2009), and the β2 toxin from *C.*

perfringens type A (Engström et al., 2003; Waters et al., 2003; Gholamiandekhordi et al., 2006; Siragusa et al., 2006; Crespo et al., 2007a; Cooper and Songer, 2010) have been implicated in NE in chickens. However, experimental induction of NE using the different strains showed that the presence of any of these toxins does not guarantee to produce the disease.

C. perfringens genes are encoded in the plasmids and chromosomal genes. Variations of NE-associated strains of *C. perfringens* may explain the differences in virulence. Three pathogenicity loci (PAL) have been characterized: NELoc1, NELoc2, and NELoc3. NELoc1 and NELoc3 are encoded in the plasmid.

Plasmids about 40 to 140 kb encode three of the four toxins, beta, iota, and epsilon, and other virulence factors, such as *β2*, *cpe*, *delta*, *netB*, *netF*, and *tpeL* (Freedman et al., 2015). The plasmids also encode bacitracin and tetracycline resistance, adhesins, and catabolic enzyme genes. Three large plasmids are associated with the NE-associated strains; in the EHE-NE18 strain, pJIR3536 encodes the netB toxin in the NELoc1 region; pJIR3565 encodes tetracycline resistance, and pJIR3537 encodes β2 in the NELoc3 region. The number of large plasmids correlates to the genetic diversity of the NE-associated strains (Bannam et al., 2011; Parreira et al., 2012). Another plasmid encodes the TpeL toxin in some NE-associated strains (Coursodon et al., 2012).

β toxin (35 kDa) was proposed to cause necrotic enteritis in humans and animals (Sakurai and Fujii, 1987; Sakurai, 1995a). *Cpe* gene is encoded in the plasmid (Sakurai and Duncan, 1978; Katayama et al., 1996). B2 toxin (CPB2) is not related to β toxin; the two only share 15% nucleotide similarity (Gibert et al., 1997). CPB2 is secreted as a 265 amino acid protein (27.7 kDa) (Bueschel et al., 2003). *Cpb2* is carried on plasmids (Gibert et al., 1997; Shimizu et al., 2002).

The *cpa* alpha-toxin gene is encoded in the chromosome (Dahiya et al., 2006). *Cpa* does not encode the virulent factor for NE (Keyburn et al., 2006). However, multiple SNPs have been found on the

cpa gene (Sheedy et al., 2004; Abildgaard et al., 2009). The *cpe* gene can present on the chromosome or plasmid (Cornillot et al., 1995; Collie and McClane, 1998). These four plasmids are present in the NE-associated strains (Keyburn et al., 2010; Lepp et al., 2013). The WERNE36 and JGS4143 contain the pJIR3536 and 9JIR3537 plasmids. CP4 and NAG-NE31 contain all four plasmids but not bacitracin resistance. CP3 contains all four plasmids and the bacitracin resistance gene.

The *netB* gene is encoded in the 85 kb plasmid associated with a 42 kb pathogenicity locus (NELoc1), a plasmid-encoded NELoc3, and a chromosomal locus (NELoc2) (Bannam et al., 2011; Lepp et al., 2013). Two alleles for the *netB* gene have been discovered that produce variants in netB toxins (Bannam et al., 2011). An SNP in the *netB* allele resulted in amino acid change A168T.

In the chromosomal genes of *C. perfringens*, the adhesion-related locus, VR-10, was found to consist of variants that may contribute to the virulence: VR-10A with a von Willebrand domain and a collagen-adhesion protein (VR-10B) (Lepp et al., 2013). VR-10A is associated with the non-poultry strains, and VR-10B is associated with the poultry strains.

TpeL of the Large Clostridial Toxins (LCT) family is present in some type A NE-associated isolates (Chalmers et al., 2008). A study showed that *netB*-positive and tpeL-positive produced NE.

In addition to the isotype of the bacteria and toxin types, the route, dosage, timing, and frequency of the experimental NE models vary among literature. The common routes of *C. perfringens* inoculation were by oral gavage or exposure to contaminated feed or litter. Previous studies inoculated the birds between 10^6 and 10^9 CFU/bird of *C. perfringens* (McReynolds et al., 2004b; Dahiya et al., 2007b; McReynolds et al., 2007b). Some studies only inoculated birds once, while others inoculated birds on multiple days at a different age. The dosages of *C. perfringens* and

coccidia vary between strains from each laboratory, and each batch of the inoculates with the laboratory. Therefore, there is not a consensus on the amount required to induce NE.

A common method of NE induction is the coinfection with field isolates and vaccine strains of *Eimeria* with *C. perfringens*. *Eimeria maxima*, *acervuline*, *tenella*, and *mitis* have been used in these models (Williams et al., 2003; Park et al., 2008; Timbermont et al., 2009; Miller et al., 2010). The dosage of *Eimeria* ranges between 10^3 to 10^4 oocysts/bird. Generally, *Eimeria* is inoculated about five days before *C. perfringens* inoculation. For vaccine strains, 10 to 24-fold higher than recommended dosage given three to one day before *C. perfringens* is used to induce NE (McReynolds et al., 2004b; Pedersen et al., 2008; Timbermont et al., 2009). Most notably, many of these studies did not have any mortality; therefore, NE models vary. This is explained by current knowledge that the *Eimeria* requires at least five days to complete the lifecycle in the host (Lal et al., 2009). Therefore, inoculation of *C. perfringens* too early after the inoculation of coccidia may not disrupt the intestine adequately to induce NE.

Nutrition, particularly high protein diets and indigestible non-starch polysaccharides (NSP), such asβ glucan and arabinoxylans, favors *C. perfringens* colonization and increase the risk of NE (Williams, 2005; Dahiya et al., 2006; McDevitt et al., 2006; Cooper and Songer, 2009). *C. perfringens* cannot synthesize many amino acids; therefore rely on breaking down tissues or digesta in the intestine using hydrolytic enzymes (Shimizu et al., 2002). Therefore, high protein diets at 24 to 38% that increase undigested protein in the intestine increased the risk in NE (Park et al., 2008; Mikkelsen et al., 2009). In addition, glycine and methionine increase alpha-toxin production by *C. perfringens* (Dahiya et al., 2007b). NSP increases digesta viscosity (Kaldhusdal and Hofshangen, 1992; Kleessen et al., 2003; Dahiya et al., 2006; McDevitt et al., 2006) and mucin

secretion. *C. perfringens* infected chickens fed high NSP diets had mortality between 26 and 35%, whereas those fed low NSP diets had mortality between 26 and 35% (Riddell and Kong, 1992a).

Gross lesions of necrotic enteritis are scored based on the infection in the small intestine. Lesions of NE can be observed throughout the small intestine but centralized in the jejunum. Lesion scoring scales varied between studies: 0 to 3 (Lovland et al., 2004; Gholamiandehkordi et al., 2007; Collier et al., 2008), 0 to 4 (Prescott et al., 1978; Brennan et al., 2003; McReynolds et al., 2004a; Cooper et al., 2009; Cooper and Songer, 2010; Cooper et al., 2010), 0 to 6 (Keyburn et al., 2006; Keyburn et al., 2008; Timbermont et al., 2009; Timbermont et al., 2010). Regardless, NE infection progresses from healthy tissues to exudates or fibrins on the intestinal tissues, then the intestine becomes thin and friable, and later ulcers, hemorrhages, and blood clots.

Several experimental NE models were used to determine the role of zinc in the infection. The coccidia and *C. perfringens* co-infection model is the favored model to induce NE to study the nutritional effects of nutrients and feed additives. This is because using high fishmeal diets or NSP to induce NE can have interactions with the nutritional treatments. Zinc has the potential to partially alleviating NE severity. However, the specific mechanism of zinc is not well understood. Zinc has multiple biological properties in cellular and immune processes in chickens. In addition, zinc has a bactericidal effect on *C. perfringens*, and it has been demonstrated that toxin production by the bacteria can be affected by zinc. Therefore, understanding the interrelationship of zinc on the intestinal cells, immune cells, and *C. perfringens* is crucial in determining the mechanism of zinc and recommending the proper concentration in the diets.

REFERENCES

Abildgaard, L., R. M. Engberg, K. Pedersen, A. Schramm, and O. Hojberg. 2009. Sequence variation in the α-toxin encoding plc gene of Clostridium perfringens strains isolated from diseased and healthy chickens. Veterinary Microbiology 136(3-4):293-299.

Akbari, M., H. Kermanshahi, H. N. Moghaddam, A. H. Moussavi, and J. T. Afshari. 2008. Effects of wheat-soybean meal based diet supplementation with vitamin A, vitamin E and zinc on blood cells, organ weights and humoral immune response in broiler chickens. J. Anim. Vet. Adv 7(3):297-304.

Al-Sheikhly, F., and R. B. Truscott. 1977. The interaction of Clostridium perfringens and its toxins in the production of necrotic enteritis of chickens. Avian diseases 21(2):256-263.

Allen, J. I., R. T. Perri, C. J. McClain, and N. E. Kay. 1983. Alterations in human natural killer cell activity and monocyte cytotoxicity induced by zinc deficiency. Journal of Laboratory and Clinical Medicine 102(4):577-589.

Allison, C., and G. T. Macfarlane. 1989. Influence of pH, nutrient availability, and growth rate on amine production by Bacteroides fragilis and Clostridium perfringens. Applied and environmental microbiology 55(11):2894-2898.

Andrews, G. K. 2008. Regulation and function of Zip4, the acrodermatitis enteropathica gene. Portland Press Limited.

Applegate, T. J., and R. Angel. 2014. Nutrient requirements of poultry publication: History and need for an update. Journal of Applied Poultry Research 23(3):567-575.

Archibald, J. 1944. Zinc in cows' milk. Journal of Dairy Science 27(4):257-261.

Aschner, M., and A. K. West. 2005. The role of MT in neurological disorders. Journal of Alzheimer's Disease 8(2):139-145.

Awad, M. M., D. M. Ellemor, R. L. Boyd, J. J. Emmins, and J. I. Rood. 2001. Synergistic effects of alpha-toxin and perfringolysin O in Clostridium perfringens-mediated gas gangrene. Infection and Immunity 69(12):7904-7910.

Baba, E., A. L. Fuller, J. M. Gilbert, S. G. Thayer, and L. R. McDougald. 1992. Effects of Eimeria brunetti infection and dietary zinc on experimental induction of necrotic enteritis in broiler chickens. Avian Diseases 36(1):59-62.

Baer, M. T., and J. C. King. 1984. Tissue zinc levels and zinc excretion during experimental zinc depletion in young men. The American Journal of Clinical Nutrition 39(4):556-570. doi: 10.1093/ajcn/39.4.556

Bafundo, K., D. Baker, and P. Fitzgerald. 1984. Zinc utilization in the chick as influenced by dietary concentrations of calcium and phytate and by Eimeria acervulina infection. Poultry Science 63(12):2430-2437.

Bannam, T. L., X.-X. Yan, P. F. Harrison, T. Seemann, A. L. Keyburn, C. Stubenrauch, L. H. Weeramantri, J. K. Cheung, B. A. McClane, and J. D. Boyce. 2011. Necrotic enteritis-derived Clostridium perfringens strain with three closely related independently conjugative toxin and antibiotic resistance plasmids. MBio 2(5):e00190-00111.

Barney, G. H., M. P. Macapinlac, W. N. Pearson, and W. J. Darby. 1967a. Parakeratosis of the tongue--a unique histopathologic lesion in the zinc-deficient squirrel monkey. Journal of Nutrition 93(4):511-517. doi: 10.1093/jn/93.4.511

Barney, G. H., M. P. Macapinlac, W. N. Pearson, and W. J. Darby. 1967b. Parakeratosis of the tongue--a unique histopathologic lesion in the zinc-deficient squirrel monkey. Nutrition 93(4):511-517. doi: 10.1093/jn/93.4.511

Barta, J. R., M. C. Jenkins, and H. D. Danforth. 1991. Evolutionary relationships of avian Eimeria species among other Apicomplexan protozoa: monophyly of the apicomplexa is supported. Molecular Biology and Evolution 8(3):345-355.

Baumann, H., and J. Gauldie. 1994. The acute phase response. Immunology today 15(2):74-80.

Beck, F. W., J. Kaplan, N. Fine, W. Handschu, and A. S. Prasad. 1997. Decreased expression of CD73 (ecto-5′-nucleotidase) in the CD8+ subset is associated with zinc deficiency in human patients. Journal of Laboratory and Clinical Medicine 130(2):147-156.

Bendtzen, K. 1980. Differential role of Zn2+ in antigen- and mitogen-induced lymphokine production. Scandinavian journal of immunology 12(6):489-492.

Berfenstam, R. 1952a. Blood zinc during pregnancy and delivery. Acta Radiologica 41

Berfenstam, R. 1952b. Studies on blood zinc; a clinical and experimental investigation into the zinc content of plasma and blood corpuscles with special reference to infancy. Acta paediatrica. Supplementum 41(87):1.

Bertrand, G., and R. Vladesco. 1921. Role of zinc in reproduction. In: Academy of Science. p 176.

Birckner, V. 1919. The Zinc Content of Some Food Products. Journal of Biological Chemistry 38(2):191-203.

Blackmon, D. M., W. J. Miller, and J. D. Morton. 1967. Zinc deficiency in ruminants. Occurrence, effects, diagnosis and treatments. Veterinary Medicine, Small Animal Clinician 62(3):265-270.

Blamberg, D. L., U. B. Blackwood, W. C. Supplee, and G. F. Combs. 1960. Effect of zinc deficiency in hens on hatchability and embryonic development. Proceedings of the Society for Experimental Biology and Medicine 104:217-220.

Bly, M. 2006. Examination of the zinc transporter gene, SLC39A12. Schizophrenia Research 81(2):321-322.

Bonaventura, P., G. Benedetti, F. Albarède, and P. Miossec. 2015. Zinc and its role in immunity and inflammation. Autoimmunity Reviews 14(4):277-285.

Bortoluzzi, C., B. Lumpkins, G. Mathis, M. França, W. King, D. Graugnard, K. Dawson, and T. Applegate. 2019a. Zinc source modulates intestinal inflammation and intestinal integrity of broiler chickens challenged with coccidia and Clostridium perfringens. Poultry science 98(5):2211-2219.

Bortoluzzi, C., B. Vieira, B. Lumpkins, G. Mathis, W. King, D. Graugnard, K. Dawson, and T. Applegate. 2019b. Can dietary zinc diminish the impact of necrotic enteritis on growth performance of broiler chickens by modulating the intestinal immune-system and microbiota? Poultry Science

Brennan, J., J. Skinner, D. Barnum, and J. Wilson. 2003. The efficacy of bacitracin methylene disalicylate when fed in combination with narasin in the management of necrotic enteritis in broiler chickens. Poultry Science 82(3):360-363.

Bridges, C. C., and R. K. Zalups. 2005. Molecular and ionic mimicry and the transport of toxic metals. Toxicology and Applied Pharmacology 204(3):274-308.

Brink, M., D. Becker, S. Terrill, and A. Jensen. 1959. Zinc toxicity in the weanling pig. Journal of Animal Science 18(2):836-842.

Bueschel, D. M., B. H. Jost, S. J. Billington, H. T. Trinh, and J. G. Songer. 2003. Prevalence of cpb2, encoding beta2 toxin, in Clostridium perfringens field isolates: correlation of genotype with phenotype. Veterinary Microbiology 94(2):121-129.

Bun, S., Y. Guo, F. Guo, F. Ji, and H. Cao. 2011. Influence of organic zinc supplementation on the antioxidant status and immune responses of broilers challenged with Eimeria tenella. Poultry Science 90(6):1220-1226.

Cao, J., J. A. Bobo, J. P. Liuzzi, and R. J. Cousins. 2001. Effects of intracellular zinc depletion on metallothionein and ZIP2 transporter expression and apoptosis. Journal of leukocyte biology 70(4):559-566.

Carpenter, A. C., J. R. Grainger, Y. Xiong, Y. Kanno, H. H. Chu, L. Wang, S. Naik, L. dos Santos, L. Wei, and M. K. Jenkins. 2012. The transcription factors Thpok and LRF are necessary and partly redundant for T helper cell differentiation. Immunity 37(4):622-633.

Caspar, M., I. Florin, and M. Thelestam. 1987. Calcium and calmodulin in cellular intoxication with Clostridium difficile toxin B. Journal of cellular physiology 132(1):168-172.

Cavell, P. A., and E. M. Widdowson. 1964. Intakes and excretions of iron, copper, and zinc in the neonatal period. Archives of Disease in Childhood 39(207):496.

Cha, J. O., J. Zhao, M. S. Yang, W. I. Kim, H. S. Cho, C. W. Lim, and B. Kim. 2018. Oocyst-shedding patterns of three Eimeria species in chickens and shedding pattern variation depending on the storage period of Eimeria tenella oocysts. Journal of Parasitology 104(1):18-22.

Chalmers, G., H. L. Bruce, D. B. Hunter, V. R. Parreira, R. R. Kulkarni, Y. F. Jiang, J. F. Prescott, and P. Boerlin. 2008. Multilocus sequence typing analysis of Clostridium perfringens isolates from necrotic enteritis outbreaks in broiler chicken populations. Journal of Clinical Microbiology 46(12):3957-3964. doi: 10.1128/jcm.01548-08

Chavakis, T., A. E. May, K. T. Preissner, and S. M. Kanse. 1999. Molecular mechanisms of zinc-dependent leukocyte adhesion involving the urokinase receptor and β2-integrins. Blood 93(9):2976-2983.

Chen, J., N. Qu, and Y. Xia. 2005. Effect of zinc on thymulin level in mice. Wei sheng yan jiu= Journal of Hygiene Research 34(4):430-432.

Chimienti, F., S. Devergnas, A. Favier, and M. Seve. 2004. Identification and cloning of a β-cell–specific zinc transporter, ZnT-8, localized into insulin secretory granules. Diabetes 53(9):2330-2337.

Chimienti, F., A. Favier, and M. Seve. 2005. ZnT-8, a pancreatic beta-cell-specific zinc transporter. Biometals 18(4):313-317.

Cho, E., J. J. Hwang, S. H. Han, S. J. Chung, J. Y. Koh, and J. Y. Lee. 2010. Endogenous zinc mediates apoptotic programmed cell death in the developing brain. Neurotoxicity Research 17(2):156-166. doi: 10.1007/s12640-009-9085-2

Cole, T. B., H. J. Wenzel, K. E. Kafer, P. A. Schwartzkroin, and R. D. Palmiter. 1999. Elimination of zinc from synaptic vesicles in the intact mouse brain by disruption of the ZnT3 gene. Proceedings of the National Academy of Sciences 96(4):1716-1721.

Collie, R. E., and B. A. McClane. 1998. Evidence that the enterotoxin gene can be episomal in Clostridium perfringens isolates associated with non-food-borne human gastrointestinal diseases. Journal of Clinical Microbiology 36(1):30-36.

Collier, C., C. Hofacre, A. Payne, D. Anderson, P. Kaiser, R. Mackie, and H. Gaskins. 2008. Coccidia-induced mucogenesis promotes the onset of necrotic enteritis by supporting Clostridium perfringens growth. Veterinary Immunology and Immunopathology 122(1-2):104-115.

Compton, M. M., and J. A. Cidlowski. 1992. Thymocyte apoptosis: a model of programmed cell death. Trends in endocrinology and metabolism: TEM 3(1):17-23.

Concordet, J. P., and A. Ferry. 1993. Physiological programmed cell death in thymocytes is induced by physical stress (exercise). American Journal of Physiology-Gastrointestinal and Liver Physiology 265(3 Pt 1):C626-629. doi: 10.1152/ajpcell.1993.265.3.C626

Cooper, K., H. Trinh, and J. G. Songer. 2009. Immunization with recombinant alpha toxin partially protects broiler chicks against experimental challenge with Clostridium perfringens. Veterinary Microbiology 133(1-2):92-97.

Cooper, K. K., and J. G. Songer. 2009. Necrotic enteritis in chickens: a paradigm of enteric infection by Clostridium perfringens type A. Anaerobe 15(1-2):55-60.

Cooper, K. K., and J. G. Songer. 2010. Virulence of Clostridium perfringens in an experimental model of poultry necrotic enteritis. Veterinary Microbiology 142(3-4):323-328.

Cooper, K. K., J. R. Theoret, B. A. Stewart, H. T. Trinh, R. D. Glock, and J. G. Songer. 2010. Virulence for chickens of Clostridium perfringens isolated from poultry and other sources. Anaerobe 16(3):289-292.

Copper, I. 2001. Dietary reference intakes for vitamin A vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. Washington, DC: The National Academies Press.

Cornillot, E., B. Saint-Joanis, G. Daube, S. i. Katayama, P. E. Granum, B. Canard, and S. T. Cole. 1995. The enterotoxin gene (cpe) of Clostridium perfringens can be chromosomal or plasmid-borne. Molecular Microbiology 15(4):639-647.

Costello, L. C., Y. Liu, J. Zou, and R. B. Franklin. 1999. Evidence for a zinc uptake transporter in human prostate cancer cells which is regulated by prolactin and testosterone. Journal of Biological Chemistry 274(25):17499-17504.

Coursodon, C. F., R. D. Glock, K. L. Moore, K. K. Cooper, and J. G. Songer. 2012. TpeL-producing strains of Clostridium perfringens type A are highly virulent for broiler chicks. Anaerobe 18(1):117-121. doi: 10.1016/j.anaerobe.2011.10.001

Cousins, R. 1989. Systemic transport of zinc, Zinc in Human Biology. Springer. p. 79-93.

Cousins, R. J., J. P. Liuzzi, and L. A. Lichten. 2006. Mammalian zinc transport, trafficking, and signals. Journal of Biological Chemistry 281(34):24085-24089.

Cousins, R. J., and R. J. McMahon. 2000a. Integrative aspects of zinc transporters. Nutrition 130(5):1384S-1387S.

Cousins, R. J., and R. J. McMahon. 2000b. Integrative aspects of zinc transporters. Journal of Nutrition 130(5):1384S-1387S.

Cowen, B. S., L. D. Schwartz, R. A. Wilson, and S. I. Ambrus. 1987. Experimentally induced necrotic enteritis in chickens. Avian Diseases 31(4):904-906.

Cox, D. H., and D. L. Harris. 1960. Effect of excess dietary zinc on iron and copper in the rat. Journal of Nutrition 70(4):514-520.

Crespo, R., D. J. Fisher, H. Shivaprasad, M. E. Fernández-Miyakawa, and F. A. Uzal. 2007a. Toxinotypes of Clostridium perfringens isolated from sick and healthy avian species. Journal of Veterinary Diagnostic Investigation 19(3):329-333.

Crespo, R., D. J. Fisher, H. Shivaprasad, M. E. Fernández-Miyakawa, and F. A. Uzal. 2007b. Toxinotypes of Clostridium perfringens isolated from sick and healthy avian species. Journal of Beterinary Diagnostic Investigation 19(3):329-333.

Dahiya, J. P., D. Hoehler, A. G. Van Kessel, and M. D. Drew. 2007a. Dietary encapsulated glycine influences Clostridium perfringens and Lactobacilli growth in the gastrointestinal tract of broiler chickens. Journal of Nutrition 137(6):1408-1414. doi: 10.1093/jn/137.6.1408

Dahiya, J. P., D. Hoehler, A. G. Van Kessel, and M. D. Drew. 2007b. Dietary encapsulated glycine influences Clostridium perfringens and Lactobacilli growth in the gastrointestinal tract of broiler chickens. Nutrition 137(6):1408-1414. doi: 10.1093/jn/137.6.1408

Dahiya, J. P., D. C. Wilkie, A. G. Van Kessel, and M. D. Drew. 2006. Potential strategies for controlling necrotic enteritis in broiler chickens in post-antibiotic era. Animal Feed Science and Technology 129(1):60-88. doi: https://doi.org/10.1016/j.anifeedsci.2005.12.003

Dardenne, M., J. M. Pleau, B. Nabarra, P. Lefrancier, M. Derrien, J. Choay, and J. F. Bach. 1982. Contribution of zinc and other metals to the biological activity of the serum thymic factor. Proceedings of the National Academy of Sciences 79(17):5370-5373.

Del Cacho, E., M. Gallego, F. López-Bernad, J. Quílez, and C. Sánchez-Acedo. 2004. Expression of anti-apoptotic factors in cells parasitized by second-generation schizonts of Eimeria tenella and Eimeria necatrix. Veterinary parasitology 125(3-4):287-300.

DePasquale-Jardieu, P., and P. J. Fraker. 1979. The role of corticosterone in the loss in immune function in the zinc-deficient A/J mouse. Journal of Nutrition 109(11):1847-1855. doi: 10.1093/jn/109.11.1847

DePasquale-Jardieu, P., and P. J. Fraker. 1980a. Further characterization of the role of corticosterone in the loss of humoral immunity in zinc-deficient A/J mice as determined by adrenalectomy. Immunogy 124(6):2650-2655.

DePasquale-Jardieu, P., and P. J. Fraker. 1980b. Further characterization of the role of corticosterone in the loss of humoral immunity in zinc-deficient A/J mice as determined by adrenalectomy. Journal of Immunology 124(6):2650-2655.

DePasquale-Jardieu, P., and P. J. Fraker. 1984a. Interference in the development of a secondary immune response in mice by zinc deprivation: persistence of effects. Nutrition 114(10):1762-1769.

DePasquale-Jardieu, P., and P. J. Fraker. 1984b. Interference in the development of a secondary immune response in mice by zinc deprivation: persistence of effects. Journal of Nutrition 114(10):1762-1769.

Dollery, C. M., J. R. McEwan, and A. M. Henney. 1995. Matrix metalloproteinases and cardiovascular disease. Circulation Research 77(5):863-868.

Dowd, P. S., J. Kelleher, and P. J. Guillou. 1986. T-lymphocyte subsets and interleukin-2 production in zinc-deficient rats. British Journal of Nutrition 55(1):59-69.

Driessen, C., K. Hirv, L. Rink, and H. Kirchner. 1994a. Induction of cytokines by zinc ions in human peripheral blood mononuclear cells and separated monocytes. Lymphokine and cytokine research 13(1):15-20.

Driessen, C., K. Hirv, L. Rink, and H. Kirchner. 1994b. Induction of cytokines by zinc ions in human peripheral blood mononuclear cells and separated monocytes. Lymphokine and cytokine research 13(1):15-20.

Edgar, S. 1954. Effect of temperature on the sporulation of oocysts of the protozoan, Eimeria tenella. Transactions of the American Microscopical Society 73(3):237-242.

El-Katcha, M., M. A. Soltan, and M. El-Badry. 2017. Effect of Dietary Replacement of Inorganic Zinc by Organic or Nanoparticles Sources on Growth Performance, Immune Response and Intestinal Histopathology of Broiler Chicken. Alexandria Journal for Veterinary Sciences 55(2)

Engel, R., R. Miller, and N. Price. 1966. Metabolic patterns in preadolescent children. XIII. Zinc balance. Zinc Metabolism:326-338.

Engström, B., C. Fermer, A. Lindberg, E. Saarinen, V. Båverud, and A. Gunnarsson. 2003. Molecular typing of isolates of Clostridium perfringens from healthy and diseased poultry. Veterinary Microbiology 94(3):225-235.

Ercan, M. T., and N. M. Bor. 1991. Phagocytosis by macrophages in zinc-deficient rats. International Journal of Radiation Applications and Instrumentation 18(7):765-768.

Fernando, M. A. 1974. Fine structure of the schizonts and merozoites of Eimeria acervulina in the chicken. The Journal of parasitology:149-159.

Ficko-Blean, E., C. P. Stuart, M. D. Suits, M. Cid, M. Tessier, R. J. Woods, and A. B. Boraston. 2012. Carbohydrate recognition by an architecturally complex α-N-acetylglucosaminidase from Clostridium perfringens. PLoS One 7(3):e33524.

Ficko-Blean, E., C. P. Stuart, and A. B. Boraston. 2011. Structural analysis of CPF_2247, a novel α-amylase from Clostridium perfringens. Proteins: Structure, Function, and Bioinformatics 79(10):2771-2777.

Foley, B., S. A. Johnson, B. Hackley, J. Smith Jr, and J. A. Halsted. 1968. Zinc content of human platelets. Proceedings of the Society for Experimental Biology and Medicine 128(1):265-269.

Follis, R. H., Jr., H. G. Day, and E. V. McCollum. 1941a. Histological Studies of the Tissues of Rats Fed a Diet Extremely Low in Zinc: Two Plates (Eleven Figures). Journal of Nutrition 22(3):223-237. doi: 10.1093/jn/22.3.223

Follis, R. H., Jr., H. G. Day, and E. V. McCollum. 1941b. Histological Studies of the Tissues of Rats Fed a Diet Extremely Low in Zinc: Two Plates (Eleven Figures). Nutrition 22(3):223-237. doi: 10.1093/jn/22.3.223

Forbes, R. M., and M. Yohe. 1960a. Zinc requirement and balance studies with the rat. Nutrition 70:53-57. doi: 10.1093/jn/70.1.53

Forbes, R. M., and M. Yohe. 1960b. Zinc requirement and balance studies with the rat. Journal of Nutrition 70:53-57. doi: 10.1093/jn/70.1.53

Fox, M. R. S., and B. N. Harrison. 1964. Use of Japanese Quail for the Study of Zinc Deficiency. Proceedings of the Society for Experimental Biology and Medicine 116(2):256-259. doi: 10.3181/00379727-116-29217

Fraker, P., M. Gershwin, R. Good, and A. Prasad. 1986. Interrelationships between zinc and immune function. In: Federation proceedings. p 1474-1479.

Fraker, P., and C. Zwickl. 1981. Immune Repair Capacity of Zinc-deficient Young-adult and Neonatal Mice. In: Federation proceedings. p 918-918.

Fraker, P. J., P. DePasquale-Jardieu, C. M. Zwickl, and R. W. Luecke. 1978. Regeneration of T-cell helper function in zinc-deficient adult mice. Proceedings of the National Academy of Sciences 75(11):5660-5664.

Fraker, P. J., S. M. Haas, and R. W. Luecke. 1977. Effect of zinc deficiency on the immune response of the young adult A/J mouse. Journal of Nutrition 107(10):1889-1895.

Fraker, P. J., K. Hildebrandt, and R. W. Luecke. 1984. Alteration of antibody-mediated responses of suckling mice to T-cell-dependent and independent antigens by maternal marginal zinc deficiency: restoration of responsivity by nutritional repletion. Journal of Nutrition 114(1):170-179.

Fraker, P. J., F. Osati-Ashtiani, M. A. Wagner, and L. E. King. 1995. Possible roles for glucocorticoids and apoptosis in the suppression of lymphopoiesis during zinc deficiency: a review. Journal of the American College of Nutrition 14(1):11-17.

Freedman, J. C., J. R. Theoret, J. A. Wisniewski, F. A. Uzal, J. I. Rood, and B. A. McClane. 2015. Clostridium perfringens type A–E toxin plasmids. Research in Microbiology 166(4):264-279.

Fujioka, M., and I. Lieberman. 1964. A Zn++ Rrequirement for Synthesis of Deoxribonucleic Acid by Rat Live. Journal of Biological Chemistry 239:1164-1167.

Fujita, K., J. Katahira, Y. Horiguchi, N. Sonoda, M. Furuse, and S. Tsukita. 2000. Clostridium perfringens enterotoxin binds to the second extracellular loop of claudin-3, a tight junction integral membrane protein. FEBS letters 476(3):258-261.

Gajula, S. S., V. K. Chelasani, A. K. Panda, V. R. Mantena, and R. R. Savaram. 2011. Effect of supplemental inorganic Zn and Mn and their interactions on the performance of broiler chicken, mineral bioavailability, and immune response. Biological trace element research 139(2):177-187.

Garnica, J., R. Achata, and P. Bravo. 1993. Physical and biochemical characteristics of alpaca semen. Animal Reproduction Science 32(1-2):85-90.

Garvy, B. A., L. E. King, W. G. Telford, L. A. Morford, and P. J. Fraker. 1993. Chronic elevation of plasma corticosterone causes reductions in the number of cycling cells of the B lineage in murine bone marrow and induces apoptosis. Immunology 80(4):587-592.

Gholamiandehkordi, A. R., L. Timbermont, A. Lanckriet, W. V. D. Broeck, K. Pedersen, J. Dewulf, F. Pasmans, F. Haesebrouck, R. Ducatelle, and F. V. Immerseel. 2007. Quantification of gut lesions in a subclinical necrotic enteritis model. Avian Pathology 36(5):375-382.

Gholamiandekhordi, A. R., R. Ducatelle, M. Heyndrickx, F. Haesebrouck, and F. Van Immerseel. 2006. Molecular and phenotypical characterization of Clostridium perfringens isolates from poultry flocks with different disease status. Veterinary Microbiology 113(1-2):143-152.

Gibert, M., C. Jolivet-Renaud, and M. R. Popoff. 1997. Beta2 toxin, a novel toxin produced by Clostridium perfringens. Gene 203(1):65-73.

Gilbert, I., and D. Taylor. 1956. The behaviour of zinc and radio-zinc in the rat. Biochimica et Biophysica Acta 21(3):545-551.

Grant-Frost, D., and E. Underwood. 1958. Zinc toxicity in the rat and its interrelation with copper. Australian Journal of Experimental Biology and Medical Science 36(4):339-346.

Gropper, S. S., and J. L. Smith. 2012. Advanced nutrition and human metabolism. Cengage Learning.

Gumulec, J., M. Raudenska, V. Adam, R. Kizek, and M. Masarik. 2014. Metallothionein–immunohistochemical cancer biomarker: a meta-analysis. PLoS One 9(1):e85346.

Gunn, S. A., and T. C. Gould. 1956. The relative importance of androgen and estrogen in the selective uptake of Zn65 by the dorsolateral prostate of the rat. Endocrinology 58(4):443-452.

Györke, A., L. Pop, and V. Cozma. 2013. Prevalence and distribution of Eimeria species in broiler chicken farms of different capacities. Parasite 20

Heller, V., and A. Burke. 1927. Toxicity of Zinc. Journal of Biological Chemistry 74:85-93.

Heth, D. A., W. M. Becker, and W. G. Hoekstra. 1966a. Effect of calcium, phosphorus and zinc on zinc-65 absorption and turnover in rats fed semipurified diets. Journal of Nutrition 88(3):331-337. doi: 10.1093/jn/88.3.331

Heth, D. A., and W. G. Hoekstra. 1965. Zinc-65 Absorption and Turnover in Rats. I. A Procedure to Determine Zinc-65 Absorption and the Antagonistic Effect of Calcium in a Practical Diet. Journal of Nutrition 85(4):367-374. doi: 10.1093/jn/85.4.367

Heth, D. A., M. L. Sunde, and W. G. Hoekstra. 1966b. Influence of dietary calcium and zinc on zinc-65 metabolism in laying hens and their progeny. Poultry Science 45(1):75-83. doi: 10.3382/ps.0450075

Ho, E., and B. N. Ames. 2002. Low intracellular zinc induces oxidative DNA damage, disrupts p53, NFκB, and AP1 DNA binding, and affects DNA repair in a rat glioma cell line. Proceedings of the National Academy of Sciences 99(26):16770-16775.

Hockenbery, D. 1995. Defining apoptosis. The American Journal of Pathology 146(1):16.

Hoekstra, W., E. Faltin, C. Lin, H. Roberts, and R. Grummer. 1967. Zinc Deficiency in Reproducing Gilts Fed a Diet high in Calcium and its Effect on Tissue Zinc and Blood Serum Alkaline Phosphatase 1, 2. Journal of Animal Science 26(6):1348-1357.

Hoekstra, W. G. 1964a. Recent Observations on Mineral Interrelationships. Federation proceedings 23:1068-1076.

Hoekstra, W. G. 1964b. Recent Observations on Mineral Interreplationships. Federation proceedings 23:1068-1076.

Hofacre, C. L. 2001. Necrotic enteritis, currently a billion dollar disease: is there anything new on the horizon. In: Proceedings of Alltech's 17th Annual Symposium Science and Technology in the Feed Industry. p 79-86.

Hong, Y. H., H. S. Lillehoj, S. H. Lee, R. A. Dalloul, and E. P. Lillehoj. 2006. Analysis of chicken cytokine and chemokine gene expression following Eimeria acervulina and Eimeria tenella infections. Veterinary Immunology and Immunopathology 114(3-4):209-223.

Hove, E., C. Elvehjem, and E. Hart. 1938. Further studies on zinc deficiency in rats. American Journal of Physiology-Legacy Content 124(3):750-758.

Hsu, J., and W. Anthony. 1970. Zinc deficiency and urinary excretion of taurine-35S and inorganic sulfate-35S following cystine-35S injection in rats. Journal of Nutrition 100(10):1189-1195.

Hsu, J., W. Anthony, and P. Buchanan. 1969. Zinc deficiency and incorporation of 14C-labeled methionine into tissue proteins in rats. Journal of Nutrition 99(4):425-432.

Hsu, J. M., W. L. Anthony, and P. J. Buchanan. 1968. Incorporation of glycine-1-14C into liver glutathione in zinc deficient rats. Proceedings of the Society for Experimental Biology and Medicine 127(4):1048-1051.

Huang, L., and S. Tepaamorndech. 2013. The SLC30 family of zinc transporters–a review of current understanding of their biological and pathophysiological roles. Molecular Aspects of Medicine 34(2-3):548-560.

Hurley, L. S., and H. Swenerton. 1966. Congenital malformations resulting from zinc deficiency in rats. Proceedings of the Society for Experimental Biology and Medicine 123(3):692-696.

Huse, M., M. J. Eck, and S. C. Harrison. 1998. A Zn2+ ion links the cytoplasmic tail of CD4 and the N-terminal region of Lck. Journal of Biological Chemistry 273(30):18729-18733.

Ibs, K.-H., and L. Rink. 2003. Zinc-altered immune function. Journal of Nutrition 133(5):1452S-1456S.

Iji, P., and D. Tivey. 1998. Natural and synthetic oligosaccharides in broiler chicken diets. World's Poultry Science Journal 54(2):129-143.

Iwata, T., G. S. Incefy, S. Cunningham-Rundles, C. Cunningham-Rundles, E. Smithwick, N. Geller, R. O'Reilly, and R. A. Good. 1981. Circulating thymic hormone activity in patients with primary and secondary immunodeficiency diseases. The American journal of medicine 71(3):385-394.

Iwata, T., G. S. Incefy, T. Tanaka, G. Fernandes, C. J. Menendez-Botet, K. Pih, and R. A. Good. 1979. Circulating thymic hormone levels in zinc deficiency. Cell Immunology 47(1):100-105.

Jackson, M., D. Jones, and R. Edwards. 1982. Tissue zinc levels as an index of body zinc status. Clinical Physiology 2(4):333-343.

Janeway, C., P. Travers, M. Walport, and M. Schlomchik. 2004. Immunobiology: The Immune System in Health and Diseases.

Jarosz, Ł., A. Marek, Z. Grądzki, M. Kwiecień, and M. Kalinowski. 2017. The effect of feed supplementation with zinc chelate and zinc sulphate on selected humoral and cell-mediated immune parameters and cytokine concentration in broiler chickens. Research in veterinary science 112:59-65.

Jeong, J., J. M. Walker, F. Wang, J. G. Park, A. E. Palmer, C. Giunta, M. Rohrbach, B. Steinmann, and D. J. Eide. 2012. Promotion of vesicular zinc efflux by ZIP13 and its implications for spondylocheiro dysplastic Ehlers–Danlos syndrome. Proceedings of the National Academy of Sciences 109(51):E3530-E3538.

Johnson, J., and W. M. Reid. 1970. Anticoccidial drugs: lesion scoring techniques in battery and floor-pen experiments with chickens. Experimental parasitology 28(1):30-36.

Johnson, P. E., C. D. Hunt, D. B. Milne, and L. K. Mullen. 1993a. Homeostatic control of zinc metabolism in men: zinc excretion and balance in men fed diets low in zinc. Am J Clin Nutr 57(4):557-565. doi: 10.1093/ajcn/57.4.557

Johnson, P. E., C. D. Hunt, D. B. Milne, and L. K. Mullen. 1993b. Homeostatic control of zinc metabolism in men: zinc excretion and balance in men fed diets low in zinc. The American journal of clinical nutrition 57(4):557-565.

Juśkiewicz, J., Z. Zduńczyk, and J. Jankowski. 2004. Selected parameters of gastrointestinal tract metabolism of turkeys fed diets with flavomycin and different inulin content. World's Poultry Science Journal 60(2):177-185.

Kagara, N., N. Tanaka, S. Noguchi, and T. Hirano. 2007. Zinc and its transporter ZIP10 are involved in invasive behavior of breast cancer cells. Cancer Science 98(5):692-697.

Kaldhusdal, M., M. Hofshagen, A. Lovland, H. Langstrand, and K. Redhead. 1999. Necrotic enteritis challenge models with broiler chickens raised on litter: evaluation of preconditions, Clostridium perfringens strains and outcome variables. FEMS immunology and medical microbiology 24(3):337-343. doi: 10.1111/j.1574-695X.1999.tb01303.x

Kaldhusdal, M., and M. Hofshangen. 1992. Barley inclusion and avoparcin supplementation in broiler diets. 2. Clinical, pathological, and bacteriological findings in a mild form of necrotic enteritis. Poultry Science 71(7):1145-1153.

Kambe, T., H. Narita, Y. Yamaguchi-Iwai, J. Hirose, T. Amano, N. Sugiura, R. Sasaki, K. Mori, T. Iwanaga, and M. Nagao. 2002. Cloning and characterization of a novel mammalian zinc transporter, ZnT-5, abundantly expressed in pancreatic beta cells. Journal of Biological Chemistry

Karsunky, H., H. Zeng, T. Schmidt, B. Zevnik, R. Kluge, K. W. Schmid, U. Dührsen, and T. Möröy. 2002. Inflammatory reactions and severe neutropenia in mice lacking the transcriptional repressor Gfi1. Nature Genetics 30(3):295.

Katahira, J., N. Inoue, Y. Horiguchi, M. Matsuda, and N. Sugimoto. 1997a. Molecular cloning and functional characterization of the receptor for Clostridium perfringens enterotoxin. Journal of Cell Biology 136(6):1239-1247.

Katahira, J., H. Sugiyama, N. Inoue, Y. Horiguchi, M. Matsuda, and N. Sugimoto. 1997b. Clostridium perfringens enterotoxin utilizes two structurally related membrane proteins as functional receptors in vivo. Journal of Biological Chemistry 272(42):26652-26658.

Katayama, S., B. Dupuy, S. Cole, G. Daube, and B. China. 1996. Genome mapping ofClostridium perfringens strains with I-CeuI shows many virulence genes to be plasmid-borne. Molecular and General Genetics MGG 251(6):720-726.

Kelleher, S. L., and B. Lönnerdal. 2002. Zinc transporters in the rat mammary gland respond to marginal zinc and vitamin A intakes during lactation. Journal of Nutrition 132(11):3280-3285.

Keyburn, A. L., J. D. Boyce, P. Vaz, T. L. Bannam, M. E. Ford, D. Parker, A. Di Rubbo, J. I. Rood, and R. J. Moore. 2008. NetB, a new toxin that is associated with avian necrotic enteritis caused by Clostridium perfringens. PLoS Pathogens 4(2):e26.

Keyburn, A. L., S. A. Sheedy, M. E. Ford, M. M. Williamson, M. M. Awad, J. I. Rood, and R. J. Moore. 2006. Alpha-toxin of Clostridium perfringens is not an essential virulence factor in necrotic enteritis in chickens. Infection and Immunity 74(11):6496-6500.

Keyburn, A. L., X.-X. Yan, T. L. Bannam, F. Van Immerseel, J. I. Rood, and R. J. Moore. 2010. Association between avian necrotic enteritis and Clostridium perfringens strains expressing NetB toxin. Veterinary Research 41(2):1-8.

Kienholz, E. W., D. E. Turk, M. L. Sunde, and W. G. Hoekstra. 1961a. Effects of zinc deficiency in the diets of hens'. Journal of Nutrition 75(2):211-221. doi: 10.1093/jn/75.2.211

Kienholz, E. W., D. E. Turk, M. L. Sunde, and W. G. Hoekstra. 1961b. Effects of zinc deficiency in the diets of hens'. Nutrition 75(2):211-221. doi: 10.1093/jn/75.2.211

King, L. E., J. W. Frentzel, J. J. Mann, and P. J. Fraker. 2005. Chronic zinc deficiency in mice disrupted T cell lymphopoiesis and erythropoiesis while B cell lymphopoiesis and myelopoiesis were maintained. Journal of the American College of Nutrition 24(6):494-502.

Kirchgessner, M. 1993. Homeostasis and homeorhesis in trace element metabolism. Trace Elements in Man and Animals 8:4-21.

Kirschke, C. P., and L. Huang. 2003. ZnT7, a novel mammalian zinc transporter, accumulates zinc in the Golgi apparatus. Journal of Biological Chemistry 278(6):4096-4102.

Kitamura, H., H. Morikawa, H. Kamon, M. Iguchi, S. Hojyo, T. Fukada, S. Yamashita, T. Kaisho, S. Akira, M. Murakami, and T. Hirano. 2006. Toll-like receptor-mediated regulation of zinc homeostasis influences dendritic cell function. Nature Immunology 7(9):971-977. doi: 10.1038/ni1373

Kleessen, B., L. Hartmann, and M. Blaut. 2003. Fructans in the diet cause alterations of intestinal mucosal architecture, released mucins and mucosa-associated bifidobacteria in gnotobiotic rats. British Journal of Nutrition 89(5):597-606.

Kondo, F. 1988. In vitro lecithinase activity and sensitivity to 22 antimicrobial agents of Clostridium perfringens isolated from necrotic enteritis of broiler chickens. Research in veterinary science 45(3):337-340.

Kratzer, F. H., P. Vohra, J. B. Allred, and P. N. Davis. 1958. Effect of zinc upon growth and incidence of perosis in turkey poults. Proceedings of the Society for Experimental Biology and Medicine 98(1):205-207.

Ku, P., D. Ullrey, E. Miller, and C. Mills. 1970. Zinc deficiency and tissue nucleic acid and protein concentration. E & S Livingstone

Kurtoğlu, F., V. Kurtoğlu, I. Celik, T. Kececi, and M. Nizamlioğlu. 2005. Effects of dietary boron supplementation on some biochemical parameters, peripheral blood lymphocytes, splenic plasma cells and bone characteristics of broiler chicks given diets with adequate or inadequate cholecalciferol (vitamin D3) content. British Journal of Poultry Science 46(1):87-96.

Kwiecień, M., A. Winiarska-Mieczan, A. Milczarek, and R. Klebaniuk. 2017. Biological Response of Broiler Chickens to Decreasing Dietary Inclusion Levels of Zinc Glycine Chelate. Biological Trace Element Research 175(1):204-213. (journal article) doi: 10.1007/s12011-016-0743-y

Kwon, H.-S., H. W. Lim, J. Wu, M. Schnölzer, E. Verdin, and M. Ott. 2012. Three novel acetylation sites in the Foxp3 transcription factor regulate the suppressive activity of regulatory T cells. Journal of Immunology 188(6):2712-2721.

Lal, K., E. Bromley, R. Oakes, J. H. Prieto, S. J. Sanderson, D. Kurian, L. Hunt, J. R. Yates, 3rd, J. M. Wastling, R. E. Sinden, and F. M. Tomley. 2009. Proteomic comparison of four Eimeria tenella life-cycle stages: unsporulated oocyst, sporulated oocyst, sporozoite and second-generation merozoite. Proteomics 9(19):4566-4576. doi: 10.1002/pmic.200900305

Lee, D.-Y., A. S. Prasad, C. Hydrick-Adair, G. Brewer, and P. E. Johnson. 1993. Homeostasis of zinc in marginal human zinc deficiency: role of absorption and endogenous excretion of zinc. Translational Research 122(5):549-556.

Lee, H. H., A. S. Prasad, G. J. Brewer, and C. Owyang. 1989. Zinc absorption in human small intestine. American Journal of Physiology-Gastrointestinal and Liver Physiology 256(1):G87-G91.

Lee, K., H. S. Lillehoj, and G. R. Siragusa. 2010. Direct-fed microbials and their impact on the intestinal microflora and immune system of chickens. The journal of poultry science:1001160039-1001160039.

Lee, S. H., H. S. Lillehoj, S. I. Jang, M. Jeong, D. K. Kim, S. Xu, S. K. Lee, J. B. Kim, H. J. Park, H. R. Kim, and D. M. Bravo. 2014. Immune and anti-oxidant effects of in ovo selenium proteinate on post-hatch experimental avian necrotic enteritis. Veterinary parasitology 206(3-4):115-122. doi: 10.1016/j.vetpar.2014.10.025

Lepp, D., J. Gong, J. Songer, P. Boerlin, V. Parreira, and J. Prescott. 2013. Identification of accessory genome regions in poultry Clostridium perfringens isolates carrying the netB plasmid. Journal of Bacteriology 195(6):1152-1166.

Lewis Jr, P., W. Hoekstra, R. Grummer, and P. Phillips. 1956. The effect of certain nutritional factors including calcium, phosphorus and zinc on parakeratosis in swine. Journal of Animal Science 15(3):741-751.

Lewis, P., W. Hoekstra, and R. Grummer. 1957a. Restricted Calcium Feeding Versus Zinc Supplementation for the Control of Parakeratosis in Swine. Journal of Animal Science 16(3):578-588.

Lewis, P., W. Hoekstra, and R. Grummer. 1957b. Restricted Calcium Feeding versus Zinc Supplementation for the Control of Parakerotosis in Swine. Journal of Animal Science 16(3):578-588.

Lin, R. S., C. Rodriguez, A. Veillette, and H. F. Lodish. 1998. Zinc is essential for binding of p56 lck to CD4 and CD8α. Journal of Biological Chemistry 273(49):32878-32882.

Liptrap, D., E. Miller, D. Ullrey, D. Whitenack, B. Schoepke, and R. Luecke. 1970. Sex influence on the zinc requirement of developing swine. Journal of Animal Science 30(5):736-741.

Liu, M., S. Bao, M. Galvez-Peralta, C. Pyle, A. Rudawsky, R. Pavlovicz, D. Killilea, C. Li, D. Nebert, and M. Wewers. 2013. ZIP8 regulates host defense through zinc-mediated inhibition of NF-kappaB. Cell Rep 3: 386–400.

Liuzzi, J. P., R. K. Blanchard, and R. J. Cousins. 2001. Differential regulation of zinc transporter 1, 2, and 4 mRNA expression by dietary zinc in rats. Journal of Nutrition 131(1):46-52.

Liuzzi, J. P., and R. J. Cousins. 2004. Mammalian zinc transporters. Annual Review of Nutrition 24:151-172.

Livingston, H. D., H. Smith, and N. Stojanovic. 1967. Simultaneous estimation of copper, zinc, cadmium and mercury in biological material by neutron activation analysis. Talanta 14(4):505-513.

Lodemann, U., R. Einspanier, F. Scharfen, H. Martens, and A. Bondzio. 2013. Effects of zinc on epithelial barrier properties and viability in a human and a porcine intestinal cell culture model. Toxicology in vitro 27(2):834-843.

Long, J. R., and R. B. Truscott. 1976. Necrotic enteritis in broiler chickens. III. Reproduction of the disease. Canadian Journal of Comparative Medicine 40(1):53-59.

Long, P. 1965. Development of Eimeria tenella in avian embryos. Nature 208(5009):509-510.

Lovell, M. A., J. L. Smith, and W. R. Markesbery. 2006. Elevated zinc transporter-6 in mild cognitive impairment, Alzheimer disease, and pick disease. Journal of Neuropathology & Experimental Neurology 65(5):489-498.

Lovland, A., M. Kaldhusdal, K. Redhead, E. Skjerve, and A. Lillehaug. 2004. Maternal vaccination against subclinical necrotic enteritis in broilers. Avian Pathology 33(1):81-90.

Luecke, R., J. Hoefer, W. Brammell, and D. Schmidt. 1957. Calcium and Zinc in Parakeratosis of Swine 1. Journal of Animal Science 16(1):3-11.

Luecke, R. W., M. E. Olman, and B. V. Baltzer. 1968. Zinc deficiency in the rat: effect on serum and intestinal alkaline phosphatase activities. Journal of Nutrition 94(3):344-350. doi: 10.1093/jn/94.3.344

Luecke, R. W., C. E. Simonel, and P. J. Fraker. 1978. The effect of restricted dietary intake on the antibody mediated response of the zinc deficient A/J mouse. Journal of Nutrition 108(5):881-887.

Lutz, R. E. 1926. The Normal Occurrence of Zinc in Biologic Materials: a Review of the Literature, and a Study of the Normal Distribution of Zinc in the Rat, Cat, and Man. Journal of Industrial Hygiene 8:177-207.

Macapinlac, M., G. Barney, W. Pearson, and W. Darby. 1967. Production of zinc deficiency in the squirrel monkey (Saimiri sciureus). Journal of Nutrition 93(4):499-510.

MacDonald, R. S. 2000. The role of zinc in growth and cell proliferation. Journal of Nutrition 130(5):1500S-1508S.

Magee, A. C., and G. Matrone. 1960. Studies on growth, copper metabolism and iron metabolism of rats fed high levels of zinc. Journal of Nutrition 72(2):233-242.

Majno, G., and I. Joris. 1995. Apoptosis, oncosis, and necrosis. An overview of cell death. The American Journal of Pathology 146(1):3.

Maret, W. 2005. Zinc coordination environments in proteins determine zinc functions. Journal of Trace Elements in Medicine and Biology 19(1):7-12.

Marikovsky, M., V. Ziv, N. Nevo, C. Harris-Cerruti, and O. Mahler. 2003. Cu/Zn superoxide dismutase plays important role in immune response. Journal of Immunology 170(6):2993-3001.

Martin, A. B., T. B. Aydemir, G. J. Guthrie, D. A. Samuelson, S.-M. Chang, and R. J. Cousins. 2013. Gastric and Colonic Zinc Transporter ZIP11 (Slc39a11) in Mice Responds to Dietary Zinc and Exhibits Nuclear Localization–3. Journal of Nutrition 143(12):1882-1888.

Mawson, C. A., and M. I. Fischer. 1953. Zinc and carbonic anhydrase in human semen. Biochemical Journal 55(4):696-700.

Mayer, L. S., P. Uciechowski, S. Meyer, T. Schwerdtle, L. Rink, and H. Haase. 2014. Differential impact of zinc deficiency on phagocytosis, oxidative burst, and production of pro-inflammatory cytokines by human monocytes. Metallomics 6(7):1288-1295.

McCance, R., and E. M. Widdowson. 1942. Mineral metabolism of healthy adults on white and brown bread dietaries. Journal of Physiology 101(1):44-85.

McDevitt, R. M., J. D. Brooker, T. Acamovic, and N. H. C. Sparks. 2006. Necrotic enteritis; a continuing challenge for the poultry industry. World's Poultry Science Journal 62(2):221-247. doi: 10.1079/WPS200593

McDougald, L., and R. Galloway. 1973. Eimeria tenella: anticoccidial drug activity in cell cultures. Experimental Parasitology 34(2):189-196.

McMahon, R. J., and R. J. Cousins. 1998. Regulation of the zinc transporter ZnT-1 by dietary zinc. Proceedings of the National Academy of Sciences 95(9):4841-4846.

McQuibban, G. A., J.-H. Gong, J. P. Wong, J. L. Wallace, I. Clark-Lewis, and C. M. Overall. 2002. Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties in vivo. Blood 100(4):1160-1167.

McReynolds, J., J. Byrd, R. Anderson, R. Moore, T. Edrington, K. Genovese, T. Poole, L. Kubena, and D. Nisbet. 2004a. Evaluation of immunosuppressants and dietary mechanisms in an experimental disease model for necrotic enteritis. Poultry Science 83(12):1948-1952.

McReynolds, J., C. Waneck, J. Byrd, K. Genovese, S. Duke, and D. Nisbet. 2009. Efficacy of multistrain direct-fed microbial and phytogenetic products in reducing necrotic enteritis in commercial broilers. Poultry Science 88(10):2075-2080. doi: 10.3382/ps.2009-00106

McReynolds, J. L., J. A. Byrd, R. C. Anderson, R. W. Moore, T. S. Edrington, K. J. Genovese, T. L. Poole, L. F. Kubena, and D. J. Nisbet. 2004b. Evaluation of immunosuppressants and dietary mechanisms in an experimental disease model for necrotic enteritis. Poultry Science 83(12):1948-1952. doi: 10.1093/ps/83.12.1948

McReynolds, J. L., J. A. Byrd, K. J. Genovese, T. L. Poole, S. E. Duke, M. B. Farnell, and D. J. Nisbet. 2007a. Dietary lactose and its effect on the disease condition of necrotic enteritis. Poultry Sciences 86(8):1656-1661. doi: 10.1093/ps/86.8.1656

McReynolds, J. L., J. A. Byrd, K. J. Genovese, T. L. Poole, S. E. Duke, M. B. Farnell, and D. J. Nisbet. 2007b. Dietary lactose and its effect on the disease condition of necrotic enteritis. Poultry Science 86(8):1656-1661. doi: 10.1093/ps/86.8.1656

Meerarani, P., G. Reiterer, M. Toborek, and B. Hennig. 2003. Zinc modulates PPARγ signaling and activation of porcine endothelial cells. Journal of Nutrition 133(10):3058-3064.

Menghini, R., L. Fiorentino, V. Casagrande, R. Lauro, and M. Federici. 2013. The role of ADAM17 in metabolic inflammation. Atherosclerosis 228(1):12-17.

Merithew, E., C. Stone, S. Eathiraj, and D. G. Lambright. 2003. Determinants of Rab5 interaction with the N terminus of early endosome antigen 1. Journal of Biological Chemistry 278(10):8494-8500.

Methfessel, A. H., and H. Spencer. 1973. Zinc metabolism in the rat. II. Secretion of zinc into intestine. Journal of applied physiology 34(1):63-67. doi: 10.1152/jappl.1973.34.1.63

Mikkelsen, L. L., J. K. Vidanarachchi, C. G. Olnood, Y. M. Bao, P. H. Selle, and M. Choct. 2009. Effect of potassium diformate on growth performance and gut microbiota in broiler chickens challenged

with necrotic enteritis. British Journal of Poultry Science 50(1):66-75. doi: 10.1080/00071660802613252

Millar, M., N. R. Vincent, and C. Mawson. 1961. An autoradiographic study of the distribution of zinc-65 in rat tissues. Journal of Histochemistry & Cytochemistry 9(2):111-116.

Millar, M. J., P. Elcoate, and C. A. Mawson. 1957. Sex hormone control of the zinc content of the prostate. Canadian Journal of Biochemistry and Physiology 35(11):865-868.

Millar, M. J., M. I. Fischer, P. V. Elcoate, and C. A. Mawson. 1958a. The effects of dietary zinc deficiency on the reproductive system of male rats. Candadian Journal of Biochemistry and Physiology 36(6):557-569.

Millar, M. J., M. I. Fischer, P. V. Elcoate, and C. A. Mawson. 1958b. The effects of dietary zinc deficiency on the reproductive system of male rats. Canadian Journal of Biochemistry and Physiology 36(6):557-569.

Millard, B., J. Bradley, and P. Long. 1971. The schizogony of Eimeria maxima in the chicken. Zeitschrift für Parasitenkunde 38(1):77-81.

Miller, E., R. Luecke, D. Ullrey, B. V. Baltzer, B. Bradley, and J. Hoefer. 1968a. Biochemical, skeletal and allometric changes due to zinc deficiency in the baby pig. Journal of Nutrition 95(2):278-286.

Miller, E. R., R. W. Luecke, D. E. Ullrey, B. V. Baltzer, B. L. Bradley, and J. A. Hoefer. 1968b. Biochemical, skeletal and allometric changes due to zinc deficiency in the baby pig. Journal of Nutrition 95(2):278-286. doi: 10.1093/jn/95.2.278

Miller, J. K., and W. J. Miller. 1960. Development of zinc deficiency in Holstein calves fed a purified diet. Journal of Dairy Science 43:1854-1856.

Miller, R. W., E. J. Skinner, A. Sulakvelidze, G. F. Mathis, and C. L. Hofacre. 2010. Bacteriophage therapy for control of necrotic enteritis of broiler chickens experimentally infected with Clostridium perfringens. Avian Diseases 54(1):33-40. doi: 10.1637/8953-060509-Reg.1

Miller, W. J., D. M. Blackmon, R. Gentry, G. Powell, and H. Perkins. 1966. Influence of Zinc Deficiency on Zinc and Dry Matter Content of Ruminant Tissues and on Excretion of Zinc1. Journal of Dairy Science 49(11):1446-1453.

Miller, W. J., J. D. Morton, W. J. Pitts, and C. M. Clifton. 1965a. Effect of Zinc Deficiency and Restricted Feeding on Wound Healing in teh Bovine. Proceedings of the Society for Experimental Biology and Medicine 118:427-430.

Miller, W. J., J. D. Morton, W. J. Pitts, and C. M. Clifton. 1965b. Effect of zinc deficiency and restricted feeding on wound healing in the bovine. Proceedings of the Society for Experimental Biology and Medicine 118:427-430.

Miller, W. J., W. J. Pitts, C. M. Clifton, and S. C. Schmittle. 1964. Experimentally produced zinc deficiency in the goat. Journal of Dairy Science 47:556-559. doi: 10.3168/jds.S0022-0302(64)88713-0

Mills, C., A. Dalgarno, R. Williams, and J. Quarterman. 1967. Zinc deficiency and the zinc requirements of calves and lambs. British Journal of Nutrition 21(3):751-768.

Miyata, S., O. Matsushita, J. Minami, S. Katayama, S. Shimamoto, and A. Okabe. 2001. Cleavage of a C-terminal peptide is essential for heptamerization of Clostridium perfringens ε-toxin in the synaptosomal membrane. Journal of Biological Chemistry 276(17):13778-13783.

Moeller, M., and H. Scott. 1958. Studies with purified diets. 3. Zinc requirement. In: Poultry Science. p 1227-1228.

Mohammadi, V., S. Ghazanfari, A. Mohammadi-Sangcheshmeh, and M. Nazaran. 2015. Comparative effects of zinc-nano complexes, zinc-sulphate and zinc-methionine on performance in broiler chickens. British poultry science 56(4):486-493.

Mohanna, C., and Y. Nys. 1999. Effect of dietary zinc content and sources on the growth, body zinc deposition and retention, zinc excretion and immune response in chickens. British Journal of Poultry Science 40(1):108-114.

Moulder, K., and M. W. Steward. 1989. Experimental zinc deficiency: effects on cellular responses and the affinity of humoral antibody. Clinical and experimental immunology 77(2):269-274.

Munyaka, P., H. Echeverry, A. Yitbarek, G. Camelo-Jaimes, S. Sharif, W. Guenter, J. House, and J. Rodriguez-Lecompte. 2012. Local and systemic innate immunity in broiler chickens supplemented with yeast-derived carbohydrates. Poultry science 91(9):2164-2172.

Murgia, C., I. Vespignani, J. Cerase, F. Nobili, and G. Perozzi. 1999. Cloning, expression, and vesicular localization of zinc transporter Dri 27/ZnT4 in intestinal tissue and cells. American Journal of Physiology-Gastrointestinal and Liver Physiology 277(6):G1231-G1239.

Nakatani, Y., M. Yamazaki, W. J. Chazin, and S. Yui. 2005. Regulation of S100A8/A9 (calprotectin) binding to tumor cells by zinc ion and its implication for apoptosis-inducing activity. Mediators of Inflammation 2005(5):280-292.

Nason, A., N. O. Kaplan, and H. A. Oldewurtel. 1953. Further studies of nutritional conditions affecting enzymatic constitution in Neurospora. Journal of Biological Chemistry 201(1):435-444.

Norton, C., and L. Joyner. 1981. Eimeria acervulina and E. mivati: oocysts, life-cycle and ability to develop in the chicken embryo. Parasitology 83(2):269-279.

NRC, N. R. C. 1994. NRC. Nutrient requirements of poultry. Washington. National Academy is Sciences

O'Brien, K. O., N. Zavaleta, L. E. Caulfield, J. Wen, and S. A. Abrams. 2000. Prenatal iron supplements impair zinc absorption in pregnant Peruvian women. Journal of Nutrition 130(9):2251-2255. doi: 10.1093/jn/130.9.2251

O'Dell, B., P. Newberne, and J. Savage. 1958. Significance of dietary zinc for the growing chicken. Journal of Nutrition 65(4):503-523.

O'hara, P., A. Newman, and E. Jackson. 1960. Parakeratosis and copper poisoning in pigs fed a copper supplement. Australian Veterinary Journal 36(5):225-229.

O'dell, B., and J. Savage. 1957. Potassium, zinc and distillers dried solubles as supplements to a purified diet. Poultry Science 36(2):459-460.

Oberleas, D., M. E. Muhrer, and B. L. O'Dell. 1966. Dietary metal-complexing agents and zinc availability in the rat. Journal of Nutrition 90(1):56-62.

Officials, A. o. A. F. C. 2013. Official Publication-Association of American Feed Control Officials. Association of American Feed Control Officials.

Olkowski, A. A., C. Wojnarowicz, M. Chirino-Trejo, and M. D. Drew. 2006. Responses of broiler chickens orally challenged with Clostridium perfringens isolated from field cases of necrotic enteritis. Research in veterinary science 81(1):99-108. doi: 10.1016/j.rvsc.2005.10.006

Olkowski, B., H. Classen, C. Wojnarowicz, and A. Olkowski. 2005. Feeding high levels of lupine seeds to broiler chickens: plasma micronutrient status in the context of digesta viscosity and morphometric and ultrastructural changes in the gastrointestinal tract. Poultry Science 84(11):1707-1715.

Olsen, S. J., L. C. MacKinon, J. S. Goulding, N. H. Bean, and L. Slutsker. 2000. Surveillance for foodborne-disease outbreaks, United States, 1993-1997.

Ott, E., W. Smith, R. Harrington, H. Parker, and W. Beeson. 1966a. Zinc Toxicity in Ruminants. IV. Physiological Changes in Tissues of Beef Cattle 1. Journal of Animal Science 25(2):432-438.

Ott, E., W. Smith, R. Harrington, M. Stob, H. Parker, and W. Beeson. 1966b. Zinc Toxicity in Ruminants. III. Physiological Changes in Tissues and Alterations in Rumen Metabolism in Lambs 1. Journal of Animal Science 25(2):424-431.

Ozeki, Y., K. Kaneda, N. Fujiwara, M. Morimoto, S. Oka, and I. Yano. 1997. In vivo induction of apoptosis in the thymus by administration of mycobacterial cord factor (trehalose 6,6'-dimycolate). Infection and Immunity 65(5):1793-1799.

Paik, H. Y., H. Joung, J. Y. Lee, H. K. Lee, J. C. King, and C. L. Keen. 1999. Serum extracellular superoxide dismutase activity as an indicator of zinc status in humans. Biological Trace Element Research 69(1):45-57.

Palmiter, R. D., T. B. Cole, and S. D. Findley. 1996a. ZnT-2, a mammalian protein that confers resistance to zinc by facilitating vesicular sequestration. The EMBO Journal 15(8):1784-1791.

Palmiter, R. D., T. B. Cole, C. J. Quaife, and S. D. Findley. 1996b. ZnT-3, a putative transporter of zinc into synaptic vesicles. Proceedings of the National Academy of Sciences 93(25):14934-14939.

Pařízek, J., J. Boursnell, M. F. Hay, A. Babicky, and D. Taylor. 1966. Zinc in the maturing rat testis. Journal of Reproduction and Fertility 12(3):501-507.

Park, S. S., H. S. Lillehoj, P. C. Allen, D. W. Park, S. FitzCoy, D. A. Bautista, and E. P. Lillehoje. 2008. Immunopathology and cytokine responses in broiler chickens coinfected with Eimeria maxima and Clostridium perfringens with the use of an animal model of necrotic enteritis. Avian Diseases 52(1):14-22. doi: 10.1637/7997-041707-Reg

Parreira, V. R., M. Costa, F. Eikmeyer, J. Blom, and J. F. Prescott. 2012. Sequence of two plasmids from Clostridium perfringens chicken necrotic enteritis isolates and comparison with C. perfringens conjugative plasmids. PLoS One 7(11):e49753. doi: 10.1371/journal.pone.0049753

Paul, W. E., and R. A. Seder. 1994. Lymphocyte responses and cytokines. Cell 76(2):241-251.

Pedersen, K., L. Bjerrum, O. E. Heuer, D. M. Lo Fo Wong, and B. Nauerby. 2008. Reproducible infection model for Clostridium perfringens in broiler chickens. Avian Diseases 52(1):34-39. doi: 10.1637/7955-022307-Reg

Pedersen, K., L. Bjerrum, B. Nauerby, and M. Madsen. 2003. Experimental infections with rifampicin-resistant Clostridium perfringens strains in broiler chickens using isolator facilities. Avian Pathology 32(4):403-411.

Pensack, J., J. Henson, and P. Bogdonoff. 1958. The effects of calcium and phosphorus on the zinc requirements of growing chickens. In: Poultry Science. p 1232-1233.

Perry, T., W. Beeson, W. Smith, and M. Mohler. 1968. Value of zinc supplementation of natural rations for fattening beef cattle. Journal of Animal Science 27(6):1674-1677.

Phan, R. T., M. Saito, Y. Kitagawa, A. R. Means, and R. Dalla-Favera. 2007. Genotoxic stress regulates expression of the proto-oncogene Bcl6 in germinal center B cells. Nature Immunology 8(10):1132.

Pimentel, J., M. Cook, and J. Greger. 1991. Immune response of chicks fed various levels of zinc. Poultry Science 70(4):947-954.

Pitts, W. J., W. J. Miller, O. T. Fosgate, J. D. Morton, and C. M. Clifton. 1966. Effect of zinc deficiency and restricted feeding from two to five months of age on reproduction in Holstein bulls. Journal of Dairy Science 49(8):995-1000. doi: 10.3168/jds.S0022-0302(66)87997-3

Pories, W. J., J. H. Henzel, C. G. Rob, and W. H. Strain. 1967. Acceleration of wound healing in man with zinc sulphate given by mouth. Lancet (London, England) 1(7482):121-124.

Pories, W. J., E. W. Schear, D. R. Jordan, J. Chase, G. Parkinson, R. Whittaker, W. H. Strain, and C. Rob. 1966. The measurement of human wound healing. Surgery 59(5):821-824.

Pountney, D., T. Dickson, J. Power, J. Vickers, A. West, and W. Gai. 2011. Association of metallothionein-III with oligodendroglial cytoplasmic inclusions in multiple system atrophy. Neurotoxicity Research 19(1):115-122.

Powell, G., W. Miller, J. Morton, and C. Clifton. 1964. Influence of dietary cadmium level and supplemental zinc on cadmium toxicity in the bovine. Journal of Nutrition 84(3):205-214.

Prasad, A. S. 2000. Effects of zinc deficiency on Th1 and Th2 cytokine shifts. The Journal of infectious diseases 182(Supplement_1):S62-S68.

Prasad, A. S., J. A. Halsted, and M. Nadimi. 1983. Nutrition classics. The American Journal of Medicine, Volume 31, 1961. Syndrome of iron deficiency anemia, hepatosplenomegaly, hypogonadism, dwarfism and geophagia. Nutrition reviews 41(7):220-223.

Prasad, A. S., S. Meftah, J. Abdallah, J. Kaplan, G. J. Brewer, J. F. Bach, and M. Dardenne. 1988. Serum thymulin in human zinc deficiency. The Journal of clinical investigation 82(4):1202-1210. doi: 10.1172/jci113717

Prasad, A. S., D. Oberleas, and J. A. Halsted. 1965. Determination of zinc in biological fluids by atomic absorption spectrophotometry in normal and cirrhotic subjects. Translational Research 66(3):508-516.

Prasad, A. S., D. Oberleas, P. Wolf, and H. P. Horwitz. 1967a. Studies on zinc deficiency: changes in trace elements and enzyme activities in tissues of zinc-deficient rats. Journal of Clinical Investigation 46(4):549-557. doi: 10.1172/jci105556

Prasad, A. S., D. Oberleas, P. Wolf, and H. P. Horwitz. 1967b. Studies on zinc deficiency: changes in trace elements and enzyme activities in tissues of zinc-deficient rats. The Journal of clinical investigation 46(4):549-557. doi: 10.1172/jci105556

Prasad, A. S., D. Oberleas, P. Wolf, J. P. Horwitz, E. Miller, and R. Luecke. 1969. Changes in trace elements and enzyme activities in tissues of zinc-deficient pigs. The American journal of clinical nutrition 22(5):628-637.

Prescott, J., R. Sivendra, and D. Barnum. 1978. The use of bacitracin in the prevention and treatment of experimentally-induced necrotic enteritis in the chicken. The Canadian Veterinary journal 19(7):181.

Qin, Y., D. Thomas, C. P. Fontaine, and R. A. Colvin. 2009. Silencing of ZnT1 reduces Zn2+ efflux in cultured cortical neurons. Neuroscience Letters 450(2):206-210.

Quarterman, J., and W. R. Humphries. 1979. Effect of zinc deficiency and zinc supplementation on adrenals, plasma steroids and thymus in rats. Life sciences 24(2):177-183.

Quarterman, J., C. F. Mills, and W. R. Humphries. 1966. The reduced secretion of, and sensitivity to insulin in zinc-deficient rats. Biochemical and Biophysical Research Communications 25(3):354-358.

Radhakrishnan, R., L. J. Walter, A. Hruza, P. Reichert, P. P. Trotta, T. L. Nagabhushan, and M. R. Walter. 1996. Zinc mediated dimer of human interferon-alpha 2b revealed by X-ray crystallography. Structure (London, England : 1993) 4(12):1453-1463.

raker, P. J. 1984. Zinc deficiency: a common immunodeficiency state. Immunologic Research 2(2):155-163.

Raman, M., S. S. Banu, S. Gomathinayagam, and G. D. Raj. 2011. Lesion scoring technique for assessing the virulence and pathogenicity of Indian field isolates of avian Eimeria species. Veterinarski arhiv 81(2):259-271.

Rao, S. R., B. Prakash, M. Raju, A. Panda, R. Kumari, and E. P. K. Reddy. 2016. Effect of supplementing organic forms of zinc, selenium and chromium on performance, anti-oxidant and immune responses in broiler chicken reared in tropical summer. Biological trace element research 172(2):511-520.

Reiterer, G., M. Toborek, and B. Hennig. 2004. Peroxisome proliferator activated receptors α and γ require zinc for their anti-inflammatory properties in porcine vascular endothelial cells. Journal of Nutrition 134(7):1711-1715.

Riddell, C., and X.-M. Kong. 1992a. The influence of diet on necrotic enteritis in broiler chickens. Avian Diseases:499-503.

Riddell, C., and X. M. Kong. 1992b. The influence of diet on necrotic enteritis in broiler chickens. Avian diseases 36(3):499-503.

Robertson, B., and M. Burns. 1963. Zinc metabolism and the zinc-deficiency syndrome in the dog. American Journal of Veterinary Research 24:997-1002.

Rosenkranz, E., C. H. Metz, M. Maywald, R. D. Hilgers, I. Weßels, T. Senff, H. Haase, M. Jäger, M. Ott, and R. Aspinall. 2016. Zinc supplementation induces regulatory T cells by inhibition of Sirt-1 deacetylase in mixed lymphocyte cultures. Molecular Nutrition & Food Research 60(3):661-671.

Saif, Y., A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan, and D. E. Swayne. 2011. Diseases of poultry. John Wiley & Sons.

Sakurai, J. 1995. Toxins of Clostridium perfringens. Reviews in Medical Microbiology 6:175-185.

Sakurai, J., and C. Duncan. 1978. Some properties of beta-toxin produced by Clostridium perfringens type C. Infection and Immunity 21(2):678-680.

Sakurai, J., and Y. Fujii. 1987. Purification and characterization of Clostridium perfringens beta toxin. Toxicon 25(12):1301-1310.

Salas, M., and H. Kirchner. 1987. Induction of interferon-gamma in human leukocyte cultures stimulated by Zn2+. Clinical Immunology and Immunopathology 45(1):139-142.

Salvesen, G. S. 2002. Caspases: opening the boxes and interpreting the arrows. Nature Publishing Group.

Salvin, S. B., B. L. Horecker, L. X. Pan, and B. S. Rabin. 1987. The effect of dietary zinc and prothymosin alpha on cellular immune responses of RF/J mice. Clinical immunology and immunopathology 43(3):281-288.

Sandstead, H. H., A. S. Prasad, A. R. Schulert, Z. Farid, A. Miale Jr, S. Bassilly, and W. J. Darby. 1967. Human zinc deficiency, endocrine manifestations and response to treatment. American Journal of Clinical Nutrition 20(5):422-442.

Sandstead, H. H., and R. A. Rinaldi. 1969. Impairment of deoxyribonucleic acid synthesis by dietary zinc deficiency in the rat. Journal of cellular physiology 73(1):81-83. doi: 10.1002/jcp.1040730111

Sandstead, H. H., and G. H. Shepard. 1968. The effect of zinc deficiency on the tensile strength of healing surgical incisions in the integument of the rat. Proceedings of the Society for Experimental Biology and Medicine 128(3):687-689.

Savage, A. K., M. G. Constantinides, J. Han, D. Picard, E. Martin, B. Li, O. Lantz, and A. Bendelac. 2008. The transcription factor PLZF directs the effector program of the NKT cell lineage. Immunity 29(3):391-403.

Schlegel, P., and W. Windisch. 2006. Bioavailability of zinc glycinate in comparison with zinc sulphate in the presence of dietary phytate in an animal model with 65Zn labelled rats. Journal of Animal Physiology and Animal Nutrition 90(5-6):216-222.

Schlicker, S. A., and D. H. Cox. 1968a. Maternal dietary zinc, and development and zinc, iron, and copper content of the rat fetus. Journal of Nutrition 95(2):287-294.

Schlicker, S. A., and D. H. Cox. 1968b. Maternal dietary zinc, and development and zinc, iron, and copper content of the rat fetus. Nutrition 95(2):287-294.

Schroeder, H. A., and A. P. Nason. 1969. Trace metals in human hair. Journal of Investigative Dermatology 53(1):71-78.

Schroeder, H. A., A. P. Nason, I. H. Tipton, and J. J. Balassa. 1967. Essential trace metals in man: zinc. Relation to environmental cadmium. Journal of Chronic Diseases 20(4):179-210.

Scott, M. L., E. R. Holm, and R. E. Reynolds. 1959. Studies on the Niacin, Riboflavin, Choline, Manganese and Zinc Requirements of Young Ringnecked Pheasants for Growth, Feathering and Prevention of Leg Disorders. Poultry Science 38(6):1344-1350. doi: 10.3382/ps.0381344

Scuderi, P. 1990. Differential effects of copper and zinc on human peripheral blood monocyte cytokine secretion. Cell Immunology 126(2):391-405.

Segal, D., E. Ohana, L. Besser, M. Hershfinkel, A. Moran, and I. Sekler. 2004. A role for ZnT-1 in regulating cellular cation influx. Biochemical and Biophysical Research Communications 323(4):1145-1150.

Sharma, J. 1997. The structure and function of the avian immune system. Acta Veterinaria Hungarica 45(3):229-238.

Sheedy, S. A., A. B. Ingham, J. I. Rood, and R. J. Moore. 2004. Highly conserved alpha-toxin sequences of avian isolates of Clostridium perfringens. Journal of Clinical Microbiology 42(3):1345-1347.

Sheikh, A., S. Shamsuzzaman, S. M. Ahmad, D. Nasrin, S. Nahar, M. M. Alam, A. Al Tarique, Y. A. Begum, S. S. Qadri, and M. I. Chowdhury. 2010. Zinc Influences Innate Immune Responses in Children with Enterotoxigenic Escherichia coli-Induced Diarrhea–4. Journal of Nutrition 140(5):1049-1056.

Shen, H., R. MacDonald, D. Bruemmer, A. Stromberg, A. Daugherty, X.-a. Li, M. Toborek, and B. Hennig. 2007. Zinc deficiency alters lipid metabolism in LDL receptor–deficient mice treated with rosiglitazone. Journal of Nutrition 137(11):2339-2345.

Shen, H., E. Oesterling, A. Stromberg, M. Toborek, R. MacDonald, and B. Hennig. 2008. Zinc deficiency induces vascular pro-inflammatory parameters associated with NF-Kb and PPAR signaling. Journal of the American College of Nutrition 27(5):577-587.

Shi, H. N., M. E. Scott, M. M. Stevenson, and K. G. Koski. 1994. Zinc deficiency impairs T cell function in mice with primary infection of Heligmosomoides polygyrus (Nematoda). Parasite immunology 16(7):339-350.

Shimizu, T., K. Ohtani, H. Hirakawa, K. Ohshima, A. Yamashita, T. Shiba, N. Ogasawara, M. Hattori, S. Kuhara, and H. Hayashi. 2002. Complete genome sequence of Clostridium perfringens, an anaerobic flesh-eater. Proceedings of the National Academy of Sciences 99(2):996-1001.

Sim, D. L., and V. T. Chow. 1999. The novel human HUEL (C4orf1) gene maps to chromosome 4p12–p13 and encodes a nuclear protein containing the nuclear receptor interaction motif. Genomics 59(2):224-233.

Simonsen, A., R. Lippe, S. Christoforidis, J.-M. Gaullier, A. Brech, J. Callaghan, B.-H. Toh, C. Murphy, M. Zerial, and H. Stenmark. 1998. EEA1 links PI (3) K function to Rab5 regulation of endosome fusion. Nature 394(6692):494.

Singh, A., M. L. Failla, and P. A. Deuster. 1994. Exercise-induced changes in immune function: effects of zinc supplementation. Journal of applied physiology 76(6):2298-2303. doi: 10.1152/jappl.1994.76.6.2298

Singh, K. P., S. I. Zaidi, S. Raisuddin, A. K. Saxena, R. C. Murthy, and P. K. Ray. 1992. Effect of zinc on immune functions and host resistance against infection and tumor challenge. Immunopharmacol Immunotoxicology 14(4):813-840. doi: 10.3109/08923979209009237

Singh, R. B., U. C. Gupta, N. Mittal, M. A. Niaz, S. Ghosh, and V. Rastogi. 1997. Epidemiologic study of trace elements and magnesium on risk of coronary artery disease in rural and urban Indian populations. Journal of the American College of Nutrition 16(1):62-67.

Singh, R. B., M. A. Niaz, S. S. Rastogi, S. Bajaj, Z. Gaoli, and Z. Shoumin. 1998. Current zinc intake and risk of diabetes and coronary artery disease and factors associated with insulin resistance in rural and urban populations of North India. Journal of the American College of Nutrition 17(6):564-570.

Siragusa, G., M. Danyluk, K. Hiett, M. Wise, and S. Craven. 2006. Molecular subtyping of poultry-associated type A Clostridium perfringens isolates by repetitive-element PCR. Journal of Clinical Microbiology 44(3):1065-1073.

Smedley, J., D. Fisher, S. Sayeed, G. Chakrabarti, and B. McClane. 2004. The enteric toxins of Clostridium perfringens, Reviews of Physiology, Biochemistry, and Pharmacology. Springer. p. 183-204.

Smirnov, A. 1948. Polargraphic method for the determination of zinc in blood erythrocytes. Biokhim. 13:79.

Smirov, A. 1948. Polyarograficheskii Method Kolichestvennogo Opredeleniya Tsinka V Eritrotsitakh Krovi. Biokhimiya 13(1):79-87.

Smith, J., S. Xiong, W. Markesbery, and M. Lovell. 2006. Altered expression of zinc transporters-4 and-6 in mild cognitive impairment, early and late Alzheimer's disease brain. Neuroscience 140(3):879-888.

Smith, S. E., and E. J. Larson. 1946. Zinc toxicity in rats; antagonistic effects of copper and liver. Journal of Biological Chemistry 163:29-38.

Solomons, N. W., and R. A. Jacob. 1981. Studies on the bioavailability of zinc in humans: effects of heme and nonheme iron on the absorption of zinc. The American Journal of Clinical Nutrition 34(4):475-482. doi: 10.1093/ajcn/34.4.475

Somers, M., and E. J. Underwood. 1969a. Ribonuclease activity and nucleic acid and protein metabolism in the testes of zinc-deficient rats. Austratlian Journal of Biological Science 22(5):1277-1282.

Somers, M., and E. J. Underwood. 1969b. Ribonuclease activity and nucleic acid and protein metabolism in the testes of zinc-deficient rats. Australian journal of biological sciences 22(5):1277-1282.

Song, J., D. Kim, C. H. Lee, M. S. Lee, C.-H. Chun, and E.-J. Jin. 2013. MicroRNA-488 regulates zinc transporter SLC39A8/ZIP8 during pathogenesis of osteoarthritis. Journal of Biomedical Science 20(1):31.

Songer, J. G. 1996. Clostridial enteric diseases of domestic animals. Clinical Microbiology Reviews 9(2):216.

Songer, J. G., and R. R. Meer. 1996. Genotyping ofClostridium perfringensby Polymerase Chain Reaction is a Useful Adjunct to Diagnosis of Clostridial Enteric Disease in Animals. Anaerobe 2(4):197-203.

Southern, L., and D. Baker. 1983a. Eimeria acervulina infection and the zinc-copper interrelationship in the chick. Poultry science 62(2):401-404.

Southern, L. L., and D. H. Baker. 1983b. Zinc toxicity, zinc deficiency and zinc-copper interrelationship in Eimeria acervulina-infected chicks. The Journal of nutrition 113(3):688-696.

Spencer, H., V. Vankinscott, I. Lewin, and J. Samachson. 1965. Zinc-65 metabolism during low and high calcium intake in man. Journal of Nutrition 86(2):169-177.

Spray, C. M., and E. M. Widdowson. 1950. The effect of growth and development on the composition of mammals. British Journal of Nutrition 4(4):332-353.

Sridhar, K., D. Nagalakshmi, D. S. Rao, and S. Rao. 2014a. Effect of dietary addition of organic zinc on performance and carcass traits in commercial broiler chicken. Indian J. Poult. Sci 49(2):155-158.

Sridhar, K., D. Nagalakshmi, D. S. Rao, and S. Rao. 2014b. Effect of dietary addition of organic zinc on performance and carcass traits in commercial broiler chicken. Indian Journal of Poultry Science 49(2):155-158.

Sridhar, K., D. Nagalakshmi, D. S. Rao, and S. R. Rao. 2015a. Effect of supplementation of graded levels of organic zinc on nutrient utilization and retention of minerals in broiler chicken. Indian J. Anim. Nutr 32(1):80-85.

Sridhar, K., D. Nagalakshmi, D. S. Rao, and S. R. Rao. 2015b. Effect of supplementation of graded levels of organic zinc on nutrient utilization and retention of minerals in broiler chicken. Indian Journal of Animal Nutrition 32(1):80-85.

Sridhar, K., D. Nagalakshmi, and S. R. Rao. 2015c. Effect of graded concentration of organic zinc (zinc glycinate) on skin quality, hematological and serum biochemical constituents in broiler chicken. Indian Journal of Anim Science 85:643-648.

Steller, H. 1995. Mechanisms and genes of cellular suicide. Science 267(5203):1445-1449.

Stevenson, J., and I. Earle. 1956. Studies on parakeratosis in swine. Journal of Animal Science 15(4):1036-1045.

Sunder, G. S., A. Panda, N. Gopinath, S. R. Rao, M. Raju, M. Reddy, and C. V. Kumar. 2008. Effects of higher levels of zinc supplementation on performance, mineral availability, and immune competence in broiler chickens. Journal of Applied Poultry Research 17(1):79-86.

Supplee, W. C. 1963. Antagonistic relationship between dietary cadmium and zinc. Science 139(3550):119-120.

Sutton, W., and V. E. Nelson. 1937. Studies on zinc. Proceedings of the Society for Experimental Biology and Medicine 36(2):211-213.

Swenerton, H., and L. S. Hurley. 1968. Severe zinc deficiency in male and female rats. Journal of Nutrition 95(1):8-18. doi: 10.1093/jn/95.1.8

Tanaka, Y., S. Shiozawa, I. Morimoto, and T. Fujita. 1990. Role of zinc in interleukin 2 (IL-2)-mediated T-cell activation. Scandinavian journal of immunology 31(5):547-552.

Taniguchi, M., A. Fukunaka, M. Hagihara, K. Watanabe, S. Kamino, T. Kambe, S. Enomoto, and M. Hiromura. 2013. Essential role of the zinc transporter ZIP9/SLC39A9 in regulating the activations of Akt and Erk in B-cell receptor signaling pathway in DT40 cells. PLoS One 8(3):e58022.

Taylor, K., H. Morgan, A. Johnson, and R. Nicholson. 2005. Structure–function analysis of a novel member of the LIV-1 subfamily of zinc transporters, ZIP14. FEBS letters 579(2):427-432.

Taylor, K. M., and R. I. Nicholson. 2003. The LZT proteins; the LIV-1 subfamily of zinc transporters. Biochimica et Biophysica Acta 1611(1-2):16-30.

Taylor, K. M., P. Vichova, N. Jordan, S. Hiscox, R. Hendley, and R. I. Nicholson. 2008. ZIP7-mediated intracellular zinc transport contributes to aberrant growth factor signaling in antihormone-resistant breast cancer Cells. Endocrinology 149(10):4912-4920.

Thévenod, F. 2010. Catch me if you can! Novel aspects of cadmium transport in mammalian cells. Biometals 23(5):857-875.

Thomas, E. A., L. B. Bailey, G. A. Kauwell, D. Y. Lee, and R. J. Cousins. 1992. Erythrocyte metallothionein response to dietary zinc in humans. Journal of Nutrition 122(12):2408-2414. doi: 10.1093/jn/122.12.2408

Thompson, D. R., V. R. Parreira, R. R. Kulkarni, and J. F. Prescott. 2006. Live attenuated vaccine-based control of necrotic enteritis of broiler chickens. Veterinary Microbiology 113(1-2):25-34. doi: 10.1016/j.vetmic.2005.10.015

Timbermont, L., A. Lanckriet, J. Dewulf, N. Nollet, K. Schwarzer, F. Haesebrouck, R. Ducatelle, and F. Van Immerseel. 2010. Control of Clostridium perfringens-induced necrotic enteritis in broilers by target-released butyric acid, fatty acids and essential oils. Avian Pathology 39(2):117-121.

Timbermont, L., A. Lanckriet, A. R. Gholamiandehkordi, F. Pasmans, A. Martel, F. Haesebrouck, R. Ducatelle, and F. Van Immerseel. 2009. Origin of Clostridium perfringens isolates determines the ability to induce necrotic enteritis in broilers. Comparative Immunology, Microbiology, Infectious Diseases 32(6):503-512. doi: 10.1016/j.cimid.2008.07.001

Tipton, I. H., and M. J. Cook. 1963. Trace elements in human tissue. II. Adult subjects from the United States. Health physics 9:103-145.

Titball, R. W., C. E. Naylor, and A. K. Basak. 1999. The Clostridium perfringensα-toxin. Anaerobe 5(2):51-64.

Todd, W. E. C. H. E. 1980. Nutrition classics. The American Journal of Physiology. Volume 107, 1934, pages 146-156. "Zinc in the nutrition of the rat" by W.R. Todd, C.A. Elvehjem and E.B. Hart. Nutrition reviews 38(4):151-154.

Torres, C. A., J. E. Peña, R. Barros, R. N. Reis, S. L. Vieira, and P. C. Nascimento. 2009. Selenium contents of eggs from broiler breeders supplemented with sodium selenite or zinc-l-selenium-methionine. Journal of Applied Poultry Research 18(2):151-157. doi: 10.3382/japr.2008-00069

Tupper, R., R. W. Watts, and A. Wormall. 1954. The incorporation of 65Zn into avian eggs. Biochemical Journal 57(2):245-255.

Turk, D. 1986. Microelements in the circulation of coccidiosis-infected chicks. Poultry science 65(11):2098-2103.

Valberg, L. S., P. R. Flanagan, and M. J. Chamberlain. 1984. Effects of iron, tin, and copper on zinc absorption in humans. The American Journal of Clinical Nutrition 40(3):536-541. doi: 10.1093/ajcn/40.3.536

Vallee, B. L., and A. Galdes. 1984. The metallobiochemistry of zinc enzymes. Advances in Enzymology and Related Areas of Molecular Biology 56:283-430.

Vallee, B. L., and J. G. Gibson. 1948. An improved dithizone method for the determination of small quantities of zinc in blood and tissue samples. J. biol. Chem 176(1):435-443.

Vallee, B. L., and J. G. Gibson. 1949. The zinc content of whole blood, plasma, leukocytes and erythrocytes in the anemias. Blood 4:455-466.

Van Campen, D. R., and P. U. Scaife. 1967. Zinc interference with copper absorption in rats. Journal of Nutrition 91(4):473-476.

Van der Sluis, W. 2000. Clostridial enteritis is an often underestimated problem. World's Poultry Science Journal 16(7):42-43.

Van Immerseel, F., J. I. Rood, R. J. Moore, and R. W. Titball. 2009. Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. Trends in Microbiology 17(1):32-36.

Van Reen, R. 1953. Effects of excessive dietary zinc in the rat and the interrelationship with copper. Archives of Biochemistry 46:337-344.

Vetterling, J. M., and D. J. Doran. 1966. Schizogony and gametogony in the life cycle of the poultry coccidium, Eimeria acervulina Tyzzer, 1929. The Journal of parasitology:1150-1157.

Vlad, M., E. Caseanu, G. Uza, and M. Petrescu. 1994. Concentration of copper, zinc, chromium, iron and nickel in the abdominal aorta of patients deceased with coronary heart disease. Journal of Trace Elements and Electrolytes in Health and Disease 8(2):111-114.

Wade, B., and A. Keyburn. 2015. The true cost of necrotic enteritis. World's Poultry Science Journal 31(7):16-17.

Wade, S., F. Bleiberg, A. Mosse, J. Lubetzki, H. Flavigny, P. Chapuis, D. Roche, D. Lemonnier, and M. Dardenne. 1985. Thymulin (Zn-facteur thymique serique) activity in anorexia nervosa patients. The American Journal of Clinical Nutrition 42(2):275-280. doi: 10.1093/ajcn/42.2.275

Wang, F., B.-E. Kim, M. J. Petris, and D. J. Eide. 2004. The mammalian Zip5 protein is a zinc transporter that localizes to the basolateral surface of polarized cells. Journal of Biological Chemistry 279(49):51433-51441.

Wang, X., Z.-Y. Wang, H.-L. Gao, G. Danscher, and L. Huang. 2006. Localization of ZnT7 and zinc ions in mouse retina—immunohistochemistry and selenium autometallography. Brain Research Bulletin 71(1-3):91-96.

Waters, M., A. Savoie, H. S. Garmory, D. Bueschel, M. R. Popoff, J. G. Songer, R. W. Titball, B. A. McClane, and M. R. Sarker. 2003. Genotyping and phenotyping of beta2-toxigenic Clostridium perfringens fecal isolates associated with gastrointestinal diseases in piglets. Journal of Clinical Microbiology 41(8):3584-3591.

Wedekind, K., and D. Baker. 1990. Zinc bioavailability in feed-grade sources of zinc. Journal of animal science 68(3):684-689.

West, A. P., G. S. Shadel, and S. Ghosh. 2011. Mitochondria in innate immune responses. Nature Reviews Immunology 11(6):389.

Westmoreland, N., N. L. First, and W. G. Hoekstra. 1967. In-vitro uptake of zinc by boar spermatozoa. J Reprod Fertil 13(2):223-228.

Wetterdal, B. 1958. Experimental studies on radioactive zinc in the male reproductive organs of the rat. Acta Radiologica 156:1-83.

Widdowson, E. M., E. McCance, and C. M. Spray. 1951. The chemical composition of the human body. Clinical Science 10:113-125.

Williams, R. B. 2005. Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. Avian Pathology 34(3):159-180. doi: 10.1080/03079450500112195

Williams, R. B., and J. K. Chesters. 1970. The effects of early zinc deficiency on DNA and protein synthesis in the rat. British Journal of Nutrition 24(4):1053-1059.

Williams, R. B., R. N. Marshall, R. M. La Ragione, and J. Catchpole. 2003. A new method for the experimental production of necrotic enteritis and its use for studies on the relationships between necrotic enteritis, coccidiosis and anticoccidial vaccination of chickens. Parasitology research 90(1):19-26. doi: 10.1007/s00436-002-0803-4

Winchurch, R. A., J. Togo, and W. H. Adler. 1987. Supplemental zinc (Zn2+) restores antibody formation in cultures of aged spleen cells. II. Effects on mediator production. European journal of immunology 17(1):127-132. doi: 10.1002/eji.1830170121

Winder, F., and J. M. Denneny. 1959. Effect of iron and zinc on nucleic acid and protein synthesis in Mycobacterium smegmatis. Nature 184(Suppl 10):742-743.

Wirth, J. J., P. J. Fraker, and F. Kierszenbaum. 1984. Changes in the levels of marker expression by mononuclear phagocytes in zinc-deficient mice. Journal of Nutrition 114(10):1826-1833. doi: 10.1093/jn/114.10.1826

Wirth, J. J., P. J. Fraker, and F. Kierszenbaum. 1989. Zinc requirement for macrophage function: effect of zinc deficiency on uptake and killing of a protozoan parasite. Immunology 68(1):114-119.

Wnek, A. P., and B. A. McClane. 1986. Comparison of receptors for Clostridium perfringens type A and cholera enterotoxins in isolated rabbit intestinal brush border membranes. Microbial Pathogenesis 1(1):89-100.

Wolff, H. 1956. Untersuchungen zur Pathophysiologie des Zinkstoffwechsels. Klinische Wochenschrift 34(15-16):409-418.

Wu, C., C. Pot, L. Apetoh, T. Thalhamer, B. Zhu, G. Murugaiyan, S. Xiao, Y. Lee, M. Rangachari, and N. Yosef. 2013. Metallothioneins negatively regulate IL-27–induced type 1 regulatory T-cell differentiation. Proceedings of the National Academy of Sciences:201211776.

Xin, A., S. L. Nutt, G. T. Belz, and A. Kallies. 2011. Blimp1: driving terminal differentiation to a T, Crossroads between Innate and Adaptive Immunity III. Springer. p. 85-100.

Xu, S., S.-H. Lee, H. S. Lillehoj, Y. H. Hong, and D. Bravo. 2015. Effects of dietary selenium on host response to necrotic enteritis in young broilers. Research in veterinary science 98:66-73.

Yang, X., X. Sun, C. Li, X. Wu, and J. Yao. 2011. Effects of copper, iron, zinc, and manganese supplementation in a corn and soybean meal diet on the growth performance, meat quality, and immune responses of broiler chickens. Journal of Applied Poultry Research 20(3):263-271.

Yin, L.-H., X.-P. Liu, L.-Y. Yi, J. Wang, Y.-J. Zhang, and Y.-F. Feng. 2017. Structural characterization of calcium glycinate, magnesium glycinate and zinc glycinate. Journal of Innovative Optical Health Sciences 10(03):1650052.

Yogesh, K., C. Deo, H. Shrivastava, A. Mandal, A. Wadhwa, and I. Singh. 2013. Growth performance, carcass yield, and immune competence of broiler chickens as influenced by dietary supplemental zinc sources and levels. Agricultural Research 2(3):270-274.

Young, R. J., H. M. Edwards, Jr., and M. B. Gillis. 1958. Studies on Zinc in Poultry Nutrition: 2. Zinc Requirement and Deficiency Symptoms of Chicks. Poultry Science 37(5):1100-1107. doi: 10.3382/ps.0371100

Yu, Y., A. Wu, Z. Zhang, G. Yan, F. Zhang, L. Zhang, X. Shen, R. Hu, Y. Zhang, and K. Zhang. 2013. Characterization of the GufA subfamily member SLC39A11/Zip11 as a zinc transporter. Journal of Nutritional Biochemistry 24(10):1697-1708.

Zeigler, T. R., M. L. Scott, E. R. Mc, R. H. Greenlaw, F. Huegin, and W. H. Strain. 1962. Radiographic studies on skeletal parts of zinc deficient pullets. Proceedings of the Society for Experimental Biology and Medicine 109:239-242.

Zekarias, B., A. A. Ter Huurne, W. J. Landman, J. M. Rebel, J. M. Pol, and E. Gruys. 2002. Immunological basis of differences in disease resistance in the chicken. Veterinary Research 33(2):109-125.

Zhang, K., G. A. McQuibban, C. Silva, G. S. Butler, J. B. Johnston, J. Holden, I. Clark-Lewis, C. M. Overall, and C. Power. 2003. HIV-induced metalloproteinase processing of the chemokine stromal cell derived factor-1 causes neurodegeneration. Nature Neuroscience 6(10):1064.

Zhang, L., Y.-X. Wang, X. Xiao, J.-S. Wang, Q. Wang, K.-X. Li, T.-Y. Guo, and X.-A. Zhan. 2017a. Effects of zinc Glycinate on productive and reproductive performance, zinc concentration and antioxidant status in broiler breeders. Biological Trace Element Research 178(2):320-326.

Zhang, L., Y. X. Wang, X. Xiao, J. S. Wang, Q. Wang, K. X. Li, T. Y. Guo, and X. A. Zhan. 2017b. Effects of Zinc Glycinate on Productive and Reproductive Performance, Zinc Concentration and

Antioxidant Status in Broiler Breeders. Biological Trace Element Research 178(2):320-326. doi: 10.1007/s12011-016-0928-4

Zhao, Y., Y. Tan, J. Dai, B. Li, L. Guo, J. Cui, G. Wang, X. Shi, X. Zhang, N. Mellen, W. Li, and L. Cai. 2011. Exacerbation of diabetes-induced testicular apoptosis by zinc deficiency is most likely associated with oxidative stress, p38 MAPK activation, and p53 activation in mice. Toxicology letters 200(1-2):100-106. doi: 10.1016/j.toxlet.2010.11.001

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CHAPTER 3

EFFECTS OF ZINC GLYCINATE ON GROWTH, IMMUNITY, AND INTESTINAL HEALTH IN BROILER CHICKENS[1, 2, 3]

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ABSTRACT

The objective of this study is to investigate the effects of dietary zinc source and concentration in non-challenged broiler chickens. Day-old broiler chicks (n = 6) were allotted to one of six treatments in a 2 x 3 factorial arrangement to examine the effects of supplemental zinc source (sulfate and glycinate) and concentration (40, 80, and 120 mg/kg) on growth performance, immunity, and intestinal health. On day 21 and 35, birds supplemented with 80 mg/kg of zinc had a higher body weight gain (BWG) of 113 g compared to the birds that were supplemented with 40 mg/kg dietary zinc. On day 35, birds supplemented with 120 mg/kg of zinc had a higher BWG of 155 g compared to the birds that were supplemented with 40 mg/kg of zinc. On day 35, birds supplemented with 120 mg/kg of zinc had a higher feed intake of 239 g compared to birds that were supplemented with 40 mg/kg. On day 21, birds supplemented with 120 mg/kg of zinc had longer villi of 63 μm compared to the birds that were supplemented with 40 mg/kg of zinc. On day 21, birds supplemented with 80 mg/kg of zinc glycinate had a higher IL-1β relative mRNA expression by 2.5 and 2.4-fold in the cecal tonsils compared to the birds that were supplemented with 40 mg/kg of zinc from zinc glycinate and 120 mg/kg of zinc from zinc sulfate, respectively. On day 21, birds supplemented with 40 mg/kg of zinc from zinc glycinate had a lower IL-10 relative mRNA expression in the cecal tonsils by 2-fold compared to the birds that were supplemented with 40 mg/kg of zinc from zinc sulfate. On day 21, birds supplemented with 120 mg/kg of zinc from zinc sulfate had a lower IL-10 relative mRNA expression in the cecal tonsils by 2-fold compared to the birds that were supplemented with 40 mg/kg of zinc from zinc sulfate. On day 35, birds supplemented with zinc glycinate had a higher IL-1β relative mRNA expression in the cecal tonsils by 1.3-fold compared to the birds that were supplemented with zinc sulfate. On day 21, birds supplemented with 80 mg/kg of zinc from zinc glycinate had a higher TGF-β relative

mRNA expression in the spleen by 1.4-fold compared to the birds that were supplemented with 40 mg/kg of zinc from zinc glycinate. On day 35, birds supplemented with 80 mg/kg of zinc from zinc glycinate had a lower LITAF relative mRNA expression in the spleen by 0.6-fold compared to birds that were supplemented with 80 mg/kg of zinc from zinc sulfate. On day 35, birds supplemented with 80 mg/kg of zinc from zinc glycinate had a higher claudin-2 relative mRNA expression in the jejunum by 2.1-fold compared to the birds that were supplemented with 80 mg/kg of zinc from zinc sulfate. These results show that zinc concentration supplementation at 120 mg/kg improves the growth performance of broiler chickens, and the growth advantage is accompanied by improvements in intestinal morphology and modulation in immunity through an altered cytokine production profile.

Keywords: gut health, immunology, minerals, nutrition, broiler chickens, zinc

List of Abbreviations: BWG, body weight gain; Cq, quantification cycle; FCR, feed conversion ratio; FI, feed intake; IL, interleukins; IFN-γ, interferon-gamma; LITAF, lipopolysaccharide-induced tumor necrosis factor-alpha factor; TGF-β, transforming growth factor-beta; ZIP-9, Zrt- and Irt-like protein-9.

INTRODUCTION

Zinc is an essential micronutrient for growth and development in chickens (O'Dell et al., 1958a). Though according to the National Research Council recommendation, the optimal total zinc in chicken diet is 40 mg/kg (NRC, 1994), broiler breeders recommend supplementing zinc at 100 mg/kg (Cobb-Vantress, 2018). Zinc deficiency decreases thymus, bursa, and spleen weight (Cui et al., 2004) and decreases cellular and humoral immune responses to pathogens (Pimentel et al., 1991; Sunder et al., 2008). In addition, zinc functions as co-factors in the antioxidant enzyme, Zn/Cu superoxide dismutase (Salim et al., 2008).

Zinc sulfate is currently one of the most commonly used inorganic zinc sources in poultry feed (Yogesh et al., 2013). Organic mineral sources, such as zinc glycinate, methionate, and proteinate, have been applied to replace inorganic minerals in animal feed (Cao et al., 2000). Zinc from zinc glycinate has higher solubility (Low et al., 1959), higher bioavailability (Schlegel and Windisch, 2006), digestibility (Bao et al., 2009; Yenice et al., 2015) and retention (Cao et al., 2002) compared to the zinc from zinc sulfate. Zinc glycinate supplementation increases the copper/zinc superoxide dismutase gene expression compared to chickens concentration zinc sulfate (Zhang et al., 2017a). The effect of zinc glycinate supplementation in broiler chickens at concentrations above 40 mg/kg on cytokine expressions has not been explored before and this study investigated the effects of zinc glycinate supplemented at 100 mg/kg. We hypothesize that zinc glycinate supplementation improves the immunity of the birds by increasing cytokine mRNA expressions; thereby improves body weight gain, feed intake, feed conversion ratio, and jejunum villi surface areas. The effects of zinc glycinate supplementation on cytokines relative mRNA expression in cecal tonsils and spleen, jejunal villi length and crypt depth, jejunum claudin-2, zonula occludens, and ZIP-9 relative mRNA expression were studied.

MATERIALS AND METHODS

Animals and Management

This study was conducted under the animal protocol approved by the Institutional Animal Care and Use Committee at the University of Georgia (IACUC Protocol: A2018 04-009-Y2-A0). The birds were housed in floor pens under standard animal husbandry practices, according to Cobb 500 management guides (Cobb-Vantress, AR, USA). The chickens were housed in 2 X 2 meter pens with fresh wood shaving litter. The temperature was maintained at 33°C on day 1, 31°C from the day 2 to 7, 27°C from day 8 to 14, 24°C from day 15 to 21, 21°C from day 22 to 28, and 19°C from day 29 to 35. Water was provided *ad libitum*.

Treatment Diets and Sample Collection

Day-old Cobb 500 off-sex male broiler chicks were randomly assigned to two zinc sources (zinc sulfate and zinc glycinate) and three supplemental zinc concentrations (40, 80, and 120 mg/kg), resulting in a 2 X 3 factorial set up of treatments. The supplemental zinc concentrations were calculated from the molecular weight of zinc sulfate and glycinate. Each treatment was replicated in six pens with eight birds per pen (n = 6). A priori power analyses were carried out utilizing the effect size from a similar study of selenium glycinate on immune status in chickens (Lee et al., 2014a) to determine the sample size required to achieve 97% power (G*Power ver. 3.0.10) (Erdfelder et al., 1996). Birds were supplemented with zinc at 40, 80, and 120 mg/kg from zinc sulfate or zinc glycinate. The experimental diets were prepared from basal diets that were consisted of a zinc-free mineral mix. Three basal diets were used: a starter diet from day 1 to 10, a grower diet from day 11 to 22, and a finisher diet from day 23 to 35 (Table 3.1). The diets with the lowest concentration of zinc were mixed first to ensure a progressive increase in zinc in subsequent mixes. Additionally, basal feed that had the zinc-free mineral mix was used to clean out the feed mill

before mixing the subsequent diets. The compositions of the experimental diets of the starter, grower, and finisher diets, except for the zinc content, were based on the Cobb 500 Performance and Nutrition Supplement Guide. The six corn-soybean based diets were iso-caloric and iso-nitrogenous (Table 3.2).

Body weight and feed consumption were measured weekly. Body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) on day 21 and 35 were calculated. Body weight and feed conversion data from the birds, including those that died before day 21 or 35 were included in calculating the body weight gain and feed intake. On day 21 and 35, samples were collected from one bird per replicate. The cecal tonsil, spleen, and jejunum tissues were snap-frozen in liquid nitrogen and stored at -80ºC until further analyses. Jejunal sections were collected, flushed using PBS, and stored in 10% neutral buffered formalin for histological analyses.

Effect of Zinc Glycinate Supplementation on Jejunum Histological Parameters

The jejunal tissue in 10% neutral buffered formalin was processed in the Poultry Diagnostic and Research Center (PDRC, Athens, GA) laboratory. The cross-sections were viewed and photographed using an Olympus IX71 microscope and analyzed using DP Controller ver. 2.1.1.183 software (Olympus America) to determine the villi length and crypt depth. Five villi per section and four sections per sample were analyzed. For each jejunum sample, villi and crypt of four sections of the slides were measured and averaged as technical replicates. Villi length to crypt depth ratios was calculated.

Effect of Zinc Glycinate Supplementation on Cytokine Relative mRNA Expression

Total RNA from cecal tonsil, spleen, and jejunum tissues were isolated using the TRIzol/chloroform method. Briefly, tissues (25 mg) were homogenized using the TissueLyser LT

(Qiagen, MD, USA) and 5 mm stainless steel beads in TRIzol for 6 min. Chloroform (200 µl) was added to the homogenate and centrifuged to fractionate the total RNA in the aqueous phase. The total RNA was precipitated in isopropanol, washed in 70% ethanol, and then resuspended in molecular-grade water. Optical density at 260 nm was used to determine RNA concentrations. RNA samples were reverse transcribed. Two micrograms of RNA were converted to cDNA in a 20 µL reaction volume containing 1X reaction buffer (50 mM Tris–HCl [pH 8.3], 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT), 10 mM DTT, 0.5 mM dNTPs, 0.5 µg of oligo(dT)15 primer, 8 units of RNAsin and 100 units of M-MLV reverse transcriptase (all from Promega) at 40°C for 1 h, and then 95°C for 10 min.Com

Relative gene expressions of pro-inflammatory cytokines (IL-1β, IFN-γ, and LITAF, anti-inflammatory cytokines (IL-10 and TGF-β) of mRNA from cecal tonsil and spleen tissues were analyzed using the CFX-96 and CFX Maestro (Bio-Rad, CA, USA). Relative gene expressions of jejunum tight junction proteins (claudin-2 and zonula occludens) and zinc importer (ZIP-9) were analyzed. The expressions were normalized to β-actin as the housekeeping gene, normalized to expressions of zinc sulfate 40 mg/kg treatment, and reported as fold-change ± SE. The primer sequences and references are listed in Table 3.3. PCR reagents and reaction conditions were optimized for each gene. Fold change from the reference was quantified as $2^{-\Delta\Delta Cq}$, where Cq is the threshold cycle defined as the cycle number when the qRT-PCR product fluorescence reaches exponentially above the background (Livak and Schmittgen, 2001).

Statistical Analysis

All data were analyzed using two-way ANOVA using SAS v.9.0 (SAS Institute, Inc., NC, USA) to determine the effects of zinc sources and concentrations on the dependent variables. When the

main effect was significant ($P < 0.05$), the differences between means were analyzed using Tukey's HSD post-hoc analysis.

RESULTS

Effect of Zinc Glycinate Supplementation on BWG, FI, and FCR

On day 21 and 35, there were no significant interactions between zinc source and supplemental zinc concentration on body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR). On day 21 and 35, birds supplemented with 80 mg/kg of dietary zinc had a higher BWG of 113 g compared to the birds that were supplemented with 40 mg/kg dietary zinc (**$P < 0.01$; Figure 3.1A). On day 35, birds supplemented with 120 mg/kg of dietary zinc had a higher BWG of 155 g compared to the birds that were supplemented with 40 mg/kg dietary zinc (**$P < 0.01$; Figure 3.1A). On day 35, birds supplemented with 120 mg/kg of dietary zinc had a higher FI of 239 g compared to birds that were supplemented with 40 mg/kg (*$P < 0.05$; Figure 3.1B). On day 21, there were no significant main effects of zinc source or concentration on FI and FCR between different treatment groups. On day 35, there were no significant main effects of zinc source or concentration on FCR between treatment groups.

Effect of Zinc Glycinate Supplementation on Jejunum Histological Parameters

On day 21 and 35, there were no significant interactions between zinc source and supplemental zinc concentration on jejunum histological parameters studied. On day 21, birds supplemented with 120 mg/kg of dietary zinc had longer villi of 63 μm compared to the birds that were supplemented with 40 mg/kg dietary zinc (*$P < 0.05$; Figure 3.2A). On day 21, there were no significant main effects of zinc source or concentration on jejunum crypt depth and villi length to crypt depth ratio between different treatment groups. On day 35, there were no significant main

effects of zinc source or concentration on jejunum villi length, crypt depth, and villi length to crypt depth ratio between treatment groups.

Effect of Zinc Glycinate Supplementation on Cytokine Relative mRNA Expression

Cecal Tonsils

On day 21, there were significant interactions between zinc source and concentration on IL-1β relative mRNA expression in the cecal tonsils (**$P < 0.01$; Figure 3.3A). On day 21, birds supplemented with 80 mg/kg of zinc from zinc glycinate had a higher IL-1β relative mRNA expression in the cecal tonsils by 2.5 and 2.4-fold compared to the birds that were supplemented with 40 mg/kg of zinc from zinc glycinate and 120 mg/kg of zinc from zinc sulfate, respectively. On day 21, there were significant interactions between zinc source and concentration on IL-10 relative mRNA expression in the cecal tonsils (**$P < 0.01$; Figure 3.3B). On day 21, birds supplemented with 40 mg/kg of zinc from zinc glycinate had a lower IL-10 relative mRNA expression in the cecal tonsils by 2-fold compared to the birds that were supplemented with 40 mg/kg of zinc from zinc sulfate. On day 21, birds supplemented with 120 mg/kg of zinc from zinc sulfate had a lower IL-10 relative mRNA expression in the cecal tonsils by 2-fold compared to the birds that were supplemented with 40 mg/kg of zinc from zinc sulfate. On day 35, there were no significant interactions between zinc source and supplemental zinc concentration on IL-1β relative mRNA expression in the cecal tonsils. On day 35, birds supplemented with zinc glycinate had a higher IL-1β relative mRNA expression in the cecal tonsils by 1.3-fold compared to the birds that were supplemented with zinc sulfate. On day 21, there were no significant main effects of zinc source or concentration on the IFN-γ, LITAF, and TGF-β relative mRNA expression in the cecal tonsils between different treatment groups. On day 35, there were no significant main effects of

zinc source or concentration on the IFN-γ, LITAF, IL-10, and TGF-β relative mRNA expression in the cecal tonsils between treatment groups.

Spleen

On day 21, there were significant interactions between zinc source and concentration on TGF-β relative mRNA expression in the spleen (*$P < 0.05$; Figure 3.4A). On day 21, birds supplemented with 80 mg/kg of zinc from zinc glycinate had a higher TGF-β relative mRNA expression in the spleen by 1.4-fold compared to the birds that were supplemented with 40 mg/kg of zinc from zinc glycinate. On day 35, there were significant interactions between zinc source and concentration on LITAF relative mRNA expression in the spleen (*$P < 0.05$; Figure 3.4B). On day 35, birds supplemented with 80 mg/kg of zinc from zinc glycinate had a lower LITAF relative mRNA expression in the spleen by 0.6-fold compared to birds that were supplemented with 80 mg/kg of zinc from zinc sulfate. On day 21, there were no significant main effects of zinc source or concentration on the IL-1β, IFN-γ, LITAF, and IL-10 relative mRNA expression in the spleen between different treatment groups. On day 35, there were no significant main effects of zinc source or concentration on the IL-1β, IFN-γ, IL-10, and TGF-β relative mRNA expression in the spleen between treatment groups.

Jejunum

On day 35, there were significant interactions between zinc source and concentration on claudin-2 relative mRNA expression in the jejunum (*$P < 0.05$; Figure 3.2B). On day 35, birds supplemented with 80 mg/kg of zinc from zinc glycinate had a higher claudin-2 relative mRNA expression in the jejunum by 2.1-fold compared to the birds that were supplemented with 80 mg/kg of zinc from zinc sulfate. On day 21, there were no significant main effects of zinc source or concentration on the claudin-2, zonula occludens (ZO-1), and ZIP-9 relative mRNA expression in

the jejunum between different treatment groups. On day 35, there were no significant main effects of zinc source or concentration on the zonula occludens and ZIP-9 relative mRNA expression in the jejunum between treatment groups.

DISCUSSION

This experiment studied the effects of zinc from zinc sulfate and zinc glycinate and concentrations on immune parameters in broiler chickens. Inorganic mineral sources, such as zinc sulfate and zinc oxide, have been traditionally used in poultry diets. Organic sources, such as zinc methionine, zinc glycinate, and zinc proteinate, has been introduced to replace inorganic zinc. Zinc conjugated to amino acids, such as glycine and methionine, have improved solubility (Cao et al., 2000) and bioavailability (Mohanna and Nys, 1999) *in vivo*.

The impact of zinc supplementation on the growth performance of the chickens was studied. Mortality was not significantly different between treatments in this study. The organic source, zinc glycinate, did not result in a significant change of growth performance in non-challenged birds compared to the inorganic source, zinc sulfate. This study determined that zinc supplementation at 120 mg/kg is optimal for the growth of broiler chickens. While some studies have demonstrated that organic zinc improved FCR in chickens (Sridhar et al., 2014b; Akhavan-Salamat and Ghasemi, 2019) and body weight gain (Akhavan-Salamat and Ghasemi, 2019), others observed no differences in the growth performance between birds that were supplemented with inorganic and organic zinc (Mohanna and Nys, 1999; Hu et al., 2013; Yogesh et al., 2013; Kwiecień et al., 2016; Tomaszewska et al., 2017; Zhang et al., 2017a; Mählmeyer et al., 2018; Bortoluzzi et al., 2019b). Three factors complicate the use of organic zinc in poultry diets. First, some studies included zinc supplementation far below the current recommendation of 100 mg/kg (Sridhar et al., 2014b; Mählmeyer et al., 2018). Second, some studies did not include the same concentrations of

inorganic and organic zinc (Sridhar et al., 2014b). Particularly, inorganic zinc was supplemented at 40 mg/kg, while organic zinc was supplemented at 30, 20, and 10 mg/kg. Third, the organic zinc sources differ between studies. For example, some studies utilized zinc glycinate (Schlegel and Windisch, 2006; Sridhar et al., 2015c; Kwiecień et al., 2016; Kwiecień et al., 2017; Yin et al., 2017; Zhang et al., 2017a), and others utilized zinc proteinate (Bortoluzzi et al., 2019b) as the organic sources. Although the addition of organic zinc is generally considered safe in animal diets, one study observed a reduction of body weight by zinc glycinate supplemented at 100 mg/kg compared to the inorganic zinc treatment at the same concentration (Kwiecień et al., 2016).

The effects of zinc supplementation on jejunum morphological parameters were studied as an indicator of intestinal health. Zinc supplementation at 120 mg/kg resulted in longer villi in this study. The jejunum is the primary site for nutrient absorption, and the increase in the villi surface area is correlated with the increase ins nutrient absorption (Hu et al., 2013). Zinc regulates cell proliferation through the insulin growth factor (IGF-I) axis (MacDonald, 2000b). Multiple transcription factors, such as the Krüppel-like factor-4 (Katz et al., 2002), GATA-4 (van der Sluis et al., 2004), and insulinoma-associate 1 (Gierl et al., 2006) that modulate the growth of intestinal cells have zinc-finger domains. The improvement in BWG and FI observed in this study was likely associated with the increase in the villi surface area. Jejunum tight junction proteins are associated with epithelial cell integrity (Ulluwishewa et al., 2011). The loss of claudin-2 mRNA amount increases tight-junction permeability in the intestine (Rosenthal et al., 2010). Claudin-2 mRNA amount on day 35 was higher in the zinc supplemented treatments at 80 mg/kg from zinc glycinate than from zinc sulfate. Hence, it can be concluded that zinc glycinate was more beneficial to intestinal health compared to zinc sulfate. ZIP-9 transporter is involved in zinc transport in the Trans-Golgi network in all tissues (Matsuura et al., 2009; Thomas et al., 2018) and TGF-β

signaling (Troche et al., 2015). ZIP-9 transporter mRNA was not affected by zinc supplementation in this study.

In the cecal tonsils, the IL-1β relative mRNA expression was higher in birds supplemented with zinc from zinc glycinate than zinc sulfate. The cytokine, IL-1β mediates inflammatory responses by immune cell activation, dendritic maturation, and phagocytosis of pathogens (Dinarello, 2011). Therefore, the increase in IL-1β production may indicate a more robust immune activation in case of an infection. The lipopolysaccharide-induced TNF factor (LITAF) is produced by chicken macrophages (Zhang et al., 1995) and is responsible for activating immune responses such as nitric oxide production (Rautenschlein et al., 1999). In the spleen, LITAF relative mRNA expression was lower in birds supplemented with zinc at 80 mg/kg from zinc glycinate than zinc sulfate. Zinc glycinate favors the adaptive immune response by increasing immune cell proliferation through IL-1β and downregulation of myeloid cells through LITAF, as suggested by previous studies (Akbari et al., 2008).

The anti-inflammatory cytokine, IL-10, is secreted by $CD4^+$ regulatory T lymphocytes (Tregs) (Shanmugasundaram and Selvaraj, 2011). Tregs dampens inflammatory responses through IL-10 to limit damages from immune cells (O'Garra et al., 2004). We observed downregulation of IL-10 in the cecal tonsils with increasing concentration of zinc sulfate, but not zinc glycinate. The decrease in IL-10 relative mRNA expression suggests an upregulation of Tregs to return immunity to homeostasis. The transforming growth factor-beta (TGF-β) has dual roles in pro-inflammatory and anti-inflammatory responses (Sanjabi et al., 2009). TGF-β promotes tolerance by suppression of naïve $CD8^+$ T-lymphocyte activation, or in the combination of IL-4 or IL-6 promotes immune activation. The TGF-β relative mRNA expression in the spleen was higher in the birds supplemented with zinc at 80 mg/kg from zinc sulfate than supplemented l mg/kg from zinc sulfate.

In conjunction with the increase in IL-1β in the cecal tonsils and a decrease in IL-10, the data suggests the immune activation role of TGF-β by zinc glycinate in this study.

In summary, this study demonstrated that the supplemental zinc concentration had a more significant effect on growth performance than the zinc source in non-challenged chickens. Supplemental zinc concentration at 120 mg/kg was optimal for growth for non-challenged broiler chickens regardless of source. We concluded that immunity was improved by zinc glycinate supplementation, particularly at 120 mg/kg. Zinc supplemented at 120 mg/kg in broiler chicken diets is optimal for non-challenged birds.

DISCLOSURES

Zinc glycinate was donated by Pancosma SA (Geneva, CH). Pancosma SA was not involved in designing and conducting the research.

LITERATURE CITED

Akbari, M., H. Kermanshahi, H. N. Moghaddam, A. H. Moussavi, and J. T. Afshari. 2008. Effects of wheat-soybean meal based diet supplementation with vitamin A, vitamin E and zinc on blood cells, organ weights and humoral immune response in broiler chickens. J. Anim. Vet. Adv 7(3):297-304.

Akhavan-Salamat, H., and H. Ghasemi. 2019. Effect of different sources and contents of zinc on growth performance, carcass characteristics, humoral immunity and antioxidant status of broiler chickens exposed to high environmental temperatures. Livestock science 223:76-83.

Bao, Y., M. Choct, P. Iji, and K. Bruerton. 2009. Optimal dietary inclusion of organically complexed zinc for broiler chickens. British Journal of Poultry Science 50(1):95-102.

Bortoluzzi, C., B. Vieira, B. Lumpkins, G. Mathis, W. King, D. Graugnard, K. Dawson, and T. Applegate. 2019. Can dietary zinc diminish the impact of necrotic enteritis on growth performance of broiler chickens by modulating the intestinal immune-system and microbiota? Poultry Science

Cao, J., P. Henry, S. Davis, R. Cousins, R. Miles, R. Littell, and C. Ammerman. 2002. Relative bioavailability of organic zinc sources based on tissue zinc and metallothionein in chicks fed conventional dietary zinc concentrations. Animal Feed Science and Technology 101(1-4):161-170.

Cao, J., P. Henry, R. Guo, R. Holwerda, J. Toth, R. Littell, R. Miles, and C. Ammerman. 2000. Chemical characteristics and relative bioavailability of supplemental organic zinc sources for poultry and ruminants. Journal of Animal Science 78(8):2039-2054.

Cobb-Vantress. 2018. Cobb 500 Broiler Perfromance and Nutrition Supplement.

Cui, H., P. Xi, D. Junliang, L. Debing, and Y. Guang. 2004. Pathology of lymphoid organs in chickens fed a diet deficient in zinc. Avian Pathology 33(5):519-524.

Dinarello, C. A. 2011. A clinical perspective of IL-1β as the gatekeeper of inflammation. European journal of immunology 41(5):1203-1217.

Erdfelder, E., F. Faul, and A. Buchner. 1996. GPOWER: A general power analysis program. Behavior research methods, instruments, & computers 28(1):1-11.

Gierl, M. S., N. Karoulias, H. Wende, M. Strehle, and C. Birchmeier. 2006. The zinc-finger factor Insm1 (IA-1) is essential for the development of pancreatic β cells and intestinal endocrine cells. Genes & development 20(17):2465-2478.

Hu, C., Z. Qian, J. Song, Z. Luan, and A. Zuo. 2013. Effects of zinc oxide-montmorillonite hybrid on growth performance, intestinal structure, and function of broiler chicken. Poultry Science 92(1):143-150.

Katz, J. P., N. Perreault, B. G. Goldstein, C. S. Lee, P. A. Labosky, V. W. Yang, and K. H. Kaestner. 2002. The zinc-finger transcription factor Klf4 is required for terminal differentiation of goblet cells in the colon. Development 129(11):2619-2628.

Kwiecień, M., A. Winiarska-Mieczan, A. Milczarek, and R. Klebaniuk. 2017. Biological Response of Broiler Chickens to Decreasing Dietary Inclusion Levels of Zinc Glycine Chelate. Biological Trace Element Research 175(1):204-213. (journal article) doi: 10.1007/s12011-016-0743-y

Kwiecień, M., A. Winiarska-Mieczan, A. Milczarek, E. Tomaszewska, and J. Matras. 2016. Effects of zinc glycine chelate on growth performance, carcass characteristics, bone quality, and mineral content in bone of broiler chicken. Livestock Science 191:43-50.

Lee, S., H. Lillehoj, S. Jang, M. Jeong, S. Xu, J. Kim, H. Park, H. Kim, E. Lillehoj, and D. Bravo. 2014. Effects of in ovo injection with selenium on immune and antioxidant responses during experimental necrotic enteritis in broiler chickens. Poultry science 93(5):1113-1121.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods 25(4):402-408.

Low, B. W., F. Hirshfeld, and F. Richards. 1959. Glycinate complexes of zinc and cadmium. Journal of the American Chemical Society 81(16):4412-4416.

MacDonald, R. S. 2000. The role of zinc in growth and cell proliferation. The Journal of nutrition 130(5):1500S-1508S.

Mählmeyer, A., J. Lindel, A. Schlagheck, B. Hildebrand, and K. Männer. 2018. Investigation on the Effect of Trace Mineral Source on Parameters of Bioavailability in Broiler Chickens. Veterinarija ir Zootechnika 76(98)

Matsuura, W., T. Yamazaki, Y. Yamaguchi-Iwai, S. Masuda, M. Nagao, G. K. Andrews, and T. Kambe. 2009. SLC39A9 (ZIP9) regulates zinc homeostasis in the secretory pathway: characterization of the ZIP subfamily I protein in vertebrate cells. Bioscience, biotechnology, and biochemistry 73(5):1142-1148.

Mohanna, C., and Y. Nys. 1999. Effect of dietary zinc content and sources on the growth, body zinc deposition and retention, zinc excretion and immune response in chickens. British Journal of Poultry Science 40(1):108-114.

NRC, N. R. C. 1994. NRC. Nutrient requirements of poultry. Washington. National Academy is Sciences

O'Dell, B., P. Newberne, and J. Savage. 1958. Significance of dietary zinc for the growing chicken. The Journal of Nutrition 65(4):503-523.

O'Garra, A., P. L. Vieira, P. Vieira, and A. E. Goldfeld. 2004. IL-10–producing and naturally occurring CD4+ Tregs: limiting collateral damage. The Journal of clinical investigation 114(10):1372-1378.

Pimentel, J., M. Cook, and J. Greger. 1991. Immune response of chicks fed various levels of zinc. Poultry Science 70(4):947-954.

Rautenschlein, S., A. Subramanian, and J. M. Sharma. 1999. Bioactivities of a tumour necrosis-like factor released by chicken macrophages. Developmental & Comparative Immunology 23(7-8):629-640.

Rosenthal, R., S. Milatz, S. M. Krug, B. Oelrich, J.-D. Schulzke, S. Amasheh, D. Günzel, and M. Fromm. 2010. Claudin-2, a component of the tight junction, forms a paracellular water channel. Journal of cell science 123(11):1913-1921.

Salim, H., C. Jo, and B. Lee. 2008. Zinc in broiler feeding and nutrition. Avian Biology Research 1(1):5-18.

Sanjabi, S., L. A. Zenewicz, M. Kamanaka, and R. A. Flavell. 2009. Anti-inflammatory and pro-inflammatory roles of TGF-β, IL-10, and IL-22 in immunity and autoimmunity. Current opinion in pharmacology 9(4):447-453.

Schlegel, P., and W. Windisch. 2006. Bioavailability of zinc glycinate in comparison with zinc sulphate in the presence of dietary phytate in an animal model with 65Zn labelled rats. Journal of Animal Physiology and Animal Nutrition 90(5-6):216-222.

Shanmugasundaram, R., and R. K. Selvaraj. 2011. Regulatory T cell properties of chicken CD4+ CD25+ cells. The Journal of Immunology 186(4):1997-2002.

Sridhar, K., D. Nagalakshmi, D. S. Rao, and S. Rao. 2014. Effect of dietary addition of organic zinc on performance and carcass traits in commercial broiler chicken. Indian J. Poult. Sci 49(2):155-158.

Sridhar, K., D. Nagalakshmi, and S. R. Rao. 2015. Effect of graded concentration of organic zinc (zinc glycinate) on skin quality, hematological and serum biochemical constituents in broiler chicken. Indian Journal of Animal Science 85:643-648.

Sunder, G. S., A. Panda, N. Gopinath, S. R. Rao, M. Raju, M. Reddy, and C. V. Kumar. 2008. Effects of higher levels of zinc supplementation on performance, mineral availability, and immune competence in broiler chickens. Journal of Applied Poultry Research 17(1):79-86.

Thomas, P., A. Converse, and H. A. Berg. 2018. ZIP9, a novel membrane androgen receptor and zinc transporter protein. General and comparative endocrinology 257:130-136.

Tomaszewska, E., S. Muszyński, P. Dobrowolski, M. Kwiecień, A. Winiarska-Mieczan, I. Świetlicka, and A. Wawrzyniak. 2017. Effect of zinc level and source (zinc oxide vs. zinc glycine) on bone mechanical and geometric parameters, and histomorphology in male Ross 308 broiler chicken. Brazilian Journal of Poultry Science 19(1):159-170.

Troche, C., S. D. Eicher, and T. J. Applegate. 2015. The influence of dietary zinc source and coccidial vaccine exposure on intracellular zinc homeostasis and immune status in broiler chickens. British Journal of Nutrition 114(2):202-212.

Ulluwishewa, D., R. C. Anderson, W. C. McNabb, P. J. Moughan, J. M. Wells, and N. C. Roy. 2011. Regulation of tight junction permeability by intestinal bacteria and dietary components. The Journal of nutrition 141(5):769-776.

van der Sluis, M., M. H. Melis, N. Jonckheere, M.-P. Ducourouble, H. A. Büller, I. Renes, A. W. Einerhand, and I. Van Seuningen. 2004. The murine Muc2 mucin gene is transcriptionally regulated by the zinc-finger GATA-4 transcription factor in intestinal cells. Biochemical and biophysical research communications 325(3):952-960.

Yenice, E., C. Mızrak, M. Gültekin, Z. Atik, and M. Tunca. 2015. Effects of organic and inorganic forms of manganese, zinc, copper, and chromium on bioavailability of these minerals and calcium in late-phase laying hens. Biological Trace Element Research 167(2):300-307.

Yin, L.-H., X.-P. Liu, L.-Y. Yi, J. Wang, Y.-J. Zhang, and Y.-F. Feng. 2017. Structural characterization of calcium glycinate, magnesium glycinate and zinc glycinate. Journal of Innovative Optical Health Sciences 10(03):1650052.

Yogesh, K., C. Deo, H. Shrivastava, A. Mandal, A. Wadhwa, and I. Singh. 2013. Growth performance, carcass yield, and immune competence of broiler chickens as influenced by dietary supplemental zinc sources and levels. Agricultural Research 2(3):270-274.

Zhang, L., Y.-X. Wang, X. Xiao, J.-S. Wang, Q. Wang, K.-X. Li, T.-Y. Guo, and X.-A. Zhan. 2017. Effects of zinc Glycinate on productive and reproductive performance, zinc concentration and antioxidant status in broiler breeders. Biological Trace Element Research 178(2):320-326.

Zhang, S., H. Lillehoj, and M. Ruff. 1995. Chicken Tumor Necrosis-Like Factor. 1. In Vitro Production by Macrophages Stimulated with Eimeria tenella or Bacterial Lipopolysaccharide. Poultry science 74(8):1304-1310.

TABLES AND FIGURES

Table 3.1.
Ingredients of experimental diets in starter, grower, and finisher phases.

Ingredient (as-fed basis)	Starter 1 to 10 days	Grower 11 to 22 days	Finisher 23 to 42 days
Yellow corn grain, %	62.82	67.77	68.60
Soybean meal (48%), %	33.27	27.95	26.37
Vegetable fat, %	1.17	1.69	2.79
Defluorinated phosphate, %	1.13	0.98	0.76
Calcium carbonate, %	0.75	0.75	0.74
†Vitamin premix, %	0.25	0.25	0.25
DL-Methionine, %	0.24	0.21	0.16
L-Lysine, %	0.16	0.17	0.15
Salt, %	0.12	0.14	0.10
‡Trace Mineral (Zinc-free) , %	0.08	0.08	0.08
Quantum Blue Phytase (5,000 FTU/g), %§	0.01	0.01	0.01

†Vitamins and minerals were provided in the form and amount described in the Cobb 500 Broiler Performance and Nutrition Supplement guide. Vitamin A: 10,000 IU; vitamin D3: 5,000 IU; vitamin E: 8,000 IU; vitamin K3: 3 mg/kg; vitamin B1 (thiamine): 3 mg/kg; vitamin B2 (riboflavin): 9 mg/kg; vitamin B6 (pyridoxine): 4 mg/kg; vitamin B12: 0.02 mg/kg; biotin: 0.15 mg/kg; choline: 500 mg/kg; folic acid: 2 mg/kg; nicotinic acid: 60 mg/kg; pantothenic acid: 15 mg/kg.
‡Manganese (from manganese sulfate): 100 mg/kg; iron (from ferrous sulfate): 40 mg/kg; copper (from copper sulfate): 15 mg/kg; iodine (from calcium iodide): 1 mg/kg; selenium (from sodium selenite): 0.35 mg/kg.
§FTU: phytase unit

Table 3.2.
Calculated nutrient compositions of the starter, grower, and finisher diets

Nutrient	Units	Starter 1 to 10 days	Grower 11 to 22 days	Finisher 23 to 42 days
Dry Matter	%	87.73	87.72	87.79
Crude Protein	%	21	18.8	18
Crude Fat	%	3.69	4.33	5.43
Crude Fiber	%	2.19	2.13	2.09
Calcium	%	0.9	0.84	0.76
Total Phosphorus	%	0.58	0.53	0.48
Available Phosphorus	%	0.45	0.42	0.38
Metabolizable Energy	kcal/kg	3,008	3,086	3,167
Methionine	%	0.58	0.52	0.47
Lysine	%	1.28	1.14	1.04
Tryptophan	%	0.28	0.24	0.23
Threonine	%	0.85	0.76	0.73
Sodium	%	0.16	0.16	0.15
Potassium	%	0.82	0.74	0.71
Chloride	%	0.15	0.16	0.15
Digestible methionine	%	0.56	0.5	0.45
Digestible cysteine	%	0.32	0.3	0.29
Digestible lysine	%	1.18	1.05	0.95
Digestible tryptophan	%	0.27	0.24	0.23
Digestible threonine	%	0.77	0.69	0.66
Digestible isoleucine	%	0.96	0.85	0.81
Digestible histidine	%	0.53	0.48	0.46
Digestible valine	%	1.05	0.94	0.9
Digestible leucine	%	1.75	1.61	1.56
Digestible arginine	%	1.33	1.17	1.12
Digestible phenylalanine	%	1.07	0.95	0.92
Digestible TSAA[†]	%	0.88	0.8	0.74

[†]Total sulfur amino acids

Table 3.3.
Primers information for quantitative real-time PCR.

Gene		Primer Sequence (5' – 3')	Accession #/Reference	Primer Efficiency
Housekeeping	β–actin	F: ACCGGACTGTTACCAACACC R: GACTGCTGCTGACACCTTCA	(Shanmugasundaram et al., 2013)	87.19%
Pro-inflammatory Cytokine	IL-1β	F: TGGGCATCAAGGGCTACA R: TCGGGTTGGTTGGTGATG	Y07922/(Hong et al., 2006)	98.29%
	IFN-γ	F: GTGAAGAAGGTGAAAGATATCATGGA R: GCTTTGCGCTGGATTCTCA	(Shanmugasundaram et al., 2013)	79.66%
	LITAF	F: ATCCTCACCCCTACCCTGTC R: GGCGGTCATAGAACAGCACT	(Luoma, 2016)	97.33%
Anti-inflammatory Cytokine	TGF-β	F: CGGGACGGATGAGAAGAAC R: CGGCCCACGTAGTAAATGAT	M31160/(Hong et al., 2006)	107.78%
	IL-10	F: CATGCTGCTGGGCCTGAA R: CGTCTCCTTGATCTGCTTGATG	(Shanmugasundaram et al., 2013)	87.02%
Tight Junction Protein	Claudin 2	F: CCTGCTCACCCTCATTGGAG R: GCTGAACTCACTCTTGGGCT	(Bortoluzzi et al., 2019b)	78.32%
	ZO-1	F: CCGTAACCCCGAGTTGGAT R: ATTGAGGCGGTCGTTGATG	(Bortoluzzi et al., 2019b)	78.01%
Zinc Importer	ZIP-9	F: CGTTCCATCTGCCTGCTGTC R: GCACCCAGAACAGTCACCAAC	(Troche et al., 2015)	88.28%

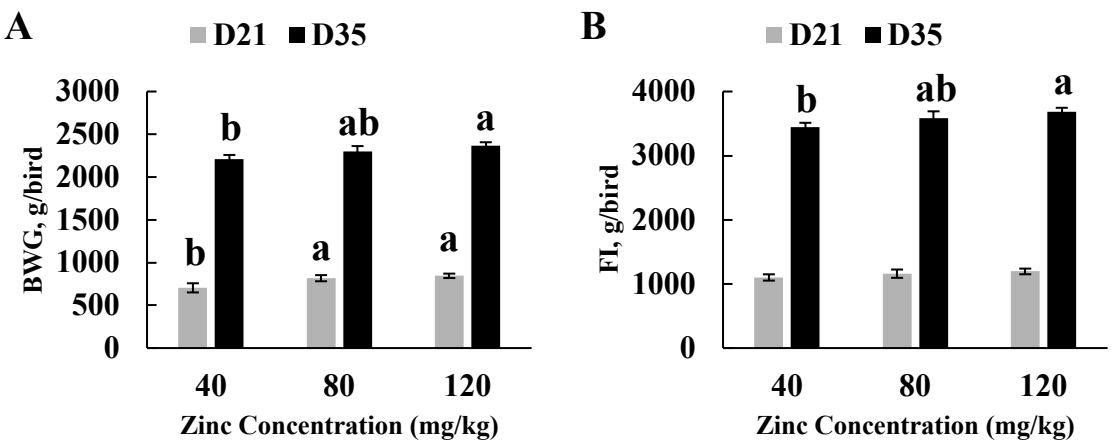

Figure 3.1. (A) The main effect of supplemental zinc concentration on body weight gain (BWG, g/bird) and **(B)** the main effect of supplemental zinc concentration on feed intake (FI, g/bird) on day 21 (grey bars) and 35 (black bars). Each bar represents the mean ± SE of 6 replicates of birds in floor pens. [a,b]Values with different letters represent significant differences of means separated by ANOVA followed by post-hoc Tukey's HSD. Results on day 21 and 35 were analyzed separately. The BWG was higher (C; $^{**}P < 0.01$) on day 21 in the 80 and 120 mg/kg treatments compared to the 40 mg/kg treatment. The BWG was higher (C; $^{*}P < 0.05$) on day 35 in the 120 mg/kg treatment compared to the 40 mg/kg treatment. The FI was higher (C; $^{*}P < 0.05$) in the 120 mg/kg treatment compared to the 40 mg/kg treatment.

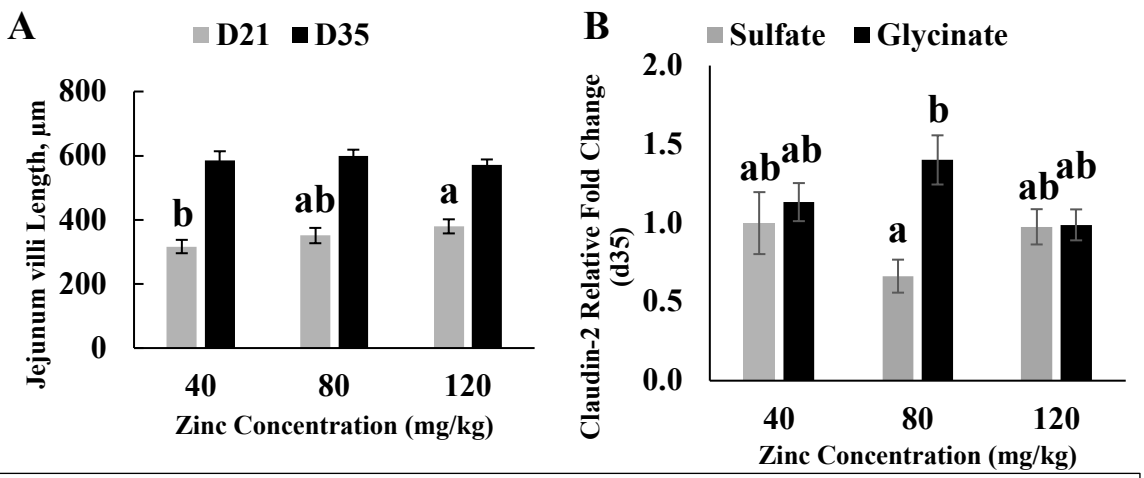

Figure 3.2. (A) The main effect of supplemental zinc concentration on jejunum villi length on day 21 (grey bars) and 35 (black bar) and **(B)** the interactions between zinc source and concentration (S x C) on claudin-2 relative mRNA expressions on day 35. Each bar represents the mean ± SE of 6 replicates of birds in floor pens. Jejunum villi length, expressed as µm, on day 21 (grey bars) and 35 (black bars) were analyzed separately. Claudin-2 mRNA expression in the jejunum expressed as fold-change normalized to beta-actin as housekeeping and normalized to the zinc sulfate at 40 mg/kg treatment. [a,b]Values with different letters represent significant differences of means separated by ANOVA followed by post-hoc Tukey's HSD. The jejunum villi length measurement was greater (C; $*P < 0.05$) on day 21 in the 120 mg/kg treatment compared to the 40 mg/kg treatment. The expression of claudin-2 in the jejunum on day 35 was greater (S x C; $*P < 0.05$) in the zinc glycinate treatment at 80 mg/kg compared to the zinc sulfate treatment at 80 mg/kg.

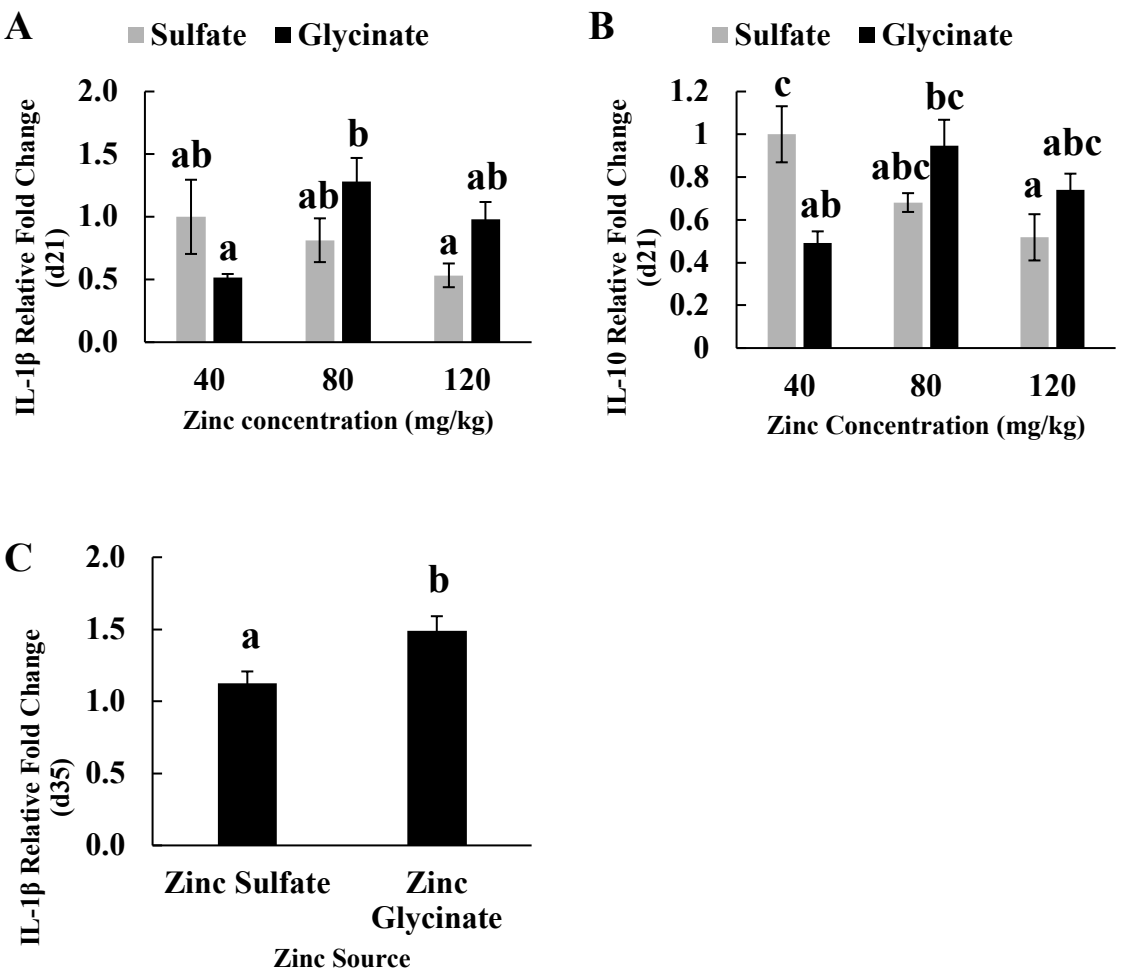

Figure 3.3. (A) The interactions between zinc source and supplemental concentration (S x C) on IL-1β relative mRNA expressions in the cecal tonsils on day 21, **(B)** the interactions between zinc source and concentration (S x C) on IL-10 relative mRNA expressions in the cecal tonsils on day 21, and **(C)** the main effect of zinc source on IL-1β relative mRNA expressions in the cecal tonsils on day 35. Each bar represents the mean ± SE of 6 replicates of birds in floor pens. The mRNA expression is expressed as fold-change normalized to beta-actin as housekeeping and normalized to the zinc sulfate at 40 mg/kg treatment. [a,b,c]Values with different letters represent significant differences of means separated by ANOVA followed by post-hoc Tukey's HSD. The expression of IL-1β in the cecal tonsils on day 21 was greater (S x C; $^{**}P < 0.01$) in the zinc glycinate treatment at 80 mg/kg compared to the zinc glycinate treatments at 40 mg/kg and zinc sulfate treatment at 120 mg/kg. The expression of IL-10 in the cecal tonsils on day 21 was lower (S x C; ** $P < 0.01$) in the zinc glycinate treatment at 40 mg/kg compared to the zinc sulfate treatment at 40 mg/kg. The expression of IL-10 in the cecal tonsils on day 21 was lower in the zinc sulfate treatment at 120 mg/kg compared to the zinc sulfate treatment at 40 mg/kg. The expression of IL-1β in the cecal tonsils on day 35 was greater ($^{*}P < 0.05$) in the zinc glycinate treatment compared to the zinc sulfate treatment.

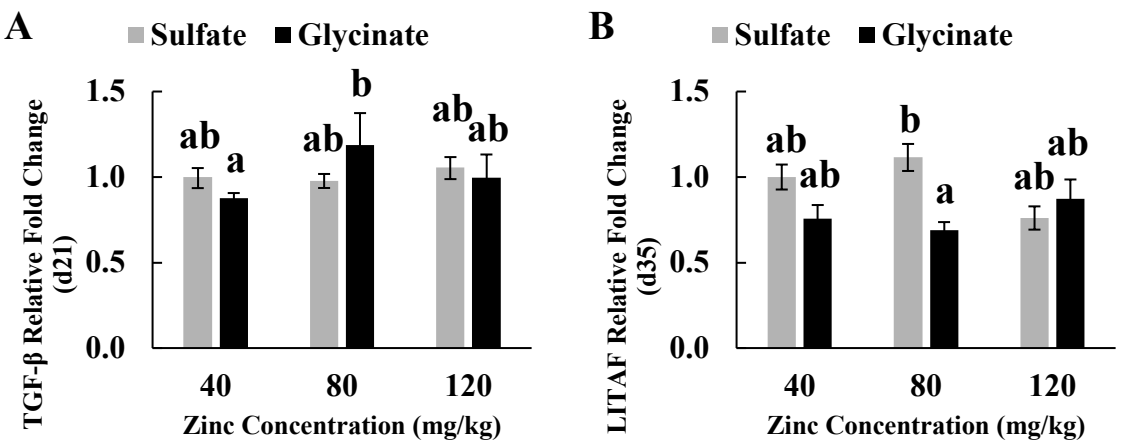

Figure 3.4. (A) The interactions between zinc source and supplemental concentration (S x C) on TGF-β relative mRNA expression in the spleen on day 21 and **(B)** the interactions between zinc source and concentration (S x C) on LITAF relative mRNA expressions in the spleen on day 35. Each bar represents the mean ± SE of 6 replicates of birds in floor pens. The mRNA expression is expressed as fold-change normalized to beta-actin as housekeeping and normalized to the zinc sulfate at 40 mg/kg treatment. [a,b]Values with different letters represent significant differences of means separated by ANOVA followed by post-hoc Tukey's HSD. The expression of TGF-β in the spleen on day 21 was greater (S x C; $*P < 0.05$) in the zinc glycinate treatment at 80 mg/kg compared to the zinc glycinate treatment at 40 mg/kg. The expression of LITAF in the spleen on day 35 was lower (S x C; $*P < 0.05$) in the zinc glycinate treatment at 80 mg/kg compared to the zinc sulfate treatment at 80 mg/kg.

CHAPTER 4

THE EFFECTS OF ZINC GLYCINATE IS NOT MEDIATED THROUGH THE ADAPTIVE IMMUNITY IN NECROTIC ENTERITIS IN BROILER CHICKENS[1, 2, 3]

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ABSTRACT

We conducted two experiments to determine the effect of an amino acid chelate, zinc glycinate (ZG), during necrotic enteritis (NE) challenge in broiler chickens. Dietary zinc supplementation has the potential to modulate immunity and alleviate necrotic enteritis, but the optimal concentration of zinc in the diets was unclear. ZG was supplemented at 40, 80, and 120 mg/kg in experiment 1 and 100, 120, 140, and 160 mg/kg in experiment 2 (n = 6). Infected birds supplemented with dietary ZG at 120 mg/kg had a higher body weight gain (BWG) of 303 g/bird compared to infected birds supplemented at 100 mg/kg (d28, $*P < 0.05$). Infected birds supplemented with ZG at 140 mg/kg had a lower feed conversion ratio (FCR) of 0.81 compared to infected birds supplemented at 100 mg/kg (d28, $*P < 0.05$). Infected birds supplemented with ZG at 40 mg/kg had higher lesion scores compared to non-challenged birds supplemented at 40 mg/kg of dietary ZG ($**P < 0.01$). Infected birds supplemented with ZG at 120 mg/kg had lower lesion scores compared to infected birds supplemented at 40 and 80 mg/kg of dietary ZG ($**P < 0.01$). Infected birds supplemented with ZG at 40 mg/kg had 8% higher mortality compared to non-challenged birds supplemented at 40 mg/kg ($*P < 0.05$). Infected birds supplemented with ZG at 80 mg/kg of dietary ZG had 8% lower mortality compared to infected birds supplemented at 40 and 120 mg/kg ($*P < 0.05$). Infected birds supplemented with ZG above 120 mg/kg had reduced lesion scores compared to infected birds supplemented at 100 mg/kg ($**P < 0.01$). Infected birds supplemented with ZG at 100 mg/kg had 27.1% higher mortality compared to non-challenged birds supplemented at 100 mg/kg ($**P < 0.01$). Infected birds supplemented with ZG at 120 mg/kg had 12.5% lower mortality compared to infected birds supplemented at 100 mg/kg ($*P < 0.05$). Cytokine expressions (IL-1β, IFN-γ, LITAF, TGF-β, and IL-10) in the cecal tonsils and spleen were not affected by ZG supplementation in NE, except that infected birds supplemented with ZG

at 160 mg/kg had a higher IFN-γ in the spleen by 1.4-fold compared to infected birds supplemented at 100 mg/kg (**$P < 0.01$). Zinc transporter (ZIP-9) and tight junction protein (Claudin-2 and zonula occludens) expression in the jejunum were not affected by ZG supplementation in NE. Anti-*Clostridium perfringens* bile IgG and serum IgA were not affected by ZG supplementation between 40 and 120 mg/kg. CD4+ T cells population in the spleen was not affected by ZG supplementation between 100 and 160 mg/kg. Infected birds supplementation at 140 mg/kg had a lower Zn/Cu SOD-1 mRNA relative expression in the cecal tonsils by 1.3-fold compared to infected birds supplemented at 120 mg/kg (*$P < 0.05$). Although growth performance was improved, and mortality and lesions were reduced by ZG supplementation, these data indicate that ZG does not alleviate NE through $CD4^+$ T helper cells or antibody response. In addition, the antioxidant enzyme, Cu/Zn-SOD-1, was involved in the alleviation of NE severity by zinc glycinate.

Keywords: Infectious disease, immunology, necrotic enteritis, nutrition, poultry, zinc

List of Abbreviations: BWG, bodyweight gain; Cq, quantification cycle; FCR, feed conversion ratio; FI, feed intake; IL, interleukins; IFN-γ, interferon-gamma; LITAF, lipopolysaccharide-induced tumor necrosis factor-alpha factor; TGF-β, transforming growth factor-beta; ZIP-9, Zrt- and Irt-like protein-9.

INTRODUCTION

Necrotic enteritis costs over US$6 billion in chicken production in the United States (Van der Sluis, 2000b; Hofacre, 2001; Wade and Keyburn, 2015b). The causative agent of necrotic enteritis is primarily the bacteria, *Clostridium perfringens* (Wilson et al., 2018), with predisposing factors such as *Eimeria* infection (Al-Sheikhly and Al-Saieg, 1980). One of the strategies to mitigate the infection is to improve mineral status to support the immunity of the birds (Lee et al., 2014a).

Zinc is an essential micronutrient for growth (O'Dell et al., 1958a) and cell-mediated immunity (Chandra and Au, 1980) in chickens. Organic sources, like zinc glycinate, increase the solubility (Low et al., 1959), bioavailability (Cao et al., 2000), digestibility (Bao et al., 2009; Yenice et al., 2015), and tissue retention (Cao et al., 2002) of zinc. Zinc from organic sources improves body weight gain (BWG), feed conversion ratio (FCR), and modulates IL-8 and IL-10 cytokine expressions in cellular immunity during infections over inorganic sources (Bortoluzzi et al., 2019a). However, the optimal concentration of zinc glycinate in broiler chicken diets against necrotic enteritis is unclear.

The objective of this study was to determine the optimal inclusion concentration of zinc glycinate in broiler chicken diets to alleviate necrotic enteritis and determine the mechanism in immunity in chickens. We supplemented zinc glycinate between 40 mg/kg and 160 mg/kg in the diets, and experimentally-induced necrotic enteritis *Eimeria* and *C. perfringens* (Hofacre et al., 2003). We hypothesize that zinc glycinate above the current recommendation of zinc supplementation at 100 mg/kg (Cobb-Vantress, 2018) reduces gross lesions from necrotic enteritis and improves growth performance after the infection by modulating cellular and humoral immunity.

MATERIALS AND METHODS

Animals, Diets, and Experimental Necrotic Enteritis Induction

Two experiments were conducted at the Southern Poultry Research Inc. (Athens, GA, USA) to investigate the effects of zinc glycinate concentrations on immunity. A priori power analysis utilizing the effect size from a similar study of selenium glycinate on immune status in chickens (Lee et al., 2014a) aided determining the sample size required to achieve 97% power (G*Power ver. 3.0.10) (Erdfelder et al., 1996). Birds were housed under standard animal husbandry practices in Petersime battery cages, and water was provided *ad libitum*. The institutional animal care and use committee (IACUC—Southern Poultry Research, Athens, GA, USA) approved our experimental protocols.

In experiment 1, day-old Cobb 500 off-sex male by-product broiler chicks (Cobb-Vantress, Cleveland, GA) were randomly assigned to the zinc glycinate treatments. Each treatment was replicated in six cages with eight birds per cage (n = 6). The birds were supplemented with zinc from zinc glycinate at 40, 80, and 120 mg/kg in experiment 1 and 100, 120, 140, and 160 mg/kg in experiment 2. The experimental diets were prepared from a basal unmedicated starter diet consisted of a zinc-free mineral mix. The diets with the lowest concentration of zinc were mixed first to ensure a progressive increase in zinc in subsequent mixes. The feed mixer was flushed using the zinc-free basal diet between mixes to prevent cross-contamination. The composition of the diet except for zinc was based on Cobb 500 Performance and Nutrition Supplement Guide (Cobb-Vantress, 2018) (Supplemental Table 4.1). The test diets were iso-caloric and iso-nitrogenous (Supplemental Table 4.2).

In both experiments, necrotic enteritis was induced by oral gavage of 5,000 oocysts/ml of Eimeria maxima on day 14, and 10^8 CFU/ml of *Clostridium perfringens* daily on days 19, 20, and 21

(Hofacre et al., 2003; Miller et al., 2010). Separately, birds (n = 6) that were supplemented with 40 mg/kg in experiment 1 and 100 mg/kg in experiment 2 were uninfected as controls. On day 21, birds were euthanized for analyses. Cecal tonsils, spleen, and jejunal tissues were snap -frozen in liquid nitrogen for relative mRNA gene expressions in both experiments. Jejunual tissues were collected for histology analysis in experiment 1. Bile and serum were collected for anti-*C. perfringens* IgA and IgG, respectively in experiment 1. Spleen tissues were collected for flow cytometry of $CD4^+$ T helper cells in experiment 2. Cecal content was collected for 16s *C. perfringens*, CPA, and netB analysis by real-time qPCR. Gross lesions (0: lowest to 3: highest) were evaluated on day 21 by a pathologist that was blinded to treatments (Hofacre et al., 2019). On day 28, the remaining birds were analyzed for recovery phase growth performance.

Effect of Zinc Glycinate Supplementation on Jejunum Histological Parameters

In experiment 1, the jejunum tissue in 10% neutral buffered formalin was processed in the Poultry Diagnostic and Research Center (PDRC, Athens, GA) laboratory. The cross-sections were viewed and photographed using an Olympus IX71 microscope and analyzed using DP Controller ver. 2.1.1.183 software (Olympus America) to determine the villi length and crypt depth. Five villi per section and four sections per sample were analyzed. For each jejunum sample, villi and crypt of four sections of the slides were measured and averaged as technical replicates. Villi length to crypt depth ratios was calculated.

Effect of Zinc Glycinate Supplementation on Anti-*C. Perfringens* Antibody Response

In experiment 1, bile and plasma were analyzed for *Clostridium perfringens*-specific IgA and IgG, respectively, using a modified enzyme-linked immunosorbent assay (ELISA) (Markazi et al., 2018; Bolek and Klasing, 2019). Briefly, *C. perfringens* pure culture was lysed using 600 μm glass beads (Sigma, St. Louis, MO) in a TissueLyser LT (Qiagen Hilder, Germany) for 5 minutes at 50

1/s frequency with repeated freeze/thaw cycles. The concentration of the antigen was estimated by spectrophotometry. High binding flat-bottom 96-well plate (Greiner Bio-one, NC, USA) was coated using the lysed antigen (100 µl of 10 µg/ml in 0.1M carbonate buffer) and incubated overnight at 4°C. The plates were washed using 0.05% Tween 20 in PBS (200 µl). The wells were blocked using 2.5% nonfat dry milk in 0.05% Tween 20 in PBS (100 µl) for one hour at room temperature to prevent non-specific binding. After washing, bile (1:10) or serum (1:200) diluted in blocking buffer (100 µl) was incubated for one hour at room temperature. After washing, HRP-labeled anti-chicken IgA (1:1000,000 v/v in blocking buffer) or IgG (1:10,000 v/v in blocking buffer) were added (100 µl). After washing, peroxidase activity was activated using a TMB substrate solution (3M sodium acetate, TMB, hydrogen peroxide) for 10 minutes. Sulfuric acid (2M, 100 µl) was added to stop the reaction. Optical density (OD) at 490 nm was measured. The measurements were normalized from the blanks. Duplicates were performed for each sample.

Effect of Zinc Glycinate Supplementation on Cytokine Relative mRNA Expression

Total RNA from cecal tonsil, spleen, and jejunum tissues were isolated using the TRIzol/chloroform method. Briefly, tissues (25 mg) were homogenized using the TissueLyser LT (Qiagen, MD, USA) and 5 mm stainless steel beads in TRIzol for 6 min. Chloroform (200 µl) was added to the homogenate and centrifuged to fractionate the total RNA in the aqueous phase. The total RNA was precipitated in isopropanol, washed in 70% ethanol, and then resuspended in molecular-grade water. Optical density at 260 nm was used to determine RNA concentrations. RNA samples were reverse transcribed. Two micrograms of RNA were converted to cDNA in a 20 µL reaction volume containing 1X reaction buffer (50 mM Tris–HCl [pH 8.3], 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT), 10 mM DTT, 0.5 mM dNTPs, 0.5 µg of oligo(dT)15 primer, 8 units of

RNAsin and 100 units of M-MLV reverse transcriptase (all from Promega) at 40°C for 1 h, and then 95°C for 10 min.

In experiments 1 and 2, relative gene expressions of pro-inflammatory cytokines (IL-1β, IFN-γ, and LITAF), anti-inflammatory cytokines (IL-10 and TGF-β) of mRNA from cecal tonsil and spleen tissues were analyzed using the CFX-96 and CFX Maestro (Bio-Rad, CA, USA). Relative gene expression of jejunum tight junction proteins (Claudin-2 and occludin) and zinc importer (ZIP-9) were analyzed. In experiment 2, Zn/Cu-SOD-1 gene expressions were measured in the cecal tonsils and spleen. The expressions were normalized to β-actin as the housekeeping gene and normalized to expressions of the zinc glycinate at 40 mg/kg infected treatment in experiment 1 and zinc glycinate at 100 mg/kg infected treatment in experiment 2. Primers and cycle conditions are described in Table 4.1. Gene expressions are reported as fold-change ± SE. Real-time qPCR reagents (PerfeCTa SYBR Green FastMix, Quantabio, MA, USA). Fold-change from the reference was quantified as $2^{-\Delta\Delta Cq}$, where Cq is the threshold cycle defined as the cycle number when the RT-qPCR product fluorescence reaches exponentially above the background (Livak and Schmittgen, 2001).

Effect of Zinc Glycinate Supplementation on Cecal Bacterial by Real-Time Quantitative-PCR

In experiment 2, cecal contents were homogenized in sterile bags. DNA was extracted using a commercial kit (QIAamp DNA Stool Mini Kit, Qiagen Hilder, Germany). The *Clostridium perfringens, netB*, and *cpa* genes were quantified using primers and cycle conditions described in Table 4.2. Expressions were normalized to 16s rRNA as the housekeeping gene and normalized to the zinc glycinate at 100 mg/kg infected treatment. The relative fold-change was calculated as described above.

Effect of Zinc Glycinate Supplementation on $CD4^+$ T cells Population

Spleen tissues were homogenized into single-cell suspensions in RPMI using 70 µm cell strainers. Lymphocytes were separated using Histopaque-1077. Cells were plated at a density of 10^6 cells/mL and then stained for $CD4^+$ T helper cells using CD4 FITC-conjugated anti-CD4 (1:300 v/v in buffer) (Shanmugasundaram and Selvaraj, 2011; Oxford and Selvaraj, 2019). After staining, the cells were washed and fixed using 0.5% paraformaldehyde in PBS and then washed before analysis. Flow cytometry data were acquired by CytoFLEX (Beckman Coulter, Inc., Brea, CA), and data were analyzed with FlowJo v9 (Treestar, Inc., San Carlos, CA).

Statistical Analysis

Growth performance (BWG, FI, and FCR), gene expression, and flow cytometry data were analyzed using one-way ANOVA using SAS v.9.0 (SAS Institute, Inc., NC, USA) to determine the effects of zinc glycinate concentrations on the dependent variables. When the main effect was significant ($P < 0.05$), the differences between means were analyzed using Tukey's HSD post-hoc analysis. Intestinal lesion scores were analyzed by Kruskal-Wallace non-parametric test with post-hoc Tukey's HSD. Mortality was analyzed by the Chi-square test with the post-hoc Mann Whitney U test.

RESULTS

Effect of Zinc Glycinate Supplementation on BWG, FI, and FCR (Table 4.3)

On day 21 in experiment 1, infected birds supplemented with ZG at 40 mg/kg had a higher feed conversion ratio (FCR) of 0.33 compared to non-challenged birds supplemented at 40 mg/kg (*$P < 0.05$). On day 28 in experiment 1, infected birds supplemented with ZG at 40 mg/kg had a higher FCR of 0.40 compared to non-challenged birds supplemented at 40 mg/kg (*$P < 0.05$). On day 21

and 28 in experiment 1, there were no significant effects of zinc glycinate on body weight gain (BWG) and feed intake (FI) between different treatment groups.

On day 21 in experiment 2, infected birds supplemented with ZG at 100 mg/kg had a lower BWG of 130 g/bird compared to non-challenged birds supplemented at 100 mg/kg (*$P < 0.05$). On day 28 in experiment 2, infected birds supplemented with ZG at 100 mg/kg had a lower in BWG of 302 g/bird compared to non-challenged birds supplemented at 100 mg/kg (*$P < 0.05$). On day 28 in experiment 2, infected birds supplemented with ZG at 120 mg/kg had a higher BWG of 303 g/bird compared to infected birds supplemented at 100 mg/kg (*$P < 0.05$). On day 28 in experiment 2, infected birds supplemented with ZG at 100 mg/ had a higher in FCR of 0.95 compared to non-challenged birds supplemented at 100 mg/kg (*$P < 0.05$). On day 28 in experiment 2, infected birds supplemented with ZG at 140 mg/kg had a lower in FCR of 0.81 compared to infected birds supplemented at 100 mg/kg (*$P < 0.05$). On day 21 in experiment 2, there were no significant effects of zinc glycinate on FI and FCR between different treatment groups. On day 28 in experiment 2, there was no significant effect of zinc glycinate on FI between different treatment groups.

Effect of Zinc Glycinate Supplementation on Gross Lesion and Mortality (Table 4.3)

On day 21 in experiment 1, infected birds supplemented with ZG at 40 mg/kg had higher lesion scores compared to non-challenged birds supplemented at 40 mg/kg (**$P < 0.01$). On day 21 in experiment 1, infected birds supplemented with ZG at 120 mg/kg had reduced lesion scores compared to infected birds supplemented at 40 and 80 mg/kg (**$P < 0.01$). On day 21 in experiment 1, infected birds supplemented with ZG at 40 mg/kg had 8% higher mortality compared to non-challenged birds supplemented at 40 mg/kg (*$P < 0.05$). On day 21 in experiment 1, infected birds supplemented with ZG at 80 mg/kg of dietary zinc glycinate had 8% lower mortality

compared to infected birds supplemented at 40 and 120 mg/kg of dietary zinc glycinate (*P < 0.05).

On day 21 in experiment 2, infected birds supplemented with ZG at 100 mg/kg had higher lesion scores compared to non-challenged birds supplemented at 100 mg/kg of dietary zinc glycinate (**P < 0.01). On day 21 in experiment 2, infected birds supplemented with ZG above 120 mg/kg had reduced lesion scores compared to infected birds supplemented at 100 mg/kg (**P < 0.01). On day 21 in experiment 2, infected birds supplemented with ZG at 100 mg/kg had 27.1% higher mortality compared to non-challenged birds supplemented at 100 mg/kg (**P < 0.01). On day 21 in experiment 2, infected birds supplemented with ZG at 120 mg/kg had 12.5% lower mortality compared to infected birds supplemented at 100 mg/kg (*P < 0.05).

Effect of Zinc Glycinate Supplementation on Jejunum Histological Parameters (Table 4.4)

On day 21 in experiment 1, infected birds supplemented with ZG at 40 mg/kg had lower villi to crypt ratio of 1.2 compared to non-challenged birds supplemented at 40 mg/kg (*P < 0.05). On day 21 in experiment 1, there were no significant effects of zinc glycinate on villi length and crypt depth between different treatment groups.

Effect of Zinc Glycinate Supplementation on Anti-*C. Perfringens* Antibody Response

On day 21 in experiment 1, there were no significant effects of zinc glycinate on anti-*C. perfringens* IgA and bile anti- *C. perfringens* IgG between different treatment groups.

Effect of Zinc Glycinate Supplementation on Cytokine Relative mRNA Expression

On day 21 in experiment 1, infected birds supplemented with ZG at 40 mg/kg had a higher IFN-γ mRNA relative expression in the cecal tonsils by 1.7-fold compared to non-challenged birds supplemented at 40 mg/kg (*P < 0.05) (Figure 4.1). On day 21 in experiment 1, infected birds

supplemented with ZG at 40 mg/kg had a higher LITAF mRNA relative expression in the cecal tonsils by 1.6-fold compared to non-challenged birds supplemented at 40 mg/kg (*$P < 0.05$). On day 21 in experiment 1, infected birds supplemented with ZG at 40 mg/kg had a lower IL-10 mRNA relative expression in the spleen by 2-fold compared to non-challenged birds supplemented at 40 mg/kg (*$P < 0.05$). On day 21 in experiment 1, infected birds supplemented with ZG at 40 mg/kg had a lower Claudin-2 mRNA relative expression in the jejunum by 1.7-fold compared to non-challenged birds supplemented at 40 mg/kg (**$P < 0.01$). On day 21 in experiment 1, infected birds supplemented with ZG at 40 mg/kg had a lower ZIP-9 mRNA relative expression in the jejunum by 1.4-fold compared to non-challenged birds supplemented at 40 mg/kg of dietary zinc glycinate (*$P < 0.05$).

On day 21 in experiment 2, infected birds supplemented with ZG at 140 mg/kg had a lower Zn/Cu SOD-1 mRNA relative expression in the cecal tonsils by 1.3-fold compared to infected birds supplemented at 120 mg/kg (*$P < 0.05$) (Figure 4.2). On day 21 in experiment 2, infected birds supplemented with ZG at 100 mg/kg of dietary zinc glycinate had a higher LITAF mRNA relative expression in the spleen by 1.7-fold compared to non-challenged birds supplemented at 100 mg/kg (**$P < 0.01$).

Effect of Zinc Glycinate Supplementation on Cecal Bacterial by Real-Time Quantitative-PCR

On day 21 in experiment 2, *C. perfringens, netB, and cpa* expressions in the ceca were not statistically different between treatments (Figure 4.3). However, we observed that *C. perfringens* and *cpa* expressions in the ceca were inversely correlated as zinc glycinate concentration increased from 100 to 160 mg/kg in infected birds.

Effect of Zinc Glycinate Supplementation on CD4+ T cells Population

On day 21 in experiment 2, there was no significant effect of zinc glycinate on $CD4^+$ T helper cells population between different treatment groups.

DISCUSSION

Zinc is an essential micronutrient that is used as a cofactor in biological processes (O'Dell et al., 1958a). The antioxidant enzyme, copper/zinc superoxide dismutase, utilizes zinc as a cofactor against oxidative stress (Tainer et al., 1983). Zinc is the structural cofactor in the zinc-finger binding domains in transcription factors such as RANTES (Song et al., 1999), Th-POK (He et al., 2005), Egr-2 (Harris et al., 2004) that involves in T cells activation. Zinc glycinate has been used against pathogens in chickens such as coccidia (Southern and Baker, 1983a), necrotic enteritis (Baba et al., 1992b), *Salmonella enteritidis* (Barbour et al., 2000; Zhang et al., 2012), and *Campylobacter jejuni* (Xie et al., 2011). This experiment studied the effects of zinc from zinc glycinate, an organic source, on necrotic enteritis in broiler chickens. Necrotic enteritis is primarily caused by uncontrolled *Clostridium perfringens* proliferation in the intestine (Long and Truscott, 1976; Al-Sheikhly and Truscott, 1977a). *Clostridial* toxins, CPA and netB, are proteases that damage the mucosal layer of the intestine, which results in necrosis of the intestine (Lee et al., 2012). A previous study demonstrated that zinc from an organic source, zinc proteinate, reduces necrotic enteritis lesions, and modulated IL-8 and IFN-γ cytokine gene expressions (Bortoluzzi et al., 2019b).

Although previous studies have shown the potential of organic zinc in enteric challenges, those studies have not been able to determine the optimal concentration of organic zinc in broiler chicken diets. In the present study, zinc supplemented at 120 mg/kg from zinc glycinate reduced gross lesion in both experiments, and zinc glycinate reduced mortality by 13 percent in experiment 2.

After the infection on day 21, zinc glycinate supplemented at 120 mg/kg tended to result in lower FCR by 0.04 in experiment 1, and BWG was 66 grams higher in experiment 2. After the recovery phase from the infection on day 28 in experiment 2, the BWG was regained by 303 grams, and the BWG was returned to the level similar to the uninfected birds by zinc glycinate supplemented at 120 mg/kg. Furthermore, zinc glycinate at supplemented 140 mg/kg had lower FCR by 0.83 after the recovery phrase. These findings demonstrated that zinc glycinate supplemented at 120 mg/kg is beneficial in alleviating necrotic enteritis in broiler chickens, and growth performance can be regained.

In a previous study, subtle differences in cytokine expressions were observed in birds supplemented with organic zinc (Bortoluzzi et al., 2019b). In the current study, we analyzed a more comprehensive list of cytokines. Necrotic enteritis resulted in changes in cytokine expressions compared to non-challenged birds. However, to our surprise, we did not observe any differences cytokine expressions in infected birds in both experiments, except IFN-γ in the spleen in experiment 2. According to conventional believes, the expression of IFN-γ should be lower rather than higher if the infection severity were lowered by zinc. Also, zinc glycinate supplemented at the concentrations between 40 and 120 mg/kg did not affect anti-*Clostridium perfringens* bile IgA and serum IgG concentrations; and concentrations between 100 and 160 mg/kg did not affect $CD4^+$ T cells population after the infection. Although cytokine responses have been demonstrated in *C. perfringens* infection (Park et al., 2008) and zinc has also been implicated in T cell response in adaptive immunity (Joshua et al., 2016) in chickens, zinc glycinate did not affect cytokines, T cell response, and antibody response in this study. Taken together, the reduction in necrotic enteritis severity in this study appears to be independent of adaptive immunity.

Aside from adaptive immunity parameters, the zinc/copper superoxide dismutase gene expression in the cecal tonsils was reduced by zinc glycinate supplemented at 140 mg/kg after the infection. Superoxide dismutase reduces oxidative stress in chickens macrophages (Gou et al., 2015). Micronutrients such as manganese have been shown to modulate superoxide dismutase in chickens (Li et al., 2011). Therefore, zinc glycinate may have more significant effects on innate immunity in necrotic enteritis.

The jejunum brush border is the primary site for zinc absorption (Tako et al., 2005). Despite the reduction of gross lesions by zinc glycinate supplemented at 120 mg/kg, jejunal villi morphologies and gene expressions of tight junction protein (claudin-2 and zonula occludens), were not affected by zinc glycinate in the infection. Zn/Fe-regulated transporter (ZRT/IRT-like or ZIP) transport zinc into the cytosol, and two have been identified in chickens (Troche et al., 2015). ZIP-9 transporter is involved in zinc transport in the Trans-Golgi network in all tissues (Matsuura et al., 2009; Thomas et al., 2018) and TGF-β signaling (Troche et al., 2015). However, jejunal ZIP-9 expression was not affected by zinc glycinate in this study. Therefore, cellular transporter of zinc into the jejunal cells may involve other zinc transporters in chickens.

CPA and netB toxins released from *C. perfringens* damage the intestinal epithelium (Sakurai, 1995b). Although netB is regarded as the primary toxin in necrotic enteritis (Keyburn et al., 2008), the CPA toxin is unique because it is a zinc-containing phospholipase-C enzyme (Si et al., 2007). In this study, the cecal *C. perfringens* 16s rRNA expression tended to be higher as zinc glycinate concentration in the diets was increased. Although the CPA toxin requires zinc, *C. perfringens* may not be able to utilize zinc glycinate to produce the toxin; as zinc glycinate concentration in the diets was increased, the *cpa* expression was lower. Future studies will be conducted to elucidate the relationship between zinc source and *C. perfringens* toxin production.

In conclusion, appropriate zinc glycinate concentrations are essential for alleviating necrotic enteritis in chickens evaluated by the improvement in growth performance, the reduction in NE gross lesions in the intestine, and reduction of mortality. The decrease in Zn/Cu SOD-1 expression in the cecal tonsils by zinc glycinate supplemented at 140 mg/kg and the lack of responses of zinc glycinate on cytokine expressions, antibody responses, and $CD4^+$ T cells population suggest that the effects of zinc glycinate in necrotic enteritis are mediated through immune mechanisms independent of cytokine responses, humoral immunity, and $CD4^+$ T cells.

DISCLOSURES

Pancosma SA (Geneva, CH) donated Zinc glycinate but was not involved in the design or conduct of the research.

LITERATURE CITED

Al-Sheikhly, F., and A. Al-Saieg. 1980. Role of coccidia in the occurrence of necrotic enteritis of chickens. Avian Diseases:324-333.

Al-Sheikhly, F., and R. Truscott. 1977. The interaction of Clostridium perfringens and its toxins in the production of necrotic enteritis of chickens. Avian Diseases:256-263.

Baba, E., A. L. Fuller, J. M. Gilbert, S. G. Thayer, and L. R. McDougald. 1992. Effects of Eimeria brunetti infection and dietary zinc on experimental induction of necrotic enteritis in broiler chickens. Avian diseases:59-62.

Bao, Y., M. Choct, P. Iji, and K. Bruerton. 2009. Optimal dietary inclusion of organically complexed zinc for broiler chickens. British Journal of Poultry Science 50(1):95-102.

Barbour, E. K., N. E. Bejjani, N. J. Daghir, O. M. Faroon, M. Bouljihad, and R. Spasojevic. 2000. Induction of Early Immunopotentiation to Fimbriae of Salmonella Enteritidis (SE) by Administering Thymulin and Zinc to SE-Vaccinated Chicken Breeders. Journal of Veterinary Medical Science 62(11):1139-1143.

Bolek, K., and K. Klasing. 2019. The effects of vaccination with keyhole limpet hemocyanin or oral administration of Salmonella enterica serovar Enteritidis on the growth performance of immunoglobulin knockout chickens. Poultry Science

Bortoluzzi, C., B. Lumpkins, G. Mathis, M. França, W. King, D. Graugnard, K. Dawson, and T. Applegate. 2019a. Zinc source modulates intestinal inflammation and intestinal integrity of broiler chickens challenged with coccidia and Clostridium perfringens. Poultry science 98(5):2211-2219.

Bortoluzzi, C., B. Vieira, B. Lumpkins, G. Mathis, W. King, D. Graugnard, K. Dawson, and T. Applegate. 2019b. Can dietary zinc diminish the impact of necrotic enteritis on growth performance of broiler chickens by modulating the intestinal immune-system and microbiota? Poultry Science

Cao, J., P. Henry, S. Davis, R. Cousins, R. Miles, R. Littell, and C. Ammerman. 2002. Relative bioavailability of organic zinc sources based on tissue zinc and metallothionein in chicks fed conventional dietary zinc concentrations. Animal Feed Science and Technology 101(1-4):161-170.

Cao, J., P. Henry, R. Guo, R. Holwerda, J. Toth, R. Littell, R. Miles, and C. Ammerman. 2000. Chemical characteristics and relative bioavailability of supplemental organic zinc sources for poultry and ruminants. Journal of Animal Science 78(8):2039-2054.

Chandra, R., and B. Au. 1980. Single nutrient deficiency and cell-mediated immune responses I. Zinc. American Journal of Clinical Nutrition 33(4):736-738.

Cobb-Vantress. 2018. Broiler Performance and Nutrition Supplement Cobb 500.

Erdfelder, E., F. Faul, and A. Buchner. 1996. GPOWER: A general power analysis program. Behavior research methods, instruments, & computers 28(1):1-11.

Gou, Z., S. Jiang, C. Zheng, Z. Tian, and X. Lin. 2015. Equol inhibits LPS-induced oxidative stress and enhances the immune response in chicken HD11 macrophages. Cellular Physiology and Biochemistry 36(2):611-621.

Harris, J. E., K. D. Bishop, N. E. Phillips, J. P. Mordes, D. L. Greiner, A. A. Rossini, and M. P. Czech. 2004. Early growth response gene-2, a zinc-finger transcription factor, is required for full induction of clonal anergy in CD4+ T cells. The Journal of Immunology 173(12):7331-7338.

He, X., X. He, V. P. Dave, Y. Zhang, X. Hua, E. Nicolas, W. Xu, B. A. Roe, and D. J. Kappes. 2005. The zinc finger transcription factor Th-POK regulates CD4 versus CD8 T-cell lineage commitment. Nature 433(7028):826-833.

Hofacre, C., T. Beacorn, S. Collett, and G. Mathis. 2003. Using competitive exclusion, mannan-oligosaccharide and other intestinal products to control necrotic enteritis. Journal of Applied Poultry Research 12(1):60-64.

Hofacre, C. L. 2001. Necrotic enteritis, currently a billion dollar disease: is there anything new on the horizon. In: Proceedings of Alltech's 17th Annual Symposium Science and Technology in the Feed Industry. p 79-86.

Hofacre, C. L., D. J. Reynolds, G. F. Mathis, B. S. Lumpkins, N. Ollis, J. A. Smith, and V. Demey. 2019. Effect of a Competitive Exclusion Culture in a Necrotic Enteritis Challenge Model in Broilers. The Journal of Applied Poultry Research

Joshua, P. P., C. Valli, and V. Balakrishnan. 2016. Effect of in ovo supplementation of nano forms of zinc, copper, and selenium on post-hatch performance of broiler chicken. Veterinary world 9(3):287.

Keyburn, A. L., J. D. Boyce, P. Vaz, T. L. Bannam, M. E. Ford, D. Parker, A. Di Rubbo, J. I. Rood, and R. J. Moore. 2008. NetB, a new toxin that is associated with avian necrotic enteritis caused by Clostridium perfringens. PLoS Pathogens 4(2):e26.

Lee, K., H. Lillehoj, M. Park, S. Jang, G. Ritter, Y. Hong, W. Jeong, H. Jeoung, D. An, and E. Lillehoj. 2012. Clostridium perfringens α-toxin and NetB toxin antibodies and their possible role in protection against necrotic enteritis and gangrenous dermatitis in broiler chickens. Avian diseases 56(1):230-233.

Lee, S., H. Lillehoj, S. Jang, M. Jeong, S. Xu, J. Kim, H. Park, H. Kim, E. Lillehoj, and D. Bravo. 2014. Effects of in ovo injection with selenium on immune and antioxidant responses during experimental necrotic enteritis in broiler chickens. Poultry science 93(5):1113-1121.

Li, S., L. Lu, S. Hao, Y. Wang, L. Zhang, S. Liu, B. Liu, K. Li, and X. Luo. 2011. Dietary manganese modulates expression of the manganese-containing superoxide dismutase gene in chickens. The Journal of Nutrition 141(2):189-194.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25(4):402-408.

Long, J. R., and R. B. Truscott. 1976. Necrotic enteritis in broiler chickens. III. Reproduction of the disease. Canadian Journal of Comparative Medicine 40(1):53-59.

Low, B. W., F. Hirshfeld, and F. Richards. 1959. Glycinate complexes of zinc and cadmium. Journal of the American Chemical Society 81(16):4412-4416.

Markazi, A., A. Luoma, R. Shanmugasundaram, M. Mohnl, G. Raj Murugesan, and R. Selvaraj. 2018. Effects of drinking water synbiotic supplementation in laying hens challenged with Salmonella. Poultry Science 97(10):3510-3518.

Matsuura, W., T. Yamazaki, Y. Yamaguchi-Iwai, S. Masuda, M. Nagao, G. K. Andrews, and T. Kambe. 2009. SLC39A9 (ZIP9) regulates zinc homeostasis in the secretory pathway: characterization of the ZIP subfamily I protein in vertebrate cells. Bioscience, biotechnology, and biochemistry 73(5):1142-1148.

Miller, R. W., E. J. Skinner, A. Sulakvelidze, G. F. Mathis, and C. L. Hofacre. 2010. Bacteriophage therapy for control of necrotic enteritis of broiler chickens experimentally infected with Clostridium perfringens. Avian Diseases 54(1):33-40. doi: 10.1637/8953-060509-Reg.1

O'Dell, B., P. Newberne, and J. Savage. 1958. Significance of dietary zinc for the growing chicken. The Journal of Nutrition 65(4):503-523.

Oxford, J. H., and R. K. Selvaraj. 2019. Effects of Glutamine Supplementation on Broiler Performance and Intestinal Immune Parameters During an Experimental Coccidiosis Infection. The Journal of Applied Poultry Research 28(4):1279-1287.

Park, S. S., H. S. Lillehoj, P. C. Allen, D. W. Park, S. FitzCoy, D. A. Bautista, and E. P. Lillehoje. 2008. Immunopathology and cytokine responses in broiler chickens coinfected with Eimeria maxima and Clostridium perfringens with the use of an animal model of necrotic enteritis. Avian Diseases 52(1):14-22. doi: 10.1637/7997-041707-Reg

Sakurai, J. 1995. Toxins of Clostridium perfringens. Review of Medical Microbiology 6:175-185.

Shanmugasundaram, R., and R. K. Selvaraj. 2011. Regulatory T cell properties of chicken CD4+ CD25+ cells. The Journal of Immunology 186(4):1997-2002.

Si, W., J. Gong, Y. Han, H. Yu, J. Brennan, H. Zhou, and S. Chen. 2007. Quantification of cell proliferation and alpha-toxin gene expression of Clostridium perfringens in the development of necrotic enteritis in broiler chickens. Appl. Environ. Microbiol. 73(21):7110-7113.

Song, A., Y.-F. Chen, K. Thamatrakoln, T. A. Storm, and A. M. Krensky. 1999. RFLAT-1: a new zinc finger transcription factor that activates RANTES gene expression in T lymphocytes. Immunity 10(1):93-103.

Southern, L., and D. Baker. 1983. Eimeria acervulina infection and the zinc-copper interrelationship in the chick. Poultry science 62(2):401-404.

Tainer, J. A., E. D. Getzoff, J. S. Richardson, and D. C. Richardson. 1983. Structure and mechanism of copper, zinc superoxide dismutase. Nature 306(5940):284.

Tako, E., P. R. Ferket, and Z. Uni. 2005. Changes in chicken intestinal zinc exporter mRNA expression and small intestinal functionality following intra-amniotic zinc-methionine administration. The Journal of nutritional biochemistry 16(6):339-346.

Thomas, P., A. Converse, and H. A. Berg. 2018. ZIP9, a novel membrane androgen receptor and zinc transporter protein. General and comparative endocrinology 257:130-136.

Troche, C., S. D. Eicher, and T. J. Applegate. 2015. The influence of dietary zinc source and coccidial vaccine exposure on intracellular zinc homeostasis and immune status in broiler chickens. British Journal of Nutrition 114(2):202-212.

Van der Sluis, W. 2000. Clostridial enteritis is an often underestimated problem. World Poultry 16(7):42-43.

Wade, B., and A. Keyburn. 2015. The true cost of necrotic enteritis. World Poult 31(7):16-17.

Wilson, K., K. Chasser, A. Duff, W. Briggs, J. Latorre, J. Barta, and L. Bielke. 2018. Comparison of multiple methods for induction of necrotic enteritis in broilers. I. The Journal of Applied Poultry Research 27(4):577-589.

Xie, Y., Y. He, P. L. Irwin, T. Jin, and X. Shi. 2011. Antibacterial activity and mechanism of action of zinc oxide nanoparticles against Campylobacter jejuni. Appl. Environ. Microbiol. 77(7):2325-2331.

Yenice, E., C. Mızrak, M. Gültekin, Z. Atik, and M. Tunca. 2015. Effects of organic and inorganic forms of manganese, zinc, copper, and chromium on bioavailability of these minerals and calcium in late-phase laying hens. Biological Trace Element Research 167(2):300-307.

Zhang, B., Y. Shao, D. Liu, P. Yin, Y. Guo, and J. Yuan. 2012. Zinc prevents Salmonella enterica serovar Typhimurium-induced loss of intestinal mucosal barrier function in broiler chickens. Avian Pathology 41(4):361-367.

FIGURES AND TABLES

Table 4.1.
Primers information for quantitative real-time PCR of gene expressions in the cecal tonsil, spleen, and jejunum tissues in experiment 1 and 2.

Gene		Primer sequence (5' – 3')	Accession #/Reference	Annealing	Extension
Housekeeping	β–actin	F: ACCGGACTGTTACCAACACC R: GACTGCTGCTGACACCTTCA	(Shanmugasundaram et al., 2013)	57.5°C, 30 s	10 s
Pro-inflammatory Cytokine	IL-1β	F: TGGGCATCAAGGGCTACA R: TCGGGTTGGTTGGTGATG	Y07922/(Hong et al., 2006)	57.5°C, 45 s	20 s
	IFN-γ	F: GTGAAGAAGGTGAAAGATATCATGGA R: GCTTTGCGCTGGATTCTCA	(Shanmugasundaram et al., 2013)		
	LITAF	F: ATCCTCACCCCTACCCTGTC R: GGCGGTCATAGAACAGCACT	(Luoma, 2016)		
Anti-inflammatory Cytokine	TGF-β	F: CGGGACGGATGAGAAGAAC R: CGGCCCACGTAGTAAATGAT	M31160/(Hong et al., 2006)		
	IL-10	F: CATGCTGCTGGGCCTGAA-3′ R: CGTCTCCTTGATCTGCTTGATG	(Shanmugasundaram et al., 2013)		
Tight Junction Protein	Claudin 2	F: CCTGCTCACCCTCATTGGAG R: GCTGAACTCACTCTTGGGCT	(Bortoluzzi et al., 2019b)	50.5°C, 45 s	20 s
	ZO-1	F: CCGTAACCCCGAGTTGGAT R: ATTGAGGCGGTCGTTGATG	(Bortoluzzi et al., 2019b)		
Zinc Importer	ZIP-9	F: CGTTCCATCTGCCTGCTGTC- R: GCACCCAGAACAGTCACCAAC	(Troche et al., 2015)	49.4°C, 45 s	20 s
Antioxidant	Zn/Cu-SOD-1	F: GGCTTGTCTGATGGAGATCAT R: GCTTGCCTTCAGGATTAAAGTG	XM_205064.1 / (Resnyk et al., 2013)	60°C, 30 s	3 s

Table 4.2.
Primers information for quantitative real-time PCR of bacterial genes in the ceca in experiment 2.

Gene		Primer sequence (5' – 3')	Accession #/Reference	Annealing	Extension
Bacteria genes	16s rRNA	F: AGAGTTTGATCCTGGCTCAG R: GACTACCAGGGTATCTAATC	(Markazi et al., 2018)	52°C, 50 s	20 s
	Clostridium perfringens	F: CGCATAACGTTGAAAGATGG R: CCTTGGTAGGCCGTTACCC	(Wise and Siragusa, 2005)		
	netB	F:CGCTTCACATAAAGGTTGGAAG GC R:TCCAGCACCAGCAGTTTTTCCT	(Bailey et al., 2013)	55°C, 45 s	
	cpa	F: TGCATGAGCTTCAATTAGGT R: TTAGTTTTGCAACCTGCTGT	(Wilson et al., 2018)		

Table 4.3.
Growth performance of birds on day 21 and 28 in experiment 1 and 2.

			Day 21					Day 28		
			BWG	FI	FCR	Lesion score	NE mortality	BWG	FI	FCR
Experiment	Zinc Glycinate Concentration	Infection	g/bird	g/bird		(0—3)	%	g/bird	g/bird	
1	40 mg/kg	Control	402	640	1.60^{b}	0.00^{a}	0.0^{a}	609	1062	1.76^{a}
	40 mg/kg	NE	342	657	1.92^{a}	1.06^{b}	8.3^{b}	469	1032	2.22^{b}
	80 mg/kg	NE	358	684	1.91^{a}	1.11^{b}	0.0^{a}	509	1104	2.20^{b}
	120 mg/kg	NE	326	603	1.87^{ab}	0.50^{c}	4.2^{ab}	502	1002	2.06^{b}
	SEM		10	16	0.05	0.26	2.0	30	21	0.11
	P value		0.08	0.41	*<0.05	**<0.01	**<0.01	0.09	0.4	*<0.05
2	100 mg/kg	Control	546^{a}	930	1.69	0.00^{a}	0.0^{a}	873^{a}	1204	1.40^{b}
	100 mg/kg	NE	416^{b}	759	1.82	1.11^{c}	27.1^{b}	571^{b}	1229	2.35^{a}
	120 mg/kg	NE	482^{ab}	831	1.74	0.67^{b}	14.6^{c}	874^{a}	1390	1.59^{ab}
	140 mg/kg	NE	428^{b}	752	1.8	0.67^{b}	12.5^{cd}	809^{ab}	1218	1.53^{b}
	160 mg/kg	NE	461^{ab}	779	1.71	0.72^{b}	10.4^{ac}	755^{ab}	1213	1.67^{ab}
	SEM		23	33	0.03	0.18	4.3	56	35	0.17
	P value		*<0.05	0.09	0.74	**<0.01	**<0.01	*<0.05	0.25	*<0.05

^{a,b,c,d}Values with different letters represent significant differences of means of BWG, FI, and FCR separated by ANOVA followed by post-hoc Tukey's HSD. Means of lesions scores were separated by Kruskal Wallace test with post-hoc Tukey's HSD. Means of NE mortality were separated by Chi-square test with post-hoc Mann Whitney test. Data in experiment 1 and 2 were analyzed separately.

Table 4.4.
Pathohistological analysis of the jejunum on day 21 in experiment 1.

	Villi (µm)	**Crypt (µm)**	**Villi:crypt**
Zinc glycinate 40 mg/kg, uninfected	362	89[a]	4.19[a]
Zinc glycinate 40 mg/kg, infected	310	118[ac]	2.65[b]
Zinc glycinate 80 mg/kg, infected	347	128[bc]	2.81[b]
Zinc glycinate 120 mg/kg, infected	333	118[ac]	2.85[b]
SEM	11	8	0.36
P value	0.51	**< 0.01	**< 0.01

[a,b,c]Values with different letters represent significant differences of means separated by ANOVA followed by post-hoc Tukey's HSD.

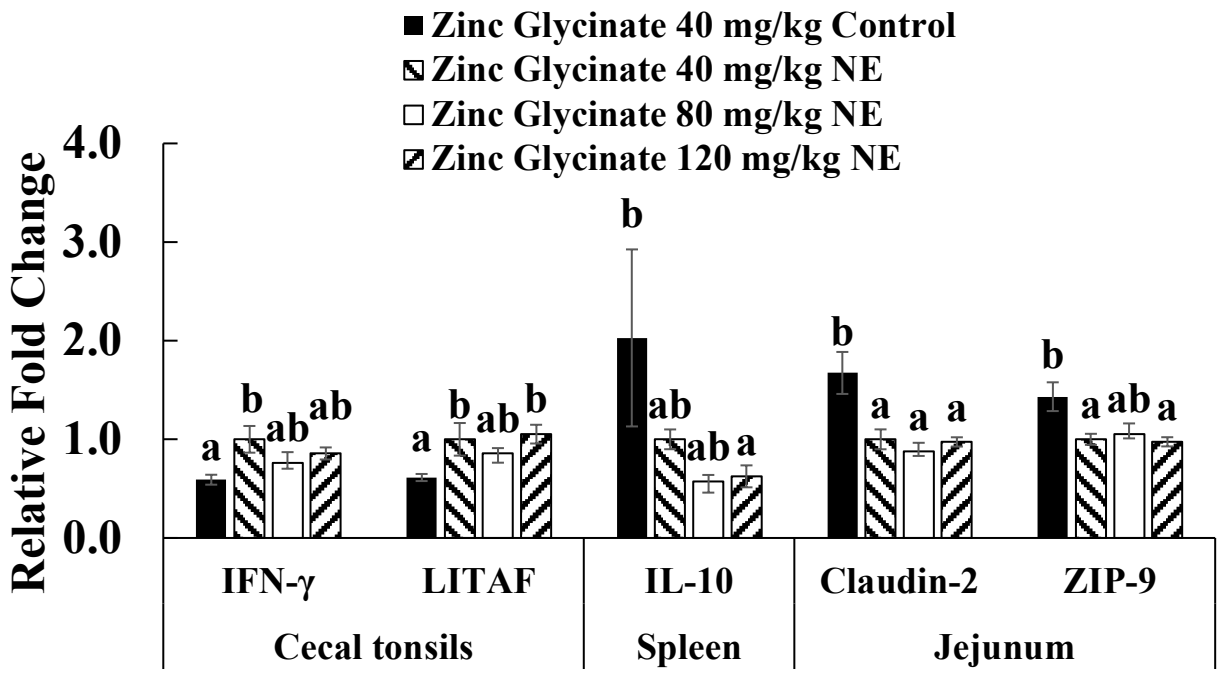

Figure 4.1. Effects of supplementation of dietary zinc glycinate at 40, 80, and 120 mg/kg on gene expression of cytokines in the cecal tonsils (IFN-γ and LITAF) and spleen (IL-10) and tight junction protein (Claudin-2) and zinc transporter (ZIP-9) in the jejunum on day 21 in experiment 1. Each bar represents the mean ± SE of 6 replicates of birds in floor pens. The mRNA expression is expressed as fold-change normalized to beta-actin as housekeeping and normalized to the zinc glycinate at 40 mg/kg uninfected treatment. [a,b]Values with different letters represent significant differences of means separated by ANOVA followed by post-hoc Tukey's HSD. The expression of IFN-γ in the cecal tonsils was higher in infected birds supplemented with 40 mg/kg of zinc glycinate compared to non-challenged birds supplemented with 40 mg/kg of zinc glycinate (*$P <$ 0.05). The expression of LITAF in the cecal tonsils was higher in infected birds supplemented with 40 mg/kg of zinc glycinate compared to non-challenged birds supplemented with 40 mg/kg of zinc glycinate (*$P < 0.05$). The expression of Claudin-2 in the jejunum was lower in infected birds supplemented with 40 mg/kg of zinc glycinate compared to non-challenged birds supplemented with 40 mg/kg of zinc glycinate (**$P < 0.01$). The expression of ZIP-9 in the jejunum was lower in infected birds supplemented with 40 mg/kg of zinc glycinate compared to non-challenged birds supplemented with 40 mg/kg of zinc glycinate (*$P < 0.05$).

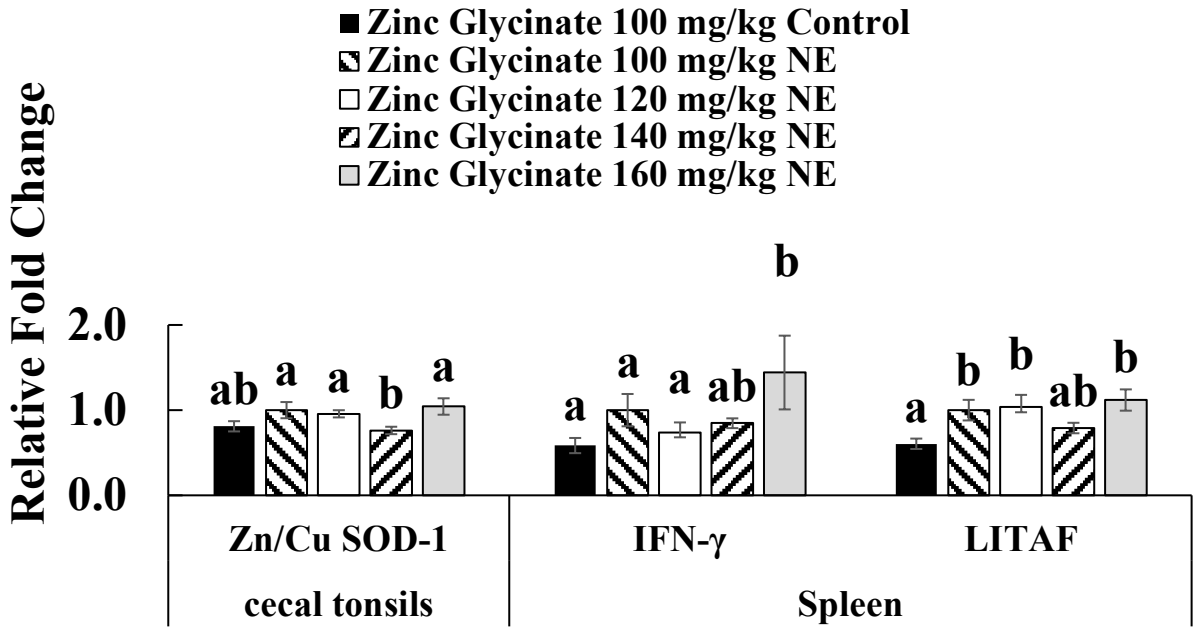

Figure 4.2. Effects of supplementation of dietary zinc glycinate at 100, 120, 140, and 160 mg/kg on gene expressions of antioxidant protein in the cecal tonsils (Zn/Cu SOD-1) and cytokines in the spleen (IFN-γ and LITAF) on day 21 in experiment 2. Each bar represents the mean ± SE of 6 replicates of birds in floor pens. The mRNA expression is expressed as fold-change normalized to beta-actin as housekeeping and normalized to the zinc glycinate at 100 mg/kg uninfected treatment. [a,b]Values with different letters represent significant differences of means separated by ANOVA followed by post-hoc Tukey's HSD. The expression of Zn/Cu SOD-1 in the cecal tonsils was lower in infected birds supplemented with 160 mg/kg of zinc glycinate compared to infected birds supplemented with 120 mg/kg of zinc glycinate (*$P < 0.05$). The expression of IFN-γ in the spleen was higher in infected birds supplemented with 100 mg/kg of zinc glycinate compared to non-challenged birds supplemented with 100 mg/kg of zinc glycinate (**$P < 0.01$).

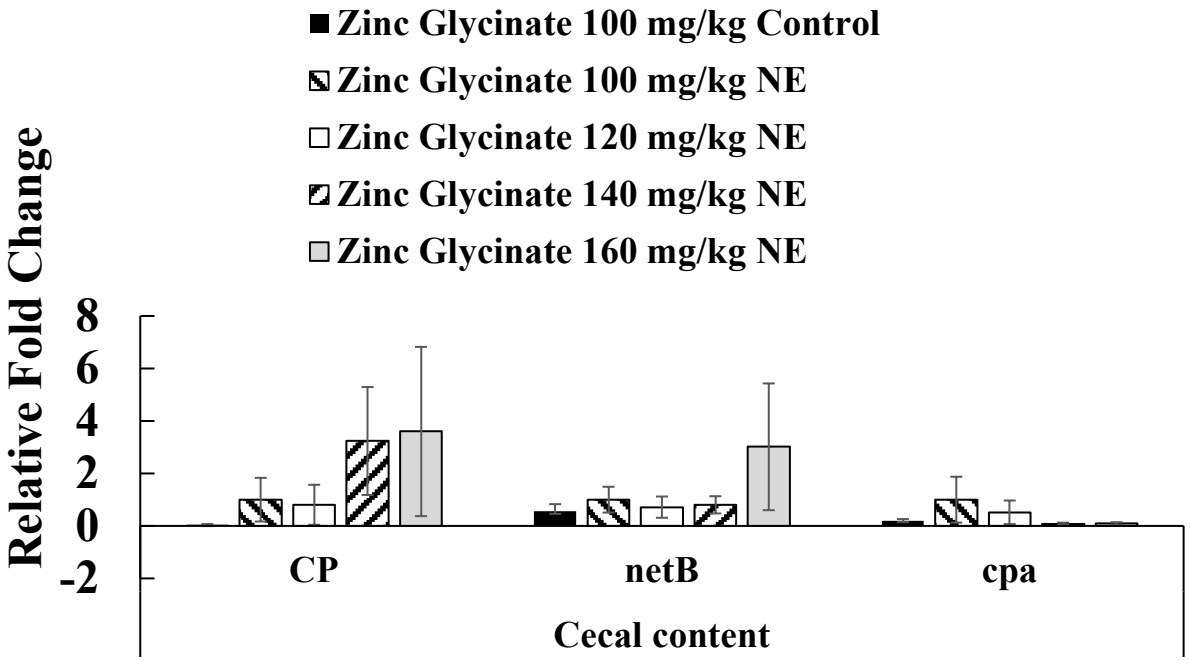

Figure 4.3. Effects of supplementation of dietary zinc glycinate at 100, 120, 140, and 160 mg/kg on gene expressions of bacteria genes (Clostridium perfringens, *netB*, and *cpa*) in the ceca on day 21 in experiment 2. Each bar represents the mean ± SE of 6 replicates of birds in floor pens. The mRNA expression is expressed as fold-change normalized to 16s rRNA as housekeeping and normalized to the zinc glycinate at 100 mg/kg uninfected treatment.

SUPPLEMENTARY MATERIALS

Supplemental Table 4.1.
Ingredients of basal experimental diets.

Ingredient (as-fed basis)	Basal 1 to 28 days
Yellow corn grain, %	62.82
Soybean meal (48%), %	33.27
Vegetable fat, %	1.17
Defluorinated phosphate, %	1.13
Calcium carbonate, %	0.75
[†]Vitamin premix, %	0.25
DL-Methionine, %	0.24
L-Lysine, %	0.16
Salt, %	0.12
[‡]Trace Mineral (Zinc-free) , %	0.08
Quantum Blue Phytase (5,000 FTU/g), %[§]	0.01

[†]Vitamins and minerals were provided in the form and amount described in the Cobb 500 Broiler Performance and Nutrition Supplement guide. Vitamin A: 10,000 IU; vitamin D3: 5,000 IU; vitamin E: 8,000 IU; vitamin K3: 3 mg/kg; vitamin B1 (thiamine): 3 mg/kg; vitamin B2 (riboflavin): 9 mg/kg; vitamin B6 (pyridoxine): 4 mg/kg; vitamin B12: 0.02 mg/kg; biotin: 0.15 mg/kg; choline: 500 mg/kg; folic acid: 2 mg/kg; nicotinic acid: 60 mg/kg; pantothenic acid: 15 mg/kg.
[‡]Manganese (from manganese sulfate): 100 mg/kg; iron (from ferrous sulfate): 40 mg/kg; copper (from copper sulfate): 15 mg/kg; iodine (from calcium iodide): 1 mg/kg; selenium (from sodium selenite): 0.35 mg/kg.
[§]FTU: phytase unit

Supplemental Table 4.2.
Calculated nutrient compositions basal diets.

Nutrient	Units	Basal 1 to 28 days
Dry Matter	%	87.73
Crude Protein	%	21
Crude Fat	%	3.69
Crude Fiber	%	2.19
Calcium	%	0.9
Total Phosphorus	%	0.58
Available Phosphorus	%	0.45
Metabolizable Energy	kcal/kg	3,008
Methionine	%	0.58
Lysine	%	1.28
Tryptophan	%	0.28
Threonine	%	0.85
Sodium	%	0.16
Potassium	%	0.82
Chloride	%	0.15
Digestible methionine	%	0.56
Digestible cysteine	%	0.32
Digestible lysine	%	1.18
Digestible tryptophan	%	0.27
Digestible threonine	%	0.77
Digestible isoleucine	%	0.96
Digestible histidine	%	0.53
Digestible valine	%	1.05
Digestible leucine	%	1.75
Digestible arginine	%	1.33
Digestible phenylalanine	%	1.07
Digestible TSAA[†]	%	0.88

[†]Total sulfur amino acids

CHAPTER 5

AN *IN VITRO* MODEL TO EVALUATE THE EFFICACY OF ZINC USING CHICKEN INTESTINAL CELLS AND MACROPHAGES[1, 2]

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ABSTRACT

Zinc is used to alleviate necrotic enteritis infection in chickens; however, there is limited information regarding the mode of action of zinc in the infection. This study aimed to determine the effects of zinc concentration and source (sulfate and glycinate) on *Clostridium perfringens*, chicken intestinal cells, and chicken macrophages (HD-11 cell line) *in vitro*. *C. perfringens* proliferation was limited by the addition of zinc at 1000 μM, but the expression of toxin genes, *cpa* and *netB*, were not affected by zinc source or concentration. Cell proliferation was measured by the MTT assay, and cell cytotoxicity was measured by the LDH assay. The proliferation of chicken intestinal primary cells was higher by the addition of zinc at 10 or 100 μM (***$P < 0.001$). After 12 hours of incubation, the cytotoxicity of the chicken intestinal primary cells was lowered by the addition of zinc at 100 μM (***$P < 0.001$). After 24 hours of incubation, the cytotoxicity of chicken intestinal primary cells was lowered by the addition of zinc at 10 μM (***$P < 0.001$). The cytotoxicity of HD-11 was lowest by the addition of zinc at 100 μM (****$P < 0.0001$). The infection resulted in higher nitric oxide production by HD-11 cells compared to uninfected cells (**$P < 0.01$). These data suggest that zinc concentration at 100 μM is beneficial regardless of the zinc source.

Keywords *Clostridium perfringens*, HD-11, in vitro, zinc sulfate, zinc glycinate, chicken intestinal cells

INTRODUCTION

Necrotic enteritis is an enteric infection in chickens caused by endotoxins from *Clostridium perfringens* (Hofacre, 2001). Zinc supplementation in broiler chicken diets alleviates intestinal damage from necrotic enteritis (Bortoluzzi et al., 2019b). Zinc is an essential nutrient that functions

as co-factors, such as the antioxidant enzyme, zinc/copper superoxide dismutase (Tainer et al., 1983). Therefore, zinc supplementation is used against a host of foodborne-illness in chickens including *Campylobacter jejuni* (Xie et al., 2011), *Clostridium perfringens* (Bortoluzzi et al., 2019b), coccidia (Baba et al., 1992a), and *Salmonella enterica* (Zhang et al., 2012). In addition, organic zinc increases zinc retention in chickens (Sridhar et al., 2015c). However, the benefits of organic zinc over inorganic zinc remain elusive (Mohanna and Nys, 1999).

Despite the demonstrated efficacy of zinc in reducing damages by the pathogens, the expression of many immune genes (IL-1β, -6, 8, -10, IFN-γ, iNOS, and NF-kB) are unaffected by zinc (Bortoluzzi et al., 2019b). Compound to that, zinc exacerbates *Clostridial* proliferation *in vitro* (Eryavuz and Dehority, 2009). The CPA toxin of *C. perfringens* is a zinc metalloprotease (Cafardi et al., 2013), and zinc decreases resistance to *C. difficile* infection in infants (Zackular et al., 2016). Therefore, it remains unclear whether zinc reduces necrotic enteritis by modulating immune responses of chickens or the proliferation of *C. perfringens* in the intestine.

Here we described an *in vitro* method for determining the effects of zinc concentration and source (sulfate and glycinate). Zinc was co-cultured with *C. perfringens*, chicken intestine primary cells, and chicken macrophages. The growth and bacterial toxin gene expression were analyzed after the co-culture. Cell cytotoxicity and viability were measured after the co-culture of zinc at the physiological concentration (< 100 μM) with chicken intestinal primary cells using the lactate dehydrogenase (LDH) assay and 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay, respectively. Cell cytotoxicity and nitric oxide activity were measured in chicken macrophages cell line (HD-11) (Beug et al., 1979).

MATERIALS AND METHODS

Animals

The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Georgia under the protocol, A2018 04-010-Y2-A2. The intestinal tissues in the chicken primary intestinal cells experiment were collected from day-old Cobb 500 off-sex male by-product broiler chicks post-mortem. The birds were housed in Petersime cages under standard animal husbandry practices. Water was provided *ad libitum*. The animals were fed a corn-soybean based diet.

Isolation and Co-culturing of *Clostridium perfringens* with Zinc

Clostridium perfringens (CP6) were field isolated from necrotic enteritis in chickens (Hofacre et al., 1998). CP6 was incubated in tryptic soy with sodium thioglycolate (TSB-T) (0.05% w/v) under anaerobic conditions. A control without zinc, zinc at 10, 100, and 1000 µM from zinc sulfate or zinc glycinate was added to the media, resulting in a 2 X 3 plus control factorial design. The zinc concentration was calculated from the molecular weight of zinc from zinc sulfate (35.5% according to manufacturer) and zinc glycinate (26% according to manufacturer). The experiment was repeated twice and each treatment was replicated four times (n = 4). Bacteria growth was measured by absorbance at OD_{600} hourly for 6 hours and confirmed by plating. At the end of the growth period, the bacteria were collected and washed in PBS.

DNA from the bacteria was extracted using the previously described method (Markazi et al., 2017). Briefly, the DNA from the bacteria was resuspended in 450 µL of 50 mM EDTA and digested with 50 µL of lysozyme (20 mg/mL) for 45 minutes at 36°C. After incubation, the supernatant was discarded after centrifugation at 14,500 x g for 5 mins. The samples were then resuspended in 600

μL lysis buffer and 32 μL of proteinase K (20 mg/mL) for 5 minutes at 80°C. The DNA pellets were extracted from the cell pellet using 5 M NaCl and isopropanol after centrifugation. The DNA pellets were washed in 100% ethanol and resuspended in molecular grade water. The expression of the toxin genes (*cpa* and *netB*) were analyzed by quantitative real time-PCR using the CFX-96 and CFX Maestro (Bio-Rad, CA, USA) and normalized to the 16s *C. perfringens* rRNA gene. The *Clostridium perfringens, netB*, and *cpa* genes were quantified using primers and cycle conditions described in Table 5.1. Fold-change from the reference was quantified as $2^{-\Delta\Delta Cq}$, where Cq is the threshold cycle defined as the cycle number when the RT-qPCR product fluorescence reaches exponentially above the background and normalized to the control group (Livak and Schmittgen, 2001).

Isolation and Co-culturing of Chicken Primary Intestinal Epithelial Cells with Zinc

The jejunum was collected from three broiler chickens (n = 3) at 28 days of age in complete media (DMEM media containing 10% FBS, 1% penicillin-streptomycin, and 2 mM glutamine). The tissues were flushed with PBS repeatedly and then minced into 5 cm sections for digestion in collagenase (0.3 mg/mL in DMEM) (Psichas et al., 2017). The fourth and fifth digestions containing the intestinal crypt were filtered through a 70 μm filter to remove debris. The remaining collagenase was washed away by centrifugation in PBS.

The LDH assay was performed to determine the cytotoxicity of the cells. The primary chicken intestinal cells were seeded in 24-well plates at approximately 80% confluency in complete media as control, or complete media containing zinc at 10 or 100 μM from zinc sulfate or zinc glycinate for 12 or 24 hours (41°C and 5% CO_2). The supernatant was collected for the LDH assay. Briefly, the phenazine methosulfate (PMS), ioinitotetrazolium chloride (INT), and diphosphopyridine nucleotide (NAD) (1:1:23 v/v/v) were mixed before the assay. Then the supernatant (1:3 v/v) was

added to the LDH reagent (1:1:1 v/v/v of 200 mM TRIS at pH 8, 50 mM lithium lactate, and PMS, INT, and NAD mixture. Maximum release was determined by lysing cells with 1% Triton X-100. Absorbance was read in the microplate reader at OD_{490}.

The MTT assay was performed to determine cell proliferation of the cells. Primary chicken intestinal cells were seeded in 96-well plates at approximately 80% confluency in 100 µL phenol-red free RPMI media containing 10% FBS and 1% penicillin-streptomycin as control, or 100 microliters control media containing zinc at 10 or 100 µM from zinc sulfate or zinc glycinate for 24 hours (41°C and 5% CO_2). The MTT reagent (20 µL) was added directly to the wells and allowed to incubate for 4 hours in the dark to accumulate formazan product, and then 100 µL DMSO was added to dissolve the product overnight. The absorbance was read in the microplate reader at OD_{575}.

Co-culturing of Chicken Macrophages Cell Line (HD-11) with Zinc

The HD-11 experiment was repeated, resulting in two occasions (n = 2). HD-11 cells were cultured at 41°C and 5% CO2 in complete media (DMEM media containing 10% FBS and 1% penicillin-streptomycin). CP6 was thawed, washed with PBS to remove DMSO, and grown TSB-T medium overnight. The bacteria were in the late log to the stationary phase before being washed thrice in PBS through centrifugation at 3000 rpm for 10 minutes. The bacteria were resuspended in complete media, OD600 taken and added to the appropriate wells at the multiplicity of infection (MOI) of 10 bacteria to one cell.

For the NO assay, a total of 50,000 cells were seeded in 96-well plates in complete media, or complete media containing zinc at 10 or 100 µM from zinc sulfate or zinc glycinate 10 or 100 µM for 24 hours. Subsequently, an MOI of 10 bacteria to one cell was added and incubated for 2 hours (41°C and 5% CO2) before excess bacteria were rinsed off with PBS twice. Finally, cells were

incubated for 24 hours in phenol-red free RPMI media containing 10% FBS and 1% penicillin-streptomycin to allow the accumulation of nitric oxide species. An equal volume of Griess reagent was added to supernatants, and absorbance read with a microplate reader at OD540. Concentrations of nitrate were compared to that of a sodium nitrate standard curve.

For the LDH assay, a total of 250,000 cells were seeded in 24-well plates in complete media, or complete media containing zinc at 10 or 100 μM from zinc sulfate or zinc glycinate for 24 hours. Subsequently, an MOI of 10 bacteria to one cell was added and incubated for 2 hours (41°C and 5% CO2) before excess bacteria were rinsed off with PBS twice. Cells were incubated for 24 hours in complete media before the supernatant was collect for LDH determination.

For the visualization of phagocytosed bacteria, after removal of media, cells were fixed with methanol for 1 minute and then subsequently stained with Giemsa for 1 minute. The excess stain was removed by gentle rinsing in PBS.

Statistical Analyses

A two-way ANOVA (JMP Pro 15) was used to examine the main effects and interaction of zinc source and concentration on the dependent variables. When main effects or main effects were significant ($P < 0.05$), differences between means were determined using Tukey's least-square means comparisons.

RESULTS AND DISCUSSION

In this novel study, we evaluated the effects of zinc concentration and source on *C. perfringens*, chicken intestinal cells, and chicken macrophages. Although *in vitro* studies have been used in murine and human cell culture models, there has not been any study on zinc using chicken derived cells. Furthermore, the potential advantage of organic over inorganic zinc has not been studied

using the *in vitro* cell culture model. The proliferation of *C. perfringens* was lower in the treatment supplemented by zinc at 1000 μM. However, the toxin genes of *C. perfringens* were not affected by zinc. We determined the enrichment of the cells by zinc at 100 μM regardless of the source.

C. perfringens proliferation with or without zinc from zinc sulfate or zinc glycinate at 0, 10, 100, or 1000 μM was evaluated by spectrophotometer at OD_{600} (Table 5.2). The expressions of the toxin genes, *cpa* and *netB*, were not statistically different between treatments. The proliferation *C. perfringens* was not enriched by zinc in this study, which is contradicted in some studies (Eryavuz and Dehority, 2009). At a high concentration of zinc beyond physiological concentration at 1000 μM, the proliferation of the bacteria was dampened. The CPA and netB toxins were analyzed by gene expression in this study because these toxins are implicated in necrotic enteritis in chickens (Sheedy et al., 2004; Si et al., 2007; Cooper et al., 2009; Keyburn et al., 2010). In addition, the CPA toxin is zinc protease (Sato and Murata, 1973). Zinc supplementation at 50 μM in bacterial culture media had lower CPA toxin production (Murata et al., 1969) but was not observed in the current study. Recent studies show bacterial resistance to heavy metals due to the accumulation in agricultural waste (Ahemad and Malik, 2011). Therefore, caution should be taken in zinc supplementation in broiler chicken diets to minimize zinc resistance by the bacteria.

Intestinal primary cells were extracted enzymatically from the jejunum of the chicken and then co-cultured with zinc. There were no significant interactions between zinc source and zinc concentration on intestinal cells MTT production. The main effect, zinc concentration ($P < 0.001$), was significant in intestinal cells MTT production. The addition of zinc at 10 or 100 μM resulted in significantly higher MTT production by the intestinal cells. Hence, zinc increased the proliferation of the intestinal cells. There were no significant interactions between zinc source and zinc concentration on intestinal cells LDH production. The main effect, zinc concentration, was

significant on intestinal cells LDH production at 12 hours ($P < 0.001$) and 24 hours ($P < 0.001$) after incubation. LDH production from the intestinal cells at 12 hours after incubation was significantly lower in the treatments supplemented with zinc at 100 μM. LDH production from the intestinal cells at 24 hours after incubation was significantly lower in the treatments supplemented with zinc at 10 μM. The major hurdle in this experiment is contamination by the microbiota of the intestine. The intestine was washed repeatedly, and the experiments were conducted within 24 hours after plated to minimize bacteria overgrowth. Furthermore, zinc is found in cell culture media in fetal bovine serum in nanomolar to the picomolar range. Therefore, completely devoid of zinc was not possible in cell culture. In this study, the viability of chicken intestinal primary cells was improved by zinc at 100 μM. This is consistent with one study on zinc sulfate in the porcine intestinal cell line (IPEC-J2) (Lodemann et al., 2013).

The HD-11 cell line is an avian leukemia virus-derived chicken macrophage-like cells (Beug et al., 1979). The zinc-finger transcription factor, Egr-1, is involved in the activation of macrophages (Krishnaraju et al., 1995). There was an interaction between zinc source and concentration on HD-11 LDH production ($P < 0.01$), and the main effect of zinc concentration was significant ($P < 0.0001$). Zinc at 100 μM had the lowest LDH production, while the LDH production of the cells with added zinc at 10 μM was between the treatments of 0 and 100 μM. Macrophages recognize antigens and mediate cytotoxic killing through nitric oxide (NO) production (Albina and Reichner, 1998). There were no significant interactions between *C. perfringens* infection, zinc source, and zinc concentration on HD-11 nitric oxide production. The main effect, infection ($P < 0.01$), was significant on HD-11 nitric oxide production. The infection resulted in significantly higher HD-11 nitric oxide production but not by zinc.

The results in this paper indicate that supplementing zinc at 100 μM promotes the growth of chicken intestinal cells and macrophages *in vitro*. Zinc sources did not affect any parameters in this study. This could explain that increasing zinc concentration in broiler chicken diets partially alleviates necrotic enteritis *in vivo,* and zinc source did not affect immune parameters in previous studies.

ACKNOWLEDGMENT

Not applicable

CONFLICT OF INTEREST

Zinc glycinate was donated by Pancosma SA (Geneva, CH). Pancosma SA was not involved in designing and conducting the research.

REFERENCES

Ahemad, M., and A. Malik. 2011. Bioaccumulation of heavy metals by zinc resistant bacteria isolated from agricultural soils irrigated with wastewater. Bacteriol. J 2(1):12-21.

Albina, J. E., and J. S. Reichner. 1998. Role of nitric oxide in mediation of macrophage cytotoxicity and apoptosis. Cancer and Metastasis Reviews 17(1):39-53.

Baba, E., A. L. Fuller, J. M. Gilbert, S. G. Thayer, and L. R. McDougald. 1992. Effects of Eimeria brunetti infection and dietary zinc on experimental induction of necrotic enteritis in broiler chickens. Avian Diseases 36(1):59-62.

Beug, H., A. von Kirchbach, G. Döderlein, J.-F. Conscience, and T. Graf. 1979. Chicken hematopoietic cells transformed by seven strains of defective avian leukemia viruses display three distinct phenotypes of differentiation. Cell 18(2):375-390.

Bortoluzzi, C., B. Vieira, B. Lumpkins, G. Mathis, W. King, D. Graugnard, K. Dawson, and T. Applegate. 2019. Can dietary zinc diminish the impact of necrotic enteritis on growth performance of broiler chickens by modulating the intestinal immune-system and microbiota? Poultry Science

Cafardi, V., M. Biagini, M. Martinelli, R. Leuzzi, J. T. Rubino, F. Cantini, N. Norais, M. Scarselli, D. Serruto, and M. Unnikrishnan. 2013. Identification of a novel zinc metalloprotease through a global analysis of Clostridium difficile extracellular proteins. PloS one 8(11)

Cooper, K., H. Trinh, and J. G. Songer. 2009. Immunization with recombinant alpha toxin partially protects broiler chicks against experimental challenge with Clostridium perfringens. Veterinary Microbiology 133(1-2):92-97.

Eryavuz, A., and B. A. Dehority. 2009. Effects of supplemental zinc concentration on cellulose digestion and cellulolytic and total bacterial numbers in vitro. Animal feed science and technology 151(3-4):175-183.

Hofacre, C., R. Froyman, B. Gautrias, B. George, M. Goodwin, and J. Brown. 1998. Use of Aviguard and other intestinal bioproducts in experimental Clostridium perfringens-associated necrotizing enteritis in broiler chickens. Avian Diseases:579-584.

Hofacre, C. L. 2001. Necrotic enteritis, currently a billion dollar disease: is there anything new on the horizon. In: Proceedings of Alltech's 17th Annual Symposium Science and Technology in the Feed Industry. p 79-86.

Keyburn, A. L., X.-X. Yan, T. L. Bannam, F. Van Immerseel, J. I. Rood, and R. J. Moore. 2010. Association between avian necrotic enteritis and Clostridium perfringens strains expressing NetB toxin. Veterinary Research 41(2):1-8.

Krishnaraju, K., H. Q. Nguyen, D. A. Liebermann, and B. Hoffman. 1995. The zinc finger transcription factor Egr-1 potentiates macrophage differentiation of hematopoietic cells. Molecular and cellular biology 15(10):5499-5507.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25(4):402-408.

Lodemann, U., R. Einspanier, F. Scharfen, H. Martens, and A. Bondzio. 2013. Effects of zinc on epithelial barrier properties and viability in a human and a porcine intestinal cell culture model. Toxicology in vitro 27(2):834-843.

Markazi, A. D., V. Perez, M. Sifri, R. Shanmugasundaram, and R. K. Selvaraj. 2017. Effect of whole yeast cell product supplementation (CitriStim®) on immune responses and cecal microflora species in pullet and layer chickens during an experimental coccidial challenge. Poultry science 96(7):2049-2056.

Mohanna, C., and Y. Nys. 1999. Effect of dietary zinc content and sources on the growth, body zinc deposition and retention, zinc excretion and immune response in chickens. British Journal of Poultry Science 40(1):108-114.

Murata, R., S. SODA, A. YAMAMOTO, H. SATO, and A. ITO. 1969. The effect of zinc on the production of various toxins of Clostridium perfringens. Japanese Journal of Medical Science and Biology 22(3):133-148.

Psichas, A., G. Tolhurst, C. A. Brighton, F. M. Gribble, and F. Reimann. 2017. Mixed Primary Cultures of Murine Small Intestine Intended for the Study of Gut Hormone Secretion and Live Cell Imaging of Enteroendocrine Cells. Journal of visualized experiments : JoVE (122):55687. doi: 10.3791/55687

Sato, H., and R. Murata. 1973. Role of zinc in the production of Clostridium perfringens alpha toxin. Infection and immunity 8(3):360-369.

Sheedy, S. A., A. B. Ingham, J. I. Rood, and R. J. Moore. 2004. Highly conserved alpha-toxin sequences of avian isolates of Clostridium perfringens. Journal of Clinical Microbiology 42(3):1345-1347.

Si, W., J. Gong, Y. Han, H. Yu, J. Brennan, H. Zhou, and S. Chen. 2007. Quantification of cell proliferation and alpha-toxin gene expression of Clostridium perfringens in the development of necrotic enteritis in broiler chickens. Appl. Environ. Microbiol. 73(21):7110-7113.

Sridhar, K., D. Nagalakshmi, and S. R. Rao. 2015. Effect of graded concentration of organic zinc (zinc glycinate) on skin quality, hematological and serum biochemical constituents in broiler chicken. Indian Journal of Animal Science 85:643-648.

Tainer, J. A., E. D. Getzoff, J. S. Richardson, and D. C. Richardson. 1983. Structure and mechanism of copper, zinc superoxide dismutase. Nature 306(5940):284.

Xie, Y., Y. He, P. L. Irwin, T. Jin, and X. Shi. 2011. Antibacterial activity and mechanism of action of zinc oxide nanoparticles against Campylobacter jejuni. Appl. Environ. Microbiol. 77(7):2325-2331.

Zackular, J. P., J. L. Moore, A. T. Jordan, L. J. Juttukonda, M. J. Noto, M. R. Nicholson, J. D. Crews, M. W. Semler, Y. Zhang, and L. B. Ware. 2016. Dietary zinc alters the microbiota and decreases resistance to Clostridium difficile infection. Nature medicine 22(11):1330.

Zhang, B., Y. Shao, D. Liu, P. Yin, Y. Guo, and J. Yuan. 2012. Zinc prevents Salmonella enterica serovar Typhimurium-induced loss of intestinal mucosal barrier function in broiler chickens. Avian Pathology 41(4):361-367.

TABLES AND FIGURES

Table 5.1.
Primers information for quantitative real-time PCR of bacterial genes in the ceca in experiment 2.

	Primer sequence (5' – 3')	Accession #/Reference	Annealing	Extension
16s rRNA Clostridium perfringens	F: CGCATAACGTTGAAAGATGG R: CCTTGGTAGGCCGTTACCC	(Wise and Siragusa, 2005)	55°C, 45 s	20 s
netB	F:CGCTTCACATAAAGGTTGGAAGGC R:TCCAGCACCAGCAGTTTTTCCT	(Bailey et al., 2013)		
cpa	F: TGCATGAGCTTCAATTAGGT R: TTAGTTTTGCAACCTGCTGT	(Wilson et al., 2018)		

Table 5.2.
Two-way ANOVA of *Clostridium perfringens* growth curve during co-culture with zinc at 0, 10, 100, or 1000 µM from zinc sulfate or zinc glycinate for 6 hours.

		Time, hours						
Source	**Concentration of zinc µM**	**0**	**1**	**2**	**3**	**4**	**5**	**6**
Control	0	0.05^{bcd}	0.06^{b}	0.13^{ab}	0.29^{ab}	0.52^{ab}	0.78^{ab}	0.75^{b}
Sulfate	10	0.06^{b}	0.06^{b}	0.13^{ab}	0.27^{ab}	0.52^{ab}	0.69^{ab}	0.78^{b}
Sulfate	100	0.06^{bc}	0.06^{b}	0.11^{bc}	0.22^{b}	0.44^{b}	0.64^{b}	0.74^{b}
Sulfate	1000	0.05^{d}	0.05^{c}	0.07^{d}	0.12^{c}	0.26^{c}	0.42^{c}	0.57^{c}
Glycinate	10	0.05^{cd}	0.06^{b}	0.12^{bc}	0.29^{a}	0.56^{a}	0.76^{ab}	0.81^{ab}
Glycinate	100	0.05^{d}	0.06^{b}	0.09^{cd}	0.23^{b}	0.48^{ab}	0.74^{ab}	0.89^{a}
Glycinate	1000	0.06^{a}	0.07^{a}	0.07^{d}	0.08^{c}	0.11^{d}	0.26^{d}	0.57^{c}
Pooled SD		0.01	0.01	0.02	0.08	0.17	0.20	0.12
		Probability						
ANOVA								
Source (S)		NS	$P < 0.0001$	$P < 0.01$	NS	NS	NS	$P < 0.01$
Concentration (C)		$P < 0.05$	NS	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
S X C		$P < 0.0001$	$P < 0.0001$	NS	$P < 0.05$	$P < 0.001$	$P < 0.0001$	$P < 0.0001$

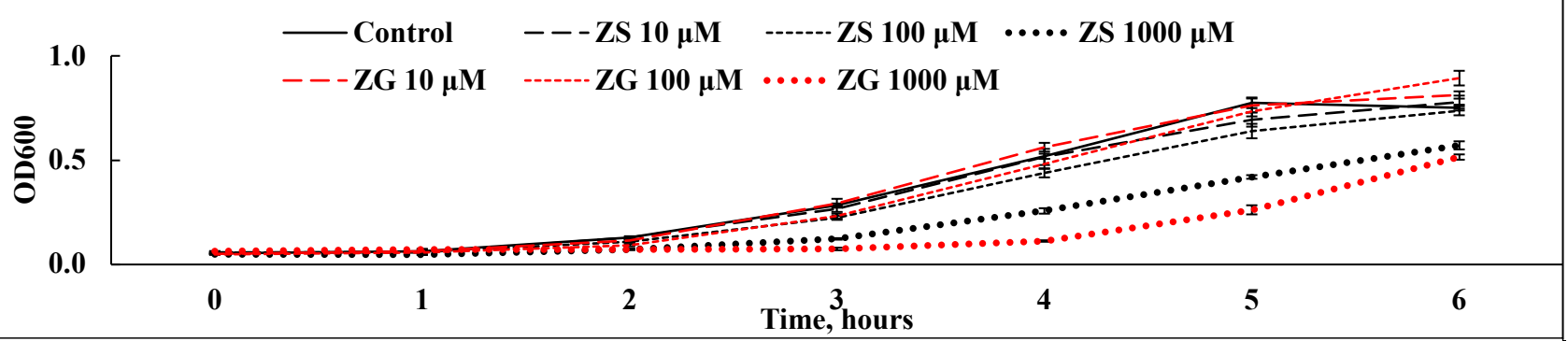

Table 5.2. Effect of dietary zinc source (S), concentration (C), and their interaction (S x C) on *C. perfringens* growth evaluated by spectrophotometer at OD_{600}. Values are the mean OD_{600} of four replicates per treatment (n = 4). [a, b, c, d]Values with different letters represent significant differences of means separated by two-way ANOVA followed by post-hoc Tukey's HSD.

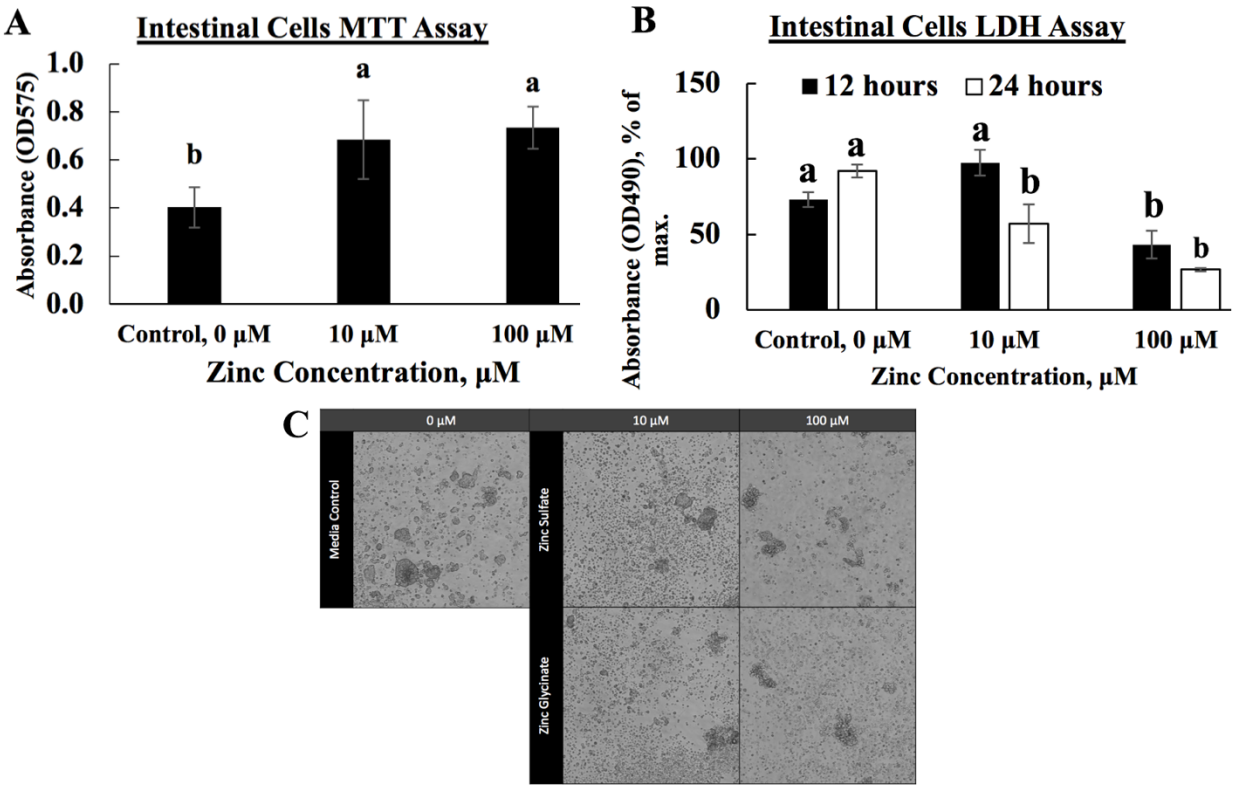

Figure 5.1. Chicken jejunum intestinal cells cell viability and cytotoxicity. (A) Effects of zinc concentration of chicken intestinal cells in the jejunum on cell proliferation using MTT assay. (B) Effects of zinc concentration on the cell cytotoxicity of chicken intestinal cells in the jejunum using LDH assay. (C) Images of jejunum crypt cells during co-culturing with or without zinc at 0, 10, or 100 µM from zinc sulfate or glycinate.

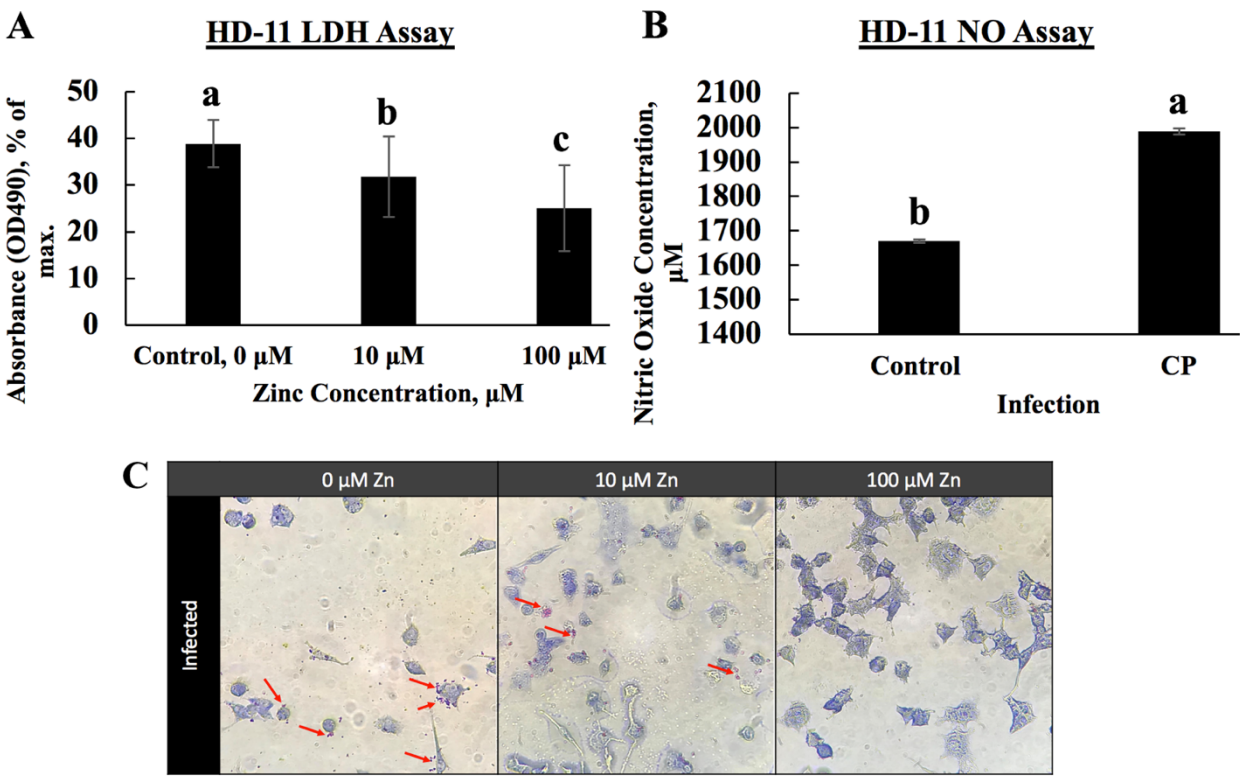

Figure 5.2. HD-11 cell cytotoxicity and NO production. (A) Effects of zinc concentration on cell cytoxicity of HD-11 cells using MTT assay. (B) Effects of zinc concentration on nitric oxide production of HD-11 cells using NO assay using Griess reagent. (C) Images of HD-11 cells infected with *C. perfringens* during co-culturing with zinc at 0, 10, or 100 µM.

CHAPTER 6

OVERALL CONCLUSION

Zinc status is a critical factor in supporting growth and immunity in animals. Necrotic enteritis infection is one of the most costly diseases in poultry production. We conducted two *in vitro* studies and one *in vivo* studies to determine the optimal supplemental zinc concentration and zinc source in broiler chicken diets against diseases such as necrotic enteritis. The findings in this study provide a guide for improving broiler chicken diets.

We demonstrated that supplementation of zinc improved body weight gain and feed intake in non-infected broiler chickens regardless of zinc source. In necrotic enteritis infected chickens, body weight gain and feed conversion ratio were improved by zinc glycinate supplemented at 120 mg/kg and 140 mg/kg, respectively. In the infected birds, the severity of infection, evaluated by gross lesions in the intestine and mortality, was partially alleviated by zinc glycinate supplemented at 120 mg/kg.

The immunological effects of zinc were not apparent in our study. In non-infected chickens, zinc glycinate supplementation demonstrated immune-modulating effects on IL-1β, IL-10, TGF-β, and LITAF expressions in immune organs. In necrotic enteritis infected chickens, the effect of zinc glycinate on IFN-γ was contradictory to previous studies. Zinc glycinate supplemented at 160 mg/kg had a higher expression of IFN-γ in the spleen than zinc glycinate supplemented at 100 mg/kg. The conventional belief is that the expression of IFN-γ would be lower at the higher concentration of zinc glycinate rather than higher, given that zinc glycinate reduced infection

severity. Zinc glycinate supplemented at 140 mg/kg resulted in lower Cu/Zn-SOD-1 in the cecal tonsils. Zinc glycinate treatments did not affect antibody and $CD4^+$ T cell responses in necrotic enteritis infected chickens. Taken together, we believe that the effects of zinc glycinate are directed through innate immunity, such as the antioxidant enzyme Cu/Zn-SOD-1.

In the *in vitro* study, zinc source had no effect on *C. perfringens*, chicken intestinal cells, and chicken macrophages. We demonstrated that zinc at 100 μM improves the viability of chicken intestinal cells and macrophages. Therefore, despite the higher bioavailability of organic zinc, we did not find any evidence that the higher bioavailability of organic zinc compared to inorganic zinc translates to biological functions in immunity. We concluded that increasing supplemental zinc concentration in broiler chicken diets to 120 mg/kg is beneficial against necrotic enteritis. We demonstrated that the benefits of zinc on necrotic enteritis severity was not modulated by antibody and $CD4^+$ T helper cell responses.

REFERENCES

Abildgaard, L., R. M. Engberg, K. Pedersen, A. Schramm, and O. Hojberg. 2009. Sequence variation in the α-toxin encoding plc gene of Clostridium perfringens strains isolated from diseased and healthy chickens. Veterinary Microbiology 136(3-4):293-299.

Ahemad, M., and A. Malik. 2011. Bioaccumulation of heavy metals by zinc resistant bacteria isolated from agricultural soils irrigated with wastewater. Bacteriol. J 2(1):12-21.

Akbari, M., H. Kermanshahi, H. N. Moghaddam, A. H. Moussavi, and J. T. Afshari. 2008. Effects of wheat-soybean meal based diet supplementation with vitamin A, vitamin E and zinc on blood cells, organ weights and humoral immune response in broiler chickens. J. Anim. Vet. Adv 7(3):297-304.

Akhavan-Salamat, H., and H. Ghasemi. 2019. Effect of different sources and contents of zinc on growth performance, carcass characteristics, humoral immunity and antioxidant status of broiler chickens exposed to high environmental temperatures. Livestock science 223:76-83.

Al-Sheikhly, F., and A. Al-Saieg. 1980. Role of coccidia in the occurrence of necrotic enteritis of chickens. Avian Diseases:324-333.

Al-Sheikhly, F., and R. Truscott. 1977a. The interaction of Clostridium perfringens and its toxins in the production of necrotic enteritis of chickens. Avian Diseases:256-263.

Al-Sheikhly, F., and R. B. Truscott. 1977b. The interaction of Clostridium perfringens and its toxins in the production of necrotic enteritis of chickens. Avian diseases 21(2):256-263.

Albina, J. E., and J. S. Reichner. 1998. Role of nitric oxide in mediation of macrophage cytotoxicity and apoptosis. Cancer and Metastasis Reviews 17(1):39-53.

Allen, J. I., R. T. Perri, C. J. McClain, and N. E. Kay. 1983. Alterations in human natural killer cell activity and monocyte cytotoxicity induced by zinc deficiency. Journal of Laboratory and Clinical Medicine 102(4):577-589.

Allison, C., and G. T. Macfarlane. 1989. Influence of pH, nutrient availability, and growth rate on amine production by Bacteroides fragilis and Clostridium perfringens. Applied and environmental microbiology 55(11):2894-2898.

Andrews, G. K. 2008. Regulation and function of Zip4, the acrodermatitis enteropathica gene. Portland Press Limited.

Applegate, T. J., and R. Angel. 2014. Nutrient requirements of poultry publication: History and need for an update. Journal of Applied Poultry Research 23(3):567-575.

Archibald, J. 1944. Zinc in cows' milk. Journal of Dairy Science 27(4):257-261.

Aschner, M., and A. K. West. 2005. The role of MT in neurological disorders. Journal of Alzheimer's Disease 8(2):139-145.

Aviagen, R. 2019. Broiler nutrition specification. Cummings Research Park

Awad, M. M., D. M. Ellemor, R. L. Boyd, J. J. Emmins, and J. I. Rood. 2001. Synergistic effects of alpha-toxin and perfringolysin O in Clostridium perfringens-mediated gas gangrene. Infection and Immunity 69(12):7904-7910.

Baba, E., A. L. Fuller, J. M. Gilbert, S. G. Thayer, and L. R. McDougald. 1992a. Effects of Eimeria brunetti infection and dietary zinc on experimental induction of necrotic enteritis in broiler chickens. Avian Diseases 36(1):59-62.

Baba, E., A. L. Fuller, J. M. Gilbert, S. G. Thayer, and L. R. McDougald. 1992b. Effects of Eimeria brunetti infection and dietary zinc on experimental induction of necrotic enteritis in broiler chickens. Avian diseases:59-62.

Baer, M. T., and J. C. King. 1984. Tissue zinc levels and zinc excretion during experimental zinc depletion in young men. The American Journal of Clinical Nutrition 39(4):556-570. doi: 10.1093/ajcn/39.4.556

Bafundo, K., D. Baker, and P. Fitzgerald. 1984. Zinc utilization in the chick as influenced by dietary concentrations of calcium and phytate and by Eimeria acervulina infection. Poultry Science 63(12):2430-2437.

Bailey, M. A., K. S. Macklin, and J. T. Krehling. 2013. Use of a Multiplex PCR for the Detection of Toxin-Encoding Genes netB and tpeL in Strains of Clostridium perfringens. ISRN veterinary science 2013

Bannam, T. L., X.-X. Yan, P. F. Harrison, T. Seemann, A. L. Keyburn, C. Stubenrauch, L. H. Weeramantri, J. K. Cheung, B. A. McClane, and J. D. Boyce. 2011. Necrotic enteritis-derived Clostridium perfringens strain with three closely related independently conjugative toxin and antibiotic resistance plasmids. MBio 2(5):e00190-00111.

Bao, Y., M. Choct, P. Iji, and K. Bruerton. 2009. Optimal dietary inclusion of organically complexed zinc for broiler chickens. British Journal of Poultry Science 50(1):95-102.

Barbour, E. K., N. E. Bejjani, N. J. Daghir, O. M. Faroon, M. Bouljihad, and R. Spasojevic. 2000. Induction of Early Immunopotentiation to Fimbriae of Salmonella Enteritidis (SE) by Administering Thymulin and Zinc to SE-Vaccinated Chicken Breeders. Journal of Veterinary Medical Science 62(11):1139-1143.

Barney, G. H., M. P. Macapinlac, W. N. Pearson, and W. J. Darby. 1967a. Parakeratosis of the tongue--a unique histopathologic lesion in the zinc-deficient squirrel monkey. Journal of Nutrition 93(4):511-517. doi: 10.1093/jn/93.4.511

Barney, G. H., M. P. Macapinlac, W. N. Pearson, and W. J. Darby. 1967b. Parakeratosis of the tongue--a unique histopathologic lesion in the zinc-deficient squirrel monkey. Nutrition 93(4):511-517. doi: 10.1093/jn/93.4.511

Barta, J. R., M. C. Jenkins, and H. D. Danforth. 1991. Evolutionary relationships of avian Eimeria species among other Apicomplexan protozoa: monophyly of the apicomplexa is supported. Molecular Biology and Evolution 8(3):345-355.

Baumann, H., and J. Gauldie. 1994. The acute phase response. Immunology today 15(2):74-80.

Beck, F. W., J. Kaplan, N. Fine, W. Handschu, and A. S. Prasad. 1997. Decreased expression of CD73 (ecto-5′-nucleotidase) in the CD8+ subset is associated with zinc deficiency in human patients. Journal of Laboratory and Clinical Medicine 130(2):147-156.

Bendtzen, K. 1980. Differential role of Zn2+ in antigen- and mitogen-induced lymphokine production. Scandinavian journal of immunology 12(6):489-492.

Berfenstam, R. 1952a. Blood zinc during pregnancy and delivery. Acta Radiologica 41

Berfenstam, R. 1952b. Studies on blood zinc; a clinical and experimental investigation into the zinc content of plasma and blood corpuscles with special reference to infancy. Acta paediatrica. Supplementum 41(87):1.

Bertrand, G., and R. Vladesco. 1921. Role of zinc in reproduction. In: Academy of Science. p 176.

Beug, H., A. von Kirchbach, G. Döderlein, J.-F. Conscience, and T. Graf. 1979. Chicken hematopoietic cells transformed by seven strains of defective avian leukemia viruses display three distinct phenotypes of differentiation. Cell 18(2):375-390.

Birckner, V. 1919. The Zinc Content of Some Food Products. Journal of Biological Chemistry 38(2):191-203.

Blackmon, D. M., W. J. Miller, and J. D. Morton. 1967. Zinc deficiency in ruminants. Occurrence, effects, diagnosis and treatments. Veterinary Medicine, Small Animal Clinician 62(3):265-270.

Blamberg, D. L., U. B. Blackwood, W. C. Supplee, and G. F. Combs. 1960. Effect of zinc deficiency in hens on hatchability and embryonic development. Proceedings of the Society for Experimental Biology and Medicine 104:217-220.

Bly, M. 2006. Examination of the zinc transporter gene, SLC39A12. Schizophrenia Research 81(2):321-322.

Bolek, K., and K. Klasing. 2019. The effects of vaccination with keyhole limpet hemocyanin or oral administration of Salmonella enterica serovar Enteritidis on the growth performance of immunoglobulin knockout chickens. Poultry Science

Bonaventura, P., G. Benedetti, F. Albarède, and P. Miossec. 2015. Zinc and its role in immunity and inflammation. Autoimmunity Reviews 14(4):277-285.

Bortoluzzi, C., B. Lumpkins, G. Mathis, M. França, W. King, D. Graugnard, K. Dawson, and T. Applegate. 2019a. Zinc source modulates intestinal inflammation and intestinal integrity of broiler chickens challenged with coccidia and Clostridium perfringens. Poultry science 98(5):2211-2219.

Bortoluzzi, C., B. Vieira, B. Lumpkins, G. Mathis, W. King, D. Graugnard, K. Dawson, and T. Applegate. 2019b. Can dietary zinc diminish the impact of necrotic enteritis on growth performance of broiler chickens by modulating the intestinal immune-system and microbiota? Poultry Science

Brennan, J., J. Skinner, D. Barnum, and J. Wilson. 2003. The efficacy of bacitracin methylene disalicylate when fed in combination with narasin in the management of necrotic enteritis in broiler chickens. Poultry Science 82(3):360-363.

Bridges, C. C., and R. K. Zalups. 2005. Molecular and ionic mimicry and the transport of toxic metals. Toxicology and Applied Pharmacology 204(3):274-308.

Brink, M., D. Becker, S. Terrill, and A. Jensen. 1959. Zinc toxicity in the weanling pig. Journal of Animal Science 18(2):836-842.

Bueschel, D. M., B. H. Jost, S. J. Billington, H. T. Trinh, and J. G. Songer. 2003. Prevalence of cpb2, encoding beta2 toxin, in Clostridium perfringens field isolates: correlation of genotype with phenotype. Veterinary Microbiology 94(2):121-129.

Bun, S., Y. Guo, F. Guo, F. Ji, and H. Cao. 2011. Influence of organic zinc supplementation on the antioxidant status and immune responses of broilers challenged with Eimeria tenella. Poultry Science 90(6):1220-1226.

Cafardi, V., M. Biagini, M. Martinelli, R. Leuzzi, J. T. Rubino, F. Cantini, N. Norais, M. Scarselli, D. Serruto, and M. Unnikrishnan. 2013. Identification of a novel zinc metalloprotease through a global analysis of Clostridium difficile extracellular proteins. PloS one 8(11)

Cao, J., J. A. Bobo, J. P. Liuzzi, and R. J. Cousins. 2001. Effects of intracellular zinc depletion on metallothionein and ZIP2 transporter expression and apoptosis. Journal of leukocyte biology 70(4):559-566.

Cao, J., P. Henry, S. Davis, R. Cousins, R. Miles, R. Littell, and C. Ammerman. 2002. Relative bioavailability of organic zinc sources based on tissue zinc and metallothionein in chicks fed conventional dietary zinc concentrations. Animal Feed Science and Technology 101(1-4):161-170.

Cao, J., P. Henry, R. Guo, R. Holwerda, J. Toth, R. Littell, R. Miles, and C. Ammerman. 2000. Chemical characteristics and relative bioavailability of supplemental organic zinc sources for poultry and ruminants. Journal of Animal Science 78(8):2039-2054.

Carpenter, A. C., J. R. Grainger, Y. Xiong, Y. Kanno, H. H. Chu, L. Wang, S. Naik, L. dos Santos, L. Wei, and M. K. Jenkins. 2012. The transcription factors Thpok and LRF are necessary and partly redundant for T helper cell differentiation. Immunity 37(4):622-633.

Caspar, M., I. Florin, and M. Thelestam. 1987. Calcium and calmodulin in cellular intoxication with Clostridium difficile toxin B. Journal of cellular physiology 132(1):168-172.

Cavell, P. A., and E. M. Widdowson. 1964. Intakes and excretions of iron, copper, and zinc in the neonatal period. Archives of Disease in Childhood 39(207):496.

Cha, J. O., J. Zhao, M. S. Yang, W. I. Kim, H. S. Cho, C. W. Lim, and B. Kim. 2018. Oocyst-shedding patterns of three Eimeria species in chickens and shedding pattern variation depending on the storage period of Eimeria tenella oocysts. Journal of Parasitology 104(1):18-22.

Chalmers, G., H. L. Bruce, D. B. Hunter, V. R. Parreira, R. R. Kulkarni, Y. F. Jiang, J. F. Prescott, and P. Boerlin. 2008. Multilocus sequence typing analysis of Clostridium perfringens isolates from necrotic enteritis outbreaks in broiler chicken populations. Journal of Clinical Microbiology 46(12):3957-3964. doi: 10.1128/jcm.01548-08

Chandra, R., and B. Au. 1980. Single nutrient deficiency and cell-mediated immune responses I. Zinc. American Journal of Clinical Nutrition 33(4):736-738.

Chavakis, T., A. E. May, K. T. Preissner, and S. M. Kanse. 1999. Molecular mechanisms of zinc-dependent leukocyte adhesion involving the urokinase receptor and β2-integrins. Blood 93(9):2976-2983.

Chen, J., N. Qu, and Y. Xia. 2005. Effect of zinc on thymulin level in mice. Wei sheng yan jiu= Journal of Hygiene Research 34(4):430-432.

Chimienti, F., S. Devergnas, A. Favier, and M. Seve. 2004. Identification and cloning of a β-cell–specific zinc transporter, ZnT-8, localized into insulin secretory granules. Diabetes 53(9):2330-2337.

Chimienti, F., A. Favier, and M. Seve. 2005. ZnT-8, a pancreatic beta-cell-specific zinc transporter. Biometals 18(4):313-317.

Cho, E., J. J. Hwang, S. H. Han, S. J. Chung, J. Y. Koh, and J. Y. Lee. 2010. Endogenous zinc mediates apoptotic programmed cell death in the developing brain. Neurotoxicity Research 17(2):156-166. doi: 10.1007/s12640-009-9085-2

Cobb-Vantress. 2018. Broiler Performance and Nutrition Supplement Cobb 500.

Cole, T. B., H. J. Wenzel, K. E. Kafer, P. A. Schwartzkroin, and R. D. Palmiter. 1999. Elimination of zinc from synaptic vesicles in the intact mouse brain by disruption of the ZnT3 gene. Proceedings of the National Academy of Sciences 96(4):1716-1721.

Collie, R. E., and B. A. McClane. 1998. Evidence that the enterotoxin gene can be episomal in Clostridium perfringens isolates associated with non-food-borne human gastrointestinal diseases. Journal of Clinical Microbiology 36(1):30-36.

Collier, C., C. Hofacre, A. Payne, D. Anderson, P. Kaiser, R. Mackie, and H. Gaskins. 2008. Coccidia-induced mucogenesis promotes the onset of necrotic enteritis by supporting Clostridium perfringens growth. Veterinary Immunology and Immunopathology 122(1-2):104-115.

Compton, M. M., and J. A. Cidlowski. 1992. Thymocyte apoptosis: a model of programmed cell death. Trends in endocrinology and metabolism: TEM 3(1):17-23.

Concordet, J. P., and A. Ferry. 1993. Physiological programmed cell death in thymocytes is induced by physical stress (exercise). American Journal of Physiology-Gastrointestinal and Liver Physiology 265(3 Pt 1):C626-629. doi: 10.1152/ajpcell.1993.265.3.C626

Cooper, K., H. Trinh, and J. G. Songer. 2009. Immunization with recombinant alpha toxin partially protects broiler chicks against experimental challenge with Clostridium perfringens. Veterinary Microbiology 133(1-2):92-97.

Cooper, K. K., and J. G. Songer. 2009. Necrotic enteritis in chickens: a paradigm of enteric infection by Clostridium perfringens type A. Anaerobe 15(1-2):55-60.

Cooper, K. K., and J. G. Songer. 2010. Virulence of Clostridium perfringens in an experimental model of poultry necrotic enteritis. Veterinary Microbiology 142(3-4):323-328.

Cooper, K. K., J. R. Theoret, B. A. Stewart, H. T. Trinh, R. D. Glock, and J. G. Songer. 2010. Virulence for chickens of Clostridium perfringens isolated from poultry and other sources. Anaerobe 16(3):289-292.

Copper, I. 2001. Dietary reference intakes for vitamin A vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. Washington, DC: The National Academies Press.

Cornillot, E., B. Saint-Joanis, G. Daube, S. i. Katayama, P. E. Granum, B. Canard, and S. T. Cole. 1995. The enterotoxin gene (cpe) of Clostridium perfringens can be chromosomal or plasmid-borne. Molecular Microbiology 15(4):639-647.

Costello, L. C., Y. Liu, J. Zou, and R. B. Franklin. 1999. Evidence for a zinc uptake transporter in human prostate cancer cells which is regulated by prolactin and testosterone. Journal of Biological Chemistry 274(25):17499-17504.

Coursodon, C. F., R. D. Glock, K. L. Moore, K. K. Cooper, and J. G. Songer. 2012. TpeL-producing strains of Clostridium perfringens type A are highly virulent for broiler chicks. Anaerobe 18(1):117-121. doi: 10.1016/j.anaerobe.2011.10.001

Cousins, R. 1989. Systemic transport of zinc, Zinc in Human Biology. Springer. p. 79-93.

Cousins, R. J., J. P. Liuzzi, and L. A. Lichten. 2006. Mammalian zinc transport, trafficking, and signals. Journal of Biological Chemistry 281(34):24085-24089.

Cousins, R. J., and R. J. McMahon. 2000a. Integrative aspects of zinc transporters. Journal of Nutrition 130(5):1384S-1387S.

Cousins, R. J., and R. J. McMahon. 2000b. Integrative aspects of zinc transporters. Nutrition 130(5):1384S-1387S.

Cowen, B. S., L. D. Schwartz, R. A. Wilson, and S. I. Ambrus. 1987. Experimentally induced necrotic enteritis in chickens. Avian Diseases 31(4):904-906.

Cox, D. H., and D. L. Harris. 1960. Effect of excess dietary zinc on iron and copper in the rat. Journal of Nutrition 70(4):514-520.

Crespo, R., D. J. Fisher, H. Shivaprasad, M. E. Fernández-Miyakawa, and F. A. Uzal. 2007a. Toxinotypes of Clostridium perfringens isolated from sick and healthy avian species. Journal of Veterinary Diagnostic Investigation 19(3):329-333.

Crespo, R., D. J. Fisher, H. Shivaprasad, M. E. Fernández-Miyakawa, and F. A. Uzal. 2007b. Toxinotypes of Clostridium perfringens isolated from sick and healthy avian species. Journal of Beterinary Diagnostic Investigation 19(3):329-333.

Cui, H., P. Xi, D. Junliang, L. Debing, and Y. Guang. 2004. Pathology of lymphoid organs in chickens fed a diet deficient in zinc. Avian Pathology 33(5):519-524.

Dahiya, J. P., D. Hoehler, A. G. Van Kessel, and M. D. Drew. 2007a. Dietary encapsulated glycine influences Clostridium perfringens and Lactobacilli growth in the gastrointestinal tract of broiler chickens. Journal of Nutrition 137(6):1408-1414. doi: 10.1093/jn/137.6.1408

Dahiya, J. P., D. Hoehler, A. G. Van Kessel, and M. D. Drew. 2007b. Dietary encapsulated glycine influences Clostridium perfringens and Lactobacilli growth in the gastrointestinal tract of broiler chickens. Nutrition 137(6):1408-1414. doi: 10.1093/jn/137.6.1408

Dahiya, J. P., D. C. Wilkie, A. G. Van Kessel, and M. D. Drew. 2006. Potential strategies for controlling necrotic enteritis in broiler chickens in post-antibiotic era. Animal Feed Science and Technology 129(1):60-88. doi: https://doi.org/10.1016/j.anifeedsci.2005.12.003

Dardenne, M., J. M. Pleau, B. Nabarra, P. Lefrancier, M. Derrien, J. Choay, and J. F. Bach. 1982. Contribution of zinc and other metals to the biological activity of the serum thymic factor. Proceedings of the National Academy of Sciences 79(17):5370-5373.

Del Cacho, E., M. Gallego, F. López-Bernad, J. Quílez, and C. Sánchez-Acedo. 2004. Expression of anti-apoptotic factors in cells parasitized by second-generation schizonts of Eimeria tenella and Eimeria necatrix. Veterinary parasitology 125(3-4):287-300.

DePasquale-Jardieu, P., and P. J. Fraker. 1979. The role of corticosterone in the loss in immune function in the zinc-deficient A/J mouse. Journal of Nutrition 109(11):1847-1855. doi: 10.1093/jn/109.11.1847

DePasquale-Jardieu, P., and P. J. Fraker. 1980a. Further characterization of the role of corticosterone in the loss of humoral immunity in zinc-deficient A/J mice as determined by adrenalectomy. Journal of Immunology 124(6):2650-2655.

DePasquale-Jardieu, P., and P. J. Fraker. 1980b. Further characterization of the role of corticosterone in the loss of humoral immunity in zinc-deficient A/J mice as determined by adrenalectomy. Immunogy 124(6):2650-2655.

DePasquale-Jardieu, P., and P. J. Fraker. 1984a. Interference in the development of a secondary immune response in mice by zinc deprivation: persistence of effects. Journal of Nutrition 114(10):1762-1769.

DePasquale-Jardieu, P., and P. J. Fraker. 1984b. Interference in the development of a secondary immune response in mice by zinc deprivation: persistence of effects. Nutrition 114(10):1762-1769.

Dinarello, C. A. 2011. A clinical perspective of IL-1β as the gatekeeper of inflammation. European journal of immunology 41(5):1203-1217.

Dollery, C. M., J. R. McEwan, and A. M. Henney. 1995. Matrix metalloproteinases and cardiovascular disease. Circulation Research 77(5):863-868.

Dowd, P. S., J. Kelleher, and P. J. Guillou. 1986. T-lymphocyte subsets and interleukin-2 production in zinc-deficient rats. British Journal of Nutrition 55(1):59-69.

Driessen, C., K. Hirv, L. Rink, and H. Kirchner. 1994a. Induction of cytokines by zinc ions in human peripheral blood mononuclear cells and separated monocytes. Lymphokine and cytokine research 13(1):15-20.

Driessen, C., K. Hirv, L. Rink, and H. Kirchner. 1994b. Induction of cytokines by zinc ions in human peripheral blood mononuclear cells and separated monocytes. Lymphokine and cytokine research 13(1):15-20.

Edgar, S. 1954. Effect of temperature on the sporulation of oocysts of the protozoan, Eimeria tenella. Transactions of the American Microscopical Society 73(3):237-242.

El-Katcha, M., M. A. Soltan, and M. El-Badry. 2017. Effect of Dietary Replacement of Inorganic Zinc by Organic or Nanoparticles Sources on Growth Performance, Immune Response and Intestinal Histopathology of Broiler Chicken. Alexandria Journal for Veterinary Sciences 55(2)

Engel, R., R. Miller, and N. Price. 1966. Metabolic patterns in preadolescent children. XIII. Zinc balance. Zinc Metabolism:326-338.

Engström, B., C. Fermer, A. Lindberg, E. Saarinen, V. Båverud, and A. Gunnarsson. 2003. Molecular typing of isolates of Clostridium perfringens from healthy and diseased poultry. Veterinary Microbiology 94(3):225-235.

Ercan, M. T., and N. M. Bor. 1991. Phagocytosis by macrophages in zinc-deficient rats. International Journal of Radiation Applications and Instrumentation 18(7):765-768.

Erdfelder, E., F. Faul, and A. Buchner. 1996. GPOWER: A general power analysis program. Behavior research methods, instruments, & computers 28(1):1-11.

Eryavuz, A., and B. A. Dehority. 2009. Effects of supplemental zinc concentration on cellulose digestion and cellulolytic and total bacterial numbers in vitro. Animal feed science and technology 151(3-4):175-183.

Fernando, M. A. 1974. Fine structure of the schizonts and merozoites of Eimeria acervulina in the chicken. The Journal of parasitology:149-159.

Ficko-Blean, E., C. P. Stuart, M. D. Suits, M. Cid, M. Tessier, R. J. Woods, and A. B. Boraston. 2012. Carbohydrate recognition by an architecturally complex α-N-acetylglucosaminidase from Clostridium perfringens. PLoS One 7(3):e33524.

Ficko-Blean, E., C. P. Stuart, and A. B. Boraston. 2011. Structural analysis of CPF_2247, a novel α-amylase from Clostridium perfringens. Proteins: Structure, Function, and Bioinformatics 79(10):2771-2777.

Foley, B., S. A. Johnson, B. Hackley, J. Smith Jr, and J. A. Halsted. 1968. Zinc content of human platelets. Proceedings of the Society for Experimental Biology and Medicine 128(1):265-269.

Follis, R. H., Jr., H. G. Day, and E. V. McCollum. 1941a. Histological Studies of the Tissues of Rats Fed a Diet Extremely Low in Zinc: Two Plates (Eleven Figures). Nutrition 22(3):223-237. doi: 10.1093/jn/22.3.223

Follis, R. H., Jr., H. G. Day, and E. V. McCollum. 1941b. Histological Studies of the Tissues of Rats Fed a Diet Extremely Low in Zinc: Two Plates (Eleven Figures). Journal of Nutrition 22(3):223-237. doi: 10.1093/jn/22.3.223

Forbes, R. M., and M. Yohe. 1960a. Zinc requirement and balance studies with the rat. Journal of Nutrition 70:53-57. doi: 10.1093/jn/70.1.53

Forbes, R. M., and M. Yohe. 1960b. Zinc requirement and balance studies with the rat. Nutrition 70:53-57. doi: 10.1093/jn/70.1.53

Fox, M. R. S., and B. N. Harrison. 1964. Use of Japanese Quail for the Study of Zinc Deficiency. Proceedings of the Society for Experimental Biology and Medicine 116(2):256-259. doi: 10.3181/00379727-116-29217

Fraker, P., M. Gershwin, R. Good, and A. Prasad. 1986. Interrelationships between zinc and immune function. In: Federation proceedings. p 1474-1479.

Fraker, P., and C. Zwickl. 1981. Immune Repair Capacity of Zinc-deficient Young-adult and Neonatal Mice. In: Federation proceedings. p 918-918.

Fraker, P. J., P. DePasquale-Jardieu, C. M. Zwickl, and R. W. Luecke. 1978. Regeneration of T-cell helper function in zinc-deficient adult mice. Proceedings of the National Academy of Sciences 75(11):5660-5664.

Fraker, P. J., S. M. Haas, and R. W. Luecke. 1977. Effect of zinc deficiency on the immune response of the young adult A/J mouse. Journal of Nutrition 107(10):1889-1895.

Fraker, P. J., K. Hildebrandt, and R. W. Luecke. 1984. Alteration of antibody-mediated responses of suckling mice to T-cell-dependent and independent antigens by maternal marginal zinc deficiency: restoration of responsivity by nutritional repletion. Journal of Nutrition 114(1):170-179.

Fraker, P. J., F. Osati-Ashtiani, M. A. Wagner, and L. E. King. 1995. Possible roles for glucocorticoids and apoptosis in the suppression of lymphopoiesis during zinc deficiency: a review. Journal of the American College of Nutrition 14(1):11-17.

Freedman, J. C., J. R. Theoret, J. A. Wisniewski, F. A. Uzal, J. I. Rood, and B. A. McClane. 2015. Clostridium perfringens type A–E toxin plasmids. Research in Microbiology 166(4):264-279.

Fujioka, M., and I. Lieberman. 1964. A Zn++ Rrequirement for Synthesis of Deoxribonucleic Acid by Rat Live. Journal of Biological Chemistry 239:1164-1167.

Fujita, K., J. Katahira, Y. Horiguchi, N. Sonoda, M. Furuse, and S. Tsukita. 2000. Clostridium perfringens enterotoxin binds to the second extracellular loop of claudin-3, a tight junction integral membrane protein. FEBS letters 476(3):258-261.

Gajula, S. S., V. K. Chelasani, A. K. Panda, V. R. Mantena, and R. R. Savaram. 2011. Effect of supplemental inorganic Zn and Mn and their interactions on the performance of broiler

chicken, mineral bioavailability, and immune response. Biological trace element research 139(2):177-187.

Garnica, J., R. Achata, and P. Bravo. 1993. Physical and biochemical characteristics of alpaca semen. Animal Reproduction Science 32(1-2):85-90.

Garvy, B. A., L. E. King, W. G. Telford, L. A. Morford, and P. J. Fraker. 1993. Chronic elevation of plasma corticosterone causes reductions in the number of cycling cells of the B lineage in murine bone marrow and induces apoptosis. Immunology 80(4):587-592.

Gholamiandehkordi, A. R., L. Timbermont, A. Lanckriet, W. V. D. Broeck, K. Pedersen, J. Dewulf, F. Pasmans, F. Haesebrouck, R. Ducatelle, and F. V. Immerseel. 2007. Quantification of gut lesions in a subclinical necrotic enteritis model. Avian Pathology 36(5):375-382.

Gholamiandekhordi, A. R., R. Ducatelle, M. Heyndrickx, F. Haesebrouck, and F. Van Immerseel. 2006. Molecular and phenotypical characterization of Clostridium perfringens isolates from poultry flocks with different disease status. Veterinary Microbiology 113(1-2):143-152.

Gibert, M., C. Jolivet-Renaud, and M. R. Popoff. 1997. Beta2 toxin, a novel toxin produced by Clostridium perfringens. Gene 203(1):65-73.

Gierl, M. S., N. Karoulias, H. Wende, M. Strehle, and C. Birchmeier. 2006. The zinc-finger factor Insm1 (IA-1) is essential for the development of pancreatic β cells and intestinal endocrine cells. Genes & development 20(17):2465-2478.

Gilbert, I., and D. Taylor. 1956. The behaviour of zinc and radio-zinc in the rat. Biochimica et Biophysica Acta 21(3):545-551.

Gou, Z., S. Jiang, C. Zheng, Z. Tian, and X. Lin. 2015. Equol inhibits LPS-induced oxidative stress and enhances the immune response in chicken HD11 macrophages. Cellular Physiology and Biochemistry 36(2):611-621.

Grant-Frost, D., and E. Underwood. 1958. Zinc toxicity in the rat and its interrelation with copper. Australian Journal of Experimental Biology and Medical Science 36(4):339-346.

Gropper, S. S., and J. L. Smith. 2012. Advanced nutrition and human metabolism. Cengage Learning.

Gumulec, J., M. Raudenska, V. Adam, R. Kizek, and M. Masarik. 2014. Metallothionein–immunohistochemical cancer biomarker: a meta-analysis. PLoS One 9(1):e85346.

Gunn, S. A., and T. C. Gould. 1956. The relative importance of androgen and estrogen in the selective uptake of Zn65 by the dorsolateral prostate of the rat. Endocrinology 58(4):443-452.

Györke, A., L. Pop, and V. Cozma. 2013. Prevalence and distribution of Eimeria species in broiler chicken farms of different capacities. Parasite 20

Harris, J. E., K. D. Bishop, N. E. Phillips, J. P. Mordes, D. L. Greiner, A. A. Rossini, and M. P. Czech. 2004. Early growth response gene-2, a zinc-finger transcription factor, is required for full induction of clonal anergy in CD4+ T cells. The Journal of Immunology 173(12):7331-7338.

He, X., X. He, V. P. Dave, Y. Zhang, X. Hua, E. Nicolas, W. Xu, B. A. Roe, and D. J. Kappes. 2005. The zinc finger transcription factor Th-POK regulates CD4 versus CD8 T-cell lineage commitment. Nature 433(7028):826-833.

Heller, V., and A. Burke. 1927. Toxicity of Zinc. Journal of Biological Chemistry 74:85-93.

Heth, D. A., W. M. Becker, and W. G. Hoekstra. 1966a. Effect of calcium, phosphorus and zinc on zinc-65 absorption and turnover in rats fed semipurified diets. Journal of Nutrition 88(3):331-337. doi: 10.1093/jn/88.3.331

Heth, D. A., and W. G. Hoekstra. 1965. Zinc-65 Absorption and Turnover in Rats. I. A Procedure to Determine Zinc-65 Absorption and the Antagonistic Effect of Calcium in a Practical Diet. Journal of Nutrition 85(4):367-374. doi: 10.1093/jn/85.4.367

Heth, D. A., M. L. Sunde, and W. G. Hoekstra. 1966b. Influence of dietary calcium and zinc on zinc-65 metabolism in laying hens and their progeny. Poultry Science 45(1):75-83. doi: 10.3382/ps.0450075

Ho, E., and B. N. Ames. 2002. Low intracellular zinc induces oxidative DNA damage, disrupts p53, NFκB, and AP1 DNA binding, and affects DNA repair in a rat glioma cell line. Proceedings of the National Academy of Sciences 99(26):16770-16775.

Hockenbery, D. 1995. Defining apoptosis. The American Journal of Pathology 146(1):16.

Hoekstra, W., E. Faltin, C. Lin, H. Roberts, and R. Grummer. 1967. Zinc Deficiency in Reproducing Gilts Fed a Diet high in Calcium and its Effect on Tissue Zinc and Blood Serum Alkaline Phosphatase 1, 2. Journal of Animal Science 26(6):1348-1357.

Hoekstra, W. G. 1964a. Recent Observations on Mineral Interrelationships. Federation proceedings 23:1068-1076.

Hoekstra, W. G. 1964b. Recent Observations on Mineral Interreplationships. Federation proceedings 23:1068-1076.

Hofacre, C., T. Beacorn, S. Collett, and G. Mathis. 2003. Using competitive exclusion, mannan-oligosaccharide and other intestinal products to control necrotic enteritis. Journal of Applied Poultry Research 12(1):60-64.

Hofacre, C., R. Froyman, B. Gautrias, B. George, M. Goodwin, and J. Brown. 1998. Use of Aviguard and other intestinal bioproducts in experimental Clostridium perfringens-associated necrotizing enteritis in broiler chickens. Avian Diseases:579-584.

Hofacre, C. L. 2001. Necrotic enteritis, currently a billion dollar disease: is there anything new on the horizon. In: Proceedings of Alltech's 17th Annual Symposium Science and Technology in the Feed Industry. p 79-86.

Hofacre, C. L., D. J. Reynolds, G. F. Mathis, B. S. Lumpkins, N. Ollis, J. A. Smith, and V. Demey. 2019. Effect of a Competitive Exclusion Culture in a Necrotic Enteritis Challenge Model in Broilers. The Journal of Applied Poultry Research

Hong, Y. H., H. S. Lillehoj, S. H. Lee, R. A. Dalloul, and E. P. Lillehoj. 2006. Analysis of chicken cytokine and chemokine gene expression following Eimeria acervulina and Eimeria tenella infections. Veterinary Immunology and Immunopathology 114(3-4):209-223.

Hove, E., C. Elvehjem, and E. Hart. 1938. Further studies on zinc deficiency in rats. American Journal of Physiology-Legacy Content 124(3):750-758.

Hsu, J., and W. Anthony. 1970. Zinc deficiency and urinary excretion of taurine-35S and inorganic sulfate-35S following cystine-35S injection in rats. Journal of Nutrition 100(10):1189-1195.

Hsu, J., W. Anthony, and P. Buchanan. 1969. Zinc deficiency and incorporation of 14C-labeled methionine into tissue proteins in rats. Journal of Nutrition 99(4):425-432.

Hsu, J. M., W. L. Anthony, and P. J. Buchanan. 1968. Incorporation of glycine-1-14C into liver glutathione in zinc deficient rats. Proceedings of the Society for Experimental Biology and Medicine 127(4):1048-1051.

Hu, C., Z. Qian, J. Song, Z. Luan, and A. Zuo. 2013. Effects of zinc oxide-montmorillonite hybrid on growth performance, intestinal structure, and function of broiler chicken. Poultry Science 92(1):143-150.

Huang, L., and S. Tepaamorndech. 2013. The SLC30 family of zinc transporters–a review of current understanding of their biological and pathophysiological roles. Molecular Aspects of Medicine 34(2-3):548-560.

Hurley, L. S., and H. Swenerton. 1966. Congenital malformations resulting from zinc deficiency in rats. Proceedings of the Society for Experimental Biology and Medicine 123(3):692-696.

Huse, M., M. J. Eck, and S. C. Harrison. 1998. A Zn2+ ion links the cytoplasmic tail of CD4 and the N-terminal region of Lck. Journal of Biological Chemistry 273(30):18729-18733.

Ibs, K.-H., and L. Rink. 2003. Zinc-altered immune function. Journal of Nutrition 133(5):1452S-1456S.

Iji, P., and D. Tivey. 1998. Natural and synthetic oligosaccharides in broiler chicken diets. World's Poultry Science Journal 54(2):129-143.

Iwata, T., G. S. Incefy, S. Cunningham-Rundles, C. Cunningham-Rundles, E. Smithwick, N. Geller, R. O'Reilly, and R. A. Good. 1981. Circulating thymic hormone activity in patients with primary and secondary immunodeficiency diseases. The American journal of medicine 71(3):385-394.

Iwata, T., G. S. Incefy, T. Tanaka, G. Fernandes, C. J. Menendez-Botet, K. Pih, and R. A. Good. 1979. Circulating thymic hormone levels in zinc deficiency. Cell Immunology 47(1):100-105.

Jackson, M., D. Jones, and R. Edwards. 1982. Tissue zinc levels as an index of body zinc status. Clinical Physiology 2(4):333-343.

Janeway, C., P. Travers, M. Walport, and M. Schlomchik. 2004. Immunobiology: The Immune System in Health and Diseases.

Jarosz, Ł., A. Marek, Z. Grądzki, M. Kwiecień, and M. Kalinowski. 2017. The effect of feed supplementation with zinc chelate and zinc sulphate on selected humoral and cell-mediated immune parameters and cytokine concentration in broiler chickens. Research in veterinary science 112:59-65.

Jeong, J., J. M. Walker, F. Wang, J. G. Park, A. E. Palmer, C. Giunta, M. Rohrbach, B. Steinmann, and D. J. Eide. 2012. Promotion of vesicular zinc efflux by ZIP13 and its implications for spondylocheiro dysplastic Ehlers–Danlos syndrome. Proceedings of the National Academy of Sciences 109(51):E3530-E3538.

Johnson, J., and W. M. Reid. 1970. Anticoccidial drugs: lesion scoring techniques in battery and floor-pen experiments with chickens. Experimental parasitology 28(1):30-36.

Johnson, P. E., C. D. Hunt, D. B. Milne, and L. K. Mullen. 1993a. Homeostatic control of zinc metabolism in men: zinc excretion and balance in men fed diets low in zinc. The American journal of clinical nutrition 57(4):557-565.

Johnson, P. E., C. D. Hunt, D. B. Milne, and L. K. Mullen. 1993b. Homeostatic control of zinc metabolism in men: zinc excretion and balance in men fed diets low in zinc. Am J Clin Nutr 57(4):557-565. doi: 10.1093/ajcn/57.4.557

Joshua, P. P., C. Valli, and V. Balakrishnan. 2016. Effect of in ovo supplementation of nano forms of zinc, copper, and selenium on post-hatch performance of broiler chicken. Veterinary world 9(3):287.

Juśkiewicz, J., Z. Zduńczyk, and J. Jankowski. 2004. Selected parameters of gastrointestinal tract metabolism of turkeys fed diets with flavomycin and different inulin content. World's Poultry Science Journal 60(2):177-185.

Kagara, N., N. Tanaka, S. Noguchi, and T. Hirano. 2007. Zinc and its transporter ZIP10 are involved in invasive behavior of breast cancer cells. Cancer Science 98(5):692-697.

Kaldhusdal, M., M. Hofshagen, A. Lovland, H. Langstrand, and K. Redhead. 1999. Necrotic enteritis challenge models with broiler chickens raised on litter: evaluation of preconditions, Clostridium perfringens strains and outcome variables. FEMS immunology and medical microbiology 24(3):337-343. doi: 10.1111/j.1574-695X.1999.tb01303.x

Kaldhusdal, M., and M. Hofshangen. 1992. Barley inclusion and avoparcin supplementation in broiler diets. 2. Clinical, pathological, and bacteriological findings in a mild form of necrotic enteritis. Poultry Science 71(7):1145-1153.

Kambe, T., H. Narita, Y. Yamaguchi-Iwai, J. Hirose, T. Amano, N. Sugiura, R. Sasaki, K. Mori, T. Iwanaga, and M. Nagao. 2002. Cloning and characterization of a novel mammalian zinc transporter, ZnT-5, abundantly expressed in pancreatic beta cells. Journal of Biological Chemistry

Karsunky, H., H. Zeng, T. Schmidt, B. Zevnik, R. Kluge, K. W. Schmid, U. Dührsen, and T. Möröy. 2002. Inflammatory reactions and severe neutropenia in mice lacking the transcriptional repressor Gfi1. Nature Genetics 30(3):295.

Katahira, J., N. Inoue, Y. Horiguchi, M. Matsuda, and N. Sugimoto. 1997a. Molecular cloning and functional characterization of the receptor for Clostridium perfringens enterotoxin. Journal of Cell Biology 136(6):1239-1247.

Katahira, J., H. Sugiyama, N. Inoue, Y. Horiguchi, M. Matsuda, and N. Sugimoto. 1997b. Clostridium perfringens enterotoxin utilizes two structurally related membrane proteins as functional receptors in vivo. Journal of Biological Chemistry 272(42):26652-26658.

Katayama, S., B. Dupuy, S. Cole, G. Daube, and B. China. 1996. Genome mapping ofClostridium perfringens strains with I-CeuI shows many virulence genes to be plasmid-borne. Molecular and General Genetics MGG 251(6):720-726.

Katz, J. P., N. Perreault, B. G. Goldstein, C. S. Lee, P. A. Labosky, V. W. Yang, and K. H. Kaestner. 2002. The zinc-finger transcription factor Klf4 is required for terminal differentiation of goblet cells in the colon. Development 129(11):2619-2628.

Kelleher, S. L., and B. Lönnerdal. 2002. Zinc transporters in the rat mammary gland respond to marginal zinc and vitamin A intakes during lactation. Journal of Nutrition 132(11):3280-3285.

Keyburn, A. L., J. D. Boyce, P. Vaz, T. L. Bannam, M. E. Ford, D. Parker, A. Di Rubbo, J. I. Rood, and R. J. Moore. 2008. NetB, a new toxin that is associated with avian necrotic enteritis caused by Clostridium perfringens. PLoS Pathogens 4(2):e26.

Keyburn, A. L., S. A. Sheedy, M. E. Ford, M. M. Williamson, M. M. Awad, J. I. Rood, and R. J. Moore. 2006. Alpha-toxin of Clostridium perfringens is not an essential virulence factor in necrotic enteritis in chickens. Infection and Immunity 74(11):6496-6500.

Keyburn, A. L., X.-X. Yan, T. L. Bannam, F. Van Immerseel, J. I. Rood, and R. J. Moore. 2010. Association between avian necrotic enteritis and Clostridium perfringens strains expressing NetB toxin. Veterinary Research 41(2):1-8.

Kienholz, E. W., D. E. Turk, M. L. Sunde, and W. G. Hoekstra. 1961a. Effects of zinc deficiency in the diets of hens'. Nutrition 75(2):211-221. doi: 10.1093/jn/75.2.211

Kienholz, E. W., D. E. Turk, M. L. Sunde, and W. G. Hoekstra. 1961b. Effects of zinc deficiency in the diets of hens'. Journal of Nutrition 75(2):211-221. doi: 10.1093/jn/75.2.211

King, L. E., J. W. Frentzel, J. J. Mann, and P. J. Fraker. 2005. Chronic zinc deficiency in mice disrupted T cell lymphopoiesis and erythropoiesis while B cell lymphopoiesis and myelopoiesis were maintained. Journal of the American College of Nutrition 24(6):494-502.

Kirchgessner, M. 1993. Homeostasis and homeorhesis in trace element metabolism. Trace Elements in Man and Animals 8:4-21.

Kirschke, C. P., and L. Huang. 2003. ZnT7, a novel mammalian zinc transporter, accumulates zinc in the Golgi apparatus. Journal of Biological Chemistry 278(6):4096-4102.

Kitamura, H., H. Morikawa, H. Kamon, M. Iguchi, S. Hojyo, T. Fukada, S. Yamashita, T. Kaisho, S. Akira, M. Murakami, and T. Hirano. 2006. Toll-like receptor-mediated regulation of zinc homeostasis influences dendritic cell function. Nature Immunology 7(9):971-977. doi: 10.1038/ni1373

Kleessen, B., L. Hartmann, and M. Blaut. 2003. Fructans in the diet cause alterations of intestinal mucosal architecture, released mucins and mucosa-associated bifidobacteria in gnotobiotic rats. British Journal of Nutrition 89(5):597-606.

Kondo, F. 1988. In vitro lecithinase activity and sensitivity to 22 antimicrobial agents of Clostridium perfringens isolated from necrotic enteritis of broiler chickens. Research in veterinary science 45(3):337-340.

Kratzer, F. H., P. Vohra, J. B. Allred, and P. N. Davis. 1958. Effect of zinc upon growth and incidence of perosis in turkey poults. Proceedings of the Society for Experimental Biology and Medicine 98(1):205-207.

Krishnaraju, K., H. Q. Nguyen, D. A. Liebermann, and B. Hoffman. 1995. The zinc finger transcription factor Egr-1 potentiates macrophage differentiation of hematopoietic cells. Molecular and cellular biology 15(10):5499-5507.

Ku, P., D. Ullrey, E. Miller, and C. Mills. 1970. Zinc deficiency and tissue nucleic acid and protein concentration. E & S Livingstone

Kurtoğlu, F., V. Kurtoğlu, I. Celik, T. Kececi, and M. Nizamlioğlu. 2005. Effects of dietary boron supplementation on some biochemical parameters, peripheral blood lymphocytes, splenic plasma cells and bone characteristics of broiler chicks given diets with adequate or inadequate cholecalciferol (vitamin D3) content. British Journal of Poultry Science 46(1):87-96.

Kwiecień, M., A. Winiarska-Mieczan, A. Milczarek, and R. Klebaniuk. 2017. Biological Response of Broiler Chickens to Decreasing Dietary Inclusion Levels of Zinc Glycine Chelate. Biological Trace Element Research 175(1):204-213. (journal article) doi: 10.1007/s12011-016-0743-y

Kwiecień, M., A. Winiarska-Mieczan, A. Milczarek, E. Tomaszewska, and J. Matras. 2016. Effects of zinc glycine chelate on growth performance, carcass characteristics, bone quality, and mineral content in bone of broiler chicken. Livestock Science 191:43-50.

Kwon, H.-S., H. W. Lim, J. Wu, M. Schnölzer, E. Verdin, and M. Ott. 2012. Three novel acetylation sites in the Foxp3 transcription factor regulate the suppressive activity of regulatory T cells. Journal of Immunology 188(6):2712-2721.

Lal, K., E. Bromley, R. Oakes, J. H. Prieto, S. J. Sanderson, D. Kurian, L. Hunt, J. R. Yates, 3rd, J. M. Wastling, R. E. Sinden, and F. M. Tomley. 2009. Proteomic comparison of four

Eimeria tenella life-cycle stages: unsporulated oocyst, sporulated oocyst, sporozoite and second-generation merozoite. Proteomics 9(19):4566-4576. doi: 10.1002/pmic.200900305

Lee, D.-Y., A. S. Prasad, C. Hydrick-Adair, G. Brewer, and P. E. Johnson. 1993. Homeostasis of zinc in marginal human zinc deficiency: role of absorption and endogenous excretion of zinc. Translational Research 122(5):549-556.

Lee, H. H., A. S. Prasad, G. J. Brewer, and C. Owyang. 1989. Zinc absorption in human small intestine. American Journal of Physiology-Gastrointestinal and Liver Physiology 256(1):G87-G91.

Lee, K., H. Lillehoj, M. Park, S. Jang, G. Ritter, Y. Hong, W. Jeong, H. Jeoung, D. An, and E. Lillehoj. 2012. Clostridium perfringens α-toxin and NetB toxin antibodies and their possible role in protection against necrotic enteritis and gangrenous dermatitis in broiler chickens. Avian diseases 56(1):230-233.

Lee, K., H. S. Lillehoj, and G. R. Siragusa. 2010. Direct-fed microbials and their impact on the intestinal microflora and immune system of chickens. The journal of poultry science:1001160039-1001160039.

Lee, S., H. Lillehoj, S. Jang, M. Jeong, S. Xu, J. Kim, H. Park, H. Kim, E. Lillehoj, and D. Bravo. 2014a. Effects of in ovo injection with selenium on immune and antioxidant responses during experimental necrotic enteritis in broiler chickens. Poultry science 93(5):1113-1121.

Lee, S. H., H. S. Lillehoj, S. I. Jang, M. Jeong, D. K. Kim, S. Xu, S. K. Lee, J. B. Kim, H. J. Park, H. R. Kim, and D. M. Bravo. 2014b. Immune and anti-oxidant effects of in ovo selenium proteinate on post-hatch experimental avian necrotic enteritis. Veterinary parasitology 206(3-4):115-122. doi: 10.1016/j.vetpar.2014.10.025

Lepp, D., J. Gong, J. Songer, P. Boerlin, V. Parreira, and J. Prescott. 2013. Identification of accessory genome regions in poultry Clostridium perfringens isolates carrying the netB plasmid. Journal of Bacteriology 195(6):1152-1166.

Lewis Jr, P., W. Hoekstra, R. Grummer, and P. Phillips. 1956. The effect of certain nutritional factors including calcium, phosphorus and zinc on parakeratosis in swine. Journal of Animal Science 15(3):741-751.

Lewis, P., W. Hoekstra, and R. Grummer. 1957a. Restricted Calcium Feeding Versus Zinc Supplementation for the Control of Parakeratosis in Swine. Journal of Animal Science 16(3):578-588.

Lewis, P., W. Hoekstra, and R. Grummer. 1957b. Restricted Calcium Feeding versus Zinc Supplementation for the Control of Parakerotosis in Swine. Journal of Animal Science 16(3):578-588.

Li, S., L. Lu, S. Hao, Y. Wang, L. Zhang, S. Liu, B. Liu, K. Li, and X. Luo. 2011. Dietary manganese modulates expression of the manganese-containing superoxide dismutase gene in chickens. The Journal of Nutrition 141(2):189-194.

Lin, R. S., C. Rodriguez, A. Veillette, and H. F. Lodish. 1998. Zinc is essential for binding of p56 lck to CD4 and CD8α. Journal of Biological Chemistry 273(49):32878-32882.

Liptrap, D., E. Miller, D. Ullrey, D. Whitenack, B. Schoepke, and R. Luecke. 1970. Sex influence on the zinc requirement of developing swine. Journal of Animal Science 30(5):736-741.

Liu, M., S. Bao, M. Galvez-Peralta, C. Pyle, A. Rudawsky, R. Pavlovicz, D. Killilea, C. Li, D. Nebert, and M. Wewers. 2013. ZIP8 regulates host defense through zinc-mediated inhibition of NF-kappaB. Cell Rep 3: 386–400.

Liuzzi, J. P., R. K. Blanchard, and R. J. Cousins. 2001. Differential regulation of zinc transporter 1, 2, and 4 mRNA expression by dietary zinc in rats. Journal of Nutrition 131(1):46-52.

Liuzzi, J. P., and R. J. Cousins. 2004. Mammalian zinc transporters. Annual Review of Nutrition 24:151-172.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods 25(4):402-408.

Livingston, H. D., H. Smith, and N. Stojanovic. 1967. Simultaneous estimation of copper, zinc, cadmium and mercury in biological material by neutron activation analysis. Talanta 14(4):505-513.

Lodemann, U., R. Einspanier, F. Scharfen, H. Martens, and A. Bondzio. 2013. Effects of zinc on epithelial barrier properties and viability in a human and a porcine intestinal cell culture model. Toxicology in vitro 27(2):834-843.

Long, J. R., and R. B. Truscott. 1976. Necrotic enteritis in broiler chickens. III. Reproduction of the disease. Canadian Journal of Comparative Medicine 40(1):53-59.

Long, P. 1965. Development of Eimeria tenella in avian embryos. Nature 208(5009):509-510.

Lovell, M. A., J. L. Smith, and W. R. Markesbery. 2006. Elevated zinc transporter-6 in mild cognitive impairment, Alzheimer disease, and pick disease. Journal of Neuropathology & Experimental Neurology 65(5):489-498.

Lovland, A., M. Kaldhusdal, K. Redhead, E. Skjerve, and A. Lillehaug. 2004. Maternal vaccination against subclinical necrotic enteritis in broilers. Avian Pathology 33(1):81-90.

Low, B. W., F. Hirshfeld, and F. Richards. 1959. Glycinate complexes of zinc and cadmium. Journal of the American Chemical Society 81(16):4412-4416.

Luecke, R., J. Hoefer, W. Brammell, and D. Schmidt. 1957. Calcium and Zinc in Parakeratosis of Swine 1. Journal of Animal Science 16(1):3-11.

Luecke, R. W., M. E. Olman, and B. V. Baltzer. 1968. Zinc deficiency in the rat: effect on serum and intestinal alkaline phosphatase activities. Journal of Nutrition 94(3):344-350. doi: 10.1093/jn/94.3.344

Luecke, R. W., C. E. Simonel, and P. J. Fraker. 1978. The effect of restricted dietary intake on the antibody mediated response of the zinc deficient A/J mouse. Journal of Nutrition 108(5):881-887.

Luoma, A. E. 2016. Effect of synbiotic and organic acid plus phytochemical product supplementation on layer production performance and immune parameters, The Ohio State University.

Lutz, R. E. 1926. The Normal Occurrence of Zinc in Biologic Materials: a Review of the Literature, and a Study of the Normal Distribution of Zinc in the Rat, Cat, and Man. Journal of Industrial Hygiene 8:177-207.

Macapinlac, M., G. Barney, W. Pearson, and W. Darby. 1967. Production of zinc deficiency in the squirrel monkey (Saimiri sciureus). Journal of Nutrition 93(4):499-510.

MacDonald, R. S. 2000a. The role of zinc in growth and cell proliferation. Journal of Nutrition 130(5):1500S-1508S.

MacDonald, R. S. 2000b. The role of zinc in growth and cell proliferation. The Journal of nutrition 130(5):1500S-1508S.

Magee, A. C., and G. Matrone. 1960. Studies on growth, copper metabolism and iron metabolism of rats fed high levels of zinc. Journal of Nutrition 72(2):233-242.

Mählmeyer, A., J. Lindel, A. Schlagheck, B. Hildebrand, and K. Männer. 2018. Investigation on the Effect of Trace Mineral Source on Parameters of Bioavailability in Broiler Chickens. Veterinarija ir Zootechnika 76(98)

Majno, G., and I. Joris. 1995. Apoptosis, oncosis, and necrosis. An overview of cell death. The American Journal of Pathology 146(1):3.

Maret, W. 2005. Zinc coordination environments in proteins determine zinc functions. Journal of Trace Elements in Medicine and Biology 19(1):7-12.

Marikovsky, M., V. Ziv, N. Nevo, C. Harris-Cerruti, and O. Mahler. 2003. Cu/Zn superoxide dismutase plays important role in immune response. Journal of Immunology 170(6):2993-3001.

Markazi, A., A. Luoma, R. Shanmugasundaram, M. Mohnl, G. Raj Murugesan, and R. Selvaraj. 2018. Effects of drinking water synbiotic supplementation in laying hens challenged with Salmonella. Poultry Science 97(10):3510-3518.

Markazi, A. D., V. Perez, M. Sifri, R. Shanmugasundaram, and R. K. Selvaraj. 2017. Effect of whole yeast cell product supplementation (CitriStim®) on immune responses and cecal microflora species in pullet and layer chickens during an experimental coccidial challenge. Poultry science 96(7):2049-2056.

Martin, A. B., T. B. Aydemir, G. J. Guthrie, D. A. Samuelson, S.-M. Chang, and R. J. Cousins. 2013. Gastric and Colonic Zinc Transporter ZIP11 (Slc39a11) in Mice Responds to Dietary Zinc and Exhibits Nuclear Localization–3. Journal of Nutrition 143(12):1882-1888.

Matsuura, W., T. Yamazaki, Y. Yamaguchi-Iwai, S. Masuda, M. Nagao, G. K. Andrews, and T. Kambe. 2009. SLC39A9 (ZIP9) regulates zinc homeostasis in the secretory pathway:

characterization of the ZIP subfamily I protein in vertebrate cells. Bioscience, biotechnology, and biochemistry 73(5):1142-1148.

Mawson, C. A., and M. I. Fischer. 1953. Zinc and carbonic anhydrase in human semen. Biochemical Journal 55(4):696-700.

Mayer, L. S., P. Uciechowski, S. Meyer, T. Schwerdtle, L. Rink, and H. Haase. 2014. Differential impact of zinc deficiency on phagocytosis, oxidative burst, and production of pro-inflammatory cytokines by human monocytes. Metallomics 6(7):1288-1295.

McCance, R., and E. M. Widdowson. 1942. Mineral metabolism of healthy adults on white and brown bread dietaries. Journal of Physiology 101(1):44-85.

McDevitt, R. M., J. D. Brooker, T. Acamovic, and N. H. C. Sparks. 2006. Necrotic enteritis; a continuing challenge for the poultry industry. World's Poultry Science Journal 62(2):221-247. doi: 10.1079/WPS200593

McDougald, L., and R. Galloway. 1973. Eimeria tenella: anticoccidial drug activity in cell cultures. Experimental Parasitology 34(2):189-196.

McMahon, R. J., and R. J. Cousins. 1998. Regulation of the zinc transporter ZnT-1 by dietary zinc. Proceedings of the National Academy of Sciences 95(9):4841-4846.

McQuibban, G. A., J.-H. Gong, J. P. Wong, J. L. Wallace, I. Clark-Lewis, and C. M. Overall. 2002. Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties in vivo. Blood 100(4):1160-1167.

McReynolds, J., J. Byrd, R. Anderson, R. Moore, T. Edrington, K. Genovese, T. Poole, L. Kubena, and D. Nisbet. 2004a. Evaluation of immunosuppressants and dietary mechanisms in an experimental disease model for necrotic enteritis. Poultry Science 83(12):1948-1952.

McReynolds, J., C. Waneck, J. Byrd, K. Genovese, S. Duke, and D. Nisbet. 2009. Efficacy of multistrain direct-fed microbial and phytogenetic products in reducing necrotic enteritis in commercial broilers. Poultry Science 88(10):2075-2080. doi: 10.3382/ps.2009-00106

McReynolds, J. L., J. A. Byrd, R. C. Anderson, R. W. Moore, T. S. Edrington, K. J. Genovese, T. L. Poole, L. F. Kubena, and D. J. Nisbet. 2004b. Evaluation of immunosuppressants and dietary mechanisms in an experimental disease model for necrotic enteritis. Poultry Science 83(12):1948-1952. doi: 10.1093/ps/83.12.1948

McReynolds, J. L., J. A. Byrd, K. J. Genovese, T. L. Poole, S. E. Duke, M. B. Farnell, and D. J. Nisbet. 2007a. Dietary lactose and its effect on the disease condition of necrotic enteritis. Poultry Sciences 86(8):1656-1661. doi: 10.1093/ps/86.8.1656

McReynolds, J. L., J. A. Byrd, K. J. Genovese, T. L. Poole, S. E. Duke, M. B. Farnell, and D. J. Nisbet. 2007b. Dietary lactose and its effect on the disease condition of necrotic enteritis. Poultry Science 86(8):1656-1661. doi: 10.1093/ps/86.8.1656

Meerarani, P., G. Reiterer, M. Toborek, and B. Hennig. 2003. Zinc modulates PPARγ signaling and activation of porcine endothelial cells. Journal of Nutrition 133(10):3058-3064.

Menghini, R., L. Fiorentino, V. Casagrande, R. Lauro, and M. Federici. 2013. The role of ADAM17 in metabolic inflammation. Atherosclerosis 228(1):12-17.

Merithew, E., C. Stone, S. Eathiraj, and D. G. Lambright. 2003. Determinants of Rab5 interaction with the N terminus of early endosome antigen 1. Journal of Biological Chemistry 278(10):8494-8500.

Methfessel, A. H., and H. Spencer. 1973. Zinc metabolism in the rat. II. Secretion of zinc into intestine. Journal of applied physiology 34(1):63-67. doi: 10.1152/jappl.1973.34.1.63

Mikkelsen, L. L., J. K. Vidanarachchi, C. G. Olnood, Y. M. Bao, P. H. Selle, and M. Choct. 2009. Effect of potassium diformate on growth performance and gut microbiota in broiler chickens challenged with necrotic enteritis. British Journal of Poultry Science 50(1):66-75. doi: 10.1080/00071660802613252

Millar, M., N. R. Vincent, and C. Mawson. 1961. An autoradiographic study of the distribution of zinc-65 in rat tissues. Journal of Histochemistry & Cytochemistry 9(2):111-116.

Millar, M. J., P. Elcoate, and C. A. Mawson. 1957. Sex hormone control of the zinc content of the prostate. Canadian Journal of Biochemistry and Physiology 35(11):865-868.

Millar, M. J., M. I. Fischer, P. V. Elcoate, and C. A. Mawson. 1958a. The effects of dietary zinc deficiency on the reproductive system of male rats. Canadian Journal of Biochemistry and Physiology 36(6):557-569.

Millar, M. J., M. I. Fischer, P. V. Elcoate, and C. A. Mawson. 1958b. The effects of dietary zinc deficiency on the reproductive system of male rats. Candadian Journal of Biochemistry and Physiology 36(6):557-569.

Millard, B., J. Bradley, and P. Long. 1971. The schizogony of Eimeria maxima in the chicken. Zeitschrift für Parasitenkunde 38(1):77-81.

Miller, E., R. Luecke, D. Ullrey, B. V. Baltzer, B. Bradley, and J. Hoefer. 1968a. Biochemical, skeletal and allometric changes due to zinc deficiency in the baby pig. Journal of Nutrition 95(2):278-286.

Miller, E. R., R. W. Luecke, D. E. Ullrey, B. V. Baltzer, B. L. Bradley, and J. A. Hoefer. 1968b. Biochemical, skeletal and allometric changes due to zinc deficiency in the baby pig. Journal of Nutrition 95(2):278-286. doi: 10.1093/jn/95.2.278

Miller, J. K., and W. J. Miller. 1960. Development of zinc deficiency in Holstein calves fed a purified diet. Journal of Dairy Science 43:1854-1856.

Miller, R. W., E. J. Skinner, A. Sulakvelidze, G. F. Mathis, and C. L. Hofacre. 2010. Bacteriophage therapy for control of necrotic enteritis of broiler chickens experimentally infected with Clostridium perfringens. Avian Diseases 54(1):33-40. doi: 10.1637/8953-060509-Reg.1

Miller, W. J., D. M. Blackmon, R. Gentry, G. Powell, and H. Perkins. 1966. Influence of Zinc Deficiency on Zinc and Dry Matter Content of Ruminant Tissues and on Excretion of Zinc1. Journal of Dairy Science 49(11):1446-1453.

Miller, W. J., J. D. Morton, W. J. Pitts, and C. M. Clifton. 1965a. Effect of Zinc Deficiency and Restricted Feeding on Wound Healing in teh Bovine. Proceedings of the Society for Experimental Biology and Medicine 118:427-430.

Miller, W. J., J. D. Morton, W. J. Pitts, and C. M. Clifton. 1965b. Effect of zinc deficiency and restricted feeding on wound healing in the bovine. Proceedings of the Society for Experimental Biology and Medicine 118:427-430.

Miller, W. J., W. J. Pitts, C. M. Clifton, and S. C. Schmittle. 1964. Experimentally produced zinc deficiency in the goat. Journal of Dairy Science 47:556-559. doi: 10.3168/jds.S0022-0302(64)88713-0

Mills, C., A. Dalgarno, R. Williams, and J. Quarterman. 1967. Zinc deficiency and the zinc requirements of calves and lambs. British Journal of Nutrition 21(3):751-768.

Miyata, S., O. Matsushita, J. Minami, S. Katayama, S. Shimamoto, and A. Okabe. 2001. Cleavage of a C-terminal peptide is essential for heptamerization of Clostridium perfringens ε-toxin in the synaptosomal membrane. Journal of Biological Chemistry 276(17):13778-13783.

Moeller, M., and H. Scott. 1958. Studies with purified diets. 3. Zinc requirement. In: Poultry Science. p 1227-1228.

Mohammadi, V., S. Ghazanfari, A. Mohammadi-Sangcheshmeh, and M. Nazaran. 2015. Comparative effects of zinc-nano complexes, zinc-sulphate and zinc-methionine on performance in broiler chickens. British poultry science 56(4):486-493.

Mohanna, C., and Y. Nys. 1999. Effect of dietary zinc content and sources on the growth, body zinc deposition and retention, zinc excretion and immune response in chickens. British Journal of Poultry Science 40(1):108-114.

Moulder, K., and M. W. Steward. 1989. Experimental zinc deficiency: effects on cellular responses and the affinity of humoral antibody. Clinical and experimental immunology 77(2):269-274.

Munyaka, P., H. Echeverry, A. Yitbarek, G. Camelo-Jaimes, S. Sharif, W. Guenter, J. House, and J. Rodriguez-Lecompte. 2012. Local and systemic innate immunity in broiler chickens supplemented with yeast-derived carbohydrates. Poultry science 91(9):2164-2172.

Murata, R., S. SODA, A. YAMAMOTO, H. SATO, and A. ITO. 1969. The effect of zinc on the production of various toxins of Clostridium perfringens. Japanese Journal of Medical Science and Biology 22(3):133-148.

Murgia, C., I. Vespignani, J. Cerase, F. Nobili, and G. Perozzi. 1999. Cloning, expression, and vesicular localization of zinc transporter Dri 27/ZnT4 in intestinal tissue and cells. American Journal of Physiology-Gastrointestinal and Liver Physiology 277(6):G1231-G1239.

Nakatani, Y., M. Yamazaki, W. J. Chazin, and S. Yui. 2005. Regulation of S100A8/A9 (calprotectin) binding to tumor cells by zinc ion and its implication for apoptosis-inducing activity. Mediators of Inflammation 2005(5):280-292.

Nason, A., N. O. Kaplan, and H. A. Oldewurtel. 1953. Further studies of nutritional conditions affecting enzymatic constitution in Neurospora. Journal of Biological Chemistry 201(1):435-444.

Norton, C., and L. Joyner. 1981. Eimeria acervulina and E. mivati: oocysts, life-cycle and ability to develop in the chicken embryo. Parasitology 83(2):269-279.

NRC, N. R. C. 1994. NRC. Nutrient requirements of poultry. Washington. National Academy is Sciences

O'Brien, K. O., N. Zavaleta, L. E. Caulfield, J. Wen, and S. A. Abrams. 2000. Prenatal iron supplements impair zinc absorption in pregnant Peruvian women. Journal of Nutrition 130(9):2251-2255. doi: 10.1093/jn/130.9.2251

O'Dell, B., P. Newberne, and J. Savage. 1958a. Significance of dietary zinc for the growing chicken. The Journal of Nutrition 65(4):503-523.

O'Dell, B., P. Newberne, and J. Savage. 1958b. Significance of dietary zinc for the growing chicken. Journal of Nutrition 65(4):503-523.

O'hara, P., A. Newman, and E. Jackson. 1960. Parakeratosis and copper poisoning in pigs fed a copper supplement. Australian Veterinary Journal 36(5):225-229.

O'dell, B., and J. Savage. 1957. Potassium, zinc and distillers dried solubles as supplements to a purified diet. Poultry Science 36(2):459-460.

O'Garra, A., P. L. Vieira, P. Vieira, and A. E. Goldfeld. 2004. IL-10–producing and naturally occurring CD4+ Tregs: limiting collateral damage. The Journal of clinical investigation 114(10):1372-1378.

Oberleas, D., M. E. Muhrer, and B. L. O'Dell. 1966. Dietary metal-complexing agents and zinc availability in the rat. Journal of Nutrition 90(1):56-62.

Officials, A. o. A. F. C. 2013. Official Publication-Association of American Feed Control Officials. Association of American Feed Control Officials.

Olkowski, A. A., C. Wojnarowicz, M. Chirino-Trejo, and M. D. Drew. 2006. Responses of broiler chickens orally challenged with Clostridium perfringens isolated from field cases of necrotic enteritis. Research in veterinary science 81(1):99-108. doi: 10.1016/j.rvsc.2005.10.006

Olkowski, B., H. Classen, C. Wojnarowicz, and A. Olkowski. 2005. Feeding high levels of lupine seeds to broiler chickens: plasma micronutrient status in the context of digesta viscosity and morphometric and ultrastructural changes in the gastrointestinal tract. Poultry Science 84(11):1707-1715.

Olsen, S. J., L. C. MacKinon, J. S. Goulding, N. H. Bean, and L. Slutsker. 2000. Surveillance for foodborne-disease outbreaks, United States, 1993-1997.

Ott, E., W. Smith, R. Harrington, H. Parker, and W. Beeson. 1966a. Zinc Toxicity in Ruminants. IV. Physiological Changes in Tissues of Beef Cattle 1. Journal of Animal Science 25(2):432-438.

Ott, E., W. Smith, R. Harrington, M. Stob, H. Parker, and W. Beeson. 1966b. Zinc Toxicity in Ruminants. III. Physiological Changes in Tissues and Alterations in Rumen Metabolism in Lambs 1. Journal of Animal Science 25(2):424-431.

Oxford, J. H., and R. K. Selvaraj. 2019. Effects of Glutamine Supplementation on Broiler Performance and Intestinal Immune Parameters During an Experimental Coccidiosis Infection. The Journal of Applied Poultry Research 28(4):1279-1287.

Ozeki, Y., K. Kaneda, N. Fujiwara, M. Morimoto, S. Oka, and I. Yano. 1997. In vivo induction of apoptosis in the thymus by administration of mycobacterial cord factor (trehalose 6,6'-dimycolate). Infection and Immunity 65(5):1793-1799.

Paik, H. Y., H. Joung, J. Y. Lee, H. K. Lee, J. C. King, and C. L. Keen. 1999. Serum extracellular superoxide dismutase activity as an indicator of zinc status in humans. Biological Trace Element Research 69(1):45-57.

Palmiter, R. D., T. B. Cole, and S. D. Findley. 1996a. ZnT-2, a mammalian protein that confers resistance to zinc by facilitating vesicular sequestration. The EMBO Journal 15(8):1784-1791.

Palmiter, R. D., T. B. Cole, C. J. Quaife, and S. D. Findley. 1996b. ZnT-3, a putative transporter of zinc into synaptic vesicles. Proceedings of the National Academy of Sciences 93(25):14934-14939.

Pařízek, J., J. Boursnell, M. F. Hay, A. Babicky, and D. Taylor. 1966. Zinc in the maturing rat testis. Journal of Reproduction and Fertility 12(3):501-507.

Park, S. S., H. S. Lillehoj, P. C. Allen, D. W. Park, S. FitzCoy, D. A. Bautista, and E. P. Lillehoje. 2008. Immunopathology and cytokine responses in broiler chickens coinfected with Eimeria maxima and Clostridium perfringens with the use of an animal model of necrotic enteritis. Avian Diseases 52(1):14-22. doi: 10.1637/7997-041707-Reg

Parreira, V. R., M. Costa, F. Eikmeyer, J. Blom, and J. F. Prescott. 2012. Sequence of two plasmids from Clostridium perfringens chicken necrotic enteritis isolates and comparison with C.

perfringens conjugative plasmids. PLoS One 7(11):e49753. doi: 10.1371/journal.pone.0049753

Paul, W. E., and R. A. Seder. 1994. Lymphocyte responses and cytokines. Cell 76(2):241-251.

Pedersen, K., L. Bjerrum, O. E. Heuer, D. M. Lo Fo Wong, and B. Nauerby. 2008. Reproducible infection model for Clostridium perfringens in broiler chickens. Avian Diseases 52(1):34-39. doi: 10.1637/7955-022307-Reg

Pedersen, K., L. Bjerrum, B. Nauerby, and M. Madsen. 2003. Experimental infections with rifampicin-resistant Clostridium perfringens strains in broiler chickens using isolator facilities. Avian Pathology 32(4):403-411.

Pensack, J., J. Henson, and P. Bogdonoff. 1958. The effects of calcium and phosphorus on the zinc requirements of growing chickens. In: Poultry Science. p 1232-1233.

Perry, T., W. Beeson, W. Smith, and M. Mohler. 1968. Value of zinc supplementation of natural rations for fattening beef cattle. Journal of Animal Science 27(6):1674-1677.

Phan, R. T., M. Saito, Y. Kitagawa, A. R. Means, and R. Dalla-Favera. 2007. Genotoxic stress regulates expression of the proto-oncogene Bcl6 in germinal center B cells. Nature Immunology 8(10):1132.

Pimentel, J., M. Cook, and J. Greger. 1991. Immune response of chicks fed various levels of zinc. Poultry Science 70(4):947-954.

Pitts, W. J., W. J. Miller, O. T. Fosgate, J. D. Morton, and C. M. Clifton. 1966. Effect of zinc deficiency and restricted feeding from two to five months of age on reproduction in Holstein bulls. Journal of Dairy Science 49(8):995-1000. doi: 10.3168/jds.S0022-0302(66)87997-3

Pories, W. J., J. H. Henzel, C. G. Rob, and W. H. Strain. 1967. Acceleration of wound healing in man with zinc sulphate given by mouth. Lancet (London, England) 1(7482):121-124.

Pories, W. J., E. W. Schear, D. R. Jordan, J. Chase, G. Parkinson, R. Whittaker, W. H. Strain, and C. Rob. 1966. The measurement of human wound healing. Surgery 59(5):821-824.

Pountney, D., T. Dickson, J. Power, J. Vickers, A. West, and W. Gai. 2011. Association of metallothionein-III with oligodendroglial cytoplasmic inclusions in multiple system atrophy. Neurotoxicity Research 19(1):115-122.

Powell, G., W. Miller, J. Morton, and C. Clifton. 1964. Influence of dietary cadmium level and supplemental zinc on cadmium toxicity in the bovine. Journal of Nutrition 84(3):205-214.

Prasad, A. S. 2000. Effects of zinc deficiency on Th1 and Th2 cytokine shifts. The Journal of infectious diseases 182(Supplement_1):S62-S68.

Prasad, A. S., J. A. Halsted, and M. Nadimi. 1983. Nutrition classics. The American Journal of Medicine, Volume 31, 1961. Syndrome of iron deficiency anemia, hepatosplenomegaly, hypogonadism, dwarfism and geophagia. Nutrition reviews 41(7):220-223.

Prasad, A. S., S. Meftah, J. Abdallah, J. Kaplan, G. J. Brewer, J. F. Bach, and M. Dardenne. 1988. Serum thymulin in human zinc deficiency. The Journal of clinical investigation 82(4):1202-1210. doi: 10.1172/jci113717

Prasad, A. S., D. Oberleas, and J. A. Halsted. 1965. Determination of zinc in biological fluids by atomic absorption spectrophotometry in normal and cirrhotic subjects. Translational Research 66(3):508-516.

Prasad, A. S., D. Oberleas, P. Wolf, and H. P. Horwitz. 1967a. Studies on zinc deficiency: changes in trace elements and enzyme activities in tissues of zinc-deficient rats. Journal of Clinical Investigation 46(4):549-557. doi: 10.1172/jci105556

Prasad, A. S., D. Oberleas, P. Wolf, and H. P. Horwitz. 1967b. Studies on zinc deficiency: changes in trace elements and enzyme activities in tissues of zinc-deficient rats. The Journal of clinical investigation 46(4):549-557. doi: 10.1172/jci105556

Prasad, A. S., D. Oberleas, P. Wolf, J. P. Horwitz, E. Miller, and R. Luecke. 1969. Changes in trace elements and enzyme activities in tissues of zinc-deficient pigs. The American journal of clinical nutrition 22(5):628-637.

Prescott, J., R. Sivendra, and D. Barnum. 1978. The use of bacitracin in the prevention and treatment of experimentally-induced necrotic enteritis in the chicken. The Canadian Veterinary journal 19(7):181.

Psichas, A., G. Tolhurst, C. A. Brighton, F. M. Gribble, and F. Reimann. 2017. Mixed Primary Cultures of Murine Small Intestine Intended for the Study of Gut Hormone Secretion and Live Cell Imaging of Enteroendocrine Cells. Journal of visualized experiments : JoVE (122):55687. doi: 10.3791/55687

Qin, Y., D. Thomas, C. P. Fontaine, and R. A. Colvin. 2009. Silencing of ZnT1 reduces Zn2+ efflux in cultured cortical neurons. Neuroscience Letters 450(2):206-210.

Quarterman, J., and W. R. Humphries. 1979. Effect of zinc deficiency and zinc supplementation on adrenals, plasma steroids and thymus in rats. Life sciences 24(2):177-183.

Quarterman, J., C. F. Mills, and W. R. Humphries. 1966. The reduced secretion of, and sensitivity to insulin in zinc-deficient rats. Biochemical and Biophysical Research Communications 25(3):354-358.

Radhakrishnan, R., L. J. Walter, A. Hruza, P. Reichert, P. P. Trotta, T. L. Nagabhushan, and M. R. Walter. 1996. Zinc mediated dimer of human interferon-alpha 2b revealed by X-ray crystallography. Structure (London, England : 1993) 4(12):1453-1463.

raker, P. J. 1984. Zinc deficiency: a common immunodeficiency state. Immunologic Research 2(2):155-163.

Raman, M., S. S. Banu, S. Gomathinayagam, and G. D. Raj. 2011. Lesion scoring technique for assessing the virulence and pathogenicity of Indian field isolates of avian Eimeria species. Veterinarski arhiv 81(2):259-271.

Rao, S. R., B. Prakash, M. Raju, A. Panda, R. Kumari, and E. P. K. Reddy. 2016. Effect of supplementing organic forms of zinc, selenium and chromium on performance, anti-oxidant and immune responses in broiler chicken reared in tropical summer. Biological trace element research 172(2):511-520.

Rautenschlein, S., A. Subramanian, and J. M. Sharma. 1999. Bioactivities of a tumour necrosis-like factor released by chicken macrophages. Developmental & Comparative Immunology 23(7-8):629-640.

Reiterer, G., M. Toborek, and B. Hennig. 2004. Peroxisome proliferator activated receptors α and γ require zinc for their anti-inflammatory properties in porcine vascular endothelial cells. Journal of Nutrition 134(7):1711-1715.

Resnyk, C. W., W. Carré, X. Wang, T. E. Porter, J. Simon, E. Le Bihan-Duval, M. J. Duclos, S. E. Aggrey, and L. A. Cogburn. 2013. Transcriptional analysis of abdominal fat in genetically fat and lean chickens reveals adipokines, lipogenic genes and a link between hemostasis and leanness. BMC genomics 14(1):557.

Riddell, C., and X.-M. Kong. 1992a. The influence of diet on necrotic enteritis in broiler chickens. Avian Diseases:499-503.

Riddell, C., and X. M. Kong. 1992b. The influence of diet on necrotic enteritis in broiler chickens. Avian diseases 36(3):499-503.

Robertson, B., and M. Burns. 1963. Zinc metabolism and the zinc-deficiency syndrome in the dog. American Journal of Veterinary Research 24:997-1002.

Rosenkranz, E., C. H. Metz, M. Maywald, R. D. Hilgers, I. Weßels, T. Senff, H. Haase, M. Jäger, M. Ott, and R. Aspinall. 2016. Zinc supplementation induces regulatory T cells by inhibition of Sirt-1 deacetylase in mixed lymphocyte cultures. Molecular Nutrition & Food Research 60(3):661-671.

Rosenthal, R., S. Milatz, S. M. Krug, B. Oelrich, J.-D. Schulzke, S. Amasheh, D. Günzel, and M. Fromm. 2010. Claudin-2, a component of the tight junction, forms a paracellular water channel. Journal of cell science 123(11):1913-1921.

Saif, Y., A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan, and D. E. Swayne. 2011. Diseases of poultry. John Wiley & Sons.

Sakurai, J. 1995a. Toxins of Clostridium perfringens. Reviews in Medical Microbiology 6:175-185.

Sakurai, J. 1995b. Toxins of Clostridium perfringens. Review of Medical Microbiology 6:175-185.

Sakurai, J., and C. Duncan. 1978. Some properties of beta-toxin produced by Clostridium perfringens type C. Infection and Immunity 21(2):678-680.

Sakurai, J., and Y. Fujii. 1987. Purification and characterization of Clostridium perfringens beta toxin. Toxicon 25(12):1301-1310.

Salas, M., and H. Kirchner. 1987. Induction of interferon-gamma in human leukocyte cultures stimulated by Zn2+. Clinical Immunology and Immunopathology 45(1):139-142.

Salim, H., C. Jo, and B. Lee. 2008. Zinc in broiler feeding and nutrition. Avian Biology Research 1(1):5-18.

Salvesen, G. S. 2002. Caspases: opening the boxes and interpreting the arrows. Nature Publishing Group.

Salvin, S. B., B. L. Horecker, L. X. Pan, and B. S. Rabin. 1987. The effect of dietary zinc and prothymosin alpha on cellular immune responses of RF/J mice. Clinical immunology and immunopathology 43(3):281-288.

Sandstead, H. H., A. S. Prasad, A. R. Schulert, Z. Farid, A. Miale Jr, S. Bassilly, and W. J. Darby. 1967. Human zinc deficiency, endocrine manifestations and response to treatment. American Journal of Clinical Nutrition 20(5):422-442.

Sandstead, H. H., and R. A. Rinaldi. 1969. Impairment of deoxyribonucleic acid synthesis by dietary zinc deficiency in the rat. Journal of cellular physiology 73(1):81-83. doi: 10.1002/jcp.1040730111

Sandstead, H. H., and G. H. Shepard. 1968. The effect of zinc deficiency on the tensile strength of healing surgical incisions in the integument of the rat. Proceedings of the Society for Experimental Biology and Medicine 128(3):687-689.

Sanjabi, S., L. A. Zenewicz, M. Kamanaka, and R. A. Flavell. 2009. Anti-inflammatory and pro-inflammatory roles of TGF-β, IL-10, and IL-22 in immunity and autoimmunity. Current opinion in pharmacology 9(4):447-453.

Sato, H., and R. Murata. 1973. Role of zinc in the production of Clostridium perfringens alpha toxin. Infection and immunity 8(3):360-369.

Savage, A. K., M. G. Constantinides, J. Han, D. Picard, E. Martin, B. Li, O. Lantz, and A. Bendelac. 2008. The transcription factor PLZF directs the effector program of the NKT cell lineage. Immunity 29(3):391-403.

Schlegel, P., and W. Windisch. 2006. Bioavailability of zinc glycinate in comparison with zinc sulphate in the presence of dietary phytate in an animal model with 65Zn labelled rats. Journal of Animal Physiology and Animal Nutrition 90(5-6):216-222.

Schlicker, S. A., and D. H. Cox. 1968a. Maternal dietary zinc, and development and zinc, iron, and copper content of the rat fetus. Nutrition 95(2):287-294.

Schlicker, S. A., and D. H. Cox. 1968b. Maternal dietary zinc, and development and zinc, iron, and copper content of the rat fetus. Journal of Nutrition 95(2):287-294.

Schroeder, H. A., and A. P. Nason. 1969. Trace metals in human hair. Journal of Investigative Dermatology 53(1):71-78.

Schroeder, H. A., A. P. Nason, I. H. Tipton, and J. J. Balassa. 1967. Essential trace metals in man: zinc. Relation to environmental cadmium. Journal of Chronic Diseases 20(4):179-210.

Scott, M. L., E. R. Holm, and R. E. Reynolds. 1959. Studies on the Niacin, Riboflavin, Choline, Manganese and Zinc Requirements of Young Ringnecked Pheasants for Growth, Feathering and Prevention of Leg Disorders. Poultry Science 38(6):1344-1350. doi: 10.3382/ps.0381344

Scuderi, P. 1990. Differential effects of copper and zinc on human peripheral blood monocyte cytokine secretion. Cell Immunology 126(2):391-405.

Segal, D., E. Ohana, L. Besser, M. Hershfinkel, A. Moran, and I. Sekler. 2004. A role for ZnT-1 in regulating cellular cation influx. Biochemical and Biophysical Research Communications 323(4):1145-1150.

Shanmugasundaram, R., and R. K. Selvaraj. 2011. Regulatory T cell properties of chicken CD4+ CD25+ cells. The Journal of Immunology 186(4):1997-2002.

Shanmugasundaram, R., M. Sifri, and R. K. Selvaraj. 2013. Effect of yeast cell product supplementation on broiler cecal microflora species and immune responses during an experimental coccidial infection. Poultry Science 92(5):1195-1201.

Sharma, J. 1997. The structure and function of the avian immune system. Acta Veterinaria Hungarica 45(3):229-238.

Sheedy, S. A., A. B. Ingham, J. I. Rood, and R. J. Moore. 2004. Highly conserved alpha-toxin sequences of avian isolates of Clostridium perfringens. Journal of Clinical Microbiology 42(3):1345-1347.

Sheikh, A., S. Shamsuzzaman, S. M. Ahmad, D. Nasrin, S. Nahar, M. M. Alam, A. Al Tarique, Y. A. Begum, S. S. Qadri, and M. I. Chowdhury. 2010. Zinc Influences Innate Immune Responses in Children with Enterotoxigenic Escherichia coli-Induced Diarrhea–4. Journal of Nutrition 140(5):1049-1056.

Shen, H., R. MacDonald, D. Bruemmer, A. Stromberg, A. Daugherty, X.-a. Li, M. Toborek, and B. Hennig. 2007. Zinc deficiency alters lipid metabolism in LDL receptor–deficient mice treated with rosiglitazone. Journal of Nutrition 137(11):2339-2345.

Shen, H., E. Oesterling, A. Stromberg, M. Toborek, R. MacDonald, and B. Hennig. 2008. Zinc deficiency induces vascular pro-inflammatory parameters associated with NF-Kb and PPAR signaling. Journal of the American College of Nutrition 27(5):577-587.

Shi, H. N., M. E. Scott, M. M. Stevenson, and K. G. Koski. 1994. Zinc deficiency impairs T cell function in mice with primary infection of Heligmosomoides polygyrus (Nematoda). Parasite immunology 16(7):339-350.

Shimizu, T., K. Ohtani, H. Hirakawa, K. Ohshima, A. Yamashita, T. Shiba, N. Ogasawara, M. Hattori, S. Kuhara, and H. Hayashi. 2002. Complete genome sequence of Clostridium

perfringens, an anaerobic flesh-eater. Proceedings of the National Academy of Sciences 99(2):996-1001.

Si, W., J. Gong, Y. Han, H. Yu, J. Brennan, H. Zhou, and S. Chen. 2007. Quantification of cell proliferation and alpha-toxin gene expression of Clostridium perfringens in the development of necrotic enteritis in broiler chickens. Appl. Environ. Microbiol. 73(21):7110-7113.

Sim, D. L., and V. T. Chow. 1999. The novel human HUEL (C4orf1) gene maps to chromosome 4p12–p13 and encodes a nuclear protein containing the nuclear receptor interaction motif. Genomics 59(2):224-233.

Simonsen, A., R. Lippe, S. Christoforidis, J.-M. Gaullier, A. Brech, J. Callaghan, B.-H. Toh, C. Murphy, M. Zerial, and H. Stenmark. 1998. EEA1 links PI (3) K function to Rab5 regulation of endosome fusion. Nature 394(6692):494.

Singh, A., M. L. Failla, and P. A. Deuster. 1994. Exercise-induced changes in immune function: effects of zinc supplementation. Journal of applied physiology 76(6):2298-2303. doi: 10.1152/jappl.1994.76.6.2298

Singh, K. P., S. I. Zaidi, S. Raisuddin, A. K. Saxena, R. C. Murthy, and P. K. Ray. 1992. Effect of zinc on immune functions and host resistance against infection and tumor challenge. Immunopharmacol Immunotoxicology 14(4):813-840. doi: 10.3109/08923979209009237

Singh, R. B., U. C. Gupta, N. Mittal, M. A. Niaz, S. Ghosh, and V. Rastogi. 1997. Epidemiologic study of trace elements and magnesium on risk of coronary artery disease in rural and urban Indian populations. Journal of the American College of Nutrition 16(1):62-67.

Singh, R. B., M. A. Niaz, S. S. Rastogi, S. Bajaj, Z. Gaoli, and Z. Shoumin. 1998. Current zinc intake and risk of diabetes and coronary artery disease and factors associated with insulin

resistance in rural and urban populations of North India. Journal of the American College of Nutrition 17(6):564-570.

Siragusa, G., M. Danyluk, K. Hiett, M. Wise, and S. Craven. 2006. Molecular subtyping of poultry-associated type A Clostridium perfringens isolates by repetitive-element PCR. Journal of Clinical Microbiology 44(3):1065-1073.

Smedley, J., D. Fisher, S. Sayeed, G. Chakrabarti, and B. McClane. 2004. The enteric toxins of Clostridium perfringens, Reviews of Physiology, Biochemistry, and Pharmacology. Springer. p. 183-204.

Smirnov, A. 1948. Polargraphic method for the determination of zinc in blood erythrocytes. Biokhim. 13:79.

Smirov, A. 1948. Polyarograficheskii Method Kolichestvennogo Opredeleniya Tsinka V Eritrotsitakh Krovi. Biokhimiya 13(1):79-87.

Smith, J., S. Xiong, W. Markesbery, and M. Lovell. 2006. Altered expression of zinc transporters-4 and-6 in mild cognitive impairment, early and late Alzheimer's disease brain. Neuroscience 140(3):879-888.

Smith, S. E., and E. J. Larson. 1946. Zinc toxicity in rats; antagonistic effects of copper and liver. Journal of Biological Chemistry 163:29-38.

Solomons, N. W., and R. A. Jacob. 1981. Studies on the bioavailability of zinc in humans: effects of heme and nonheme iron on the absorption of zinc. The American Journal of Clinical Nutrition 34(4):475-482. doi: 10.1093/ajcn/34.4.475

Somers, M., and E. J. Underwood. 1969a. Ribonuclease activity and nucleic acid and protein metabolism in the testes of zinc-deficient rats. Australian journal of biological sciences 22(5):1277-1282.

Somers, M., and E. J. Underwood. 1969b. Ribonuclease activity and nucleic acid and protein metabolism in the testes of zinc-deficient rats. Austratlian Journal of Biological Science 22(5):1277-1282.

Song, A., Y.-F. Chen, K. Thamatrakoln, T. A. Storm, and A. M. Krensky. 1999. RFLAT-1: a new zinc finger transcription factor that activates RANTES gene expression in T lymphocytes. Immunity 10(1):93-103.

Song, J., D. Kim, C. H. Lee, M. S. Lee, C.-H. Chun, and E.-J. Jin. 2013. MicroRNA-488 regulates zinc transporter SLC39A8/ZIP8 during pathogenesis of osteoarthritis. Journal of Biomedical Science 20(1):31.

Songer, J. G. 1996. Clostridial enteric diseases of domestic animals. Clinical Microbiology Reviews 9(2):216.

Songer, J. G., and R. R. Meer. 1996. Genotyping ofClostridium perfringensby Polymerase Chain Reaction is a Useful Adjunct to Diagnosis of Clostridial Enteric Disease in Animals. Anaerobe 2(4):197-203.

Southern, L., and D. Baker. 1983a. Eimeria acervulina infection and the zinc-copper interrelationship in the chick. Poultry science 62(2):401-404.

Southern, L. L., and D. H. Baker. 1983b. Zinc toxicity, zinc deficiency and zinc-copper interrelationship in Eimeria acervulina-infected chicks. The Journal of nutrition 113(3):688-696.

Spencer, H., V. Vankinscott, I. Lewin, and J. Samachson. 1965. Zinc-65 metabolism during low and high calcium intake in man. Journal of Nutrition 86(2):169-177.

Spray, C. M., and E. M. Widdowson. 1950. The effect of growth and development on the composition of mammals. British Journal of Nutrition 4(4):332-353.

Sridhar, K., D. Nagalakshmi, D. S. Rao, and S. Rao. 2014a. Effect of dietary addition of organic zinc on performance and carcass traits in commercial broiler chicken. Indian Journal of Poultry Science 49(2):155-158.

Sridhar, K., D. Nagalakshmi, D. S. Rao, and S. Rao. 2014b. Effect of dietary addition of organic zinc on performance and carcass traits in commercial broiler chicken. Indian J. Poult. Sci 49(2):155-158.

Sridhar, K., D. Nagalakshmi, D. S. Rao, and S. R. Rao. 2015a. Effect of supplementation of graded levels of organic zinc on nutrient utilization and retention of minerals in broiler chicken. Indian J. Anim. Nutr 32(1):80-85.

Sridhar, K., D. Nagalakshmi, D. S. Rao, and S. R. Rao. 2015b. Effect of supplementation of graded levels of organic zinc on nutrient utilization and retention of minerals in broiler chicken. Indian Journal of Animal Nutrition 32(1):80-85.

Sridhar, K., D. Nagalakshmi, and S. R. Rao. 2015c. Effect of graded concentration of organic zinc (zinc glycinate) on skin quality, hematological and serum biochemical constituents in broiler chicken. Indian Journal of Animal Science 85:643-648.

Sridhar, K., D. Nagalakshmi, and S. R. Rao. 2015d. Effect of graded concentration of organic zinc (zinc glycinate) on skin quality, hematological and serum biochemical constituents in broiler chicken. Indian Journal of Anim Science 85:643-648.

Steller, H. 1995. Mechanisms and genes of cellular suicide. Science 267(5203):1445-1449.

Stevenson, J., and I. Earle. 1956. Studies on parakeratosis in swine. Journal of Animal Science 15(4):1036-1045.

Sunder, G. S., A. Panda, N. Gopinath, S. R. Rao, M. Raju, M. Reddy, and C. V. Kumar. 2008. Effects of higher levels of zinc supplementation on performance, mineral availability, and immune competence in broiler chickens. Journal of Applied Poultry Research 17(1):79-86.

Supplee, W. C. 1963. Antagonistic relationship between dietary cadmium and zinc. Science 139(3550):119-120.

Sutton, W., and V. E. Nelson. 1937. Studies on zinc. Proceedings of the Society for Experimental Biology and Medicine 36(2):211-213.

Swenerton, H., and L. S. Hurley. 1968. Severe zinc deficiency in male and female rats. Journal of Nutrition 95(1):8-18. doi: 10.1093/jn/95.1.8

Tainer, J. A., E. D. Getzoff, J. S. Richardson, and D. C. Richardson. 1983. Structure and mechanism of copper, zinc superoxide dismutase. Nature 306(5940):284.

Tako, E., P. R. Ferket, and Z. Uni. 2005. Changes in chicken intestinal zinc exporter mRNA expression and small intestinal functionality following intra-amniotic zinc-methionine administration. The Journal of nutritional biochemistry 16(6):339-346.

Tanaka, Y., S. Shiozawa, I. Morimoto, and T. Fujita. 1990. Role of zinc in interleukin 2 (IL-2)-mediated T-cell activation. Scandinavian journal of immunology 31(5):547-552.

Taniguchi, M., A. Fukunaka, M. Hagihara, K. Watanabe, S. Kamino, T. Kambe, S. Enomoto, and M. Hiromura. 2013. Essential role of the zinc transporter ZIP9/SLC39A9 in regulating the activations of Akt and Erk in B-cell receptor signaling pathway in DT40 cells. PLoS One 8(3):e58022.

Taylor, K., H. Morgan, A. Johnson, and R. Nicholson. 2005. Structure–function analysis of a novel member of the LIV-1 subfamily of zinc transporters, ZIP14. FEBS letters 579(2):427-432.

Taylor, K. M., and R. I. Nicholson. 2003. The LZT proteins; the LIV-1 subfamily of zinc transporters. Biochimica et Biophysica Acta 1611(1-2):16-30.

Taylor, K. M., P. Vichova, N. Jordan, S. Hiscox, R. Hendley, and R. I. Nicholson. 2008. ZIP7-mediated intracellular zinc transport contributes to aberrant growth factor signaling in antihormone-resistant breast cancer Cells. Endocrinology 149(10):4912-4920.

Thévenod, F. 2010. Catch me if you can! Novel aspects of cadmium transport in mammalian cells. Biometals 23(5):857-875.

Thomas, E. A., L. B. Bailey, G. A. Kauwell, D. Y. Lee, and R. J. Cousins. 1992. Erythrocyte metallothionein response to dietary zinc in humans. Journal of Nutrition 122(12):2408-2414. doi: 10.1093/jn/122.12.2408

Thomas, P., A. Converse, and H. A. Berg. 2018. ZIP9, a novel membrane androgen receptor and zinc transporter protein. General and comparative endocrinology 257:130-136.

Thompson, D. R., V. R. Parreira, R. R. Kulkarni, and J. F. Prescott. 2006. Live attenuated vaccine-based control of necrotic enteritis of broiler chickens. Veterinary Microbiology 113(1-2):25-34. doi: 10.1016/j.vetmic.2005.10.015

Timbermont, L., A. Lanckriet, J. Dewulf, N. Nollet, K. Schwarzer, F. Haesebrouck, R. Ducatelle, and F. Van Immerseel. 2010. Control of Clostridium perfringens-induced necrotic enteritis in broilers by target-released butyric acid, fatty acids and essential oils. Avian Pathology 39(2):117-121.

Timbermont, L., A. Lanckriet, A. R. Gholamiandehkordi, F. Pasmans, A. Martel, F. Haesebrouck, R. Ducatelle, and F. Van Immerseel. 2009. Origin of Clostridium perfringens isolates determines the ability to induce necrotic enteritis in broilers. Comparative Immunology, Microbiology, Infectious Diseases 32(6):503-512. doi: 10.1016/j.cimid.2008.07.001

Tipton, I. H., and M. J. Cook. 1963. Trace elements in human tissue. II. Adult subjects from the United States. Health physics 9:103-145.

Titball, R. W., C. E. Naylor, and A. K. Basak. 1999. The Clostridium perfringensα-toxin. Anaerobe 5(2):51-64.

Todd, W. E. C. H. E. 1980. Nutrition classics. The American Journal of Physiology. Volume 107, 1934, pages 146-156. "Zinc in the nutrition of the rat" by W.R. Todd, C.A. Elvehjem and E.B. Hart. Nutrition reviews 38(4):151-154.

Tomaszewska, E., S. Muszyński, P. Dobrowolski, M. Kwiecień, A. Winiarska-Mieczan, I. Świetlicka, and A. Wawrzyniak. 2017. Effect of zinc level and source (zinc oxide vs. zinc glycine) on bone mechanical and geometric parameters, and histomorphology in male Ross 308 broiler chicken. Brazilian Journal of Poultry Science 19(1):159-170.

Torres, C. A., J. E. Peña, R. Barros, R. N. Reis, S. L. Vieira, and P. C. Nascimento. 2009. Selenium contents of eggs from broiler breeders supplemented with sodium selenite or zinc-l-selenium-methionine. Journal of Applied Poultry Research 18(2):151-157. doi: 10.3382/japr.2008-00069

Troche, C., S. D. Eicher, and T. J. Applegate. 2015. The influence of dietary zinc source and coccidial vaccine exposure on intracellular zinc homeostasis and immune status in broiler chickens. British Journal of Nutrition 114(2):202-212.

Tupper, R., R. W. Watts, and A. Wormall. 1954. The incorporation of 65Zn into avian eggs. Biochemical Journal 57(2):245-255.

Turk, D. 1986. Microelements in the circulation of coccidiosis-infected chicks. Poultry science 65(11):2098-2103.

Ulluwishewa, D., R. C. Anderson, W. C. McNabb, P. J. Moughan, J. M. Wells, and N. C. Roy. 2011. Regulation of tight junction permeability by intestinal bacteria and dietary components. The Journal of nutrition 141(5):769-776.

Valberg, L. S., P. R. Flanagan, and M. J. Chamberlain. 1984. Effects of iron, tin, and copper on zinc absorption in humans. The American Journal of Clinical Nutrition 40(3):536-541. doi: 10.1093/ajcn/40.3.536

Vallee, B. L., and A. Galdes. 1984. The metallobiochemistry of zinc enzymes. Advances in Enzymology and Related Areas of Molecular Biology 56:283-430.

Vallee, B. L., and J. G. Gibson. 1948. An improved dithizone method for the determination of small quantities of zinc in blood and tissue samples. J. biol. Chem 176(1):435-443.

Vallee, B. L., and J. G. Gibson. 1949. The zinc content of whole blood, plasma, leukocytes and erythrocytes in the anemias. Blood 4:455-466.

Van Campen, D. R., and P. U. Scaife. 1967. Zinc interference with copper absorption in rats. Journal of Nutrition 91(4):473-476.

van der Sluis, M., M. H. Melis, N. Jonckheere, M.-P. Ducourouble, H. A. Büller, I. Renes, A. W. Einerhand, and I. Van Seuningen. 2004. The murine Muc2 mucin gene is transcriptionally regulated by the zinc-finger GATA-4 transcription factor in intestinal cells. Biochemical and biophysical research communications 325(3):952-960.

Van der Sluis, W. 2000a. Clostridial enteritis is an often underestimated problem. World's Poultry Science Journal 16(7):42-43.

Van der Sluis, W. 2000b. Clostridial enteritis is an often underestimated problem. World Poultry 16(7):42-43.

Van Immerseel, F., J. I. Rood, R. J. Moore, and R. W. Titball. 2009. Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. Trends in Microbiology 17(1):32-36.

Van Reen, R. 1953. Effects of excessive dietary zinc in the rat and the interrelationship with copper. Archives of Biochemistry 46:337-344.

Vetterling, J. M., and D. J. Doran. 1966. Schizogony and gametogony in the life cycle of the poultry coccidium, Eimeria acervulina Tyzzer, 1929. The Journal of parasitology:1150-1157.

Vlad, M., E. Caseanu, G. Uza, and M. Petrescu. 1994. Concentration of copper, zinc, chromium, iron and nickel in the abdominal aorta of patients deceased with coronary heart disease. Journal of Trace Elements and Electrolytes in Health and Disease 8(2):111-114.

Wade, B., and A. Keyburn. 2015a. The true cost of necrotic enteritis. World's Poultry Science Journal 31(7):16-17.

Wade, B., and A. Keyburn. 2015b. The true cost of necrotic enteritis. World Poult 31(7):16-17.

Wade, S., F. Bleiberg, A. Mosse, J. Lubetzki, H. Flavigny, P. Chapuis, D. Roche, D. Lemonnier, and M. Dardenne. 1985. Thymulin (Zn-facteur thymique serique) activity in anorexia nervosa patients. The American Journal of Clinical Nutrition 42(2):275-280. doi: 10.1093/ajcn/42.2.275

Wang, F., B.-E. Kim, M. J. Petris, and D. J. Eide. 2004. The mammalian Zip5 protein is a zinc transporter that localizes to the basolateral surface of polarized cells. Journal of Biological Chemistry 279(49):51433-51441.

Wang, X., Z.-Y. Wang, H.-L. Gao, G. Danscher, and L. Huang. 2006. Localization of ZnT7 and zinc ions in mouse retina—immunohistochemistry and selenium autometallography. Brain Research Bulletin 71(1-3):91-96.

Waters, M., A. Savoie, H. S. Garmory, D. Bueschel, M. R. Popoff, J. G. Songer, R. W. Titball, B. A. McClane, and M. R. Sarker. 2003. Genotyping and phenotyping of beta2-toxigenic Clostridium perfringens fecal isolates associated with gastrointestinal diseases in piglets. Journal of Clinical Microbiology 41(8):3584-3591.

Wedekind, K., and D. Baker. 1990. Zinc bioavailability in feed-grade sources of zinc. Journal of animal science 68(3):684-689.

West, A. P., G. S. Shadel, and S. Ghosh. 2011. Mitochondria in innate immune responses. Nature Reviews Immunology 11(6):389.

Westmoreland, N., N. L. First, and W. G. Hoekstra. 1967. In-vitro uptake of zinc by boar spermatozoa. J Reprod Fertil 13(2):223-228.

Wetterdal, B. 1958. Experimental studies on radioactive zinc in the male reproductive organs of the rat. Acta Radiologica 156:1-83.

Widdowson, E. M., E. McCance, and C. M. Spray. 1951. The chemical composition of the human body. Clinical Science 10:113-125.

Williams, R. B. 2005. Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. Avian Pathology 34(3):159-180. doi: 10.1080/03079450500112195

Williams, R. B., and J. K. Chesters. 1970. The effects of early zinc deficiency on DNA and protein synthesis in the rat. British Journal of Nutrition 24(4):1053-1059.

Williams, R. B., R. N. Marshall, R. M. La Ragione, and J. Catchpole. 2003. A new method for the experimental production of necrotic enteritis and its use for studies on the relationships between necrotic enteritis, coccidiosis and anticoccidial vaccination of chickens. Parasitology research 90(1):19-26. doi: 10.1007/s00436-002-0803-4

Wilson, K., K. Chasser, A. Duff, W. Briggs, J. Latorre, J. Barta, and L. Bielke. 2018. Comparison of multiple methods for induction of necrotic enteritis in broilers. I. The Journal of Applied Poultry Research 27(4):577-589.

Winchurch, R. A., J. Togo, and W. H. Adler. 1987. Supplemental zinc (Zn2+) restores antibody formation in cultures of aged spleen cells. II. Effects on mediator production. European journal of immunology 17(1):127-132. doi: 10.1002/eji.1830170121

Winder, F., and J. M. Denneny. 1959. Effect of iron and zinc on nucleic acid and protein synthesis in Mycobacterium smegmatis. Nature 184(Suppl 10):742-743.

Wirth, J. J., P. J. Fraker, and F. Kierszenbaum. 1984. Changes in the levels of marker expression by mononuclear phagocytes in zinc-deficient mice. Journal of Nutrition 114(10):1826-1833. doi: 10.1093/jn/114.10.1826

Wirth, J. J., P. J. Fraker, and F. Kierszenbaum. 1989. Zinc requirement for macrophage function: effect of zinc deficiency on uptake and killing of a protozoan parasite. Immunology 68(1):114-119.

Wise, M. G., and G. R. Siragusa. 2005. Quantitative detection of Clostridium perfringens in the broiler fowl gastrointestinal tract by real-time PCR. Appl. Environ. Microbiol. 71(7):3911-3916.

Wnek, A. P., and B. A. McClane. 1986. Comparison of receptors for Clostridium perfringens type A and cholera enterotoxins in isolated rabbit intestinal brush border membranes. Microbial Pathogenesis 1(1):89-100.

Wolff, H. 1956. Untersuchungen zur Pathophysiologie des Zinkstoffwechsels. Klinische Wochenschrift 34(15-16):409-418.

Wu, C., C. Pot, L. Apetoh, T. Thalhamer, B. Zhu, G. Murugaiyan, S. Xiao, Y. Lee, M. Rangachari, and N. Yosef. 2013. Metallothioneins negatively regulate IL-27–induced type 1 regulatory T-cell differentiation. Proceedings of the National Academy of Sciences:201211776.

Xie, Y., Y. He, P. L. Irwin, T. Jin, and X. Shi. 2011. Antibacterial activity and mechanism of action of zinc oxide nanoparticles against Campylobacter jejuni. Appl. Environ. Microbiol. 77(7):2325-2331.

Xin, A., S. L. Nutt, G. T. Belz, and A. Kallies. 2011. Blimp1: driving terminal differentiation to a T, Crossroads between Innate and Adaptive Immunity III. Springer. p. 85-100.

Xu, S., S.-H. Lee, H. S. Lillehoj, Y. H. Hong, and D. Bravo. 2015. Effects of dietary selenium on host response to necrotic enteritis in young broilers. Research in veterinary science 98:66-73.

Yang, X., X. Sun, C. Li, X. Wu, and J. Yao. 2011. Effects of copper, iron, zinc, and manganese supplementation in a corn and soybean meal diet on the growth performance, meat quality, and immune responses of broiler chickens. Journal of Applied Poultry Research 20(3):263-271.

Yenice, E., C. Mızrak, M. Gültekin, Z. Atik, and M. Tunca. 2015. Effects of organic and inorganic forms of manganese, zinc, copper, and chromium on bioavailability of these minerals and calcium in late-phase laying hens. Biological Trace Element Research 167(2):300-307.

Yin, L.-H., X.-P. Liu, L.-Y. Yi, J. Wang, Y.-J. Zhang, and Y.-F. Feng. 2017. Structural characterization of calcium glycinate, magnesium glycinate and zinc glycinate. Journal of Innovative Optical Health Sciences 10(03):1650052.

Yogesh, K., C. Deo, H. Shrivastava, A. Mandal, A. Wadhwa, and I. Singh. 2013. Growth performance, carcass yield, and immune competence of broiler chickens as influenced by dietary supplemental zinc sources and levels. Agricultural Research 2(3):270-274.

Young, R. J., H. M. Edwards, Jr., and M. B. Gillis. 1958. Studies on Zinc in Poultry Nutrition: 2. Zinc Requirement and Deficiency Symptoms of Chicks. Poultry Science 37(5):1100-1107. doi: 10.3382/ps.0371100

Yu, Y., A. Wu, Z. Zhang, G. Yan, F. Zhang, L. Zhang, X. Shen, R. Hu, Y. Zhang, and K. Zhang. 2013. Characterization of the GufA subfamily member SLC39A11/Zip11 as a zinc transporter. Journal of Nutritional Biochemistry 24(10):1697-1708.

Zackular, J. P., J. L. Moore, A. T. Jordan, L. J. Juttukonda, M. J. Noto, M. R. Nicholson, J. D. Crews, M. W. Semler, Y. Zhang, and L. B. Ware. 2016. Dietary zinc alters the microbiota and decreases resistance to Clostridium difficile infection. Nature medicine 22(11):1330.

Zeigler, T. R., M. L. Scott, E. R. Mc, R. H. Greenlaw, F. Huegin, and W. H. Strain. 1962. Radiographic studies on skeletal parts of zinc deficient pullets. Proceedings of the Society for Experimental Biology and Medicine 109:239-242.

Zekarias, B., A. A. Ter Huurne, W. J. Landman, J. M. Rebel, J. M. Pol, and E. Gruys. 2002. Immunological basis of differences in disease resistance in the chicken. Veterinary Research 33(2):109-125.

Zhang, B., Y. Shao, D. Liu, P. Yin, Y. Guo, and J. Yuan. 2012. Zinc prevents Salmonella enterica serovar Typhimurium-induced loss of intestinal mucosal barrier function in broiler chickens. Avian Pathology 41(4):361-367.

Zhang, K., G. A. McQuibban, C. Silva, G. S. Butler, J. B. Johnston, J. Holden, I. Clark-Lewis, C. M. Overall, and C. Power. 2003. HIV-induced metalloproteinase processing of the

chemokine stromal cell derived factor-1 causes neurodegeneration. Nature Neuroscience 6(10):1064.

Zhang, L., Y.-X. Wang, X. Xiao, J.-S. Wang, Q. Wang, K.-X. Li, T.-Y. Guo, and X.-A. Zhan. 2017a. Effects of zinc Glycinate on productive and reproductive performance, zinc concentration and antioxidant status in broiler breeders. Biological Trace Element Research 178(2):320-326.

Zhang, L., Y. X. Wang, X. Xiao, J. S. Wang, Q. Wang, K. X. Li, T. Y. Guo, and X. A. Zhan. 2017b. Effects of Zinc Glycinate on Productive and Reproductive Performance, Zinc Concentration and Antioxidant Status in Broiler Breeders. Biological Trace Element Research 178(2):320-326. doi: 10.1007/s12011-016-0928-4

Zhang, S., H. Lillehoj, and M. Ruff. 1995. Chicken Tumor Necrosis-Like Factor. 1. In Vitro Production by Macrophages Stimulated with Eimeria tenella or Bacterial Lipopolysaccharide. Poultry science 74(8):1304-1310.

Zhao, Y., Y. Tan, J. Dai, B. Li, L. Guo, J. Cui, G. Wang, X. Shi, X. Zhang, N. Mellen, W. Li, and L. Cai. 2011. Exacerbation of diabetes-induced testicular apoptosis by zinc deficiency is most likely associated with oxidative stress, p38 MAPK activation, and p53 activation in mice. Toxicology letters 200(1-2):100-106. doi: 10.1016/j.toxlet.2010.11.001