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Alternation of zinc metabolism and meat quality under different challenge situations of Broilers

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Alternation of zinc metabolism and meat quality under different challenge situations of Broilers

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Abstract

Zinc, as an essential trace element, is involved in many metabolic processes in broilers. Zinc is also associated with the antioxidant defense system and immune function. Therefore, different sources of zinc are added to feed to meet the nutritional requirements of broilers and to improve production performance. However, studies on alterations in zinc metabolism under different challenge states and associations with antioxidant function, inflammatory response, intestinal health and meat quality are still limited. Therefore, we selected three common broiler challenge models for our study: a heat stress model, an immune challenge (lipopolysaccharide exposure) model and a necrotic enteritis (*NE*) model. After evaluating alterations in zinc homeostasis in broilers under these challenge models, we explored the function of zinc in mitigating the inflammatory response and impaired intestinal health in challenged broilers. Moreover, we validated the negative effects of *NE* on meat quality and the ameliorative effects of zinc.

Firstly, to investigate the effects of heat stress on systemic zinc metabolism in broilers (Chapter III), 7 days of heat stress was induced in broilers starting at 28 days of age. The effects of heat stress on growth performance, serum and tissue zinc levels, antioxidant capacity, and zinc transporter expression in the liver and jejunum were examined, while the microbial composition of caecal content was also analyzed. The results showed a negative effect of heat-induced oxidative stress on growth performance and caecum microbial composition in broilers. Heat stress altered zinc homeostasis, leading to the redistribution of zinc in tissues, as evidenced by increased zinc concentrations in the jejunum, liver and tibia. The remodeling of zinc homeostasis is mediated by zinc transporter proteins, and metallothionein (MT) also plays a key role in this process. We conclude that broiler chickens alter systemic zinc homeostasis by regulating zinc transporters and MT in the liver and jejunum to resist oxidative stress brought about by heat stress.

Based on the observation that heat stress led to a disruption in zinc homeostasis and after characterization of zinc transporter function, Chapter IV focused on investigating the impact of an immune challenge from lipopolysaccharide (LPS) exposure on zinc homeostasis and regulation of zinc glycine. A broiler embryo model was used and a two-factor (LPS exposure and zinc glycinate) grouping was performed for the study. Zinc concentrations in the serum, tibia, and tissues were assayed, in addition to measuring levels of inflammatory cytokines, antioxidant status, intestinal barrier function, zinc-related enzyme activities, and zinc transporter-related gene expression. The results showed that LPS exposure caused an inflammatory response and oxidative stress in the jejunum and liver, contributing to the mobilization of zinc transport proteins and MT. As a result, hypozincaemia and remodeling of systemic zinc homeostasis were observed. Zinc glycinate is involved in the regulation of zinc homeostasis, it improves the antioxidant capacity of the embryo and inhibits the production of pro-inflammatory factors via the TLR4/NF-κB pathway. As such, it is able to attenuate inflammatory responses and intestinal barrier damage induced by an

immune challenge.

In the last study, the *NE* model was established to evaluate the effects of different dietary zinc sources (zinc glycinate and zinc sulfate) on growth performance, zinc concentrations in various tissues, intestinal health, and immune function in challenged broilers (Chapter V). The results showed that the *NE* challenge increased the feed conversion ratio (FCR) in broilers from days 22-35, while zinc glycinate reduced the FCR from days 1-21. The reduced intestinal lesion scores and up-regulated jejunal barrier gene levels by the zinc glycinate diets reflected the improvement in intestinal health by zinc glycinate under the *NE* challenge. Both zinc glycinate and zinc sulfate reduced the upregulation of intestinal permeability due to the *NE* challenge. The two sources of zinc reduced the level of malondialdehyde (MDA) and gene expression levels of interleukin 1β (IL-1β) in the jejunum, and increased the level of immunoglobulin A (IgA) in the jejunum under *NE* challenge, suggesting that zinc has an impact on antioxidant capacity and the improvement of inflammatory responses in *NE* challenge states. An *NE* challenge and dietary zinc facilitated the regulation of systemic zinc homeostasis by zinc transporter proteins and MT, as evidenced by significant changes in liver and jejunal zinc transporter proteins and *MT* gene expression. In conclusion, an *NE* challenge causes inflammatory damage and oxidative stress in broilers, disrupts intestinal barrier function, reduces growth performance, and leads to remodeling of zinc homeostasis. Two zinc sources alleviated *NE* challenge-induced oxidative stress and intestinal inflammation, with zinc glycinate performing better in reducing the FCR and improving intestinal barrier function.

Chapter VI focused on the effects of the dietary addition of different sources of zinc on meat quality and lipid metabolism in broilers under an *NE* challenge. Zinc glycinate increased the proportion of broiler leg muscle and also attenuated the decrease in carcass percentage of broilers under *NE* challenge. Both sources of zinc reduced pectoral muscle shear and decreased pectoral muscle yellowness b*. Moreover, the zinc glycinate was shown to reduce pectoral muscle cooking losses. The *NE* challenge also increased the pH of the pectoral muscle at 45 min and 24 h after slaughter. Both the *NE* challenge and zinc supplementation affected lipid peroxidation in broilers as evidenced by the fact that *NE* resulted in an up-regulation of the TBARS value of the pectoral muscle after 24 h of storage, while both sources of zinc significantly reduced the TBARS value of the pectoral muscle after five days of storage. The non-targeted metabolomic results showed that zinc glycinate affected fatty acid biosynthetic pathways, whereas the *NE* challenge also up-regulated a fatty acid synthesis pathway, down-regulated a fatty acid oxidation pathway and affected an unsaturated fatty acid biosynthetic pathway. The gene expression level of the peroxisome proliferator activated receptor-alpha (*PPAR-α*) and acyl CoA oxidase 1 (*ACOX1*), involved in liver fatty acid bio-oxidation, was down-regulated due to the *NE* challenge, and both sources of zinc up-regulated the liver fatty acid synthesis gene acetyl coenzyme A hydroxylase (*ACC*). Zinc glycinate increased the proportion of polyunsaturated fatty acids in the pectoral muscle by increasing the content of C20:2 (eicosadienoic acid) compared to the zinc-free treatment, and C20:3n3 (dihydro-α-linolenic acid) compared to the zinc sulfate treatment. These results confirm

that an *NE* challenge exacerbates lipid peroxidation and affects fatty acid composition in the pectoral muscle. We conclude that both an *NE* challenge and zinc supplementation affect lipid metabolism and therefore meat quality in broilers.

The general conclusion is that different challenges cause negative effects on broiler growth performance and cause remodeling of zinc homeostasis. Challenge and zinc remodeled zinc homeostasis through zinc transporter proteins and MT in the liver and intestine. Zinc attenuated the inflammatory response and impairment of intestinal barrier function induced by immune challenge through the TLR4/NF-κB signaling pathway. *NE* challenge led to a decrease in meat quality, and zinc improved meat quality by ameliorating meat coloration and lipid peroxidation, whereas both *NE* challenge and zinc affected meat quality by modulating liver lipid metabolic processes. Zinc glycinate showed a superior performance in improving broiler growth performance and intestinal barrier function compared to zinc sulfate, which was attributed to the better bioavailability of zinc glycinate.

Keywords: Broilers; Chicken embryos; Intestinal health; LPS; Metallothionein; *NE* challenge; Oxidative stress; Zinc homeostasis.

Résumé

Le zinc, un oligoélément essentiel, est impliqué dans de nombreux processus métaboliques chez les poulets de chair. Le zinc est associé également au système de défense antioxydant et à la fonction immunitaire. Par conséquent, différentes sources de zinc sont ajoutées aux aliments pour satisfaire aux exigences nutritionnelles des poulets de chair et pour améliorer leur capacité de production. Cependant, les études sur les modifications du métabolisme du zinc sous différents états de défi et les associations avec la fonction antioxydante, la réponse inflammatoire, la santé intestinale et la qualité de la viande restent encore limitées. Par conséquent, nous avons choisi pour notre étude trois modèles communs de défi pour les poulets de chair: un modèle du stress thermique, un modèle de défi immunitaire (l'exposition aux lipopolysaccharides) et un modèle d'entérocolite nécrosante (*EN*). Après avoir évalué les changements de l'homéostasie du zinc chez les poulets de chair sous ces modèles de défi, nous avons examiné la fonction du zinc dans l'atténuation de la réponse inflammatoire et de la santé intestinale diminuée chez des poulets de chair soumis aux défis. De plus, nous avons validé les effets négatifs de l'*EN* sur la qualité de la viande et les effets amélioratifs du zinc.

D'abord, pour enquêter sur les effets du stress thermique sur le métabolisme systémique du zinc chez les poulets de chair (Chapitre III), 7 jours de stress thermique ont été provoqué chez les poulets dès l'âge de 28 jours. Les effets du stress thermique sur la performance de croissance, les niveaux de zinc dans le sérum et les tissus, la capacité antioxydante, et l'expression de transporteurs de zinc dans le foie et le jéjunum ont été examinés, tandis que la composition microbienne du contenu caecal a été aussi analysée. Les résultats ont démontré un effet négatif du stress oxydant induit par la chaleur sur la performance de croissance et la composition microbienne du cæcum. Le stress thermique a modifié l'homéostasie du zinc, ce qui a conduit à la redistribution du zinc dans les tissus, attestée par l'augmentation des concentrations de zinc dans le jéjunum, le foie et le tibia. Le remodelage de l'homéostasie du zinc est facilité par les protéines transporteuses du zinc, et la métallothionéine (MT), qui a la capacité de liaison du zinc, et joue à cet effet aussi un rôle clé dans ce processus. Dans cette section, nous concluons que les poulets de chair modifient l'homéostasie systémique du zinc en régulant les transporteurs de zinc et la MT dans le foie et le jéjunum pour résister au stress oxydatif provoqué par le stress thermique.

Fondé sur l'observation que le stress thermique entraînait une perturbation de l'homéostasie du zinc chez les poulets de chair, et après la caractérisation de la fonction du transporteur de zinc, le Chapitre IV se concentre sur l'étude de l'impact d'un défi immunitaire dû à l'exposition aux lipopolysaccharides (LPS) sur l'homéostasie du zinc et la régulation de la glycine de zinc. Un modèle d'embryon de poulet de chair a été utilisé, et un regroupement à deux facteurs (l'exposition aux LPS et à la glycine de zinc) a été réalisé pour l'étude. Les concentrations de zinc dans le sérum, le tibia et les tissus ont été analysées, en plus de mesurer les cytokines inflammatoires, le statut antioxydant, la fonction de barrière intestinale, les activités enzymatiques liées au zinc, et l'expression génique liée aux transporteurs de zinc. Les résultats ont montré que l'exposition

aux LPS provoquait une réponse inflammatoire et un stress oxydatif dans le jéjunum et le foie, contribuant à la mobilization des protéines de transport du zinc et de la MT. En conséquence, une hypozincémie et un remodelage de l'homéostasie systémique du zinc ont été observés. Le glycinate de zinc est impliqué dans la régulation de l'homéostasie du zinc. Il améliore la capacité antioxydante de l'embryon et inhibe la production de facteurs pro-inflammatoires via la voie TLR4/NF-κB. Il est à cet effet capable d'atténuer les réponses inflammatoires et les dommages à la barrière intestinale induits par un défi immunitaire.

Dans l'étude finale, le modèle *EN* a été établis pour évaluer les effets de différentes sources alimentaires de zinc (glycinate de zinc et sulfate de zinc) sur la performance de croissance, les concentrations de zinc dans les tissus, la santé intestinale et la fonction immunitaire chez les poulets de chair soumis au défi (Chapitre V). Les résultats ont montré que le défi d'*EN* augmentait l'indice de consommation (IC) chez les poulets de chair pendant les jours 22-35, tandis que le glycinate de zinc réduisait l'IC pendant les jours 1-21. La réduction des scores de lésions intestinales et la régulation à la hausse des gènes de la barrière jéjunale grâce aux régimes de glycinate de zinc ont reflété l'amélioration de la santé intestinale par le glycinate de zinc sous le défi *EN*. Le glycinate de zinc et le sulfate de zinc on tous deux réduit la régulation à la hausse de la perméabilité intestinale à cause du défi *EN*. Les deux sources de zinc on réduit le niveau de malondialdéhyde (MDA) et les niveaux d'expression génique de l'interleukine 1β (IL-1β) dans le jéjunum, et ont augmenté le niveau d'immunoglobuline A (IgA) dans le jéjunum sous le défi *EN*. Ceci suggère que le zinc a un impact sur la capacité antioxydante et sur l'amélioration des réponses inflammatoires sous un défi *EN*. Un défi *EN* et du zinc alimentaire ont facilité la régulation de l'homéostasie systémique du zinc par les protéines transporteuses du zinc et la MT, ce qui est attesté par des changements significatifs dans les protéines transporteuses du zinc hépatiques et jéjunales et l'expression génique de la *MT*. En conclusion, un défi *EN* cause des dommages inflammatoires et un stress oxydatif chez les poulets de chair, perturbe la fonction de la barrière intestinale, réduit la performance de croissance, et entraîne un remodelage de l'homéostasie du zinc. Deux sources de zinc ont atténué le stress oxydatif et l'inflammation intestinale induits par le défi *EN*, le glycinate de zinc étant plus efficace pour réduire l'IC et améliorer la fonction de barrière intestinale.

Le Chapitre VI se concentre sur les effets de la supplémentation alimentaire de différentes sources de zinc sur la qualité de la viande et le métabolisme de poulets de chair soumis à un défi *EN*. Le glycinate de zinc a augmenté la proportion de muscle de la cuisse des poulets de chair et a de plus atténué la diminution du pourcentage de carcasse de poulets de chair soumis au défi *EN*. Les deux sources de zinc ont toutes deux réduit le cisaillement des muscles pectoraux et diminué le jaunissement b* des muscles pectoraux. D'ailleurs, il a été démontré que le glycinate de zinc réduisait les pertes à la cuisson des muscles pectoraux. Le défi *EN* a également augmenté le pH du muscle pectoral 45 min et 24 h après l'abattage. Le défi de *EN* et la supplémentation en zinc ont tous deux affecté la peroxydation lipidique chez les poulets de chair, ce qui est attesté par la régulation à la hausse de la valeur TBARS du muscle pectoral après 24 h de stockage due à

l'*EN*, tandis que les deux sources de zinc ont réduit de manière significative la valeur TBARS du muscle pectoral après 5 jours de stockage. Les résultats des métabolomiques non ciblés ont montré que le zinc glycinate affectait les voies biosynthétiques des acides gras, tandis que le défi *EN* régulait également à la hausse une voie de synthèse des acides gras, régulait à la baisse une voie d'oxydation des acides gras et affectait une voie biosynthétique des acides gras insaturés. L'expression des gènes pour le récepteur alpha activé par les proliférateurs de peroxysomes (*PPAR-α*) et l'acyl CoA oxydase 1 (*ACOX1*), impliqués dans la bio-oxydation hépatique des acides gras a été régulée à la baisse à cause du défi *EN*, et les deux sources de zinc ont régulé à la hausse le gène de synthèse hépatique des acides gras l'hydroxylase d'acétyl coenzyme A. Le glycinate de zinc a augmenté la proportion d'acides gras polyinsaturés dans le muscle pectoral en augmentant la teneur en C20:2 (acide eicosadiénoïque) par rapport au traitement sans zinc, et en C20:3n3 (acide dihydro-α-linolénique) par rapport au traitement du sulfate de zinc. Ces résultats confirment qu'un défi de *EN* exacerbe la peroxydation lipidique et influence la composition des acides gras dans le muscle pectoral. Nous concluons qu'un défi de *EN* et la supplémentation en zinc peuvent tous affecter le métabolisme lipidique, et par conséquent la qualité de la viande chez les poulets de chair.

La conclusion générale est que différents défis ont des effets négatifs sur les performances de croissance des poulets de chair et entraînent un remodelage de l'homéostasie du zinc. Le défi et le zinc ont remodelé l'homéostasie du zinc par l'intermédiaire des protéines transporteuses de zinc et des MT dans le foie et l'intestin. Le zinc a atténué la réponse inflammatoire et l'altération de la fonction de barrière intestinale induite par le défi immunitaire par le biais de la voie de signalisation TLR4/NF-κB. L'ingestion de *NE* a entraîné une diminution de la qualité de la viande, et le zinc a amélioré la qualité de la viande en améliorant la coloration de la viande et la peroxydation des lipides, tandis que l'ingestion de *NE* et le zinc ont affecté la qualité de la viande en modulant les processus métaboliques des lipides du foie. Le glycinate de zinc s'est avéré plus efficace que le sulfate de zinc pour améliorer les performances de croissance des poulets de chair et la fonction de la barrière intestinale, ce qui a été attribué à la meilleure biodisponibilité du glycinate de zinc.

Mots-clés: Poulets de chair; Embryons de poulet; Santé intestinale; LPS; Métallothionéine; Défi *NE*; Stress oxydatif; Homéostasie du zinc.

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Chuanpi Xiao

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List of abbreviations

ACC: Acetyl coenzyme A hydroxylase
ACOX1: Acyl CoA oxidase 1
ADFI: Average daily feed intake
ADG: Average daily gain
ALT: Alanine aminotransferase
AST: Aspartate aminotransferase
BW: Body weight
CAT: Catalase
CPT-1: Carnitine palmitoyl transferase 1
Cu,Zn-SOD: Copper-zinc superoxide dismutase
CV: Coefficient of variation
DMT1: Divalent metal transporter 1
FAS: Fatty acid synthase
FCR: Feed conversion ratio
FITC-D: Fluorescein isothiocyanate dextran
GADPH: Glyceraldehyde-3-phosphate dehydrogenase
GLU: Glucose
GSH-px: Glutathione peroxidase
HDL: Low-density lipoprotein
HSP: Heat shock protein
IFN-γ: Interferon-γ
IgA: Immunoglobulin A
IgY: Immunoglobulin Y
IL-1β: Interleukin 1β
IL-4: Interleukin 4
IL-6: Interleukin 6
IL-10: Interleukin 10
INOS: Inducible nitric oxide synthase
LDL: Low density lipoprotein
LPS: Lipopolysaccharide
MDA: Malondialdehyde
MT: Metallothionein
MTF-1: Metal transcription factor-1
MUFA: Monounsaturated fatty acids
NE: Necrotic enteritis
NF-κB: Nuclear factor kappa light chain enhancer of B cells
OTUs: Operational taxonomic units

PCA: Principal component analysis

PPAR-α: Peroxisome proliferator activated receptor-alpha

PUFA: Polyunsaturated fatty acids

PVDF: Polyvinylidene difluoride

ROS: Reactive oxygen species

RSD: Relative standard deviation

SCD-1: Stearoyl coenzyme A desaturase-1

SEM: Standard error of the mean

SFA: Saturated fatty acids

T-AOC: Total antioxidant capacity

T-SOD: Total superoxide dismutase

TBARS: Thiobarbituric acid reactive substances

TCHO: Total cholesterol

TG: Triglycerides

TLR: Toll-like receptor

TNF-α, Tumor necrosis factor-α

WHC: Water holding capacity

ZIP: Zinc-regulated transporters (Zrts), iron-regulated transporter (Irt)-like protein

Zn-Gly: Zinc glycinate

ZnT: Zinc transporter

ZO-1: Zonula occludens 1

Chapter I

General introduction

1. Abstract

Modern broilers are selected for rapid growth and converting protein efficiently. However, excessive nutrient metabolism brings potential physiological stresses and pathological conditions that could result in heat stress, inflammation, and reduced meat quality. Zinc plays an important role in various metabolic processes in broilers such as bone formation, protein and DNA synthesis, immunity and wound healing. Zinc from different sources has been added to the diet of broilers to meet the nutritional requirement and to improve their growth. The status of zinc as an essential trace element has been widely recognized, but research on the underlying mechanism of the positive effects of zinc on growth performance and poultry health is still lacking. However, it is believed that these effects may be due to the alleviation of inflammation and oxidative stress. This general introduction concludes the current status and research progress of zinc supplementation in broiler nutrition, including zinc's role in reducing inflammation and oxidative stress and in improving intestinal health in broilers. In addition, the application of different sources of zinc in broilers is systematically summarized.

2. Advances in zinc research

2.1. Biological characteristics of zinc

Zinc plays an important role as a trace element in the growth process of animals. First of all, zinc is a key component involved in the physiological functions of metabolism-related enzymes, such as RNA polymerase, DNA polymerase, lactate dehydrogenase, and alkaline phosphatase, affecting nutrient digestion and absorption as well as cell division and apoptosis (Saper et al. 2009). Furthermore, zinc also regulates appetite and feed intake through the modulation of gustatory hormones, which regulate the metabolism of oral mucosal epithelial cells to enhance taste sensitivity (Maret 2013). Zinc levels are also closely related to the synthesis, storage and secretion of other hormones in the body. For example, a zinc deficiency may negatively regulate growth hormone to affect the synthesis and release of insulin-like growth factor and androgens, which ultimately leads to slow growth and development of animals (Hambidge et al. 2010).

Zinc is a typical Type 2 nutrient, which means it is directly required by the animal metabolism but cannot be stored in large quantities and therefore demands a daily intake (Pompano et al. 2021). The "zinc pool" is often used to describe the zinc that is absorbed and stored in the body and consists of three components: plasma zinc, zinc stored in different tissues and zinc bound to metallothionein (MT) in cells (Ruttkay-Nedecky et al. 2013). Dietary zinc concentrations cause rapid changes in plasma zinc concentrations, with adjustments becoming smaller as plasma zinc level reaches a critical concentration. It cannot be ignored that the process of redistribution of zinc from plasma to tissues is affected by the presence of stress and infection, and under the influence of hormones. Plasma zinc concentrations are not only related to dietary intake, but also to metabolic

redistribution and the amount of zinc in the zinc pool. Previous research has indicated that MT level is increased in the liver during inflammation or stress, while blood zinc levels are reduced, resulting in hypozincemia. This demonstrates that MT may be the key to zinc redistribution due to infection, wounding, and stress (Oteiza 2012).

In poultry, zinc is primarily distributed in muscle and bone, with approximately 50-60% found in muscle and around 30% involved in bone formation. The zinc content in other tissues decreases progressively in the following order: feathers, male reproductive organs, liver, heart and kidneys (Wu et al. 2019).

2.2. The application of zinc in poultry

The importance of zinc in animal health has been widely reported and since zinc cannot be stored in large quantities in living organisms, it needs to be routinely added to the feed to fulfill normal physiological functions (Qin et al. 2017). The effective functions of zinc in poultry have been characterized in several ways. Zinc is involved in the synthesis of collagenase, and zinc deficiency leads to reduced efficiency of collagen synthesis and turnover and osteoblast activity in the bones of poultry, resulting in symptoms of poor skin quality, stiff bones, and enlarged ankles (Duffy et al. 2023). Since zinc forms a soluble complex with insulin that facilitates insulin's performance, zinc plays a vital role in animal growth by influencing the anabolism of proteins and fatty acids (Lowe et al. 2024). In addition, zinc contributes to the antioxidant system through the formation of copper-zinc superoxide dismutase (Cu,Zn-SOD) and MT, which in turn affects the meat quality of broilers (Alian et al. 2023). These studies have shown that zinc has a positive effect on growth performance and meat quality in broilers through its involvement in the animal's metabolism and through improving antioxidant capacity.

Poultry feeding a low or no zinc diet for one to two weeks will show zinc deficiency. Zinc deficiency in poultry is characterized by loss of appetite and a decreased feed intake. Since zinc is involved in the metabolism of B vitamins and essential fatty acids, zinc deficiency has similar symptoms to deficiencies in these nutrients, reflected in skin and corneal lesions (Jomova et al. 2022). Zinc-deficient laying hens show poor reproduction and cause a decrease in egg production and egg quality. Broiler chickens or chickens in the grower phase with zinc deficiencies exhibit stunted growth, scaly skin, and poor feather development. Zinc deficiency may be caused by low levels of zinc in the diet or by nutritional antagonism or competitive inhibition due to high levels of phytate or other divalent metal elements in the diet (Huang et al. 2019). While the NRC specifies a minimum content of 40 mg/kg of zinc in feed for chickens, the basal diet contains about 25 mg/kg of zinc, which does not meet the needs and requires additional supplementation (Naz et al. 2019). For the commercial feed, the European Food Safety Authority (EFSA) specified an upper limit of 100 mg/kg of zinc in compound feed (Duffy et al. 2023), while the Chinese Ministry of Agriculture issued Notice No.2625, setting a maximum limit of 120 mg/kg of zinc in compound feed for broilers. Zinc toxicity due to high zinc levels is uncommon as the tolerated dose of zinc in poultry is as high as 1000-2000 mg/kg. However, excessive zinc or zinc toxicity results in ruffled feathers, enlarged livers, kidneys

and spleens, and exudative quality disease. In breeding and laying birds, zinc toxicity exhibits symptoms similar to those of zinc deficiency, including ovarian atrophy and decreased egg production (Dewar et al. 1983).

Animal nutrition researchers commonly add zinc to feed at doses higher than required to cover the normal development and reproduction of livestock and poultry. Due to the low absorption and utilization of zinc from traditional sources, a large amount of zinc is excreted through the feces. Large accumulations of zinc in the environment are toxic to soil and plants (Romeo et al. 2014). Excessive levels of Zn in soil inhibit plant root development, resulting in reduced yields of sorghum and corn, and long-term accumulation of zinc may alter soil microbiota, potentially affecting organic matter decomposition and ecological cycling processes (Alkhtib et al. 2020). In surface water, the biotic ligand model (BLM) has been used to evaluate the toxicity of Zn. The concentration of zinc bound to biotic ligands is directly proportional to the toxic effects, and high concentrations of zinc could have toxic effects on aquatic organisms (Bodar et al. 2005). Over the years, environmental concerns have led to attempts to lower zinc levels in feed that may pose potential risks to animal health. The key to solving this problem is to reduce the total amount of zinc added to feeds by applying zinc sources with higher bioavailability. In recent years, new chemical compound constructions such as amino acid chelated zinc and nano-zinc have been utilized in animal nutrition, and zinc from non-inorganic sources has gradually gained widespread acceptance due to their higher bioavailability and low toxicity to animals. In addition, research has shown that organic zinc of moderate chelating strength has the highest relative efficiency, while the addition of phytase of microbial origin to feeds can improve the bioavailability of zinc (Schlegel et al. 2010). Researchers have examined the digestibility of different sources of zinc in broilers and confirmed that the apparent digestibility of inorganic zinc ranged from 22.99%-32.3%, while the digestibility of amino acid chelated organic zinc ranged from 33.54%-36.4% (Grande et al. 2020; Sadeq et al. 2018). Tibia zinc deposition was also used to assess the biological utilization of zinc in broilers, and tibia zinc deposition suggested that the bioavailability of organic zinc was 177%-206% of inorganic zinc (Star et al. 2012).

3. Challenges in the broiler production

3.1. Effect of heat stress on broilers

Stress is a non-specific biological response to an adverse environmental stimulus, and is the process by which a stressor goes from merely eliciting an individual's awareness of a potential challenge to prompting that individual's active response (Gaidica et al. 2020). The occurrence of stress in animals is influenced by a variety of factors, including genetic traits, external environment, and production and reproduction conditions (Acharya et al. 2022). In the process of livestock and poultry farming, various stress triggers can be categorized as vaccination stress, weaning stress, heat stress, cold stress, transportation stress and slaughter stress, according to the respective different stress triggers. Undesirable stressors lead to clinical symptoms such as loss of appetite,

emotional irritability, growth retardation and decreased immunity in animals (Akinyemi et al. 2021). Stress is a double-edged sword, as its generation serves to activate an animal's innate defense mechanism, enabling swift resistance against the detrimental impacts of stress. However, prolonged exposure to a stressful state or excessive intensity of stress can result in metabolic disturbances, leading to organ inflammation with potentially fatal consequences. Specifically for heat stress, the underlying cause is the breaking of the balance between the animal's own heat production and heat dissipation, and this imbalance often arises from a variety of factors, such as air temperature and humidity, animal characteristics, and thermal radiation (Rostagno 2020). The livestock industry is significantly affected by heat stress, which engenders substantial economic losses annually (Gonzalez-Rivas et al. 2020).

Although the effects of heat stress caused by non-ideal environmental problems are similar across all species of animals, poultry are particularly sensitive to heat stress, and especially broilers are prone to lower productivity and health issues due to heat stress (Lu et al. 2023). Factors that make broilers susceptible to heat stress include the fact that broilers selected for superior performance are more metabolically active, resulting in a greater susceptibility to heat stress due to the large amount of heat production in growing broilers. The well-developed feathers, missing sweat glands and higher body temperatures of broilers also contribute to their increased sensitivity to heat stress. In response to heat stress, poultry exhibit reduced locomotion and feeding behaviors, while showing increased water intake, excessive panting, and extended periods of rest (Khan et al. 2023). The activation of the hypothalamic-pituitary-adrenal axis in poultry experiencing heat stress leads to heightened glucocorticoid levels in the circulatory system, consequently elevating blood glucose levels as a means of combating stress (Du et al. 2023).

Heat stress can cause a number of negative effects on broilers. First of all, heat stress suppresses the immune function of broilers, which is reflected in the decrease of the relative weight of the thymus and spleen and the decrease in the immunoglobulin content in the serum (Hirakawa et al. 2020). In addition, broilers under heat stress increase heart rate and cardiac load through the sympathetic control of the adrenal axis, confirming the adverse effects of heat stress on the heart of poultry. The intestine, an important organ in maintaining the stability of the internal environment, is also challenged by heat stress, which can damage the intestinal mucosa. Due to this disruption, bacteria and endotoxins can enter the circulatory system through the intestinal mucosa, leading to negative effects. Previous investigation has demonstrated that heat stress causes the breakage of the villi of the small intestine and increased intestinal permeability in broilers, which in turn causes an inflammatory response within the organism. The negative impact of heat stress on the respiratory system and kidneys is attributed to the absence of sweat glands in broilers, which necessitates reliance on respiration for heat dissipation. High respiration rates cause large amounts of carbon dioxide to be eliminated from the bloodstream, leading to respiratory alkalosis. The loss of large amounts of body fluids due to heat stress causes kidney ischemia, and large amounts of toxic substances can accumulate and cause kidney damage (Tang et al. 2018).

Given the growing emphasis on broiler welfare within the livestock industry

and advancements in farming techniques, the adoption of various strategies to mitigate heat stress has become widely accepted. Standardized farming practices employ external insulation and fan installations to mitigate heat stress in broilers by either insulating heat or enhancing air flow rates (Goel 2021). The application of feed additives, particularly vitamin C and vitamin E, which possess antioxidant properties, has garnered significant attention for their role in combating heat stress in broilers. These additives aid in safeguarding cell membrane structures by facilitating the recovery of free radicals (Wang et al. 2023). Various plant extracts have demonstrated the ability to enhance the well-being of broilers by mitigating the presence of reactive oxygen species during periods of heat stress (Jimoh et al. 2022). Additionally, research has demonstrated that the inclusion of zinc in poultry feed can safeguard the intestinal epithelial villi and ameliorate compromised intestinal barrier function caused by heat stress (Hu et al. 2023a).

3.2. Effect of bacterial infections on broilers

The industrial farming practices promote the efficient production of broilers, while high density stocking leads to a higher susceptibility to infections by pathogenic microorganisms. Consequently, the overall health and immune function of broilers become crucial in combating bacterial infections. Common bacterial diseases in broilers include *Escherichia coli* (*E. coli*), *Salmonella*, *Clostridium perfringens*, and *Staphylococcus* (Swelum et al. 2021). The control of bacterial diseases is particularly important for ensuring the health of broilers and avoiding subsequent economic losses. Management strategies employed for bacterial diseases in broilers encompass the implementation of biosecurity measures, utilization of antibiotics, and administration of vaccines (Abd El-Hack et al. 2022). In the past, broiler farmers relied on antibiotics to kill pathogenic bacteria in the gut and reduce intestinal inflammation, which in turn improved broiler performance and lowered mortality. However, with the increase in animal resistance due to antibiotic misuse and the emergence of superbugs in the environment, the move to supplement feed with antibiotics to fight disease-causing bacteria and promote animal growth is now a thing of the past. In contrast, vaccines have the disadvantages of high specificity and cost, while commonly used attenuated vaccines have the risk of reversal of virulence and recombinant vaccines have the disadvantage of short duration of effect. (Romanutti et al. 2020). The absence of antibiotics has resulted in compromised gut health, increased occurrence of necrotic enteritis (*NE*), and reduced productivity in broilers (Manafi et al. 2019). Broiler health and their immune system play an important role in fighting bacterial infections. Therefore, nutritional strategies to alleviate immune stress in poultry and to modulate the animal's innate immune response to inflammation have become a widespread strategy.

E. coli is an aerobic, gram-negative bacterium that colonizes the mucosal surfaces of the broiler intestine. Most kinds of E. coli are not pathogenic to broilers, but 10-15% are nonetheless pathogenic which are classified as *avian pathogenic E. coli* (APEC) (Roth et al. 2019). APEC induces diarrhea and systemic infections in broilers following a decline in immune function or damage to the intestinal barrier. The hallmark of APEC-induced intestinal inflammation is an abnormal increase in the infiltration of immune cells, including neutrophils,

macrophages, T cells, and B cells (Sun et al. 2015). APEC possesses the ability to secrete endotoxins, resulting in harm to the intestinal epithelium and detachment of intestinal epithelial cells, thereby causing hemorrhage in the intestinal wall and elevating intestinal permeability. Research has demonstrated that toll-like receptors (TLRs) identify APEC, triggering the activation of the immune-related signaling pathway and prompting the production of pro-inflammatory factors (Cao et al. 2022). APEC infection also induces bursal atrophy, inhibits B-cell proliferation and differentiation, and consequently suppresses humoral immune function in poultry (Kathayat et al. 2021).

Salmonella is the main pathogen causing bacterial food poisoning and a common pathogenic bacterium in poultry production. As a complex bacterium belonging to the Enterobacteriaceae family, *Salmonella* consists of two species and six subspecies, including more than 2,500 serovars (Andino et al. 2015). In poultry, a distinction must be made between infections with host-specific salmonella serotypes and non-host-specific salmonella serotypes, and only subtypes *Salmonella gallinarum* and *Salmonella pullorum* cause clinical symptoms for broilers (Kasier et al. 2000). Broilers generally grow for only 6 weeks and are more susceptible to *Salmonella* infection during the first two weeks after hatching due to poor development of the intestinal tract and immune function (Totton et al. 2012). Broilers infected with *Salmonella* suffer from slow growth, depression and diarrhoeal symptoms. *Salmonella* induces enteritis and disrupts intestinal tight junctions after colonizing the intestinal tract of poultry. Pathogenic *Salmonella* has been largely controlled by a range of biosecurity strategies in Europe and North America, but continues to cause economic losses in poultry in Asia and South America (Kasier et al. 2000). On the contrary, the most common Salmonella serotypes found in modern poultry production such as *Salmonella*. *Enteritidis*, *Salmonella Typhimurium*, *Salmonella Virchow*, *Salmonella Hadar* and *Salmonella Infantis* are non-host-specific salmonella serotypes. These serotypes only rarely induce clinical signs in poultry but are of great food hygiene and economic importance because they are main causative agents of zoonoses in humans. Both *E. coli* and *Salmonella* belong to Gram-negative bacteria, and lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria (Chapman et al. 2005). Following a bacterial infection in broilers that results in damage to the intestinal barrier, LPS will enter the circulatory system through the intestinal barrier causing an inflammatory response and an imbalance in the antioxidant system that induces immune stress.

Strategies to prevent bacterial diseases in the antibiotic-free era rely on reducing broiler stress, improving the farming environment and adopting nutritional strategies to maintain gut health. Employing effective biosecurity management practices and staying away from stress and immunological challenges will enable broilers to reach their full genetic potential to offset the negative impact of antibiotic withdrawal on production performance.

3.3. Effect of necrotic enteritis on broilers

NE is an intestinal inflammatory disease caused by *Clostridium perfringens*, which is mainly characterized by fibrosis and necrosis of the intestinal epithelial

tissue. The clinical manifestations of *NE* in broilers are in the form of purulent bloody stools or mixed bloody stools, often co-occurring with coccidial infections and coccidiosis (Collier et al. 2008). Broilers with *NE* show clinical signs of diarrhea, anorexia, disheveled feathers, and mucosal shedding of bloody stools. The clinical signs of *NE* develop into subclinical cases after a period of time. The mortality rate of subclinical cases is not high, but the destruction of the intestinal structure can lead to reduced nutrient absorption and a slower weight gain in broilers, resulting in significant economic losses (Lee et al. 2011). The administration of anticoccidial drugs and antibiotics in the feed used to play an important role in the control of *NE*. However, the misuse of antibiotic drugs has led to severe bacterial resistance, and the use of antibiotics in poultry has been severely restricted globally, resulting in a gradual increase in the incidence of *NE*, which has become an important disease threatening the broiler industry.

Coccidial infection and diet are important determinants of the pathogenesis and severity of *NE*. The disruption of the intestinal barrier function caused by the proliferation of *Eimeria coccidia* in the intestine is considered to be an important cause of *NE* (Li et al. 2022). Due to this disruption of the intestinal epithelium, plasma proteins in the intestinal wall of the host enter the intestinal tract and provide nutrients for the proliferation of *Clostridium perfringens* (Rochell et al. 2016). When *NE* is modeled in the laboratory, *Clostridium perfringens* is often used in conjunction with a 100-fold dose of a live *Eimeria coccidia* vaccine for oral administration (Song et al. 2022). The presence of animal proteins and non-starch polysaccharides in broiler diets has been found to be associated with an increased probability of *NE*. This is likely due to the fact that non-starch polysaccharides contribute to increased chow viscosity and prolonged gut transit time, providing more opportunities for *Clostridium perfringens* colonization (Kim et al. 2022). *Clostridium perfringens* exhibits an inability to autonomously produce amino acids and primarily depends on hydrolytic enzymes to acquire nutrients from host tissues. However, a substantial quantity of animal protein sources, such as fishmeal and meat meal, could effectively cater to the preference of *Clostridium perfringens* for animal proteins (Bortoluzzi et al. 2019). The impact of dietary factors on *NE* remains a topic of active research, with ongoing investigations into potential causative factors.

Prior to the 21st century, the majority of research aimed at the treatment of *NE* focused on the application of antibiotics, but the global prohibition on antibiotic use has led to a surge in bacterial disease outbreaks (Smith 2019). Based on the existing research, the primary methods of controlling the spread of *NE* involve bolstering the animal's immune response, managing the pathogen, and modifying the composition of feed ingredients. Vaccines increase the animal-specific immune response to attenuate or resist coccidiosis and prevent *NE*. Biosecurity measures, such as good disinfection of litter, cleaning of broiler houses after slaughter and decontamination of breeders are all effective means of disease control (Imam et al. 2021). Feed adjustment can be accomplished through modifications in ingredient type and composition, as well as the inclusion of supplementary additives like enzymes, probiotics, essential oils and organic zinc (Broom 2017). Decreasing the protein proportion in the feed and employing exogenous enzymes to diminish feed intake can effectively mitigate the

proliferation of *Clostridium perfringens* in confined spaces (Abd El-Hack et al. 2022). While complete safeguarding against *NE* in broilers cannot be attained solely through feed strategies, the risk management of *NE* can be achieved by manipulating the intestinal environment, promoting gut health and reducing the incidence and severity of *NE*.

4. Benefits of zinc for broilers

Zinc is considered an additional trace element to be added to broiler diets. Zinc is the second most abundant trace element after iron in animal tissues. Since free zinc reacts with water to form insoluble zinc hydroxide in plasma and cytoplasm, zinc exists in complexes with proteins in cells and blood (Kaur et al. 2014). Zinc cannot be stored in the body for long periods and daily intake of zinc through feed is necessary. Ingredients commonly used in animal feed such as corn, wheat, soybean meal, fishmeal and bran are good sources of zinc, and in practice, premixes with a fixed zinc content are added to feeds to provide adequate zinc for livestock. Broilers do not show signs of zinc deficiency when the zinc content of the diet reaches 40mg/kg, and an additional 40 mg/kg- 80 mg/kg is often added to obtain a better production performance (Naz et al. 2019).

Inorganic and organic forms of zinc are the more common sources provided in the diet, while nano-zinc is also utilized. Organic forms of zinc have a higher bioavailability due to the presence of independent amino acid ligands, which make it easier to avoid a reaction with phytic acid in the digestive tract to produce complex precipitates. However, the amount of zinc added may be a more critical factor in broiler performance than the source. Research has shown that the administration of both organic and inorganic zinc at 80 mg/kg enhanced broiler performance and economic efficiency through an improved feed conversion ratio (FCR) in broilers. However, it is undeniable that the organic form of zinc offers superior advantages over zinc sulfate when added at lower levels (Ogbuewu et al. 2023). Given the demand for green farming and the tightening of pollution emission policies, research on the application of organic zinc has emerged in recent years (Świątkiewicz et al. 2019). These studies have focused on the effects of different sources of zinc on broiler performance, the bioavailability of zinc, antioxidant capacity, immune status, intestinal health and meat quality.

4.1. Zinc and antioxidant function in broilers

As mentioned above, broilers in industrial production are susceptible to stress due to their lifelong state of rapid metabolism, which is dictated by the nature of the bird (Tallentire et al. 2016). Oxidative stress is a metabolic dysfunction that leads to oxidative damage to cells and tissues by promoting the oxidation of lipid molecules, and free radicals are key to this process (Lushchak 2014). Under normal physiological conditions, free radicals, as a redox-active group of atoms, have beneficial effects on the metabolism, whereas under stress conditions, excess free radicals cause irreversible oxidative damage to biomolecules such as lipids, nucleic acids and proteins (Ncho et al. 2023). The Kelch-like ECH-associated protein 1 (Keap1) /Nuclear factor erythroid 2-related factor 2 (Nrf2) /Antioxidant Response Element (ARE) pathway is the most important antioxidant pathway in

animals, which is responsible for regulating the expression of superoxide dismutase (SOD), glutathione peroxidase (GSH-px) and catalase (CAT) (Wang et al. 2022). Zinc assumes a critical role in the structural configuration of Keap1, which dissociates from Nrf2 upon the onset of oxidative stress, thereby triggering the activation of the antioxidant pathway and enhancing the antioxidant capacity of animals by upregulating the expression of various antioxidant enzymes (Feng et al. 2020). Zinc is a structural component of Cu,Zn-SOD, an enzyme that promotes the breakdown of two free radicals and reduces the toxicity of reactive oxygen species (ROS). The results of a past study by Zhu et al. demonstrated that 60 mg/kg of zinc in the diet had a significant impact on reducing serum malondialdehyde (MDA) levels and increasing GSH-px activity in broilers, suggesting that zinc has the potential to enhance the antioxidant enzyme system (Zhu et al. 2022).

MT is a cysteine-rich, low molecular weight protein that participates in the reduction of hydroxyl radicals and neutralizes free radicals generated under stress conditions. As a metal-binding protein, MT has a strong binding affinity for zinc ions, being able to bind up to seven zinc ions, and plays a key role in the regulation of zinc homeostasis. Zinc has been observed to stimulate the production of MT, which in turn exerts its important role in cellular resistance to oxidative stress (Formigari et al. 2007). When oxidative stress occurs, the oxidation of disulfide bonds in the MT structure is accompanied by the release of zinc ions. These released zinc ions are involved in the synthesis of the antioxidant enzyme Cu,Zn-SOD, which helps protecting cells from free radical oxidation (Rodriguez-Menendez et al. 2018). In addition, it was shown that the Nrf2 pathway, which is related to antioxidant function, is also important for the induction and expression of MT (Yang et al. 2023). The gene expression level of *MT* was up-regulated in the liver of broilers subjected to oxidative stress induced by high temperatures, and in this way, it could regulate zinc homeostasis in broilers (Xiao et al. 2022). These results confirm the involvement of MT as part of the zinc pool in resistance to oxidative stress.

The transportation and storage of zinc are dependent on MT and two families of transporter proteins, the zinc-regulated transporters (Zrts), iron-regulated transporter (Irt)-like protein (Zip) family and the zinc transporter (ZnT) family. Recent research has indicated that zinc transporter proteins may have the ability to mitigate cellular damage caused by oxidative stress. For instance, ZnT7 has been found to enhance the survival of the osteoblast cell line MC3T3-E when exposed to oxidative stress, potentially through the induction of intracellular zinc accumulation (Liang et al. 2013). The down-regulation of Zip5 and Zip14 in hepatocytes during chronic alcohol exposure has been observed to disrupt the dynamic equilibrium of zinc in the liver (Sun et al. 2014). These results highlight the potential for alterations in zinc status in various pathological and physiological conditions, which can be attributed to the modulation of antioxidant enzymes, the binding affinity of MT for zinc, and the expression of different zinc transporter proteins. Since such studies have been carried out less frequently in poultry, the exact mechanisms are worth exploring in broilers.

4.2. Zinc and immune function in broilers

Zinc is involved in humoral and cellular immunity and influences the development of immune organs and ultimately the immune function of the animal. Zinc deficiency or excess zinc both affect the activity of various zinc-containing enzymes and the nucleotide metabolism of lymphocytes in the bone marrow. The level of lymphocyte proliferation, differentiation and maturation correlates with the normal physiological function of immune organs such as the spleen and thymus (Broere et al. 2019). As a major contributor to cellular immunity, T lymphocytes mature in the thymus, while zinc deficiency negatively affects DNA transferase activity and thymosin, interfering with T lymphocyte maturation and reducing cellular immune function (Hojyo et al. 2016). Zinc also has an important effect on B cell-mediated humoral immunity. Zinc deficiency leads to the apoptosis of the precursors of B lymphocytes and reduces the number of mature B lymphocytes, while zinc supplementation has a positive effect on promoting antibody synthesis (Gammoh et al. 2017). It has been shown that the addition of organic zinc to broiler feed increases the levels of several immunoglobulins, heralding the positive effect of zinc on humoral immunity (Jarosz et al. 2017). Numerous scientific studies have demonstrated that zinc can modulate immune function by regulating the production and release of cytokines, including interleukin 1β (IL-1β) and interferon (IFN), which are pro-inflammatory factors, as well as by inhibiting the secretion of tumor necrosis factor-α (TNF-α) (Faghfouri et al. 2021). Additionally, a zinc deficiency will result in a reduction in lymphoid cell counts and the atrophy of immune organs such as the bursa (Akbari Moghaddam Kakhki et al. 2018).

It has been shown that zinc acts as an immunostimulant to increase the weight of immune organs in broilers, while increasing overall broiler body weight and decreasing the feed conversion ratio. Compared to diets with low zinc supplementation, feeding diets supplemented with an additional 80 mg/kg of zinc sulfate increased antibody titer in broiler serum (Gajula et al. 2011). In addition, animals under stress were also able to enhance immunity and have better survival rates with zinc supplementation (Hu et al. 2023a). Studies on the application of dietary zinc to examine the immune competence of broilers usually apply disease models, such as a *Salmonella* infection model or *NE* model. Broilers under immune challenge or in stressful conditions are more sensitive to the immune-enhancing effects of zinc, which may be related to zinc's ability to resist oxidative stress and its direct involvement in a broiler's metabolism through the formation of a range of synthetic enzymes (He et al. 2019).

4.3. Zinc and its influence on carcass traits and meat quality

The modern broiler industry aims to provide high quality animal protein. As a commodity, both extrinsic and intrinsic characteristics of chicken meat influence consumer choice. Extrinsic characteristics can be understood as skin quality, carcass ratio and color, while intrinsic characteristics of chicken meat include nutritional composition, pH, water holding capacity (WHC), shelf life and cooking characteristics (Marchewka et al. 2023). All these characteristics belong to the carcass traits and meat quality indicators of broilers.

The skin quality of broiler chickens affects the appearance of the meat, while the carcass ratio is directly related to economic efficiency. Skin-broken poultry meat requires longer processing times and procedures. As the skin serves as a protective layer of chicken meat, skin-broken broilers are often bruised, which severely reduces the economic value of the meat (Salim et al. 2012). Zinc is an essential nutrient for collagen synthesis and a deficiency of zinc reduces the amount of collagen in the skin. The close correlation between skin strength and collagen, as well as the involvement of zinc in the formation of keratin, abundantly present in the skin, dictates that zinc intake plays an important role in the maintenance of skin health (Ogawa et al. 2016). A related study showed that the addition of 60 mg/kg of organic zinc had no effect on production performance but improved carcass quality by increasing skin toughness (Tronina et al. 2007). Another study revealed that the pectoral muscle rate, leg muscle rate, pectoral muscle fat ratio and leg muscle fat percentage of broilers were significantly improved by dietary supplementation of 40 mg/kg of zinc glycinate compared to an equivalent amount of inorganic zinc (Kwiecień et al. 2016). However, a different study revealed that dietary zinc supplementation did not exert a significant influence on carcass production or the proportions of each component (Abhishek Sahoo et al. 2016). Nevertheless, the majority of studies confirm that zinc supplementation has a positive impact on both skin quality and carcass performance indicators, thereby enhancing carcass quality. The color of chicken meat holds significant importance in consumer preference, and zinc is believed to contribute to the preservation of chicken meat color stability because of its antioxidant properties and ability to bind to myoglobin. The present research on the impact of zinc on the color of chicken meat has produced varying outcomes, primarily involving a decrease in yellowness values (Qudsieh et al. 2018) and either an increase (Yang et al. 2016) or no significant alteration (Sevim et al. 2021) in redness values when zinc is added. The variations observed across different studies may be attributed to disparities in farming practices, dietary nutrient compositions, and breeds.

Improvements in the intrinsic characteristics of chicken meat due to zinc include, among others, increased WHC, longer storage life and improved cooking losses. In addition, several studies have been conducted in recent years examining the association between dietary factors and chicken meat nutrient composition, suggesting that zinc may alter the fatty acid composition of chicken meat by improving lipid oxidation. The mechanism by which zinc improves the WHC of chicken meat involves its antioxidant capacity and its ability to regulate lipid metabolism. By avoiding oxidative damage, zinc helps increasing the WHC of the meat (Khajeh Bami et al. 2020). The thiobarbituric acid reactive substances (TBARS) value, which reflects the MDA content of meat, is significantly associated with the longevity of chicken meat. Improving the antioxidant function of animals directly improves the MDA content and positively affects meat quality (Alian et al. 2023).

Most of the synthesis and metabolism of fats in broilers occurs in the liver, not in adipose tissue. Dietary fat and carbohydrate are exogenous sources of fat that are metabolized in the liver along with stored fat (Zaefarian et al. 2019). After digestion and absorption of fats in the small intestine, dietary fatty acids reach the

liver after entering the portal circulatory system as very low density lipoproteins (VLDL). Lipoproteins in the form of chylomicrons consist of 90% triglycerides, as well as free cholesterol, phospholipids, and lipoproteins, and these chylomicrons are stored and metabolized by the liver. The liver is the major organ for the synthesis of triglycerides, phospholipids and cholesterol, which make up lipoproteins. Based on the different ratios of lipids and proteins contained, the lipoproteins synthesized by the liver are classified as VLDL, low density lipoproteins (LDL), intermediate density lipoproteins (IDL), and high density lipoproteins (HDL) (Hermier et al. 1997). The liver not only stores triglycerides and fats, but also metabolizes and synthesizes fatty acids into lipoproteins and phospholipids, catalyzed by a range of enzymes. The expression level and enzyme activity of hepatic fat synthase (FAS) serving as crucial biological foundations for the regulation of fat metabolism (Alves-Bezerra et al. 2017). It was demonstrated that zinc upregulates the expression of the FAS gene to increase the amount of fatty acid synthesis in the liver and to change the fatty acid composition of the liver (Liu et al. 2019). Although zinc has been shown to affect meat quality and enhance fatty acid metabolism, there is still a lack of evidence as to how zinc affects meat quality by influencing fatty acid metabolism and therefore meat quality.

4.4. Zinc and intestinal health

Intestinal health could be interpreted as the ability of the intestine to isolate exogenous substances in the intestine such as pathogenic microorganisms and various antigenic substances so that they can no longer invade the host and damage the intestine. Intestinal health depends on the integrity of the intestinal mucosal barrier, which consists mainly of the intestinal mucosal physical barrier, chemical barrier and microbiological barrier as shown in Figure 1-1 (Minton 2022). The intestinal barrier prevents harmful substances from entering the bloodstream from the intestinal epithelial cells, causing systemic inflammation and immune responses. The physical barrier consists of intestinal epithelial cells and cell-directed tight junctions, where the intestinal epithelium consists of cuprocytes and endocrine cells with secretory functions, as well as Panniculus cells (Konig et al. 2016). The chemical barrier includes intestinal secretions and cytokines. Intestinal secretions contain enzymes, mucus and immune proteins that neutralize acids and break down food while fighting pathogenic microbes. The microbial barrier refers to the community of microorganisms normally present in the intestine, known as the intestinal bacterial community. Microorganisms form a symbiotic relationship with the host, assisting in the digestion of food and the synthesis of vitamins, while inhibiting the growth of pathogenic microorganisms (Ghosh et al. 2021). Intestinal permeability is a direct indicator of intestinal health and is often measured using an oral probe method, which consists mainly of FITC-dextran with different molecular weights or mannitol (Gilani et al. 2021). An orally administered FITC-dextran molecule is large enough not to cross the intestinal epithelial barrier into the bloodstream unless inflammation and loss cause impairment of barrier function (Li et al. 2019b). Various factors, such as stress, nutrition, and infection caused by pathogenic bacteria, contribute to the determination of intestinal permeability (Di Vincenzo et al. 2024). Stress induces

the production of toxic metabolites in intestinal mucosal cells, resulting in damage and increased permeability. The invasion of pathogenic microorganisms in the intestine causes inflammation, and leads to excessive apoptosis of epithelial cells, while inadequate or unbalanced dietary nutrient intake can cause nutritional disorders, leading to impaired intestinal barrier function (De Santis et al. 2015). These factors can directly or indirectly cause immunocompromise in the organism, due to the damage of the intestinal epithelial barrier function, the increase of intestinal permeability, and can thus cause intestinal infections or systemic immune response.

There is a consensus that zinc has an important role in improving intestinal health in poultry, mainly through maintaining mucosal integrity, increasing the activity of intestinal digestive enzymes, and improving the balance of intestinal microbiota (Jing et al. 2009). It has also been reported that zinc reduces the number of inflammatory cells by mediating the regulation of intestinal substance transport reducing chloride ion secretion (Sunuwar et al. 2017). Feeding zinc-containing diets promotes small intestinal villus height in broilers, implying a higher nutrient absorption; a benefit that appears to be amplified by organic zinc (Ogbuewu and Mbajiorgu 2023). The underlying mechanism for the morphological benefits of zinc on intestinal tissue is unclear and may be related to the involvement of zinc in the synthesis of several enzymes as well as its inhibitory function on inflammation.

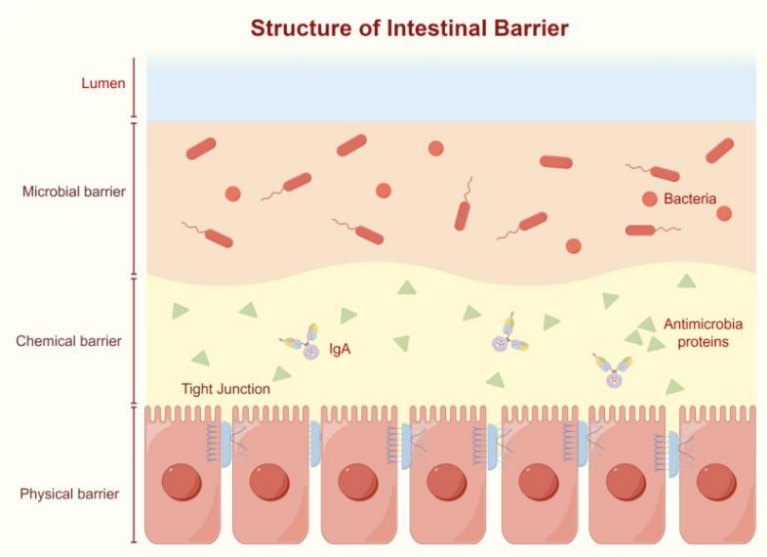

Figure 1- 1: Structure of intestinal barrier

5. Zinc metabolism

5.1. Zinc absorption and transport

We described in the previous section that zinc needs to be supplemented daily

to maintain zinc homeostasis. It is believed that, after entering the intestine, zinc absorption occurs by both intercellular diffusion and transcellular transport, and the latter, proposed in 1975, has been widely disseminated and subjected to extensive scientific validation (Cousins 1985). Zinc transporter proteins in the apical and basolateral membrane cells of the intestinal epithelium play an important role in zinc absorption (Lodemann et al. 2015).

The process of zinc transport in the intestine involves the uptake of zinc from the intestinal lumen into the cytoplasm by the parietal membrane of the intestinal epithelial cell, the transport and storage of zinc in the cell, the passage of zinc from the cytoplasm through the basement membrane into the portal blood, and the entry of zinc into the circulatory system where it binds to albumin (Handing et al. 2016). The process of zinc absorption in the intestine is shown in Figure 1-2 (Maares et al. 2020). Further work showed that the binding of zinc from the intestinal lumen to the parietal membrane of intestinal epithelial cells is a rapid, carrier-independent, unsaturated diffusion process. In contrast, the uptake of zinc into the cytoplasm by the apical membrane of the intestinal epithelial cells is a slow, carrier-involved, passive diffusion process (Kiela et al. 2016). Early research showed that the divalent metal transporter 1 (DMT-1), a cationic transporter with a broad binding function for divalent metal ions, is involved in zinc uptake in the intestine. With the discovery of two families of transporter proteins, SLC39A (Zip) and SLC30A (ZnT), it has been confirmed that zinc uptake by intestinal epithelial cells is a carrier-regulated physiological process (Wang et al. 2010). ZnT proteins assist in the transport of zinc ions from the cytoplasm to the organelle or extracellularly. The opposite occurs with the Zip proteins that are responsible for the transport of zinc ions from the organelle or extracellularly to the cytoplasm. The mechanisms of zinc transporter protein signaling are still under investigation (Bin et al. 2018). In the intestine, MT directly regulates cytoplasmic and organelle zinc metabolism and storage, and intracellular zinc homeostasis is achieved by MT binding or releasing zinc ions. As the main transporter controlling the entry of zinc from the intestinal epithelium into the bloodstream, ZnT1 is responsible for transporting zinc from the cytoplasm across the basement membrane into the portal bloodstream, regulating cellular zinc levels, and also preventing cellular zinc toxicity caused by the accumulation of high zinc concentrations (Laity et al. 2007). Zinc in the blood is bound to ligands such as albumin, transferrin, α2-macroglobulin and amino acids to participate in the metabolism as soluble small molecules (Bal et al. 2013). Zinc is absorbed and transported in the ionic form, but bioavailable organic zincs have ligand bonds, which makes them differ in their water solubility, e.g., zinc methionine is insoluble in water, whereas zinc glycinate is soluble in water. Since there is no clear evidence to explain the higher bioavailability of organic zinc, there are hypotheses suggesting that organic forms of zinc are also thought to pass through absorption pathways shared with amino acids (Shi et al. 2024).

Zinc undergoes primarily hepatic metabolism, with approximately 35% of zinc being absorbed by the liver via the portal vein and subsequently released into the bloodstream (Akdas et al. 2020). Variations in tissue-specific turnover efficiencies and rates are also observed for zinc. Organs such as the liver, kidneys, and pancreas exhibit efficient zinc turnover, while muscle and blood cells display

lower rates and zinc metabolism is further diminished in the bones and nervous system. Zinc present in bones and hair, due to chronic binding, exhibits minimal participation in the metabolic processes (Glutsch et al. 2019). The feces is the main route of zinc excretion, and urine, sweat, and livestock products such as milk and eggs also contribute to zinc efflux (Cummings et al. 2009). For broilers, most of the absorbed zinc is retained in the muscle and the unabsorbed zinc is excreted out in the feces. It has been shown that zinc transporter proteins play a key role in the process of mobilizing zinc from the circulatory system to the immune system through a complex self-defense system to increase intracellular free zinc ions to counteract inflammatory and antioxidant responses in broiler chickens under stress or inflammatory states, which can be verified by serum low zinc status and changes in gene expression of zinc transporter carriers (Yu et al. 2022). The mechanism of this complex physiological regulation is still unclear, but there is no doubt that MT plays an important role in this physiological process as a zinc ion concentration sensing and regulating protein.

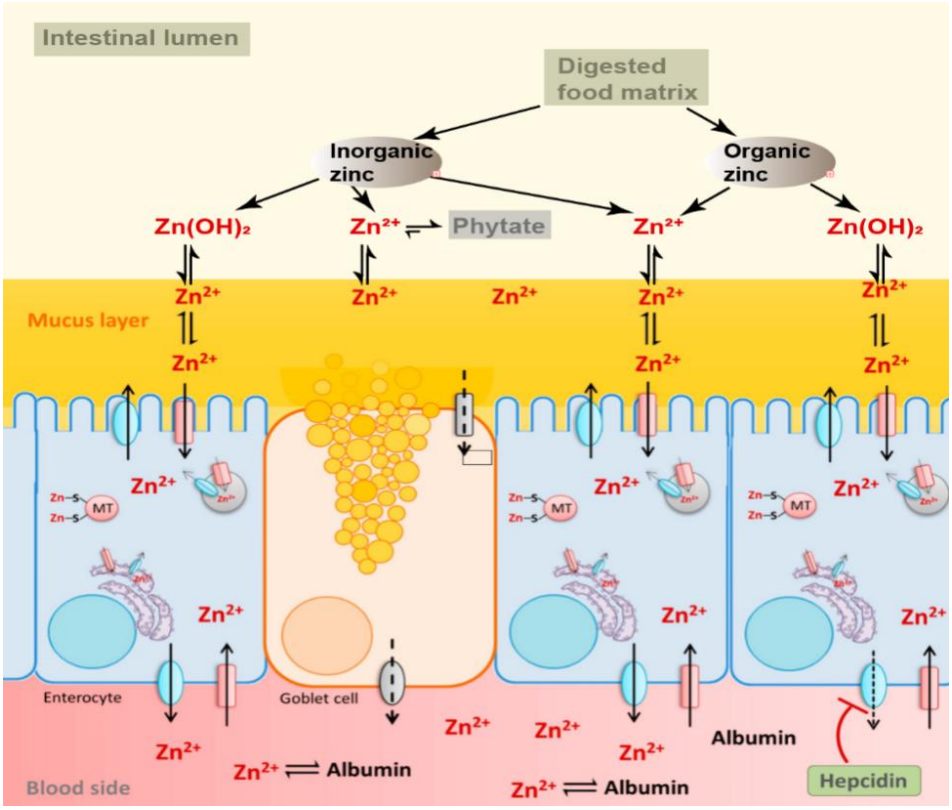

Figure 1-2: Zinc absorption and transport in the intestine

From Maares et al., 2020

5.2. Factors affecting zinc absorption

The absorption of zinc is influenced primarily by the concentration of zinc in

the food, with the intestinal availability of dietary zinc also playing a crucial role. The investigation of factors impacting zinc absorption remains a prominent subject of research, encompassing both dietary factors and the characteristics of the organism. In the case of poultry, zinc is present in the digestive tract as a low molecular supplement and in a bound form with macromolecular ligands derived from food sources (Lonnerdal 2000). Therefore, the availability of zinc is closely associated with its state of presence, which is regulated by a combination of diet composition and intestinal environment.

Dietary factors encompass the dietary composition, as research suggests that the bioavailability of zinc (referring to the absorption of zinc by the intestinal epithelial cells and subsequent release into the bloodstream) can reach up to 30% in refined grain-based diets devoid of hulls, while unrefined grain-based diets exhibit a bioavailability of approximately 20% (Bultosa et al. 2013). The type of protein ingredient in the diet and the level of phytic acid also affect the bioavailability of zinc, due to the fact that the level of phytic acid in the digested food matrix influences the concentration of free zinc in the intestine to the extent that the concentration of absorbable zinc is affected (Figure 1-3) (Katimba et al. 2024). Phytic acid forms stable precipitating substances with zinc in the intestinal environment, reducing zinc availability. Digestive enzymes are beneficial for zinc absorption, breaking down food proteins into peptides and amino acids, increasing the solubility of the chowder in the intestine and improving the bioavailability of zinc (Cousins 2010). Minerals can have a great influence on zinc absorption, with calcium being a macronutrient that affects zinc absorption due to the ability of zinc ions to be adsorbed by precipitation from complexes formed by calcium ions and phytic acid (Zhang et al. 2022). Iron and copper, on the other hand, share transporter carriers and there is competitive absorption inhibition. It is worth noting that excessive zinc intake has a more pronounced impact on iron metabolism than excessive iron intake has on zinc metabolism (Knez et al. 2017).

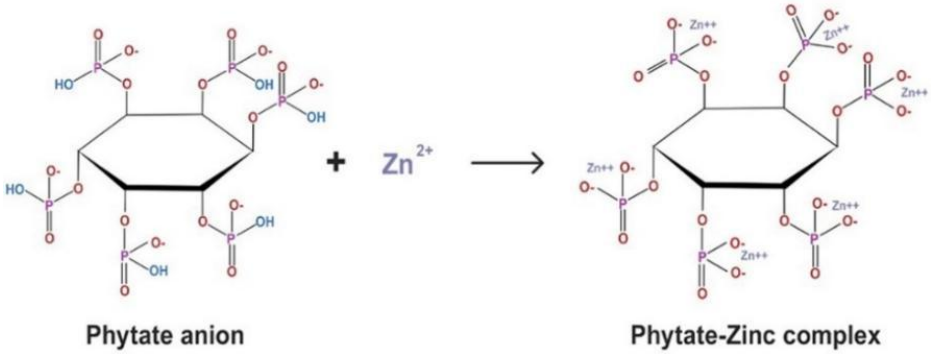

Figure 1-3: Structure of phytate and chelating possibilities with zinc.

From Katimba et al., 2024

5.3. Zinc from different sources

Due to the instability of the zinc pool, zinc homeostasis needs to be maintained by daily intake of zinc from the diet. Since the zinc content of bulk ingredients in the feed is not sufficient to cover the nutritional requirements of the animals, additional micronutrient premixes containing zinc need to be added. Zinc is typically incorporated into premixes for various animals in the form of sulfates and organic complexes, with zinc oxide being the preferred choice for piglets (Philippi et al. 2023). Organo-chelates are compounds in which metal ions form coordination bonds with amino acids, the presence of which makes the compounds more difficult to dissociate in most cases, resulting in better stability than sulfates. In addition, some types of organo-chelates have good solubility in solution, resulting in them being easier to handle in organisms or chemical reactions (Byrne et al. 2022). The coordination number of organo-zincs depends specifically on the nature of the zinc ion and the organic ligand, and the diversity allows them to function in different environments and reaction conditions. It is worth noting that organometallic chelates, in which the metal ligand bonds form a cyclic structure, are specialized complexes, with better stability (Lei et al. 2022).

Organic forms of zinc are thought to have higher biological availability compared to zinc sulfate, and researchers have attributed this to the fact that organic forms of zinc are absorbed in a different way. It has been hypothesized that zinc complexes with amino acids and small peptides may share uptake pathways with these ligands, thereby supporting the intact uptake absorption hypothesis and the competing uptake hypothesis. The intact absorption hypothesis posits that zinc forms metal ion chelates through covalent or ionic bonding with amino acid ligands. These chelates enter the intestinal lumen without undergoing hydrolysis at the parietal membrane of the small intestinal mucosa. Subsequently, they traverse the parietal membrane, mucosal cells, and basement membranes to reach the bloodstream as intact entities (Li et al. 2019a). Study on broilers have shown that the use of zinc complexed with amino acids increases zinc levels in the tibia, predicting higher bioavailability of organic zinc compared to inorganic forms (Zhu et al. 2022). Zinc absorption is also promoted when the feed is additionally supplemented with small molecules such as amino acids and small peptides (Duan et al. 2023). It needs to be further investigated whether these small molecules chelated with zinc are absorbed under intact molecular form. The competitive absorption hypothesis highlights that trace elements chelated with ligands can circumvent the inhibitory effect of other ligands present in the intestinal lumen. This enables them to directly reach the mucosal absorption site within the intestinal cells, where they are absorbed into the bloodstream as ions through the intestinal epithelial cells (Ashaolu et al. 2023). Further verification is required to determine whether organic zinc is completely dissociated and absorbed, or partially dissociated with the remaining portion being absorbed as a complete complex, under the influence of ligand bonding. Recent research indicates a significant reduction in the absorption of zinc methionine chelate when the methionine transporter is blocked in an intestinal model, suggesting that zinc amino acids may be absorbed through the amino acid absorption system (Hu et al. 2023b).

6. Conclusion

This general introduction discusses the critical role of zinc in modern broiler nutrition and its impact on stress, inflammation and intestinal health. We summarized the biology of zinc, particularly the research on zinc applications in poultry, as well as common stresses and challenges in the broiler industry. In response to these issues, the introduction also suggests application options for different sources of zinc in feed to improve stress resistance and immune system function in broilers. By comprehensively exploring the nutritional role and application prospects of zinc, insights are provided for optimizing broiler feeding strategies.

Chapter II

Aims and outline of the thesis

1. Aims of the thesis

Due to the rapid growth rate and efficient nutrient conversion of broiler chickens, chicken meat has emerged as a crucial animal product for human consumption. However, the intense nutrient metabolism of broilers results in elevated body heat production and physiological stress, potentially leading to heat stress and inflammation. Furthermore, the susceptibility to pathogens for broilers in the early stage due to the developing intestinal barrier function and a still-establishing intestinal microbiota exacerbate health risks, ultimately diminishing their growth performance and survival rates. As previously noted in the "General Introduction" section, zinc, an essential trace element, is involved in numerous metabolic processes in broilers. Despite the acknowledged significance of zinc, there remains a lack of research elucidating its mechanisms in enhancing poultry performance and health. Our hypothesis speculates that the observed mechanism may be attributed to the ability of zinc to alleviate inflammation and oxidative stress, consequently enhancing broiler growth performance. The objectives of this thesis were: (1) to examine alterations in zinc homeostasis in broiler chickens subjected to heat stress, inflammation, and necrotic enteritis, three important challenge models in the poultry sector; (2) to elucidate the function and mechanism of zinc in reducing inflammatory response and affecting intestinal health in broilers; (3) to investigate alterations in meat quality and the role and mechanism of zinc in enhancing meat quality in the presence of inflammatory challenges.

2. Outline of the thesis

To achieve the research objectives, three experiments were conducted and outlined within the context of action (Figure 2-1). Chapter III specifically investigated the impact of a heat stress challenge on zinc metabolism in broiler systems, hypothesizing that heat stress may alter zinc transport and deposition in broiler tissues. To address this hypothesis, broilers aged 28 days were subjected to an environmental heat stress (ambient temperature of 26 °C for the control group and 36 °C for the heat stress group), and various parameters including growth performance, zinc levels in serum and tissues, and antioxidant capacity were assessed. The gene expression of zinc transporters in the liver and jejunum was assessed and confirmed by Western blot analysis. Additionally, the microbial composition of the cecum was determined.

Reference: Xiao, C., Kong, L., Pan, X., Zhu, Q., Song, Z., and Everaert, N. (2022). High temperature-induced oxidative stress affects systemic zinc homeostasis in broilers by regulating zinc transporters and metallothionein in the liver and jejunum [J]. *Oxidative Medicine and Cellular Longevity*, 2022.

Based on the observation that heat stress led to a disruption in zinc homeostasis in broilers and after characterization of zinc transporter function, Chapter IV focused on investigating the impact of immune challenge from lipopolysaccharide (LPS) exposure on zinc homeostasis and regulation of zinc glycine. A broiler embryo model was used. Broiler fertilized eggs were allocated into four groups: CON (control group, injected with 0.5 mL of saline), LPS (LPS group, injected

with 0.5 mL of saline solution containing 32 μg of LPS), Zn-Gly (zinc glycinate group, injected with 0.5 mL of saline solution containing 80 μg of zinc glycinate) and Zn-Gly+LPS (zinc glycinate and LPS groups, injected with the same amount of zinc glycine and LPS saline solution). Eggs were incubated under standard conditions, *in ovo* feeding technique was implemented on embryonic day 17.5, and sample collection was performed after the following 12 hours. The study analyzed zinc concentrations in serum, bone, and various tissues, as well as levels of inflammatory cytokines, antioxidant status, intestinal barrier function, zinc-related enzyme activities, and zinc transport-related gene expression.

Reference: Xiao, C., Comer, L., Pan, X., Everaert, N., Schroyen, M., and Song, Z. (2024). Zinc glycinate alleviates LPS-induced inflammation and intestinal barrier disruption in chicken embryos by regulating zinc homeostasis and TLR4/NF-κB pathway [J]. *Ecotoxicology and Environmental Safety*, 272, 116111.

Chapters V and VI explored changes in zinc metabolism of broilers with necrotic enteritis and the potential of different zinc sources to enhance defense mechanisms through zinc metabolism, thereby mitigating inflammatory responses, compromising intestinal health, and reducing meat quality associated with necrotic enteritis. The broilers were allocated to six treatment groups, which included a negative dietary control group (NT, receiving a basal diet without supplemental zinc), an inorganic zinc-treated group (IT, receiving a diet with an additional 60 mg/kg of zinc sulfate), an organic zinc-treated group (OT, receiving a diet with an additional 60 mg/kg of zinc glycinate), a negative control group challenged with necrotic enteritis (CNT, challenged with NT feed), a group challenged with necrotic enteritis without supplemental zinc (CIT, challenged with IT feed), and a group challenged with necrotic enteritis with supplemental organic zinc (COT, challenged with OT feed). The broilers in the three challenge groups were orally inoculated with a 30-fold dose of coccidiostat vaccine and broth cultures containing *Clostridium perfringens*. Chapter V explores the impact of various dietary zinc sources on growth performance, zinc concentrations in various tissues, intestinal health, and immune function in broilers facing challenges. Chapter VI shifts focus to meat quality, exploring the effects of necrotic enteritis on antioxidant capacity and meat quality in broilers, as well as analyzing the non-targeted metabolome and fatty acid composition of chicken meat.

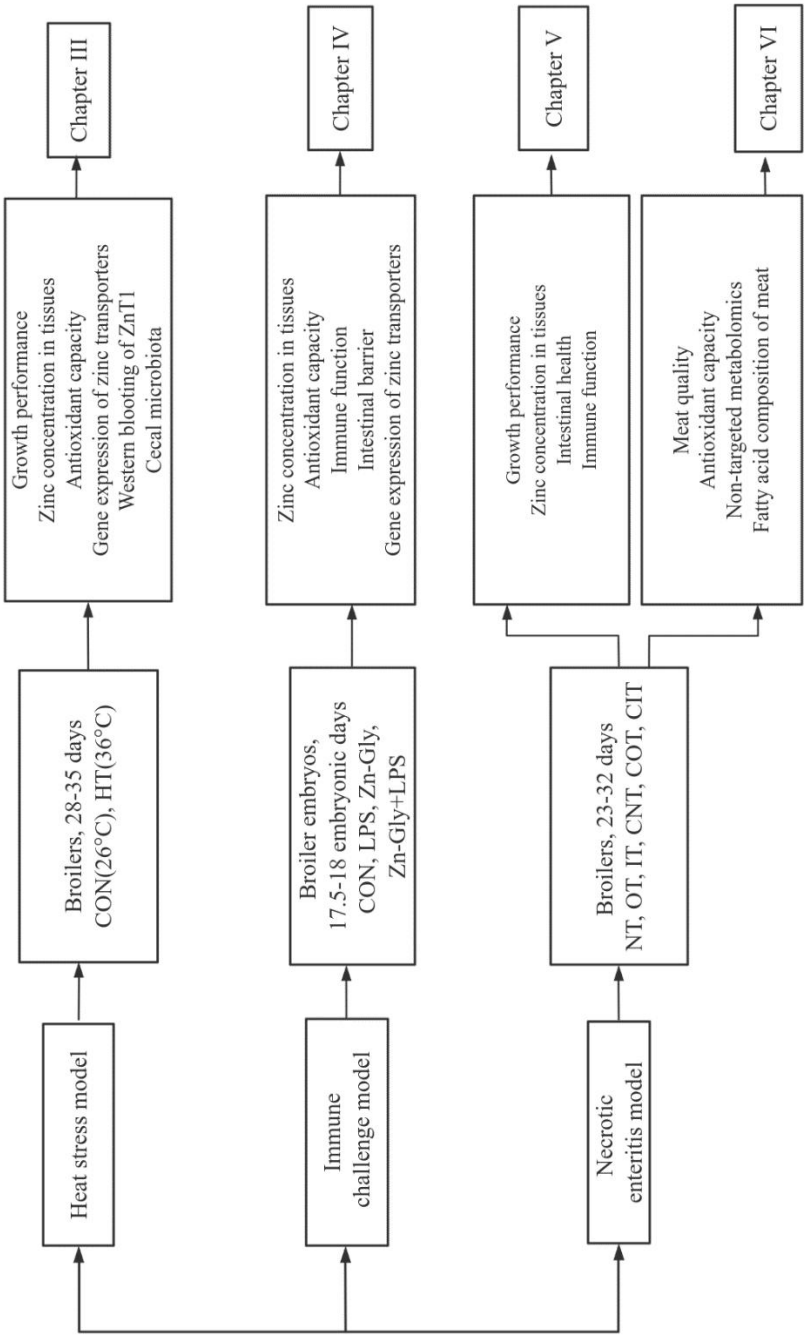

Figure 2-1: The technical route of the thesis

Chapter III

High temperature-induced oxidative stress affects systemic zinc homeostasis in broilers by regulating zinc transporters and metallothionein in the liver and jejunum

This chapter is based on the following publication:

Chuanpi Xiao, Linglian Kong, Xue Pan, Qidong Zhu, Zhigang Song, and Nadia Everaert

High temperature-induced oxidative stress affects systemic zinc homeostasis in broilers by regulating zinc transporters and metallothionein in the liver and jejunum

Oxidative Medicine and Cellular Longevity, 2022(1), 1427335.

Chapter III. High temperature-induced oxidative stress affects systemic zinc homeostasis in broilers by regulating zinc transporters and metallothionein in the liver and jejunum

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Keywords: Broiler; Metallothionein; Oxidative stress; ZIP; ZnT; Zinc homeostasis.

1. Abstract

To investigate the change in zinc homeostasis of broilers under heat stress, 512 broiler chickens were raised to the age of 28 days. The broilers were then assigned to heat stress and normal temperature (36 °C vs. 26 °C) groups for 7 days. The results showed that oxidative stress induced by high temperature had a negative effect on the growth performance of broilers. Heat stress altered zinc homeostasis and led to a redistribution of zinc in broilers, which was reflected in increased zinc concentrations in the jejunum, liver, and tibia. Upregulation of the expression of the zinc exporter *ZnT1* and importers *ZIP8* and *ZIP14* in the jejunum indicated that more zinc was absorbed and transported from the jejunum into the blood, while the liver increased its capacity to hold zinc through upregulation of metallothionein (*MT*) expression, which was achieved by reducing *ZnT1* expression and upregulating the expression of the importer *ZIP3*. The pathway was mediated by zinc transporters, but the capacity of MT to chelate and release zinc ions also played a crucial role. The mechanism of alterations in zinc homeostasis under heat stress was revealed by the changes in zinc transporters and MT levels in the intestine and liver. Heat stress also altered cecal microbial diversity and reduced the relative abundances of *Bilophila* and *Dialister*. In conclusion, broilers altered systemic zinc homeostasis through the regulation of zinc transporters and MT in the liver and jejunum to resist oxidative stress induced by high temperature.

2. Introduction

With global warming and the aggravation of the greenhouse effect, heat stress has become a challenge that cannot be ignored in animal husbandry. In poultry production, broilers have shown great growth potential with the improvement of modern breeding technology (Tallentire et al. 2016). However, the increased growth rate will accelerate metabolism and likely lead to heat stress; the lack of sweat glands in broilers is not conducive to heat dissipation. Heat stress could cause physiological system disorders in broilers, including immune system damage, respiratory alkalosis, and hormone secretion disorders (Liu et al. 2020). These phenomena could lead to oxidative stress by affecting mitochondrial function and changing reactive oxygen species (ROS) levels, thereby causing oxidative damage to proteins and lipids and changes in the levels of oxidative stress markers, such as malondialdehyde (MDA), glutathione peroxidase (GSH-px), and superoxide dismutase (SOD) (Emami et al. 2020). When heat stress occurs, the heat shock protein (HSP) family begins to act as a stress indicator and cell protector (Uerlings et al. 2018). A large number of studies have shown that the HSP70 protein is related to the immune function of birds, such as controlling the transcription of various genes in response to immune stimulation by regulating the activity of nuclear factor kappa light chain enhancer of B cells (NF-κB) (Kumada et al. 2019).

Zinc, an essential trace element, participates in a variety of enzymatic reactions and affects various biological processes, such as digestion, absorption, and metabolism of nutrients in animals (Wu 2017). Therefore, the metabolism and

homeostasis of zinc is a complex physiological process. An increasing amount of evidence indicates that many protein families, such as metallothionein (MT) and zinc-regulated transporters (Zrts), iron-regulated transporter (Irt)-like proteins (ZIPs) and zinc transporters (ZnTs), play major roles in zinc metabolism and homeostasis (Kambe et al. 2004). MT is a family of low-molecular metal-binding proteins that are rich in cysteine and usually bind zinc in cells to serve as a zinc reservoir (Baltaci et al. 2018). MT is not only a zinc reservoir but also a potential diagnostic biological marker of intracellular zinc content. MT also participates in a variety of physiological processes, including oxidative stress and immune function (Mocchegiani et al. 2011). ZIPs belong to the SLC39A family and transport zinc into the cytoplasm from extracellular or cellular organelles, while ZnTs belong to the SLC30A family and transport zinc from the cytoplasm to the outside of the cell, functioning in zinc mobilization across biological membranes. Thus far, 10 ZnT transporters and 14 ZIP transporters have been discovered (Kambe et al. 2015). In an inflammatory or oxidative stress state, the level of liver MT can rise rapidly, while the expression of the zinc finger protein A20 is altered. Moreover, NF-κB is negatively regulated to inhibit the production of inflammatory cytokines (Prasad et al. 2011).

Adding zinc to feed has positive effects on animal growth and immunity. According to the National Research Council (NRC, 1994), the requirement of zinc for broilers is 40 ppm, and approximately 100 ppm of zinc from different sources is typically added to feed to optimize animal performance (Gheisari et al. 2011). The underlying mechanisms could be attributed to altered intestinal histomorphology and reduced inflammation and oxidative stress (Bortoluzzi et al. 2020) resulting in improved growth of broilers. Increasing lines of evidence indicate that zinc is redistributed in animals under conditions of oxidative stress and immune challenge (Sahin et al. 2009). However, changes in zinc homeostasis in broilers under heat stress conditions and the regulatory mechanism have not been reported.

In this study, we investigated the changes in systemic zinc homeostasis and the regulatory mechanism in broilers under oxidative stress and inflammation caused by heat stress. The role of zinc transporters and metallothioneins in the regulation of zinc metabolism under heat stress conditions was innovatively revealed.

3. Materials and methods

All experimental procedures were approved by the Ethics Committee of Shandong Agricultural University and performed in accordance with the Guidelines for Experimental Animals of the Ministry of Science and Technology (Beijing, People's Republic of China). All feeding and euthanasia procedures were performed with full consideration of animal welfare.

3.1. Experimental design and animal management

A total of 512 28-day-old male broiler chicks (Arbor Acre) with similar weights were randomly divided into two treatment groups, each of which included 16 replicates (cages) and 16 birds per cage. The two treatments were as follows: half of the birds were assigned to temperature treatment at 36 ± 1 °C (heat stress group,

HT) for 7 days; birds in the other treatment group were raised at a normal temperature of 26 ± 1 °C (CON). All birds were given free access to pellet feed and water during the rearing period. The basal diet composition and nutrition levels of the basal diet are listed in Table 3-1, the diet was formulated according to the "Compound Feed for Egg Laying Chickens and Broilers" (GB/T 5916-2020) in the People's Republic of China. During the first 3 days, the average relative humidity was maintained at approximately 70% and was maintained between 55% and 65% thereafter. According to the Arbor Acres broiler management handbook (2018), the birds are exposed to 23 hours of light and 1 hour of darkness for the first week of life, which changes to 20 hours of light and 4 hours of darkness beginning on the seventh day. All broilers were vaccinated by means of drinking water. Newcastle disease (ND) and infectious bronchitis (IB) vaccines were administered on day 6 and infectious bursal disease (IBD) vaccine on day 12. The use of antibiotics was strictly prohibited to ensure the effectiveness of the experiment.

Table 3- 1: Composition and nutritional levels of the basal diet

Item	1-21 days	22-35 days
Ingredient (%)		
Corn	49.98	54.9
Soybean meal (46%)	35.75	30.5
Corn protein flour (60%)	3.80	3.10
Salt	0.28	0.28
Limestone	1.75	1.62
Dicalcium phosphate	1.55	1.40
Soybean oil	5.10	6.50
Vitamin premix	0.05	0.05
Mineral premix	0.20	0.20
Choline chloride (50%)	0.10	0.10
Methionine (99%)	0.35	0.35
Lysine (70%)	0.80	0.75
Threonine (98.5%)	0.29	0.25
Phytase (20000 U)	0.02	0.02
Total	100	100
Nutritional level		

Metabolizable energy	3100 (kcal/kg)	3200 (kcal/kg)
Crude protein	23.5	21.0
Lysine	1.39	1.20
Methionine+cystine	1.02	0.92
Calcium	1.00	0.90
Total phosphorus	0.99	0.90
Available phosphorus	0.50	0.45

Provided per kilogram of compound diet: vitamin A, 12000 IU; vitamin D3, 5000 IU; vitamin E, 80 mg; vitamin K, 3.2 mg; vitamin B1, 3.2 mg; vitamin B2, 8.6 mg; nicotinic acid, 65 mg; pantothenic acid, 20 mg; vitamin B6, 4.3 mg; biotin, 0.22 mg; folic acid, 2.2 mg; vitamin B12, 0.017 mg; I, 1.50 mg; Fe, 80 mg; Mn, 120 mg; Se, 0.3 mg; Cu, 16 mg; and Zn, 110 mg. The nutrition level was calculated.

3.2. Growth performance

At 35 days of age, feed consumption was recorded to calculate the average daily feed intake (ADFI) for each replicate. The birds were weighed to calculate the average daily gain (ADG). The feed conversion ratio (FCR) was defined as ADFI: ADG. Mortality data were recorded and included in the FCR calculation.

3.3. Sample collection

One bird was randomly selected from each cage at 35 days of age after the measurement of growth performance. Blood samples were collected intravenously with a sterile syringe from the wing, placed in a glass tube without anticoagulant, and centrifuged at 3000 rpm at 4 °C for 10 min after being left to stand for 30 min. Serum was obtained and stored at -20 °C for biochemical analysis. The birds were euthanized by cervical dislocation after obtaining blood samples. Approximately 2 cm segments were excised from the mid-jejunum (from the entry point of the bile duct to Meckel's diverticulum), flushed repeatedly with a cold saline solution, and immediately immersed in a 4% paraformaldehyde solution for histological examination. Tissue samples of approximately 1 g to 2 g were collected from the jejunum and liver, rapidly frozen in liquid nitrogen, and stored at -80 °C for further analysis. Tibia from both sides were dissected carefully and stored at -20 °C until analysis. All birds selected were fasted for eight hours before the procedure.

3.4. Analysis of oxidative stress and cytokines in serum

The total SOD (T-SOD) activity and MDA, and endotoxin levels were measured in the serum by using diagnostic kits purchased from Nanjing Jiancheng Biotechnology Institute (Nanjing, China). All determination procedures were performed strictly according to the manufacturer's instructions. The intraassay coefficient of variation (CV) was less than 5%, and the interassay CV was less than 8%.

3.5. Zinc and metallothionein concentrations

The serum concentration of zinc was measured by an inductively coupled plasma optical emission spectroscopy instrument (ICAP 7000, Thermo, USA). Each serum sample (75 μL) was placed in a centrifuge tube with 600 μL of HNO3 (5%) and 75 μL of hydrogen peroxide (30%). The centrifuge tubes were placed in a water bath at 60 °C for 2 h, and 750 μL of HNO3 (5%) was added. The mixed solution was centrifuged for 15 min (8000 r/min), and the supernatant was stored at 4 °C until measurement. The concentrations of zinc in the tibia, liver, jejunum and cecal contents were evaluated via flame atomic absorption spectrometry (SpectrAA 50/55, Warman Corporation, Palo Alto, CA, USA). The tibiae were boiled in deionized water for 10 min, soaked in ether for 96 h, degreased, and dried at 105 °C to constant weight. The liver and jejunum tissues were freeze dried for 24 h and weighed. The samples from the tibia, liver, and jejunum were ashed in a muffle oven (550-600 °C for 24 h). Ash content was measured and expressed as dry degreased weight. The ash of the sample was dissolved with 0.6 mol/L hydrochloric acid and filtered. The solution was stored at 4 °C before determination.

The concentrations of MT in the liver and jejunum were measured using ELISA kits (MLBIO Co., Shanghai, China) according to the manufacturer's instructions. The inter- and intraassay CVs were less than 10 %.

3.6. Total RNA extraction and real-time PCR

Total RNA in the jejunum was extracted with TRIzol reagent (Invitrogen, San Diego, USA). The concentration and purity of each RNA sample were detected using a NanoDrop spectrophotometer (ND-2000, Thermo Scientific, Wilmington, USA). RNA integrity was detected by 1% agarose gel electrophoresis. Reverse transcription of 1 μg of total RNA was performed using a PrimeScript® RT reagent kit (RR047A, TaKaRa, Japan). RT-PCR analysis was performed to determine gene expression by using TB Green Premix Ex Taq (RR820A, Takara, Japan) in an ABI 7500 Real-Time PCR System (Thermo Scientific, Wilmington, USA). The reaction program included the following: predenaturation at 95 °C for 10 s, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing and extension at 60 °C for 40 s. Each reaction was repeated in triplicate wells, and the primer sequences are shown in Table 3-2. The amplification efficiencies of the primers were calculated using a standard curve. The specificities of the amplified products were verified by the melting curve. The geometric mean of the expression of *β-actin* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used to normalize the expression of the target genes. Relative gene expression levels of each target gene were analyzed using the $2^{-\Delta\Delta ct}$ method.

Table 3- 2: Nucleotide sequences of real-time PCR primers

Gene	Accession number	Primer sequence, 5'→ 3'	Product size (bp)
ZnT1	NM_001389457.1	F: CTTCGCTTAGCATTTCTT R: TCTCCGATTTAGTCCTTCT	75
DMT1	NM_001128102.2	F: AGCCGTTCACCACTTATTTCG R: GGTCCAAATAGGCGATGCTC	129
ZIP3	NM_144564.4	F: GGGCACTTTCTTGTTCATCACC R: GCAGCATAACCCAGCACCAG	105
ZIP8	XM_040671236.1	F: TGTAAATGTCTCGGTGGG R: CAAGATGGCTATGGAGGT	159
ZIP14	XM_040689606.1	F: GTTCTGCCCCGCTGTCCT R: GGTCTGCCCTCCTCCGTCT	96
MT	NM_205275.1	F：GCAACAACTGTGCCAAGGGC R: TTTCGTGGTCCCTGTCACCC	138
MTF-1	XM_015297695.3	F: CCTGGTTCAACTCCTATGC R: TCAAACGGCTTCTCCTTA	278
NF-λB	NM_205129	F：GTGTGAAGAAACGGGAACTG R: GGCACGGTTGTCATAGATGG	203
A20	XM_003640919.2	F：GACATCGTGCTAACAGCTTGGA R: AGAAAAGAGGTATCAGGCACAAC	141
S100A9	NM_001305151.1	F: TTGAGAAGCAGCTTGCCAACTAC R: TGCTGTTGCTGGTGGTCCTC	187
HSP70	NM_001006685.1	F: TCTCATCAAGCGTAACACCAC R: TCTCACCTTCATACACCTGGAC	104
β-actin	NM_205518.1	F: ATGTGGATCAGCAAGCAGGAGTA R: TTTATGCGCATTTATGGGTTTTGT	127
GADPH	NM_204305	F: ACATGGCATCCAAGGAGTGAG R: GGGGAGACAGAAGGGAACAGA	266

MT: metallothionein; *ZIP*: zinc-regulated transporter, iron-regulated transporter-like protein; *ZnT*: zinc transporter; *DMT1*: divalent metal transporter 1; *MTF-1*: metal transcription factor-1; *NF-κB*: nuclear factor kappa-B; *HSP70*: heat shock protein 70; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase.

3.7. Western blot analysis

Frozen liver and jejunum samples were crushed into powder in liquid nitrogen, and protein extraction was performed using a total protein extraction kit (CW Biotech, Beijing, China) as specified by the manufacturer's instructions. After the protein concentration of the sample was determined using the BCA protein analysis kit (Beyotime Institute of Biotechnology, Beijing, China), a 40 μg protein sample was loaded into a 10% sodium dodecyl sulfate-polyacrylamide gel for electrophoresis (New Cell & Molecular Biotech Co, Suzhou, China). The protein was separated in electrophoresis buffer at a constant voltage of 150 mV and then transferred to a polyvinylidene difluoride (PVDF) membrane (Invitrogen, Carlsbad, CA, USA) with transfer buffer (Beyotime Institute of Biotechnology, Beijing, China) at a constant current of 400 mA. After membrane transfer, the PVDF membrane was immersed in a blocking buffer for 0.5 h and then incubated overnight with primary antibodies at 4 °C. Primary antibodies against ZnT1 (1:500) and GAPDH (1:2000) were purchased from Bioss Technology Inc. After three washes with TBST buffer, a secondary horseradish peroxidase-conjugated antibody (Beyotime Institute of Biotechnology, Beijing, China) was incubated at room temperature for 1 h. Finally, the protein blot strips were visualized in a gel imager using ECL kits (Beyotime Institute of Biotechnology, Beijing, China), and signals were quantified using the measurement software in the imager. GAPDH protein was used as an internal control for all the immunoblotting bands to obtain the corresponding expression of the target proteins.

3.8. 16S rRNA gene amplicon sequencing

Samples of cecal contents were collected on ice after slaughter and immediately stored in a -80 °C freezer for subsequent analysis. Microbial DNA extraction from cecum contents was performed according to the instructions of the E.Z.N.A.® soil kit (Omega Bio-tek, Norcross, GA, USA). The DNA concentration and purity were measured using a NanoDrop 2000 spectrophotometer, and the quality of DNA extraction was determined using 1% agarose gel electrophoresis. PCR amplification of the V3-V4 variable region of the bacterial 16S rRNA gene was performed using the 338F and 806R primers. The PCR conditions consisted of an initial denaturing program for 3 min at 95 °C, 27 cycles (95 °C for 30 s, 55 °C annealing for 30 s, and 72 °C for 30 s), and a final extension step at 72 °C for 10 min (PCR instrument: GeneAmp9700 produced by ABI). PCRs were performed in a 20 μL mixture: 4 μL of 5X FastPfu buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of primer (5 μM), 0.4 μL of FastPfu polymerase and 10 ng of DNA template. PCR products were recovered using a 2% agarose gel, purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor™-ST (Promega, USA).

Purified amplicons were pooled in equimolar amounts and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, USA) according to standard protocols. Quantitative Insights Into Microbial Ecology 2 (QIIME2) software was used for quality screening of the raw sequences, and quality filtering and pruning, denoising, merging and chimera removal of the demultiplexed sequence of each sample were carried out to obtain the amplicon

sequence variation feature table. According to the 338F/806R primers, the database obtained in the previous step was pruned to the V3-V4 region to obtain the species classification table. After all contaminating mitochondrial and chloroplast sequences were removed, appropriate methods, including ANCOM, ANOVA, Kruskal-Wallis test, and LEfSe, were used to identify bacteria with differential abundances between samples and groups. QIIME2 core diversity was used to calculate the horizontal alpha diversity index of feature sequences, including observed operational taxonomic units (OTUs), Chao1 abundance estimator, Shannon diversity index and Faith's phylogenetic diversity index. The beta diversity index included Bray-Curtis, unweighted UniFrac and weighted UniFrac indices, which were used to evaluate the structural changes in microbial communities between samples and are presented in principal coordinate analysis (PCoA) and nonmetric dimensional scaling (NMDS) diagrams. Partial least squares discriminant analysis in R software was used as a monitoring model to reveal the relationship between the microbial community and sample category. The R package "Vegan" redundancy analysis method was used to reveal potential associations between microbial communities and related environmental factors. Spearman rank correlation coefficients were calculated using co-occurrence analysis to show associations between species based on the relative abundances of major microbial species in the samples. The parameters used in the analysis were set as the defaults.

3.9. Statistical analysis

Data are presented as the mean ± SD. All data were analyzed through normal distribution determination. Growth performance was analyzed on a replicate (per cage) basis while other indicators were analyzed on an individual basis. Differences in the treatments were analyzed with a t-test in SPSS 22.0 (SPSS. Inc., Chicago, USA). Significance was set as $P < 0.05$.

4. Results

4.1. Growth performance

Heat stress had a significant effect on growth performance. As shown in Figure 3-1, heat stress significantly reduced the ADFI and ADG ($P < 0.05$) and increased the FCR and mortality of 35-day-old broilers ($P < 0.05$).

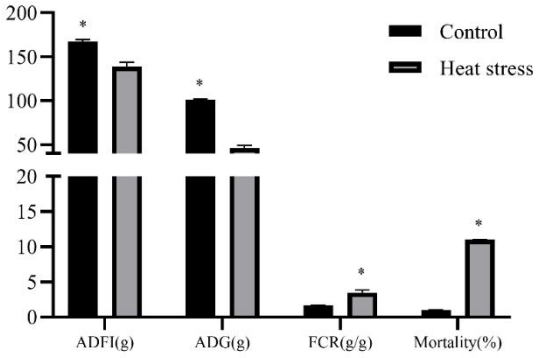


Figure 3- 1: Effects of heat stress on growth performance. ADG: average daily gain; ADFI: average daily feed intake; FCR: feed conversion ratio

4.2. Analysis of oxidative stress in serum

The heat stress group had higher T-SOD ($P = 0.047$) and endotoxin ($P = 0.015$) levels in serum (Table 3-3). No significant difference was found in serum MDA levels ($P = 0.949$) between the heat stress and normal temperature groups.

Table 3- 3: Effects of heat stress on serum oxidative parameters

Temperature	Control	Heat stress	*P*-value
MDA (nmol/mL)	3.11±0.13	3.09±0.27	0.949
T-SOD (U/mL)	100.46 ± 7.48^{b}	120.35 ± 5.27^{a}	0.047
Endotoxin (EU/mL)	0.32 ± 0.03^{b}	0.47 ± 0.05^{a}	0.015

MDA: malondialdehyde; T-SOD: total superoxide dismutase. Values are expressed as the mean ± SD (n = 8). Different superscripts (a, b) in the same line indicate significant differences ($P < 0.05$).

4.3. Zinc concentration in tissues and cecal contents

As shown in Figure 3-2a, the concentration of zinc increased in the jejunum, liver, and tibia in response to heat stress ($P < 0.05$), while the opposite result was observed in the cecal contents. However, heat stress had no significant effects on the serum concentration of zinc. The results indicated that zinc was redistributed under heat stress.

4.4. MT concentration in tissues

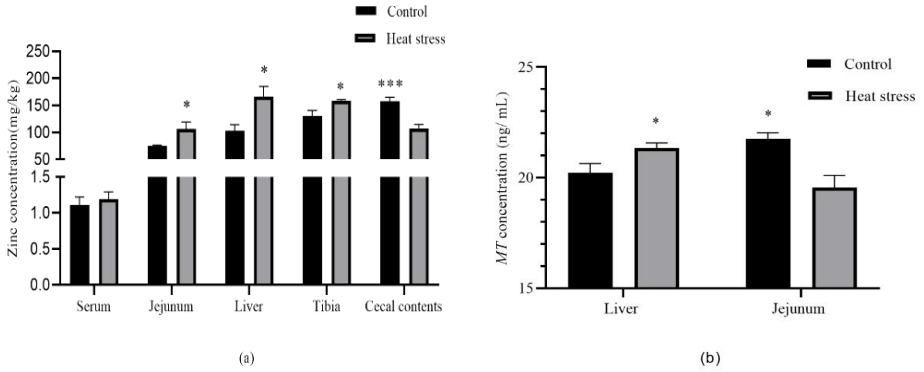
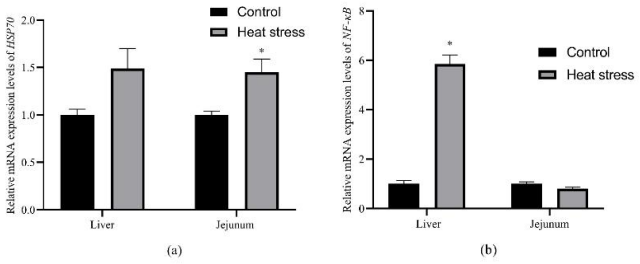


Figure 3-2: Zinc and metallothionein concentrations in different tissues of broilers were affected by heat stress. (a) Zinc concentrations in the serum, jejunum, liver, tibia and cecal contents of broilers; (b) MT concentrations in the liver and jejunum of broilers. MT: metallothionein

Figure 3-2b summarizes the MT concentration in the liver and jejunum. Heat stress increased the MT concentration in the liver ($P = 0.049$). Moreover, the MT concentration in the jejunum was decreased under heat stress conditions ($P = 0.003$).

4.5. mRNA expression of jejunal and liver genes

Figure 3-3a shows the mRNA expression of *HSP70* in the jejunum and liver after heat stress. We also determined the mRNA expression levels of the immune and inflammatory genes calprotectin *NF-κB* and *S100A9* (Figure 3-3, b-c). In general, the expression of the *HSP70* gene in the jejunum was upregulated after heat stress ($P < 0.05$). Moreover, the expression levels of *S100A9* and *NF-κB* in the liver were upregulated ($P < 0.05$).



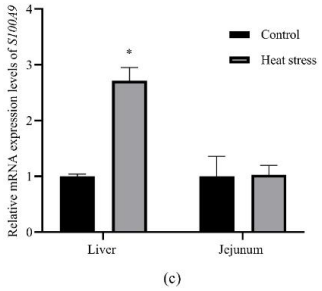


Figure 3-3: Influence of heat stress on the gene expression of *HSP70* (a), *NF-κB* (b), and *S100A9* (c) in the liver and jejunum. *HSP70*: heat shock protein 70; *NF-κB*: nuclear factor kappa light chain enhancer of B cells.

Figure 3-4 shows that heat stress altered zinc metabolism in the liver and jejunum, which plays a crucial role in regulating systemic zinc homeostasis. The expression of the zinc transporters *ZnT1*, *ZIP8*, and *ZIP14* was downregulated ($P < 0.05$), and that of the divalent metal transporter 1 (*DMT1*) showed the same trend ($P < 0.05$) in the liver. The mRNA expression of the zinc importer *ZIP3* was significantly upregulated ($P < 0.05$) in the livers of heat-stressed broilers (Figure 3-4a). Moreover, heat stress increased the gene expression levels ($P < 0.05$) of zinc transporters (*ZnT1*, *ZIP8*, and *ZIP14*) and *DMT1* in the jejunum (Figure 3-4b). Heat stress resulted in

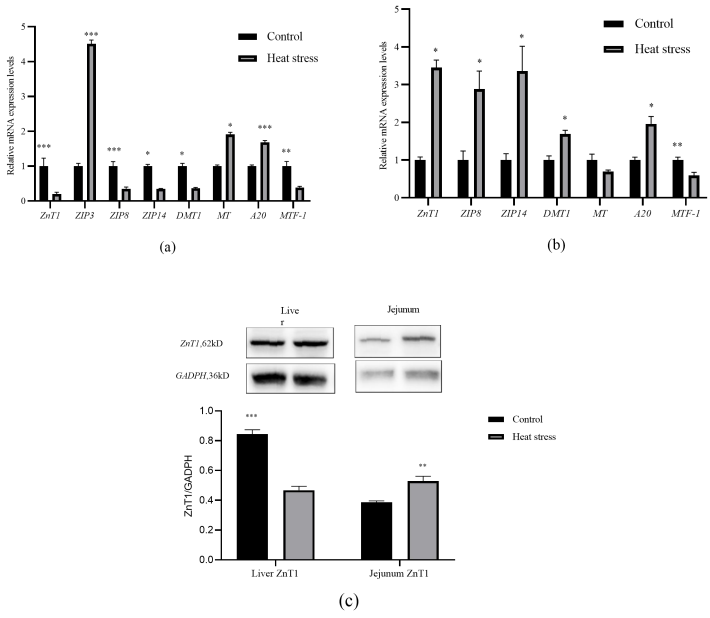


Figure 3-4: Influence of heat stress on gene and protein expression in the liver and jejunum. (a) Zinc transporter- and zinc transporter regulation-related genes in the liver; (b)

zinc transporter- and zinc transporter regulation-related genes in the jejunum; (c) protein expression of ZnT1 in the liver and jejunum. *MT*: metallothionein; *ZIP*: zinc-regulated transporter, iron-regulated transporter-like protein; *ZnT*, zinc transporter; *DMT1*: divalent metal transporter 1; *MTF-1*: metal transcription factor-1.

Higher gene expression levels of *MT* and the zinc finger protein *A20* ($P < 0.05$) in the liver, and *A20* expression showed the same trend in the jejunum. The expression of metal-binding transcription factor-1 (*MTF-1*) was downregulated ($P < 0.05$) in both organs.

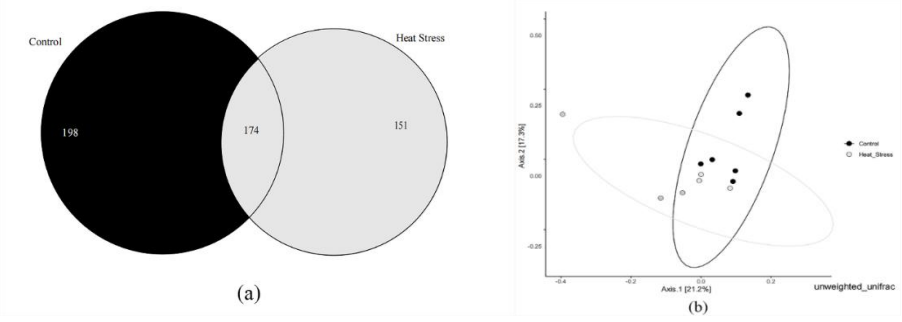
4.6. Protein expression of jejunal and liver genes

As shown in Figure 3-4c, heat stress elevated the protein expression of ZnT1 in the liver but decreased the protein expression of ZnT1 in the jejunum ($P = 0.013$). This result confirmed the same trend as the gene expression results.

4.7. Composition and community diversity of the cecal microbiota

In 35-day-old broilers, 174 OTUs were identical in the cecal contents of both the heat stress group and normal temperature group, while 198 and 151 OTUs were unique to the normal temperature group and heat stress group, respectively (Figure 3-5a). β diversity analysis using unweighted UniFrac distance did not show specific clustering between the two treatments (Figure 3-5b). As shown in Figure 3-5c, heat stress significantly decreased the Chao1, PD, Shannon, and Simpson indices of α diversity ($P < 0.05$).

Phylum level-based analyses showed that over 95% of the cecal microbiota in both treatment groups was dominated by three major phyla: *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* (Figure 3-6). There was no significant effect of heat stress on the relative abundance of the cecum microbial community at the phylum level. Taxonomic analysis of the relative abundances of the 20 predominant genera in each group was used to confirm specific changes in the microbial community (Figure 3-7a). The results showed that heat stress reduced the relative abundances of *Bilophila* and *Dialister* at the genus level (Figure 3-7b).



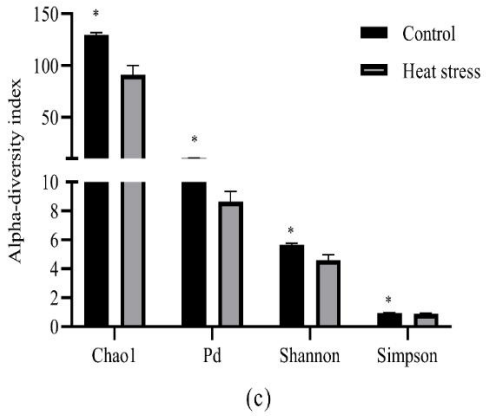


Figure 3-5: Heat stress altered the community diversity of the cecal microbiota. (a) Venn diagram based on the OTU level. (b) PCoA plots of β diversity based on OTUs. (c) α diversity based on the Chao1, PD, Shannon, and Simpson indices.

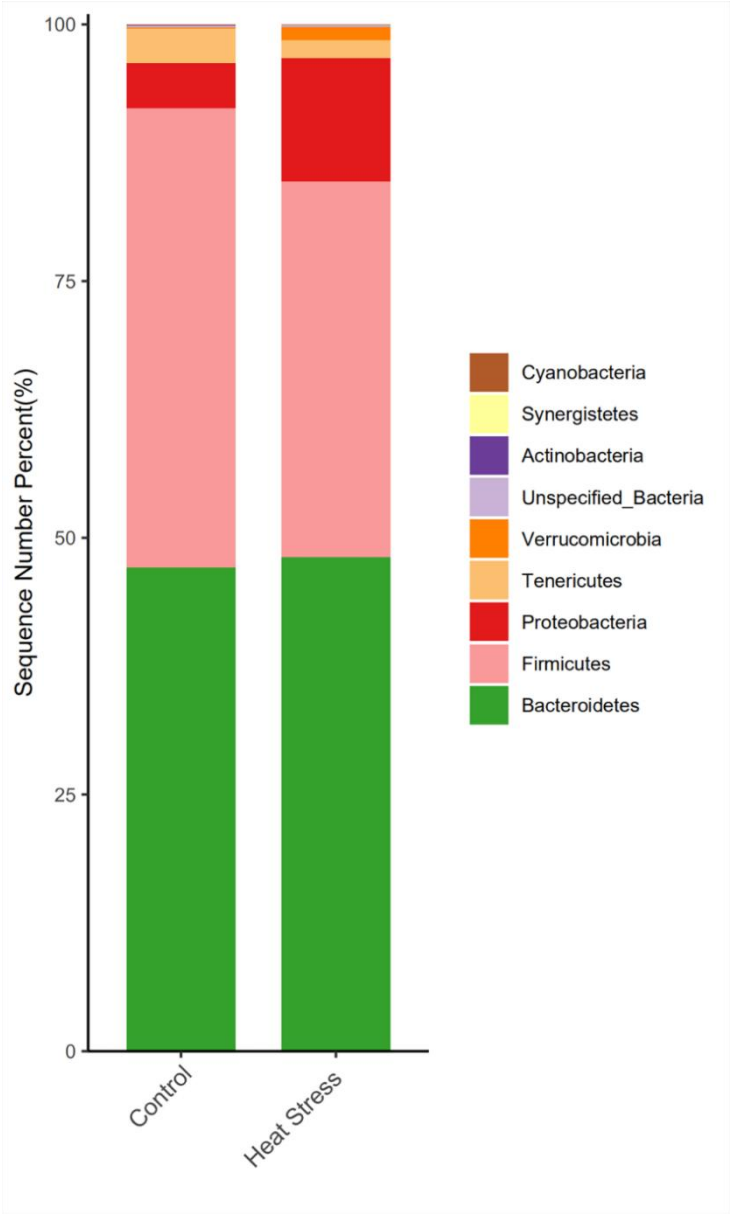


Figure 3-6: Average relative abundances of predominant bacteria at the phylum level in cecal contents.

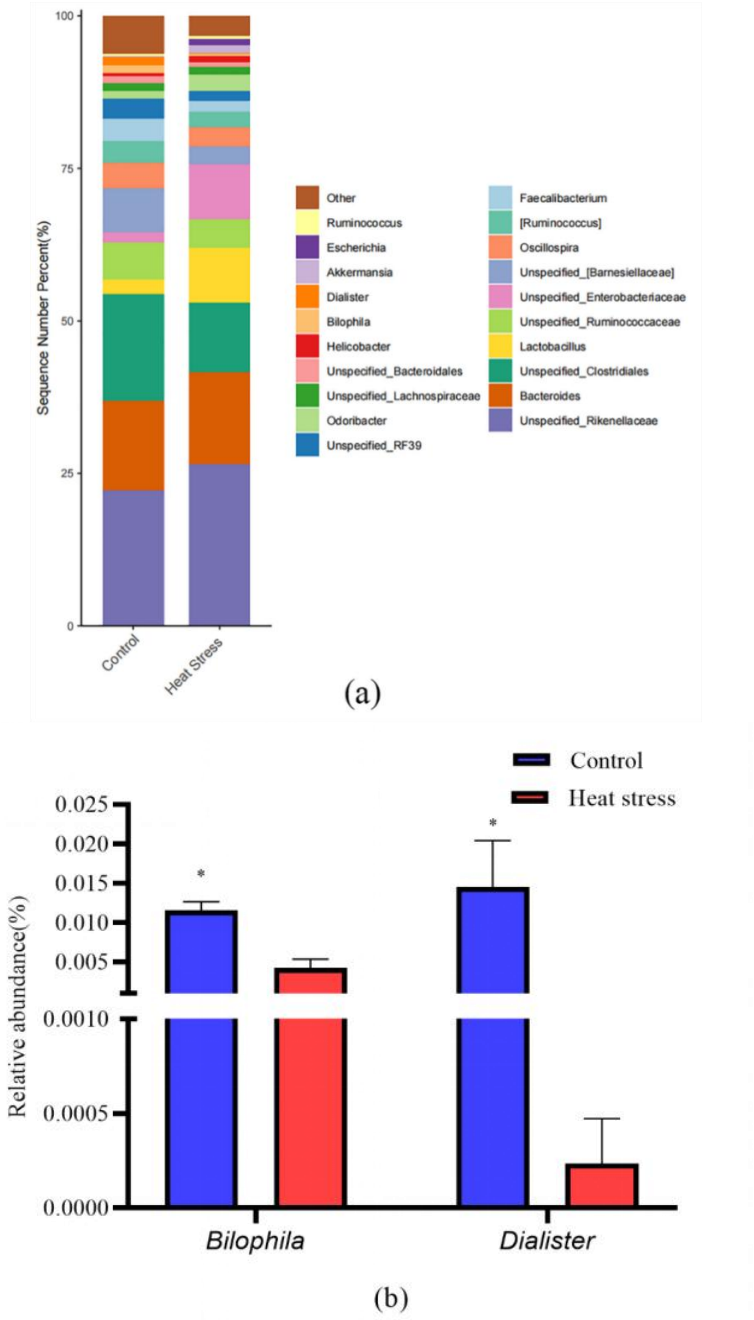


Figure 3-7: Alteration of the cecal microbiota at the genus level. (a) Average relative abundances of predominant bacteria (top 20) at the genus level in cecal contents. (b) Relative abundances of *Bilophila* and *Dialister*.

5. Discussion

Heat stress causes a variety of physiological changes, such as oxidative stress and immune function suppression, leading to increased mortality and decreased feed efficiency, body weight, and feed intake (Wasti et al. 2020). In the present study, heat stress decreased broiler performance, as manifested by the decrease in ADFI and ADG. High temperatures increased mortality in broilers from 1% to 11%, while feed conversion rates more than doubled. The effect of heat stress has been reported to be responsible for the decrease in feed intake as well as layer weight, feed utilization, and egg production and quality (Deng et al. 2012). Birds need to breathe to release heat at high ambient temperatures. A previous study indicated that eating is restricted during heat stress because birds cannot simultaneously breathe and eat. Therefore, birds spend more time panting than eating when exposed to high temperatures (Mack et al. 2013). Another reason is that hyperthermic birds aim to reduce metabolic heat by eating less to decrease heat generation (Belhadj Slimen et al. 2016).

High temperatures can cause oxidative stress. The levels of MDA and T-SOD can effectively assess the antioxidant balance of animals (Attia et al. 2020). The activity of antioxidant enzymes in the liver and serum significantly increased with increasing environmental temperature (Tan et al. 2010). In the present study, heat stress increased the T-SOD content in broiler serum as a protective response to oxidative stress (Thomas 2000). Exposure to acute heat stress (35 °C, 3 h) resulted in increased liver lipid peroxidation in broilers. However, heat stress did not affect the serum MDA level of broilers, which may be related to the duration of exposure to heat stress. Under heat stress conditions, the blood flow distribution changes from the visceral capillaries to the peripheral capillaries, resulting in a rapid drop in body temperature (Shakeri et al. 2020). However, reduced visceral blood flow may lead to hypoxia in gastrointestinal tissues (Hall et al. 2001), oxidative stress damage, and increased permeability to pathogens and related endotoxins (Shakeri et al. 2019). Heat stress increases the serum endotoxin (ET) content, suggesting increased intestinal permeability.

HSP is a protein chaperone in cells that senses oxidative damage and plays a role in cell protection under various stresses (Cong et al. 2017). Consistent with the present study, previous work has reported that the expression of HSP70 increased in the presence of oxidative stress (Tang et al. 2018). The NF-κB pathway is a ubiquitous participator in the expression of proinflammatory cytokines, such as IL-1β, IL-6, IL-8, and TNF-α, after an inflammatory challenge with lipopolysaccharide (LPS) (Pires et al. 2018). Heat stress can activate the expression of NF-κB in the liver and jejunum of birds, leading to the secretion of a series of proinflammatory cytokines (Orhan et al. 2012). Calprotectin is also a biomarker of inflammation and belongs to the S100 protein family, whose expression is elevated in the gut and liver following inflammatory infection (Abildtrup et al. 2015). In the present study, the expression levels of *NF-κB* and *S100A9* were upregulated in the liver, similar to those of *HSP70* in the jejunum.

As a classical type 2 nutrient, zinc should be ingested frequently, and its homeostasis must be regulated accurately (Baer et al. 1984). After heat stress, zinc in the liver was redistributed, and MT gene expression in the liver was

upregulated, indicating that the liver increased zinc storage in response to oxidative stress, as demonstrated by the increase in zinc content in the liver, tibia and jejunum. In the carrier-mediated process of zinc absorption (Condomina et al. 2002), zinc can be absorbed into intestinal epithelial cells through the enterocyte apical membrane by DMT1 and ZIP family transporters (Hojyo et al. 2016) and then stored for binding with metallothionein. The zinc exporter ZnT1 is located at the basolateral membrane and contributes to the transport of zinc from the intestine into the portal vein circulation. The liver plays an important role in zinc homeostasis due to its rich content and fast exchange in the metabolism of zinc (Krebs et al. 2001). The regulation of transporter proteins is necessary for the process of zinc uptake by the liver. The importer ZIP3 is involved in the transport of zinc from serum to the liver, where zinc is stored in cells after binding to metallothionein and transported out of the liver by the exporter ZnT1 (Dempski 2012).

Our results suggested that zinc was redistributed in the jejunum and liver after heat stress. The expression levels of zinc importers (*DMT1*, *ZIP8*, *ZIP14*) and the exporter *ZnT1* increased in the jejunum, indicating that more zinc was absorbed from the intestinal tract as evidenced by a decrease in the concentration of zinc in the intestinal contents. More interestingly, the expression of *ZIP3* increased and that of *ZnT1* decreased, which meant that the liver needed more zinc reserves, as reflected by the higher contents of liver MT and zinc. Heat stress altered zinc homeostasis in chickens, as reflected in the increased zinc contents in tissues and bone. The adjustment of zinc transporter levels in different organs played an incredible role in increasing the ability of zinc to be absorbed in the intestine, while the liver, as a major organ of metabolism, had an increased ability to store zinc. Oxidative stress and immune challenge process are accompanied by a decrease in serum zinc concentration and an increase in liver zinc concentration providing antioxidant defense system (Liuzzi et al. 2005). Similar to the results after seven days of *Salmonella* infection in broilers (Wu et al. 2020), we did not find a decreasing trend in serum zinc concentrations after heat stress, which should be attributed to the adaptation of broilers to temperature and adequate zinc content in the feed.

Accumulating evidence shows that oxidative stress can alter the expression of zinc transporters (Marreiro et al. 2017). ZIP14 directly or indirectly promotes intestinal zinc absorption and regulates zinc metabolism in response to inflammatory stimuli in the liver (Aydemir et al. 2012). Oxidative stress induced by chronic alcohol exposure can downregulate the expression of the zinc transporter ZIP14 in mouse hepatocytes (Sun et al. 2014). In aged cardiomyocytes, the ZIP8 level has been found to decrease with increasing ROS levels (Olgar et al. 2019). ZIP8 and ZIP14 are configured differently from the other members of the ZIP family because a histidine is replaced by glutamic acid (Fujishiro et al. 2012), which may be the key to their immunity and antioxidant roles. As the only zinc-sensitive transcription factor, MTF-1 is involved in zinc concentration sensing and MT regulation (Stuart et al. 1985). MT not only regulates the concentration of free zinc in cells but also affects the production of ROS in cells to resist stress by regulating the expression of NF-κB (Wu et al. 2017). Zinc concentration affects the expression of the zinc finger protein A20, which in turn changes the ability of

NF-κB to assemble with DNA and reduces the inflammatory response caused by oxidative stress (Morgan et al. 2011). Heat stress decreased the expression of *MTF-1* and increased the expression of *A20* in the liver and jejunum, indicating that the increase in the tissue zinc concentration downregulated the expression of the sensing gene *MTF-1*, while the zinc finger protein *A20* activated the anti-inflammatory effect of *NF-κB*. These modifications support the physiological significance of greater accumulation of zinc in tissues for the promotion of zinc-mediated antioxidant actions.

The cecal microbiota resists inflammatory bowel disease and metabolic disorders and helps maintain normal barrier function and antioxidant capacity (Shehata et al. 2021). Our results confirm that heat stress alters the microbial composition of the broiler cecum and that lower levels of intestinal microbes may be associated with the negative effects of heat stress on intestinal health and microbial community stability. We did not find significant correlations between heat stress and the cecum microbiota at the phylum level, similar to previous findings in yellow-feathered broilers (Huang et al. 2021). At the genus level, heat stress treatment reduced the relative abundances of *Bilophila* and *Dialister*. *Bilophila* is involved in the anaerobic metabolic pathway that converts the substrate taurine, which is abundant in the gut microbiota, into the toxic metabolite hydrogen sulfide. A reduction in the relative abundance of *Bilophila* predicts a weakening of intestinal immune stimulation and the inflammatory response after heat stress treatment (Peck et al. 2019). In the gut microbiota of pigs fed higher levels of lysine-chelated zinc diets, Dialister had a high relative abundance, promoting the decarboxylation of succinate to propionate (Pieper et al. 2020). Propionic acid is a short-chain fatty acid (SCFA), and zinc can result in the enrichment of beneficial SCFAs by increasing the abundance of corresponding bacteria in the gut, thereby influencing the composition of the microbiota to promote zinc absorption by the host (Reed et al. 2018). In our study, heat stress enhanced intestinal absorption of zinc via transporter proteins, leading to a low-zinc environment in the gut and resulting in a decrease in the relative abundance of *Dialister*. Therefore, the reduction in cecum microbial abundance in broiler chickens due to heat stress is associated with an attenuated inflammatory response and a low-zinc environment in the intestine.

6. Conclusion

Heat stress could cause oxidative stress and immune challenge in broilers, which can be reflected in the decrease in production performance. Our results confirmed the remodeling of zinc homeostasis in broilers under heat stress and identified the role of zinc transporters and MT in the liver and jejunum during this process. The effects of heat stress on the gut microbiota seem to be involved in the redistribution of zinc.

Chapter IV

Zinc glycinate alleviates LPS-induced inflammation and intestinal barrier disruption in chicken embryos by regulating zinc homeostasis and TLR4/NF-κB pathway

This chapter is based on the following publication:

Chuanpi Xiao, Luke Comer, Xue Pan, Nadia Everaert, Martine Schroyen, and Zhigang Song

Zinc glycinate alleviates LPS-induced inflammation and intestinal barrier disruption in chicken embryos by regulating zinc homeostasis and TLR4/NF-κB pathway

Ecotoxicology and Environmental Safety, 2024, 272 :1161111.

Chapter IV. Zinc glycinate alleviates LPS-induced inflammation and intestinal barrier disruption in chicken embryos by regulating zinc homeostasis and TLR4/NF-κB pathway

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Keywords: Chicken embryos; Immune challenge; *In ovo* feeding; Lipopolysaccharide; Zinc.

1. Abstract

The effect of an immune challenge induced by lipopolysaccharide (LPS) exposure on systemic zinc homeostasis and the modulation of zinc glycinate (Zn-Gly) was investigated using a chicken embryo model. 160 Arbor Acres broiler fertilized eggs were randomly divided into 4 groups: CON (control group, injected with saline), LPS (LPS group, injected with 32 μg of LPS saline solution), Zn-Gly (zinc glycinate group, injected with 80 μg of zinc glycinate saline solution) and Zn-Gly+LPS (zinc glycinate and LPS group, injected with the same content of zinc glycinate and LPS saline solution). Each treatment consisted of eight replicates of five eggs each. An *in ovo* feeding procedure was performed at 17.5 embryonic days and samples were collected after 12 hours. The results showed that Zn-Gly attenuated the effects of LPS challenge-induced upregulation of pro-inflammatory factor interleukin 1β (IL-1β) level (P =0.003). The LPS challenge mediated zinc transporter proteins and metallothionein (MT) to regulate systemic zinc homeostasis, with increased expression of the jejunum zinc export gene zinc transporter protein 1 (*ZnT-1*) and elevated expression of the import genes divalent metal transporter 1 (*DMT1*), Zrt- and Irt-like protein 3 (*Zip3*), *Zip8* and *Zip14* (P < 0.05). A similar trend could be observed for the zinc transporter genes in the liver, which for ZnT-1 mitigated by Zn-Gly supplementation (P =0.01). Liver *MT* gene expression was downregulated in response to the LPS challenge (P =0.004). These alterations caused by LPS resulted in decreased serum and liver zinc levels and increased small intestinal, muscle and tibial zinc levels. Zn-Gly reversed the elevated expression of the liver zinc finger protein *A20* induced by the LPS challenge (P =0.025), while Zn-Gly reduced the gene expression of the pro-inflammatory factors *IL-1β* and *IL-6*, decreased toll-like receptor 4 (*TLR4*) and nuclear factor kappa-B p65 (*NF-κB p65*) (P < 0.05). Zn-Gly also alleviated the LPS-induced downregulation of the intestinal barrier gene Claudin-1. Thus, LPS exposure prompted the mobilization of zinc transporter proteins and MT to perform the remodeling of systemic zinc homeostasis, Zn-Gly participated in the regulation of zinc homeostasis and inhibited the production of pro-inflammatory factors through the TLR4/NF-κB pathway, attenuating the inflammatory response and intestinal barrier damage caused by an immune challenge.

2. Introduction

Intensive poultry farming facilitates the efficient production of animal protein, yet has nonetheless led to increased challenges for birds, including susceptibility to pathogenic microorganisms, environmental pollution, and factors related to food which has resulted in immune challenges and threats of oxidative stress (Van Hoeck et al. 2020, Zhang et al. 2023). Under these circumstances, the immune system of poultry plays a crucial role in combating bacterial infections, particularly in the global context of increasing concerns regarding antimicrobial resistance and the prohibition of antibiotic additives in feed (Hood-Pishchany et al. 2020, Palmer et al. 2020). Therefore, the application of feed additives to alleviate immune stress in poultry and regulate innate immune responses to inflammation has become an effective strategy (Das et al. 2021).

As an important component of many enzymes in animals, zinc is known to influence a variety of physiological processes (Liu et al. 2023). As such, it is commonly added to feeds to improve animal growth and performance, which is related to its function in improving intestinal barrier function and reducing inflammatory responses (Shimizu et al. 2020, Nguyen et al. 2021). Zinc amino acid chelates have for instance been widely used in recent years due to their higher biological availability (Farhadi Javid et al. 2021). The widespread application of zinc is due to a number of physiological functions in its participation. The three components, zinc bound to metallothionein (MT) with low affinity, zinc bound to organelles and cytoplasmic free zinc, constitute the zinc pool after zinc absorption by tissue cells and removal of the fraction bound to metalloenzymes as structural components or cofactors (Nolin et al. 2019, Endo et al. 2020). Zinc homeostasis is a complex physiological homeostasis regulated by a family of MT, zinc-regulated transporters (Zrts), iron-regulated transporter (Irt)-like proteins (Zips) and a family of zinc transporter proteins (ZnTs) (Betrie et al. 2021). A previous study showed that zinc homeostasis in broilers under heat stress was regulated by zinc transporter proteins and metallothionein (Xiao et al. 2022). However, the effects and mechanisms of immune challenges on zinc metabolism in birds remain elusive.

As a major component of Gram-negative bacteria's outer membrane, lipopolysaccharide (LPS) induces immune stress by causing an imbalance in the inflammatory response and antioxidant system (Izadparast et al. 2022). Recognized by host toll-like receptors (TLRs), LPS activates the downstream nuclear factor kappa-B (NF-κB) pathway which triggers the intrinsic immune response and promotes the secretion of pro-inflammatory cytokines, causing an inflammatory response and disrupting intestinal barrier function (Ciesielska et al. 2021, Peace and O'Neill 2022). With its accessibility and ease of handling, the *in ovo* feeding is an attractive way for research in the fields of nutrition and toxicology (Kadam et al. 2013, Retes et al. 2018). Past studies have confirmed that broiler embryos begin to ingest amniotic fluid through the oral cavity at embryonic day 17 (E17) (Pan et al. 2023), and *in ovo* feeding through the amniotic cavity at late hatching has been applied to an LPS exposure model. Therefore, this study aimed to evaluate the effect of zinc glycinate (Zn-Gly) on zinc homeostasis under LPS exposure and the ameliorative effects on the inflammatory response, oxidative stress and intestinal barrier function.

3. Materials and methods

All experimental procedures were approved by the Ethics Committee of Shandong Agricultural University (No. SDAUA-2022-50) and conducted in accordance with the Guide for Laboratory Animals of the Ministry of Science and Technology (Beijing, People's Republic of China). The experiment was conducted in Shandong Agricultural University (Taian, China).

3.1. Experimental design and treatments

The optimal embryonic age for sampling needed to be determined first by a pilot study prior to *in ovo* injection. The challenge model for *in ovo* feeding of

LPS and the dose of LPS has been reported in past study, identifying 32 μg and 12 h as the dose and sampling time for LPS, respectively (Kong et al. 2023). Therefore, we still required standardization of sampling times only by specifying the length of time required for zinc to be able to be utilized in the embryos. The age of the injected embryos and the dose of zinc were sourced from past studies about zinc *in ovo* administration, and 40 Arbor Acres (AA) broiler fertilized eggs of similar weights were weighed, labeled, and incubated, before 0.5 mL of Zn-Gly solution (at a zinc concentration of 160 μg/mL) was injected into the amniotic cavity at E17.5 (Meriwether et al. 2010). Yolk samples were collected before injection and after 6, 12, and 24 hours of continued incubation for a zinc content assay.

A total of 160 AA broiler fertilized eggs with similar weights (71.5 ± 2 g) from 60 weeks old female breeders were weighed and labeled for the formal experiment, and all eggs were randomly divided into four treatment groups (abbreviated as CON, LPS, Zn-Gly and Zn-Gly+LPS) with eight replicates of five eggs each. At E17.5, 0.5 mL of saline, LPS solution (64 μg/mL), Zn-Gly solution (160 μg/mL), or a mixture of LPS and Zn-Gly solution were injected.

3.2. Eggs and incubation management

All eggs were purchased from Taian Liuhe Broiler Company (Taian, China), stored at 20 °C for 24 h and sterilized by fumigation with formaldehyde gas before being transferred to an automatic incubator (Jiayu Electronic Technology Company, Dezhou, China) for incubation with a relative humidity of 60% and a temperature of 37.8 °C. At E17.5, we candled all the fertilized eggs in warm environment, discarded the dead embryos, injected *in ovo*, and incubated the remaining fertilized eggs until samples were collected.

3.3. Solution preparation and in ovo injection procedure

The reagent grade Zn-Gly ($C_4H_8N_2O_4Zn$) was prepared at Jining HeShi Biological Co., Ltd. According to the manufacturer's procedure, the molar ratio of 2:1 glycine and alkaline zinc carbonate were dissolved in water in a reaction kettle and then heated to 80 °C for 40 min to produce zinc glycinate mother liquor. The Zn-Gly product was obtained by centrifugation to extract the crystals and then dried. The zinc content was measured and found to be 21.2% while the chelation strength Qf value was 11.2. The solution was diluted with saline to obtain a theoretical value of 160μg/mL. All prepared solutions were filtered through a 0.22 μm acetate filter and verified for Zn-Gly solution to obtain a zinc content of 157.8 μg/mL, thus determining that the actual zinc content of the 0.5 mL of zinc glycinate solution used for injection was 78.9 μg. LPS reagent (L2880, Sigma-Aldrich I, St. Louis, USA) was purchased from a supplier. The LPS reagent is derived from *E. coli* (055:B5) and is purified to obtain a powder with a purity higher than 97%.

The protocol was conducted as previously described (Uni et al. 2005). The eggs were candled and the location of the air chamber was marked using a pencil, the shell was sterilized with alcohol swabs after which a 1 mm diameter hole was drilled in the middle, while 0.5 mL solution of different treatments was injected

into the amniotic cavity by a needle of twenty-one gauge. The depth of injection was 2 cm and the operation was assisted by illumination. Immediately after injection, the hole was covered with paraffin and the return the eggs to the incubator. The entire procedure was performed in a laminar flow system, and all solutions were freshly prepared and then heated to 37 °C to prevent embryonic cold shock. The entire procedure of each egg took no more than 15 minutes outside the incubator.

3.4. Sample collection

The timing of sample collection was determined according to the results of the pretest and the Taipan Blue stain localization method. Briefly, yolk samples were collected from pre-tested chicken embryos and their yolk zinc content was measured to infer the utilization of the zinc solution by the embryos. The results showed that the yolk zinc content was 713.38 μg, 700.32 μg, 707.43 μg and 680.98 μg before and 6, 12 and 24 h after injection, respectively. It was possible to determine that the exogenous zinc was efficiently absorbed by the embryos after 12 h of injection. Combined with the photographs after *in ovo* injection with 0.5 mL of Taipan Blue staining solution (dilution 1:500) as described and at 12 h (Figure 4-1), it was shown that the solution was completely utilized by the embryos after 12 h of *in ovo* injection. Therefore, 12 h was chosen as the sampling time point after *in ovo* injection.

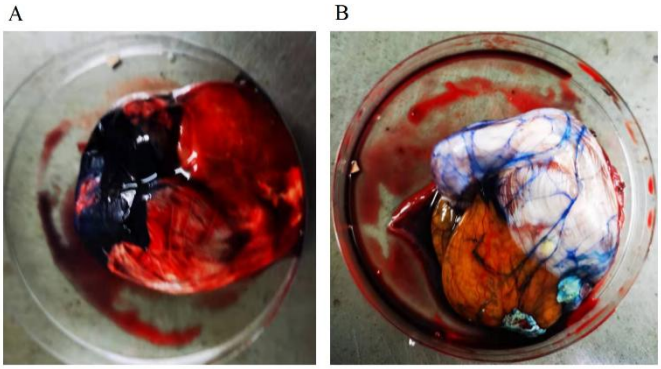

Figure 4-1: Embryo anatomy after 0h (A) and 12h (B) of *in ovo* feeding Taipan Blue stain solution.

Eight samples per treatment were obtained for analysis of various indicators. Samples were collected at E18 and four embryos per replicate were randomly selected for execution with the neck transfer method. The blood was drawn from the embryonic heart using a sterile syringe and approximately 0.2 mL of blood was transferred into a tube without anticoagulant. After 30 min of settling, the upper layer of fluid was separated by centrifugation using a speed of 3,000 × *g* for 10 min to obtain the serum. The liver and small intestine were immediately removed, stored in a freezer at -80 °C after being rapidly frozen in liquid nitrogen. After removing the skin and bones from the right thigh, the meat was removed

and stored at -80 °C after being stored at room temperature. Samples from every three consecutive embryos with same weight were combined into one sample for zinc content analysis. Jejunum and liver samples were obtained from one additional chicken embryo in each replicate, rapidly snap-frozen in liquid nitrogen and stored at -80 °C for subsequent index analysis.

3.5. Determination of zinc concentration

After soaking in ether for 96 hours, the tibia were degreased, dried at 105 °C, then ash in a muffle furnace (550 °C, 24 h) at a constant weight. The ashed samples were dissolved using 5 mL of hydrochloric acid solution with a concentration of 6 mol/L and then fixed in a 25 mL volumetric flask for measurement. The samples of small intestine, liver and leg muscle were freeze-dried, and 0.2 g dried powder was added to 2 mL of nitric acid (70%) and 1 mL of hydrogen peroxide solution (30%), placed in a 50 mL plastic centrifuge tube and digested in a water bath at 95 °C for 6 h. After cooling, the samples were fixed in a 25 mL volumetric flask and filtered for measurement. The zinc content of tissues was analyzed by an inductively coupled plasma spectrometer (ICAP 7000, Thermo, Waltham, MA, USA).

3.6. Detection of immune parameters and zinc-related enzyme activity

The levels of immune factors interleukin 1β (IL-1β), interleukin 6 (IL-6), interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) in the jejunum and liver were measured with enzyme-linked immunosorbent assay kits from MLBIO (Shanghai, China). Zinc metabolism-related markers alkaline phosphatase (ALP) activity, MT levels and copper-zinc superoxide dismutase (Cu,Zn-SOD) activity were also assayed using kits from the same company. According to the manufacturer's instructions, all assay procedures were performed strictly and the intra-batch coefficient of variation (CV) of the data was 5% and the inter-batch CV was 8%.

3.7. Oxidative status assay

Catalase (CAT) activity, total antioxidant capacity (T-AOC) levels, inducible nitric oxide synthase (iNOS) activity and malondialdehyde (MDA) levels were measured in the jejunum and liver using kits provided by Nanjing Jiancheng Institute of Biotechnology (Nanjing, China) for the assessment of antioxidant capacity.

3.8. RNA isolation and real-time quantitative PCR

We isolated total RNA from the jejunum and liver using Trizol reagent (Invitrogen, San Diego, USA). NanoDrop spectrophotometers (ND-2000, Thermo Scientific, Wilmington, USA) were used to measure the concentration and purity of each RNA sample. An agarose gel electrophoresis of 1% was used to test for RNA integrity. A PrimeScript® RT kit (RR047A, TaKaRa) was used to reverse transcribe 1 g of total RNA. To determine gene expression, RT-PCR analysis was performed using the TB Green Premix Ex Taq (RR820A, Takara, Japan) in an ABI 7500 real-time PCR system (Thermo Scientific, Wilmington, USA). Each

reaction was repeated in three wells, and the first sequence is shown in Table 4-1. Each reaction consisted of: predenaturation at 95 °C for 10 s, denaturation at 95 °C for 5 s, and annealing and extension at 60 °C for 40 s. Based on a standard curve from the software, the primer amplification efficiencies were calculated. Melting curve analysis verified that the amplification products were specific. To analyze the relative gene expression levels for all the target genes, we normalized with the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression.

Table 4-1: Nucleotide sequences of real-time PCR primers

Gene	Accession number	Primer sequence, 5'→ 3'	Reference
IL-1β	NM_204524.1	GGTCAACATCGCCACCTACA CATACGAGATGCAAACCAGCAA	Kong et al. 2022
1L-6	NM_204628.1	AAATCCCTCCTCGCCAATCT CCCTCACGGTCTTCTCCATAAA	
TLR4	NM_001030693.1	AGGCACCTGAGCTTTTCCTC TACCAACGTGAGGTTGAGCC	
NF-κB p65	NM_001396038.1	CAGCCCATCTATGACAACCG TCAGCCCAGAAACGAACCTC	
ZO-1	XM_015278981.2	CTTCAGGTGTTTCTCTTCCTCCTCTC CTGTGGTTTCATGGCTGGATC	
Occludin	NM_205128.1	TCATCGCCTCCATCGTCTAC TCTTACTGCGCGTCTTCTGG	
Claudin-1	NM_001013611.2	CTGATTGCTTCCAACCAG CAGGTCAAACAGAGGTACAAG	
MT	NM_205275.1	GCAACAACTGTGCCAAGGGC TTTCGTGGTCCCTGTCACCC	
Cu,Zn-SOD	NM_205064.1	TTGTTCTGATGGAGATCATGGCTTC TGCTTGCCTTCAGGATTAAAGTGAG	Li et al. 2015
MTF-1	XM_015297695.3	CCTGGTTCAACTCCTATGC TCAAACGGCTTCTCCTTA	
A20	XM_003640919.2	GACATCGTGCTAACAGCTTGGA AGAAAAGAGGTATCAGGCACAAC	
ZnT1	NM_001389457.1	CTTCGCTTAGCATTTCTT TCTCCGATTTAGTCCTTCT	
DMT1	NM_001128102.2	AGCCGTTCACCACTTATTTCG GGTCCAAATAGGCGATGCTC	Wu et al. 2020
Zip8	XM_040671236.1	TGTAAATGTCTCGGTGGG CAAGATGGCTATGGAGGT	
Zip14	XM_040689606.1	GTTCTGCCCCGCTGTCCT	

GADPH	NM_204305.1	GGTCTGCCCTCCTCCGTCT GCCCAGAACATCATCCCA GGGGAGACAGAAGGGAACAGA	Kong et al. 2023

TLR4: toll-like receptor 4; *NF-κB p65*: nuclear factor kappa-B p65; *ZO-1*: zonula occludens 1; *MTF-1*: metal transcription factor-1; *ZnT*: zinc transporter; *Zip*: zinc-regulated transporter, iron-regulated transporter-like protein; *MT*: metallothionein; *Cu,Zn-SOD*: copper-zinc superoxide dismutase; *GADPH*: glyceraldehyde-3-phosphate dehydrogenase.

3.9. Data analysis

All data were expressed as mean and total standard error of the mean, and data were counted and analyzed in replicates. Data were analyzed using the Shapiro-Wilk test (95% confidence interval) using the software SPSS 22.0 (SPSS. Inc., Chicago, USA) followed by two-way ANOVA with general linear model, and Tukey's post-hoc test was applied to determine differences between groups. Data were considered statistically significantly different when $P < 0.05$.

4. Results

4.1. Inflammatory cytokine levels

Table 4-2: Effects of *in ovo* feeding of zinc glycine on immune parameters of lipopolysaccharide-challenged embryos

Item	**Liver** (pg/mL)				**Jejunum** (pg/mL)			
	IL-1β	**IL-6**	**IFN-γ**	**TNF-α**	**IL-1β**	**IL-6**	**IFN-γ**	**TNF-α**
CON	479.23^{b}	29.07^{b}	49.33^{b}	68.65	512.43^{ab}	25.69^{b}	54.64^{b}	63.02
LPS	542.16^{a}	41.73^{a}	69.21^{a}	63.17	561.53^{a}	35.09^{a}	66.25^{a}	71.26
Zn-Gly	550.94^{a}	36.96^{ab}	52.03^{b}	65.24	466.16^{b}	32.03^{ab}	61.26^{ab}	68.41
Zn-Gly+LPS	522.79^{ab}	39.61^{a}	64.05^{a}	65.68	475.53^{b}	30.45^{ab}	58.94^{ab}	69.96
Pooled SEM	24.98	4.12	2.63	4.52	14.23	1.95	2.45	5.03
Zinc								
No zinc	510.70	35.41	59.27	65.91	536.98	30.39	60.45	67.14
Gly-Zn	536.87	38.29	58.04	65.46	470.85	31.24	60.10	69.19
LPS Challenge								
No Challenge	515.09	33.02	50.68	66.95	489.30	28.86	57.95	65.72
Challenge	532.48	40.67	66.63	64.43	518.53	32.77	62.59	70.61
P value								
Zn-Gly	0.076	0.062	0.286	0.802	< 0.001	0.308	0.811	0.124
LPS	< 0.001	< 0.001	< 0.001	0.166	< 0.001	< 0.001	0.001	0.029
Interaction	0.01	< 0.001	0.002	0.106	0.003	< 0.001	< 0.001	0.339

Values are expressed as the mean pooled SEM (n = 8). Means in a row with no common

superscript are significantly different ($P < 0.05$), SEM: standard error of the mean, IL-1β: interleukin 1β; IL-6: interleukin 6; IFN-γ: interferon gamma; TNF-α: tumor necrosis factor alpha.

The effects of experimental treatments on cytokine levels are shown in Table 4-2. A significant interaction between Zn-Gly and the LPS challenge was observed in the liver and jejunal cytokines of embryos， where exogenous zinc injection significantly reduced jejunal IL-1β content in challenged embryos ($P = 0.003$). The LPS exposure increased the levels of IL-1β, IL-6 and IFN-γ in the liver ($P < 0.05$),

Table 4-3: Effects of *in ovo* feeding of zinc glycine on oxidative status of lipopolysaccharide-challenged embryos

Item	Liver				Jejunum			
	CAT	**T-AOC**	**iNOS**	**MDA**	**CAT**	**T-AOC**	**iNOS**	**MDA**
	pg/mg prot	nmol/mg prot	nmol/mg prot	nmol/mg prot	pg/mg prot	nmol/mg prot	nmol/mg prot	nmol/mg prot
CON	16.14[a]	0.19	0.88	1.58	19.38	0.18	0.23	1.07
LPS	10.69[c]	0.06	0.63	1.9	18.03	0.19	0.34	2.08
Zn-Gly	12.98[b]	0.16	1.26	1.76	17.34	0.19	0.21	2.06
Zn+LPS	11.31[c]	0.09	0.71	2.55	15.54	0.2	0.26	2.25
Pooled SEM	1.92	0.01	0.18	0.42	2.36	0.03	0.04	0.32
Zinc								
No zinc	13.42	0.13	0.76	1.74	18.71	0.19	0.29	1.58
Gly-Zn	12.15	0.13	0.99	2.16	16.44	0.20	0.24	2.16
Challenge								
No	14.56	0.18	1.07	1.67	18.36	0.19	0.22	1.57
Yes	11.00	0.08	0.67	2.23	16.79	0.20	0.30	2.17
P value								
Zn-Gly	0.025	0.896	0.101	0.064	0.262	0.829	0.062	0.057
LPS	0.001	0.001	0.27	0.009	0.428	0.82	0.012	0.033
Interaction	0.003	0.232	0.08	0.244	0.908	0.41	0.313	0.134

Values are expressed as the mean pooled SEM (n = 8). Means in a row with no common superscript are significantly different ($P < 0.05$), SEM: standard error of the mean; CAT: catalase; T-AOC: total superoxide dismutase; iNOS: inducible nitric oxide synthase; MDA: malondialdehyde.

The same trend was found in the jejunum. In addition, the main effect of Zn-Gly was significant, reducing the contents of jejunal IL-1β ($P < 0.001$).

4.2. Antioxidant status

Table 4-3 presents the effect of Zn-Gly on the antioxidant capacity of the embryonic liver and jejunum as a result of the LPS challenge. The interaction

showed that Zn-Gly did not alleviate the effect of LPS challenge on embryonic antioxidant enzyme activity. The main effect showed that the LPS challenge reduced CAT activity and T-AOC levels, and elevated MDA levels in the liver, while contributing to a significant increase in the jejunal iNOS activity and MDA level ($P < 0.05$). Whereas, Zn-Gly did not show a significant positive effect on the antioxidant capacity of embryos.

4.3. Zinc concentration in serum, tibia and tissues

As shown in Figure 4-2, Zn-Gly caused a redistribution of zinc in embryonic tibia, serum and tissues under LPS challenge. A significant interaction between Zn-Gly and the LPS challenge on zinc content was observed in the tibia ($P < 0.001$) and small intestine ($P = 0.038$). Moreover, the LPS challenge upregulated the tibial zinc content ($P = 0.04$), while Zn-Gly alleviated the LPS challenge-induced increase in the zinc concentration of the tibia. Both exogenous zinc ($P = 0.014$) and the LPS challenge ($P = 0.037$) resulted in the upregulation of zinc concentrations in the small intestine, while the LPS challenge enhanced the increase in small intestine zinc due to exogenous zinc. In addition, the LPS challenge reduced zinc levels in the serum and liver but increased zinc levels in the thigh meat ($P < 0.05$). Zn-Gly supplementation increased zinc content in the serum ($P = 0.004$).

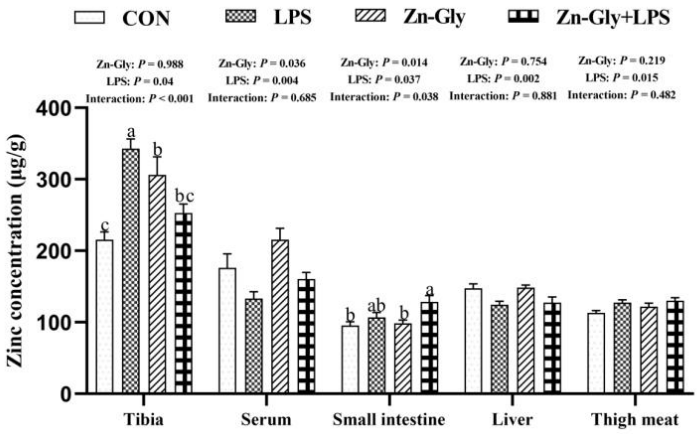

Figure 4-2: Effects of *in ovo* feeding of zinc glycine on zinc concentrations in the tibia, serum and tissues of lipopolysaccharide-challenged embryos. Means with no common superscripts differ significantly ($P < 0.05$).

4.4. Zinc-related enzyme activity and MT

To further clarify the mechanism of zinc redistribution under LPS challenge, ALP activity (Figure 4-3A), MT concentration (Figure 4-3B), and Cu,Zn-SOD activity (Figure 4-3C) were examined in the liver and jejunum. There was a significant interaction between Zn-Gly and LPS challenge on the jejunal ALP

activity (P = 0.04), with the LPS challenge attenuating the upregulation of ALP activity by Zn-Gly. Zn-Gly treatment resulted resoundingly in elevated jejunal (P = 0.018) and liver (P = 0.047) ALP activity, while LPS exhibited a significant negative effect in liver (P = 0.043). In contrast, there was no significant interaction between the two factors on the levels of MT in the jejunum and liver (P > 0.05), Zn-Gly treatment down-regulated the levels of MT in the jejunum (P = 0.01) and liver (P = 0.003) tissues, and the LPS challenge showed a similar reduction in the levels of MT in the liver (P < 0.001). The effect of Zn-Gly and LPS challenge on Cu,Zn-SOD activity in the embryonic liver caused a significant interaction (P < 0.001), with LPS inhibiting the Zn-Gly-induced upregulation of Cu,Zn-SOD activity. In addition, the main effect showed that LPS challenge caused an upregulation of embryonic jejunal Cu,Zn-SOD activity (P = 0.05).

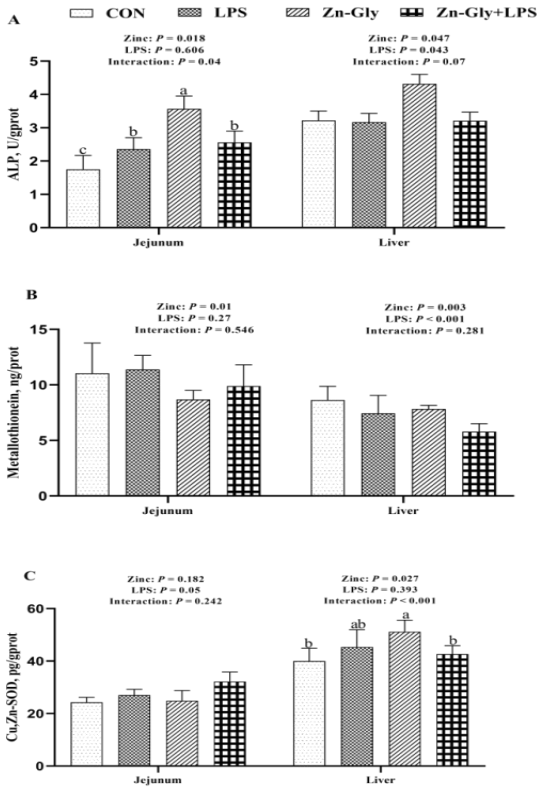

Figure 4-3: Effects of *in ovo* feeding of zinc glycine on ALP activity, metallothionein concentration and Cu,Zn-SOD activity in the jejunum and liver of lipopolysaccharide-challenged embryos. Means with no common superscripts differ significantly (P < 0.05). ALP: alkaline phosphatase; Cu,Zn-SOD: copper-zinc superoxide dismutase.

4.5. Zinc transport-related gene expression

The regulatory effects of Zn-Gly injection on the major zinc importing and zinc exporting genes in the embryonic liver and jejunum under LPS challenge are shown in Figure 4-4A and Figure 4-5A respectively. In the liver, a significant interaction between Zn-Gly and the LPS challenge on the gene expression of *ZnT-1*, divalent metal transporter 1 (*DMT1*), and *Zip3* ($P < 0.05$) indicated that Zn-Gly attenuated the enhanced effect of LPS on the liver zinc transport genes *ZnT-1*. While the main effect analysis showed that LPS challenge significantly upregulated the gene expression of embryonic liver *ZnT-1*, *DMT1*, *Zip3* and *Zip8* ($P < 0.05$), exogenous zinc injection similarly upregulated the expression of *DMT1* ($P = 0.017$). In addition, the main effect showed that LPS challenge resulted in elevated expression of the zinc export gene *ZnT-1*, with the zinc import genes *DMT1*, *Zip3*, *Zip8* and *Zip14* reflecting the same trend ($P < 0.05$). *In ovo* feeding with Zn-Gly similarly upregulated the expression of the zinc export gene *ZnT-1* ($P = 0.05$) and the zinc import gene *DMT1* ($P = 0.017$).

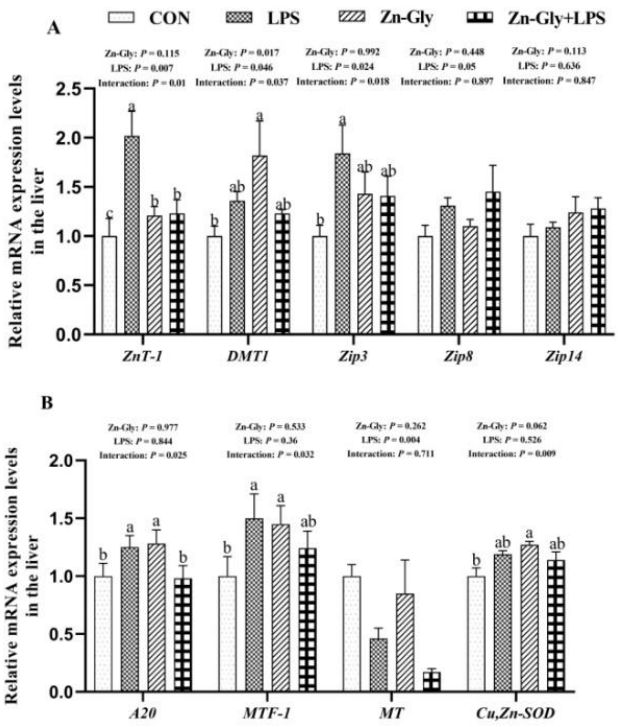

Figure 4-4: Effects of *in ovo* feeding of zinc glycine on the gene expression levels of zinc transporter (A) and zinc regulation-related genes (B) in the liver of lipopolysaccharide-challenged embryos. Means with no common superscripts differ significantly ($P < 0.05$). *ZnT*: zinc transporter; *DMT1*: divalent metal transporter 1; *ZIP*: zinc-regulated transporters (Zrts), iron-regulated transporter (Irt)-like protein; *MTF-1*: metal transcription factor-1; *MT*: metallothionein; *Cu,Zn-SOD*: Copper-zinc superoxide dismutase.

Having demonstrated that Zn-Gly and LPS challenge regulated the embryonic zinc transport-related genes, the embryonic liver and jejunal zinc transport regulatory gene *A20* was also examined, alongside metal transcription factor-1 (*MTF-1*), and zinc concentration markers *MT* and *Cu,Zn-SOD* gene expression, as shown in Figure 4-4B and Figure 4-5B.

The significant interaction between *in ovo* feeding with Zn-Gly and the LPS challenge was observed in the liver for the gene expression of *A20*, *MTF-1* and *Cu,Zn-SOD* ($P < 0.05$), while no interaction was observed for this series of gene expression in the jejunum. An upregulation of the liver *A20* gene expression after LPS injection was observed, while Zn-Gly injection suppressed the alteration. Main effects analysis showed that LPS decreased the gene expression of liver *MT* ($P = 0.004$) and upregulated expression of jejunal *MTF-1* ($P = 0.005$) and *Cu,Zn-SOD* ($P = 0.031$). Additionally, Zn-Gly upregulated the gene expression of jejunal *A20* and decreased expression of *MT* ($P < 0.001$).

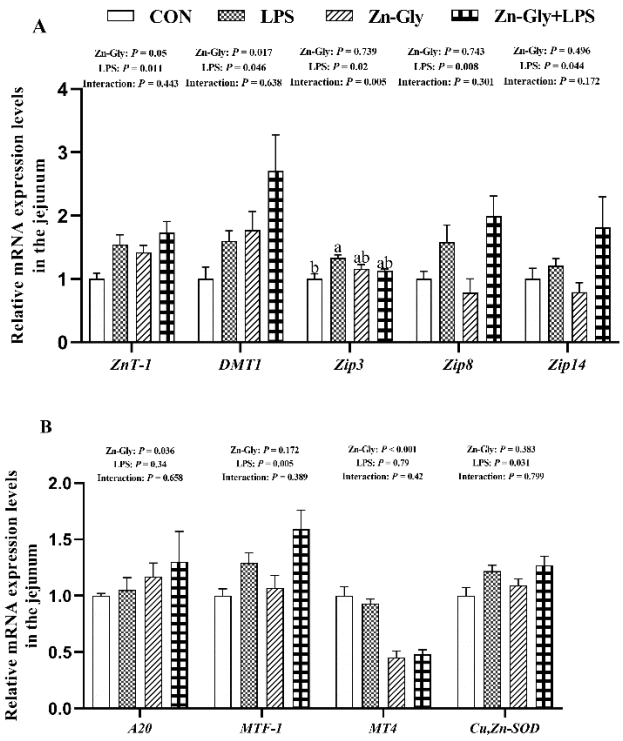

Figure 4-5: Effects of *in ovo* feeding of zinc glycine on the gene expression levels of zinc transporter (A) and zinc regulation-related genes (B) in the jejunum of lipopolysaccharide-challenged embryos. Means with no common superscripts differ significantly ($P < 0.05$). *ZnT*: zinc transporter; *DMT1*: divalent metal transporter 1; *ZIP*: zinc-regulated transporter, iron-regulated transporter-like protein; *MTF-1*: metal transcription factor-1; *MT*: metallothionein; *Cu,Zn-SOD*: Copper-zinc superoxide dismutase.

4.6. TLR4/NF-κB pathway

Figure 4-6A shows that the interaction between Zn-Gly and LPS challenge significantly affected the liver *TLR4* ($P = 0.022$) and nuclear factor kappa-B p65 (*NF-κB p65*) ($P < 0.001$), and *IL-1β* ($P = 0.022$). Embryos in the LPS-treated group had higher levels of liver *TLR4*, *NF-κB p65* and *IL-1β* gene expression than those of control embryos ($P < 0.05$). The main effect showed that LPS significantly upregulated the gene expression of embryonic liver *TLR4*, *NF-κB p65*, *IL-1β* and *IL-6* ($P < 0.05$).

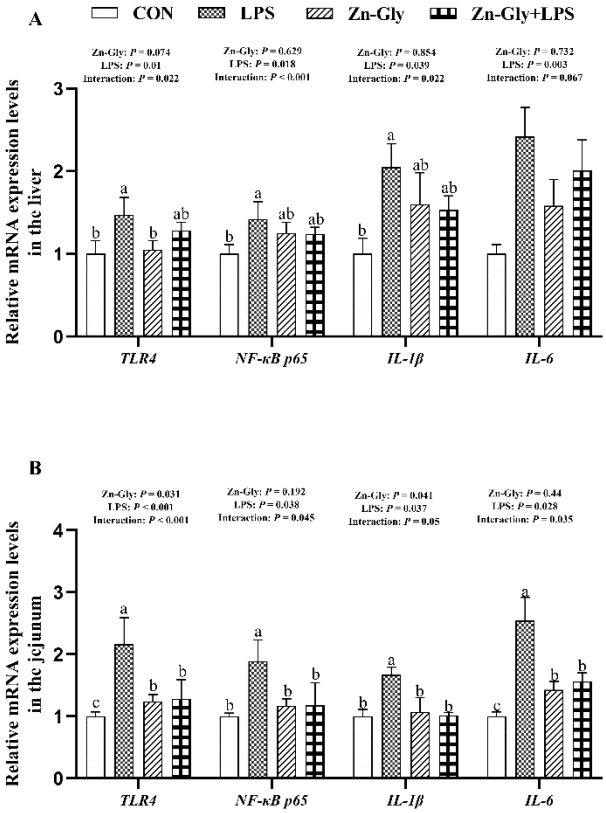

Figure 4-6: Effects of *in ovo* feeding of zinc glycine on the gene expression levels of TLR4 /NF-κB p65 pathway in the liver and jejunum. Means with no common superscripts differ significantly ($P < 0.05$). *TLR4*: toll-like receptor 4; *NF-κB p65*: nuclear factor kappa-B p65.

In the embryonic jejunum, a significant interaction between Zn-Gly and LPS exposure was observed in *TLR4* ($P < 0.001$), *NF-κB p65* ($P = 0.045$), *IL-1β* ($P = 0.05$) and *IL-6* ($P = 0.035$) (Figure 4-6B). Zn-Gly significantly increased the expression of *TLR4* and *IL-6* compared to the control group. The gene expression

of *TLR4*, *NF-κB p65*, *IL-1β* and *IL-6* was down-regulated in the Zn-Gly+LPS group compared to the LPS group ($P < 0.05$). Furthermore, the significant main effect of LPS in the jejunum was consistent with that seen in the liver, exhibiting upregulation of all pathway genes, while Zn-Gly downregulated gene expression of *TLR4* and *IL-1β*.

4.7. Expression of jejunal barrier genes

Figure 4-7 presented the effect of the experimental treatments on the expression of genes responsible for the embryonic jejunal barrier is presented. The interaction between Zn-Gly and LPS challenge significantly affected the gene expression of Claudin-1 ($P = 0.035$). Supplementation with Zn-Gly rescued the reduction in Claudin-1 expression caused by LPS. Main effects analysis showed that LPS challenge downregulated Claudin-1, Occludin and zonula occludens 1 (*ZO-1*) gene expression in the embryonic jejunum ($P < 0.05$), while Zn-Gly showed a significant upregulation effect on all these genes ($P < 0.05$). These data suggested that *in ovo* feeding with Zn-Gly ameliorated the decreased intestinal barrier function due to LPS treatment of avian embryos.

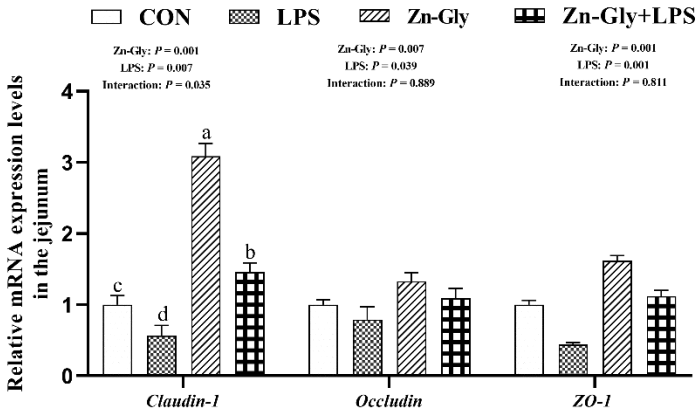

Figure 4-7: Effects of *in ovo* feeding of zinc glycine on the gene expression levels of intestinal barrier genes in the jejunum of lipopolysaccharide-challenged embryos. Means with no common superscripts differ significantly ($P < 0.05$). *ZO-1*: zonula occludens-1.

5. Discussion

Past study showed that zinc is a classical type 2 essential trace nutrient consumed frequently by animals and maintained dynamically in their tissues to sustain regular metabolic functions (Kim et al. 2023). Zinc is added to feed to cover nutritional requirements, improve gut health and enhance innate immune defenses in animals, reducing the pro-inflammatory response to infection (Oh et al. 2021, Ogbuewu and Mbajiorgu 2023). However, the alteration of zinc metabolism in animals undergoing immune challenge and the association of changes with immune regulation have not been explored hitherto. The purpose of this study was to investigate systemic alterations in zinc homeostasis under LPS exposure and

the effects of zinc-glycine on innate immunity in broiler embryos.

Research has shown that the integrity of the intestinal barrier plays an important role in protecting animals from pathogens (Camilleri 2019). By decreasing the expression of tight junction proteins, LPS affects intestinal permeability and impairs intestinal barrier function (Pothuraju et al. 2018). Moreover, LPS exposure leads to impaired intestinal barrier function whereupon it invades the circulatory system before circulating through the hepatic portal system to reach the liver. As a result, this exposure triggers a hepatic inflammatory response and activates the production of inflammatory cytokines IL-1β, IL-6, IFN-γ, and TNF-α (Arab et al. 2018). In this study, LPS exposure caused downregulation of the expression of the gut barrier genes Claudin-1, Occludin, and *ZO-1*, and upregulation of the levels of immune factors such as IL-1β, IL-6, and IFN-γ in the embryonic liver and jejunum, which is similar to previous findings (Bavananthasivam et al. 2019). Zinc acts as a second messenger for immune cells not only to regulate cell-mediated immune function but also as an antioxidant and anti-inflammatory agent (Prasad 2013). It has been shown to significantly reduce the levels of the inflammatory factors IL-1β, IL-6 and TNF-α to block the production of cytokine storms (Wu et al. 2022). Zinc deficiency is known to induce the secretion of IL-1β through activation of macrophage NLRP3 (NOD-, LRR- and pyrin domain-containing 3) inflammasomes followed by inflammatory response (Summersgill et al. 2014). We demonstrated that Zn-Gly reversed the up-regulation of pro-inflammatory factor IL-1β in the jejunum and enhanced the expression levels of barrier genes under LPS challenge. This would suggest that Zn-Gly is capable of attenuating the aforementioned inflammatory response by protecting against the potential loss of intestinal tight junction proteins as a result of LPS exposure.

The toll-like receptor family is the initiator of innate immunity, and TLR4 is the most characteristic member of the family that recognizes lipopolysaccharides and subsequently triggers a series of cellular signals ultimately activating the NF-κB signaling pathway (Doyle and O'Neill 2006). It regulates the inflammatory response by binding to nucleotide sequences upstream of pro-inflammatory cytokines and chemokine genes (Zhang et al. 2017). We confirmed the activation of the embryonic TLR4/NF-κB pathway by LPS, which is consistent with previous work (Xiong et al. 2022). Interaction effect showed that Zn-Gly reversed the upregulation of *TLR4* and *NF-κB p65* expression induced by LPS exposure and modulated the expression of the pro-inflammatory factors *IL-1β* and *IL-6* in the jejunum. This suggests that Zn-Gly alleviates the LPS-induced inflammatory response through the TLR4/NF-κB pathway. However, the NF-κB signaling pathway also induced the zinc import protein *Zip8* to promote an increase in intracellular zinc levels, which in turn inhibited the release of IκB kinase to counteract oxidative stress (Liu et al. 2013). IL-6 was also shown to upregulate the expression of *Zip14* in the liver in murine models to mobilize free zinc to counteract inflammatory responses (Liuzzi et al. 2005). Weakened *NF-κB p65* expression due to zinc supplementation has also been shown to be associated with improved intestinal barrier function (Chen et al. 2021). Thus, Zn-Gly regulates the LPS-induced inflammatory responses and protects against impairment of intestinal barrier function due to LPS exposure via the TLR4/NF-κB pathway, as

evidenced by its involvement in zinc homeostasis remodeling and inhibition of proinflammatory cytokines.

Stimulation of cells by LPS induces excess production of reactive oxygen species (ROS) by the NADPH oxidase system, which activates the antioxidant system and regulates the expression of antioxidant enzymes (Zhang et al. 2019, Yang et al. 2020). ROS production is a double-edged sword that both kills invading pathogens and causes oxidative damage to tissues during the inflammatory response (Lauridsen 2019). The occurrence of oxidative stress represents a disruption in the balance between antioxidant and pro-oxidant factors in animals (Li et al. 2017). MDA and iNOS are biomarkers of lipid damage caused by the oxidative stress (Al-Hakeim et al. 2023). For this study, LPS exposure up-regulated the levels of MDA in the liver and jejunum as well as the jejunal iNOS. The activation of the antioxidant enzyme system is essential to maintain oxidative stress homeostasis and LPS caused a decrease in the liver antioxidant enzymes of CAT and T-AOC (Forman and Zhang 2021). In agreement with previous findings in birds, this study indicates that LPS challenge induces oxidative stress (Pang et al. 2023). By stabilizing protein sulphydryl structures to avoid oxidation and engaging in metal-catalyzed reactions, zinc participates in antioxidant processes to ensure inhibition of inflammation and oxidative stress before cytokine storms are induced (Nakamura et al. 2019, Liao et al. 2021). In the present study, exogenous zinc didn't show a positive effect on the antioxidant capacity of the embryos, which may be related to the stage at which the oxidative reaction occurs.

An acute inflammatory or stress status of the organism is accompanied by a loss of homeostasis of essential ions such as potassium, calcium, selenium and zinc, which affects the efficiency of the metalloenzyme antioxidant defense system against oxidative stress (Borkowski et al. 2011). The inflammatory response due to an LPS challenge is known to cause hypozincemia, due to the coordinated ectopic movement of zinc ions in the blood towards damaged tissues to increase free zinc ions inside cells to combat inflammation (Kirsten et al. 2015). Nutritional interventions have been found to be an effective addition to the pharmacological treatment of acute inflammation (Chua et al. 2012). In previous studies, low blood zinc levels have also been observed in broilers challenged with *Salmonella* or *Coccidioides* (He et al. 2019, Wu et al. 2020). The jejunum is the main site of zinc absorption, with zinc being absorbed by the intestine through transport protein-mediated ion channels and entering the portal venous system to the liver (Kambe et al. 2015, Hennigar et al. 2022). Benefiting from the abundant zinc reserves and the rapidity of its ion exchange capacity, the liver is crucial in the regulation of zinc homeostasis (Stamoulis et al. 2007). After 12 hours of LPS challenge, zinc was redistributed across embryonic tissues, with reduced zinc levels in the serum and liver in contrast to increased zinc levels in intestinal and muscle tissues as well as increased zinc deposition in the tibia. The expression of the liver zinc export gene *ZnT-1* and the import genes *DMT1*, *Zip3* and *Zip8* was up-regulated and a similar trend was identified in the jejunum. As the exogenous zinc was injected, the expression of the liver and jejunum zinc import gene *DMT1* and the jejunum zinc export gene *ZnT-1* were also up-regulated and the gene expression of jejunum *MT* was down-regulated. The administration of Zn-Gly and

LPS had a mutually suppressive effect on embryonic zinc transporter *ZnT-1* gene expression. It has been suggested that embryos in an immune-challenged state increase the rate of zinc transport through the regulation of zinc transport genes and metallothionein leading to a remodeling of zinc homeostasis, with zinc supplementation alleviating this trend.

Zinc homeostasis is directly regulated by zinc transporter proteins and MT, with cysteine-rich MT acting as intracellular zinc buffers to maintain the normal concentration (Jarosz et al. 2017). Theoretically, zinc intake leads to an increase in MT level of tissue cells, and the increased MT inhibits zinc uptake through signaling. It appears that the MT level is not the only marker of zinc homeostasis, as MT undergoes complex signaling, receiving both metal-regulated transcription factor (MTF-1), which regulates zinc concentration signaling, and nuclear factor E2-related factor 2 (Nrf2), which regulates the redox pathway (Hübner and Haase 2021). A member of the nucleic acid exonuclease family, ALP, is an enzyme containing two zinc ions and has the function of dephosphorylating endotoxins to attenuate biotoxicity (Zaher et al. 2020). Cu,Zn-SOD enzyme is an antioxidant enzyme directly involved in the resistance of zinc to oxidative stress (Qi et al. 2019). The present study demonstrated that an LPS challenge down-regulated the liver MT and ALP levels and caused an up-regulation of jejunum Cu,Zn-SOD level in terms of the protein content and gene expression levels, suggesting that LPS exposure induced the release of free zinc from liver MT, which together with ALP resisted the inflammatory response induced by LPS. The jejunum, as an important immune organ, is also involved in the antioxidant process through the enzyme Cu,Zn-SOD. Zn-Gly increased ALP levels in the embryonic liver and jejunum and down-regulated MT levels, suggesting that more zinc was mobilized for the body's defense mechanisms, which was also reflected in the inhibition of LPS-induced increases in Cu,Zn-SOD levels by Zn-Gly.

Through the overall regulation of ubiquitin-dependent signaling pathways, zinc finger protein A20 (TNFAIP3) might serve as a susceptibility gene for inflammatory diseases (Li et al. 2016). A20 regulates inflammatory factors by regulating zinc concentration, with zinc supplementation having been shown to reduce inflammatory factor gene expressions by regulating *A20* level (Shembade and Harhaj 2012, Maares and Haase 2016). As an upstream gene of *MT*, *MTF-1* also modulates *MT* expression and thus participates in the anti-inflammatory response of the body by sensing zinc concentration (Jia et al. 2021). The present study reveals the effects of LPS exposure and zinc supplementation on these two zinc-sensitive genes. The imbalance in zinc homeostasis induced by LPS challenge leads to elevated embryonic liver *A20* and *MTF-1* expression, while Zn-Gly supplementation inhibited the adjustment of *A20* gene expression. A study in rats demonstrated that zinc was capable of preventing inflammatory responses Inhibiting the NF-κB pathway through upregulation of A20, while MTF-1 modulates MT to regulate zinc concentration through its modulation (Yan et al. 2016, Chen et al. 2020). Thus, the reconfiguration of zinc homeostasis and the treatment of Zn-Gly against LPS challenge-induced inflammatory responses are inextricably linked to two zinc signaling sensing factors, A20 and MTF-1.

6. Conclusion

In conclusion, this study demonstrated that the systemic zinc homeostasis in broiler embryos was modulated by immune challenge and the positive effects of exogenous zinc. In response to an LPS challenge, zinc transporter proteins and MT were involved in the remodeling of host zinc homeostasis. Zn-Gly mediated the inflammatory response and impairment of intestinal barrier function induced by LPS exposure by participating in the regulation of zinc homeostasis and inhibiting the production of pro-inflammatory cytokines via the TLR4/NF-κB signaling pathway.

Chapter V

The effect of dietary zinc on growth performance, intestinal health and zinc metabolism of broilers under necrotic enteritis challenge

Chapter V. The effect of dietary zinc on growth performance, intestinal health and zinc metabolism of broilers under necrotic enteritis challenge

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1. Abstract

This study was conducted to investigate the effect of dietary zinc from different sources on growth performance, gut health and zinc metabolism of broilers challenged with necrotic enteritis (*NE*). 432 1-day-old Arbor Acres (AA) male broilers were divided into three dietary treatments (basal diet, 60 mg/kg zinc glycinate and 60 mg/kg zinc sulfate) with 12 replicates of 12 chickens each. Half of the replicates of each dietary treatment were subjected to *NE* challenge from day 23. The animal experiment lasted 35 days. The results showed that *NE* challenge significantly increased feed conversion ratio (FCR) in broilers from 22-35 days and zinc glycinate decreased FCR from 1-21 days. Zinc glycinate significantly reduced intestinal lesion scorings in the duodenum and ileum under *NE* challenge, and both sources of zinc attenuated intestinal permeability under challenge condition. The expression level of the jejunal barrier gene Occludin was down-regulated in response to the challenge, whereas zinc glycinate diet increased the gene expression levels of jejunal Occludin and Claudin1, irrespective of whether the animals underwent *NE* challenge. In the jejunum, zinc glycinate and zinc sulfate reduced MDA concentration and *IL-1β* gene expression levels, while increasing IgA levels during *NE* challenge. In addition, *NE* challenge mediated zinc transporter proteins and metallothionein (MT) to regulate systemic zinc homeostasis, as reflected by the fact that *NE* challenge up-regulated the gene expression of jejunal *ZnT1*, *Zip8*, *Zip14*, and *MT*, and the gene expression level of the liver *ZnT1*. Zinc glycinate increased *ZnT1* gene expression in the liver under challenge, and both sources of zinc significantly reduced gene expression levels of *Zip8* in the liver. These modifications were responsible for the down-regulation of zinc levels in serum, tibia, pectoral muscle and cecum content of broilers under *NE* challenge and the up-regulation of zinc level in the liver, whereas zinc glycinate showed positive effects on zinc levels in serum, tibia, pectoral muscle and liver. In conclusion, *NE* challenge led to inflammatory damage and oxidative stress in broilers, disrupting gut barrier function, reducing growth performance and leading to the remodeling of zinc homeostasis. Dietary zinc alleviated *NE* challenge-induced oxidative stress and intestinal inflammation, and zinc glycinate showed better results in decreasing the FCR and improving the intestinal barrier function.

2. Introduction

Necrotic enteritis *(NE*) caused by *Clostridium perfringens* is a common intestinal disease in broiler chickens (Yang et al. 2022). In the context of a widespread global ban on the use of antibiotics in feed, the clinical and subclinical incidence and detrimental effects of the disease are again being amplified (Timbermont et al. 2011). *Clostridium perfringens* colonizes the intestine and causes loss of intestinal mucosa, resulting in an inflammatory response and oxidative stress, which ultimately leads to reduced body weight and increased mortality in broilers, severely affecting the economic performance (Abd El-Hack et al. 2022).

Zinc is an essential trace element for broilers, that plays an active role in bone

formation, protein and DNA synthesis, immunity and wound healing (Hall et al. 2022). By participating in the composition of metallo-antioxidant enzymes and acting as a second messenger for immune cells, zinc participates in the antioxidant and immune response in the body (Dardenne 2002). Due to excellent selective breeding, modern broilers grow faster and metabolize nutrients more vigorously than ever before, which dictates higher levels of nutritional requirements. As a nutritional supplement, zinc is added to feed to enhance the antioxidant capacity of broilers, reduce inflammatory responses and avoid zinc deficiencies that can lead to delayed feather development (Naz et al. 2016).

The regulation of zinc metabolism in animals is a complex process, and several protein families play important roles in zinc metabolism and homeostasis, such as metallothionein (MT), zinc-regulated transporters (Zrts), iron-regulated transporter (Irt)-like proteins (Zips) and zinc transport proteins (ZnTs) (Mocchegiani et al. 2010). MTs are a family of low-molecular metal-binding proteins that normally bind zinc in cells to act as "zinc reservoirs" (Baltaci et al. 2018). Our past studies have demonstrated that broilers mobilize circulating zinc to damaged tissues through a complex self-defense system to increase intracellular free zinc ions to counter inflammatory and antioxidant responses in response to stress or inflammation (Xiao et al. 2024). Zinc transporter proteins, MT and their upstream zinc ion concentration-sensing regulatory proteins play a key role in this process (Xiao et al. 2022). Therefore, using zinc as a nutritional tool to intervene in the inflammatory response, oxidative stress and intestinal damage in broilers, caused by *NE*, is an effective strategy. In contrast, amino acid chelated zinc is usually considered as a zinc source with higher biological availability, characterized by the avoidance of insoluble precipitates with phytic acid in the digestive tract, which reduces the efficiency of absorption (Farhadi Javid et al. 2021).

Studies have been conducted on the effects of zinc on the improvement of intestinal health in broiler chickens, but research on the changes of zinc metabolism in broiler chickens under *NE* conditions has never been studied to date. The underlying mechanism on how exogenous zinc enhances self-defense mechanisms to counteract the inflammatory response and the impaired intestinal health caused by *NE* is still unknown. This study aimed to investigate: (1) the changes of zinc metabolism in broiler chickens in a *NE* model; (2) the role and mechanism of different sources of zinc on the improvement of inflammatory response and intestinal health.

3. Materials and methods

All experimental procedures were approved by the Ethics Committee of Shandong Agricultural University (No. SDAUA-2022-50) and conducted in accordance with the Guide for Laboratory Animals of the Ministry of Science and Technology (Beijing, People's Republic of China).

3.1. Animals and management

A total of 432 1-day-old Arbor Acres (AA) male chicks of similar body weight were randomly divided into three dietary treatments: NT group (basal diet), OT

group (basal diet with 60 mg/kg zinc glycinate), IT group (basal diet with 60 mg/kg zinc sulfate), with 12 replicates of 12 broilers per treatment. After examining growth performance statistics on day 21, broilers from each dietary treatment group were evenly divided into challenge and non-challenge groups, ensuring that initial body weights of the same dietary treatments were close to each other at 21 days of age. Starting at 21 days of age, there were six replicates for each treatment group and the treatment design is shown in Table 5-1.

Table 5-1: Treatments of different diets and *NE* challenge

Treatment	Zn supplementation	NE challenge
NT	No zinc added	No
OT	60 mg/kg zinc glycinate	No
IT	60 mg/kg zinc sulfate	No
CNT	No zinc added	Yes
COT	60 mg/kg zinc glycinate	Yes
CIT	60 mg/kg zinc sulfate	Yes

NT: negative feed treatment, chicken fed with basal diet; OT: zinc glycinate feed treatment, chicken fed with basal diet containing 60 mg/kg zinc glycinate; IT: zinc sulfate feed treatment, chicken fed with basal diet containing 60 mg/kg zinc sulfate; CNT: negative feed treatment with necrotic enteritis challenge, chicken fed with basal diet and challenged with necrotic enteritis; COT: zinc glycinate feed treatment with necrotic enteritis challenge, chicken fed with basal diet containing 60 mg/kg zinc glycinate and challenged with necrotic enteritis; CIT: zinc sulfate feed treatment with necrotic enteritis challenge, chicken fed with basal diet containing 60 mg/kg zinc sulfate and challenged with necrotic enteritis.

The entire experimental period lasted for 35 days and the birds were kept in cages with the size of 70cm × 70cm × 40cm with feeding and watering provided ad libitum. Crushed pellets were fed for the first 21 days of the experiment and pellets were fed from the 22nd day. The basal diet (Table 5-2) and zinc additives were in accordance with the recommendations of "Compound Feed for Egg Laying Chickens and Broilers" (GB/T 5916-2020) in the People's Republic of China. The zinc levels of the diets formulated according to the requirements of the different experimental treatments were examined with ICP-MS (Table 5-3). During feed formulation, additional glycine was added to the other 4 groups to the same level as the zinc glycinate group to balance the amino acids.

Broilers were reared in an automated environmentally controlled house where the room temperature was raised to 32 °C 48 hours prior to the arrival of the chicks, and the house temperature was gradually lowered over the course of the experiment until it reached 24 °C at 21 days of age and then this temperature was maintained. Humidity was kept at an average of 70% for the first three days of the trial and 55% to 65% thereafter. According to the Arbor Acres broiler

management handbook (2018), the birds are exposed to 23 hours of light and 1 hour of darkness for the first week of life, which changes to 20 hours of light and 4 hours of darkness beginning on the seventh day. To ensure the validity of the experiment, no antibiotics and vaccines were used during the entire period except the coccidiostat vaccine used in the establishment of the *NE* model.

Table 5-2: Composition and nutritional levels of the basal diet（air-dry basis）

Item	**d 1-21**	**d 22-35**
Ingredient (%)		
Corn	49.98	54.90
Soybean meal (46%)	35.75	30.50
Corn protein flour (60%)	3.80	3.10
Salt	0.28	0.28
Limestone	1.75	1.62
Dicalcium phosphate	1.55	1.40
Soybean oil	5.10	6.50
Vitamin premix	0.05	0.05
Mineral premix	0.20	0.20
Choline chloride (50%)	0.10	0.10
Methionine (99%)	0.35	0.35
Lysine (70%)	0.80	0.75
Threonine (98.5%)	0.29	0.25
Phytase (20000 U)	0.02	0.02
Total	100	100
Nutritional level		
Metabolizable energy	3100 (kcal/kg)	3200 (kcal/kg)
Crude protein	23.50	21.00
Lysine	1.39	1.20
Methionine+ cystine	1.02	0.92
Calcium	1.00	0.90
Total phosphorus	0.99	0.90
Available phosphorus	0.50	0.45

Provided per kilogram of compound diet: vitamin A, 12000 IU; vitamin D3, 5000 IU; vitamin E, 80 mg; vitamin K, 3.2 mg; vitamin B1, 3.2 mg; vitamin B2, 8.6 mg; nicotinic acid, 65 mg; pantothenic acid, 20 mg; vitamin B6, 4.3 mg; biotin, 0.22 mg; folic acid, 2.2 mg; vitamin B12, 0.017 mg; I, 1.50 mg; Fe, 80 mg; Mn, 120 mg; Se, 0.3 mg; Cu, 16 mg.

The nutrition level was calculated.

Table 5-3: Analyzed zinc concentrations in the diet

Treatment	**Zinc concentration** (mg/kg) (d 1 to 21)	**Zinc concentration** (mg/kg) (d 22 to 35)
NT/ CNT	28.95	29.54
IT/ CIT	91.25	89.88
OT/ COT	90.65	90.32

NT: negative feed treatment, chicken fed with basal diet; OT: zinc glycinate feed treatment, chicken fed with basal diet containing 60 mg/kg zinc glycinate; IT: zinc sulfate feed treatment, chicken fed with basal diet containing 60 mg/kg zinc sulfate; CNT: negative feed treatment with necrotic enteritis challenge, chicken fed with basal diet and challenged with necrotic enteritis; COT: zinc glycinate feed treatment with necrotic enteritis challenge, chicken fed with basal diet containing 60 mg/kg zinc glycinate and challenged with necrotic enteritis; CIT: zinc sulfate feed treatment with necrotic enteritis challenge, chicken fed with basal diet containing 60 mg/kg zinc sulfate and challenged with necrotic enteritis.

3.2. Establishment of the NE model

Referring to past study (Guaragni et al. 2020), the *NE* model was performed starting from day 23 of age by administering coccidiostat vaccine (containing live attenuated oocysts of *Eimeria tenella* PTMZ strain, *E. necatrix* PNHZ strain, *E. maxima* PMHY strain, and *E. acervulina* PAHY strain; Foshan Standard Bio-Tech Co., Ltd., Foshan, China) of 30 times of normal immunological requirement orally to each broiler in the challenge group on day 23 and by instilling 1 mL of broth culture containing *Clostridium perfringens* (1×10^9 CFU/mL) daily from day 26 to day 32. The strain was purchased from China Veterinary Culture Collection Center (Beijing, China), which was isolated from the intestine of broilers clinically diagnosed with *NE* and identified as NetB toxin-positive strain A, No. CVCC2030 (Du et al. 2021). 1 mL of the strain was incubated in 250 mL of thioglycolate broth medium in an anaerobic cabinet at 37 °C for 24 h. Then 1 mL of the culture was inoculated into 1 L of thioglycolate broth medium supplemented with 10 g/L starch and 15 g/L peptone for 24 h. The broilers were fasted for 8 hours prior to the oral supplementation. Broilers in the non-challenged groups were orally administered with PBS or broth culture to balance the stress response caused by catching and supplementation.

3.3. Growth performance

Feed consumption was recorded to count average daily feed intake (ADFI), all chickens were weighed on days 21 and 35 to calculate average daily gain (ADG), and ADFI/ADG was used to calculate feed conversion ratio (FCR) for each stage. Dead chickens were weighed and recorded for FCR value correction and mortality rate.

3.4. Sample collection

Sample collection was performed at 35 days of age. Two chickens per replicate were randomly selected for blood sample collection from the wing vein using a sterile syringe. Blood samples were placed in anticoagulant-free glass tubes for 30 minutes, centrifuged at 3000 rpm in a 4°C environment, and serum was obtained and stored in a -20 °C freezer. After blood collection, the chickens were euthanized by cervical dislocation method for sample collection: liver, jejunum, pectoral muscle, unilateral tibia and cecum contents were collected and stored at -20 °C to measure zinc content. Samples of liver and jejunum were collected and stored at -80 °C for molecular and biochemical index assays.

3.5. Evaluation of intestinal health

Fluorescein isothiocyanate dextran (FITC-D, Sigma, 53557) was used to assess the intestinal permeability by oral administration 24 h after *NE* challenge procedure. Blood was collected 2.5 h after 2 mL of PBS solution containing FITC-D (1.1 g/mL) was given to each broiler, and the blood was centrifuged to separate the serum after standing at room temperature. The serum was diluted 1:1 with PBS and the amount of FITC-D in the serum was measured at 485 nm and 528 nm emission wavelengths, and the intestinal permeability values were obtained from the standard curve. No fasting before this process was conducted.

Intestinal lesion scoring was performed at day 35, observed and photographed, and the scores were set to 0-4 according to: 0 being a healthy intestine, 1 containing a few bleed spots, 2 having more bleed spots and a thinner intestinal thickness or mucosal detachment, 3 having a large number of bleed spots and a very thin intestinal wall, and 4 being a chicken that had died (Song et al. 2023). Typical 0-3 scoring intestines are shown in Figure 5-1.

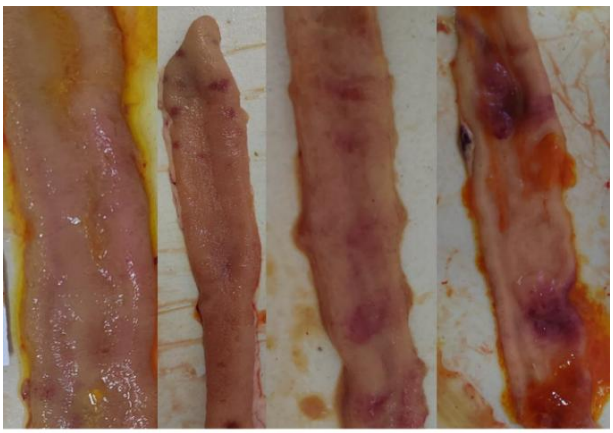

Figure 5- 1: Intestinal lesion scoring standard. Intestinal lesion scores were set to 0-4 according to severity: 0 being a healthy intestine, 1 containing a few bleed spots, 2 having more bleed spots and a thinner intestinal thickness or mucosal detachment, 3 having a large number of bleed spots and a very thin intestinal wall, and 4 being a chicken that had died.

To further determine the effect of *NE* challenge on the impairment of intestinal health and improvement of zinc, gene expression assays were performed for the intestinal barrier function genes *ZO-1*, Occludin and Claudin 1 in the jejunum.

3.6. Determination of zinc content

To determine serum zinc concentration using ICP-MS, 75 μL of serum sample was added to a 5 mL centrifuge tube, 600 μL of nitric acid (5%) and 75 μL of hydrogen peroxide (30%) were added and the samples were held in a 60 °C water bath for 2 h. Then, 750 μL of nitric acid (5%) was added and the samples were shaken. The mixture was centrifuged at 8000 rpm for 15 min, and the supernatant was stored at 4°C until further analysis.

The tibia were stripped of soft tissue and boiled in deionized water for 10 min, defatted by immersion in ether for 96 h, then dried at 105 °C to a constant weight and ashed in a muffle furnace (550-600 °C, 24 h) and expressed as dry defatted weight. The ashed samples were dissolved using 5 mL of hydrochloric acid solution at a concentration of 6 mol/L and then fixed in a 25 mL volumetric flask to be measured.

The samples of jejunum, heart, liver, pectoral muscle, feces and intestinal contents were freeze-dried, obtaining about 0.2 g of dry powder, 2 mL of nitric acid (70%) and 1mL of hydrogen peroxide solution (30%) was added, after which they were placed in a 50 mL plastic centrifuge tube in a 95°C water bath for 6 hours. Samples were cooled and then filtered, in a 25 mL volumetric flask after fixing the volume for measurement. Bones, tissues, feed and cecum contents were analyzed for zinc content by flame atomic absorption spectrometry (SpectrAA 50/55, Warian Corporation, Palo Alto, CA, USA).

3.7. Antioxidant capacity and immunological indicators

Malondialdehyde (MDA), total antioxidant capacity (T-AOC), catalase (CAT), and glutathione peroxidase (GSH-px) activities were measured in the liver and jejunum. The kits were purchased from Nanjing Jiancheng Institute of Biotechnology (Nanjing, China) and tested according to the manufacturer's instructions. The levels of interleukin 1β (IL-1β), IL-6, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), immunoglobulin A (IgA) and immunoglobulin Y (IgY) in serum and jejunal tissues were measured by enzyme-linked immunosorbent assay (ELISA) kits from MLBIO (Shanghai, China) to assess the immune function of broiler chickens. All assay procedures were performed in strict accordance with the manufacturer's instructions. The intra-batch coefficient of variation (CV) of the data was < 5% and the inter-batch CV was < 8%.

3.8. Zinc metabolism and transport-related enzyme activities

The activity of alkaline phosphatase (ALP) and copper-zinc superoxide dismutase (Cu,Zn-SOD) in the jejunum and liver was measured using the kits from the Nanjing Jiancheng Institute of Biotechnology, and the concentration of MT in liver homogenates was determined using an ELISA kit (MLBIO Co., Shanghai, China) according to the manufacturing instructions. The intra-batch CV of the data was < 5% and the inter-batch CV was < 8%.

3.9. Gene expression of the jejunum and liver

Total RNA from the jejunum and liver was extracted with Trizol reagent (Invitrogen, San Diego, USA). The concentration and purity of each RNA sample was measured using a NanoDrop spectrophotometer (ND-2000, Thermo Scientific, Wilmington, USA). 1% agarose gel electrophoresis was used to detect RNA integrity. Reverse transcription of 1 μg of total RNA was performed using PrimeScript® RT reagent kit (RR047A, TaKaRa, Japan). RT-PCR was performed using TB Green PreMix Ex Taq (RR820A, TaKaRa, Japan) in an ABI 7500 Real-Time PCR Detection System (Life Technologies) to determine relative gene expression. The reaction procedure consisted of pre-denaturation at 95 °C for 10 s, denaturation at 95 °C for 5 s for a total of 40 cycles, and finally an annealing extension at 60 °C for 34 s. Primer sequences are demonstrated in Table 5-4. *β-actin* and glyceraldehyde-3-phosphate dehydrogenase (*GADPH*) were used as housekeeping genes and the mean value of the housekeeping genes was used to normalize the expression of the target genes. The relative expression levels of each target gene were analyzed by the $2^{-\Delta\Delta ct}$ method.

Table 5-4: Nucleotide sequences of real-time PCR primers

Gene	Accession number	Primer sequence, 5'→ 3'	Reference
IL-1β	NM_204524.1	GGTCAACATCGCCACCTACA CATACGAGATGCAAACCAGCAA	Kong et al. 2022
1L-6	NM_204628.1	AAATCCCTCCTCGCCAATCT CCCTCACGGTCTTCTCCATAAA	
TLR4	NM_001030693.1	AGGCACCTGAGCTTTTCCTC TACCAACGTGAGGTTGAGCC	
NF-κB p65	NM_001396038.1	CAGCCCATCTATGACAACCG TCAGCCCAGAAACGAACCTC	
ZO-1	XM_015278981.2	CTTCAGGTGTTTCTCTTCCTCCTCTC CTGTGGTTTCATGGCTGGATC	
Occludin	NM_205128.1	TCATCGCCTCCATCGTCTAC TCTTACTGCGCGTCTTCTGG	
Claudin-1	NM_001013611.2	CTGATTGCTTCCAACCAG CAGGTCAAACAGAGGTACAAG	
MT	NM_205275.1	GCAACAACTGTGCCAAGGGC TTTCGTGGTCCCTGTCACCC	Li et al. 2015
Cu,Zn-SOD	NM_205064.1	TTGTTCTGATGGAGATCATGGCTTC TGCTTGCCTTCAGGATTAAAGTGAG	

MTF-1	XM_015297695.3	CCTGGTTCAACTCCTATGC TCAAACGGCTTCTCCTTA	
A20	XM_003640919.2	GACATCGTGCTAACAGCTTGGA AGAAAAGAGGTATCAGGCACAAC	
ZnT1	NM_001389457.1	CTTCGCTTAGCATTTCTT TCTCCGATTTAGTCCTTCT	
DMT1	NM_001128102.2	AGCCGTTCACCACTTATTTCG GGTCCAAATAGGCGATGCTC	Wu et al. 2020
Zip8	XM_040671236.1	TGTAAATGTCTCGGTGGG CAAGATGGCTATGGAGGT	
Zip14	XM_040689606.1	GTTCTGCCCCGCTGTCCT GGTCTGCCCTCCTCCGTCT	
GPR39	NM_001080105.1	CAGTTCACATCATGGAGGGGC AGCGGTCAGGTACAACGAAA	Hu et al.2024
β-actin	L08165	CAGACATCAGGGTGTGATGG TCAGGGGCTACTCTCAGCTC	Kong et al. 2023
GADPH	NM_204305.1	GCCCAGAACATCATCCCA GGGGAGACAGAAGGGAACAGA	

TLR4: toll-like receptor 4; *NF-κB p65*: nuclear factor kappa-B p65; *ZO-1*: zonula occludens 1; *GPR39*: G protein coupled receptor 39; *MTF-1*: metal transcription factor-1; *ZnT*: zinc transporter; *Zip*: zinc-regulated transporter, iron-regulated transporter-like protein; *MT*: metallothionein; *Cu,Zn-SOD*: copper-zinc superoxide dismutase; *GADPH*: glyceraldehyde-3-phosphate dehydrogenase.

3.10. Data Analysis

All data were expressed as mean and total standard error of the mean (SEM), and data were counted and samples were analyzed in replicates. Data were analyzed using the Shapiro-Wilk test (95% confidence interval) performed by the software SPSS 22.0 (SPSS. Inc., Chicago, USA) followed by a two-way ANOVA with general linear model, and Tukey's post-hoc test was applied to determine differences between groups. Data were considered statistically significantly different when $P < 0.05$.

4. Results

4.1. Growth performance

The effect of dietary zinc on growth performance of broilers challenged with *NE* from days 1-21 and 22-35 is demonstrated in Table 5-5. On days 1-21, zinc glycinate significantly decreased FCR in broilers ($P = 0.027$), while zinc did not

affect final BW, ADFI, ADG and survival rate at this period ($P > 0.05$). The interaction between dietary zinc and *NE* challenge was not reflected in the growth performance from 22-35 days ($P > 0.05$). Main effects showed that FCR of birds was significantly increased by *NE* challenge ($P = 0.002$), and challenge did not affect final BW, ADFI, ADG and survival rate ($P > 0.05$). Dietary factor did not cause significant differences in growth performance of chickens in the last two weeks ($P > 0.05$).

4.2. Intestinal health status

Figure 5-2 shows the effect of *NE* challenge and zinc on intestinal lesion scores (Figure 5-2A), intestinal permeability (Figure 5-2B), and jejunal barrier gene expression (Figure 5-2C). A significant interaction of zinc and *NE* challenge was observed for lesion scorings of the duodenum ($P = 0.039$) and ileum ($P = 0.032$), with zinc glycinate significantly reducing intestinal lesion scorings of the duodenum and ileum under *NE* challenge. Main effects showed that all intestinal lesions were significantly exacerbated by *NE* challenge ($P < 0.001$), whereas zinc glycinate significantly reduced intestinal lesions in the ileum ($P = 0.013$). Meanwhile, zinc and *NE* challenge also showed a significant interaction ($P < 0.001$) in serum FITC-D concentration, with both zinc glycinate and zinc sulfate diets reducing serum FITC-D concentration under *NE* challenge. Serum FITC-D concentrations were significantly upregulated by *NE* challenge ($P < 0.001$), whereas both sources of zinc reduced serum FITC-D concentrations ($P < 0.001$). Zinc and *NE* challenge did not cause a significant interaction on jejunal barrier gene expression. *NE* challenge resulted in a significant down-regulation of gene expression levels of Occludin ($P = 0.044$). Both zinc glycinate and zinc sulfate diets significantly increased jejunal Claudin1 ($P = 0.001$) gene expression level, and zinc glycinate also upregulated the gene expression level of Occludin ($P = 0.033$) gene expression level compared to the zinc-free treatment.

4.3. Antioxidant status

As shown in Table 5-6, antioxidant parameters in the serum and jejunum were assayed to evaluate the effect of zinc on the antioxidant status of broilers under *NE* challenge. A significant interaction of zinc and *NE* challenge on antioxidant status was observed for jejunal MDA, with both dietary zinc glycinate and zinc sulfate

Table 5-5: Effect of dietary zinc on growth performance of necrotic enteritis challenged broilers

	Item	**Final BW**, g	**ADFI**, g/day	**ADG**, g/day	**FCR**, g/g	**Survival rate**, %
1-21d	NT	793.53	49.32	36.62	1.34[a]	97.50
	OT	792.49	47.53	37.41	1.26[b]	98.33
	IT	778.82	48.06	36.65	1.31[ab]	100
	Pooled SEM	45.54	2.49	1.45	0.06	0.39
	P value	0.693	0.27	0.471	0.027	0.149
22-35d	NT	1856.22	143.77	122.53	1.17	95.83
	OT	1894.35	140.43	126.35	1.11	95.83
	IT	1956.02	150.81	128.42	1.18	95.83
	CNT	1858.08	139.99	114.39	1.23	91.67
	COT	1936.08	148.82	121.48	1.23	94.44
	CIT	1932.44	147.33	121.38	1.22	94.44
	Pooled SEM	118.87	9.51	10.72	0.07	5.33
	Diet					
	Negative	1857.39	141.88	118.46	1.20	93.75
	Gly-Zn	1915.57	144.63	123.91	1.17	95.14
	$ZnSO_4$	1945.90	149.23	121.38	1.19	95.14
	NE Challenge					

No Challenge	1906.20	145.37	125.93	1.16[b]	95.83
Challenge	1908.66	145.38	119.09	1.23[a]	93.52
P value					
Diet	0.275	0.223	0.352	0.420	0.778
NE Challenge	0.883	0.911	0.093	0.002	0.218
Interaction	0.828	0.265	0.941	0.366	0.778

Values are expressed as the mean and pooled SEM (n = 6). Means in a row with no common superscript are significantly different ($P < 0.05$). Lowercase letters indicate significantly different interactions and challenge effects, uppercase letters indicate significantly different dietary effects. SEM: standard error of the mean; BW: body weight; ADFI: average daily feed intake; ADG: average daily gain; FCR: feed conversion ratio; NT: negative feed treatment, chicken fed with basal diet; OT: zinc glycinate feed treatment, chicken fed with basal diet containing 60 mg/kg zinc glycinate; IT: zinc sulfate feed treatment, chicken fed with basal diet containing 60 mg/kg zinc sulfate; CNT: negative feed treatment with necrotic enteritis challenge, chicken fed with basal diet and challenged with necrotic enteritis; COT: zinc glycinate feed treatment with necrotic enteritis challenge, chicken fed with basal diet containing 60 mg/kg zinc glycinate and challenged with necrotic enteritis; CIT: zinc sulfate feed treatment with necrotic enteritis challenge, chicken fed with basal diet containing 60 mg/kg zinc sulfate and challenged with necrotic enteritis.

significantly decreasing jejunal MDA concentrations under *NE* challenge ($P = 0.005$). Main effects showed that *NE* challenge significantly increased serum MDA concentration ($P = 0.045$) and decreased serum GSH-px ($P = 0.001$) and jejunal CAT ($P = 0.002$) activity and as well as T-AOC values ($P = 0.001$). Both additional zinc-added diets significantly increased serum T-AOC ($P = 0.004$), jejunal CAT activity ($P = 0.001$) and GSH-px activity ($P = 0.026$) compared to the zinc-free diet.

Table 5-6: Effect of dietary zinc on oxidative status of necrotic enteritis challenged broilers

Item	Serum				Jejunum			
	MDA, nmol/mL	CAT, pg/mL	TAOC, Mmol/mL	GSH-px, ng/mL	MDA, nmol/mg prot	CAT, U/mg prot	T-AOC, U/mg prot	GSH-px, U/mg prot
NT	0.07	1.96	0.16	41.10	0.18^{c}	4.55	0.15	15.75
OT	0.05	1.91	0.17	46.35	0.20^{c}	5.61	0.17	16.05
IT	0.05	2.13	0.18	36.60	0.22^{bc}	5.57	0.16	17.55
CNT	0.08	2.08	0.15	27.75	0.40^{a}	3.17	0.12	5.70
COT	0.07	1.90	0.18	30.90	0.25^{bc}	3.96	0.10	19.05
CIT	0.06	1.83	0.19	35.85	0.29^{b}	4.82	0.06	19.20
Pooled SEM	0.01	0.26	0.03	10.50	0.06	1.23	0.02	3.45
Diet								
Negative	0.07	2.02	0.16^{B}	34.50	0.28	3.86^{B}	0.14	10.35^{B}
Gly-Zn	0.06	1.91	0.18^{A}	38.55	0.22	4.78^{A}	0.13	18.00^{A}
$ZnSO_4$	0.05	1.98	0.19^{A}	36.15	0.26	5.20^{A}	0.11	18.45^{A}
NE Challenge								
No Challenge	0.06^{b}	2.00	0.18	41.40^{a}	0.20^{b}	5.24^{a}	0.16^{a}	16.50
Challenge	0.07^{a}	1.94	0.17	31.50^{b}	0.32^{a}	3.98^{b}	0.10^{b}	14.85
P value								
Diet	0.072	0.518	0.004	0.490	0.078	0.001	0.167	0.026
NE Challenge	0.045	0.411	0.350	0.001	0.001	0.002	0.001	0.460
Interaction	0.922	0.094	0.427	0.085	0.005	0.425	0.068	0.063

Values are expressed as the mean and pooled SEM (n = 6). Means in a row with no common superscript are significantly different ($P < 0.05$). Lowercase letters indicate significantly different interactions and challenge effects, uppercase letters indicate significantly different dietary

effects. SEM: standard error of the mean; MDA: malondialdehyde; CAT: catalase; T-AOC: total superoxide dismutase; GSH-px: glutathione peroxidase. The abbreviations of the treatment groups are the same as the previous ones.

Table 5-7: Effect of dietary zinc on immune parameters of necrotic enteritis challenged broilers

Item	Serum				Jejunum			
	IL-1β, pg/mL	IL-6, pg/mL	INF-γ, pg/mL	TNF-α, pg/mL	IL-1β, pg/mgprot	IL-6, pg/mgprot	INF-γ, pg/mgprot	TNF-α, pg/mgprot
NT	863.10	32.95	84.00	70.55	686.30	27.90	85.05	77.95
OT	804.60	34.70	84.55	71.40	614.20	25.50	76.70	70.55
IT	854.30	33.85	83.15	74.85	616.75	26.80	85.15	72.45
CNT	913.85	39.05	94.50	76.10	715.10	35.60	83.15	81.60
COT	782.90	37.55	86.60	75.05	698.65	29.30	84.85	75.00
CIT	878.75	35.45	80.60	70.25	653.75	25.90	91.25	76.95
Pooled SEM	77.15	4.20	8.10	5.50	65.50	3.80	7.00	5.85
Diet								
Negative	863.10	32.95	84.00	70.55	715.10^{A}	35.60^{A}	83.15^{B}	81.60^{A}
Gly-Zn	804.60	34.70	84.55	71.40	698.65^{B}	29.30^{B}	84.85^{B}	75.00^{B}
$ZnSO_4$	854.30	33.85	83.15	74.85	653.75^{B}	25.90^{B}	91.25^{A}	76.95^{B}
NE								
No	840.67	33.83^{b}	83.90	72.27	639.08^{b}	26.73^{b}	82.30^{b}	73.65^{b}
Challenge	858.50	37.35^{a}	87.23	73.80	689.17^{a}	30.27^{a}	86.42^{a}	77.85^{a}
P value								
Diet	0.010	0.648	0.093	0.549	0.035	0.024	0.021	0.003
NE	0.459	0.020	0.220	0.927	0.015	0.034	0.015	0.012

Interaction	0.477	0.405	0.146	0.121	0.458	0.111	0.117	0.971

Values are expressed as the mean and pooled SEM (n = 6). Means in a row with no common superscript are significantly different ($P < 0.05$). Lowercase letters indicate significantly different interactions and challenge effects, uppercase letters indicate significantly different dietary effects. SEM: standard error of the mean; IL-1β: interleukin 1 beta; IL-6: interleukin 6; IFN-γ: interferon gamma; TNF-α: tumor necrosis factor alpha. The abbreviations of the treatment groups are the same as the previous ones.

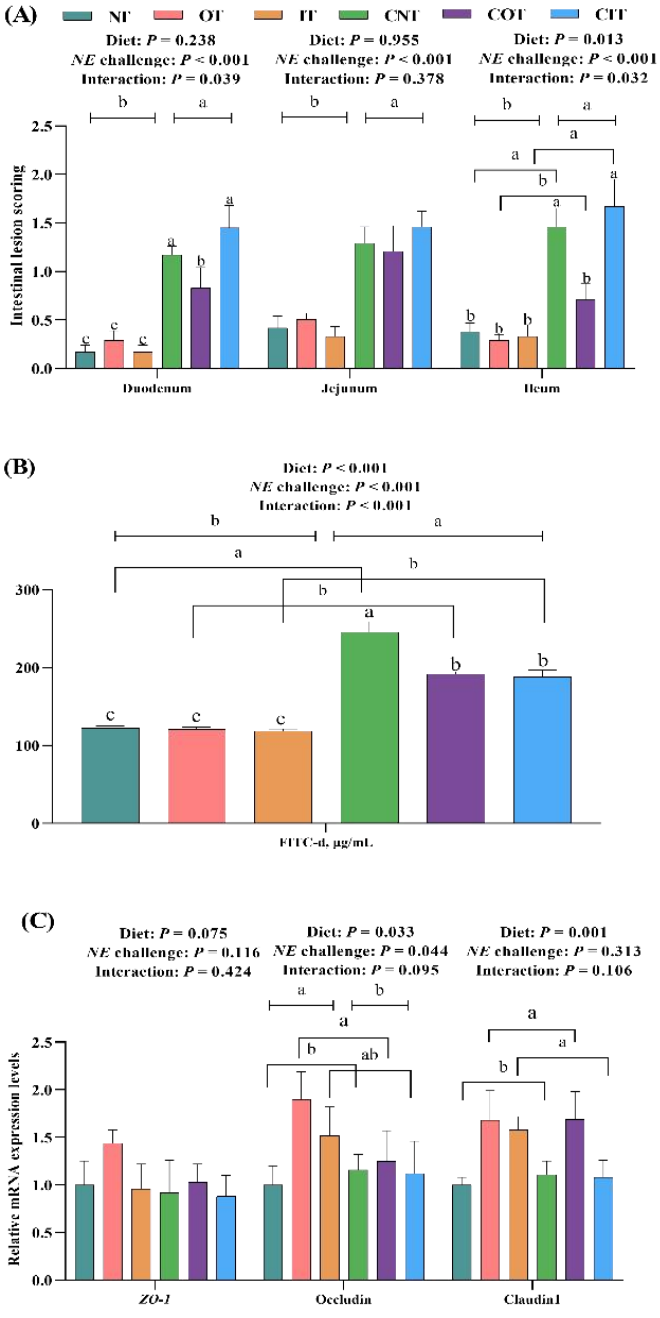

Figure 5-2: Effect of dietary zinc on intestinal lesion scoring (A), intestinal permeability (B) and Jejunum barrier gene expression (C) of necrotic enteritis challenged broilers.

Means with no common superscripts differ significantly ($P < 0.05$), with lowercase letters indicating significantly different interactions and uppercase letters indicating significantly different dietary effects. NT: negative feed treatment, chicken fed with basal diet; OT: zinc glycinate feed treatment, chicken fed with basal diet containing 60 mg/kg zinc glycinate; IT: zinc sulfate feed treatment, chicken fed with basal diet containing 60 mg/kg zinc sulfate; CNT: negative feed treatment with necrotic enteritis challenge, chicken fed with basal diet and challenged with necrotic enteritis; COT: zinc glycinate feed treatment with necrotic enteritis challenge, chicken fed with basal diet containing 60 mg/kg zinc glycinate and challenged with necrotic enteritis; CIT: zinc sulfate feed treatment with necrotic enteritis challenge, chicken fed with basal diet containing 60 mg/kg zinc sulfate and challenged with necrotic enteritis; FITC-d: fluorescein isothiocyanate dextran; *ZO-1*: zonula occludens 1.

4.4. Cytokines and immunoglobulin levels

The effect of dietary zinc on cytokine and immunoglobulin levels in broilers under *NE* challenge are shown in Tables 5-7 and 5-8, respectively. The interaction showed that both zinc glycinate and zinc sulfate significantly increased jejunal IgA level in response to *NE* challenge ($P = 0.004$). *NE* challenge resulted in a significant up-regulation of serum pro-inflammatory factor IL-6 level ($P = 0.02$), jejunal pro-inflammatory factor IL-1β ($P = 0.015$) and IL-6 ($P = 0.034$) levels. Jejunal INF-γ ($P = 0.015$) and TNF-α ($P = 0.012$) levels also showed a significant up-regulation, and serum IgA level was significantly downregulated ($P < 0.001$). Zinc glycinate significantly down-regulated serum IL-1β ($P = 0.01$) and jejunal INF-γ ($P = 0.021$) levels, whereas jejunal IL-1β ($P = 0.035$), IL-6 ($P = 0.024$), and TNF-α ($P = 0.003$) levels were significantly down-regulated by the effect of both zinc-added diets. In addition, both zinc glycinate and zinc sulfate intake significantly increased serum IgA ($P = 0.015$) and IgY ($P = 0.017$) levels.

4.5. Zinc concentration

Zinc concentrations in serum, tibia, pectoral muscle, jejunum, liver, and cecum contents are displayed in Table 5-9. Significant interaction between zinc and *NE* challenge on zinc concentrations was not observed ($P > 0.05$). Zinc levels in broiler serum ($P = 0.004$), tibia ($P < 0.001$), pectoral muscle ($P = 0.002$), and cecum content ($P = 0.046$) were decreased due to the challenge, whereas zinc levels in liver were increased ($P = 0.001$). Zinc glycinate significantly increased zinc levels in serum, tibia, pectoral muscle and liver of broilers compared to zinc-free diets, whereas zinc sulfate diets also showed a significant increase in serum zinc levels in broilers ($P < 0.05$). Interestingly, the cecum contents of broilers given the zinc sulfate diet had higher zinc concentrations compared to broilers fed the zinc-free and zinc glycinate diets.

4.6. Zinc-related enzyme activities and MT concentration

To investigate the effect of *NE* challenge and dietary zinc on zinc-related enzyme activities, Table 5-10 shows the levels of ALP activity, Cu,Zn-SOD activity and MT in the liver and jejunum. A significant interaction between dietary zinc and *NE* challenge was not observed ($P > 0.05$). Main effects showed

that Cu,Zn-SOD activity was increased in liver (P = 0.038) and jejunum (P = 0.007) under *NE* challenge. ALP activity in jejunum showed the same significant effect (P = 0.019). Dietary factors did not significantly affect ALP activity, Cu,Zn-SOD activity and MT concentration ($P > 0.05$).

Table 5-8: Effect of dietary zinc on the concentration of immunoglobulins of necrotic enteritis challenged broilers

Item	Serum		Jejunum	
	IgA, pg/mL	IgY, pg/mL	IgA, pg/mgprot	IgY, pg/mgprot
NT	350.30	2063.40	334.15^{a}	2420.85
OT	379.30	2331.70	331.3^{a}	2345.70
IT	362.25	2279.50	312.85^{ab}	2009.40
CNT	284.30	1836.80	245.95^{c}	2283.10
COT	335.65	2164.75	315.60^{ab}	2365.35
CIT	347.15	2179.35	286.45^{b}	2389.10
Pooled SEM	44.45	187.15	39.25	274.75
Diet				
Negative	284.30^{B}	1836.80^{B}	245.95^{B}	2283.10
Gly-Zn	335.65^{A}	2164.75^{A}	315.60^{A}	2365.35
$ZnSO_4$	347.15^{A}	2179.35^{A}	286.45^{B}	2389.10
NE Challenge				
No Challenge	363.95^{a}	2224.87	326.10^{a}	2258.65
Challenge	322.37^{b}	2060.30	282.67^{b}	2345.85
P value				
Diet	0.015	0.017	0.012	0.270
NE Challenge	< 0.001	0.175	< 0.001	0.322
Interaction	0.227	0.909	0.004	0.063

Values are expressed as the mean and pooled SEM (n = 6). Means in a row with no common superscript are significantly different ($P < 0.05$). Lowercase letters indicate significantly different interactions and challenge effects, uppercase letters indicate significantly different dietary effects. SEM: standard error of the mean; IgA: immunoglobulin A; IgY: immunoglobulin Y. The abbreviations of the treatment groups are the same as the previous ones.

Table 5-9: Effect of dietary zinc on zinc concentrations in the serum, tibia, tissues and cecum content of necrotic enteritis challenged broilers

Item	Serum, μg/mL	Tibia, μg/g	Pectoral muscle, μg/g	Jejunum, μg/g	Liver, μg/g	Cecum content, μg/g
NT	2.92	218.18	33.52	98.37	83.95	115.04
OT	3.42	233.34	37.94	91.24	98.12	148.29
IT	3.26	224.34	31.91	91.65	88.18	174.67
CNT	2.39	200.14	26.92	92.88	97.69	113.70
COT	3.01	224.73	32.69	87.07	105.43	132.04
CIT	2.96	189.06	31.07	97.92	91.38	158.10
Pooled SEM	0.58	24.87	5.86	12.53	13.11	29.24
Diet						
Negative	2.66^{B}	209.16^{B}	29.89^{B}	95.51	90.20^{B}	114.37^{C}
Gly-Zn	3.22^{A}	229.03^{A}	35.08^{A}	89.25	101.97^{A}	140.98^{B}
$ZnSO_4$	3.11^{A}	206.70^{B}	31.38^{B}	94.92	92.27^{B}	166.38^{A}
NE Challenge						
No Challenge	3.20^{a}	225.00^{a}	34.79^{a}	93.75	89.43^{b}	147.27^{a}
Challenge	2.79^{b}	204.46^{b}	30.32^{b}	92.95	100.36^{a}	135.41^{b}
P value						
Diet	0.004	0.006	0.005	0.183	0.008	0.001
NE Challenge	0.004	< 0.001	0.002	0.710	0.001	0.046
Interaction	0.778	0.190	0.199	0.224	0.646	0.468

Values are expressed as the mean and pooled SEM (n = 6). Means in a row with no common superscript are significantly different ($P < 0.05$). Lowercase letters indicate significantly different interactions and challenge effects, uppercase letters indicate significantly different dietary effects. SEM: standard error of the mean. The abbreviations of the treatment groups are the same as the previous ones.

Table 5-10: Effect of dietary zinc on ALP activity, metallothionein concentration and Cu,Zn-SOD activity of necrotic enteritis challenged broilers

Item	Liver			Jejunum		
	ALP, U/gprot	Cu,Zn-SOD, pg/gprot	Metallothioneine, ng/gprot	ALP, U/gprot	Cu,Zn-SOD, pg/gprot	Metallothioneine, ng/gprot
NT	20.21	45.11	66.00	2.28	8.89	47.81
OT	27.13	44.74	66.77	2.39	9.65	63.43
IT	19.82	40.92	66.00	1.93	10.42	53.97
CNT	27.43	43.89	59.31	2.29	10.68	50.99
COT	22.40	50.39	63.02	2.71	10.41	53.06
CIT	22.30	54.36	62.23	2.84	11.11	55.78
Pooled SEM	4.71	8.69	9.65	0.55	1.29	8.10
Diet						
Negative	23.55	44.45	62.40	2.28	9.78	49.40
Gly-Zn	24.95	47.79	64.88	2.54	10.03	58.72
$ZnSO_4$	20.96	47.64	64.26	2.38	10.74	54.81
NE Challenge						
No Challenge	22.39	43.74^{b}	66.24	2.21^{b}	9.62^{b}	54.66
Challenge	24.04	49.04^{a}	61.40	2.61^{a}	10.71^{a}	53.16
P value						
Diet	0.408	0.557	0.860	0.428	0.103	0.061
NE Challenge	0.480	0.038	0.166	0.019	0.007	0.434
Interaction	0.122	0.120	0.918	0.103	0.389	0.140

Values are expressed as the mean and pooled SEM (n = 6). Means in a row with no common superscript are significantly different ($P < 0.05$). Lowercase letters indicate significantly different interactions and challenge effects, uppercase letters indicate significantly different dietary effects. SEM: standard error of the mean; ALP: alkaline phosphatase; Cu,Zn-SOD: copper-zinc superoxide dismutase. The abbreviations of the treatment groups are the same as the previous ones.

4.7. Zinc transport-related gene expression

Figures 5-3A and 5-4A present the effect of zinc from different sources on the regulation of zinc import genes and zinc export genes in the liver and jejunum of broiler chickens under *NE* challenge. In the liver, dietary zinc and *NE* challenge significantly interacted on gene expression of the zinc export gene *ZnT1*. Zinc glycinate diet significantly increased *ZnT1* gene expression compared to zinc sulfate diet in the liver under *NE* challenge ($P = 0.034$). The main effect indicated that both additional zinc-added diets significantly decreased gene expression levels of *Zip8* in the liver ($P < 0.05$). In the jejunum, there was no significant interaction ($P > 0.05$) between the two sources of zinc on jejunal zinc transporter-related genes. *NE* challenge up-regulated gene expression of jejunal *ZnT1* ($P = 0.021$), *Zip8* ($P = 0.046$) and *Zip14* ($P = 0.003$). Zinc did not significantly affect the expression level of jejunal zinc transporter genes ($P > 0.05$).

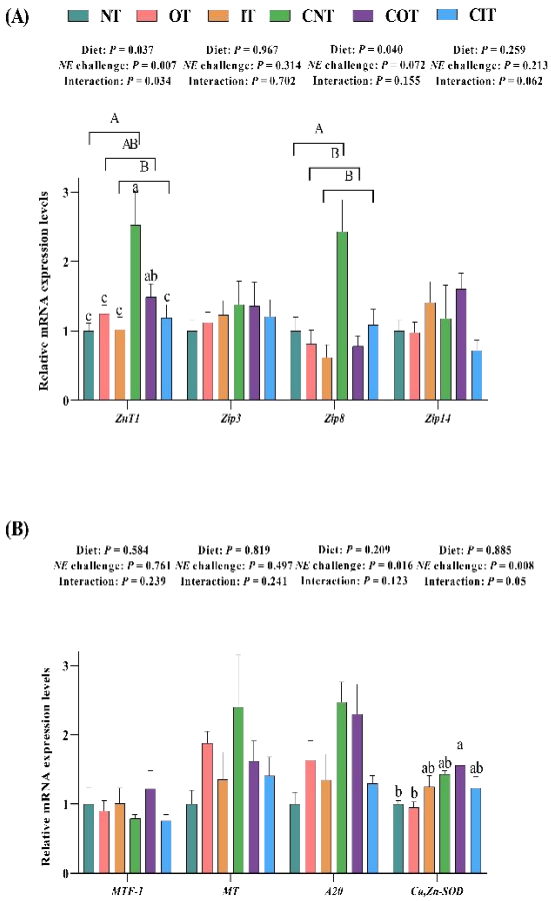

Figure 5- 3: Effect of dietary zinc on the gene expression levels of zinc transporter (A) and zinc regulation-related genes (B) in the liver of necrotic enteritis challenged broilers. Means with no common superscripts differ significantly ($P < 0.05$), with lowercase letters

indicating significantly different interactions and uppercase letters indicating significantly different dietary effects. *ZnT*: zinc transporter; *ZIP*: zinc-regulated transporter, iron-regulated transporter-like protein; *MTF-1*: metal transcription factor-1; *MT*: metallothionein; *Cu,Zn-SOD*: Copper-zinc superoxide dismutase. The abbreviations of the treatment groups are the same as the previous ones.

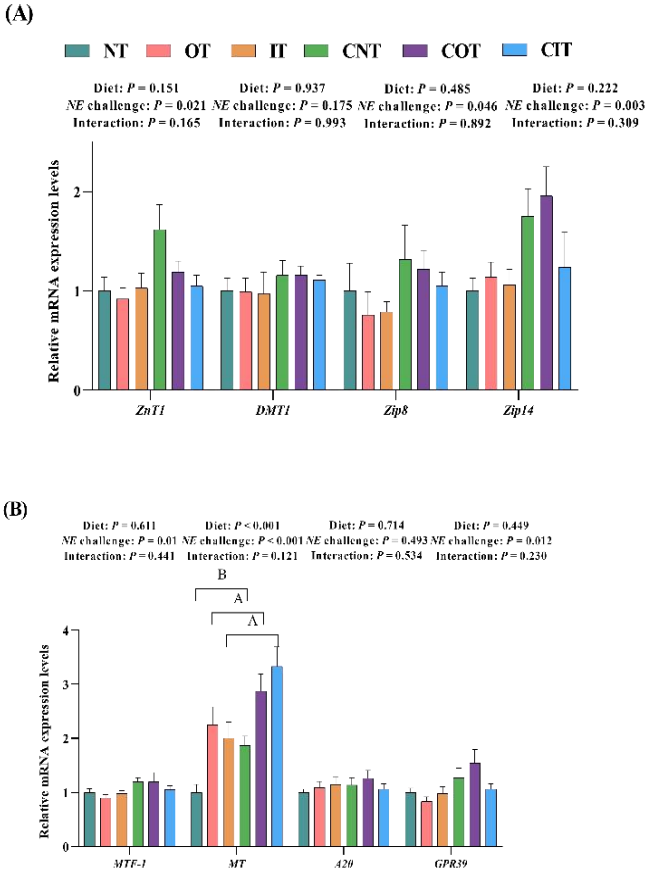

Figure 5-4: Effect of dietary zinc on the gene expression levels of zinc transporter (A) and zinc regulation-related genes (B) in the jejunum of necrotic enteritis challenged broilers. Means with no common superscripts differ significantly ($P < 0.05$), with lowercase letters indicating significantly different interactions and uppercase letters indicating significantly different dietary effects. *ZnT*: zinc transporter; *ZIP*: zinc-regulated transporter, iron-regulated transporter-like protein; *MTF-1*: metal transcription factor-1; *MT*: metallothionein; *GPR39*: G protein coupled receptor 39. The abbreviations of the treatment groups are the same as the previous ones.Figures 3B and 4B show the effect of different sources and doses of dietary zinc on zinc transport-regulated genes in the liver and jejunum under challenge. Zinc-related markers *MT*, *Cu,Zn-SOD* gene expression were also analyzed. The interaction effect showed that zinc

glycinate diet significantly increased the liver *Cu,Zn-SOD* gene expression (P = 0.05) under *NE* challenge compared to the zinc-free diet. The main effect showed that *NE* challenge up-regulated gene expression level of *A20* (P = 0.016) in the liver, with no significant effect of dietary factors (P > 0.05). For jejunum, significant interaction effects of dietary zinc and *NE* challenge on zinc transport regulation and zinc-zinc concentration markers were not observed in jejunum (P > 0.05). Gene expression levels of the metal regulated transcription factor *MTF1* (P = 0.01), *MT* (P < 0.001) and the G protein-coupled receptor 39 (*GPR39*) (P = 0.05) were significantly up-regulated under *NE* challenge, whereas the two additional zinc-added diets significantly increased gene expression levels of jejunal *MT* (P < 0.001).

4.8. Jejunal inflammation gene expression

As shown in Figure 5-5, the interaction between zinc and *NE* challenge significantly affected the gene expression levels of *IL-1β* in the jejunum, with zinc glycinate and zinc sulfate significantly down-regulating the gene expression level of *IL-1β* compared to the zinc-free diet under *NE* challenge. In the jejunum, gene expression levels of toll-like receptor 4 (*TLR4*) (P = 0.004), nuclear factor kappa-B p65 (*NF-κB p65*) (P = 0.001) and *TNF-α* (P = 0.003) were significantly up-regulated due to the challenge. Compared with the zinc-free diet, the zinc glycinate and zinc sulfate diets significantly lowered the gene expression levels of jejunal *TLR4* (P = 0.008) and *NF-κB p65* (P = 0.003), while the zinc sulfate diet significantly lowered the gene expression level of *TNF-α* (P = 0.013).

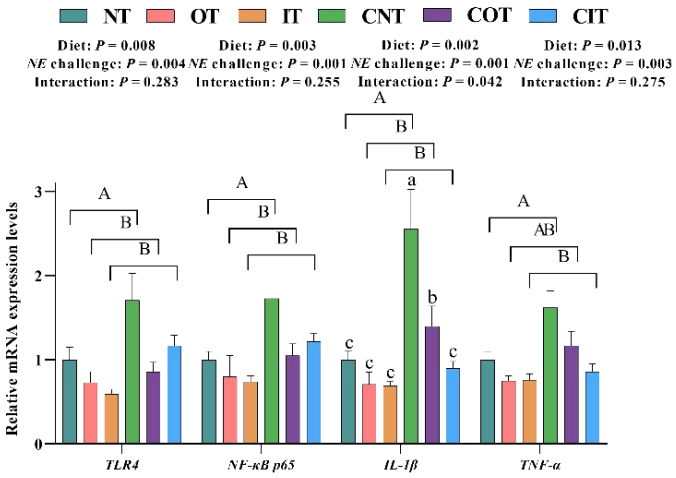

Figure 5- 5: Effect of dietary zinc on the gene expression levels of TLR4/NF-κB p65 pathway and TNF-α in the jejunum of necrotic enteritis challenged broilers. Means with no common superscripts differ significantly (P < 0.05), with lowercase letters indicating significantly different interactions and uppercase letters indicating significantly different dietary effects. *TLR4*: toll-like receptor 4; *NF-κB p65*: nuclear factor kappa-B p65; *IL-1β*: interleukin 1 beta; *TNF-α*: tumor necrosis factor alpha. The abbreviations of the treatment groups are the same as the previous ones.

5. Discussion

NE is a disease caused by *Clostridium perfringens* in the intestinal tract, which commonly occurs in broilers between 2-5 weeks of age (Gautam et al. 2024). Coccidia infection damages the integrity of the intestinal mucosa, causing plasma proteins to enter the intestinal tract, exacerbating the proliferation of *Clostridium perfringens* and releasing a variety of exotoxins, inducing systemic inflammation (Muneeb et al. 2024). In the field of poultry research, different challenges and dietary factors are used for the creation of *NE* challenge models. Intestinal lesion scoring and intestinal barrier function are often used to assess the usability of the model (Fathima et al. 2022). A study by Ningsih et al. (2023) has shown that *NE* leads to intestinal lesions, increases mortality in broilers and results in decreased growth performance (Ningsih et al. 2023). The higher abundance of *Clostridium perfringens* determined in the cecal contents from *NE* challenged broilers is also a marker of *NE* modeling, which has been presented by a recent work from our lab (Song et al. 2023). The clinical form of *NE* leads to an acute infection and results in symptoms such as depression, dehydration, diarrhea, and feather disarray, and presenting with reduced feed intake and high mortality rate, while most of diseased broilers exhibit the sub-clinical form (Shamshirgaran et al. 2024). The sub-clinical form of *NE* causes productivity inhibition and does not lead to higher mortality, but it is often accompanied by a reduction in FCR (Moore 2024). A study has confirmed that *NE* challenge may reduce the body weight and FCR of broilers, but has no significant effect on mortality (Xue et al. 2018). Another study shows that the body weight and mortality of challenged broilers are not significantly different from the control group after the *NE* model was performed two weeks, but FCR showed significant difference (Keerqin et al. 2017). These evidences suggest that the increased mortality is not a marker for judging the successful establishment of the *NE* challenge model, and the decrease in productive performance may be caused by intestinal damage and infections due to sub-clinical form of *NE* challenge. In our study, the negative effect of *NE* challenge on intestinal lesion scores and FCR suggests successful modeling of a sub-clinical *NE* challenge and a negative effect of *NE* on growth performance. Zinc is added to the diet to meet the nutritional requirements of broilers and to improve immune function and intestinal health. A study by Liu et al. (2011) shows that 60 mg/kg zinc supplementation resulted in an increase in daily feed intake and daily weight gain, while amino acid chelated zinc showed better bioavailability (Liu et al. 2011). *NE* challenge may alter the nutrient absorption in the intestine, requiring the application of nutritional strategies to ameliorate the immune challenges and intestinal damage. Owing to the positive effects of zinc on intestinal immunity and epithelial cell growth, it has been shown that zinc supplementation could inhibit the elevation of inflammatory markers and thereby improve intestinal inflammation and growth performance decline due to *NE* (Bortoluzzi et al. 2019b). In this study, FCR in 21-day-old broilers was decreased by zinc glycinate, but none of the different sources of zinc had a beneficial effect on the growth performance of broilers (22-35days) after challenge, which is the identical result of a study by He et al. (2019).

Zinc regulates cell-mediated immune function as a messenger for immune cells, while cytokine levels in the intestine and blood are markers of inflammation or infection (Kim et al. 2021). Immunoglobulins mediate the specific immunity of animals against harmful substances entering the system and IgA and IgY are the main immunoglobulins for birds that play a key role in B-cell-mediated adaptive immunity (Wlaźlak et al. 2023). To assess the effect of zinc on immune function in *NE*-infected broilers, we examined levels of cytokines and immunoglobulins in the jejunum and serum. Jejunal and serum pro-inflammatory factor levels were up-regulated in response to *NE* and IgA levels were down-regulated, suggesting that the infection resulted in inflammatory infections. In the present study, both sources of zinc alleviated the inflammatory response by decreasing the level of the pro-inflammatory factor IL-1β. In addition, zinc glycinate and zinc sulfate significantly increased jejunal IgA levels under *NE* challenge, indicating that zinc enhanced *NE* challenge-induced inflammatory response. These trends of *NE* challenge-induced changes in inflammatory factor levels and immunoglobulin levels are identical to those of our past research (Song et al. 2023). The TLR/NF-κB signaling pathway is a key signaling pathway in the innate inflammatory response (Feng et al. 2023). TLR is widely involved in the recognition of pathogen-associated molecular patterns (PAMPS) that initiate natural immunity and host defense system (Sahoo 2020). After recognizing pathogens, TLR releases cellular signals that activate NF-κB signaling to produce a range of pro-inflammatory cytokines that modulate the inflammatory response. In this study, *NE*-infected broilers increased gene expression levels of jejunal *TLR4* and *NF-κB* and promoted the downstream pro-inflammatory factor gene *TNF-α*. In addition, both sources of zinc were able to inhibit gene expression levels of a range of key factors in the pathway, indicating that zinc inhibited the inflammatory response induced by *NE* challenge.

Upon inflammatory stimulation of tissue cells, the NADPH oxidase system is triggered to produce excess reactive oxygen species (ROS), which activates a complex antioxidant enzyme self-defense system (Chandel 2021). ROS causes lipid oxidation, damages cell membrane structure and induces MDA production (Khan et al. 2023). CAT is the key endogenous antioxidant enzyme that converts hydrogen peroxide into water and oxygen to avoid oxidative damage. The index of T-AOC is the sum of antioxidant substances and antioxidant enzymes and represents the antioxidant capacity of an animal. In addition, the main function of GSH-px is to scavenge lipid hydroperoxides and hydrogen peroxide and to protect cell membranes by catalyzing reduced glutathione (Xie et al. 2022). *NE* challenge increased the intestinal and serum concentrations of MDA and decreased the concentrations of antioxidant substances GSH-px and CAT, whereas zinc application increased the intestinal and serum antioxidant concentrations. These results suggest that the *NE* challenge induced oxidative stress, which is consistent with previous finding in birds (Song et al. 2023). Zinc participates in catalytic reactions to promote antioxidant processes through the involvement of biomolecules that make up enzymes. In the current study, both dietary zinc glycinate and zinc sulfate significantly reduced jejunal MDA concentration under challenge, showing that zinc alleviated *NE* challenge-induced intestinal oxidative stress.

The intestinal barrier function is an important factor in preventing the invasion of pathogens, and intestinal permeability measures intestinal barrier function (Khoshbin et al. 2020). Toxins and pathogens will cross the disrupted intestinal barrier, leading to a systemic immune response (Kayama et al. 2020). Gut permeability increases after the gut barrier is disrupted. FITC-d molecules in the intestine enter the blood circulation through the disrupted intestinal epithelium (Liu et al. 2021). The concentration of FITC-d in serum after oral administration of FITC-d is commonly used to assess the level of intestinal barrier dysfunction and intestinal mucosal damage. We found that *NE* infection resulted in increased serum FITC-d concentration, and dietary zinc was found to decrease serum FITC-d concentration under *NE* challenge. It was demonstrated that the *NE* challenge disrupted the intestinal barrier in broilers, leading to an increase in intestinal permeability. Dietary zinc thus mitigated challenge-induced intestinal damage. The intestinal lesion score is often used as an assessment of intestinal health (Keerqin et al. 2017). *NE* induces acute inflammation in the intestinal tract leading to intestinal epithelial damage resulting in intestinal bleeding and intestinal thinning. We found an increase in the intestinal lesion score by *NE* and a protective effect of zinc glycinate. Recent research has shown that organic zinc improves intestinal health in *Clostridium perfringens*-infected birds by reducing intestinal permeability, which is similar to our results (Bortoluzzi et al. 2019a). Intestinal permeability is affected by tight junction proteins and function, and *Clostridium perfringens* was shown to affect the intestinal barrier by binding to tight junction proteins (Zhang et al. 2023). In our study, the gene expression of tight junction proteins was altered in the infected intestines, as evidenced by a down-regulated gene expression level of Occludin. In contrast, the zinc glycinate increased the expression levels of jejunal Occludin and Claudin1, suggesting a positive effect of organic zinc on the intestinal barrier.

The intestine is not only the organ for nutrient absorption, but also serves an important immunomodulatory function. According to our past work, zinc transport in the broiler intestine showed specific adjustments during inflammation and stress (Xiao et al. 2024). The liver is an important metabolic organ for zinc and both the liver and the intestine play a key role in zinc homeostasis. Inflammatory responses in tissues are accompanied by intense ionic metabolism, clinical findings on humans have shown that diseases such as respiratory inflammation and acute nephritis cause hypozincaemia due to coordinated ectopic translocation of zinc ions from the blood to damaged tissues to increase intracellular free zinc ions to counteract inflammation (Arleth et al. 2020). *NE* similarly resulted in lower zinc level in broiler serum, predicting hypozincaemia due to inflammation. Notably, infection led to an up-regulation of zinc concentration in the liver, suggesting that inflammation accelerates the mobilization of zinc in the liver, which is the same as past result in broilers (Bortoluzzi et al. 2019b). A decrease in zinc content in chicken meat and tibia was also observed, a phenomenon attributed to the depletion of zinc during the resistance to infection and the body's reduction of zinc content in tissues in the face of challenging conditions to avoid zinc utilization by pathogens (Troche et al. 2015). We observed a significant effect of specifically zinc glycinate compared to basal diet, particularly in terms of lower zinc residuals in gut contents and a

significant increase of zinc concentration in tissues. However, we did not observe a significantly better effect of zinc sulfate compared to the basal diet in most tissues, suggesting a higher biological availability of zinc in the amino acid chelated form.

Zinc is absorbed into intestinal cells at the apical membrane of the small intestine and released at the intestinal basement membrane, where the zinc binds to albumin in plasma and enters the circulatory system (Hunt et al. 2008). Zinc transporter proteins and MT are intracellular transporters and storage tools for zinc ions, keeping the normal range of intracellular zinc concentration by maintaining zinc homeostasis (Hu 2021). An MT molecule is able to bind seven zinc atoms, and due to the abundant thiol groups, MT has the function of intracellular zinc concentration regulation (Coyle et al. 2002). We found that the *NE* challenge resulted in upregulation of the expression of the zinc export gene *ZnT1* and the zinc import genes *ZiP8* and *Zip14* in the jejunum, demonstrating that the challenge increased intestinal zinc absorption. The upregulation of intestinal MT might indicate an increase in free zinc concentration in cells. Zinc glycinate up-regulated the gene expression level of *ZnT1* in the liver under *NE* challenge, highlighting the rapid transport of zinc by the liver in the challenged state and the advantage of glycine. In addition, zinc supplementation did not cause significant alteration of zinc transporter protein genes in the jejunum, perhaps owing to the longer duration of the challenge model and the faster metabolic rate of zinc as a Type2 nutrient (Petroni et al. 2021).

Zinc homeostasis is mediated by complicated signaling, with factors such as diet, inflammation and oxidative stress affecting zinc concentration regulation. MTF-1 is involved in zinc metabolism by receiving zinc concentration signals and controlling the expression of metal transport-related proteins such as MT (Hubner et al. 2021). Evidence has demonstrated that oxidative stress activates MTF-1 and facilitates zinc transport (Marreiro et al. 2017). Zinc supplementation activates zinc finger protein A20 (TNFAIP3) and inhibits the production of pro-inflammatory factors (Schwartz et al. 2020). The zinc import protein ZiP8 is associated with the concentration of TNF-α, and upstream NF-κB signaling is able to directly lead to the activation of this zinc import protein (Liu et al. 2013). GPR39 is a zinc-sensing receptor expressed on the apical membrane of intestinal epithelial cells, and zinc concentration regulates the expression of GPR39, and GPR39 activation also enhances the expression of intestinal barrier proteins (Pongkorpsakol et al. 2019). Zinc is also directly involved in antioxidant enzyme resistance to oxidative stress through its participation in the composition of Cu,Zn-SOD (Wang et al. 2023). In addition, the ALP, which is capable of degrading endotoxins, also contains two zinc atoms. The results of the present study revealed that *NE* challenge caused up-regulation of jejunal *MTF-1* and *GPR39* gene expression, which may be associated with increased intestinal absorption of zinc, and also induced elevated Cu,Zn-SOD and ALP concentrations. High zinc concentration in the liver in response to *NE* challenge up-regulated the level of *A20*. Moreover, zinc glycinate enhanced Cu,Zn-SOD activity after *NE* challenge, predicting that zinc is mobilized to participate in the antioxidant defense mechanism of the organism.

6. Conclusion

Overall, *NE* challenge induced by *Clostridium perfringens* and coccidia caused inflammatory damage and oxidative stress, disrupting the intestinal barrier function and increasing the FCR of broilers. Dietary zinc supplementation could alleviate *NE* challenge-induced oxidative stress and intestinal inflammation. Zinc glycinate showed better improvement in FCR and intestinal barrier function in broilers compared to zinc sulfate, probably due to its higher biological availability. Zinc transporter proteins and MT were involved in the process of remodeling zinc homeostasis in response to an *NE* challenge.

Chapter VI

Zinc improves meat quality by modulating lipid metabolism in necrotic enteritis challenged broilers

Chapter VI. Zinc improves meat quality by modulating lipid metabolism in necrotic enteritis challenged broilers

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1. Abstract

The objective of this study was to investigate the effect of dietary zinc from different sources on meat quality and lipid metabolism in broilers challenged with necrotic enteritis (*NE*). 432 1-day-old commercial Arbor Acres (AA) male broilers were randomly divided into six treatments with six replicates of 12 chicks each according to a two-factor design of dietary zinc (basal diet, 60 mg/kg zinc glycinate and 60 mg/kg zinc sulfate) * *NE* challenge (control or *NE*). The *NE* challenge was conducted from day 23 of age and the experiment lasted for 40 days. The results showed that zinc glycinate increased leg muscle proportion, and also improved carcass proportions under *NE* challenge. In contrast, a zinc-free diet led to an increase in bursal index under *NE* challenge. Both sources of zinc had an improved effect on meat quality under *NE* challenge, with zinc sulfate significantly reducing pectoral muscle cooking loss and zinc glycinate increasing pectoral muscle shear force. The main effect showed that both sources of zinc significantly down-regulated the yellowness b* of the pectoral muscle compared to the zinc-free treatment. The *NE* challenge increased the pH of pectoral muscle 45 min and 24 h after slaughter. These results suggest a negative effect of *NE* challenge and a beneficial effect of dietary zinc on meat quality. The *NE* challenge resulted in an upregulation of thiobarbituric acid reactive substances (TBARS) of pectoral muscle after 24 hours storage, and both sources of zinc significantly decreased TBARS after five days storage. The non-targeted metabolomic result showed that zinc glycinate affected fatty acid biosynthesis pathways, and the *NE* challenge also up-regulated a fatty acid synthesis pathway, down-regulated a fatty acid oxidation pathway and significantly affected unsaturated fatty acid biosynthesis pathways. The expression of liver fatty acid bio-oxidation genes peroxisome proliferator activated receptor-alpha (*PPAR-α*) and acyl CoA oxidase 1 (*ACOX1*) were down-regulated, and the expression of the synthesis gene sterol regulatory element-binding protein 1c (*SREBP-1C*) was up-regulated in response to the *NE* challenge. The liver fatty acid synthesis gene acetyl coenzyme A hydroxylase (*ACC*) was significantly down-regulated by both sources of zinc. These modulations may have caused a significant increase in broiler serum total cholesterol (TCHO) and low-density lipoprotein (LDL) levels due to the *NE* challenge, and zinc glycinate and zinc sulfate lowered serum glucose (GLU) level. These results confirm the negative effects of the *NE* challenge on meat quality in broilers, with *NE* exacerbating lipid peroxidation and affecting fatty acid composition in the pectoral muscle. Moreover, zinc glycinate raised the proportion of polyunsaturated fatty acids in the pectoral muscle, increasing the content of C20:2 (eicosadienoic acid) compared to the zinc-free treatment and C20:3n3 (dihomo-α-linolenic acid) compared to the zinc sulfate treatment. We conclude that both the *NE* challenge and zinc supplementation affect meat quality by influencing lipid metabolism in broilers to modulate lipid peroxidation and fatty acid composition.

2. Introduction

Clostridium perfringens-induced necrotic enteritis (*NE*) is a common intestinal

disease in broilers and infection with one or more species of coccidia is often the causative agent and exacerbates the clinical features of *NE* (Moore 2024). The pathogenic mechanism of *Clostridium perfringens* is that the pathogen proliferates in the gut and releases a variety of exotoxins (Timbermont et al. 2011). Coccidia infections disrupt the integrity of the intestinal mucosa and the intestinal microbiota balance, leading to increased intestinal permeability along with toxins entering the bloodstream (Daneshmand et al. 2022, Fathima et al. 2022). The ban on antibiotics in feed has caused resistance issues that have limited the use of anti-coccidia drugs, leading to a steady increase in *NE* incidences worldwide, and resulting in huge economic losses (Broom 2017).

Chicken meat is one of the most consumed meat products in the world (Maharjan et al. 2021). Highly heritable rapid growth genetic traits and the intensive industrial broiler production system have brought an increase in chicken meat production, but this has also led to a decline in meat quality and carcass characteristics (Nasr et al. 2021). Stocking densities, farming styles, and nutritional factors have an impact on meat quality and carcass characteristics (Marchewka et al. 2023). Benefiting from the key role minerals play in an animal's metabolic processes, zinc is added to the feed for the promotion of animal growth performance and health status (Philippi et al. 2023). Zinc can affect fatty acid synthesis and lipid metabolism by directly regulating the activity of enzymes involved in fatty acid metabolism such as fatty acid synthase (FAS), acetyl coenzyme A hydroxylase (AAC) and stearoyl coenzyme A desaturase-1 (SCD-1) (He et al. 2023). Zinc deficiency indirectly affects muscle growth by hindering the nucleic acid synthesis process involved in amino acid metabolism (Bogdanis et al. 2023). Zinc has been reported to improve meat quality by reducing drip loss and improving lipid oxidation in broilers and reducing the proportion of woody, PSE meat (De Grande et al. 2022). Amino acid chelated zinc is considered a more bioavailable source of zinc than inorganic zinc sources due to its resistance to phytic acid in the digestive tract and better performance in improving meat quality (Stiles et al. 2024).

The liver is the major organ for lipid synthesis and metabolism in birds, and nutritional and disease factors may lead to disorders of lipid metabolism (Calik et al. 2024). The occurrence of *NE* disrupts the intestinal barrier and causes intestinal inflammation along with systemic acute infections in broilers, resulting in clinical disorders such as acute splenitis and abnormalities in liver function (Shah et al. 2023). It has been observed that an *NE* challenge influences lipid metabolic processes in broilers by regulating fatty acid synthesis and metabolic processes in the liver (Zhou et al. 2016). Zinc has been proven to mitigate the negative effects of *NE* by modulating the immune function and microbial composition of the intestine (Bortoluzzi et al. 2019). There are numerous studies on the effects of zinc on meat quality and the alleviation of *NE* by zinc, but research on the changes in meat quality characteristics of broiler chickens under *NE* challenge and the modulatory effects of zinc is still lacking. This study aimed to investigate the question of whether exogenous zinc intake could affect chicken meat quality by altering lipid metabolism in the *NE* challenged state.

3. Materials and methods

All experimental procedures were approved by the Ethics Committee of Shandong Agricultural University (No. SDAUA-2022-50) and conducted in accordance with the Guide for Laboratory Animals of the Ministry of Science and Technology (Beijing, People's Republic of China).

3.1. Animals and management

A total of 432 1-day-old Arbor Acres (AA) male chicks of similar body weight were randomly divided into three dietary treatments: NT group (basal diet), OT group (basal diet with 60 mg/kg zinc glycinate), IT group (basal diet with 60 mg/kg zinc sulfate), with 12 replicates of 12 broilers per treatment. Broilers in each dietary treatment group were evenly divided into challenge and non-challenge groups, ensuring that the initial body weights within each dietary treatment were closely matched at 21 days of age. Starting at 21 days of age, there were six replicates for each treatment group. The experiment followed a two-factor design of dietary zinc × *NE* challenge, that is, NT (negative feed treatment, chicken fed with basal diet), OT (organic zinc treatment, chicken fed with basal diet containing 60 mg/kg zinc glycinate), IT (inorganic zinc treatment, chicken fed with basal diet containing 60 mg/kg zinc sulfate), CNT (negative feed treatment with *NE* challenge, chicken fed with basal diet and challenged with *NE*), COT (organic zinc treatment with *NE* challenge, chicken fed with basal diet containing 60 mg/kg zinc glycinate and challenged with *NE*) and CIT (inorganic zinc treatment with *NE* challenge, chicken fed with basal diet containing 60 mg/kg zinc sulfate and challenged with *NE*). The entire experimental period lasted for 40 days, and the birds were kept in cages with the size of 70 cm × 70 cm × 40 cm with feed and water provided ad libitum. Crushed pellets were fed for the first 21 days of the experiment and pellets were fed from the 22nd day on. The basal diet (Table 6-1) and zinc additives were in accordance with the recommendations of the “Compound Feed for Egg Laying Chickens and Broilers” (GB/T 5916-2020) in the People's Republic of China. The zinc levels of the diets formulated according to the requirements of the different experimental treatments were examined with ICP-MS (Table 6-2). During feed formulation, additional glycine was added to the other 4 groups to the same level as the zinc glycinate group to balance the amino acids.

Table 6-1: Composition and nutritional levels of the basal diet（air-dry basis）

Item	d 1-21	d 22-40
Ingredient (%)		
Corn	49.98	54.90
Soybean meal (46%)	35.75	30.50
Corn protein flour (60%)	3.80	3.10
Salt	0.28	0.28
Limestone	1.75	1.62
Dicalcium phosphate	1.55	1.40

Soybean oil	5.10	6.50
Vitamin premix	0.05	0.05
Mineral premix	0.20	0.20
Choline chloride (50%)	0.10	0.10
Methionine (99%)	0.35	0.35
Lysine (70%)	0.80	0.75
Threonine (98.5%)	0.29	0.25
Phytase (20000 U)	0.02	0.02
Total	100	100
Nutritional level		
Metabolizable energy	3100 (kcal/kg)	3200 (kcal/kg)
Crude protein	23.50	21.00
Lysine	1.39	1.20
Methionine+cystine	1.02	0.92
Calcium	1.00	0.90
Total phosphorus	0.99	0.90
Available phosphorus	0.50	0.45

Provided per kilogram of compound diet: vitamin A, 12000 IU; vitamin D3, 5000 IU; vitamin E, 80 mg; vitamin K, 3.2 mg; vitamin B1, 3.2 mg; vitamin B2, 8.6 mg; nicotinic acid, 65 mg; pantothenic acid, 20 mg; vitamin B6, 4.3 mg; biotin, 0.22 mg; folic acid, 2.2 mg; vitamin B12, 0.017 mg; I, 1.50 mg; Fe, 80 mg; Mn, 120 mg; Se, 0.3 mg; Cu, 16 mg. The nutrition level was calculated.

Broilers were reared in an automated environmentally controlled house where the room temperature was raised to 32 °C 48 hours before the arrival of the chicks, and the house temperature was gradually lowered over the course of the experiment until it reached 24 °C at 21 days of age and then this temperature was maintained. Humidity was kept at an average of 70% for the first three days of the trial and 55% to 65% thereafter. According to the Arbor Acres broiler management handbook (2018), the birds are exposed to 23 hours of light and 1 hour of darkness for the first week of life, which changes to 20 hours of light and 4 hours of darkness beginning on the seventh day. To ensure the validity of the experiment, no antibiotics and vaccines were used during the entire period except the coccidiostat vaccine used in the establishment of the *NE* model.

Table 6-2: Analyzed zinc concentrations in the diet

Treatment	**Zinc concentration** (mg/kg) (d 1 to 21)	**Zinc concentration** (mg/kg) (d 22 to 40)
NT/ CNT	28.95	29.54
IT/ CIT	91.25	89.88
OT/ COT	90.65	90.32

NT: negative feed treatment, chicken fed with basal diet; OT: zinc glycinate feed treatment, chicken fed with basal diet containing 60 mg/kg zinc glycinate; IT: zinc sulfate

feed treatment, chicken fed with basal diet containing 60 mg/kg zinc sulfate; CNT: negative feed treatment with necrotic enteritis challenge, chicken fed with basal diet and challenged with necrotic enteritis; COT: zinc glycinate feed treatment with necrotic enteritis challenge, chicken fed with basal diet containing 60 mg/kg zinc glycinate and challenged with necrotic enteritis; CIT: zinc sulfate feed treatment with necrotic enteritis challenge, chicken fed with basal diet containing 60 mg/kg zinc sulfate and challenged with necrotic enteritis.

3.2. Establishment of the NE model

Referring to past study (Guaragni et al. 2020), the *NE* model was performed starting from day 23 of age by administering coccidiostat vaccine (containing live attenuated oocysts of *Eimeria tenella* PTMZ strain, *E. necatrix* PNHZ strain, *E. maxima* PMHY strain, and *E. acervulina* PAHY strain; Foshan Standard Bio-Tech Co., Ltd., Foshan, China) of 30 times of normal immunological requirement orally to each broiler in the challenge group on day 23 and by instilling 1 mL of broth culture containing *Clostridium perfringens* (1×10^9 CFU/mL) daily from day 26 to day 32. The strain was purchased from China Veterinary Culture Collection Center (Beijing, China), which was isolated from the intestine of broilers clinically diagnosed with *NE* and identified as NetB toxin-positive strain A, No. CVCC2030 (Du et al. 2021). 1 mL of the strain was incubated in 250 mL of thioglycolate broth medium in an anaerobic cabinet at 37 °C for 24 h. Then 1 mL of the culture was inoculated into 1 L of thioglycolate broth medium supplemented with 10 g/L starch and 15 g/L peptone for 24 h. The broilers were fasted for 8 hours prior to the oral supplementation. Broilers in the non-challenged groups were orally administered with PBS or broth culture to balance the stress response caused by catching and supplementation.

3.3. Sample collection

Sample collection was performed at 40 days of age. Blood samples were collected from the wing vein using a sterile syringe from two randomly selected chickens in each replicate after 8 hours of fasting. The blood samples were placed in glass tubes without anticoagulant for 30 min, centrifuged at 3000 rpm for 10 min at 4 °C, and serum was separated and stored in a -20 °C freezer. After blood collection, chickens were euthanized by cervical dislocation for sample collection: pectoral muscle and liver samples were collected and stored at -80 °C for molecular and biochemical indexes.

3.4. Carcass characteristics

At 40 days of age, broilers were weighed and slaughtered, and birds were soaked in hot water at 60 °C for 60 s to facilitate feather stripping. The weight of liver, heart, spleen and bursa were weighed and recorded, abdominal fat, pectoral and leg muscles from the same side were weighed after being removed completely. Ratios or indexes were expressed as percentage of the body weight before slaughter.

3.5. Meat quality

Pectoral muscle samples kept at 4 °C for 24 h were used for meat quality

analysis. The pH values were determined with a meat pH meter (Star, Matthaus, Germany). pH was measured at three similar locations for each sample and the mean value was taken as the final value for that sample. For 45 min after broilers were slaughtered, the measured value was recorded as pH45min; for 24 h after slaughter, the value was recorded as pH24h. Meat color measurements were performed at three positions on the same side with a colorimeter (CR-10 PLUS, Konica Minota, Japan), and the results were expressed as luminance (L*), redness (a*), and yellowness (b*) values. The hue angle (Hue) was calculated according to arctan (b*/a*) and expressed as h*, the color saturation Chroma (C*) = $((a^*)^2 + (b^*)^2)^{0.5}$, and the value was taken as the average of the measurements. Approximately 5 g of the sample was weighed and hung in an air-filled plastic bag at 4 °C for 24 h. The meat was weighed to measure the drip loss value after drying the surface of meat with filter paper. 5-10 g meat pieces from each breast muscle were obtained and the meat samples were heated in a thermostatic water bath at 80 °C with the samples completely submerged in the water and a thermometer was inserted into the center of the meat samples, heated until the center of the meat samples reached a temperature of 70 °C and removed and placed in a refrigerator at 4 °C for 12 hours then weighed and recorded. Percentages were applied as values for the assessment of cooking losses. After the cooking loss determination, shear force was examined with reference to our previous work (Wang et al. 2024). Chicken meat samples were cut into six uniform pieces in terms of size and thickness. Shear force was measured perpendicular to the muscle fibers using a Texture Analyser model TA-XT2i (Stable Micro Systems, UK). The final shear force value was calculated as the average of the six measurements. The parameters were set to a pre-test speed of 2.0 mm/s, a test speed of 1.0 mm/s, and a post-test speed of 5.0 mm/s over a distance of 23.0 mm. The peak shear force was recorded as Warner-Bratzler Shear Force (WBSF) when cutting through the core.

3.6. Lipid peroxidation in meat

Lipid peroxidation of meat was assessed by thiobarbituric acid reactive substances (TBARS) as described by (Valentini et al. 2020). Samples of 1 g meat were obtained from several randomly selected locations on pectoral muscles after 24 h of storage in a freezer at 4 °C and after 5 days of storage, chopped and added to 4 mL of purified water and homogenized thoroughly, then 4 mL of 10% trichloroacetic acid was added and mixed. After being filtered, 1 mL filtrate was mixed with 0.25 mL of the thiobarbituric acid solution in a water bath at 80 °C for 90 min. The samples were measured by a spectrophotometer at 532 nm after cooling to room temperature. The standard curve was plotted with 1,1,3,3-tetramethoxypropane (TMP) as the standard of TBARS, and the value of TBARS was calculated.

3.7. Serum and liver biochemical indicators

The serum concentrations of glucose (GLU), total cholesterol (TC), triglycerides (TG), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) were analyzed with an automatic biochemistry analyzer (7020, Hitachi, Tokyo, Japan). TC and TG levels in liver tissue were measured using kits

provided by the Nanjing Jiancheng Biotechnology Institute (Nanjing, China). The procedure was performed strictly according to the manufacturer's instructions. The intra-batch coefficient of variation (CV) of the data did not exceed 5% and the inter-batch CV did not exceed 8%.

3.8. Metabolites profile analysis of liquid chromatography mass spectrometer (LC-MS)

Metabolites were extracted from meat samples of the treatments (NT, OT, CNT, COT) and detected by LC-MS. An accurate 0.2 g of meat sample was obtained and 1 mL of tissue extract (methanol and chloroform were mixed in a ratio of 9:1 and 25% water was mixed thoroughly again) was added. The samples were thoroughly ground and then sonicated for 30 min, ice-water bath for 30 min, and then centrifuged at 12000 rpm for 10 min at 4 °C. The supernatant was carefully obtained and concentrated and dried. Add 0.2 mL of 50% acetonitrile solution, filter and store in a special vial for mass spectrometry analysis. An aliquot of each of the samples to be tested was mixed into a quality control (QC) sample for monitoring the instrument and the confidence of the data.

Chromatography was carried out with an ACQUITY UPLC ® HSS T3 (Waters, Milford, MA, USA) using a Vanquish UHPLC System (Thermo Fisher Scientific, USA) for LC analysis. Specific parameters were 2.1 mm × 100 mm × 1.8 μm. 2 μL of solution was injected from the detection vial into the sampler at 40 °C, maintaining a flow rate of 0.3 mL/min. The mobile phases for the LC-ESI (+)-MS analysis consisted of 0.1% formic acid acetonitrile (B2) and 0.1% formic acid (A2). Separations were performed at the following gradients: 0-1 min, 8% B2; 1-8 min, 8%-98% B2; 8-10 min, 98% B2; 8-10 min, 98% B2; 10-10.1 min, 98%-8% B2; 10.1-12 min, 8% B2. LC-ESI (-)-MS analysis was performed using acetonitrile (B3) and 5 mM ammonium formate (A3). The separation gradient was as follows: 0-1 min, 8% B3; 1-8 min, 8%-98% B3; 8-10 min, 98% B3; 8-10 min, 98% B3; 10-10.1 min, 98%-8% B3; 10.1-12 min, 8% B3.

Mass spectrometric detection of metabolites was performed by Orbitrap Exploris 120 (Thermo Fisher Scientific, USA). An ESI ion source was used, with positive and negative ion modes for data acquisition. The conditions of the ion source were set as follows: sheath gas pressure (40 arb), aux gas flow (10 arb), spray voltage at 3.50 kV and -2.50 kV for ESI (+) and ESI (-), respectively, capillary temperature (325°C), MS1 range (m/z 100-1000), MS1 resolving mode (m/z 100-1000), and MS1 resolving mode (m/z 100-1000. z 100-1000), MS1 resolving power (60000 FWHM), 4 times of data dependant scans per cycle, MS/MS resolving power (15000 FWHM), normalized collision energy (30%), dynamic exclusion time (automatic).

3.9. Fatty acid profile detection with gas chromatography mass spectroscopy (GC-MS)

The composition of fatty acids in chicken meat from all treatments was determined by GC-MS and analyzed by fatty acid detection with a triple quadrupole gas chromatograph. A mixed standard solution of thirty-seven fatty acid methyl esters (CRM47885, Sigma-Aldrich, USA) was chosen for the

calibration curve to determine the fatty acid species in the samples. The sample was grinded by liquid nitrogen in a cryomill, 1g (accurate to 0.001) of the dehydrated samples was weighed in a stoppered tube, and 0.7 mL of potassium hydroxide solution (10 mol/L) and 5.3 mL of chromatographic methanol were added, and then the sample was heated at 55 ℃ for 90 min, and the samples were shaken and mixed every 20 min. After the heating process was completed and cooled to room temperature, 600 μL of concentrated sulfuric acid (12 mol/L) was added and mixed, followed by heating at 55°C for 90 min, and the sample was also shaken and mixed every 20 min. After cooling, 3 mL of n-hexane and 1 mL of saturated sodium chloride solution were added to the reaction solution for extraction and rinsing, vortexed for 5 min to mix thoroughly, and then centrifuged at 2600 g for 5 min at room temperature. The supernatant was aspirated, filtered through 0.45 μm organic phase filter membrane and placed into 2 mL of sample, and then tightened the cap of the vials and stored at -20℃ until determination.

GC-MS (TQ8030, SHIMADZU, Japan) was set up according to the following conditions: Agilent HP-innowax capillary column (30 mm × 0.25 mm × 0.25 mm); high purity helium (carrier gas) column flow rate of 1 mL/min; temperature of the inlet port of the sample port of 260 ℃; injection volume of 1 μL; and set the shunt ratio of 25: 1. Temperature rising procedure: initial temperature 50 ℃, hold for 1min, increase temperature to 100 ℃ at 25 ℃ / min, hold for 2 min, then increase temperature to 250 ℃ at 4 ℃ / min, hold for 10 min.

3.10. Fatty acid synthesis and metabolic gene expression in the liver

Total RNA from the liver was extracted with Trizol reagent (Invitrogen, San Diego, USA). The concentration and purity of each RNA sample was measured using a NanoDrop spectrophotometer (ND-2000, Thermo Scientific, Wilmington, USA). 1% agarose gel electrophoresis was used to detect RNA integrity. Reverse transcription of 1 μg of total RNA was performed using PrimeScript® RT reagent kit (RR047A, TaKaRa, Japan). RT-PCR was performed using TB Green PreMix Ex Taq (RR820A, TaKaRa, Japan) in an ABI 7500 Real-Time PCR Detection System (Life Technologies) to determine relative gene expression.

Table 6-3: Primer sequences used for real-time quantitative PCR

Gene	Accession Number	Primer sequence, 5′→ 3′	Reference
FAS	J03860	CTATCGACACAGCCTGCTCCT CAGAATGTTGACCCCTCCTACC	Zhou et al. 2016
ACC	NM_205505	AATGGCAGCTTTGGAGGTGT TCTGTTTGGGTGGGAGGTG	
SREBP-1C	AY029224	GAGGAAGGCCATCGAGTACA GGAAGACAAAGGCACAGAGG	
CPT-1	AY675193	CAATGAGGTACTCCCTGAAA CATTATTGGTCCACGCCCTC	
PPAR-α	AF163809	TGGACGAATGCCAAGGTC	

ACOX1	NM_001006205	GATTTCCTGCAGTAAAGGGTG ATGTCACGTTCACCCCATCC	Kong et al. 2023
β-actin	L08165	AGGTAGGAGACCATGCCAGT CAGACATCAGGGTGTGATGG	
GADPH	NM_204305.1	TCAGGGGCTACTCTCAGCTC GCCCAGAACATCATCCCA	
		CGGCAGGTCAGGTCAACA	

FAS: fatty acid synthase; ACC: acetyl-CoA carboxylase; SREBP-1C: sterol regulatory element binding protein 1c; CPT-1: carnitine palmitoyl transferase 1; PPAR-α: peroxisome proliferator activated receptor alpha; ACOX1: acyl CoA oxidase 1; GADPH: glyceraldehyde-3-phosphate dehydrogenase.

The reaction procedure consisted of pre-denaturation at 95 °C for 10 s, denaturation at 95 °C for 5 s for a total of 40 cycles, and finally an annealing extension at 60 °C for 34 s. Primer sequences are demonstrated in Table 6-3. *β-actin* and glyceraldehyde-3-phosphate dehydrogenase (*GADPH*) were used as housekeeping genes and the mean value of the housekeeping genes was used to normalize the expression of the target genes. The relative expression levels of each target gene were analyzed by the $2^{-\Delta\Delta ct}$ method.

3.11. Data Analysis

Data apart from metabolite profile analyses were expressed as mean and total standard error of the mean (SEM), and data were counted and samples were analyzed in replicates. Data were analyzed using the Shapiro-Wilk test (95% confidence interval) performing by the software SPSS 22.0 (SPSS. Inc., Chicago, USA) followed by a two-way ANOVA with general linear model, and Tukey's post-hoc test was applied to determine differences between groups. Data were considered statistically significantly different when $P < 0.05$.

The original mass spectrometry downlink files were converted to mz-XML file format by the MS Convert tool in Proteowizard package (v3.0.8789) (Rasmussen et al. 2022). The XCMS (v3.12.0) software package was used for peak detection, peak filtering and peak alignment to obtain the quantitative list of substances. Support vectors based on the QC samples were employed for regression correction to eliminate systematic errors. Substances with CV less than 30% in the QC samples were then retained for subsequent analysis. The R package Ropls was adopted to perform principal component analysis (PCA) on the sample data, and the model was checked for overfitting with the replacement method of the examination. Accurate mass spectra from the public database Human Metabolome Database (HMDB) and mass spectrometry comparison were used for identification, and metabolites were analyzed for function and pathway with the MetaboAnalyst software package (Xia et al. 2011).

4. Results

4.1. Analysis of carcass characteristics

Table 6-4 presents the effect of dietary zinc on carcass characteristics and organ index of broilers under *NE* challenge. The dietary and challenge interaction significantly affected bursa index and carcass ratio, with zinc-free diet increasing bursa index and zinc glycinate increasing the carcass ratio under challenge ($P < 0.001$). Main effects showed that zinc glycinate increased leg muscle ratio compared to the negative control group ($P = 0.017$).

4.2. Meat quality analysis

The results in Tables 6-5 and 6-6 show the effects of dietary zinc on pectoral muscle processing quality and meat color of broilers under *NE* challenge. The interaction of zinc and *NE* challenge significantly affected cooking loss and shear force, with zinc sulfate reducing cooking loss in pectoral muscle under *NE* challenge ($P = 0.028$), while zinc glycinate increased shear force in broiler meat under challenge condition ($P < 0.001$). The pH values of pectoral muscles 45 min ($P < 0.001$) and 24 h ($P = 0.036$) after slaughter were elevated due to *NE* challenge. The significant interaction of zinc and *NE* challenge did not affect meat color ($P > 0.05$). Both sources of zinc significantly down-regulated pectoral muscle yellowness b* ($P = 0.029$), while *NE* challenge down-regulated h^o of pectoral muscle ($P = 0.045$).

4.3. The TBARS value of meat

Figure 6-1 presents the effect of zinc on the level of lipid peroxidation in pectoral muscle of broilers challenged with *NE* through the value of TBARS. The interaction between zinc and challenge did not significantly affect the meat TBARS value ($P > 0.05$). Both sources of zinc significantly reduced TBARS after five days storage of pectoral muscle ($P = 0.015$), suggesting that zinc mitigated lipid peroxidation in meat samples. The *NE* challenge up-regulated TBARS after 24 hours of storage in pectoral muscle ($P = 0.004$), which predicted that the *NE* challenge exacerbated lipid peroxidation in pectoral muscle.

4.4. Analysis of serum and liver biochemical parameters

Tables 6-7 and 6-8 show the effect of zinc on serum biochemical parameters and liver function indexes related to lipid metabolism in broilers under *NE* challenge. There was no significant interaction observed between zinc and *NE* challenge ($P > 0.05$). Both zinc glycinate and zinc sulfate reduced glucose in serum ($P = 0.013$). Compared to the other two dietary treatments, zinc glycinate significantly increased serum TG level ($P = 0.047$). *NE* challenge significantly raised the levels of TCHO ($P = 0.048$) and LDL ($P = 0.005$) and lowered the level of TG ($P = 0.013$) in the serum. *NE* challenge showed the same trend as serum for TG and TCHO levels in the liver, with TG level being downregulated ($P = 0.004$) and TCHO showing the opposite alteration ($P = 0.004$).

Table 6-4: Effect of dietary zinc on carcass indexes of necrotic enteritis challenged broilers

Items	**Liver**, %	**Hear**, %	**Spleen**, %	**Bursa**, %	**Abdominal fat**, %	**Pectorales**, %	**Leg muscle**, %	**Carcass**, %
NT	1.93	0.56	0.11	0.13^{b}	0.63	19.53	13.59	77.48^{b}
OT	1.92	0.59	0.11	0.22^{a}	0.37	18.54	14.81	77.05^{b}
IT	1.84	0.57	0.10	0.15^{b}	0.55	19.28	13.89	77.59^{b}
CNT	1.89	0.60	0.11	0.19^{a}	0.63	19.62	14.51	77.49^{b}
COT	1.81	0.55	0.09	0.19^{a}	0.35	20.19	14.81	78.82^{a}
CIT	1.89	0.59	0.10	0.12^{b}	0.75	19.08	14.58	77.67^{b}
Pooled SEM	0.12	0.09	0.01	0.02	0.06	1.69	0.96	1.32
Diet								
Negative	1.90	0.58	0.11	0.16^{B}	0.64	19.57	14.12^{B}	77.49
Gly-Zn	1.87	0.57	0.10	0.20^{A}	0.36	19.32	14.81^{A}	77.90
$ZnSO_4$	1.86	0.58	0.10	0.13^{B}	0.65	19.18	14.25^{B}	77.63
NE Challenge								
No Challenge	1.89	0.57	0.11	0.16	0.52	19.12	14.17	77.36^{b}
Challenge	1.86	0.58	0.10	0.17	0.58	19.63	14.63	77.97^{a}
P value								
Diet	0.512	0.962	0.450	< 0.001	0.125	0.762	0.017	0.466
NE Challenge	0.306	0.861	0.145	0.897	0.651	0.240	0.190	0.041
Interaction	0.140	0.317	0.229	< 0.001	0.708	0.179	0.210	0.031

Values are expressed as the mean and pooled SEM (n = 6). Means in a row with no common superscript are significantly different ($P < 0.05$). Lowercase letters indicate significantly different interactions and challenge effects, uppercase letters indicate significantly different dietary effects. SEM: standard error of the mean. The abbreviations of the treatment groups are the same as the previous ones.

Table 6-5: Effect of dietary zinc on the meat quality of pectoral muscle of necrotic enteritis challenged broilers

Items	**pH45min**	**pH24h**	**Cooking loss**, %	**Drip loss**, %	**Shear force**, N
NT	6.44	6.44	14.88^{a}	3.01	28.52^{a}
OT	6.45	6.35	13.89^{ab}	2.70	21.04^{c}
IT	6.52	6.37	14.19^{a}	2.87	23.77^{bc}
CNT	6.57	6.46	14.03^{ab}	2.67	26.53^{ab}
COT	6.51	6.44	14.96^{a}	2.92	26.74^{ab}
CIT	6.54	6.40	12.54^{b}	2.46	22.84^{c}
Pooled SEM	0.09	0.11	1.72	0.27	4.16
Diet					
Negative	6.50	6.45	14.41^{A}	2.83	27.48^{A}
Gly-Zn	6.48	6.40	14.45^{A}	2.81	24.01^{B}
$ZnSO_4$	6.53	6.38	13.33^{B}	2.67	23.44^{B}
NE Challenge					
No Challenge	6.46^{b}	6.38^{b}	14.30	2.86	24.44
Challenge	6.54^{a}	6.43^{a}	13.80	2.68	25.88
P value					
Diet	0.138	0.075	0.046	0.604	< 0.001
NE Challenge	< 0.001	0.036	0.253	0.224	0.298
Interaction	0.083	0.487	0.028	0.168	< 0.001

Values are expressed as the mean and pooled SEM (n = 6). Means in a row with no common superscript are significantly different ($P < 0.05$). Lowercase letters indicate significantly different interactions and challenge effects, uppercase letters indicate significantly different dietary effects. SEM: standard error of the mean. The abbreviations of the treatment groups are the same as the previous ones.

Table 6-6: Effect of dietary zinc on the meat color of necrotic enteritis challenged broilers

Items	**L***	**a***	**b***	**C***	**h°**
NT	55.91	3.82	7.98	8.48	64.56
OT	54.67	4.41	7.53	8.92	62.98
IT	55.14	3.94	7.90	9.01	65.23
CNT	54.70	4.23	8.67	9.79	62.55
COT	56.42	3.95	7.70	8.70	62.95
CIT	55.63	3.85	7.24	7.98	62.58
Pooled SEM	2.09	0.65	0.84	0.76	3.05
Diet					
Negative	55.30	4.00	8.34^{A}	8.05	63.61
Gly-Zn	55.49	4.17	7.63^{B}	8.82	62.96
$ZnSO_4$	55.37	3.89	7.57^{B}	8.49	63.82
NE Challenge					
No Challenge	55.25	4.06	7.80	8.81	64.32^{a}
Challenge	55.52	3.99	7.86	8.75	62.70^{b}
P value					
Diet	0.940	0.375	0.029	0.234	0.629
NE Challenge	0.533	0.773	0.809	0.946	0.045
Interaction	0.106	0.123	0.184	0.010	0.389

Values are expressed as the mean and pooled SEM (n = 6). Means in a row with no common superscript are significantly different ($P < 0.05$). Lowercase letters indicate significantly different interactions and challenge effects, uppercase letters indicate significantly different dietary effects. SEM: standard error of the mean; L*: lightness; a*: redness; b*: yellowness; C*: chroma; h^o: hue. The abbreviations of the treatment groups are the same as the previous ones.

Table 6-7: Effect of dietary zinc on serum biochemical parameters of necrotic enteritis challenged broilers

Items	**GLU**, mmol/L	**TCHO**, mmol/L	**TG**, mmol/L	**HDL**, mmol/L	**LDL**, mmol/L
NT	11.53	2.81	0.61	2.03	0.47
OT	10.48	2.58	0.63	2.04	0.55
IT	10.83	2.55	0.52	1.89	0.51
CNT	11.47	2.74	0.43	2.05	0.57
COT	10.83	2.83	0.55	1.97	0.86
CIT	10.01	2.93	0.47	2.13	0.61
Pooled SEM	0.92	0.30	0.10	0.25	0.11
Diet					
Negative	11.50^{A}	2.78	0.52^{B}	2.04	0.53
Gly-Zn	10.67^{B}	2.71	0.59^{A}	1.91	0.61
$ZnSO_4$	10.56^{B}	2.78	0.49^{B}	2.03	0.57
NE Challenge					
No Challenge	10.95	2.65^{b}	0.58^{a}	1.92	0.51^{b}
Challenge	10.95	2.84^{a}	0.49^{b}	2.05	0.61^{a}
P value					
Diet	0.013	0.814	0.047	0.380	0.189
NE Challenge	0.560	0.048	0.013	0.118	0.005
Interaction	0.323	0.156	0.712	0.509	0.972

Values are expressed as the mean and pooled SEM (n = 6). Means in a row with no common superscript are significantly different ($P < 0.05$). Lowercase letters indicate significantly different interactions and challenge effects, uppercase letters indicate significantly different dietary effects. SEM: standard error of the mean; GLU: glucose; TCHO: total cholesterol; TG: triglycerides; HDL: high density lipoprotein; LDL: low density lipoprotein. The abbreviations of the treatment groups are the same as the previous ones.

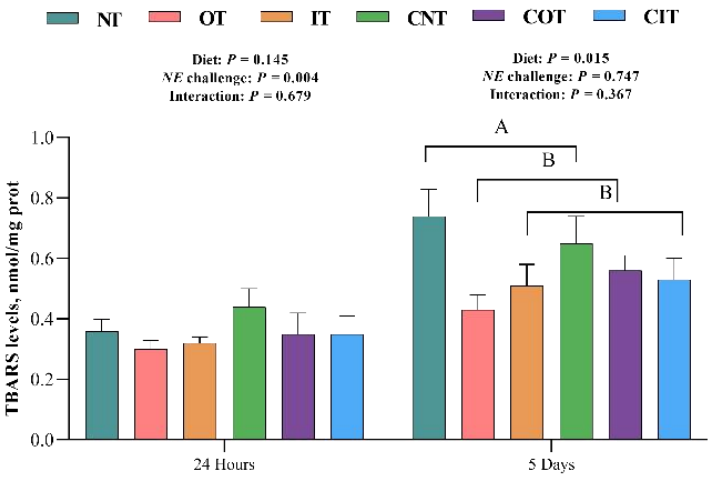

Figure 6-1: Effect of dietary zinc on the TBARS levels in the pectoral muscle of necrotic enteritis challenged broilers. Means with no common superscripts differ significantly ($P < 0.05$), with lowercase letters indicating significantly different interactions and uppercase letters indicating significantly different dietary effects. TBARS: thiobarbituric acid reactive substances; NT: negative feed treatment, chicken fed with basal diet; OT: zinc glycinate feed treatment, chicken fed with basal diet containing 60 mg/kg zinc glycinate; IT: zinc sulfate feed treatment, chicken fed with basal diet containing 60 mg/kg zinc sulfate; CNT: negative feed treatment with necrotic enteritis challenge, chicken fed with basal diet and challenged with necrotic enteritis; COT: zinc glycinate feed treatment with necrotic enteritis challenge, chicken fed with basal diet containing 60 mg /kg zinc glycinate and challenged with necrotic enteritis; CIT: zinc sulfate feed treatment with necrotic enteritis challenge, chicken fed with basal diet containing 60 mg /kg zinc sulfate and challenged with necrotic enteritis.

Table 6-8: Effect of dietary zinc on the triglycerides and total cholesterol contents of the liver of necrotic enteritis challenged broilers

Items	**TG**, nmol/g prot	**TCHO**, nmol/g prot
NT	0.17	0.20
OT	0.15	0.21
IT	0.15	0.20
CNT	0.11	0.22
COT	0.11	0.23
CIT	0.09	0.24
Pooled SEM	0.01	0.03

Diet		
Negative	0.14	0.21
Gly-Zn	0.13	0.22
$ZnSO_4$	0.12	0.22
NE Challenge		
No Challenge	0.15^{a}	0.20^{b}
Challenge	0.10^{b}	0.23^{a}
P value		
Diet	0.532	0.656
NE Challenge	0.004	0.004
Interaction	0.820	0.369

Values are expressed as the mean and pooled SEM (n = 6). Means in a row with no common superscript are significantly different ($P < 0.05$). Lowercase letters indicate significantly different interactions and challenge effects, uppercase letters indicate significantly different dietary effects. SEM: standard error of the mean; TG: triglycerides; TCHO: total cholesterol. The abbreviations of the treatment groups are the same as the previous ones.

4.5. LC-MS metabolite profile analysis

To verify the effect of zinc and *NE* challenge on meat metabolites and metabolic pathways in broilers, LC-MS metabolite profiling was implemented. Considering the economic cost, only samples from NT, OT, CNT and COT groups were examined.

Figures 6-2 and 6-3 illustrate the quality control of the samples, exhibiting consistent retention time points and ion intensities at ESI+ and ESI. PCA (principal component analysis) and RSD (relative standard deviation) are commonly performed statistical analyses in non-targeted metabolomics (Figures 6-4 and 6-5). The proportion of characteristic peaks with RSD less than 30% reached 65% to prove the reliability of the data. The analysis of Figure 6-4A and Figure 6-5A with regard to the PCA analysis reveals 24.9% of the total variation in positive ion mode and 16.1% of the total variation in negative ion mode, showing that the samples have a tendency to cluster. Figure 6-4B shows that the RSD value of the samples in the positive ion mode was 84.8%, while Figure 6-5B confirms that the RSD value of the samples in the negative ion mode was 77.2%.

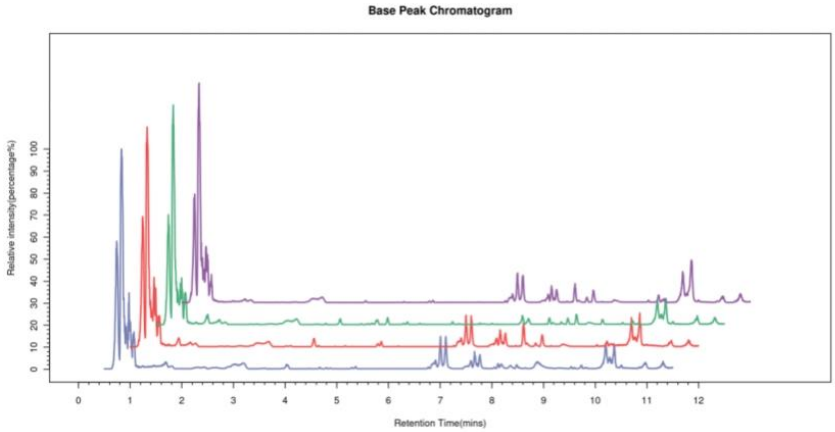

Figure 6-2: The TIC overlaps spectrum of QC samples using LC-MS at positive electrospray ionization mode.

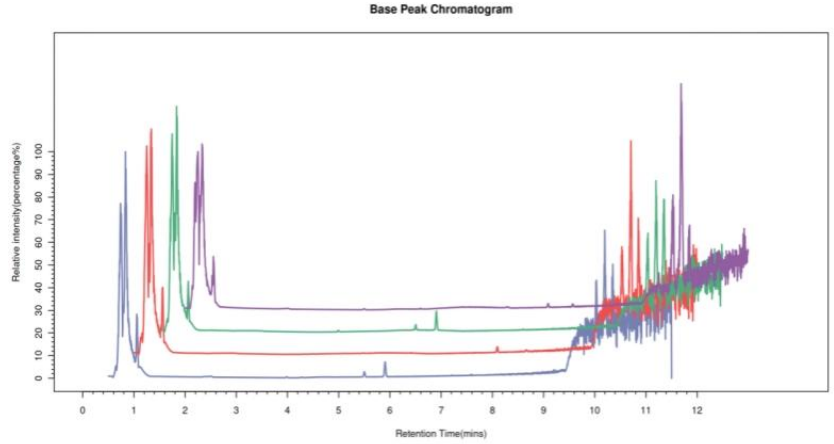

Figure 6-3: The TIC overlaps spectrum of QC samples using LC-MS at negative electrospray ionization modeAll differential metabolites obtained from the positive and negative modes were counted and screened according to the criteria of VIP (variable importance on projection) >1 and $P < 0.05$. A total of twelve metabolites were up-regulated and eighteen metabolites were down-regulated in the OT group as compared to the NT

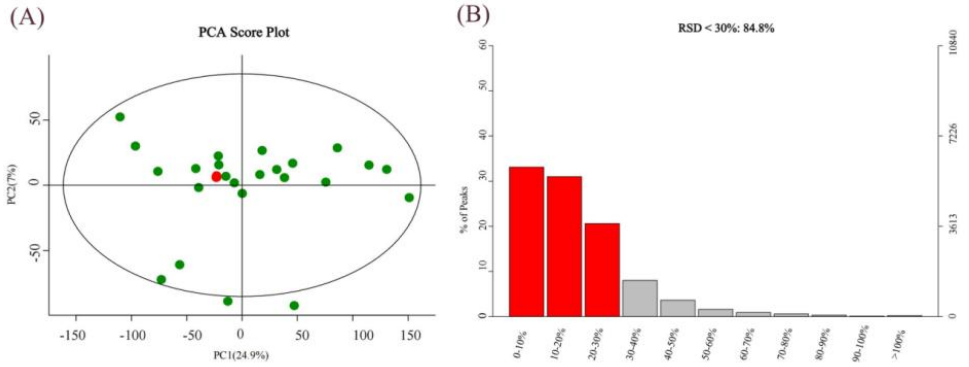

Figure 6-4: The PCA score plot (A) and RSD (B) of samples using LC-MS at positive electrospray ionization mode. PCA: principal component analysis; RSD: relative standard deviation.

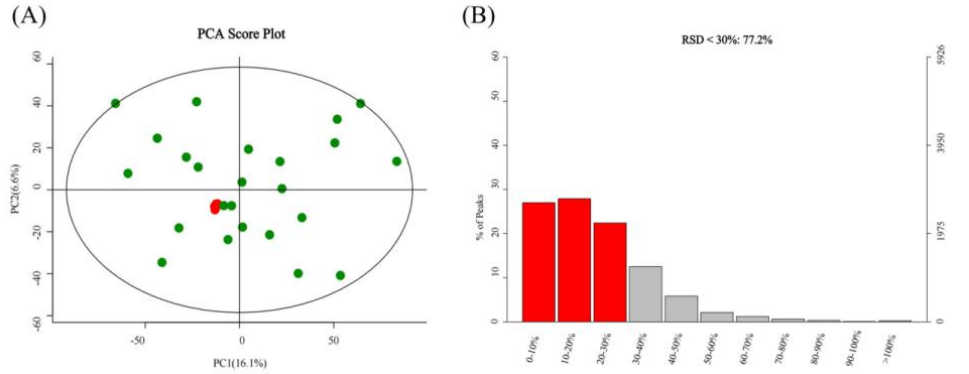

Figure 6-5: The PCA score plot (A) and RSD (B) of samples using LC-MS at negative electrospray ionization mode. PCA: principal component analysis; RSD: relative standard deviation.

Twenty-four metabolites were up-regulated and eleven metabolites were down-regulated in the COT group compared to the OT group. The metabolites were identified by the HMDB database and pathway analysis was performed for up- and down-regulated metabolites. The comparison of NT and OT is displayed in Figures 6-6 to 6-9. Figure 6-6 shows that fatty acid biosynthesis metabolism has one up-regulated pathway and one down-regulated pathway, respectively. The fatty acid biosynthesis pathways of these two groups also showed significant differences (Figure 6-7). According to the results in Figures 6-8 and 6-9, zinc glycinate up-regulated oleic acid content and down-regulated palmitoleic acid content in pectoral muscle compared to the NT group.

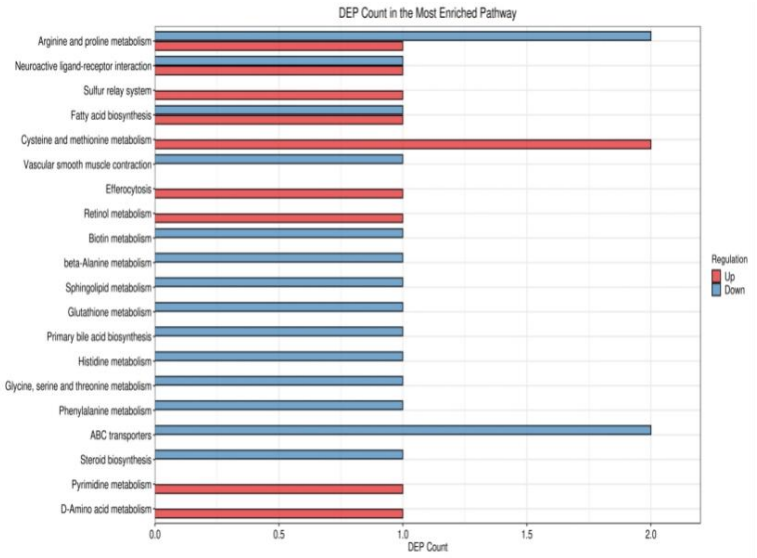

Figure 6-6: Effect of dietary zinc on the number of enriched metabolic pathways in the pectoral muscle of broilers. Comparison of the number of metabolite enrichment pathways between NT and OT groups. DEP: differential expressed proteins; NT: negative feed treatment, chicken fed with basal diet; OT: zinc glycinate feed treatment, chicken fed with basal diet containing 60 mg/kg zinc glycinate.

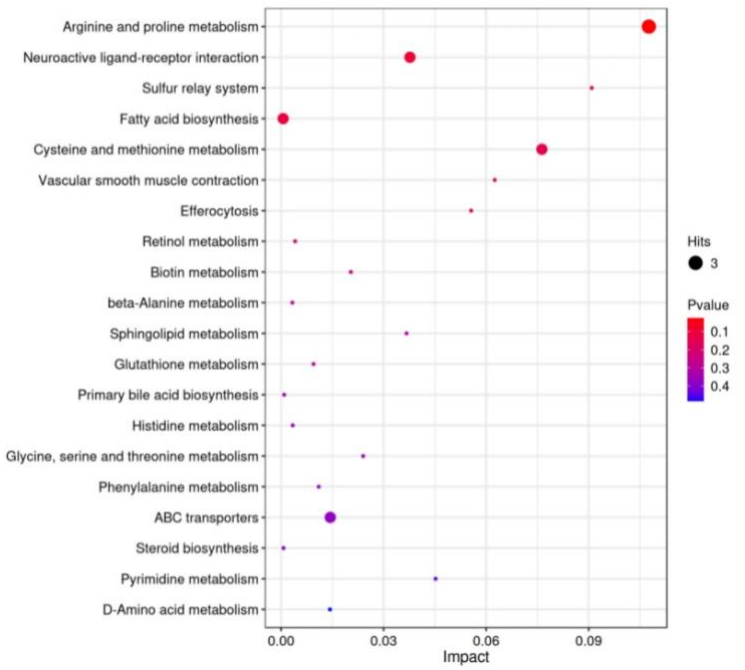

Figure 6-7: The bubble plots of factors influencing metabolic pathways by dietary zinc in

the pectoral muscle of broilers. Comparison of the degree of KEGG pathway enrichment in the NT and OT groups. Each bubble represents a metabolic pathway and the size of the bubble represents the impact factor; the redder the bubble color, the more significant the metabolite enrichment; the impact value means the level of impact on metabolic pathways, with larger values indicating that the differential metabolite has a greater impact on the target pathway; Hits indicate the overall number of differential metabolic pathways, with the largest area circle representing three differential metabolic pathways, the medium area circle representing two differential metabolic pathways, and the smallest circle representing one differential metabolic pathway. NT: negative feed treatment, chicken fed with basal diet; OT: zinc glycinate feed treatment, chicken fed with basal diet containing 60 mg/kg zinc
glycinate.

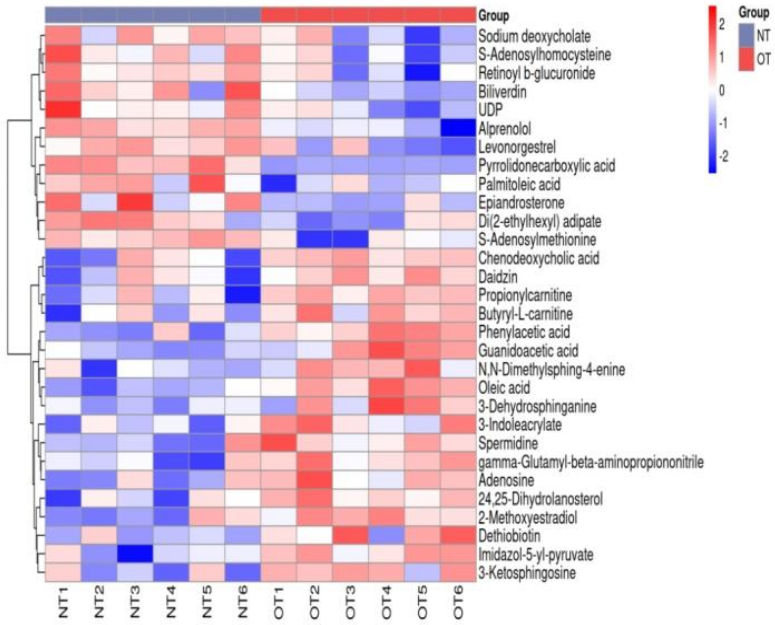

Figure 6-8: The heatmap of the effect of dietary zinc on metabolite profiles in the pectoral muscle of broilers. Comparison of metabolites between NT and OT groups. NT: negative feed treatment, chicken fed with basal diet; OT: zinc glycinate feed treatment, chicken fed with basal diet containing 60 mg/kg zinc
glycinate.

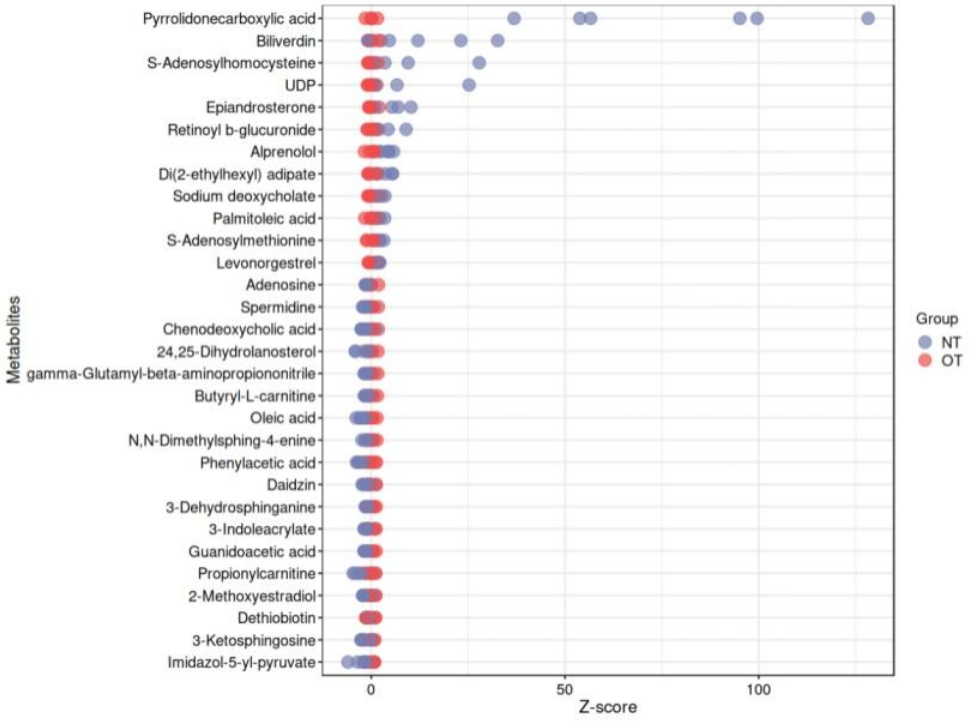

Figure 6-9: Effect of dietary zinc on the relative levels of differential metabolites in the pectoral muscle of broilers. Comparison of the relative levels of metabolites in the NT and OT groups. The closer to the right side, the higher the relative content of the current metabolite in that sample. NT: negative feed treatment, chicken fed with basal diet; OT: zinc glycinate feed treatment, chicken fed with basal diet containing 60 mg/kg zinc glycinate.

The results for the comparison of OT and COT were similar to the previous conclusions that there was one upregulated and one downregulated pathway for fatty acid biosynthesis metabolism, and the fatty acid biosynthesis pathways of the two groups showed significant differences. Challenge downregulated the oleic acid content in the pectoral muscle and upregulated the palmitoleic acid content which is presented in Figures 6-10 to 6-13. In addition, Figure 6-11 shows that *NE* challenge also affected the unsaturated fatty acid biosynthesis pathway and the linoleic acid metabolism pathway.

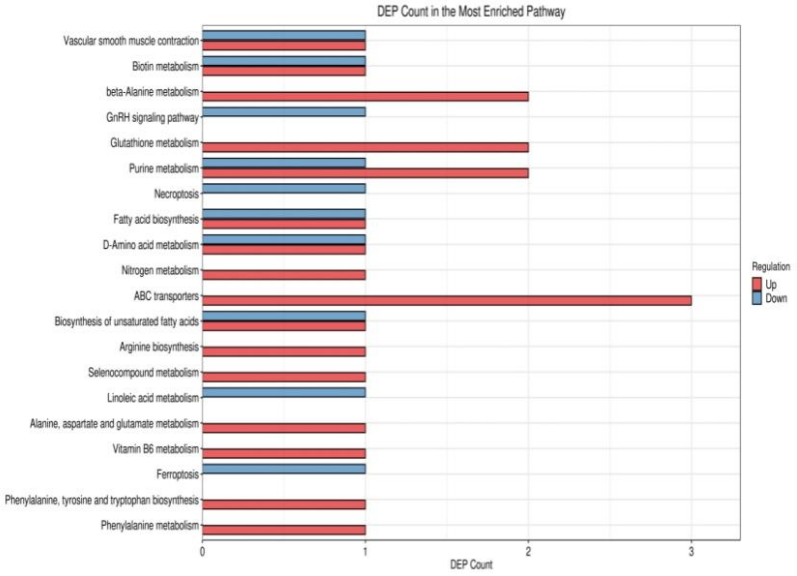

Figure 6-10: Effect of necrotic enteritis challenge on the number of enriched metabolic pathways in the pectoral muscle of broilers. Comparison of the number of metabolite enrichment pathways between OT and COT groups. DEP: differential expressed proteins; OT: zinc glycinate feed treatment, chicken fed with basal diet containing 60 mg/kg zinc glycinate; COT: zinc glycinate feed treatment with necrotic enteritis challenge, chicken fed with basal diet containing 60 mg/kg zinc glycinate and challenged with necrotic enteritis.

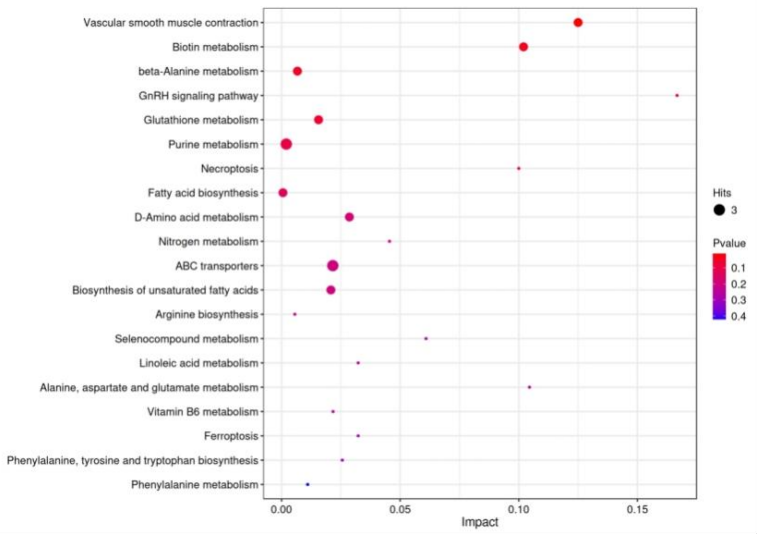

Figure 6-11: Effect of necrotic enteritis challenge on the bubble plots of factors influencing metabolic pathways in the pectoral muscle of broilers. Comparison of the degree of KEGG pathway enrichment in the OT and COT groups. Each bubble represents

a metabolic pathway and the size of the bubble represents the impact factor; the redder the bubble color, the more significant the metabolite enrichment; the impact value means the level of impact on metabolic pathways, with larger values indicating that the differential metabolite has a greater impact on the target pathway; Hits indicate the overall number of differential metabolic pathways, with the largest area circle representing three differential metabolic pathways, the medium area circle representing two differential metabolic pathways, and the smallest circle representing one differential metabolic pathway. OT: zinc glycinate feed treatment, chicken fed with basal diet containing 60 mg/kg zinc glycinate; COT: zinc glycinate feed treatment with necrotic enteritis challenge, chicken fed with basal diet containing 60 mg/kg zinc glycinate and challenged with necrotic enteritis.

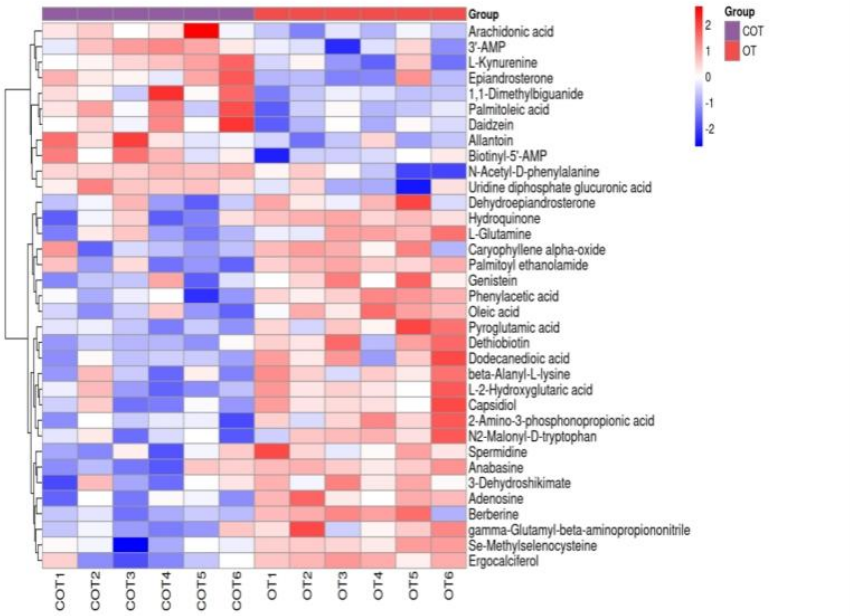

Figure 6-12: Effect of necrotic enteritis challenge on the heatmap of metabolite profiles in the pectoral muscle of broilers. Comparison of metabolites between OT and COT groups. OT: zinc glycinate feed treatment, chicken fed with basal diet containing 60 mg/kg zinc glycinate; COT: zinc glycinate feed treatment with necrotic enteritis challenge, chicken fed with basal diet containing 60 mg/kg zinc glycinate and challenged with necrotic enteritis.

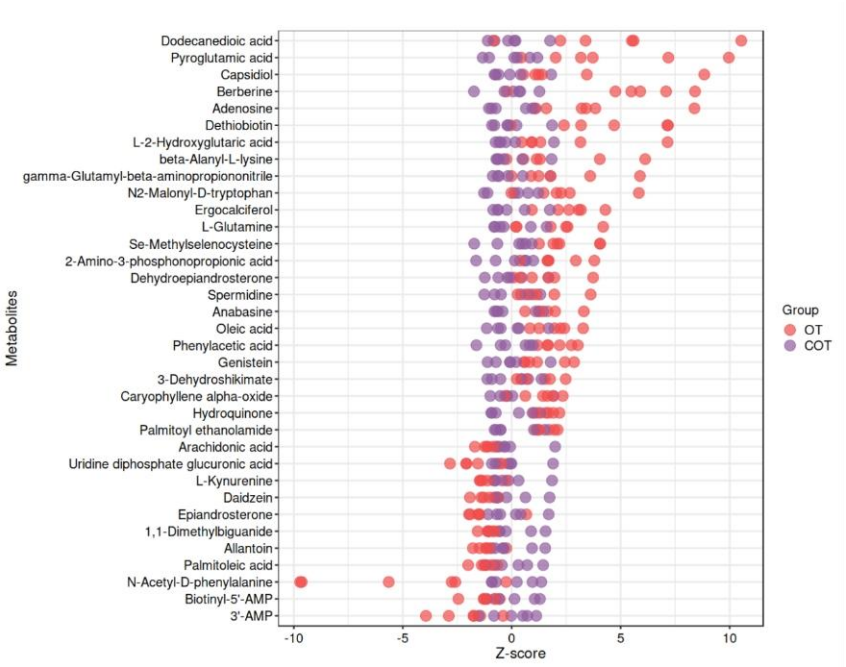

Figure 6-13: Effect of necrotic enteritis challenge on the relative levels of differential metabolites in the pectoral muscle of broilers. Comparison of the relative levels of metabolites in the OT and COT groups. OT: zinc glycinate feed treatment, chicken fed with basal diet containing 60 mg/kg zinc glycinate; COT: zinc glycinate feed treatment with necrotic enteritis challenge, chicken fed with basal diet containing 60 mg/kg zinc glycinate and challenged with necrotic enteritis.

4.6. Fatty acid profiles in meat

After confirming by non-targeted metabolomics assays that both zinc glycinate and *NE* challenge affected the fatty acid synthesis and metabolism, we examined the fatty acid profiles of pectoral muscle from all treatments with GC-MS and classified the fatty acids according to different fatty acid classifications. Table 6-9 presents the effect of zinc on saturated fatty acid (SFA) content in broiler pectoral muscle under *NE* challenge, and significant interactions between dietary zinc and *NE* challenge were not observed ($P > 0.05$). The main effect showed that *NE* challenge resulted in downregulation of C20:0 (arachidic acid) content in the pectoral muscle ($P = 0.002$). The results of monounsaturated and polyunsaturated fatty acids content in meat were presented in Tables 6-10 and 6-11. The interaction of zinc and challenge had no significant effect ($P > 0.05$) on the content of the monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). Zinc glycinate significantly increased C20:2 (eicosadienoic acid) content in meat compared to zinc-free treatment ($P = 0.037$). Zinc glycinate treatment significantly increased C20:3n3 (dihomo-α-linolenic acid) content in meat compared to zinc sulfate treatment ($P = 0.029$). Moreover, *NE* challenge significantly reduced C20:3n3 content in pectoral muscle ($P = 0.017$). Although

dietary and challenge factors affected the content of several saturated and polyunsaturated fatty acids, they did not significantly affect the overall proportions of SFA, MUFA and PUFA in pectoral muscle (Table 6-12).

Table 6-9: Effect of dietary zinc on the saturated fatty acids profile in the meat of necrotic enteritis challenged broilers

Items	**C12:0**	**C14:0**	**C15:0**	**C16:0**	**C17:0**	**C18:0**	**C20:0**
NT	0.06	0.71	0.19	13.34	0.49	14.52	0.60
OT	0.06	0.69	0.19	13.73	0.52	15.20	0.53
IT	0.07	0.89	0.22	14.02	0.54	15.26	0.33
CNT	0.07	0.85	0.22	13.73	0.54	14.76	0.28
COT	0.06	0.80	0.20	14.01	0.51	14.97	0.24
CIT	0.06	0.73	0.19	13.69	0.53	15.21	0.25
Pooled SEM	0.01	0.14	0.02	0.43	0.05	0.54	0.02
Diet							
Negative	0.06	0.78	0.20	13.53	0.51	14.64	0.44
Gly-Zn	0.06	0.74	0.20	13.87	0.52	15.09	0.39
$ZnSO_4$	0.06	0.81	0.20	13.85	0.53	15.23	0.23
NE Challenge							
No Challenge	0.06	0.76	0.20	13.69	0.52	14.99	0.49^{a}
Challenge	0.06	0.79	0.20	13.81	0.52	14.98	0.26^{b}
P value							
Diet	0.465	0.633	0.795	0.206	0.830	0.091	0.161
NE Challenge	0.799	0.613	0.720	0.507	0.763	0.964	0.002
Interaction	0.183	0.066	0.074	0.202	0.567	0.684	0.237

Values are expressed as the mean and pooled SEM (n = 6). Means in a row with no common superscript are significantly different ($P < 0.05$). Lowercase letters indicate significantly different interactions and challenge effects, uppercase letters indicate significantly different dietary effects. SEM: standard error of the mean; C12:0 = lauric acid; C14:0 = myristic acid; C15:0 = pentadecanoin acid; C16:0 = palmitic acid; C17:0 = margaric acid; C18:0 = stearic acid; C20:0 = arachidic acid. The abbreviations of the treatment groups are the same as the previous ones.

Table 6-10: Effect of dietary zinc on the monounsaturated fatty acids profile in the meat of necrotic enteritis challenged broilers

Items	C16:1n7	C17:1n7	C18:1n9c	C20:1	C22:1n9
NT	2.87	0.19	20.66	0.61	0.10
OT	2.20	0.16	21.09	0.58	0.09
IT	2.94	0.10	20.82	0.67	0.11
CNT	3.08	0.07	21.15	0.68	0.08
COT	2.88	0.08	20.57	0.58	0.08
CIT	2.60	0.11	21.19	0.56	0.08
Pooled SEM	0.72	0.01	0.50	0.09	0.01
Diet					
Negative	2.97	0.13	20.90	0.65	0.09
Gly-Zn	2.54	0.12	20.83	0.58	0.09
$ZnSO_4$	2.77	0.11	21.01	0.61	0.09
NE Challenge					
No Challenge	2.67	0.15^{a}	20.86	0.62	0.10^{a}
Challenge	2.60	0.09^{b}	20.97	0.61	0.08^{b}
P value					
Diet	0.523	0.779	0.798	0.791	0.480
NE Challenge	0.560	0.030	0.204	0.422	0.008
Interaction	0.413	0.189	0.590	0.195	0.295

Values are expressed as the mean and pooled SEM (n = 6). Means in a row with no common superscript are significantly different ($P < 0.05$). Lowercase letters indicate significantly different interactions and challenge effects, uppercase letters indicate significantly different dietary effects. SEM: standard error of the mean; C16:1n7 = palmitolytic acid; C17:1n7 = margaroleic acid; C18:1n9c = oleic acid; C20:1 = gadoleic acid; C22:1n9 = erucic acid. The abbreviations of the treatment groups are the same as the previous ones.

Table 6-11: Effect of dietary zinc on the polyunsaturated fatty acids profile in the meat of necrotic enteritis challenged broilers

Items	C18:2n6t	C18:3n6	C18:3n3	C20:2	C20:3n6	C20:4n6	C20:3n3	C20:5n3	C22:2
NT	19.57	0.69	3.77	2.77	2.18	11.98	0.48	0.83	3.41
OT	19.49	0.81	3.74	3.15	2.34	11.50	0.49	0.78	2.67
IT	19.60	0.71	3.88	2.85	2.53	10.42	0.45	0.78	2.85
CNT	20.03	0.74	4.15	2.78	2.33	9.67	0.42	0.76	3.60
COT	19.96	0.64	3.79	2.93	2.45	10.86	0.48	0.79	3.14
CIT	19.86	0.65	3.68	2.70	2.35	11.20	0.40	0.76	3.24
Pooled SEM	0.98	0.10	0.47	0.26	0.25	1.66	0.06	0.09	0.65
Diet									
Negative	19.80	0.71	3.96	2.78^{B}	2.25	10.84	0.45^{AB}	0.79	3.50
Gly-Zn	19.73	0.72	3.76	3.04^{A}	2.40	11.18	0.48^{A}	0.78	2.91
$ZnSO_4$	19.73	0.68	3.78	2.77^{B}	2.44	10.81	0.42^{B}	0.77	3.04
NE Challenge									
No Challenge	19.55	0.74	3.80	2.92	2.35	11.30	0.47^{a}	0.80	2.98
Challenge	19.95	0.67	3.87	2.81	2.38	10.58	0.43^{b}	0.77	3.24
P value									
Diet	0.822	0.647	0.688	0.037	0.311	0.308	0.029	0.845	0.176
NE Challenge	0.606	0.142	0.712	0.263	0.817	0.887	0.017	0.474	0.202
Interaction	0.900	0.125	0.522	0.664	0.382	0.214	0.711	0.689	0.902

Values are expressed as the mean and pooled SEM (n = 6). Means in a row with no common superscript are significantly different ($P < 0.05$). Lowercase letters indicate significantly different interactions and challenge effects, uppercase letters indicate significantly different dietary effects. SEM: standard error of the mean; C18:2n6t = linoleic acid; C18:3n6 = gamma-linoleic acid; C18:3n3 = alpha-linoleic acid; C20:2 = eicosadienoic acid; C20:3n6 = dihomo-γ-linolênico acid; C20:4n6 = arachidonic acid; C20:3n3 = dihomo-α-linolenic acid; C20:5n3 = eicosadienoic acid; C22:2 = docosadienoic acid. The abbreviations of the treatment groups are the same as the previous ones.

Table 6-12: Effect of dietary zinc on the classification of fatty acids profile in the meat of necrotic enteritis challenged broilers

Items	Total SFA	Total MUFA	Total PUFA
NT	29.90	24.41	45.69
OT	30.92	24.12	44.97
IT	31.31	24.63	44.06
CNT	30.43	25.06	44.52
COT	30.79	24.19	45.03
CIT	30.65	24.54	44.82
Pooled SEM	0.68	0.96	1.25
Diet			
Negative	30.16	24.74	45.11
Gly-Zn	30.85	24.16	45.00
$ZnSO_4$	30.98	24.59	44.44
NE Challenge			
No Challenge	30.71	24.39	44.91
Challenge	30.62	24.60	44.79
P value			
Diet	0.250	0.806	0.601
NE Challenge	0.711	0.385	0.595
Interaction	0.149	0.733	0.341

Values are expressed as the mean and pooled SEM (n = 6). Means in a row with no common superscript are significantly different ($P < 0.05$). Lowercase letters indicate significantly different interactions and challenge effects, uppercase letters indicate significantly different dietary effects. SEM: standard error of the mean; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. The abbreviations of the treatment groups are the same as the previous ones.

4.7. Lipid metabolism-related gene expression in the liver

To investigate the reasons for the regulation of fatty acid content in meat and lipid metabolism in birds, gene expression levels of fatty acid synthesis (Figure 6-14A) and fatty acid oxidation (Figure 6-14B) genes were examined in the liver. The interaction of dietary zinc and *NE* challenge did not affect liver fatty acid synthesis gene expression levels ($P > 0.05$). As compared to the negative control, both zinc glycinate and zinc sulfate significantly decreased the gene expression level of liver *ACC* ($P = 0.049$). Meanwhile, *NE* challenge upregulated the expression level of sterol regulatory element binding protein 1c (*SREBP-1C*) ($P = 0.003$). The interaction indicated that zinc glycinate significantly decreased the expression level of liver *PPAR-α* in response to *NE* challenge ($P = 0.011$), while overall liver *PPAR-α* expression level was also down-regulated due to challenge ($P = 0.001$).

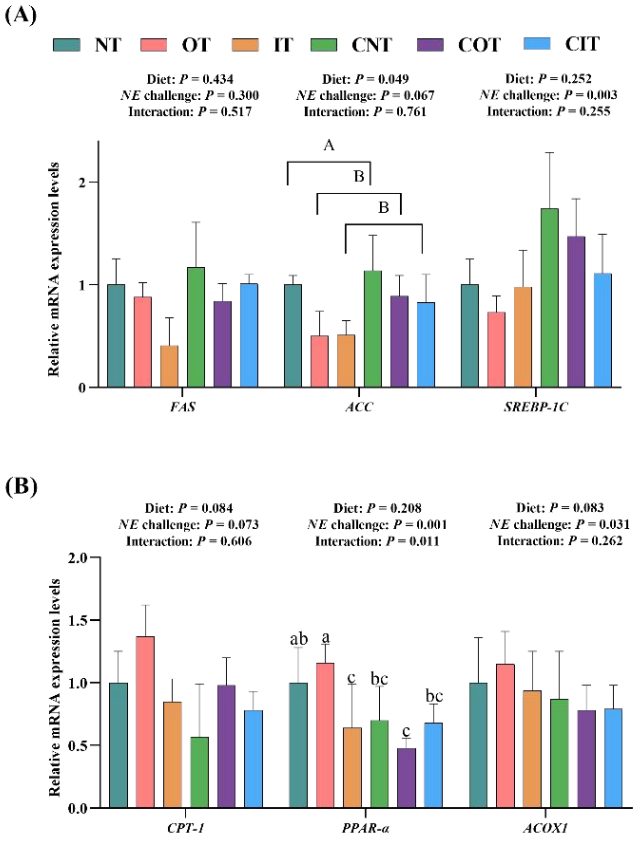

Figure 6-14: Effect of dietary zinc on the gene expression levels of fatty acid synthesis (A) and oxidation (B) in the liver of necrotic enteritis challenged broilers. Means with no common superscripts differ significantly ($P < 0.05$), with lowercase letters indicating significantly different interactions and uppercase letters indicating significantly different dietary effects. *FAS*: fatty acid synthase; *ACC*: acetyl-CoA carboxylase; *SREBP-1C*: sterol regulatory element binding protein 1c; *CPT-1*: carnitine palmitoyl transferase 1; *PPAR-α*: peroxisome proliferator activated receptor-alpha; *ACOX1*: acyl CoA oxidase 1. The abbreviations of the treatment groups are the same as the previous ones.

5. Discussion

Despite the benefits of intensive livestock farming with the support of modern poultry breeding technology, such as labor savings and a large amount of animal protein to the market, the higher stocking density and more vigorous metabolism in the broiler industry resulted in more pathogen susceptibility in broilers (Van Limbergen et al. 2020). One such pathogen is *Clostridium perfringens* and the subsequent *NE* that establishes after infection directly affects gut health of broilers by disrupting the intestinal barrier function, hindering nutrient absorption,

and finally impacting growth performance (Goo et al. 2024). Based on past work, *NE* challenge model established with the same source and dosage of coccidia vaccine and the NetB toxin-positive type A field *Clostridium perfringens* (CVCC2030) has been proven to be effective (Song et al. 2023). Damage to the intestine induced by coccidial infection leads to plasma infiltration into the intestinal lumen, which promotes the colonization of *Clostridium perfringens* and the manifestation of clinical or sub-clinical form of *NE*, which finally leads to a decrease in broiler production performance (Fathima et al. 2022). Nutritional status and diseases could affect muscle development and consequently the carcass ratio of poultry. It was found that 30 mg/kg of organic zinc increased carcass percentage in broilers (Rossi et al. 2007). However, there is also a report revealing that different sources of zinc did not affect carcass ratio and abdominal fat percentage in broilers (Qudsieh et al. 2018). In our work, the main effect of diet showed that 60 mg/kg of zinc glycinate increased the leg muscle percentage and the interaction showed that zinc glycinate increased carcass ratio under *NE* challenge, suggesting that zinc promotes anabolism and improves meat production efficiency in broilers. In general, the metabolism of *NE* challenged broilers causes a systemic immune response due to the disruption of the intestinal barrier, consuming energy to maintain the barrier function (Emami et al. 2019). In contrast, birds increase the uptake of glucose in the gut and the translocation of glucose from the liver to ensure the energy supply (Mayorga et al. 2020). High dietary energy concentration reduces carcass yield, especially high energy paired with low protein diets leads to lower carcass percentage and increased body fat in broilers, whereas feeding diets with high protein and energy nutrient concentrations improves carcass yield (Mir et al. 2017). In the present study, we applied a high-energy and high-protein diet, which may have ensured enough nutrient supply during the *NE* challenge period. In addition, we found significant alterations in bursal organ index of the increasing bursal index in zinc-free fed broilers due to *NE* challenge. The bursa is an important immune organ for birds and zinc has the function of promoting the growth of immune organs. Furthermore, acute inflammation induced by immune challenges in the state of zinc deficiency leads to edema of the bursal, which explains these results (Bilal et al. 2023).

Quantitative parameters of meat quality include sensory parameters of meat and processing characteristics. Sensory parameters include the color, texture, odor and flavor of the meat, while processing characteristics of meat include shear force, water holding capacity (WHC) and pH value (Barbut et al. 2022). Consumers usually make decisions based on sensory experiences when purchasing chicken, such as tenderness and color (Pellattiero et al. 2020). Excessive yellowness b* represents an undesirable color, and the reduction of b* by zinc shows the benefit of zinc on sensory characteristics in meat quality (Marchewka et al. 2023). Evidence suggests that there is a highly significant positive correlation between muscle pH and meat color due to an increased rate of conversion of glycogen to lactic acid after slaughter (Mir et al. 2017). Hydrolysis of myogenic fibers in chicken meat after slaughter leads to weakening of muscle fibers, affecting the integrity of muscle fibers, and a significant increase in the mobility and proportion of water outside the muscle fibers, resulting in increased toughness (Li

et al. 2016). Research showed that improving the antioxidant capacity of meat increases the WHC of meat, and dietary zinc has antioxidant capacity (Skřivanová et al. 2017). Zinc significantly reduced the TBARS value of pectoral meat, suggesting that zinc reduces the level of lipid peroxidation in chicken meat by improving the oxidative status. Lipid peroxidation is the process by which reactive oxygen species are converted to non-free radical lipid breakdown species, and this process amplifies reactive oxygen species hazards through a chain reaction (Asido et al. 2024). Zinc glycinate reduced cooking loss in pectoral muscles, while zinc sulfate decreased cooking loss under NE challenge. Additionally, both zinc sources lowered shear force, indicating that zinc improves the WHC of the meat. The lipid peroxidation status of meat directly reflects the level of meat quality and affects the shelf-life of the meat. *NE* challenge increased the lipid peroxidation status of chicken meat after 24 hours of storage, while dietary zinc improved the lipid peroxidation status of chicken meat after 5 days of storage, confirming the negative effect of *NE* challenge on lipid peroxidation and the ameliorating effect of zinc on lipid peroxidation.

Research has confirmed that lipid metabolism in broilers could be regulated by nutritional methods, which in turn modulates the fatty acid composition of chicken meat and improves meat quality (Poorghasemi et al. 2013). Unsaturated fatty acids are considered beneficial to human health, especially PUFA helps reduce the risk of cardiovascular disease (Zarate et al. 2017). Chicken is an important source of PUFA, but poultry meat is prone to lipid peroxidation due to the instability of unsaturated fatty acids. Up to now, there is no published work to show the association of *NE* challenge and zinc with the fatty acid composition of chicken meat. We confirmed the predominance of PUFA in the fatty acid composition of chicken meat by analyzing the fatty acid composition. The application of zinc glycinate increased the eicosadienoic acid and dihomo-α-linolenic acid ratios, confirming that zinc plays an active role in the synthesis of PUFA, which has the same function as other antioxidant substances such as glycerol monolaurate (Valentini et al. 2020). The *NE* challenge down-regulated the proportion of dihomo-α-linolenic acid in chicken meat, suggesting that *NE* induced lipid peroxidation and affected the PUFA biosynthetic pathway, which is consistent with the untargeted metabolomics analysis. The *NE* challenge also reduced the arachidic acid level in chicken meat, suggesting an effect of immune challenge on fatty acid anabolism. Non-targeted metabolomic analyses were also used for the first time to study the effect of *NE* challenge on chicken meat, with quality control analyses showing the validity and stability of the assay results, while PCA and RSD analyses demonstrated the reproducibility and reliability of the data. Notably, the results of the non-targeted metabolomics analysis made evident that fatty acid metabolic synthesis was affected by both, *NE* challenge and zinc, while a range of amino acid metabolic pathways were also shown to be affected, and these pathways also play important roles in meat quality regulation. We may refine these studies in the future.

An earlier study by Wu et al. (2018) has shown that *NE* leads to concurrent liver inflammation and affects liver function, thus *NE* might alter the fatty acid composition of chicken meat by affecting lipid metabolism (Wu et al. 2018). Blood biochemicals such as GLU, TG, TC, LDL and HDL levels reflect the

metabolism and absorption of carbohydrates, proteins and fats in broilers (Hu et al. 2021). The amount of TCHO in the blood reflects the level of lipids, and abnormal increases in TCHO have been linked to the development of a large number of diseases, including inflammation of the liver and heart and blood vessel disease (Lauridsen et al. 2018). The levels of GLU and TG in the serum are regulated by diet and could indicate the fat deposition condition in poultry (Velasco et al. 2010). LDL contains a higher content of cholesterol and excessive LDL increases the risk of vascular and cardiac diseases, while HDL could prevent similar diseases by participating in reverse cholesterol transport (Hernáez et al. 2019). In the present study, the *NE* challenge increased liver and serum TCHO levels, decreased TG levels, and increased serum LDL level, suggesting that *NE* induced abnormalities in liver lipid metabolism and increased the risk of lipid metabolism-related diseases. In contrast, zinc glycinate decreased serum GLU and increased TG level. These results might suggest that zinc mobilizes the liver to catabolize more cholesterol and promote lipid metabolism, which was also confirmed in data on fatty acid synthesis and oxidative gene expression in the liver. The ACC is a key enzyme in fatty acid synthesis, SREBP-1C is an important cholesterol synthesis regulatory protein, and increased *PPAR-α* and *ACOX1* gene expression promotes fatty acid oxidation (Kim et al. 2017). The present study demonstrated that *NE* challenge resulted in downregulation of *PPAR-α* and *ACOX1* gene expression, as well as upregulation of *SREBP-1C*, matching a previous result (Zhou et al. 2016). In addition, both sources of zinc could inhibit fatty acid synthesis by down-regulating gene expression in the liver *ACC*. Since the broiler diet is a typical high energy diet, the inhibition of the ACC pathway would prevent excessive fat deposition in the liver by regulating lipid metabolism disorders (Pang et al. 2021). These results suggest that *NE* leads to dysfunction of liver lipid metabolism and induces hyperlipidemia in broilers, and dietary zinc has a beneficial effect on lipid metabolism.

6. Conclusion

The current study demonstrated that *NE* challenge affected carcass characteristics, exacerbated lipid peroxidation and altered the fatty acid composition of chicken meat, ultimately leading to a decrease in meat quality. Both zinc sources lead to improved meat quality by modifying meat color and lipid peroxidation. The regulation of meat quality is related to the function of zinc and *NE* challenge through alteration of lipid metabolism.

Chapter VII

General Discussion, General Conclusion and Perspective

1. General discussion

1.1. Introduction

Zinc, an essential trace element, is widely distributed in the blood, bones and tissues of animals. Zinc regulates biological processes by participating in the composition of enzymes and hormones. Therefore, zinc plays a key role in the entire growth cycle of broilers, including feather development, maintenance of feed intake, nutrient absorption and muscle growth. As zinc is involved in the antioxidant system and protein synthesis, a zinc deficiency results in slower growth and poor bone development in broilers, while intrinsic immune function and intestinal health suffer a serious decline.

As a typical Type 2 nutrient, zinc cannot be stored in the body in large quantities, which means that a consistent intake of zinc from the diet is required to avoid a deficiency. We measured the zinc level in the basal diet in Chapter V and the result was approximately 30 mg/kg, which is below the National Research Council (NRC) recommended minimum standard of 40 mg/kg. The poultry production industry tends to add higher levels to feed for improving broiler performance and health. As such, Aviagen has set its broiler zinc requirement level at 110 mg/kg (Nguyen et al. 2021). Most of the zinc in animal feed will be excreted into the environment via feces, with excess zinc being retained in water and soil and entering the ecological cycle. A broiler will take about 4 kg of compound feed in its lifetime, with a zinc content of about 0.4 g, about 0.3 g of which will enter the environment through feces, which means that about 240 kg of zinc is excreted annually on a farm of 100,000 broilers. Due to concerns about environmental pollution caused by excess zinc, the European Food Safety Authority (EFSA) recommends an upper limit for total zinc of a maximum content of 150 mg/kg in complete feed for pigs, rabbits and salmon, and 100 mg/kg for cattle and poultry (Duffy et al. 2023), and the value is 120mg/kg in China. Animal experiments were conducted in China, the zinc content of broilers in the complete diets of the *NE* study was approximately 90 mg/kg and 110 mg/kg for heat stress study, which is below the authorized maximum concentration for zinc in China. This is below the upper limit of zinc content in poultry feed. The limited addition of zinc has prompted the search for more efficient sources of zinc to improve the efficiency of zinc absorption in animals. The efficiency of zinc absorption in the intestine and the availability of zinc in tissues, called bioavailability, is influenced by several factors. Among these, the effect of different dietary sources of phytate and different dietary sources of zinc has been widely discussed. Studies have shown that organic sources of zinc have a higher bioavailability, but the effects of different sources and doses of zinc on broiler production performance have not been consistent. So, there should be other factors influencing zinc requirement and absorption. Now that in most countries and regions around the globe antibiotics are no longer allowed to be unlimited add to the feed for disease treatment and growth promotion, has the decline in growth performance and increased immune challenge affected the requirement for zinc in broilers? In addition, better modern breeding techniques have increased the growth rate of broilers, which is accompanied by an increased metabolic rate, causing oxidative stress and reduced meat quality. Could the application of zinc

alleviate these negative effects?

With the above questions in mind, the research for this doctoral project was started. In the first study (Chapter III) we investigated changes in zinc homeostasis in broilers under heat stress compared to those at ambient temperature. In this chapter, we compared growth performance, zinc retention, levels of zinc transport and storage proteins as well as microbiota composition. In the second study (Chapter IV), the effects of immune challenge induced by a lipopolysaccharide (LPS) exposure on systemic zinc homeostasis of broiler embryos and the regulatory role of zinc glycinate were assessed. In the last study (Chapters V and VI), the objective was to investigate the effects of necrotic enteritis (*NE*) challenge on zinc metabolism and meat quality in broiler chickens, as well as the regulatory roles of zinc sulfate and zinc glycinate on the inflammatory response, intestinal health and meat quality.

1.2. Exploring the effects of zinc on the growth and health status of broilers under challenged status

1.2.1. Challenges for broilers throughout their lifespan

The development of poultry embryos is dependent on the nutrients in the fertilized egg, with the yolk and egg white containing similar proportions of protein, while fat and other nutrients are mainly derived from the yolk (Tona et al. 2022). The utilization of nutrients in the yolk determines the nutritional requirement of the embryo, and in particular the energy provided by the yolk in the days prior to hatching largely determines the hatchability of the embryo. Embryos may also be mineral-limited in the last few days before hatching due to high consumption in the early stage of incubation (Uni et al. 2012). We demonstrated in Chapter IV that zinc supplementation of broiler embryos promoted intestinal barrier development and improved the immune status of the intestine. The immune system of broiler begins to develop during the embryonic period, and maternal antibodies from the yolk sac continue to protect chicks during the first week after incubation. LPS has been shown to induce an innate immune response in E18 broiler embryos, inducing the expression of pro-inflammatory cytokines in the blood and jejunum (Kong et al. 2023). In our study we aimed to investigate the improvement of zinc on LPS challenge induced immune response and intestinal damage, while both LPS and zinc injections were performed based on some pre-test results. When we try to perform two injections in the pre-test, the hatchability of chicken embryos decreases dramatically, even to less than 40% in some groups. In addition, the yolks of E17.5 embryo eggs still had deposited zinc and the injection of zinc was applied as a supplement. Our results confirmed the validity of zinc and LPS 12 hours after injection. It is worth mentioning that the results of the pre-test revealed that the effect of LPS on embryos was still detected by higher serum pro-inflammatory cytokines after one week of hatching, while zinc after injection no longer caused significant alteration in tissue zinc concentration in chicks at one week after hatching, which may be due to the rapid metabolism of zinc as a Type 2 nutrient.

The beginning of the second week till the end of the third week is also a critical period of intestinal development, and this has a temporal crossover with the

maturation of the immune system, as the intestine also provides important immune support for broilers (Lilburn et al. 2015). At this stage, broilers face a high number of gut health issues and complex etiologies, including infections of bacteria and parasites infections, of which coccidia is a prevalent one. Intestinal diseases cause damage to the intestinal barrier, reduced nutrient digestibility and increased death rates (Tian et al. 2016). More importantly, nearly half the proportion of *NE*-infected broilers exhibited a sub-clinical form of the disease, showing a long recovery period, during which reduced nutrient digestibility causes great economic losses (Moore 2024). Chapter V examined the negative effects of an *NE* challenge and confirmed that an *NE* challenge induced increased inflammation and oxidative stress in broilers, disrupting gut barrier function. *NE* challenge did not result in an increased mortality rate but caused an increase in FCR, which may be due to intestinal damage and infection caused by sub-clinical form of *NE*. Most experiments on *NE* challenge have detected reduced production performance and increased intestinal lesions, while mortality rates ranging from 2% to 50% have been reported (Paiva et al. 2014). The variation in mortality rates due to *NE* challenge may be related to environment and feed (Palliyeguru et al. 2014).

Broilers experience a significant increase in feed intake from their fifth week of life, accompanied by accelerated body weight growth, implying rapid nutrient uptake and metabolic rates. The accelerated metabolism combined with the high density of farming is not conducive to heat dissipation, and the lack of sweat glands in broilers is a physiological characteristic that can easily lead to heat stress in broilers. Therefore, we established a heat stress model for broilers during the fifth week of growth in Chapter III, confirming that heat stress induced oxidative stress and immune challenge, disrupted the gut microbial community, and finally led to reduced performance and increased mortality in broilers.

Thus, broilers face a range of challenges at different stages from hatching to finishing, and we aimed to investigate how zinc ameliorated the losses caused by these different challenges. In this thesis, we employed three challenge models to simulate the challenges of broilers at different stages, including the heat stress broiler model, the LPS broiler embryonic egg model, and the *NE* model. Heat stress in commercial broilers often occurs in the later stages of the breeding process and is closely related to the stocking density and the environment (Liu et al. 2020). Since 2007, the EU regulation on stocking density for broilers has been limited to a maximum of 42 kg/m^2, while broilers in organic production systems are to be stocked at as low as 20 kg/m^2 (Xiao et al. 2023). In many developing countries with a shortage of animal protein, where animal welfare regulations are not sufficiently robust, broilers are farmed at higher density. In addition, in some hot summer areas it is difficult to keep the broiler's body temperature stable under high humidity, and these two factors may lead to the occurrence of heat stress. LPS from *E. coli* is a potent stimulator of the immune system and is widely used in a variety of animal and cellular models to imitate gram-negative bacterial infections (Zhang et al. 2024). LPS is released as part of the outer membrane of gram-negative bacteria following bacterial colonization or lysis, leading to the name endotoxin. LPS activates the innate immune system and triggers an immune response primarily by interacting with TLR4 on immune cells such as

macrophages and dendritic cells (Ciesielska et al. 2021). For broiler studies, LPS is often used to simulate avian pathogenic *E. coli* infection, so we applied LPS reagents derived from *E. coli* O55:B5 and used embryonic eggs for immune challenge modeling. Compared with animal infection models, the embryonic egg model has the advantages of being more standardized, reproducible, and easy to obtain samples. Oral LPS would imitate the inflammatory response in two ways. Firstly, LPS activates immune cells in the gut, such as macrophages and dendritic graph cells, leading to the production of pro-inflammatory cytokines by intestinal tissues, and LPS that pass through the intestinal barrier into the bloodstream causes systemic inflammation and immune response (Di et al. 2024). The *NE* model was constructed by administering *Clostridium perfringens* orally after pre-infection with *Eimeria coccidia*, which disrupts the intestinal epithelium and increases intestinal mucus, providing nutrients for *Clostridium perfringens* and promoting rapid colonization and increased pathogenicity (Song et al. 2023). We chose a wild type A *Clostridium perfringens* identified as Net B toxin-positive isolated from the broiler intestinal track as the attacking material, and the fact that the wild-type strain with a NetB mutant complementary to the NetB+ plasmid is thought to be able to induce *NE* ensures the validity of the model (Keyburn et al. 2008). In common with the LPS model, *Clostridium perfringens* is recognized by pathogen recognition receptors present on intestinal epithelial cells and cells of the innate immune system, activating the innate immune system. In addition, *Clostridium perfringens* activates B-cell and T-cell mediated adaptive immunity (Fathima et al. 2022).

1.2.2. Zinc and intestinal health

Broilers are selected for efficient production of animal protein, and thus modern broilers have a low feed conversion ratio (FCR). The intestine is key to the efficient absorption of nutrients in broilers, and good intestinal health is critical to the economic value of broilers, as approximately 70% of the cost of broiler production is derived from feed costs. In addition, as an important immune organ, the gut provides a physical line of defense against invading pathogens (Zhu et al. 2021). Intestinal barrier (IB) and intestinal permeability (IP) are standard parameters employed to assess intestinal health. IB consists of a physical barrier composed of intestinal epithelial cells, tight junction proteins, and mucins, a chemical barrier composed of cytokines and immune cells, and a microbial barrier composed of microbiota. The increase in IP means that the intestinal barrier is more permeable and more harmful substances pass through the intestine into the circulatory system (Yegani et al. 2008).

In this thesis, both an LPS challenge and an *NE* challenge led to a decrease in jejunal barrier function, and *NE* also led to an increase in intestinal lesion scores, while heat stress reduced the diversity of cecum microbes in broilers, suggesting that IB function was impaired by the challenge. Similarly, according to the results in Chapter V, *NE* challenge increased FITC-d concentrations in broiler serum after oral administration of FITC-d, suggesting that the *NE* challenge increased IP. Interaction effects indicated that zinc glycinate reduced the increased ileal lesion score due to *NE* and the decreased expression of Claudin-1 due to LPS, whereas both zinc glycinate and zinc sulfate reduced FITC-d concentrations in the serum

of challenged broilers. These results suggest that in the challenged state, zinc glycinate improved IB and IP, whereas zinc sulfate only improved IP function. Therefore, organic zinc seems to be better at improving gut health. However, in any case, we can conclude that intestinal health is impaired whether by heat stress, Gram-negative infections (LPS challenge) or *NE* challenge, which is manifested by reduced intestinal barrier function, microbiota disruption, and increased IP, and that zinc, and especially zinc glycinate, protects against impaired intestinal health due to these challenges.

1.3. Role of zinc in challenging status

Zinc is an important nutrient required for the growth of broilers and is involved in metabolic functions as a cofactor for many enzymes. Zinc has an excellent performance in antioxidant function, as it participates in the formation of superoxide dismutase (SOD). Metallothionein (MT) has a zinc binding function and neutralizes free radicals generated by oxidative stress by providing a zinc ion donor (Formigari et al. 2007). We observed that broilers suffered from oxidative stress in all challenge models described in this thesis. Under *NE* challenge, both zinc glycinate and zinc sulfate reduced malondialdehyde (MDA) concentration in the jejunum, showing improvement in oxidative damage due to the *NE* challenge. However, zinc did not improve the antioxidant capacity of the embryos under LPS challenge, which may be due to the underdeveloped gut barrier of the embryos, and since the yolk sac assumed most of the energy metabolism functions of the liver at this stage, it is implied that the antioxidant system of the chicken embryos was not fully developed. It is also worth discussing whether different doses of dietary zinc can cause a reduction in oxidative stress. Although there is no concentration gradient for zinc in Chapter V, we can still make some reflections: the addition of an extra 60 mg/kg of both sources of zinc was able to increase serum T-AOC levels and jejunal CAT and GSH-px activities compared to the basal dietary treatment containing 30 mg/kg of zinc, suggesting an effective effect of higher zinc levels on antioxidant capacity.

Zinc is closely related to both innate and adaptive immune functions. Research has shown that zinc plays a role in the differentiation of natural killer (NK) cells and the maturation of macrophages, and zinc also has an important influence on the formation and differentiation of T lymphocytes (Weyh et al. 2022). Our study focuses on the immunomodulatory role of zinc. In Chapters IV and V, we analyzed the content of inflammatory cytokines, immunoglobulin levels and the NF-κB pathway in relation to the supplementation of zinc in the challenged state. Zinc glycinate reduced the level of pro-inflammatory factor IL-1β in chick embryos challenged with LPS and modulated the LPS-induced inflammatory response through the TLR4/NF-κB pathway. In contrast, both zinc glycinate and zinc sulfate significantly increased IgA levels in the jejunum under *NE* challenge later in life. These results confirm the activation and promotion of zinc on the immune function in embryos and broilers under challenged status.

Heat stress has been well studied on broiler meat quality, and the negative effects of heat stress on meat quality include a decrease in water holding capacity (WHC), alteration of myogenic regulatory factors, which in turn affects protein synthesis, and an increase in fat synthesis leading to poorer meat quality (Nawaz

et al. 2021). The effects of heat stress on meat quality in broilers may be caused by a decrease in feed intake resulting in low glycogen reserves and lipid damage from oxidative stress. Study have shown that oxidative stress induced by an LPS challenge reduced WHC and increased shear force in chicken meat (Wei et al. 2022). So far, no published study has been conducted examining the effect of an *NE* challenge on meat quality. The results in Chapter VI suggest that *NE* negatively affects the chicken meat color and zinc sulfate was able to ameliorate the cooking losses resulting from an *NE* challenge. In addition, *NE* caused lipid peroxidation of the meat and the supplementation with zinc was able to reduce the lipid peroxidation value of the pectoral muscle after five days of storage. Zinc glycinate also significantly down-regulated the expression level of the fatty acid oxidation gene *PPAR-α* in the liver under *NE* challenge. These findings suggest that the *NE* challenge induced a decrease in meat quality and that the underlying reason for the positive effect of zinc might be due to its function in antioxidant capacity and fatty acid metabolism.

1.4. Zinc Bioavailability

Zinc bioavailability is usually determined by zinc retention and zinc digestibility in the anterior cecum, and zinc-related markers such as MT also validate zinc retention at the cellular level (Hall and King. et al. 2023). Zinc bioavailability is influenced by a complex set of factors such as diet composition, health status and age of the animal. In this study, we examined zinc levels in serum, tibia, tissues and cecum contents for the assessment of zinc bioavailability, as well as zinc-related protein and enzyme markers, with the aim of evaluating the effects of different sources of zinc in different challenged states of broilers with regard to zinc bioavailability.

1.4.1. Advantages of organic zinc

Zinc has been added to animal feed as a trace element for decades, with sources of zinc including inorganic and organic zinc, and in recent years nano-zinc oxide has been frequently discussed. There are various inorganic forms of zinc, mainly including zinc oxide, zinc acetate and zinc sulfate, while organic zinc refers to amino acid chelated zinc, commonly known as zinc glycinate and zinc methionine (Wedekind et al. 1992). In our study, we used zinc sulfate as a source of inorganic zinc and zinc glycinate of moderate chelating strength as a source of organic zinc, both of which are widely used in broiler feed based on lower prices and standardized processing (Duffy et al. 2023). It is often assumed that organic zinc has a better bioavailability due to the fact that organic zinc avoids the adsorption of zinc ions by phytate molecules in the coleoptile of seeds and grains during intestinal passage, and there are hypotheses suggesting that organic zinc could be absorbed via the amino acid absorption pathway in the intestinal tract.

The ability of zinc glycinate to reduce FCR in broilers during the first three weeks was demonstrated in chapter V, and zinc glycinate supplementation also showed an improved intestinal barrier function. Zinc glycinate has a better bioavailability from the point of view of tibia retention, and zinc levels in the cecum content confirm the superiority of zinc glycinate over zinc sulfate. In Chapter VI, we also find that zinc glycinate has a better performance in terms of

improving the function of lipid oxidation genes in the liver and meat quality. These properties are due to the fact that zinc glycinate has probably a better efficiency of being absorbed and therefore more zinc is utilized by the tissues. In addition, we cannot ignore the fact that the effect of zinc glycinate on gut health may also be responsible for improved production performance. Based on the above benefits, organic zinc is recommended in broiler feed.

1.4.2. "Real" Zinc Requirement

We investigated the effects of different challenge models, such as broiler heat stress, embryonic LPS infection and *NE* challenge models, on broiler zinc homeostasis in Chapters III-V, respectively. As we discussed previously, zinc has antioxidant and immunomodulatory functions, so we want to clarify whether zinc requirements are elevated in the challenged state, which can serve as a guiding value for the level of zinc to be added to the diet. Firstly, zinc needs to be absorbed in the intestinal lumen and the efficiency of digestion and absorption of zinc from different sources in the gut indicates the extent to which zinc is available at the dietary level. In a challenged state, zinc is needed to improve gut barrier function more than in a healthy state, and thus additional zinc is needed. Concentrations of zinc and zinc markers in blood and tissues can reflect the zinc requirements (Hall et al. 2022).

Tibia zinc level content, a commonly used marker of zinc absorption rate, was increased and the liver MT level was up-regulated under heat stress state, suggesting that more zinc is mobilized in the liver due to the broiler's resistance to heat stress induced by high temperatures. Hypozincaemia was induced by both the LPS challenge and the *NE* challenge implying that blood zinc was depleted in large quantities during the challenged state. These pieces of evidence suggest that zinc requirements increase in the challenged state of poultry. Changes in zinc requirements in broilers under different challenges are hard to quantify without specific data because the absorption capacity of the intestine and the rate of zinc transport in the blood and liver are limited by several factors, but the reasons for the increase in zinc requirements deserve to be discussed. Intestinal challenges cause damage to the intestinal epithelium, disruption of the integrity of the barrier function, and reduced nutrient absorption, so better absorption efficiency and higher levels of zinc above the nutrient requirement are necessary. A range of oxidative stress and immune responses occur in challenged broilers, and the involvement of zinc in key enzymes involved in oxidative stress and in regulating immune function suggests that the "real" requirement for zinc is increased.

Serum zinc is a commonly used biomarker, but it fluctuates widely. Normal zinc supplementation increases serum zinc concentrations, but high zinc intake attenuates the extent to which serum zinc levels are raised. When the level of zinc intake is low, the body reduces endogenous zinc consumption to maintain plasma zinc level (Piacenza et al. 2021). A recent study suggests that serum cytokine levels associated with immune function may be more sensitive markers, with dietary zinc increasing cytokine and thymosin activity levels without changes in blood zinc concentrations (Costarelli et al. 2014). Apart from these, circulating fatty acids, zinc transporter protein expression and hair zinc content have also been used to assess the effect of dietary zinc. So how is zinc requirement assessed

at the individual level? Zinc requirements are met based on several aspects: intestinal absorption, blood transport and cellular consumption. As a mediator of zinc transport and a zinc reservoir for tissues, serum zinc remains an important reference indicator of the efficiency of intestinal zinc absorption as well as zinc consumption.

1.5. Zinc homeostasis and immunity in broilers

1.5.1. Zinc transport and storage

Zinc transporter proteins play a crucial role in maintaining zinc homeostasis and keeping the balance between zinc uptake and excretion. The intestine serves as an organ for zinc digestion and absorption, and zinc is absorbed through the intestinal epithelium into the portal vein to bind to albumin in the blood, a process that is carried out by zinc-regulated transporters (Zrts), iron-regulated transporter (Irt)-like proteins (Zips) on the parietal membrane of epithelial cells and zinc transporter proteins (ZnTs) on the basolateral membrane (Qin et al. 2013). Different transporter proteins have different functions in the zinc transport mechanism, with differences arising from the proportion of transporter proteins in different tissues and the different modes of transport of different transporter proteins, and these studies are currently being continuously updated (Yin et al. 2023). The homeostatic regulation of zinc also depends on the presence of MT with zinc affinity, which is involved in the release and storage of intracellular zinc ions when zinc requirement increases or decreases. MT is regulated by the metal responsive element binding transcription factor (MTF-1), which acts as a zinc sensor and senses zinc concentration within the cell (Jia et al. 2021). The results of Chapter III showed that heat stress upregulated the gene expression of *ZnT1*, *Zip8*, and *Zip14* in the jejunum, suggesting that heat stress enhances intestinal zinc transport. The liver upregulated the expression of *Zip3* and *MT* to increase zinc storage to resist oxidative stress. In addition, the expression of zinc transporter proteins and zinc intake were also correlated, with zinc glycinate increasing gene expression of *ZnT1* in the jejunum of broiler embryos. However, the results of Chapter V showed that zinc supplementation did not significantly affect the level of jejunal zinc transporter gene expression in broiler chickens. Zinc transporter proteins are regulated by a complex mechanism with many influencing factors, which may be related to the basal zinc concentration in the diet. *NE* challenge upregulated zinc transporter genes in the jejunum, as well as *MTF-1* and *MT* gene expression. The effect of the *NE* challenge on zinc transporter protein and *MT* expression levels was due to reduced cellular zinc concentrations resulting from oxidative stress. Thus, MT and zinc transporter proteins play important roles in zinc digestion and absorption, storage and regulation of zinc concentration.

1.5.2. Association of zinc homeostasis and immunity

Zinc homeostasis is closely related to the regulation of the immune and antioxidant systems. During inflammation and stress, zinc is transferred from the blood to the liver to activate the immune system to fight pathogens, a mechanism that has not yet been clearly explained (Wessels et al. 2015). It has been shown that *Zip14* is upregulated during this process in response to inflammation, and a

knockout of the *Zip14* gene in mice delays leukocytosis during immune stress, preventing the accumulation of zinc in the liver. It has also been shown that hypozincaemia due to chronic malnutrition is often accompanied by an accumulation of inflammatory factors and ROS, leading to tissue damage. This suggests the importance of zinc and zinc homeostasis for immune regulation. We verified this conclusion on challenged poultry, where the *NE* challenge was able to up-regulate the gene expression level of *Zip14* and zinc glycinate was able to mitigate the increase in cytokine IL-1β level under *NE* challenge, confirming the relevance of zinc homeostasis-associated zinc transporter proteins and zinc supplementation for immune function.

Zinc concentration in tissues also influences immune function regulation. A series of zinc finger proteins have been noted to be associated with immune function by mechanisms that inhibit the activation of the NF-κB pathway. One of these zinc finger proteins with a typical function is A20 (TNF-α inducible protein 3, TNFAIP3). Zinc supplementation leads to the upregulation of mRNA- and DNA-specific binding of A20 and decreases the expression of IL-1β and TNF-α (Prasad et al. 2011). Our results in Chapters IV and V confirm this view. In addition, increased liver zinc concentrations due to challenge-induced remodeling of zinc homeostasis can also illustrate the close association between zinc homeostasis and immune regulation. Inflammatory responses induce ROS production, leading to oxidative stress. Both dietary zinc and challenge factors could cause changes in zinc homeostasis, with zinc synthesizing SOD in direct resistance to oxidative stress and intracellular MT releasing zinc ions involved in oxidative stress and immune response.

2. General conclusion

This thesis explored the effects of common challenges in broiler production on broiler performance, zinc homeostasis, immune function and meat quality, and elucidated the important role of zinc during challenges in a broiler's life. Firstly, we confirmed the adverse effects of heat stress and *NE* challenges on broiler performance, as evidenced by reduced growth performance. In addition, zinc homeostasis was remodeled, suggesting that zinc plays a key role in adapting to external challenges. Specifically, each of these challenge models resulted in increased zinc requirements and modulation of zinc homeostasis, with zinc transporter proteins and MT playing a key role in this process, and zinc finger proteins A20 and MTF-1 also functioning to regulate zinc homeostasis. Zinc attenuated the inflammatory response and impairment of intestinal barrier function induced by immune challenge through the TLR4/NF-κB signaling pathway. The *NE* challenge resulted in reduced meat quality, zinc improved meat quality by better meat color and lipid peroxidation, and both, the *NE* challenge and zinc impacted meat quality by modulating the function of the liver lipid metabolism. Organic zinc was more bioavailable, and zinc glycinate performed better in improving growth performance and intestinal barrier function in broilers at 60 mg/kg of zinc additions.

3. Perspectives

Although the present thesis confirmed the bioavailability advantage of zinc glycinate, the mechanism of the improvement of intestinal barrier function by zinc glycinate lacks sufficient knowledge. In the future, the mechanism of action of the pathways involved could be verified by cellular experiments. In tissues, zinc transporter proteins are regulated in response to stress or challenge, and the timing at which this adjustment occurs after the onset of stress and its duration in relation to nutritional immunity are issues worth investigating. It is also valuable to investigate how different sources of zinc regulate zinc homeostasis through zinc transporter proteins and MT expression in the intestine.

The study of meat quality is at the crossroads of the animal and food science industries, and we demonstrated that zinc and *NE* challenge affect meat quality by altering the function of lipid metabolism in the broiler liver. The main nutrition of meat is from protein, and meat processing characteristics and quality are also influenced by protein characteristics. Metabolomics results also reveal a range of amino acid anabolic pathways that are influenced by *NE* challenge and zinc, and this tuning may alter meat quality-related characteristics such as WHC and tenderness by affecting the type of muscle fibers, and the impact of these nutritional and challenge effects on meat quality deserves further investigation.

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Appendices

Scientific publications

Scientific publications

Adapted from the thesis:

1. **Xiao, C**., L. Kong, X. Pan, Q. Zhu, Z. Song & N. Everaert (2022). High Temperature-Induced Oxidative Stress Affects Systemic Zinc Homeostasis in Broilers by Regulating Zinc Transporters and Metallothionein in the Liver and Jejunum. *Oxid Med Cell Longev*, 2022, 1427335. doi:10.1155/2022/1427335. (**Chapter III**)

2. **Xiao, C**., L. Comer, X. Pan, N. Everaert, M. Schroyen & Z. Song (2024). Zinc glycinate alleviates LPS-induced inflammation and intestinal barrier disruption in chicken embryos by regulating zinc homeostasis and TLR4/NF-kappaB pathway. *Ecotoxicol Environ Saf*, 272, 116111. doi: 10.1016/j.ecoenv.2024.116111. (**Chapter IV**)

Other publications:

1. **Xiao, C**., Zhang, L., Zhang, B., Kong, L., Pan, X., Goossens, T., & Song, Z. (2023). Dietary sodium butyrate improves female broiler breeder performance and offspring immune function by enhancing maternal intestinal barrier and microbiota. *Poult Sci*, 102(6), 102658.

2. **Xiao, C**., Li, K., Teng, C., Wei, Z., Li, J., Zhang, S & Zhong, R. (2023). Dietary Qi-Weng-Huangbo powder enhances growth performance, diarrhoea and immune function of weaned piglets by modulating gut health and microbial profiles. *Front Immunol*, 14, 1342852.

3. **Xiao, C**., Zhu, Q., Comer, L., Pan, X., Everaert, N., Schroyen, M & Song, Z. (2023). Dietary 25-hydroxy-cholecalciferol and additional vitamin E improve bone development and antioxidant capacity in high-density stocking broilers. *J Anim Sci*, 101, skad369.