Dietary zinc source affects performance and intestinal health in broilers

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“Some beautiful paths can’t be discovered without getting lost.”

Erol Ozan

Annatachja De Grande (2020)

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PREFACE

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In order to meet the growing demand for poultry meat, broiler production has been intensified in the last two decades. Genetic selection for fast growing and feed-efficient hybrids characterized by high breast meat yield has led to an increased susceptibility of these production animals to diseases and various stressors. Development of the digestive system and maintenance of its integrity is of key importance in order to support the rapid growth of muscles and bones and to ensure overall performance. Management and nutrition play in important role in broiler's growth and health and dietary supplementation of vitamins and trace minerals is of interest in order to maximize performance. This thesis focusses on the dietary supplementation of zinc and the effect of providing zinc either as an inorganic or as an organic source. In the past, zinc was mainly supplemented to poultry diets as an inorganic source (ZnO, $ZnSO_4$, $ZnCl_2$). Recently, the supplementation of zinc as an organic source has gained interest because of the increased bioavailability as compared to inorganic zinc sources. Efficient zinc supplementation, through highly bioavailable zinc sources, is of interest to support animals health and growth and to reduce ecotoxicity. In order to evaluate the use of a more readily available zinc source, a first study (chapter 1) was performed to compare the effect of supplementation with either $ZnSO_4$ (inorganic zinc source) and a zinc amino acid complex (ZnAA) on growth performance, intestinal morphology, microbiota composition and oxidative stress parameters in plasma. In parallel with this performance study a digestibility study was performed in order to evaluate the effect of zinc source on nutrient digestibility and faecal zinc excretion. In a second study (chapters 2 and 3) the interaction of zinc source and vitamin E level was studied and effects on performance, intestinal health and meat quality were monitored when a chronic cyclic heat stress model was applied in the finisher phase. The main findings are then discussed and compared to information available in literature in the general discussion section.

GENERAL INTRODUCTION

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1.1 Broiler production and its challenges

For decades broilers have been selected for increased weight, higher breast yield and improved feed conversion ratio (Zuidhof et al., 2014). This in order to meet the growing customer demand for broiler meat, and in parallel to optimize broiler production and to increase profits for farmers (Zuidhof et al., 2014, Petracci et al., 2015). The last two decades an increased consumer preference for chicken meat, as compared to other types of muscle food, has been observed (Petracci et al., 2019). The increased consumption of chicken meat can be explained by its relative low-cost and highly appreciated sensory and nutritional properties (Estevez, 2015, Petracci et al., 2015, Wideman et al., 2016). Chicken meat is considered to be healthier as compared to red meats, which have been associated with carcinogenesis (Alisson-Silva et al., 2016, Bouvard et al., 2015, Rombouts et al., 2017). In addition, poultry meat complies with most religious and cultural principles. Intentional genetic selection of fast growing broiler hybrids has led to an increased susceptibility to various stressors including infectious, nutritional and environmental stressors (Cheema et al., 2003, Dierick et al., 2019, Liverani et al., 2013). Moreover, the high growth rates are strongly correlated with meat quality defects such as wooden breast, white striping and spaghetti meat (Petracci et al., 2019). The increased feed intake which is characteristic for the fast growing hybrids, also increases water consumption, which contributes to wet litter issues. The ban on antimicrobial growth promotors in the European Union has led to increased concerns on gut health issues due to dysbiosis and poor digestibility of feed. Impaired gut health can lead to wet litter, foot pad dermatitis and as a consequence to impaired animal welfare. As a consequence of global warming, the intensive broiler production faces constantly increasing environmental temperatures. High environmental temperatures negatively affects growth,

performance and meat quality and may lead to increased mortality. This leads to huge economic losses worldwide(Lara and Rostagno, 2013a, Lara and Rostagno, 2013b).

The last few years an increased interest of the consumer in sustainable animal production and welfare has been observed. As broilers are very efficient in conversion of feed to muscle mass and since broiler production uses less land and water for both farming and feed production, it is more sustainable and has a lower impact as compared to pork and cattle meat production (Flachowsky et al., 2017). Therefore the main challenges in modern poultry production not only constitute of disease control, prevention of gut health issues and control of environmental conditions (e.g., heat stress) but also includes maintenance of high meat quality.

1.1.1 Diseases in broiler production

Diseases interfere with the normal functioning of the cells, tissues, organs and whole body systems. Diseases resulting from nutrient deficiencies or consumption of toxic substances are referred to as non-infectious diseases and cannot be passed from bird to bird. Infectious or contagious diseases are caused by micro-organisms that include parasites, fungi, protozoa, bacteria and viruses. Oxidative stress is a characteristic feature of many infectious and non-infectious diseases (Swayne, 2013). Oxidative stress is defined as a disturbance of the balance between the production of free radicals and antioxidant defence systems, which leads to an imbalance between pro- and antioxidants (Betteridge, 2000).

Most occurring viral infections are Gumboro, Newcastle disease, Avian influenza and Marek's disease (Swayne, 2013). Viral infections can be divided in low pathogenic and high pathogenic classes based on the severity of the illness and the risk of spreading. Common bacteria causing problems in broiler production are *Salmonella sp.*, *Escherichia coli*, *Campylobacter sp.*, *Clostridium perfringens*, *Enteroccocus cecorum* and *Mycoplasma*.

Coccidiosis is a disease that is caused by protozoan parasites of the genus *Eimeria*, developing within the intestine of most domestic and wild animals and birds (Swayne, 2013). It is considered as the most economically important parasitic condition in poultry production. Seven species of *Eimeria* (*E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox and E. tenella*) are recognized as pathogenic for poultry (DeGussem, 2007). The infection pressure is related to the immune status of the birds, management and stocking density. Disease control can, depending on the pathogen, consist of vaccination strategy, good management and strict biosecurity measurements and in some cases antimicrobials.

1.1.2 Thermal stress

Due to climate change more animals are subjected to environmental stress (heat and cold stress) (Lara and Rostagno, 2013a). The intensive selection for increased performance in broilers has led to a higher metabolic heat production and increased susceptibility to high ambient temperatures (Mohammed et al., 2018, He et al., 2018c). Moreover, high stocking densities also cause thermal stress. Heat stress occurs when the amount of heat that is produced in the animal's body exceeds the capacity to dissipate heat. This causes an increase of the body core temperature and affects birds' physiology, albeit as a consequence of high environmental temperatures (Farag and Alagawany, 2018, Nawab et al., 2018, Sahin et al., 2009). It is well established that heat stress induces oxidative stress. During heat stress, highly reactive radicals are produced in tissues and can modify proteins, lipids and nucleic acids, which might eventually lead to cell death due to oxidative damage (Akbarian et al., 2016).

Heat stress can be either acute or chronic. Acute heat stress refers to a short and rapid rise in ambient temperature, whereas chronic heat stress refers to exposure to a high ambient temperature over a long period of time. Chronic heat stress can either be continuous or cyclic (Akbarian et al., 2016). Both acute and chronic heat stress have detrimental effects on overall health, which leads to impaired performance in broilers (Zhang et al., 2012). Moreover, it has

been reported that heat stress has a negative impact on intestinal morphology and gut barrier integrity in broilers (Zhang et al., 2017, Song et al., 2014, Wu et al., 2018), which are key elements in health and performance (Ducatelle et al., 2018). Additionally, heat stress deteriorates meat quality which leads to even more economic losses. Due to the global warming, the broiler industry has to face constantly increasing temperatures, therefore a lot of research is invested in both physical cooling strategies and nutritional interventions as a preventive measure (e.g., pad cooling and atomizing nozzles). Physical cooling strategies can increase fixed costs and genetic selection is a long term process (He et al., 2018b). Nutritional interventions by adding supplements to the feed do not require expensive investments nor structural changes to the feed composition and therefore constitute an attractive additional strategy on top of the aforementioned strategies. Cold stress also negatively impacts growth and meat quality (Ipek and Sahan, 2006, Dadgar et al., 2012) and significantly increases the incidence and severity of necrotic enteritis lesions as compared to non-challenged birds (Tsiouris et al., 2015).

1.1.3 Meat quality defects

In order to fulfill the increasing demand for poultry meat, selection programs have been carried out to improve the production traits of broilers. Hybrids with high growth rate and breast-yield have been developed. In the last ten years meat quality defects such as wooden breast, white striping and spaghetti meat emerged together with this evolution towards increased productivity (Figure 1). White striping can be macroscopically observed as white striations in the direction of the muscle fibers in both breast and thigh muscles. The microscopic evaluation of white striations reveals an accumulation of lipids and connective tissue (Kuttappan et al., 2013). Wooden breast myopathy seems to start as a focal lesion at the age of two weeks and develops as a widespread fibrotic injury. Both pectoralis major and minor can be affected and are characterized by a pale appearance and hardened consistency.

Microscopic evaluation shows necrotic and degenerating muscle fibers, connective tissue accumulation and infiltration of inflammatory cells (Sihvo et al., 2014). Recently Baldi et al. (2018) reported an additional myopathy, referred to as spaghetti meat, which is characterized by an overall impaired integrity of the pectoralis major muscle. The term spaghetti meat is referring to the separation of the muscle fibers composing the muscle tissue, resembling the appearance of the popular pasta. White striping and wooden breast may vary in severity and white striping can co-occur with both wooden breast and spaghetti meat.

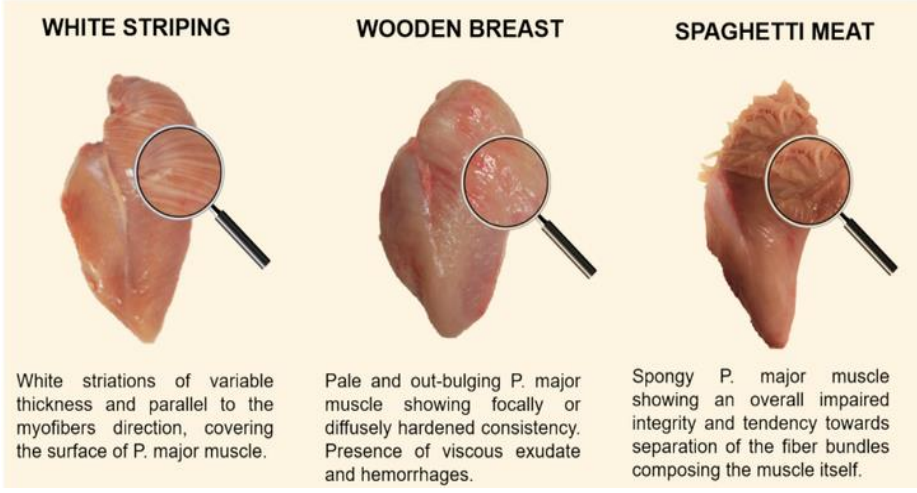


Figure 1: Macroscopic appearance and main common traits of broiler Pectoralis major affected by breast meat abnormalities.

These myopathies have been be linked to rapid muscle growth, insufficient vascularization and oxidative stress, which may lead to tissue degeneration (Kuttappan et al., 2012a, Papah et al., 2018, Sihvo et al., 2018, Soglia et al., 2019). A study performed by Alnahhas et al. (2016) showed that white striping prevalence was correlated with increasing body weight and high breast-yield hybrids. A strong genetic determination was found for the development of wooden breast and white striping, comprising a complex etiology and polygenic inheritance (Alnahhas et al., 2016, Mutryn et al., 2015, Papah et al., 2018, Pampouille et al., 2018, Zambonelli et al., 2016). The genetic predisposition is characterized by an increased muscle

fiber diameter and length, increased myofiber number, reduction in capillary density and capillary to fiber ratio and reduced numbers of satellite cells (Alnahhas et al., 2016, Clark and Velleman, 2016, Daughtry et al., 2017). Muscle fiber hypertrophy and decreased capillary density lead to reduced oxygenation and nutrient transport. Moreover, a decreased number of satellite cells impairs muscle regeneration (Petracci et al., 2019). So, a number of studies shows that breast myopathies are correlated with the genetic selection towards increased body weight and breast meat yield. Thus, this implies that reducing growth rate might be able to reduce the incidence and severity of these myopathies. In contrast, Bailey et al. (2015) found low genetic correlation between breast myopathies and body weight/breast yield, and that there is also a very important role laid out for management and dietary factors. The latter could explain why a high variation in incidence and severity has been observed between different flocks.

All of the aforementioned myopathies affects chemical composition and, functional and sensory properties of the meat. Functional properties of meat are ascribed to the ability of myofibrillar proteins to hold water, emulsify lipids and form stable gels. The functionality of meat proteins depends on the protein composition, the three-dimensional structure and the complex fibrillary architecture (Pearce et al., 2011). Disturbance of protein composition and integrity impairs the ability to interact with other biomolecules (such as lipids) and water (Estevez, 2011). Moreover, oxidative damage to proteins may also impair water holding capacity and subsequently impair the ability to form emulsions and gels. Meat of affected breasts is particularly characterized by a low water holding capacity (Petracci et al., 2019). Wooden breast is characterized by impaired marinade uptake and increased drip and cooking losses (Dalgaard et al., 2018, Mudalal et al., 2015, Soglia et al., 2016, Kuttappan et al., 2012a). Spaghetti meat is characterized by a decreased water holding capacity and an increased protein oxidation, which results in a strongly impaired functionality. Breast meat

presenting myopathies has an increased fat, moisture and collagen content and a decreased protein content, which lowers the nutritional value (Petracci et al., 2014, Baldi et al., 2018). No harmful chemical substances have been found, although the three myopathies are also associated with increased levels of lipid and protein oxidation products, which might be noxious to humans (Esterbauer, 1993, Estevez, 2011, Estevez and Luna, 2017). Recently, Boerboom et al. (2018) reported that arginine conversion into citrulline (e.g., arginine-nitric oxide pathway) was one of the most impaired metabolic pathways in breasts with severe white striping. As l-arginine could be converted stoichiometrically into citrulline and nitric oxide by means of the enzyme nitrix oxide synthethase (Khajali and Wideman, 2010). Nitric oxide has been identified as a possible vasodilator that could enhance blood flow to the muscles and can improve oxygen supply and removal of catabolites (Khajali and Wideman, 2010). Therefore, dietary supplementation of arginine has recently gained more interest in order to mitigate the occurrence and severity of breast meat myopathies in broilers (Zampiga et al., 2019). For instance, Zampiga et al. (2019) showed that an increased supplementation of L-arginine with approximately 30% as compared to the recommended dose for fast growing broilers (Aviagen, 2014) was able to attenuate the incidence and severity of WS and SM abnormalities, while it had no significant effect on WB.

Measures to alleviate occurrence and severity of the above described myopathies have mainly focused on nutritional interventions. Dietary supplementation with antioxidants (e.g., vitamin E and selenium) and organic trace minerals (e.g., zinc) (Sirri et al., 2016, Kuttappan et al., 2012b, Sihvo et al., 2017), can reduce muscle fiber oxidative stress associated with breast abnormalities (Petracci et al., 2019). However, nutritional interventions do not always have the anticipated effect, the reduction of severity and prevalence of the aforementioned myopathies are mainly an indirect result of decreased growth, slaughter weight and/or decreased breast yield (Livingston et al., 2019, Meloche et al., 2018a). Management factors

that modify the growth rate have been explored in the past. Kuttappan et al. (2012a)used a low energy diet and showed that the percentage of wooden breast could be lowered from 75% to 53% in broilers of 54 days old by lowering the energy value of the diets (0.5% poultry fat as compared to 6%). Another strategy in order to control the growth rate is to restrict the feed intake, both quantitative (Meloche et al., 2018b, Trocino et al., 2015) or time limited feed restriction (Livingston et al., 2019) has been explored to control growth and to reduce the occurrence of breast myopathies. Livingston et al. (2019) showed that the rate and severity of WS and WB could be reduced in feed restricted male chickens at the age of 42 days. However, it has been shown that early feed restriction has a negative effect on the incidence of breast meat myopathies due to the compensatory growth during the refeeding period compared to birds fed ad lib during the whole period (Trocino et al., 2015). Gratta et al. (2019) investigated the effect of early and late feed restriction and found no effect on myopathy occurrence at slaughter age and suggested that myofibril degeneration (which is at the base of breast myopathies) slows down when growth is reduced, but restarts as soon as the growth rate increases again. So, feeding strategies to control growth based on the reduction of nutrient intake do not consistently control the degree of myopathies.

1.2 Oxidative stress

As mentioned above, oxidative stress can be caused by infectious diseases and other stressors such as heat stress (Akbarian et al., 2016, Lykkesfeldt and Svendsen, 2007). Moreover, nutrient imbalances (such as micronutrient deficiencies) or harmful products (such as fat peroxides) can enhance oxidative stress (Lauridsen, 2019, Ravindran et al., 2016). Oxidative stress plays an important role in the onset of wooden breast, white striping and spaghetti meat (Petracci et al., 2019).

Oxidative stress can either be evaluated by (1) directly measuring reactive species, (2) measuring the resulting damage to biomolecules, and (3) detection of antioxidant levels. It seems logical to directly measure reactive oxygen or nitrogen species, however many reactive species are very unstable and therefore difficult to measure. As a consequence, measuring damage inflicted to biomolecules (such as DNA, RNA, proteins and lipids) is preferred. Markers for oxidative damage, such as malondialdehyde (end product of lipid peroxidation), tend to be extremely stable and provide a more reliable method to evaluate oxidative stress. Another possible approach is to measure levels and/or activity of antioxidant enzymes such as catalase, glutathione peroxidase and superoxide dismutase (Katerji et al., 2019).

1.2.1 Oxidative stress at the cellular level

Reactive oxygen and nitrogen species are produced as by-products during normal cell respiration, but are also generated during enzymatic reactions as summarized in (Figure 2) (Balaban et al., 2005).

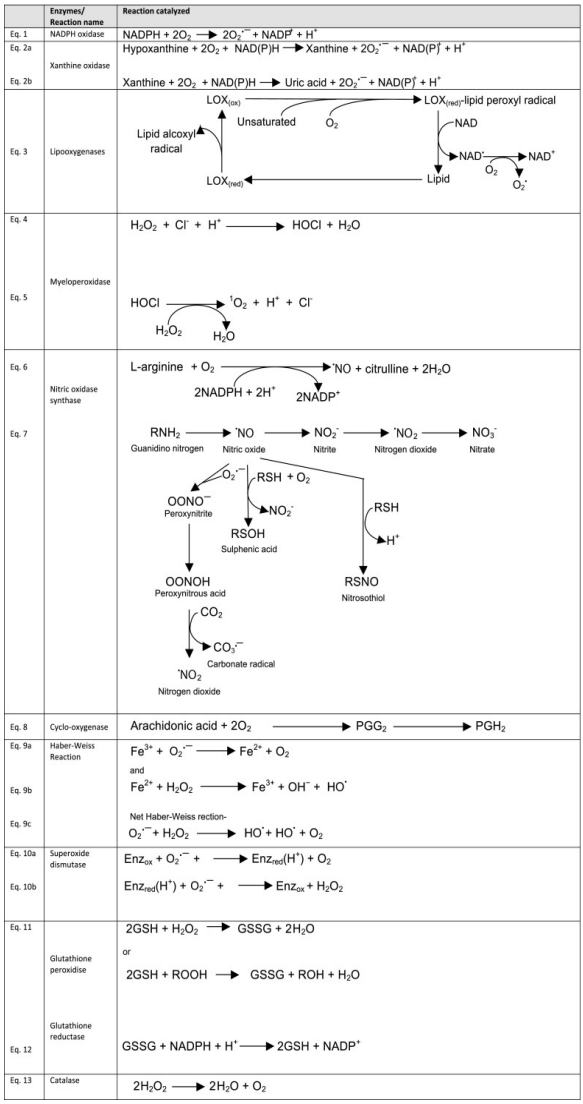


Figure 2: Major endogenous oxidative enzymatic reactions (Bhattacharyya et al., 2014)

Reactive oxygen species (ROS) include radical compounds such as superoxide (O_2^-), hydroxyl radicals (OH^-), lipid peroxides and reactive non-radical compounds including oxygen (O_2), hydrogen peroxide (H_2O_2), chloramines (RNHCl) and ozone (O_3) (Bedard and Krause, 2007). Reactive Nitrogen Species (RNS) comprise of reactive radical compounds such as nitric oxide (NO), nitrogen dioxide (NO_2) and non-reactive compounds such as peroxynitrite ($ONOO^-$) and dinitrogen trioxide (N_2O_3) (Bhattacharyya et al., 2014). In the healthy bird ROS and RNS are kept within physiological ranges by various enzyme systems. Under these conditions reactive species are not harmful and are strongly regulated by antioxidant mechanisms. Free radicals also work as physiological mediators and signalling molecules as illustrated in Figure 2 (Bhattacharyya et al., 2014). For a more in depth explanation on the role of ROS and RNS in normal physiological and pathophysiological conditions the reader is referred to Valko et al. (2007).

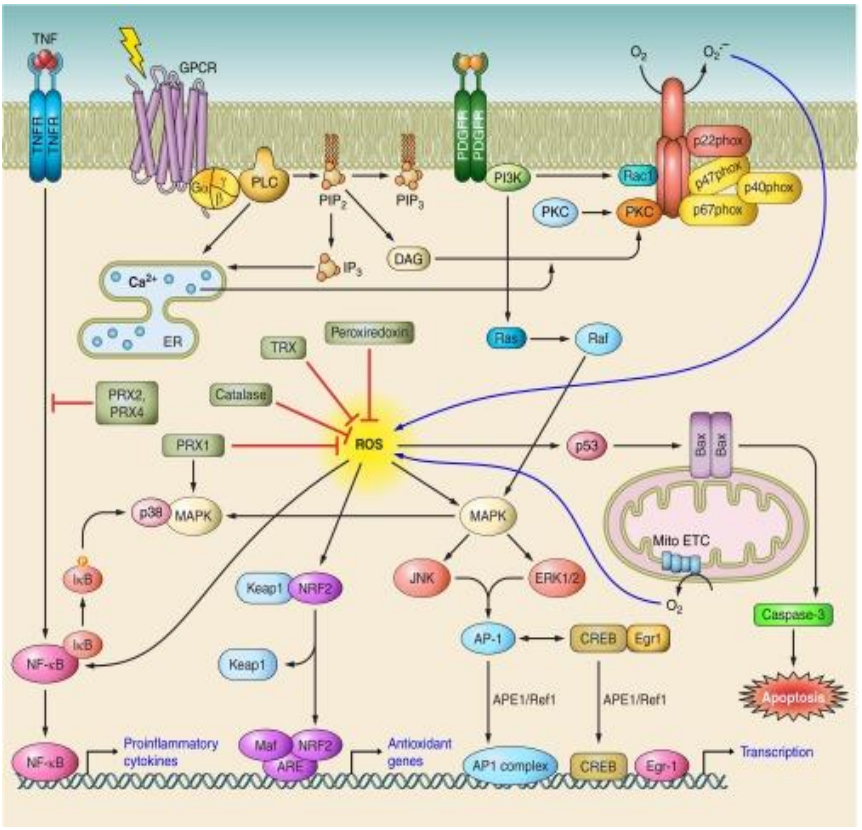


Figure 2: Schematic depiction of multiple signaling pathways that generate ROS and the intracellular events activated by ROS accumulation. Upon activation, G protein-coupled receptors (GPCRs) activate phospholipase C (PLC) leading to the activation of protein kinase C (PKC) molecules. Platelet-derived growth factor receptors (PDGFRs) activate phosphoinositide 3-kinase leading to activation of ras-related C3 botulinum toxin substrate 1 (RAC1). Both RAC1 and PKC activate membrane-bound receptors leading to membrane relocation and assembly of various components of phagocytic NADPH oxidases. Mitochondrial electron transport chain (mito ETC) is another robust source of intracellular ROS generation. ROS in turn lead to enhanced production of (APE1/Ref1) and activation of several signaling events including p53-mediated apoptotic events, mitogen-activated protein kinase (MAPK) pathways, NF-E2-related factor (NRF2)-mediated activation of genes containing antioxidant response element (ARE), and nuclear factor-κB (NF-κB). Transcription factors including AP1, NF-κB, cAMP response element-binding (CREB), and early growth response (EGR) protein, induced by these signaling events are kept in the active and reduced form by APE1/Ref1. Thus ROS signaling events play a central role in regulation of proinflammatory events, cell cycle, proliferation, and cell death. Antioxidant defense enzymes such as catalase, thioredoxins (TRX), peroxidases, and peroxiredoxins (PRX) contribute to preventing excessive levels of ROS from accumulating at the cellular and tissue level.

1.2.2 Oxidative stress as a consequence of various stressors in broiler production

Oxidative stress due to heat stress manifests in all body parts, with mitochondrial dysfunction as the major underlying mechanism (Figure 3) (Slimen et al., 2014, Akbarian et al., 2016). Acute heat stress initially increases mitochondrial substrate oxidation and electron transport activity, which results in excessive superoxide production (Akbarian et al., 2016). In a later stage of acute heat stress, down-regulation of avian uncoupling proteins exaggerates oxidative stress and leads to mitochondrial dysfunction and tissue damage (Akbarian et al., 2016). In case of acute heat stress, antioxidant enzyme activities are typically upregulated, whereas, in case of chronic heat stress an alteration in antioxidant activities is observed combined with depletion of antioxidant reserves (Akbarian et al., 2016). Moreover, chronic heat stress leads to lowered mitochondrial metabolic oxidative capacity and up-regulation of avian uncoupling protein (Akbarian et al., 2016). Extensive production of ROS in mitochondria damages proteins, lipids and DNA and reduces the efficiency of energy generation of the cell. A chain reaction of oxidized molecules extracting electrons from other molecules, ultimately results in extensive tissue damage (Akbarian et al., 2016). Lipid peroxides induce oxidative stress and inflammation which can be detrimental for intestinal health, immunity, growth and development (Engberg et al., 1996).

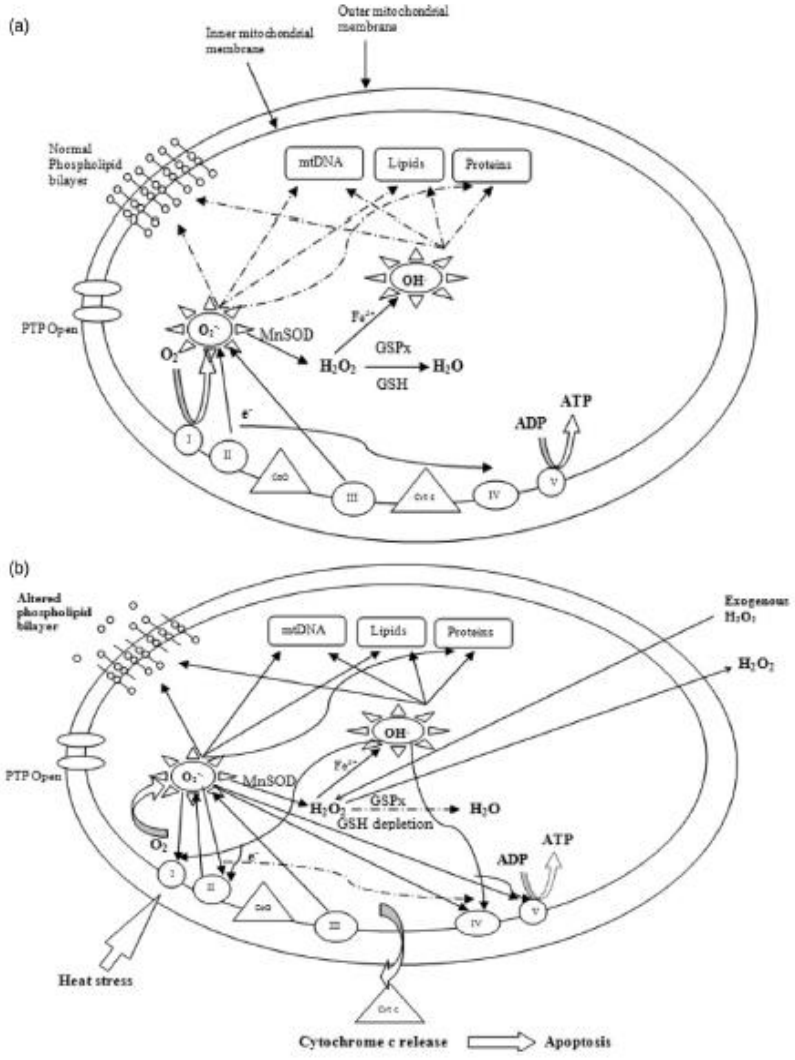


Figure 3: Generation and targets of reactive oxygen species in mitochondria. (a) A schematic diagram of ROS generation and their targets under thermal neutral conditions. Solid arrows indicate generation and diffusion of molecules. Dashed arrows indicate attenuated/blocked reactions. (b) An illustration of ROS generation and their targets under heat stress conditions. GSH depletion leads to increasing O.2 and OH. production, and therefore mitochondrial damage. From Slimen et al., 2014

Oxidative stress plays a dual role in infections. On the one hand it can protect against invading microorganisms (Lauridsen, 2019). On the other hand, oxidative stress occurring during inflammation promotes dysbiosis by promoting the outgrowth of specific taxa and decreasing microbial diversity in the gut (Hu et al., 2019). Intestinal inflammation is characterized by leukocyte infiltration, which is accompanied with the generation of reactive oxygen and nitrogen species (Weiss and Hennet, 2017a). The resulting oxidative stress exerts antimicrobial effects on strictly anaerobic bacteria, which leads to decreased microbial diversity (Lupp et al., 2007). Next to killing anaerobic bacteria, oxidative stress also promotes the selective growth of nitrate and tetrathionate respiring bacterial groups (Winter et al., 2010). Sulfate reducing bacteria are widespread in the gut and produce hydrogen sulphide and thiosulfate which can be oxidized to tetrathionate in the presence of ROS. An increase in tetrathionate in the gut promotes growth of certain *Enterobactericeae* including *Salmonella* and *Citrobacter* (Hensel et al., 1999). Additionally, the reaction of nitric oxide with superoxide anion yields peroxynitrile, which in turn can isomerize to nitrate, and this nitrate can be utilized by *Escherichia coli* (Winter et al., 2010, Gresse et al., 2017).

1.2.3 The antioxidant defence system

The antioxidant defence system, which is supposed to prevent oxidative stress mediated damage, acts on three levels (Ighodaro, 2018, Surai et al., 2018). The first level consists of three major antioxidant enzymes, namely, superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase, which are responsible for radical detoxification at the very beginning of their formation. Metal binding proteins also belong to the first level , since free iron and copper are catalysers of free radical formation. The second level of the antioxidant defence system comprises mainly of free radical scavenging antioxidants (vitamin E, ascorbic acid, glutathione, uric acid, etc.) with vitamin E being the major biological antioxidant in cell membranes. The third level of defence deals with damage repair of molecules or removal. Terminally damaged cells should be removed fast in order to prevent damage to other cells/tissues (Ighodaro, 2018). Members of the antioxidant defence system are located in all cell compartments (e.g., nucleus, mitochondria, cytosol) and are tissue specifically expressed (e.g., glutathione peroxidase has different isoforms with GPx2 primarily expressed in intestinal tissue) (Surai et al., 2018). Both internally synthesized antioxidants and antioxidants supplied in the diet belong to the antioxidant defence system. Many antioxidant enzymes are stress inducible and their expression and activity depends on stress intensity (Surai et al., 2018). Antioxidant enzymes have minerals as co-factors in order to be able to deal effectively with free radicals. Selenium is a co-factor for enzymes belonging to the family of the glutathione peroxidases and thioredoxin reductases. Zinc, manganese and copper are indispensable for the family of the superoxide dismutases and iron is the co-factor of the catalases enzyme family. Therefore these trace minerals need to be supplied in sufficient amounts to support the activity of these antioxidant enzymes. Vitamins (A, E and C) and phytobiotics are also frequently supplied in the feed as they are also known for their antioxidant properties (Akbarian et al., 2016, Horvath and Babinszky, 2019).

1.3 Zinc: an essential trace element

1.3.1 Functions of zinc provided at physiological levels

Although the first reports on the importance of zinc in organisms already date from the late 1800's (Prasad, 1984), it took until 1930 before the importance of zinc in animals was recognized (Todd et al., 1934). In 1960, it was reported that zinc might also be essential for human health (Prasad, 1991). Since then, there has been a growing interest in zinc biology and zinc supplementation in order to prevent zinc deficiency. Nowadays, it is well established that zinc is an essential trace element for all forms of life, and plays an important role in several biological processes (Frassinetti et al., 2006). Zinc contributes to the synthesis, stability and catalytic activity of proteins, to the nucleic acid metabolism, and to the function of the immune system (Maret, 2017b). At cellular level, it is involved in growth, proliferation, differentiation, integrity, respiration and cell death. Additionally, zinc plays an important function in wound healing and restoring the integrity of damaged tissues (Bonaventura et al., 2015b, Faa et al., 2008a). This myriad of functions demonstrates the major role of zinc in cell biology and the importance of strictly regulated zinc homeostasis. Zinc has a structural, catalytic or co-catalytic role in many proteins, therefore a disruption of the zinc homeostasis has an impact on a large number of biological processes with implications for the physiology of different organs and tissue (Ranaldi et al., 2013b).

Zinc supports the tertiary structure of enzymes in a manner analogous to disulphide bridges. As zinc also supports the tertiary structure in close proximity of the metal binding site, it can influence enzyme activity (Auld, 2009). In the catalytic function of enzymes, zinc is involved in the bond making or breaking step, and the co-catalytic function affects the catalysis and stabilization of the conformation of the active site (Vallee and Auld, 1993). Zinc also functions as an intracellular signalling molecule and plays a role in communication between cells (neurotransmission). Moreover, zinc can convert extracellular signals into intracellular

signals, and can act as a second messenger (Yamasaki et al., 2007). However, zinc is not a classical second messenger because it is not a short lived metabolite and has also many other functions. It also has a major role in genomic stability, due to its antioxidant effects, participation in DNA repair and DNA damage response and finally in the synthesis of molecules that are necessary for DNA methylation (Oteiza, 2012). So, zinc has a key role in the regulation of proliferation and differentiation of cells. As a consequence, tissues with a rapid cell turn-over such as the skin and the gastro-intestinal tract mucosa and immune system are most affected by an inadequate zinc supply (Bonaventura et al. 2015, Faa et al. 2008). To conclude, zinc is present in all enzyme classes, as well in transcription and replication factors, which implicates that zinc is active in all levels of cellular signal transduction. Therefore it is difficult to build a comprehensive list of the biological functions, in the next paragraphs (3.2. Zinc and oxidative stress and 3.3. Zinc and the immune system) its roles in antioxidant and immune defence system are highlighted. Additionally, the possible role of zinc in microbiota composition is discussed.

1.3.2 The role of zinc as an antioxidant

The generation of ROS is inherent to aerobic cell metabolism (Schieber and Chandel, 2014). It is well established that zinc plays different roles in protecting biological structures from damage by free radicals (Oteiza, 2012). It maintains adequate levels of metallothioneins, which are excellent ROS scavengers due to the high cysteine content (Ruttkay-Nedecky et al., 2013). Zinc is a co-factor of superoxide dismutase, which catalyses the dismutation of the superoxide radical and inhibits nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, causing reduction of the generated ROS (Prasad, 2014b). Furthermore, it is a protective compound for thiol groups and it prevents interaction between thiol groups and other chemical groups which may result in the loss of enzyme activity due to conformational changes. Moreover, zinc antagonizes redox-active transition metals (such as Fe and Cu).

Membrane associated iron and copper catalyse the generation of radicals from lipid peroxides and subsequently lead to protein and DNA damage eventually resulting in tissue damage.

1.3.3 The role of zinc in the immune system

The immune system is markedly susceptible to changes in zinc levels, because zinc plays a role in almost all immunological events (Gammoh and Rink, 2017). The immune response comprises of two mechanisms: the innate and the adaptive immune response. The cells of the innate immune system are the first cells to encounter invading pathogens. Amongst these first responders are polymorphonuclear cells, macrophages and natural killer cells. Chemotaxis and phagocytosis of polymorphonuclear cells is stimulated when zinc is supplemented. After phagocytosis pathogens are being destroyed by the activity of NADPH oxidases, which are inhibited by both zinc deficiency or excess (DeCoursey et al., 2003, Hasegawa et al., 2000). Moreover, before macrophages can mature into tissue resident cells, circulating monocytes need to be attracted to a specific tissue and adhere to the endothelial cells. It has been shown *in vitro*, that this process of adhesion is augmented by zinc supplementation (Haase and Rink, 2014). Additionally, recognition of the major histocompatibility complex class 1 by natural killer cells and the lytic activity of natural killer cells are influenced by zinc depletion (Haase et al., 2014).

Zinc deficiency is associated with an increased production of proinflammatory cytokines and impairs the adaptive immune response by causing thymic atrophy. Consecutively, T-cell lymphopenia and reduction of premature and immature B-cells occurs, which eventually leads to reduced antibody production (Haase et al., 2014). Zinc has an important role in the development of the T-helper 1 response, which is characterized by a cascade of cytokine secretion that stimulates immuno competent effector cells (Jarosz et al., 2017c).

Proper functioning of the immune system in poultry, ensures protection against infection by pathogenic organisms. Low zinc status increases susceptibility to infections and inflammation (Wong et al., 2013). There is also evidence that the animal's plasma proteins bind zinc in an attempt to prevent utilization of zinc by pathogens (Ibs and Rink, 2003). Troche et al. (2015) showed that exposure to coccidiosis increases the expression of zinc transporters (ZIP9 and ZIP13) and lowers intracellular zinc in cecal tonsils. This is most probably a defence mechanism of the cell. Current reports show that intracellular zinc homeostasis is critically associated with the signalling events in immune cells and that the intracellular zinc concentrations in these immune cells is regulated by changes in the expression of specific zinc transporters (Giacconi et al., 2012). Macrophages induced with lipopolysaccharide (LPS) also show changes in intracellular zinc, which are mediated by alterations in the expression level of the ZIP14 zinc receptor gene. Thus, alterations of zinc transporter expression due to LPS could potentially affect zinc homeostasis, and therefore might contribute to dysregulation of the immune system and chronic inflammation (Sayadi et al., 2013).

Damage inflicted to the intestinal mucosa might impair zinc uptake, resulting in a low zinc status, and therefore might also impair the immune response. Therefore it is reasonable to argue that when the absorptive capacity of the intestine is impaired due to an enteric infection, a more readily available source of zinc may be needed. It has been shown in previous studies that the expression of zinc transporters, responsible for regulating zinc homeostasis, are influenced by the zinc source in the presence of a challenge (necrotic enteritis) (Troche et al., 2015; He et al., 2018).

1.3.4 The role of zinc in microbiota composition

The structure and functionality of the intestinal microbiota is essential for maintaining intestinal and overall health. Intestinal health comprises morphological integrity, physiological functions of the gastro-intestinal tract (e.g., digestion and absorption of nutrients), tissue metabolism, energy balance, developed barrier function, efficient immune response, inflammatory balance, and adequate microbiota. Bacteria need trace minerals to support essential biological processes (Davis et al., 2009b). This is supported by the fact that 5% of the bacterial proteome is constituted of zinc binding proteins (Andreini et al., 2008). Dietary trace minerals, such as zinc, are rapidly complexed to host-derived molecules. Therefore, bacteria need to compete with their hosts to obtain the amount of trace minerals they need to survive. Additionally, there also is competition for zinc between bacterial species (Gielda and DiRita, 2012). Zinc uptake in bacteria is regulated by the zinc uptake regulator (Zur), which regulates intracellular zinc levels in bacteria by controlling zinc uptake. Zur represses the transcription of bacterial genes associated with zinc import, when zinc is abundant. Alternatively, in the absence of zinc, the transcription of these genes is upregulated (Outten et al., 2001). Under low zinc conditions, zinc is brought into bacteria through the ZnuABC transporter (Hantke, 2001). The ZnuABC transport system has a high affinity for zinc, and is important for virulence and host colonization in several bacterial pathogens, including *Escherichia coli*, *Salmonella enterica* serovar Typhimurium and *Campylobacter jejuni* (Campoy et al., 2002, Davis et al., 2009a, Lu et al., 1997, Patzer and Hantke, 1998).

Gut microbes may significantly affect metabolic processes, including the zinc metabolism (Reed et al., 2015, Zackular et al., 2016). During infection, host inflammatory responses limit microbial access to zinc ions (Lopez and Skaar, 2018). This metal withholding in the intestinal tract seems to affect both pathogenic and beneficial bacteria, and is orchestrated by metal chelating proteins. In human medicine, these metal chelating proteins, such as

lactoferrin and calprotectin, are often used as markers for gut inflammation (Walsham and Sherwood, 2016). This defense mechanism is referred to as nutritional immunity, which is defined as a process by which a host organism sequesters trace minerals in an effort to limit pathogenicity during infection. Paneth cells play an important role in this process, because zinc can be mobilized into intracellular secretory vesicles.

Circulating concentrations of minerals, such as iron and zinc, decline rapidly and dramatically during inflammation response upon infection (Hennigar and McClung, 2016). Hypozincemia has been considered as an effective strategy to limit pathogens from acquiring sufficient zinc for infection and proliferation in mice (Liuzzi et al., 2005). Similar results were observed in broilers challenged with *Salmonella* (Wu et al., 2019). Wu et al (2019) observed that hypozincemia and redistribution of zinc to the liver reduced the zinc bioavailability for *Salmonella*. In turn, this limited the replication and virulence gene expression of *Salmonella* (Campoy et al., 2002, Ammendola et al., 2007). These results indicate that zinc bioavailability at the intestinal level may significantly impact microbiota composition. Therefore, it is of interest to evaluate the effect of different zinc sources, characterized by differences in bioavailability, on bacterial community.

1.3.5 Zinc deficiency and toxicity

Both zinc excess as zinc deficiency can have detrimental effects on cells and tissues in both human and animals, therefore it is important to maintain physiological levels of zinc in the cell. Zinc deficiency can be caused by insufficient dietary intake, low bioavailability, interaction with other components and diseases or genetic predisposition. Minor changes in the bioavailability can already have clinical consequences and these will be most distinct in tissues with a high cell turn-over such as the skin, gastro-intestinal mucosa or the immune system (Ranaldi et al., 2013b, Faa et al., 2008a, Bonaventura et al., 2015b). Zinc might have cytotoxic effects when the zinc transport system fails and the buffering capacity has been exceeded, however this is very unlikely to happen as intracellular zinc levels are strongly regulated (Bonaventura et al., 2015b). This work focusses on supplementation of zinc within the physiological ranges and therefore effects due to excessive or insufficient zinc levels in feed do not fall within the scope of this work and will not be discussed in detail.

1.4 The benefits of zinc exist within a "Goldilocks zone"

1.4.1 The importance of a tight homeostatic control of free zinc ions

An excess of free zinc ions is cytotoxic; therefore, intracellular ionic zinc concentrations need to be tightly controlled (Krezel and Maret, 2016). The concentration of free zinc ions in the cytosol of the cells has been estimated to be in the range of a few hundred picomoles under steady state conditions (Krezel and Maret, 2006). This means they can only fluctuate in a very narrow range to avoid deficiency and toxicity. Although free zinc ions can be cytotoxic, recent research has revealed that they also have regulatory functions as zinc signals (Haase et al., 2015). Due to the importance of these zinc signals, there is the need for a tight homeostatic regulation within a very narrow zone, also referred to as a "Goldilocks zone" (Maret, 2017a).

Efficient zinc buffering is achieved by the binding of zinc to high affinity proteins and by transport processes that control the concentration of zinc (Figure 4). Zinc ions are hydrophilic and cannot permeate across the cytoplasmic membrane and the membranes of the intracellular compartments. Both cellular and whole body zinc is tightly regulated by zinc importers, zinc transporters, transcription factor MTF-1 (metal response element binding transcription factor-1) and metallothioneins (Kambe et al., 2015). However, next to zinc buffering, a second system helps to maintain zinc homeostasis, which is called "zinc muffling". During this process, excess zinc ions are transported via a still unidentified zinc transporter from the cytosol to a vesicular compartment. This allows cells to cope with larger zinc fluctuations. Importantly, both zinc buffering and zinc muffling systems have only a limited capacity, and when this capacity is exceeded, cell toxicity occurs (Colvin et al., 2010, Krezel and Maret, 2016). The number of zinc transporters is surprisingly high for the simple control of the homeostasis of a metabolite (Maret, 2017b). Recent advances in zinc biology have revealed

that zinc has an important regulatory function and that the change in free zinc ions acts as a regulatory signal. As zinc can be cytotoxic if not properly controlled, it needs a system to control these transient changes in free zinc concentration. Therefore, many additional transporter proteins exist and are necessary for: 1) the subcellular distribution of zinc, 2) the control of zinc homeostasis in organelles, and 3) the generation and control of zinc signals (Maret, 2017).

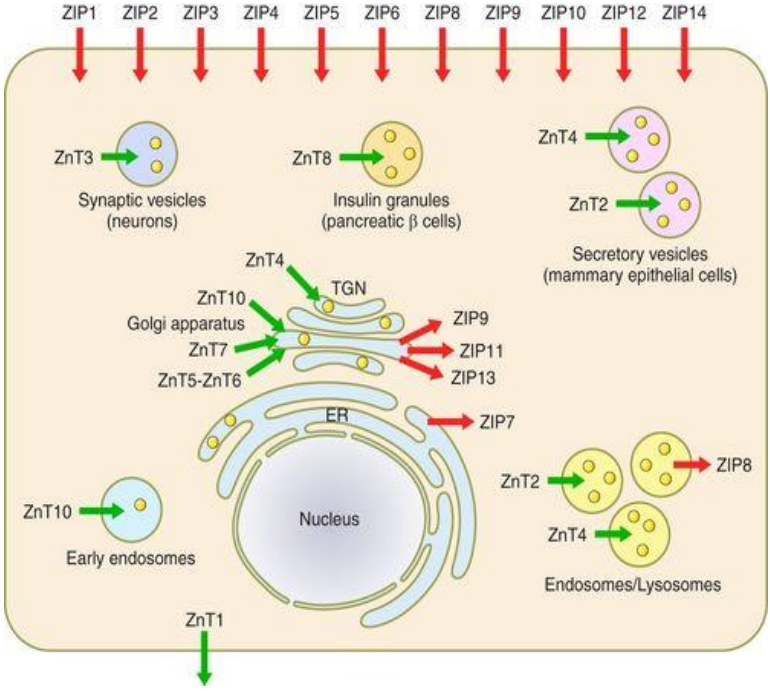


Figure 4: Localization and direction of ZIP (red arrows) and ZnT (green arrows) transporters. This figure illustrates a static view of their localization, because most ZnT and ZIP transporters dynamically change their subcellular localization in response to various stimuli. The cytosolic zinc is transported into or out of the different subcellular compartments, including the cell-specific secretory compartments such as synaptic vesicles or insulin granules. TGN, trans-Golgi network From: Kambe et al., 2015

1.4.2 The dual character of zinc: antioxidant or prooxidant?

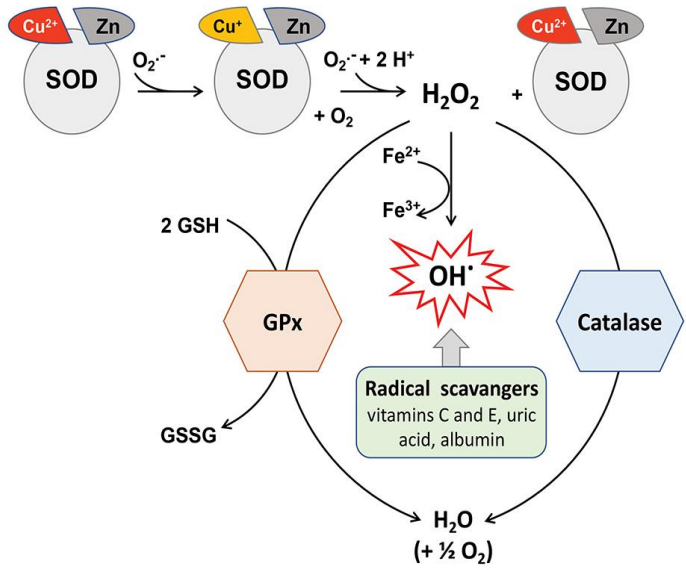
Zinc is a divalent metal and, unlike other metals such as iron or copper, zinc is a stable divalent cation and does not directly undergo redox reactions, owing to its filled d-shell. Hence, zinc ions cannot donate or receive a free electron, and as such is redox inert. However, this does not imply that zinc does not have antioxidant properties (Lee, 2018), this was discussed in paragraph "3.1. The role of zinc as an antioxidant". Numerous findings have linked zinc to the induction of enzymes involved in antioxidant defense systems and with antioxidant potential (Bray and Bettger, 1990, Jarosz et al., 2017c). Signaling with oxidative species (e.g., ROS and RNS) can release zinc ions from proteins, which links the redox metabolism to the zinc metabolism, and thus converts redox signals into zinc signals (Krezel and Maret, 2006). As zinc itself is not redox active, it will not directly bind ROS, RNS or other radicals (Bray & Bettger, 1990). The role of zinc as an antioxidant is evidenced by an increase in oxidative stress in case of disease or zinc deficiency. In case of low zinc, the increase of radicals might be attributed to a decreased activity of key enzymes, such as copper/zinc superoxide dismutase (Cu/Zn-SOD). Nevertheless, it must be noted that there is no correlation between Cu/Zn-SOD activity and dietary zinc intake or tissue zinc concentration. Alternatively, absence of zinc ions may lead to inactive proteins or misfolded proteins prone to aggregation (Bray & Bettger, 1990). Additionally, disturbance of induction of metallothioneins by zinc ions may be one of the mechanisms by which zinc exerts its antioxidant properties. Metallothioneins are able to neutralize hydroxyl radicals, even 300-fold more efficiently than glutathione. Metallothioneins bind zinc ions with high affinity and even more tightly, and at a higher concentration in comparison with other proteins (Maret, 2000). Complexation with zinc ions (and also cadmium) protects metallothioneins from degradation (Maret, 1994). A lower protection of the sulfhydryl groups of proteins against oxidation by zinc ions, might also play a role. Zinc ions can compete with other redox-active

ions for certain binding sites; due to this competition, the ability to transfer electrons and produce ROS, is induced. In case of zinc deficiency, there will be less competition, and subsequently more radical production (Barbato et al., 2007, Prasad, 2014a). Moreover, the indirect involvement of zinc as an antioxidant in many reactions has been reported. The zinc ion is a stabilizer of macromolecules and biological membranes, and minimizes their oxidative/peroxidative damage (O'Dell, 2000). Antioxidants such as gluthatione, SOD, and hemeoxygenase, can be induced by zinc ions ((Maret, 2003, Jarosz et al., 2017c). In contrast with the antioxidant properties of zinc, several prooxidant effects have been reported and are associated with increased zinc ions levels. Even if zinc overload only occurs rarely, it causes oxidative stress due to the suppression of metabolism and mitochondrial functions. The release of zinc ions from the binding sites of proteins such as metallothioneins, and/or from deposits in organelles such as mitochondria and lysosomes, will lead to secondary oxidative stress initiated by mitochondria and oxidant producing systems. Excess of free zinc ions cause oxidative stress, and eventually lead to cellular death, which has been observed in endothelial cells (Salazar et al., 2017). Moreover, tissue damage which that leads to a local increased release of zinc ions can lead to cytotoxic effects on neighboring cells. It is clear that the role of zinc as a prooxidant is induced by other factors that induce innate oxidative stress. So, an abrupt increase in zinc ions will most probably be the consequence of highly oxidative conditions or the failure of the zinc homeostasis. When considering the pro- or antioxidant effects of zinc, the time window needs to be taken in consideration. The enzymatic antioxidant systems will have a certain lag time to be able to respond after induction, while non-enzymatic systems are not largely affected by zinc. Inorganic zinc salts such as $ZnSO_4$, are absorbed by zinc transporters as described in paragraph 5.1., while ZnAA are absorbed by amino acid transporters. While inorganic zinc salts dissociate and then bind to the metal transporter to be transported to the cytosol of the cells, ZnAA bind with their amino acid

ligand to amino acid transporters and are transported as a complex. As zinc salts need to dissociate to be absorbed, there is a short time frame in which free zinc ions exist at the level of the intestinal epithelium. In this short time frame, they can bind to antagonists (phytate, fiber or other minerals) which render them unavailable for absorption, or they can exert cytotoxic/prooxidant effects.

1.4.3 Zinc and vitamin E, two important antioxidants that interact with each other's function?

As mentioned in the introduction of this thesis, oxidative stress is the underlying mechanism of frequently occurring stressors in poultry production (e.g., intestinal dysbiosis, heat stress and high stocking densities). Therefore, supplying antioxidants in the feed is important to ensure maximum growth and health (Pompeu et al., 2018). Both zinc and vitamin E, commonly added components in poultry diets, exert antioxidant properties. Vitamin E is a component of, and important antioxidant in cell membranes (Traber and Atkinson, 2007). Zinc also plays an important role in the stabilization of cellular membranes (O'Dell, 2000). As shown in Figure 5, both zinc and vitamin E play an important role in the antioxidant cascade and their function is related through the Zn/Cu SOD (Janciauskiene, 2020). One could question whether these antioxidants re-inforce or counteract each other's function.



Catalytic removal of free radicals and reactive species by superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and thiol-specific antioxidants. Myeloperoxidase (MPO) uses peroxide as a substrate via peroxidation or chlorination reaction to produce hypochloric acid (HOCl) and other toxic substances against different types of pathogens.

SOD=superoxide dismutase; GPx=glutathione peroxidase; MPO=myeloperoxidase; HOCl=hypochloric acid

Figure 5: Defense mechanism against free radicals: important role for both zinc and vitamin E. From: Janciauskiene (2020)

A limited number of studies looked into the interaction between zinc level and vitamin E. Bettger et al. (1980) investigated the effect of supplementing vitamin E to broilers fed a zinc deficient diet (5 mg/kg), and concluded that vitamin E might have helped to alleviate the negative effects of the zinc deficiency on skin lesions and oxidative status. Downs et al. (2000) showed that the supplementation of both vitamin E (48 mg/kg) and ZnAA (40 mg/kg) significantly reduced the severity and occurrence of cellulitis in broilers as compared to a non-supplemented control group. Combining both was also more efficient than when vitamin E or zinc were supplemented separately. Cardoso et al. (2006) investigated the effect of the supplementation of vitamin E (0, 12, 120 mg/kg) and chelated zinc (0, 40 and 400 mg/kg) on the humoral immune response upon Newcastle disease vaccination in broilers. Results of this study showed that the humoral immunological response was improved when higher levels of chelated zinc and vitamin E were provided. Positive effects were seen by individual

supplementation of zinc and vitamin E, but also in the combination (Cardoso et al., 2006). However, positive effects were no longer observed at the combination of the highest concentration of vitamin E (120 mg/kg) with zinc. A study of Sahin et al. (2006) performed in Japanese quails reared under heat stress, showed a positive interaction between vitamin E (0, 125, 250 mg/kg) and zinc picolinate (0, 30, 60 mg/kg) on final body weight and malondialdehyde levels in serum and liver. Kakhki et al. (2016) and Kakhki et al. (2017) did not find an effect between vitamin E (0, 150, 300 mg/kg) and zinc methionine (0, 60, 120 mg/kg) on performance and carcass yield. Pompeu et al. (2018) performed a meta-analysis on the effect of supplementation of vitamin E on performance, carcass traits, meat quality and immune response in male broilers and showed no relationship between vitamin E and growth. However, there were indications that vitamin E does play an important role in protecting unsaturated fatty acids and lipid meat from oxidation, and has an important function in the regulation of the immune response (Pompeu et al., 2018).

1.5 Zinc in broiler production

1.5.1 The need for zinc supplementation

Due to the absence of a specialized zinc store a daily intake of zinc is necessary to retain a steady state to allow zinc to maintain and support it's numerous functions (Bonaventura, benedetti et al. 2015). As zinc is an essential micronutrient it has to be provided to animals by in-feed supplementation (Ranaldi et al., 2013). Zinc can be supplied as oxide, sulphate or as organic complexed form. For metals the sulphate form is considered to be the reference source (Cano-Sacho et al., 2014).

Feedstuffs (grains, legumes) used in poultry rations contain insufficient zinc and therefore zinc needs to be supplemented. Providing insufficient zinc in broiler feed leads to loss of appetite and as a consequence to reduced feed intake. The reduced feed intake but also insufficient support of cellular metabolism for growth leads to decreased growth rates.

Moreover, zinc plays also an important role in feathering. Therefore delayed feather development resulting in frayed feathers are often observed in case of inadequate zinc supply. Furthermore, zinc is indispensable for bone development in broilers. In case of insufficient dietary zinc uptake various bone abnormalities, skeletal malformation, poor bone mineralization and stunted growth have been observed (Kidd et al., 1996). On the other hand, an excess of dietary zinc intake also seems to negatively affect the bone mineralization (Underwood, 1981, Burrell et al., 2004). The recommended level of zinc for broilers is 40 mg/kg feed (NRC, 1994). Nevertheless, several studies report improvement of body weight gain and feed conversion efficiency when levels higher than 40 mg/kg of zinc are provided. Burrell et al. (2004) and Jahanian et al. (2008) observed a linearly increasing body weight gain and the highest weight gain was observed with 80 mg/kg supplemental zinc. Feng et al. (2010) observed an increased final weight and average daily gain up to 60 mg/kg of supplementary zinc. This positive effects above the recommended level can probably be attributed to the role of zinc in various enzymatic activities involved in nutrient absorption and digestibility (Kucuk et al., 2003).

Until the beginning of the years 2000, high safety margins between requirements and supplemented dietary concentrations were applied in order to prevent zinc deficiency. This was favoured by the relatively low cost of trace minerals, which only comprise 0.2% of the total feed cost. Supplementing high levels of zinc imposes negative effects on the environment in areas of intensive animal production (Coppenet et al., 1993). Accumulation of heavy metals in the soil can cause toxicity to plants and microorganisms and encourage unfavourable pathogens in soil and water (Coppenet et al., 1993). European legislation has therefore limited the supplementation of trace minerals in feeds and reduced the level of zinc in total feed to 120 mg/kg feed for broilers (EU 2016/1095). In piglets, sows, rabbits and all fish species other than salmonids the maximum level of zinc in total feed was set at 150

mg/kg, in salmonids and milk replacers for calves at 180 mg/kg and in cats and dogs at 200 mg/kg. Zinc deficiency does not commonly occur in production animals like broilers because sufficient zinc is supplemented. Therefore effects due to inadequate zinc supply do not fall within the scope of this work.

1.5.2 Bioavailability of zinc

Zinc homeostasis is primarily regulated by the intestinal epithelial cells by absorption of exogenous zinc contained in the feed and by excretion of endogenous zinc. Adjustments in zinc absorption and excretion in the gastrointestinal tract are the primary means of maintaining zinc homeostasis in the human body (Faa et al. 2008). Cellular uptake of zinc and intracellular distribution is coordinated by several membrane-associated proteins (as shown in Figure 4). The amount of zinc absorbed depends on different factors such as zinc concentration in the feed, the zinc source, presence of dietary inhibitors and physiological status (infection, stress,…) which might increase the demand for absorbed zinc. More important than the zinc concentration in the feed is the bioavailability of zinc, which refers to the proportion of the ingested zinc that is absorbed and utilised in the animal. Calcium, iron and phytate are the main dietary components which can decrease zinc bioavailability. Calcium can form insoluble complexes with phytate and zinc and consequently have an inhibitory effect on zinc absorption. Zinc and iron compete during intestinal absorption, when both trace elements are supplemented at commonly used levels, there is evidence that an excess of iron inhibits zinc absorption and that excess zinc inhibits iron uptake (Solomons, 1986, Whittaker, 1998).

Additionally, the chemical form in which zinc is supplied to the diet is also important for the bioavailability. Zinc can be supplied either as an inorganic salt or as an organic complex. Sulphates, oxides, chlorides and carbonates constitute the inorganic sources of zinc, whereas organic sources consist of zinc chelated to amino acids or hydrolysed proteins. Chelated zinc

sources are attached through a coordinated bond with an organic ligand. Depending on the type of ligand attached to the mineral, the bond strength and the ligand to mineral ratio, the relative bioavailability and efficacy might differ (Khatun et al., 2019). In recent years organic zinc sources have been increasingly used, because they are believed to have a higher bioavailability than the inorganic zinc sources. When inorganic zinc sources are fed and reach the upper intestinal tract they tend to dissociate due to the low pH environment. These dissociated minerals might interact with other minerals or with other dietary components in the digesta, which turn them unavailable for absorption across the small intestine (Wedekind and Baker, 1990, Leeson and Summers, 2001). The improved bioavailability of organic trace minerals may be attributed to their stability in the upper gastrointestinal tract which allows the minerals to reach the small intestine where they are absorbed. However, not all organic trace minerals are stable at low pH, which can affect their bioavailability.

1.5.3 Different zinc sources and their effect on broiler performance and overall health parameters

Although there is an overall consensus that organic zinc sources have a higher bioavailability than inorganic zinc sources (Świątkiewicz et al., 2014), there is no consensus on the effects on performance and overall health parameters. As shown in table 1, there are different types of inorganic and organic zinc sources, which have different structures. Most of the organic minerals marketed are classified as complexes, chelates, or proteinates (Owens et al., 2009). Chelation refers to a special type of complex formed between a ligand and a metal ion. To be classified as a chelate, a ligand or chelating agent must contain a minimum of 2 functional groups (oxygen, nitrogen, amino, hydroxyl), each capable of donating a pair of electrons to combine (via coordinate covalent bonding) with a metal, and must form a heterocyclic ring structure with the metal. Not all metal complexes are chelates, amino acid complexes for example do not fall within this category (Owens et al., 2009).

Due to these differences in chemical structure it is possible that supplementation with different types of organic zinc sources has a different effect on performance or on intestinal health parameters (Figure 6). Indeed, the differences in trace mineral sources and their effectiveness mainly lie in the type of chemical bond that binds the metal to its ligand. Sulphate trace minerals contain a metal ion (e.g., zinc) which is bound to a sulphate ion through an ionic bond. This ionic bond dissociates easily in aqueous environment, releasing a free metal ion which is free to interact with other nutrients. Organic and hydroxy trace minerals contain covalent bonds, which are more difficult to break. This stronger covalent bond prevents early release of the metal ion in the feed or digestive tract.

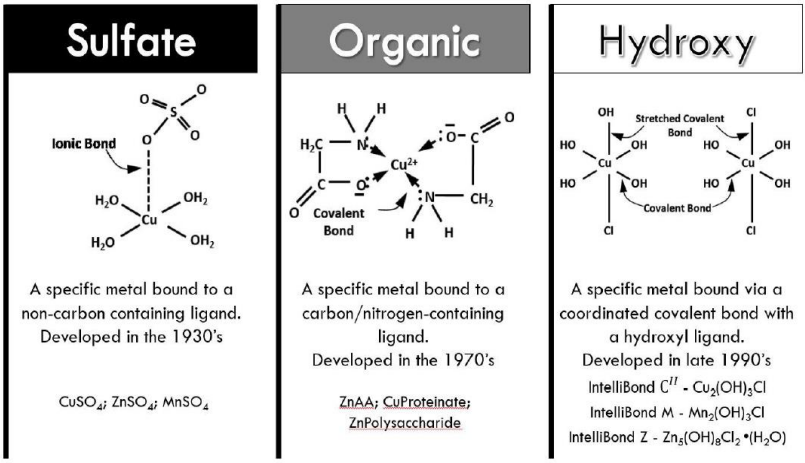


Figure 6: Chemical characteristics from different trace mineral sources. From: Kvidera (2020)

Ionic and covalent bonds are the two main chemical bonds and the key difference is that one atom essentially donates an electron to another atom in an ionic bond, while electrons are shared between atoms in a covalent bond (Figure 7). Therefore, in ionic bonds atoms dissociate in their ions in water, whereas, covalent bonds may dissolve in water but do not dissociate in their ions (Linus, 1960, Laidler, 1993, Langmuir, 1919).

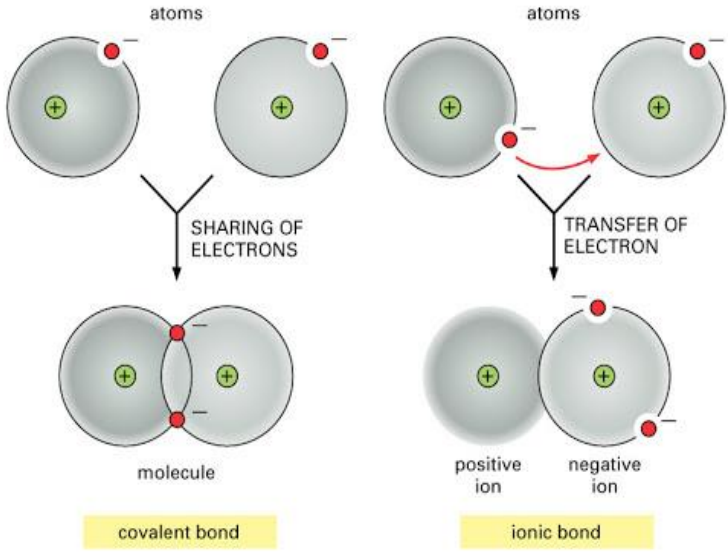


Figure 7: Illustration of electron organization in a covalent or ionic bond. From: (Wergin, 2006)

Several studies have evaluated the replacement of multiple inorganic trace minerals by organic trace minerals and have reported a positive impact on bioavailability, performance and overall health parameter (Aksu et al., 2010, Bao et al., 2007, De Marco et al., 2017, Favero et al., 2013, Gheisari et al., 2011, Hajilari et al., 2019, Sirri et al., 2016, Olukosi et al., 2018). However, based on these results it is difficult to evaluate the effect of the replacement of one specific trace mineral (in this case zinc) and to deduce what the mechanism of improvement might be. As this does not fall within the scope of this work, the focus will be on reviewing literature comparing the effects of supplementation of different zinc sources (Table 2).

Table 1: Common used inorganic and organic zinc sources

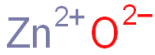
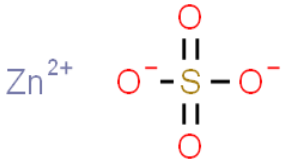
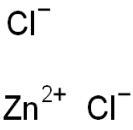
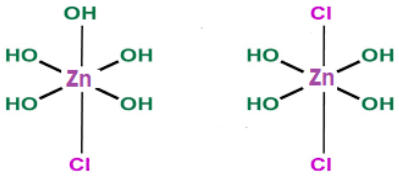
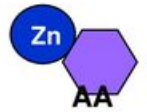
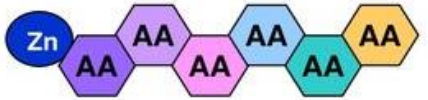
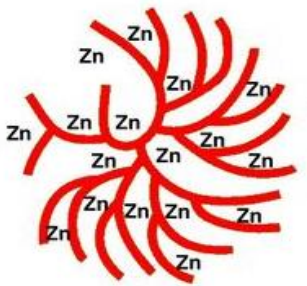
Zinc sources	Chemical structure	Type of bond and minimal zinc content
Inorganic zinc sources		
Zinc oxide (ZnO)		Ionic bond 72 %
Zinc sulphate ($ZnSO_4$)		Ionic bond 34 %
Zinc chloride ($ZnCl_2$)		Ionic bond 46.1 %
Hydroxy zinc ($ZnOH_nCl_n$)		Ionic bond

Table 1 (continued): Common used inorganic and organic zinc sources

Zinc sources	Chemical structure	Additional information on type of bond and minimal zinc content
Organic zinc sources		
Metal (specific amino acid) complex (e.g., zinc methionine)		Product resulting from complexing a soluble metal salt with a specific amino acid. Minimum zinc content: 10%
Metal poly amino acid complex		Product resulting from complexing a soluble metal salt with an amino acid(s). Minimum zinc content:10%
Metal Amino Acid Chelate	 	Product resulting from the reaction of a metal ion from a soluble metal salt with amino acids with a mole ratio of one mole of metal to one to three (preferably two) moles of amino acid to form coordinate covalent bond. Minimum zinc content: 15 %

Metal proteinate		Product resulting from the chelation of a soluble salt with amino acids and/or partially hydrolyzed protein. Minimum zinc content: 10 % (min 85 % chelated)
Metal Polyscacharide complex		Product resulting from complexing of a soluble salt with a polysaccharide solution. Minimum zinc content: not defined

1.5.3.1 Zinc amino acid complexes as an organic zinc source compared to inorganic zinc sources

A study performed by Wedekind et al. (1992) indicated that zinc provided as zinc-methionine had a higher bioavailability than zinc provided as $ZnSO_4$ or ZnO in male chicks. Burrel et al. (2004) compared the effect of providing Zn as $ZnSO_4$ or as zinc amino acid complex or the equal combination of the two in a total concentration of 0,20,40 or 80 mg/kg. They showed an improved zinc utilization (lower excretion) when zinc was supplied as zinc amino acid complex. In this study the importance of zinc supplementation was confirmed, as performance linearly improved with increasing level of zinc provided in the feed, however this improvement was independent of the zinc source. Star et al. (2012) confirmed that Zn provided from ZnAA is more readily available as compared to Zn provided from $ZnSO_4$ and also showed that this did not translate in improved performance. However, these studies were designed to evaluate bioavailability and therefore the experimental design of these studies was not ideal to evaluate effects on performance (Burrel et al., 2004; Star et al., 2012). A study performed by Hess et al. (2001) showed that adding 40 mg/kg of Zn from zinc amino acid complex (Zn-Methionine, Zn-Lysine, Zn-Methionine/Lysine) on top of 55 mg/kg of inorganic zinc improved feed efficiency and body weight. Jahanian et al. (2008) evaluated the effect of providing Zn form $ZnSO_4$ or Zn-Methionine complex at different levels (40,80,120 mg/kg). Supplementation with ZnMet (up to 120 mg/kg) increased feed intake and also improved body weight gain in all experimental stages and resulted in a higher carcass yield and breast percentage. Zakaria et al. (2017a) performed a similar study comparing a control group receiving 80 mg/kg of ZnO with a treatment group receiving 42 mg/kg of Zn provided as Zn-Methionine on top of 80 mg/kg of ZnO. Growth performance (body weight, body weight gain and feed conversion ratio), carcass yield and meat quality were not significantly different between treatments. However, supplementation of Zn-Methionine on top of ZnO significantly

($P<0.05$) decreased mortality from 4.8% to 1.25% (Zakaria et al., 2017). Additionally, an increased zinc serum level was observed in birds receiving Zn-Methionine on top of ZnO (Zakaria et al., 2017b). Kakhki et al. (2018) evaluated the effect of supplementation of alfa-tocopherylacetate (0,150 or 300 mg/kg) and zinc amino acid complex (0, 100, 200 or 400 mg/kg) in 3x4 experimental design and showed no effects on growth performance of dietary treatments nor an interaction effect. However, zinc levels in both serum and liver linearly increased with increasing concentration of zinc (Kakhi et al., 2018).

1.5.3.2 Zinc glycine chelates as an organic zinc source compared to inorganic zinc sources

A study performed by Feng et al. (2010), in which a non-supplemented control group was compared with a positive control group receiving Zn from $ZnSO_4$ (120 mg/kg) and 4 groups receiving Zn from zinc glycine chelate (30, 60, 90, 120 mg/kg) evaluated effects on performance and immunological and hematological parameters. Zinc supplementation above 60 mg/kg, independent of the zinc source, showed a positive impact on performance and immunological parameters as compared to non-supplemented control group. This experimental set-up was also used by Ma et al. (2011b) and this study showed an effect on oxidative stress parameters (decreased MDA level, increased activity of SOD and GPx activities in liver extracts) and intestinal morphology when 90 or 120 mg/kg was supplemented as compared to the non-supplemented control group. Jarosz et al. (2017a) reports a positive impact of supplementation of zinc glycine chelates (100 mg/kg) as compared to $ZnSO_4$ (100 mg/kg) on the cellular and humoral immune response. Kwiecien et al. (2017) compared a negative control group (no zinc supplemented), a positive control (100 mg/kg Zn from $ZnSO_4$) with 3 treatment groups receiving either 25, 50 or 100 mg/kg of Zn as zinc glycine chelate. Effect on anti-oxidant parameters, hematological indices and zinc excretion were evaluated. Supplementation of 100 mg/kg of zinc glycine chelates increased Zn/Cu SOD activity in liver extracts as compared to the non-supplemented control group and

decreased zinc excretion in feaces as compared to negative and positive control group. Additionally, an increased percentage of red blood cells was observed when 100 or 50 mg/kg of zinc glycine chelates were provided as compared to other groups. Levkut et al. (2017) performed a study in which a non-supplemented control groups contained (approximately 30 mg/kg of zinc) was compared with either supplementation of 30 or 70 mg/kg of $ZnSO_4$ or supplementation with 30 or 70 mg/kg of zinc glycine chelate. In the starter and grower period all birds were fed the basal control diet and supplemented feed was only fed in the finisher period. This study showed that inorganic zinc (independent of concentration) increased villus length and surface in jejunum after a diet with a low dose of zinc was fed, whereas, this effect was only observed for 70 mg/kg of zinc glycine chelates. A study performed by Jarosz et al. (2017b) showed that supplemental zinc glycine chelates (100 mg/kg) activate cellular and humoral immune response which leads to enhanced resistance to infections as compared to un-supplemented control group and supplementation with $ZnSO_4$ (100 mg/kg).

1.5.3.3 Other organic zinc sources compared to inorganic zinc sources

A study performed by Saenmahayak et al. (2012) comparing a non-supplemented control diet with either a diet supplemented with 40 mg/kg of $ZnSO_4$ or 40 mg/kg of complexed Zn (no further details provided on type of organic zinc source) or 40 mg/kg of both zinc sources did not show an effect on growth performance and carcass yield. However, a significant decrease in drip loss was found between the groups fed 40 mg/kg of $ZnSO_4$ or 40 mg/kg of complexed zinc. In an earlier study performed by Saenmahayak et al. (2010) where the supplementation of 80 mg/kg of $ZnSO_4$ was compared to supplementation with 40 mg/kg of $ZnSO_4$ combined with 40 mg/kg or 80 mg/kg of complexed zinc showed an improvement of body weight and feed conversion when $ZnSO_4$ (80 mg/kg) was partially replaced by complexed zinc (40 mg/kg $ZnSO_4$ and 40 mg/kg of complexed zinc). Further increasing the amount of complexed zinc to

the diet (40 mg/kg of $ZnSO_4$ and 80 mg/kg of complexed zinc) showed an improvement of breast meat yield as compared to supplementation with 40 mg/kg of $ZnSO_4$.

Chand et al. (2020) has compared an organic and inorganic zinc source (no further information on type of source) at an inclusion level of either 50 mg/kg and 60 mg/kg supplemental zinc (basal feed contained 40 mg/kg zinc) and showed that organic zinc at an inclusion rate of 50 mg/kg resulted in improved performance, increased villus length and goblet cell count in ileum section.

In contrast, Manangi et al. (2012)evaluated the effect of the replacement of 100 mg/kg of Zn from $ZnSO_4$ by 30 mg/kg of Zn-2-hydroxy-4-methylthiol-butanoic acid (as an organic source) and reported no significant differences in performance, carcass yield or tibia zinc content.

Table 2: Overview of the available literature evaluating the effects of the supplementation with different inorganic and organic zinc sources in broilers.

Inorganic zinc sources		
Zinc oxide (ZnO)	• Positive effect on performance as compared to non-supplemented control group.	Mwangi et al. (2017)
Zinc sulphate ($ZnSO_4$)	• Positive effect on performance up to 80 mg/kg of supplemental zinc, further increase up to 160 or 320 mg/kg did nog further improve performance. • No effect on growth performance, up to 20 mg/kg increased deposition in bone and a linear increase of zinc deposition in breast meat with increasing zinc supplementation from 0 to 120 mg/kg.	Sunder et al. (2008) Zhang et al. (2018)
Zinc chloride ($ZnCl_2$)	• Increase of body weight compared to ZnO, $ZnSO_4$ and $ZnCO_3$ at a level of 40 mg/kg.	Sunder et al. (2009)

Table 2 (continued): Overview of the available literature evaluating the effects of the supplementation with different inorganic and organic zinc sources in broilers

Organic zinc sources		
Metal (specific amino acid) complex	• Zinc methionine did not improve performance parameters and meat quality, but stimulates immune response as compared to non-supplemented control group. • Zinc methionine (40, 80 or 120 mg/kg) increased feed intake and body weight gain in all experimental periods and decreased feed conversion ratio in the first week. Carcass and breast percentages were increased with zinc methionine supplementation. • Zinc from zinc-methionine has a higher bioavailability than zinc from zinc-sulphate or zinc-oxide	Kakhki et al. (2016), Kakhki et al. (2017), Kakhki et al. (2018) Jahanian et al. (2008) Wedekind et al. (1992)
Metal poly amino acid complex	• Improved bioavailability as compared to $ZnSO_4$. Zinc source did not affect performance. • Different levels of ZnAA did not affect performance under heat stress conditions. Increasing ZnAA form 34 mg/kg to 181 mg/kg improved cellular and humoral immune response. • Zinc supplemented either as zinc sulphate, or zinc amino acid complex or the combination of both at a level of 20, 40 or 80 mg/kg did not influence performance of broilers. Zinc source did not affect performance, but an improved utilization of zinc amino acid complexes was observed, which indicates a higher bioavailability. • Promotes growth as compared to non-supplemented control group, but no effect on carcass traits and meat quality. • ZnAA supplementation up to 60 mg/kg improves performance and antioxidant stability in liver and thigh muscles as compared to non-supplemented control group.	Star et al. (2012) Bartlett and Smith (2003) Burrel et al. (2004) Liu et al. (2011) Liu et al. (2015)

Table 2 (continued): Overview of the available literature evaluating the effects of the supplementation with different inorganic and organic zinc sources in broilers

Organic zinc sources		
Metal Amino Acid Chelate	• Supplementation with zinc glycine chelates lowers liver MDA concentration, increases Cu/Zn SOD activity and improves intestinal morphology as compared to non-supplemented control group. • Zinc glycine chelates (25 and 50 mg/kg) increase weight gain as compared to non-supplemented control group and increases breast muscles weight as compared to non-supplemented control group and 100 mg/kg ZnO. • Supplemental zinc glycine chelates (100 mg/kg) activate cellular and humoral immune response which leads to enhanced resistance to infections as compared to un-supplemented control group and supplementation with $ZnSO_4$ (100 mg/kg). • Supplementation with 70 mg/kg of zinc glycine chelate in finisher period after a low zinc dose diet was fed improved villus length and surface.	Ma et al. (2011; 2017) Kwiecien et al. (2016) Jarosz et al. (2017b) Levkut et al. (2017)
Metal proteinate	• Positive effect on performance as compared to non-supplemented control group. • Positive effect on body weight, feed conversion and increased jejunal villus length under challenge with Clostridium perfringens and coccidian, as compared to $ZnSO_4$. • Promotes growth as compared to non-supplemented control group, but no effect on carcass traits and meat quality. • Supplementation up to 60 mg/kg improves performance and antioxidant stability in liver and thigh muscles as compared to non-supplemented control group.	Ao et al. (2009) Mwangi et al. (2017: Bortoluzzi et al. (2019b), Bortoluzzi et al. (2019a) Liu et al. (2011) Liu et al. (2015)
Metal Hydroxy Acid Chelate	• Hydroxychloride zinc and coppers improves gain to feed ratio and breast meat yield as compared to $ZnSO_4$ and $CuSO_4$ supplementation.	Olukosi et al. (2018)

The majority of the performed studies indicate that that organic zinc may have beneficial effects over inorganic zinc. However, the reported effects on live performance, slaughter efficiency and other health parameters are variable for the different organic zinc sources (Table 2). One could speculate that the different types of organic zinc sources have different characteristics due to a different chemical structure and therefore lead to different results. Liu et al. (2011, 2015) evaluated the effect of three different organic zinc sources (zinc amino acid complex, and 2 zinc proteinates) with different chelation strength and did not find an effect of zinc source on performance and carcass yield. Huang et al. (2009) compared the relative bioavailability of $ZnSO_4$ and three organic zinc sources with different chelation strength. Differences in bioavailability amongst organic zinc sources and a relation between chelation strength and bioavailability was shown in this study (Huang et al., 2009). In another study performed by (Huang, 2013) was shown that organic zinc sources with different chelation strength also resulted in different bioavailability in the presence of high phytate levels in the feed.

Many studies compare different zinc supplements with a non-supplemented control group. The negative impact of an inadequate zinc level is extensively described in literature. Comparing zinc supplementation (independent of the source) with a non-supplemented control group, which may be zinc-deficient, will only show that zinc supplementation is essential for good performance and animal health. Although the necessity for zinc supplementation has been extensively show, additional research is necessary to asses zinc requirements in fast growing birds under various challenging conditions and to evaluate the effect of different zinc sources with different bioavailability's.

SCIENTIFIC AIMS

SCIENTIFIC AIMS

Zinc is one of the most important trace elements in animal diets. Several studies confirm the importance of adequate zinc supply in broiler production, as reviewed by Park et al. (2004). In the past zinc was typically supplied as an inorganic source such as zinc oxide or zinc sulphate. The use of organic zinc sources have gained interest due to their improved bioavailability (Burrell et al., 2004, Salim et al., 2008, Star et al., 2012, Świątkiewicz et al., 2014). Although there seems to be an overall consensus that organic zinc sources have better bioavailability as compared to inorganic zinc sources, studies investigating the effects of zinc on performance and other parameters such as immune status often yield inconsistent results. One of the reasons may be the lack of detailed scientific data regarding effects of the different zinc sources on host functions at the cellular and molecular level. Also, in many studies inorganic zinc sources are only partially replaced by organic zinc sources, or are not included at the same level, or all inorganic trace elements are replaced by organic trace elements. There are only a few studies directly comparing the effects of supplying zinc either as an inorganic or organic source at the same inclusion level. Despite the importance of a balanced microbiota composition in the gut to ensure maximum growth, intestinal health and animal welfare (Kogut, 2019), there is no information available on the effect of zinc source on microbiota composition in broilers. Information on the effect of zinc source on intestinal health and integrity, without previously compromising intestinal integrity via coccidial challenge (Troche et al., 2015) or inducing necrotic enteritis (Bortoluzzi et al., 2019b, Bortoluzzi et al., 2019a) or creating a zinc depleted environment (Levkut et al., 2017), is limited.Therefore this doctoral thesis was performed to elucidate the difference between a specific inorganic ($ZnSO_4$) and organic (zinc amino acid complex, ZnAA) zinc source included in the diet at the same level and within the range of the recommended physiological levels in broilers.

The specific aims of this work are:

It has been reported that ZnAA have an increased bioavailability as compared to $ZnSO_4$. It could be possible that a more readily available zinc sources improves intestinal health and better supports digestive enzymes. Therefore, a digestibility study was performed in order to Evaluate the effect of zinc-amino acid complexes as compared to zinc sulphate in a digestibility study in order to elucidate whether the form in which zinc is supplied influences the digestibility (Chapter 1).

Zinc has an important function in supporting intestinal integrity, development of epithelial cells and has been ascribed antioxidant capacities, therefore the following parameters will be monitored in order to evaluate whether positive effects reported in literature could be ascribed to effects on these parameters. Additionally, effects on microbiota composition will be monitored because it has been reported in other species that zinc can modulate microbiota composition. Therefore a performance study was performed in order to evaluate the effect of supplementation of zinc sulphate or zinc amino acid complexes on performance parameters under a mild nutritional stress. In this performance study, effects on intestinal morphology, microbiota composition and oxidative stress parameters in blood were also evaluated, in order to gain more insights on how zinc source could affect these parameters (Chapter 1).

As zinc is an important antioxidant, the effects on performance, intestinal health and meat quality were explored in a model which is known to induce oxidative stress. Additionally, the interaction with another important antioxidant was explored. Therefore an experiment was performed in order to evaluate the effect of supplementation of zinc sulphate or zinc amino acid complexes on performance, intestinal health and meat quality under a heat stress challenge applied in the finisher period (Chapters 2 and 3). Additionally, the interaction of zinc source with vitamin E was explored (Chapters 2 and 3).

CHAPTER 1

CHAPTER 1:

DIETARY ZINC SOURCE IMPACTS INTESTINAL MORPHOLOGY AND OXIDATIVE STRESS IN YOUNG BROILERS

Adapted from: Dietary zinc source impacts intestinal morphology and oxidative stress in young broilers. De Grande A., Leleu S., Delezie E., Rapp C., De Smet S., Goossens E., Haesebrouck F., Van Immerseel F., Ducatelle R.

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ABSTRACT

Zinc is an essential nutritional trace element for all forms of life as it plays an important role in numerous biological processes. In poultry, zinc is provided by in-feed supplementation. In the past zinc was mainly supplemented as zinc oxide or zinc sulfate. Alternatively, zinc can be supplemented as organic sources, which are characterized by using an organic ligand, that may be an amino acid, peptide or protein to bind zinc and have a higher bioavailability than inorganic zinc sources. There are limited number of studies directly comparing the effects of inorganic vs. organic zinc sources on performance and intestinal health in broilers. Therefore, a digestibility and a performance study were conducted to evaluate and compare the effect of an amino acid-complexed zinc source vs. an inorganic zinc source on intestinal health. The experiment consisted of two treatments: either a zinc amino acid complex or zinc sulphate were added to a wheat - rye based diet at 60 mg/kg Zn, with 10 replicates (34 broilers per pen) per treatment. Effects on performance, intestinal morphology, microbiota composition and oxidative stress were measured. Supplementing zinc amino acid complexes improved the zinc digestibility coefficient as compared to supplementation with zinc sulfate. Broilers supplemented with zinc amino acid complexes had a significantly decreased lower feed conversion ratio in the starter phase compared to birds supplemented with zinc sulfate. A significantly higher villus length was observed in broilers supplemented with zinc amino acid complexes at day 10 and day 28. Supplementation with zinc amino acid complexes resulted in a decreased abundance of several genera belonging to the phylum of Proteobacteria. Plasma malondialdehyde levels and glutathione peroxidase activity showed an improved oxidative status in broilers supplemented with zinc amino acid complexes. In conclusion, zinc supplied in feed as amino acid complex is more readily absorbed, potentially conferring a protective effect on villus epithelial cells in the starter phase.

INTRODUCTION

Zinc is an essential nutritional trace element for all forms of life as it plays an important role in numerous biological processes (Faa et al., 2008a, Ranaldi et al., 2013a, Bonaventura et al., 2015a). Zinc not only contributes to the synthesis, stability and catalytic activity of many proteins (Stefanidou et al., 2006), it also influences nucleic acid metabolism and immunological responses. Moreover, it plays an important role in wound healing and in restoring the integrity of damaged tissues (Batal et al., 2001, Jahanian and Rasouli, 2015). Zinc also has antioxidant effects (Gammoh and Rink, 2017) as it is a cofactor of the Cu/Zn superoxide dismutase, which plays a crucial role in the protection of cells against oxygen radicals (Oteiza, 2012). Finally, zinc ensures normal growth, health and fertility, development of bones and feathers and regulates appetite in broilers (Kwiecien et al., 2017, Shao et al., 2014).

Cellular zinc homeostasis is strictly regulated by uptake and elimination of zinc through specialized transporters and by sequestration of zinc by carrier proteins such as metallothioneins (Bonaventura et al., 2015). Even minor changes in zinc homeostasis can lead to clinical consequences which are most distinct in tissues with a high cell turn-over, such as the skin, the gastro-intestinal mucosa and the immune system (Bonaventura et al., 2015). Due to the absence of a specialized zinc storage system, a daily intake of zinc through the diet is necessary to ensure the homeostasis that allows zinc to maintain and support its numerous functions (Bonaventura et al., 2015). In plants, zinc is mostly bound to phytate, forming an insoluble complex that hampers absorption. Adding phytases to poultry diets is common practice in order to make a portion of the complexed zinc available (Lönnerdal, 2000, Tamim and Angel, 2003). Nevertheless, additional supplementation is recommended to meet the dietary requirements as described by the NRC (1994) (Ma et al., 2011a, Mohanna and Nys,

1999b). A diet without supplemental zinc provides insufficient zinc, therefore supplementing broiler diets with zinc is common industry practice (Sunder et al., 2008).

Although, there are many studies available concerning the importance of zinc for human and animal health, information on whether zinc solely benefits the host or whether there is also an effect on microbiota composition is scarce (Lopez and Skaar, 2018). Bacteria also express several proteins to regulate zinc intake, which proves their necessity for this trace mineral (Outten et al., 2001). It is likely that there is a competition for zinc between the intestinal epithelium cells and bacteria present in the gastro-intestinal tract. In previous studies, it has already been shown that low intestinal zinc concentrations in the intestinal content may shift the microbiota composition away from beneficial bacteria, and may favor the expansion of pathogenic bacteria (Reed et al., 2015, Starke et al., 2014, Lopez and Skaar, 2018). Therefore, it is likely that dietary changes in zinc intake or changes in zinc bioavailability, shape the intestinal microbial population.

The source of zinc in the feed impacts the absorption rate of zinc. Mainly inorganic zinc, such as ZnO and $ZnSO_4$ is supplemented in poultry feed (Summers, 1997). In organic zinc sources zinc is coupled to an organic ligand, typically an amino acid, peptide or protein and these have a higher bioavailability than inorganic zinc sources (Swiatkiewicz et al., 2014, Star et al., 2012). Research has already been conducted to evaluate the effects of different zinc sources on bioavailability in broilers, but there is little information available in the literature on effects on performance directly comparing supplementation with $ZnSO_4$ vs. zinc amino acid complexes (ZnAA) included at the same level in the feed. Moreover, to the best of our knowledge, no information is available in the literature regarding the effects of zinc source in broilers on intestinal health and microbiota composition. Therefore, the aim of the present study was to compare zinc amino acid complexes as opposed to inorganic $ZnSO_4$ in the feed of broilers, focusing on performance and on intestinal and general health parameters.

MATERIALS & METHODS

All experimental procedures in this study were in compliance with the European guidelines for the care and use of animals in research (Directive 2010 : 63 : EU) and were approved by the Ethical committee of the Research Institute for Agriculture, Fisheries and Food (ILVO), Merelbeke, Belgium under authorization number 2016 : 301.

Digestibility Study

Birds used for the digestibility study were housed under the same conditions as the broilers of the performance study up to day nine. From day nine on, the broilers were housed in digestibility cages. The digestibility study consisted of six replicates per treatment with four or five male broilers (Ross 308) per replicate. After a five-day adaptation period the balance period was executed during five consecutive days between 14 and 18 days of age according to the European reference method of Bourdillon et al. (1990). Total feed intake was monitored during this balance period and excreta were collected daily and stored at -20°C. Excreta was pooled per cage and were mixed to prepare a representative sample and subsequently freeze dried and grounded. Dry matter (EC 1971), crude protein (ISO 5983-2, 2005) and crude fat (ISO 6492, 1999) were determined from the pooled samples according to ISO standards to calculate apparent digestibility coefficients. Zinc content in feed and excreta (2 samples per treatment were analyzed in duplicate) was determined with Inductive Coupled Plasma-Mass Spectrometry after microwave destruction in closed recipients (Agilent 7500ce ICP-MS, Agilent Technologies, Santa-Clara, California, USA), according to the method described by Ashoka et al. (2009).

Performance Study

Experimental Design and Dietary Treatments. A total of 680 one - day - old male Ross 308 broilers were randomly allocated to 20 floor pens (10 pens per treatment and 34 broilers per pen) in an alternating block design, one replicate per treatment in each block.

Broilers were housed on solid floor covered with wood shavings (2.38 kg/m²). Up to day seven, the broilers were subjected to a light schedule of 23 hours light and 1 hour dark. From day seven onwards the animals were subjected to a light schedule of 18 hours light and six hours dark. Broilers were orally vaccinated with Paracox®-5 (Intervet UK Ltd., Milton Keynes, UK) on day three. Dietary treatments included a wheat - rye based diet (Table 1) supplemented with 60 mg/kg Zn either as $ZnSO_4$ ($ZnSO_4.7H_2O$, Sigma-Aldrich, St. Louis, USA) or 60 mg/kg Zn as zinc amino acid complexes (ZnAA, Availa®Zn, Zinpro Corporation, Eden Prairie, USA) (Table 2). Availa®Zn is a zinc chelate based on single amino acids from hydrolysed soy protein and zinc bound in a one to one molar ratio. The wheat - rye based diet contained a high level of crude protein (23%, 21%, 20% for starter, grower and finisher diet respectively) and non-starch polysaccharides (15%) (NSP) without the addition of NSP enzymes in order to create a nutritional challenge at the intestinal level. This challenge model was applied in order to assist in the understanding of the mode of action, by increasing response of the bird and was already proven to be effective in the past (Teirlynck et al., 2009, De Maesschalck et al., 2015b) All experimental feeds contain zinc levels that comply with the dietary needs as described by the NRC (1994). The starter diet was fed from day zero up to day 10 and was provided in a crumbled form. The grower and finisher diets were fed as pellets from day 10 up to day 28 and from day 28 up to day 36, respectively. Feed and drinking water were provided ad libitum. The levels of zinc present in the total feed were measured by Inductive Coupled Plasma-Mass Spectrometry after microwave destruction in closed recipients (Agilent 7500ce ICP-MS, Agilent Technologies, Santa-Clara, California, USA), according to the method described by Ashoka et al. (2009) (Table 2). The total amount of zinc is delivered by zinc naturally present in the feed ingredients and by supplemented $ZnSO_4$ or ZnAA.

Table 1: Dietary composition of the diets

	Starter diet	Grower diet	Finisher diet
Ingredient (%)			
Wheat	49.29	55.62	59.00
Rye	5.00	5.00	5.00
Soybean meal (48)	29.37	23.16	20.11
Soybeans	7.50	7.50	7.50
Rapeseed meal	2.00	2.00	2.00
Animal fat	2.50	2.60	2.70
Soy oil	1.00	1.00	1.00
Vitamin + trace (vitamix)§	1.000	1.000	1.000
$CaCO_3$	0.820	0.908	0.826
Di-Ca-phosphate	0.650	0.361	0.107
NaCl	0.264	0.226	0.268
Na-bicarbonate	0.104	0.157	0.101
L-Lys-HCl	0.160	0.175	0.154
DL-Methonine	0.256	0.208	0.167
L-threonine	0.071	0.064	0.049
Phytase‡	0.020	0.020	0.020
Calculated nutrient composition			
Crude protein (%)	23.00	21.00	20.00
Crude fat (%)	6.43	6.41	6.46
Non-soluble polysaccharides (%)	15.37	15.00	14.83
Metabolisable energy (MCal/kg)	2.63	2.70	2.75
Dig. Lysine (%)	1.12	1.03	0.95
Dig. Methionine + Cysteine (%)	0.86	0.77	0.71
Dig. Threonine (%)	0.75	0.67	0.62
Dig. Valine (%)	0.89	0.81	0.76
Ca (%)	0.85	0.80	0.70
Available P (%)	0.40	0.35	0.31
NaCl + KCl (mEq/kg)	254	226	213
Linoleic acid (18:2) (%)	2.10	2.07	2.06

§ Provided per kg of diet: Vitamin A (retinylacetate 3a672a, 10 000 IU), vitamin D3 (E671, 3000 IU), vitamin E (all-rac-α-tocopherol acetate, 50IU), vitamin K (2.5 mg), Vitamin B1 (thiamine mononitrate, 2 mg), riboflavin (5 mg), Calcium D-pantothenate (15 mg), vitamin B6 (4 mg), vitamin B12 (0.025 mg), niacinamide (30 mg), folic acid (1 mg), biotin (0.2 mg); choline (choline chloride, 689.7 mg), Cu ($CuSO_4.5H_2O$, 12 mg), Mn ($MnSO_4.H_2O$, 95.9 mg), Fe ($FeSO_4.H_2O$), 49.2 mg; I (KI, 1.2 mg), Se (Na_2SeO_3, 0.4 mg), Sepioliet (7.0 mg), Propylgallate (2.0 mg), BHT (3.0 mg). ‡Ronozyme ® NP. 10 000 FYT/g.

Table 2: Analyzed zinc concentrations in the diets for broilers (mg/kg, as-fed basis)

Zinc source	**Analyzed Zn (mg/kg)**		
	Starter	Grower	Finisher
$ZnSO_4$	115 ± 1.4	104 ± 5.9	97 ± 1.3
ZnAA	129 ± 4.2	112 ± 4.9	109 ± 12.0

ZnAA: zinc amino acid complexes

Data represent mean ± standard deviation expressed in mg/kg

Mortality was recorded daily and this information was used to correct the performance parameters. At day 10, 28 and 36 all broilers and feed left overs were weighed per pen to determine body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR). At the same time point, three broilers per pen were euthanized by an intravenous overdose of sodium pentobarbital 20% (Kela NV, Hoogstraten, Belgium) and venous blood samples were collected in serum and heparin tubes (Vacutest Plast, Kima, Arzergrande, Italy). Tissue samples of the duodenal loop (where the duodenum is rounding the pancreas) were collected for fixation in four percent of formaldehyde at day 10 and 28. Content of ileum and cecum was collected aseptically (day 10) and stored at – 20 °C. At the end of the trial, litter quality (per pen, scores ranging from zero to three). Footpad and hock lesions were scored (of eight birds per pen, scores ranging from zero to four) based on the Welfare quality assessment protocols (Welfare Quality Consortium, 2009) (Table 3).

Table 3: Score system for litter quality and lesion scoring

Score system litter quality				
Score 0	Score 1	Score 2	Score 3	
Dry or nearly sticky litter	Litter starts to stick	Litter starts to clot	The litter is very greasy and forms a 'cake' like structure	
Score foot pad lesions and hock burns				
Score 0	Score 1	Score 2	Score 3	Score 4
No damage or inflammation	Minor damaging	Intermediate damaging	Severe damage and clear inflammation	Severe damage and clear inflammation of larger areas

Intestinal Morphology. Duodenum sections were selected to determine intestinal morphology, because the duodenum is the most important site for absorption of nutrients (also for zinc) and in this section of the gut the villi are the longest. Formalin fixed intestinal segments of the duodenum were dehydrated in xylene, embedded in paraffin and sectioned in four µm slides for hematoxylin-eosin staining and CD3 immunohistochemistry. The sections were automatically (Shandon Varistain-Gemini, Thermofisher Scientific, Cheshire, UK) deparaffinized in xylene and rehydrated in isopropylene, 95% ethanol and 50% ethanol and stained with hematoxylin and eosin. The sections were examined using a light microscope (Leica DM LB2 Digital, Leica Microsystems, Wetzlar, Germany). Villus length was measured from the tip of the villus to the crypt-villus junction. Crypt depth was measured from the crypt base up to the crypt-villus junction. Measurements were performed on 12 random selected duodenal villi and crypts per section (one section per animal) using Leica DM LB2 Digital and a computer based image analysis program, Leica Application Suite version 4.1. The average villus length and crypt depth were calculated per animal. The thickness of the tunica muscularis was determined at 20 locations per section (one section per animal) of which the average was calculated per animal. The above mentioned measurements were performed for three birds per pen (10 pens per treatment).

CD3 Immunohistochemistry. CD3 immunohistological staining of duodenal sections was performed as described by Aguirre et al. (2019). Slides were analyzed with Leica DM LB2 Digital and a computer based image analysis program LAS V4.1 (Leica Application Suite V4, Germany). The CD_3+ area percentage in the duodenal tissue was quantified using three representative fields of view per section (one section per animal) in three birds per pen (10 pens per treatment).

Microbiota Composition. DNA was extracted from the cecal and ileal content of broilers aged 10 days using the CTAB method as previously described (De Maesschalck et al., 2015a). Amplification and sequencing of the V3-V4 regions of the 16S rRNA gene was done by Macrogen (Seoul, South-Korea), using the primers S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21, as described by Klindworth et al. (2013), extended with Illumina specific adaptors. Amplicons were sequenced in a single run using Illumina MiSeq v3 technology (2 x 300 bp, paired-end) and using 30% PhiX DNA as spike-in. Demultiplexing of the amplicon dataset and deletion of the barcodes was done by the sequencing provider (Macrogen). The sequences were processed using a pipeline combining PANDAseq (Masella et al., 2012) and QIIME (v1.9.1). The paired-end sequences were assembled using PANDAseq, with a quality threshold of 0.9 and length cut-off values for the merged sequences between 400 and 500 bp. Open-reference operational taxonomic unit (OTU) picking was performed at 97% sequence similarity using USEARCH (v6.1) and converted to an OTU table (Edgar, 2010). OTU taxonomy was assigned against the Silva database (v123, clustered at 97% identity) using the PyNast algorithm with QIIME default parameters (Caporaso et al., 2010, Quast et al., 2013). OTUs with a total abundance below 0.01% of the total sequences were discarded (Bokulich et al., 2013). Alpha rarefaction curves were generated using QIIME and a subsampling depth of 10.000 reads was selected. No samples were eliminated following subsampling.

Bioinformatics and Statistical Analysis of 16S rRNA Gene Amplicon Data. Further analysis of alpha diversity (Observed OTUs, Chao1 richness estimator and Shannon diversity estimator) and beta diversity (Bray-Curtis dissimilarities) were performed using the phyloseq (McMurdie and Holmes, 2013) pipeline in R (v3.4.3). Normality of the alpha diversity data was tested using the Shapiro-Wilk test and subsequently a t-test was used for normal distributed data. Differences in beta diversity were examined using the ANOSIM function from the Vegan package. Differences in relative abundance at the phylum level were assessed using the two-sided Welch t-test from the mt wrapper in phyloseq. To detect differentially abundant taxa between the different diet groups, DESeq2 was applied on the non-rarified community composition data for either cecal or ileal communities (Love et al., 2014). Significant differences were calculated using a Wald test followed by a Benjamini-Hochberg multiple hypothesis correction. For all tests, a P-value < 0.05 was considered significant and considered as tendency at $0.05< P < 0.1$.

Metabolic Function Prediction of the Microbial Communities. To gain more insight into the possible functional pathways of the microbial communities in the ileum, the functional composition was predicted using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; (Langille et al., 2013) PICRUSt uses precomputed ancestral state reconstructions based on the Greengenes database. Therefore, OTU picking was reperformed as described above with following modifications: closed-reference OTU picking was used, and OTU taxonomy was assigned against the Greengenes database (v 13.5) (DeSantis et al., 2006) after which the OTU counts were normalized by their expected 16s copy number using QIIME (Angly et al., 2014, Kembel et al., 2012). Metagenome predictions were performed against the KEGG database (Kyoto Encyclopedia of Genes and Genomes; (Kanehisa and Goto, 2000)). The resulting function prediction (KEGG orthologues) was analyzed using the HUMAnN2 algorithm to get KEGG modules and

pathways (http://huttenhower.sph.harvard.edu/humann2). Alpha diversity (Observed OTUs, Chao1 richness estimator and Shannon diversity estimator) was determined using the phyloseq package. Bray-Curtis dissimilarity was used to quantify the difference in predicted KEGG orthologues (KO) populations using the phyloseq package. Differences in Bray-Curtis dissimilarity were examined using the anosim function from the vegan package as described above. Differentially abundant KOs were identified using DESeq2 analysis and plotted in a heatmap using the pheatmap package in R. Differentially abundant modules are identified using the phyloseq mt wrapper (Wilcoxon test with multiple hypothesis correction and calculation of the false discovery rate).

Biochemical analyses. Serum zinc concentrations were determined as described by van Riet et al. (2015). Shortly, serum samples were mixed with an equal volume of trichloroacetic acid to deproteinate samples before centrifugation at 10 000 x g for 10 minutes. The remaining supernatant was used within 2h to determine serum zinc concentrations. Therefore the deproteinated serum was diluted five times with a color reagent (Randox kit ZN2341, Randox laboratories limited, Crumlin, UK), and incubated 5 minutes at 25°C. Absorbance was measured at 560 nm. Serum zinc concentration was calculated from a zinc standard calibration curve. Malondialdehyde (MDA) concentration in plasma was measured by the reaction of MDA with thiobarbituric acid as described by (Vossen et al., 2011). The absorbance of the colored complex was measured spectrophotometrically at 532 nm. A standard curve with 1,1,3,3-tetramethoxypropane was used and the thiobarbituric acid reactive substance (TBARS) concentration was expressed in nmol MDA per ml of plasma. Glutathione peroxidase activity (GPx) was determined in plasma samples by measuring the oxidation of nicotinamide adenine dinucleotide phosphate in the presence of reduced glutathione and hydrogen peroxide. A decrease in absorbance was kinetically monitored at 340 nm for 5 min as described by Vossen et al. (2011).

Statistical Analysis

Statistical analysis was performed in R for Windows (version 3.5.1). The pen was considered as the experimental unit for all analyzed variables except for the nutrient digestibility, where the digestibility cage was considered as the experimental unit. All data were checked for outliers and normality of the residuals. Normality of the sample distribution was performed with the Kolmogorov-Smirnov test. Data were compared using an independent samples t-test. Mortality and lesion scores (foot pad lesions and hock burn) and litter quality were analyzed using an ordered logistic regression model with treatment as a fixed factor and animal or pen as the experimental unit. Statistical analysis on the gut microbiota was performed using R, as described above. The differences were considered statistically significant at $P \leq 0.05$ and considered as tendency at $0.05 < P < 0.1$.

RESULTS

Digestibility

A higher apparent zinc digestibility coefficient (P=0.02) was observed for broilers fed a diet supplemented with ZnAA (N=5) compared to broilers fed a diet supplemented with $ZnSO_4$ (N=6) (36.4 vs 32.5%) (Table 4). Zinc contents were determined in all experimental diets in order to exclude that there was a difference in zinc concentration in the diets of the different treatments (Table 2). There were no distinct differences in zinc concentration between treatments, so the difference in apparent digestibility coefficient is not due to a difference in zinc intake. There were no differences found in feed intake or amount of excreta (P>0.01) nor were there significant differences in proximate nutrient digestibility.

Table 4: Effect of supplementation with $ZnSO_4$ or ZnAA on digestibility parameters and calculated apparent digestibility coefficients in broilers

	$ZnSO_4$	**ZnAA**	**P-value**
Parameters			
Feed intake (FI) (g)	1256 ± 49	1168 ± 90	0.059
Wet excreta (WE) (g)	1797 ± 72	1720 ± 146	0.271
WE/FI*	0.300 ± 0.02	0.304 ± 0.01	0.731
Apparent digestibility coefficients (%)			
Gross energy	72.0 ± 2.3	71.8 ± 1.7	0.878
Crude protein	58.2 ± 2.9	57.1 ± 2.2	0.512
Crude fat	78.8 ± 2.5	77.0 ± 4.3	0.423
Zinc	32.7 ± 1.3	36.4 ± 2.7	**0.020**

ZnAA: zinc amino acid complexes

Data represent mean ± standard deviation ($ZnSO_4$, N=6; ZnAA, N=5)

Performance Parameters

Initial BW of one-day old chickens was on average 42 g ($ZnSO_4$) and 41 g (ZnAA) and did not significantly differ between treatments (data not shown). A decreased feed conversion ratio (FCR) was observed for the starter (d0-10) period for broilers fed the diet supplemented with ZnAA (P = 0.03) (Table 5). Moreover, there was a trend for a higher body weight from d0-10 and improved FCR from d0-28 for broilers supplemented with ZnAA as compared to $ZnSO_4$. There was no effect of the source of supplemented zinc on the performance parameters during the overall period. Dietary treatment did not affect mortality rates, litter quality or lesion scores (data not shown).

Table 5: Effect of supplementation with $ZnSO_4$ or ZnAA on performance in broilers

Period		**BW (g/animal)**	**BWG (g/animal/day)**	**FI (g/animal/day)**	**FCR**
0-10 days	$ZnSO_4$	284.7 ± 5.6	24.4 ± 0.5	28.6 ± 0.9	1.172 ± 0.018
	ZnAA	290.5 ± 5.8	25.0 ± 0.5	28.7 ± 0.9	1.149 ± 0.012
	P-value	0.063	0.061	0.860	**0.029**
10-28 days	$ZnSO_4$	1572 ± 38	71.5 ± 2.0	104.2 ± 3.2	1.456 ± 0.022
	ZnAA	1597 ± 40	72.6 ± 2.0	104.7 ± 2.8	1.441 ± 0.017
	P-value	0.207	0.283	0.720	0.167
28-36 days	$ZnSO_4$	2423 ± 43	106.4 ± 3.1	171.6 ± 3.9	1.613 ± 0.051
	ZnAA	2456 ± 46	107.4 ± 3.7	174.7 ± 3.9	1.627 ± 0.046
	P-value	0.188	0.487	0.170	0.405
0-28 days	$ZnSO_4$		54.7 ± 1.4	77.2 ± 2.2	1.411 ± 0.019
	ZnAA		55.6 ± 1.4	77.5 ± 2.0	1.394 ± 0.014
	P-value		0.207	0.733	0.073
0-36 days	$ZnSO_4$		67.3 ± 1.2	98.2 ± 2.4	1.459 ± 0.019
	ZnAA		68.2 ± 1.3	99.1 ± 2.1	1.453 ± 0.016
	P-value		0.187	0.405	0.375

ZnAA: zinc amino acid complexes. Body weight (BW) was determined at the last day of each period (d10, d28 & d36). Body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) were determined at the end of the three periods. Data represent mean ± standard deviation (N=10).

Table 6: Effect of supplementation of $ZnSO_4$ or ZnAA on intestinal morphology and T-cell abundance in broilers on day 10 and day 28 in duodenal sections

	$ZnSO_4$	**ZnAA**	**P-value**
Day 10			
Villus length (VL)	1206 ± 221	1308 ± 155	**0.046**
Crypt depth (CD)	314.5 ± 76.6	301.6 ± 62.1	0.494
Ratio (VL:CD)	3.99 ± 1.05	4.52 ± 1.04	**0.049**
Thickness *T. muscularis*	94.8 ± 30.4	91.3 ± 21.8	0.626
$CD3^+$	7.68 ± 2.51	6.32 ± 1.61	0.167
Day 28			
Villus length (VL)	1489 ± 230	1667 ± 262	**0.012**
Crypt depth (CD)	313.6 ± 62.3	311.4 ± 63.2	0.896
Ratio (VL:CD)	5.06 ± 1.13	5.80 ± 1.47	**0.046**
Thickness *T. muscularis*	132.8 ± 36.3	129.7 ± 35.1	0.758
$CD3^+$	12.29 ± 2.82	11.54 ± 1.70	0.480

ZnAA: zinc amino acid complexes. Analysis based on 10 measurements per section per bird for villus length (µm) and crypt depth (µm) or 3 microscopic fields per section for CD3+ measurements (area %). Data represent mean ± standard deviation (N=10).

Intestinal Morphology and T-cell Abundance

Duodenal villus length and crypt depth were measured and villus length/crypt depth ratio were calculated at the end of the starter (day 10) and grower period (day 28). At the end of the starter period and the grower period an increase in villus length ($P < 0.05$) and villus length to crypt depth ratio ($P < 0.05$) was observed in broilers fed a diet supplemented with ZnAA as compared to birds fed a diet supplemented with $ZnSO_4$ (Table 6). The supplementation of different zinc sources did not affect the thickness of the tunica muscularis. The amount of CD3 positive T-cells was determined in duodenal sections as a marker for intestinal inflammation. No changes were observed in the duodenal T-cell abundance at the end of the starter and grower period.

Blood Parameters

Zinc levels were determined in serum samples collected from broilers included in the performance trial. No differences in serum zinc levels were observed between dietary treatments at different ages (Table 7).

Table 7: Effect of supplementation with $ZnSO_4$ or ZnAA on serum zinc levels (ug/dL) in broilers measured on day 10, 28 and 36

	$ZnSO_4$	**ZnAA**	**P-value**
Serum zinc level			
Day 10	212.7 ± 7.4	216.2 ± 5.2	0.703
Day 28	206.6 ± 5.7	195.6 ± 5.0	0.160
Day 36	210.4 ± 8.2	210.2 ± 13.1	0.987

ZnAA: zinc amino acid complexes

Data represent mean ± standard deviation (N=10) and are expressed in µg/dl

Malondialdehyde (MDA) concentration and glutathione peroxidase activity (GPx) were measured in plasma to evaluate oxidative status. MDA is commonly known as a marker for oxidative stress and is an end product of lipid-peroxidation. Glutathione peroxidase plays an important role in the cascade which protects cells from oxidative damage. A lower MDA level ($P < 0.01$) was observed at the end of the starter period level in broilers fed a diet

supplemented with ZnAA (Table 8). There was no difference in plasma MDA level at slaughter age. However, a lower plasma GPx (P = 0.02) was observed at slaughter age in broilers supplemented with ZnAA as compared to broilers supplemented with $ZnSO_4$ (Table 8).

Table 8: Effect of supplementation with $ZnSO_4$ or ZnAA supplementation on plasma malondialdehyde concentration (MDA, mmol/L) (day 10, 28 and 36) and effect on plasma glutathione peroxidase activity (GPx, µmol/min. mL) at day 36

	$ZnSO_4$	**ZnAA**	**P-value**
MDA day 10	16.72 ± 0.90	15.08 ± 1.16	**0.007**
MDA day 28	13.88 ± 2.72	14.32 ± 1.85	0.678
MDA day 36	12.64 ± 0.60	12.00 ± 0.76	0.140
GPx day 36	0.72 ± 0.04	0.60 ± 0.15	**0.021**

ZnAA: zinc amino acid complexes. Data represent mean ± standard deviation (N=10, expressed as mmol/L for MDA and expressed as µmol/min.ml plasma for GPx).

Microbiota Composition

Influence on the Cecal and Ileal Microbial Diversity. No differences were observed in either the cecal or ileal bacterial richness or diversity (Figure 1), which was determined by calculating the number of observed OTUs, the estimated OTU richness (Chao1) and the estimated community diversity (Shannon index).

Bray-Curtis dissimilarity was used to investigate beta diversity between either the cecal or ileal microbiota in broilers fed a diet supplemented with $ZnSO_4$ or ZnAA complexes (Figure 2). A trend for a changing microbial composition was observed in the ileum (ANOSIM statistic R = 0.1177, P = 0.069), but no differences could be observed in the cecum (ANOSIM statistic R = 0.002333, P = 0.447).

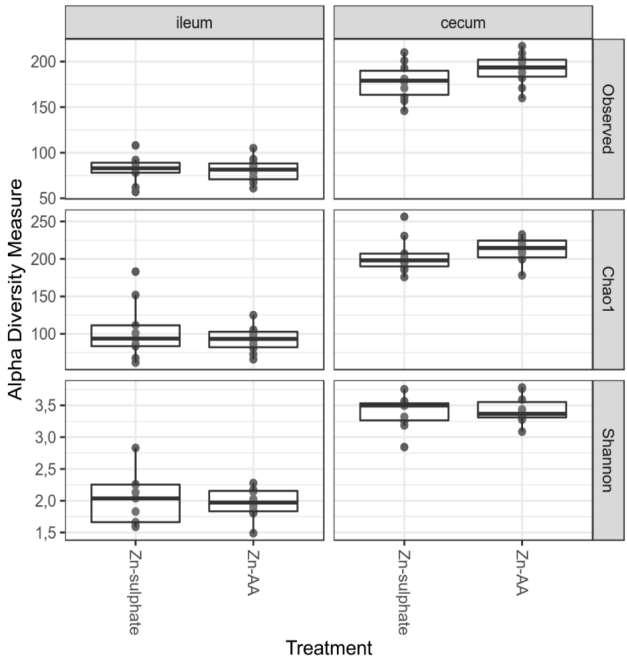

Figure 1: Alpha diversity metrics of the ileal and cecal microbial community from birds fed a diet supplemented with $ZnSO_4$ or ZnAA complexes. Observed: observed OTUs, Chao1: estimated OTU richness and Shannon: estimated community diversity. ZnAA: zinc amino acid complexes

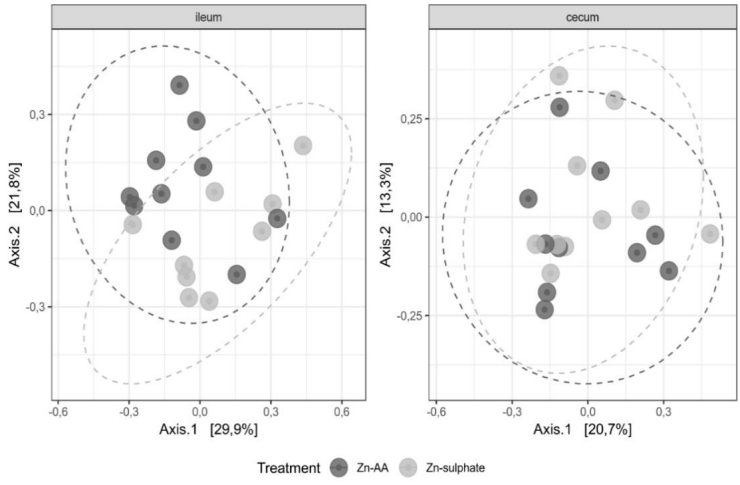

Figure 2: Principle coordinate analysis (PCoA) plot with of Bray Curtis dissimilarities of the ileal or cecal microbiota of broilers fed either the $ZnSO_4$ or ZnAA (zinc amino acid complexes) supplemented diet. Each dot represents a single chicken.

Influence on the Taxonomic Composition of the Microbiota. The ileal microbiota was characterized by a high abundance of Firmicutes (94.4% in the $ZnSO_4$ supplemented group, 96.6% in the ZnAA supplemented group), followed by Actinobacteria as the second most abundant phylum (1.0% and 2.6% respectively). The phylum Proteobacteria accounted for 3.2% in the ileal content of the broilers supplemented with $ZnSO_4$, whereas a relative abundance of 0.7% was observed in broilers supplemented with ZnAA (Figure 3). In the cecum Firmicutes (82.6% or 82.8% for birds supplemented with $ZnSO_4$ or ZnAA respectively) and Bacteroidetes (9.1% or 11.6% for birds supplemented with $ZnSO_4$ or ZnAA respectively) were the most abundant phyla. The phylum Proteobacteria accounted for 4.3% of the total sequences found in the ceca of birds fed a diet supplemented with $ZnSO_4$, whereas a relative abundance of 2.4% was observed in the ceca from birds receiving a diet supplemented with ZnAA. Dietary treatment did not significantly affect the above mentioned differences in both ileum and cecum.

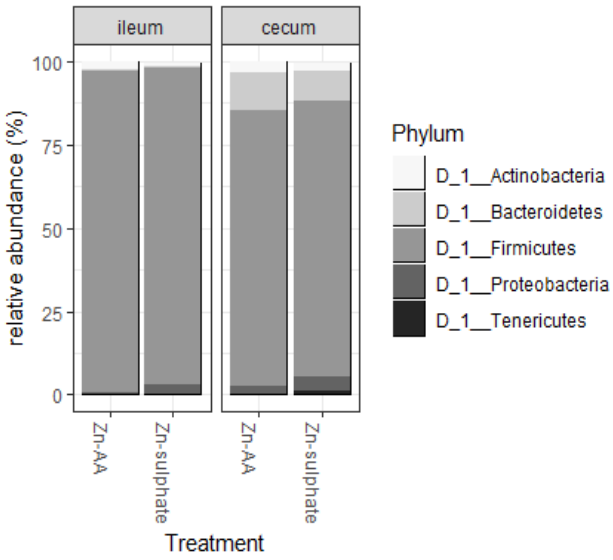

Figure 3: Relative abundance (%) of the 4 most abundant phyla in the ileum or cecum from broilers fed a diet supplemented with a $ZnSO_4$ or ZnAA (zinc amino acid complexes)

Differentially abundant genera (Table 9) in the cecal and ileal microbial composition were identified using DESeq2. In the ileal content the relative abundance of 13 genera was decreased in broilers fed a diet supplemented with ZnAA compared to broilers fed a diet compared to $ZnSO_4$. The other genera belonged to the families *Lachnospiraceae*, *Ruminococcaceae* and *Streptococcaceae* which belong to the phylum Firmicutes and the families *Helicobacteraceae, Sphinghomonadaceae, Comamonadaceae, Burkholderiaceae* and *Pseudomonaceae* which belong to the phylum Proteobacteria. In the cecal content of broilers fed a diet supplemented with ZnAA the abundancy of the genus *Defluviitaleaceae UCG-011* was decreased compared to broilers fed a diet supplemented with $ZnSO_4$.

Influence on Metabolic Function Prediction of the Microbial Communities. Alpha diversity did not show any significant differences. Bray-Curtis dissimilarity was used to quantify the difference in predicted KEGG orthologues (KO) between treatments populations (ANOSIM statistic R = 0.138, P = 0.043). In total 1215 differentially abundant KEGG orthologues (KO) were identified using DESeq2 and plotted in a heatmap (Figure 4).

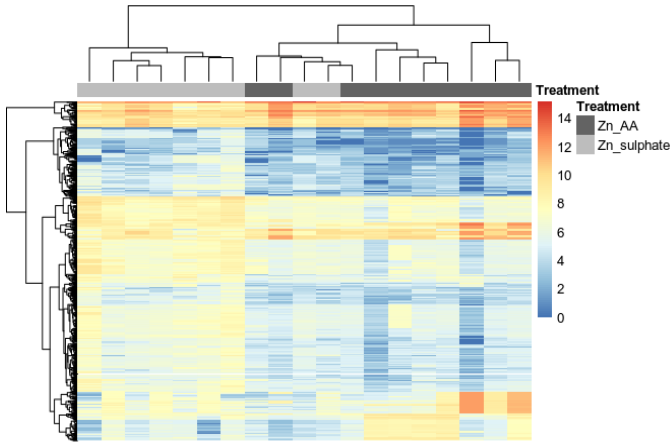

Figure 4: Differentially abundant KEGG orthologues (KO) were identified using DESeq2 and plotted in a heatmap

Samples were clustered based on the similarity of these KOs and resulted in clustered samples according to the treatment (Figure 4). In order to get more insight into the physiological processes this KOs contribute to, all predicted KOs are grouped into KEGG modules. Two modules were significantly affected ($P < 0.05$ and $FDR < 0.05$) and for another five modules there was a tendency ($P < 0.01$ and $FDR < 0.05$) (Table 10).

Some systems which are characterized by oxidative processes like Complex IV cytochrome c oxidase and pyruvate ferredoxin oxidoreductase are enriched in $ZnSO_4$ group (Furdui and Ragsdale, 2000). Putrescine transport system is also enriched in $ZnSO_4$ group. Putrescine is a cellular polyamine produced by both eukaryotic and prokaryotic cells and which is produced by some bacteria (mainly *Escherichia coli*) in response to oxidative stress (Shah and Swlatlo, 2008). Capsular polysaccharide transport system and Microcin C transport system are enriched in $ZnSO_4$ group and are mainly produced by enterobacteria as part of their defense mechanism triggered in stress conditions (He et al., 2016, Severinov and Nair, 2012) (Figure 5).

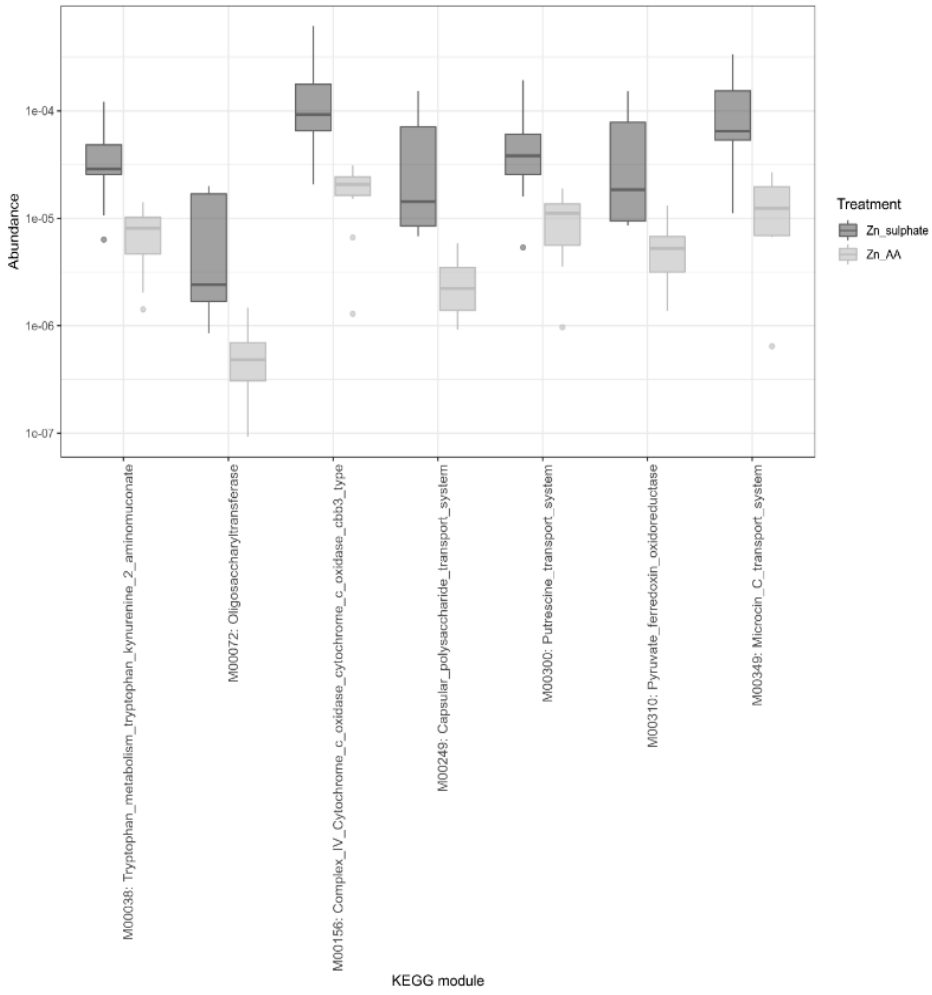

Figure 5: Significantly enriched modules in broilers fed a diet supplemented with $ZnSO_4$ compared to broilers fed a diet supplemented with ZnAA

Table 9: Significant differences in genus level abundance in the ileal and cecal microbiota from broilers fed $ZnSO_4$ or ZnAA (zinc amino acid complexes) supplemented. The taxonomic classification, the mean relative abundance and the log2 fold change (of the DESeq2 normalized abundance of each species are shown).

Phylum	Class	Order	Family	Genus	Mean relative abundance (%) ZnAA	Mean relative abundance (%) $ZnSO_4$	Log2 fold change	Adjusted p-value
Ileum								
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	uncultured	0.039	0.499	-3,35	<0.001
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	*Blautia*	0.020	0.158	-3,20	0.001
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	*Lachnospiraceae NK4A136 group*	0.018	0.176	-5,20	0.001
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Butyriciccocus*	0.007	0.086	-3.70	<0.001
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Anaerotruncus*	0.001	0.025	-4.96	0.001
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	uncultured	0.004	0.026	-3.49	0.002
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Subdoligranulum*	0.047	0.560	-2.97	0.025
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Streptococcus*	0.021	0.872	-3.932	0.002
Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	*Helicobacter*	0.019	0.510	-4.78	<0.001
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*	0.013	0.313	-4.87	<0.001
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	*Delftia*	0.045	0.455	-3.41	<0.001
Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	*Ralstonia*	0.030	0.384	-4.35	<0.001
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	0.023	0.475	-2.90	0.025
Cecum								
Firmicutes	Clostridia	Clostridiales	Defluviitaleaceae	*Defluviitaleaceae UCG-011*	0.003	0.002	-5.93	<0.001

Table 10: Significantly enriched modules in broilers fed a diet supplemented with $ZnSO_4$ compared to broilers fed a diet supplemented with ZnAA

$ZnSO_4$ enriched	Functional description	Adjusted p-value	FDR
M00249	Capsular_polysaccharide_transport_system	0.016	0.03
M00156	Complex_IV_Cytochrome_c_oxidase_ cytochrome_c_oxidase_cbb3_type	0.042	0.03
M00310	Pyruvate_ferredoxin_oxidoreductase	0.053	0.03
M00038	Tryptophan_metabolism_tryptophan_ kynurenine_2_aminomuconate	0.063	0.03
M00349	Microcin_C_transport_system	0.072	0.03
M00072	Oligosaccharyltransferase	0.072	0.03
M00300	Putrescine_transport_system	0.089	0.04

DISCUSSION

In the present study we observed a higher apparent zinc retention for ZnAA complexes compared to $ZnSO_4$, which is in line with the higher bioavailability of ZnAA reported by Star et al. (2011). Supplementation with ZnAA complexes did not influence the digestibility of other nutrients which is in accordance with studies where the supplementation of zinc-glycine chelates was compared to $ZnSO_4$ (Ma et al., 2011; Kwiecien et al., 2016). The bioavailability of zinc supplements is affected by competition with other minerals or inhibition by antagonists present in the diet (Lönnerdal, 2000; Sauer et al. 2017). Therefore it is important to supply a zinc source which is taken up by a route which is not inhibited or saturated by Zn and other trace metals. It has been shown that ZnAA complexes are taken up by amino-acid transporters as opposed to zinc salts (e.g., $ZnSO_4$, $ZnCl_2$) which are taken up by zinc transporters (Sauer et al., 2017, Gao et al., 2014). The latter can be inhibited by zinc uptake antagonists (Lönnerdal, 2000). This alternative supply route of zinc might explain the higher bioavailability of ZnAA. Although ZnAA are characterized by an increased bioavailability, no differences in zinc serum levels were observed, which is in accordance with previous observations (Mohanna and Nys, 1999a, Zakaria et al., 2017a, Abd El-Hack et al., 2018). The fact that no differences in zinc serum level could be observed can either be explained by the strict homeostatic control. Alternatively, this could be explained by an increased deposition of zinc in tissues, because broilers fed ZnAA showed an improved growth in the starter period, which resulted in an increased apparent zinc retention, as observed in the digestibility study.

To our knowledge no literature is available about the effect of supplementation of different zinc sources on the microbiota composition in broilers. A study conducted by Ishaq et al. (2019a) in yearling rams revealed that supplementation of ZnAA supplementation alters bacterial communities compared to the supplementation of $ZnSO_4$. In the present study the microbial composition in the ileum did not differ in bacterial diversity or richness but does

show a trend towards a changing microbial composition. Single genera belonging the phylum Firmicutes were reduced when supplementing ZnAA without affecting the overall relative abundance of the phylum. Several genera belonging to the phylum of Proteobacteria were less abundant in the ileum content of the group supplemented with ZnAA compared to the $ZnSO_4$ group and overall relative abundance was also reduced. Expansion of Proteobacteria has been proposed as a microbial signature of gut dysbiosis and epithelial dysfunction (Litvak et al., 2017, Weiss and Hennet, 2017b) and this may partly explain the lower villus length and villus length to crypth depth ratio observed in broilers supplemented with $ZnSO_4$ as compared to ZnAA.

This study showed that the use of ZnAA instead of $ZnSO_4$ significantly reduced FCR. This decrease in FCR is not due to an increased feed intake, but seems to be an effect of increased weight gain when zinc is provided as ZnAA complexes as opposed to $ZnSO_4$. A performance study conducted by (Saenmahayak et al., 2010) showed that partially replacement $ZnSO_4$ supplementation by ZnAA supplementation was able to improve body weight and FCR. Jahanian et al. (2008) showed that supplementation with Zn-methionine improved performance as compared to $ZnSO_4$ in broilers.

Supplementation with ZnAA increased villus length and villus length to crypt depth ratio up to day 28. The crypts constantly renew the epithelial cells lining the intestinal lumen by migration of new cells from the crypts to the villus tip. During this migration cells mature and become more efficient in nutrient absorption. The villous epithelial cells come directly in contact with the lumen content and are therefore prone to damage, which often results in an increased loss of villous epithelial cells in cases of intestinal health problems (Zhang et al., 2015). The improved villus length, without accompanying increase in crypt depth may indicate that there is less villous epithelial cell loss at the villus tip compared to supplementation with $ZnSO_4$. An increased villus length is associated with an increased

digestion and absorption of nutrients, and an increase of brush border enzymes and nutrient transport systems (Awad et al., 2017b). According to Collett (2012b) the intestinal surface is directly proportional to digestive and absorptive efficiency and thus also to feed conversion efficiency. Taking this in consideration the improved villus morphology may partly explain the lowered FCR during the starter and grower phase when supplementing with ZnAA complexes

This study showed that supplementation with ZnAA complexes seems to alleviate oxidative stress by decreased MDA plasma levels and reduction in GPx activities. Malondialdehyde (MDA) has been determined as a marker for oxidative stress, as it is the most important end product in the chain reaction of lipid peroxidation caused by radicals (Del Rio et al., 2005). This study showed a decrease of plasma MDA levels in broilers fed a diet supplemented with ZnAA complexes at day 10 (end of starter phase). This might indicate a beneficial impact of ZnAA complexes on the oxidative status in broilers in the starter phase. At slaughter age (day 36) no differences in plasma MDA levels were found, but supplementation with ZnAA complexes showed a significantly lower activity for glutathione peroxidase activity in the plasma indicating a lower need for antioxidant activity for birds supplemented with ZnAA to maintain the same oxidative status. Analysis of the metabolic function prediction of the microbial communities, shows enrichment of pathways involved in oxidative reactions in the group of $ZnSO_4$ supplemented broilers. A hypothesis could be that in the group supplemented with $ZnSO_4$ more pro-oxidative molecules were present, which led to a higher activity of the GPx in the plasma of broilers fed a diet supplemented with $ZnSO_4$ compared to broilers supplemented a diet with ZnAA. The observation of metabolic pathways responding to oxidative stress in the intestinal microbiota is in line with this hypothesis. A study conducted by Ma et al. (2011) showed that zinc supplementation can decrease MDA levels in liver extracts compared to a non-supplemented control group, which confirms the importance of

zinc supplementation, although Ma et al. (2011) did not find differences between $ZnSO_4$ and ZnAA supplementation in liver extracts.

In conclusion, zinc supplied in feed as amino-acid-complex is characterized by an increased apparent zinc retention and significantly enhances broiler performance during the early life. ZnAA complex supplementation results in an increased villus length and villus length-to crypt depth ratio, indicating an improved intestinal morphology. Moreover, a decreased abundance of several genera belonging to the phylum Proteobacteria was observed when supplementing ZnAA complexes, indicating a positive impact on intestinal health. A reduction in plasma MDA levels and GPx activity indicates that supplementation with ZnAA complexes might have a positive impact on oxidative status compared to supplementation with $ZnSO_4$. These results open opportunities for further studies, where the effect of supplementation of ZnAA complexes on intestinal health and performance under more challenging conditions should be investigated.

CHAPTER 2

CHAPTER 2:

DIETARY ZINC SOURCE AFFECTS PERFORMANCE AND INTESTINAL HEALTH PARAMETERS IN MALE BROILERS REARED UNDER HIGH TEMPERATURES

Adapted from: Dietary zinc source affects performance and intestinal health parameters in male broilers reared under high temperatures. De Grande A., R. Ducatelle., Delezie E., Rapp C., De Smet S., Michiels J.., Haesebrouck F., Van Immerseel F., Leleu S.

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ABSTRACT

The objective of this study was to evaluate the interaction of zinc source ($ZnSO_4$ vs. zinc amino acid complex) and vitamin E level (50 IU/kg vs. 100 IU/kg) on performance and intestinal health of broilers exposed to a temperature challenge in the finisher period. A total of 1224 day old male Ross 308 broilers were randomly distributed among 4 dietary treatments (9 replicates of 34 birds/treatment). Dietary treatments were organized in a 2x2 factorial arrangement: two sources of zinc, 60 mg/kg of Zn as $ZnSO_4.7H_2O$ or 60 mg/kg of Zn as zinc amino acid complexes (ZnAA) combined with two levels of vitamin E (50 or 100 IU/kg). From day 28 until day 36 (finisher period) all birds were subjected to chronic cyclic high temperatures (32°C ± 2°C and RH 55-65% for 6h daily). The combination of ZnAA and 50 IU/kg of vitamin E improved weight gain in the starter (day 0-10), finisher (day 28-36) and overall period (day 0-36) and feed conversion ratio in starter (day 0-10) and finisher phase (day 28-36). Providing Zn as ZnAA significantly improved villus length and villus/crypt ratio in the starter, grower and finisher period and decreased infiltration of T-lymphocytes and ovotransferrin leakage in the finisher period. In conclusion, replacing $ZnSO_4$ with chelated zinc (ZnAA) positively impacts intestinal morphology in broilers and also improves performance when vitamin E is supplemented at 50 IU/kg in feed. Interestingly, under the conditions of this study, positive effects of ZnAA on performance do not occur when vitamin E is supplemented at 100 IU/kg in feed. Moreover, providing zinc as zinc amino acid complex reduces intestinal inflammation in the finisher period when broilers are subjected to a temperature challenge.

INTRODUCTION

High environmental temperatures are a common problem in poultry raised in tropical and subtropical regions, but can also cause issues in temperate regions during the summer months (Farag and Alagawany, 2018, Habashy et al., 2017). Poultry is highly susceptible to high ambient temperatures because of the lack of sweat glands and the feathering (Sohail et al., 2012, Lara and Rostagno, 2013a). Moreover, the intensive selection for increased performance in broiler chickens raised for meat production has led to a higher metabolic heat production and increased susceptibility to high ambient temperatures (Mohammed et al., 2018, He et al., 2018c). When the environmental temperature is raised considerably above the thermoneutral zone of the animal heat stress occurs (Farag and Alagawany, 2018, Nawab et al., 2018, Sahin et al., 2009).

Global warming has drawn more attention towards heat stress, because of its detrimental impact on animal health and performance (He et al., 2018a). In addition, heat stress is associated with increased mortality, which leads to considerable losses in the poultry industry (He et al., 2018b, Nawab et al., 2018). Heat stress can be acute or chronic, which both have detrimental effects on overall health (Zhang et al., 2012). Acute heat stress refers to a short and rapid rise in ambient temperature, whereas chronic heat stress refers to exposure to a high ambient temperature over a long period of time. Chronic heat stress can either be continuous or cyclic (Akbarian et al., 2016).

The negative effects of high ambient temperatures on performance can be mainly attributed to the decreased feed intake. Additionally, heat stress negatively impacts intestinal morphology and gut barrier integrity in broilers (Zhang et al., 2017, Song et al., 2014, Wu et al., 2018). Intestinal integrity is considered to be a key element in health and performance (Ducatelle et al., 2018). Disturbance of intestinal health can lead to intestinal mucosal barrier damage. Measuring the villus length, crypt depth and villus/crypt ratio remains the gold standard in

order to asses intestinal mucosal damage. Intestinal integrity can be assessed by measuring the expression level of tight junction genes or leakage of plasma proteins into the intestinal content (Awad et al., 2017a). Fecal ovotransferrin has been identified as a marker for gut barrier failure in broiler chickens and can be used to assess the efficacy of additives or strategies that reduce intestinal damage (Goossens et al., 2018). Ovotransferrin acts as an acute phase protein in birds and has been described as a serum marker for stress, infection or inflammation in broilers (Rath et al., 2009, Cray et al., 2009, Ibrahim et al., 2000). A fecal marker to assess intestinal barrier failure is of great interest, however, ovotransferrin is rapidly degraded by proteases in the feces (Goossens et al., 2018), therefore ileum content constitutes a good alternative. Poor intestinal health has been associated with immune cell infiltration in the mucosal wall (Morampudi et al., 2014). An *in vivo* gut damage trial performed by De Meyer et al. (2019) showed an increased infiltration of CD_3^+ T-lymphocytes in duodenal sections of challenged broilers as compared to the control group and could therefore be used as a marker for intestinal inflammation.

In-feed supplementation of minerals and vitamins with anti-oxidative activity has been suggested as a nutritional intervention to alleviate the negative impact of high ambient temperatures in broiler production (Harsini et al., 2012, Kucuk et al., 2003, Liao et al., 2018). Zinc is an essential trace element which exerts many functions in various biological processes and acts as an important structural and catalytic compound of various metalloenzymes (Ranaldi et al., 2013a, Bonaventura et al., 2015a, Oteiza, 2012). One of the most important functions of zinc is its role in the antioxidant defence system (Sahin et al., 2009). It has been reported that vitamin E might alleviate negative effects of heat stress because of its lipid soluble antioxidant capacities (Attia et al., 2017a, Sahin et al., 2002b). Moreover, it has the capacity to eliminate free radicals in the membranes of cells and subcellular organelles exposed to heat stress (Imik et al., 2012). Thus, dietary supplementation with a combination

of zinc and vitamin E may improve heat tolerance. In broiler diets, zinc is commonly supplemented as inorganic zinc sulphate or zinc oxide, but there are also organic zinc sources available that are characterized by an improved bioavailability (Star et al., 2012, Swiatkiewicz et al., 2014). The beneficial effects of dietary zinc and vitamin E in heat stressed poultry separately have been extensively investigated, however limited information is available on the interaction effect. To the best of our knowledge, interactions of zinc source and vitamin E level have not been investigated under heat stress conditions in broilers.

As the requirements of both vitamins and trace minerals may increase during heat stress, the objective of this study was to test the hypothesis that inclusion of a more readily available zinc source and an a higher level of vitamin E in the diet might mitigate the adverse effects of high ambient temperatures (Chand et al., 2014, Farag and Alagawany, 2018, Sahin et al., 2009). The temperature challenge, which closely mimics an actual heat wave, was applied the finisher period, as the birds are more susceptible to high ambient temperatures in this life stage (Ashraf et al., 2013).

MATERIALS & METHODS

All experimental procedures in this study were in compliance with the European guidelines for the care and use of animals in research (Directive 2010 : 63 : EU) and were approved by the Ethical committee of the Research Institute for Agriculture, Fisheries and Food (ILVO), Merelbeke, Belgium under authorization number 2017 : 308.

Animals, Diets and Experimental design

A total of 1224 day old male Ross 308 broilers (Belgabroed hatchery, Merksplas, Belgium) were randomly allocated to 36 floor pens (9 pens per treatment and 34 broilers per pen) in alternating block design, with one replicate per treatment in each block. Treatments were organized in a 2x2 factorial arrangement with two sources of zinc (Zn), 60 mg/kg of Zn as $ZnSO_4$ ($ZnSO_4.7H_2O$, Sigma-Aldrich, St. Louis, USA) or 60 mg/kg of Zn as zinc amino acid

complexes (ZnAA; Availa®Zn, Zinpro Corporation, Eden Prairie, USA) combined with two levels of vitamin E (50 or 100 IU/kg). Availa®Zn is a zinc chelate based on single amino acids from hydrolysed soy protein and zinc bound in a one to one molar ratio.

The broilers were fed a wheat-rye based diet which contained a high level of crude protein and non-starch polysaccharides (NSP, 15%) without the addition of NSP enzymes in order to create a nutritional challenge at the intestinal level (Table1). One batch of basal diet was manufactured at the research facilities of ILVO and then divided in four equal portions which were mixed together with a premix (Research Diet Services BV, Utrecht, The Netherlands) containing the correct supplemental zinc source (60 mg/kg $ZnSO_4$ or ZnAA) and vitamin E level(50 or 100 IU/kg). After mixing the basal diet with the premix, the feed (500 kg/h, 65°C) was pelleted at the research facilities of ILVO. All dietary treatments contained zinc levels that comply with the dietary needs as described by NRC (1994). A vitamin E level of 50 IU/kg is recommended in the Ross 308 manual (Aviagen, 2018) and was set as the standard dose, 100 IU/kg was selected as elevated level of vitamin E. The total levels of zinc included in the experimental diets were determined using Inductively coupled plasma atomic emission spectroscopy with a method derived from NEN 15763 , ISO 21033 and ISO 27085 at ECCA laboratory (Merelbeke, Belgium) (Table 2). The total levels of vitamin E included in the experimental diets were determined according to Claeys et al. (2016) (Table 2). A three-phase feeding scheme was applied and dietary treatments were applied in all phases. The starter diet was fed from day zero up to day 10 and was provided in a crumbled form. The grower and finisher diets were fed as pellets from day 10 up to day 28 and from day 28 up to day 36, respectively. Feed and drinking water were provided ad libitum.

Table 1: Dietary composition of the diets

	Starter diet	Grower diet	Finisher diet
Ingredient (%)			
Wheat	48.54	54.45	57.65
Rye	5.00	5.00	5.00
Soybean meal (48)	30.18	24.39	21.61
Soybeans	7.50	7.50	7.42
Rapeseed meal	2.00	2.00	2.00
Animal fat	2.50	2.60	2.70
Soy oil	1.00	1.00	1.00
Vitamin + trace mineral mix§	1.000	1.000	1.000
$CaCO_3$	0.707	0.788	0.702
Di-Ca-phosphate	0.737	0.456	0.206
NaCl	0.272	0.235	0.278
Na-bicarbonate	0.104	0.145	0.087
L-Lys-HCl	0.134	0.144	0.121
DL-Methonine	0.260	0.213	0.172
L-threonine	0.064	0.056	0.040
Phytase‡	0.020	0.020	0.020
Calculated nutrient composition			
Crude protein (%)	23.00	21.00	20.00
Crude fat (%)	6.46	6.41	6.50
Non-soluble polysaccharides (%)	14.66	14.21	13.98
Metabolisable energy (MCal/kg)	2.63	2.69	2.74
Dig. Lysine (%)	1.15	1.03	0.95
Dig. Methionine + Cysteine (%)	0.86	0.77	0.71
Dig. Threonine (%)	0.75	0.67	0.62
Dig. Valine (%)	0.89	0.81	0.76
Ca (%)	0.85	0.80	0.70
Available P (%)	0.40	0.35	0.31
NaCl + KCl (mEq/kg)	267	247	213
Linoleic acid (18:2) (%)	2.10	2.08	2.06

§ Provided per kg of diet: Vitamin A (retinylacetate 3a672a), 10 000 IU; vitamin D3 (E671), 3000 IU; vitamin E (dl-α-tocopherol acetate), 50 IU (T1+T2) or 100 IU (T3+T4); vitamin K, 2,5 mg; Vitamin B1 (thiamine mononitrate), 2 mg; riboflavin, 5 mg; Calcium D-pantothenate, 15 mg; vitamin B6, 4 mg; vitamin B12, 0.025 mg; niacinamide, 30 mg; folic acid, 1 mg; biotin, 0.2 mg; choline (choline chloride), 689.7 mg; Cu ($CuSO_{4.5}H_2O$), 20 mg; Mn ($MnSO_4.H_2O$), 95.9 mg; Fe ($FeSO_4.H_2O$), 49.2 mg; I (KI), 1.2 mg; Se (Na_2SeO_3), 0.4 mg; HSepioliet, 7.0 mg; Propylgallate, 2.0 mg; BHT, 3.0 mg; Zn (T1+T3, $ZnSO_{4.7}H_2O$, T2+T4, Availa®Zn), 60 mg. ‡Ronozyme ® NP. 10 000 FYT/g

Table 2: Analyzed zinc (mg/kg) and vitamin E (IU/kg) concentrations in the different dietary treatments (T) for broilers (as-fed basis)

	Added source/levels			Analyzed zinc levels			Analyzed vitamin E levels		
T	Zinc source	Zinc	Vit E	Starter	Grower	Finisher	Starter	Grower	finisher
1	$ZnSO_4$	60	50	94	88	90	73	82	67
2	ZnAA	60	50	91	90	91	67	86	69
3	$ZnSO_4$	60	100	89	91	89	121	145	105
4	ZnAA	60	100	90	98	90	113	119	104

ZnAA (zinc amino acid complex); Vit E (Vitamin E, dl-α-tocopheryl acetate)

Broilers were housed on solid floor covered with wood shavings (2.5 kg/m²). Up to day 7, the broilers were subjected to a light schedule of 23 hours light and one hour darkness. From day 7 onwards the animals were subjected to a light schedule of 18 hours light and six hours darkness. The temperature was kept at 29°C during the first week of the experiment and reduced thereafter until a final temperature of 22°C was reached at day 28. From day 28 until day 36 (slaughter age) the temperature and relative humidity (RH) in the stable were raised up to 32°C ± 2°C and 55-65% respectively, during 3h and subsequently maintained for 6h before cooling down again to initial temperature of 22°C. The temperature and RH were constantly monitored and adjusted accordingly. In this experimental design a thermoneutral control group was not incorporated, because this was practically not feasible in our research facilities. The broilers were spray vaccinated at the hatchery against Newcastle Disease (Poulvac NDW, Zoetis, Zaventem, Belgium). Broilers were vaccinated with Paracox®-5 via oral gavage (MSD UK Ltd., Milton Keynes, UK) on day three. At the age of 15 days the vaccination against Newcastle disease was repeated with Nobilis ND Clone 30 (MSD international B.V., Boxmeer, The Netherlands), which was provided in the drinking water.

Growth Performance, Mortality and Sampling

Mortality and culled birds were recorded daily. Culled birds were not included in the mortality calculations (except when they were removed because of illness). Mortality corrected growth performance was calculated using the number of 'broiler days' (number of broilers multiplied with the number of days alive). At day 10, 28 and 36, all broilers and feed left overs were weighed per pen to determine body weight, body weight gain, feed intake and feed conversion ratio. At the same time point, three broilers per pen were randomly selected euthanized by an intravenous overdose of sodium pentobarbital 20% (Kela NV, Hoogstraten, Belgium) and venous blood samples were collected in serum and heparin tubes (Vacutest Plast, Kima, Arzergrande, Italy). Tissue samples of the duodenal loop were collected for fixation in 4% of formaldehyde. Content of ileum was collected aseptically (stored at -20 °C).

Evaluation of Intestinal Health

Tissue samples of the duodenal loop were embedded in paraffin, sectioned and stained with haematoxylin and eosin for histology and with an antibody specific to CD3 for immunohistochemistry as previously described (Chapter 1). Villus length, crypt depth and villus length to crypt depth ratio were determined in duodenum sections stained with haematoxylin and eosin, using a Leica DM LB2 Digital microscope and a computer based image analysis program, Leica Application Suite V4.1. Intestinal inflammation was evaluated by measuring the infiltration of CD3 positive cells in duodenal sections as described by Aguirre et al. (2019). Additionally, ileal content was used for determination of ovotransferrin concentration. Ileum contents were pooled per pen (n=9 per treatment group) and further processed as described by Goossens et al. (2018) to determine ovotransferrin concentration with enzyme-linked immunosorbent assay (ELISA) (Chicken Ovotransferrin ELISA, KT-530, Kamiya Biomedical Company, Tukwila, USA). The ELISA was performed according to the instructions of the manufacturer.

Serum Zinc Concentrations

Serum zinc concentrations were determined as described by van Riet et al. (2015). Briefly, serum samples were mixed with an equal volume of trichloroacetic acid to deproteinate samples before centrifugation at 10 000 x g for 10 minutes. The remaining supernatant was used within 2h to determine serum zinc concentrations. Therefore the deproteinated serum was diluted five times with a color reagent (Randox kit ZN2341, Randox laboratories limited, Crumlin, UK), and incubated 5 minutes at 25°C. Absorbance was measured at 560 nm. Serum zinc concentration was calculated from a zinc standard calibration curve.

Statistical analysis

Statistical analysis was performed in R for Windows (version 3.5.1). All data were evaluated for the presence of outliers and normality of the residuals. Performance, intestinal health parameters and serum zinc levels were analysed by a General Linear Model (GLM)) with 'Zinc source' and 'Vitamin E level' as fixed factors and block as a random factor (factorial analysis, pen as experimental unit). Mortality was analysed with logistic regression with 'Zinc Source' and 'Vitamin E level' as fixed factors and block as a random factor (factorial analysis, pen as experimental unit). In the two-factorial analyses, when there was no significant interaction, only the main effects were taken into account. In case of a significant interaction a Tukey test was performed in order to compare the treatments. In the tables, data with different superscripts differ significantly ($P<0.05$). The differences were considered statistically significant at $P \leq 0.05$ and considered as tendency at $0.05 < P < 0.1$.

RESULTS

Performance and Mortality

A significant interaction between zinc source and vitamin E level was found with respect to, growth and other performance parameters ($P < 0.05$). Replacing $ZnSO_4$ by ZnAA at a vitamin E level of 50 IU/kg increased mean body weight at day 10 (end of the starter period) ($P = 0.001$), body weight gain $P < 0.001$) and lowered feed conversion ratio ($P = 0.011$) during the starter period (day0-10). However, performance parameters did not differ when $ZnSO_4$ was replaced by ZnAA at a level of 100 IU/kg of vitamin E (Table 3&4). Dietary treatments did not affect growth and other performance parameters during the grower period (day 10-28) (Table 3&4).

During the finisher period, when the heat stress model was applied, a higher weight gain ($P = 0.009$) and a lower feed conversion ratio ($P = 0.025$) was obtained by replacing $ZnSO_4$ by ZnAA when supplementing 50 IU/kg of vitamin E. However, this effect was not observed at a vitamin E level of 100 IU/kg ((Table 3&4)

For the entire period, a higher mean body weight ($P = 0.013$) and a higher weight gain ($P = 0.013$) was observed when $ZnSO_4$ was replaced by ZnAA at a level of 50 IU/kg of vitamin E (Table 3&4).

Dietary treatments did not affect mortality rates during the starter and grower period. During the finisher period there was a trend for a main effect of the vitamin E level with a higher mortality when 100 IU/kg was administered (Table 5).

Table 3: Effect of supplemental Zn source and vitamin E level on mean body weight (g/animal) of broilers of different ages (n=9 per dietary treatment)

		Mean body weight (g/animal)			
Zn source	Vit E level (IU/kg)	Day 0	Day 10	Day 28	Day 36
$ZnSO_4$	50	45.3	332[b]	1865	2711[b]
ZnAA	50	45.6	349[a]	1912	2786[a]
$ZnSO_4$	100	45.6	334[b]	1872	2730[b]
ZnAA	100	45.7	331[b]	1871	2697[b]
SEM		0.28	3.40	18.13	22.24
Means of main effects					
$ZnSO_4$		45.4	333	1869	2720
ZnAA		45.6	339	1887	2739
	50	45.5	340	1884	2746
	100	45.6	333	1871	2713
Source of probabilities					
Source x level		0.850	**0.001**	0.188	**0.013**
Zn Source		0.359	0.029	0.684	0.847
Vit E level		0.161	0.013	0.791	0.879

ZnAA (zinc amino acid complex); Vit E (Vitamin E); SEM (standard error of the mean)

[a,b] Within column values with different superscripts differ significantly ($P \leq 0.05$)

Table 4: Effect of supplemental Zn source and vitamin E level (IU/kg) on feed intake, weight gain and feed conversion ratio (n=9)

		Daily weight gain (g/animal/day)				Daily feed intake (g/animal/day)				Feed conversion ratio			
Zn Source	Vit E level (IU/kg)	Day 0-10	Day 10-28	Day 28-36	Day 0-36	Day 0-10	Day 10-28	Day 28-36	Day 0-36	Day 0-10	Day 10-28	Day 28-36	Day 0-36
$ZnSO_4$	50	28.7^{b}	85.2	105.6^{ab}	75.3^{b}	33.9^{ab}	124.1	194.2	114.6	1.183^{b}	1.456	1.842^{b}	1.523
ZnAA	50	30.4^{a}	86.8	110.2^{a}	77.4^{a}	34.7^{a}	126.5	194.0	116.0	1.144^{a}	1.458	1.764^{a}	1.500
$ZnSO_4$	100	28.9^{b}	85.4	107.3^{ab}	75.8^{b}	33.9^{ab}	125.0	194.1	115.1	1.174^{b}	1.464	1.810^{b}	1.518
ZnAA	100	28.5^{b}	85.5	103.2^{b}	74.9^{b}	33.5^{b}	124.1	191.4	113.9	1.172^{b}	1.451	1.855^{b}	1.521
SEM		0.34	0.95	1.71	0.62	0.32	1.02	1.24	0.70	0.01	0.01	0.03	0.01
Means of main effects													
$ZnSO_4$		28.7	85.3	106.5	75.5	33.9	124.5	194.1	114.8	1.178	1.460	1.826	1.520
ZnAA		29.4	86.0	106.5	76.0	34.0	125.2	192.6	114.8	1.158	1.457	1.813	1.511
	50	29.5	85.8	107.8	76.2	34.3	125.2	194.1	115.2	1.164	1.461	1.805	1.512
	100	28.7	85.5	105.3	75.3	33.7	124.5	192.7	114.5	1.173	1.457	1.833	1.519
Source of probability													
Source x level		**<0.001**	0.425	**0.009**	**0.013**	**0.045**	0.101	0.456	0.316	**0.011**	0.446	**0.025**	0.147
Zn Source		0.030	0.365	0.643	0.315	0.523	0.470	0.743	0.110	0.005	0.535	0.546	0.280
Vit E level		0.010	0.589	0.448	0.103	0.032	0.482	0.125	0.930	0.162	0.995	0.246	0.410

ZnAA (zinc amino acid complex); Vit E (Vitamin E); SEM (standard error of the mean)

[a,b] Within column values with different superscripts differ significantly ($P \leq 0.05$)

Table 5: Effect of supplemented zinc source and vitamin E level on mortality (expressed as mean ± SD) (n=9)

		Mortality (%)			
Zn Source	Vit E level (IU/kg)	Day 0-10	Day 10-28	Day 28-36	Day 0-36
$ZnSO_4$	50	0.78 ± 0.43	2.82 ± 0.86	1.04 ± 0.47	4.17 ± 1.10
ZnAA	50	1.73 ± 0.73	2.67 ± 0.84	1.62 ± 0.67	5.53 ± 1.35
$ZnSO_4$	100	0.78 ± 0.42	2.64 ± 0.83	2.34 ± 0.83	5.22 ± 1.30
ZnAA	100	1.72 ± 0.73	2.50 ± 0.80	3.64 ± 1.07	6.90 ± 1.59
Means main effects					
$ZnSO_4$		0.79 ± 0.37	2.73 ± 0.72	1.56 ± 0.53	4.67 ± 1.06
ZnAA		1.73 ± 0.60	2.58 ± 0.69	2.43 ± 0.68	6.18 ± 1.29
	50	1.17 ± 0.47	2.74 ± 0.72	1.30 ± 0.49	4.80 ± 1.09
	100	1.16 ± 0.47	2.57 ± 0.69	2.92 ± 0.73	6.01 ± 1.26
Source of probability					
Source x level		0.127	0.226	0.249	0.543
Zn Source		0.141	0.874	0.294	0.224
Vit E level		0.994	0.848	**0.070**	0.332

ZnAA (zinc amino acid complex); Vit E (Vitamin E)

Intestinal Morphology and Inflammation

Regardless of vitamin E level, birds fed a diet supplemented with ZnAA had a higher villus length ($P < 0.05$) and villus length to crypt depth ratio ($P < 0.05$) in duodenum sections as compared to birds fed a diet supplemented with $ZnSO_4$ on day 10, 28 and 36. A main effect of zinc source was also observed for the infiltration of CD_3 positive T-lymphocytes in duodenum sections on day 36, with a lower infiltration of CD_3 positive T-lymphocytes for birds fed a diet supplemented with ZnAA compared to $ZnSO_4$ ($P = 0.023$) (Table 6).

A trend towards a lower concentration of ovotransferrin in ileum content was observed at the end of the heat stress period (day 36) for birds fed a diet supplemented with ZnAA in comparison to birds fed a diet supplemented with $ZnSO_4$ ($P = 0.094$) (Table 7), independent of vitamin E level. The ovotransferrin concentration increased with almost a 10-fold after the period in which the temperature challenge was applied.

Table 6: Effect of supplemental Zn source and vitamin E level on villus length (VL, µm), crypt depth (CD, µm), villus length to crypt depth ratio (Ratio) and infiltration of CD_3 positive cells in duodenal sections (expressed as area %) (n=9)

Zn source	Vit E level (IU/kg)	Day 10				Day 28				Day 36			
		VL	**CD**	**Ratio**	**CD_3**	**VL**	**CD**	**Ratio**	**CD_3**	**VL**	**CD**	**Ratio**	**CD_3**
$ZnSO_4$	50	1191	254	4.96	6.87	1509	248	6.54	12.7	1568	225	7.45	12.5
ZnAA	50	1413	279	5.43	6.72	1841	241	8.28	11.8	1713	222	8.19	11.6
$ZnSO_4$	100	1210	273	4.81	7.25	1523	252	6.66	13.3	1610	227	7.40	13.8
ZnAA	100	1369	274	5.37	8.00	1645	211	8.33	11.4	1683	208	8.72	10.8
SEM		17.11	5.93	0.11	0.18	34.89	2.23	0.06	1.37	23.84	4.43	0.20	1.35
Means of main effects													
$ZnSO_4$		1201	264	4.89	7.06	1516	250	6.60	13.0	1589	227	7.43	13.1
ZnAA		1391	277	5.40	7.36	1743	227	8.30	11.6	1698	214	8.46	11.2
	50 IU/kg	1302	267	5.20	6.80	1675	245	7.41	12.3	1641	223	7.82	12.0
	100 IU/kg	1289	274	5.09	7.63	1584	232	7.50	12.4	1647	218	8.06	12.2
Source of probabilities													
Source x level		0.183	0.235	0.079	0.397	0.295	0.397	0.951	0.701	0.382	0.246	0.388	0.189
Zn source		**<0.001**	0.221	**0.003**	0.564	**0.028**	0.244	**0.003**	0.301	**0.009**	0.120	**0.003**	**0.023**
Vit E level		0.596	0.517	0.073	0.120	0.366	0.542	0.880	0.938	0.885	0.539	0.478	0.756

ZnAA (zinc amino acid complex); Vit E (Vitamin E); SEM (standard error of the mean)

Table 7: Effect of supplemental zinc source and vitamin E level on ovotransferrin concentration in ileum content (µg/g) (n=9)

Zn Source	Vit E level (IU/kg)	Day 10	Day 28	Day 36
$ZnSO_4$	50	1.47	1.84	19.25
ZnAA	50	2.35	2.94	16.44
$ZnSO_4$	100	1.78	2.22	20.99
ZnAA	100	1.61	2.02	15.53
SEM		0.13	1.11	1.81
Means of main effects				
$ZnSO_4$		1.62	2.03	20.12
ZnAA		1.98	2.48	15.99
	50 IU/kg	1.91	2.39	17.84
	100 IU/kg	1.70	2.12	18.27
Source of probabilities				
Source x level		0.194	0.194	0.582
Zn Source		0.369	0.364	0.094
Vit E level		0.589	0.584	0.859

ZnAA (zinc amino acid complex); Vit E (Vitamin E); SEM (standard error of the mean)

Serum Zinc Concentrations

In birds of 10 and 28 days of age there were no significant differences in zinc serum levels between treatment groups. However, at the end of the heat stress period (day 36) significantly higher concentrations of serum zinc were observed for birds fed a diet with 100 IU/kg of vitamin E, independent of the zinc source (P = 0.031) (Table 8).

Table 8: Effect of supplemental zinc source and vitamin E level on zinc serum levels (µg/dL) of broilers of different ages (n=9)

Zn Source	Vit E level (IU/kg)	Day 10	Day 28	Day 36
$ZnSO_4$	50	305.4	319.7	264.4
ZnAA	50	254.7	284.1	255.5
$ZnSO_4$	100	260.7	299.2	295.8
ZnAA	100	284.7	293.1	282.9
SEM		1.56	1.73	0.92
Means of main effects				
$ZnSO_4$		283.1	309.5	280.1
ZnAA		269.7	288.6	269.2
	50 IU/kg	280.1	301.9	260.0
	100 IU/kg	272.7	296.2	289.4
Source of probabilities				
Source x level		0.633	0.674	0.985
Zn Source		0.353	0.995	0.668
Vit E level		0.882	0.597	**0.031**

ZnAA (zinc amino acid complex); Vit E (Vitamin E); SEM (standard error of the mean)

DISCUSSION

In the present study, maximum growth was observed when more readily available ZnAA complexes were combined with a vitamin E level of 50 IU/kg. In contrast to earlier findings, the broilers fed a diet with a higher inclusion of vitamin E (100 IU/kg) did not perform better than the broilers fed a diet supplemented with 50 IU/kg of vitamin E. Rehman et al. (2017) reported a positive effect of vitamin E (250 mg/kg) supplementation under heat stress conditions. Additionally, Sahin et al. (2006) showed that combining vitamin E (125 or 250 mg/kg) and zinc picolinate reduces the negative effects of heat stress on body weight and feed efficiency in Japanese quail. Therefore we expected that a higher inclusion rate of vitamin E might improve performance. Niu et al. (2009) found that different levels of vitamin E (0,100 and 200 mg/kg) did not influence growth and feed intake in chronic cyclic heat stressed broilers, but FCR was improved for broilers fed 100 mg/kg of vitamin E, but did not further improve at 200 mg/kg dosage.

The combination of ZnAA and 50 IU/kg of vitamin E improved weight gain in the starter, finisher and overall period (day 0-36) and feed conversion ratio in starter and finisher period. The starter period is a challenging period for young broilers because of the transition from protein-rich yolk to carbohydrate rich feed, switching to aerial breathing and starting effective thermoregulation. Broiler chicks benefit from highly digestible ingredients during the early phase in order to fully reach their genetic potential. Moreover, during this period the immune system is still immature, because the acquired immune system still needs to be developed and birds need to rely on maternal antibodies supplied via the yolk sac (Korver et al., 2012).

In the finisher period a temperature challenge was applied, which has a negative impact on growth. The presence of challenging conditions for the birds in both starter and finisher period might explain why effects of dietary treatments were only observed during these periods and not during the grower period.

The faster growth observed in the absence of increased feed intake might indicate that nutrients were used more efficiently. One likely explanation for this is the positive effect of ZnAA on intestinal morphology, as also shown in a previous study (Chapter 1). At the level of the intestinal epithelial barrier, heat stress can lead to a reduction in the proliferation rate of intestinal epithelial cells, gut barrier failure and cellular oxidative stress (Marchini et al., 2016). Zinc plays an important role in tissues with a fast cell turn-over such as the intestinal epithelium (Bonaventura et al., 2015a, Faa et al., 2008b). It has been shown in previous studies that crypt depth is increased and villus length and villus length to crypt depth ratio are decreased in the jejunum and ileum of broilers reared under chronic cyclic heat stress (Zhang et al., 2017, Song et al., 2014, Wu et al., 2018). In this study, it was observed that the supplementation of ZnAA increased villus length and villus length to crypt depth ratio in the duodenum under temperature challenge. An increase in villus length implicates an increase in intestinal surface, which is directly proportional to digestive and absorptive efficiency and thus also to feed conversion efficiency (Collett, 2012a). Therefore, the increased villus length might partly explain the positive effects of ZnAA on performance parameters. As the positive effect of ZnAA on intestinal morphology is already present during starter and grower period this might confer a protective effect against gut damage as a result of the temperature challenge in the finisher period. As no differences in crypt depth were observed, the increase in villus length cannot merely be explained by an increased renewal of epithelial cells from the pool of crypt based stem cells, but could be the consequence of a decreased loss of villous epithelial cells (Star et al., 2012). Zinc supplied as ZnAA complex to human enterocytes is

taken up by amino acid transporters, as opposed to zinc salts (such as $ZnCl_2$, $ZnSO_4$) which are taken up by Zn transporters (Sauer et al., 2017, Gao et al., 2014). It is likely that the uptake of Zn as ZnAA complexes is a process that will not be compensated by a homeostatic regulation of the amino acid transporters and given the abundance of amino acid transporters will not be easily saturated (Sauer et al., 2017). Delivering Zn as Zn salts decreases Zn absorption in the presence of higher levels of Zn in the diet which is probably because of the saturation of Zn transporters (Lee et al., 1989, Lonnerdal, 2000). Moreover, ZnAA compounds are also better protected from factors (such as Ca, Cu, phytic and folic acid) that act antagonistically in pathways responsible for uptake of Zn (Sauer et al., 2017). Indeed, in Chapter 1 an increased Zn digestibility was observed for ZnAA as compared to $ZnSO_4$. The uptake of ZnAA via amino acid transporters thus allows intracellular enrichment of zinc. Therefore it is possible that Zn from ZnAA is more efficiently used in metabolic processes in the epithelial cells. In the present study, dietary zinc source did not affect serum zinc levels, which is in accordance with previous studies (Star et al., 2012). Serum zinc levels are poor indicators of zinc bioavailability as a result of the tight homeostatic control and therefore differences in serum zinc levels of broilers fed adequate levels of zinc were not anticipated.

Another possible explanation for the positive effects of ZnAA on performance and villus morphology might be through a protective effect of ZnAA against oxidative stress induced inflammation. Oxidative stress and increased inflammation have been proposed as underlying mechanisms of the negative effects of high ambient temperatures (Akbarian et al., 2016, Mujahid et al., 2007, Sahin et al., 2002a, Xie et al., 2015). Ovotransferrin has been identified as a marker for gut barrier failure in broiler chickens (Goossens et al., 2018) and can be used to assess the efficacy of additives or strategies that reduce intestinal damage. At the end of the temperature challenge, ileal ovotransferrin tended to be lower in broilers fed a diet supplemented with ZnAA compared to broilers fed a diet supplemented with $ZnSO_4$,

independently of the vitamin E level. This suggests decreased gut barrier leakage as a result of an improved intestinal integrity. It has been shown that ZnAA complexes improve intestinal barrier function in heat stressed pigs (Mayorga et al., 2018, Pearce et al., 2015). Additionally, the infiltration of T-lymphocytes into intestinal tissue of broilers supplemented with ZnAA was also decreased compared to broilers fed $ZnSO_4$ at the end of the temperature challenge. A decreased infiltration of CD3 positive T-lymphocytes indicates a decreased stimulation of the immune system of the intestinal tract. It seems that broilers supplemented with ZnAA are more efficient in counteracting stress-induced intestinal inflammation. It has been shown that the nutritional cost of inflammation is high and has a negative impact on weight gain (Humphrey and Klasing, 2004).

In conclusion, replacing $ZnSO_4$ with chelated zinc (ZnAA) positively impacts intestinal morphology in broilers and also improves performance when vitamin E is supplemented at a level of 50 IU/kg, but was not ameliorated at a level of 100 IU/kg. Moreover, providing zinc as zinc amino acid complex reduces intestinal inflammation in the finisher period when broilers are subjected to a temperature challenge. Interestingly, under the conditions of this study, positive effects of ZnAA on performance do not occur when vitamin E is supplemented at 100 IU/kg in feed. The interaction between zinc source and vitamin E level needs to be further investigated in order to elucidate the underlying mechanism.

CHAPTER 3

CHAPTER 3:

EFFECTS OF DIETARY ZINC SOURCE ON CARCASS YIELD AND MEAT QUALITY IN MALE BROILERS REARED UNDER HIGH ENVIRONMENTAL TEMPERATURES

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ABSTRACT

The objective of this study was to evaluate whether the interaction of zinc source ($ZnSO_4$ vs. zinc amino acid complex) and vitamin E level (50 IU/kg vs. 100 IU/kg) might alleviate the negative effects on meat quality and yield of broilers exposed to chronic cyclic heat stress in the finisher phase. A total of 1224 day-old male Ross 308 broilers were randomly distributed among four dietary treatments. Each treatment contained nine replicates of 34 birds, housed in floor pens in a temperature and lighting controlled room. Treatments were organized in a 2x2 factorial arrangement: two sources of zinc**,** 60 mg/kg of Zn as $ZnSO_4$ or 60 mg/kg of Zn as zinc amino acid complexes (ZnAA), combined with two levels of vitamin E (50 or 100 IU/kg). From day 28 until day 36 (finisher phase), all birds were subjected to chronic cyclic heat stress (32°C ± 2°C for 6h daily). In the present study, it was observed that replacing $ZnSO_4$ by ZnAA, increased breast meat weight and yield of broilers reared under chronic cyclic heat stress conditions, whereas total slaughter yield was not affected. However, the increased breast meat yield is mainly attributed to the increased live weight of the selected broilers. Moreover, it was observed that replacing $ZnSO_4$ by ZnAA, resulted in breast meat with a lower drip and thawing loss and a higher marinade uptake. In conclusion, replacing $ZnSO_4$ with more readily available ZnAA, can improve breast meat yield and increase water holding capacity of breast meat of broilers exposed to chronic cyclic heat stress at the end of the production cycle. Moreover, the beneficial effects of ZnAA on breast meat yield and quality seem to be independent of the vitamin E level.

INTRODUCTION

Heat stress is a major concern in poultry production because it has a profound effect on animal health and performance. Modern broiler breeds display reduced heat tolerance because of the high metabolism associated with a low feed conversion and rapid growth (He et al., 2018b, Lara and Rostagno, 2013b). Moreover, chronic heat stress leads to deterioration of meat quality by changing the aerobic metabolism and by increasing glycolysis and fat deposition (Lu et al., 2017, Petracci et al., 2014, Wang et al., 2017). Consequently, the meat from broilers reared under high environmental temperatures, is characterized by a pale color, low water holding capacity (WHC) and therefore also increased cook and drip losses (Wang et al. 2017). Heat stress induces oxidative stress (Akbarian et al., 2016, Slimen et al., 2014), which may in turn lead to protein oxidation and denaturation. Protein denaturation leads to decreased ability of proteins to bind water and thus to a poor water holding capacity and increased drip loss (Traore et al., 2012). The impaired WHC is detrimental for the valorization of broiler meat which is further processed by marination, tumbling and cooking (Zaboli et al. 2019). Supplementation of vitamin A, C and E can improve heat tolerance ability and animal performance during heat stress (He et al., 2018; Rheman et al., 2017, Khan et al., 2011). Some antioxidant minerals, including chromium, selenium (Gitoee et al., 2018, Attia et al., 2017b, Torki et al., 2015), and zinc also (Chand et al., 2014; Sahin et al., 2009) are used to prevent negative effects of heat stress. Zinc is an essential component of many enzymes, and it has both structural and catalytic functions in metalloenzymes. Furthermore, zinc is required for normal immune function as well as proper skeletal development and maintenance (Sahin et al., 2009). One of the most important functions of zinc is its antioxidant role and its participation in the antioxidant defense system. An increased level of reactive oxygen species is one of the main causes of decreased meat quality due to heat stress (Zaboli et al., 2019). In broiler diets, $ZnSO_4$ and ZnO are two of the main inorganic zinc sources. There are also

organic zinc sources available that are characterized by an improved bioavailability (Star et al., 2012, Swiatkiewicz et al., 2014). A more readily available zinc source might be more efficient in reducing the adverse effects of heat stress. To the best of our knowledge, there is no information available concerning the effect of different zinc sources, as opposed to different zinc levels, on meat quality of broilers subjected to a temperature challenge. Therefore, the objective of this study was to evaluate the interaction of zinc source ($ZnSO_4$ vs. zinc amino acid complex) and vitamin E level (50 IU/kg vs. 100 IU/kg) might alleviate the negative effects on meat quality and yield of broilers exposed to chronic cyclic heat stress in the finisher phase.

MATERIALS & METHODS

Experimental Design

The experimental design and dietary composition was already described chapter 2. Briefly, a total of 1224 day-old male Ross 308 broilers (Belgabroed, Merksplas, Belgium) were randomly allocated to 36 floor pens (9 pens per treatment and 34 broilers per pen) in an alternating block design, with one replicate per treatment in each block. The broilers were subjected to a chronic cyclic heat stress model (consecutively 32°C ± 2°C and a relative humidity of 55-65% for 6h daily) from day 28 to day 36 (finisher phase). Dietary treatments were organized in a 2x2 factorial design with two sources of zinc (**Zn**), 60 mg/kg of Zn as $ZnSO_4$ ($ZnSO_4.7H_2O$, Sigma-Aldrich, St. Louis, USA) or 60 mg/kg of Zn as zinc amino acid complexes (**ZnAA**; Availa®Zn, Zinpro Corporation, Eden Prairie, USA), and two levels of vitamin E (50 or 100 IU/kg; dl-α-tocopheryl acetate) were provided in a wheat-rye based diet. The starter diet was fed from day 0 up to day 10 and was provided as a crumble. The grower and finisher diets were fed as pellets from day 10 up to day 28 and from day 28 up to day 36, respectively. Feed and drinking water were provided ad libitum. Broilers were fasted for 8 hours prior to transportation to the slaughterhouse.

Slaughter Yield and Meat Quality Analysis

On day 37, three broilers per pen were selected, and transported to the slaughterhouse to be commercially slaughtered. Carcasses were immediately chilled after processing. Slaughter yield was determined approximately 24h after slaughter (108 birds in total, 27 from each treatment group). Carcasses were weighed without neck and feet. The broilers were manually dissected by trained personnel to determine carcass. To obtain parts yield, the drumstick and thigh (leg quarter) and wings were removed and weighed. The breast skin was removed and weighed together with the waste. The breast (both Pectoralis major and minor) were removed by cutting laterally at the wing joint and gently pulling from carcass. The remaining skin and skeletal rack were removed and weighed together with waste. From these measurements the percentage of breast, thigh, drumstick and wing was calculated as a percentage of post chill carcass weight.

Figure 1: Illustration of dissected carcass to determine slaughter yield.

All yields were expressed as percentage of carcass weight, except carcass yield which is expressed as a percentage of the live body weight. The different meat quality parameters were determined using breast (Pectoralis major muscles) (Figure 2). .

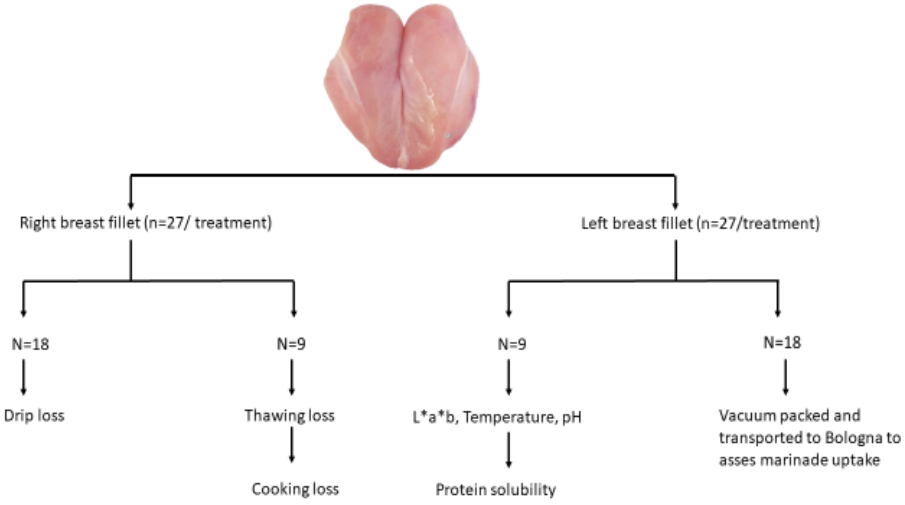

Figure 2: Overview of meat quality parameters determined on left and right breast fillet

Left breast fillets (n=9 per group) were weighed and colour measurements were performed using a Miniscan EZ colorimeter (Hunterlab, Reston, VA) to record CIE L* (lightness), a* (redness), and b* (yellowness) values. Temperature and pH ultimate were measured using a Portamess® 910 (Knick, Berlin, Germany). Following these measurements, breast fillets were vacuum packed and stored at -20°C in order to determine protein solubility at a later time point. The remaining left breast fillets (n=18 per group) were vacuum packed and transported to the University of Bologna (Italy, Cesena, Department of Agricultural and Food Sciences) in order to determine marinade uptake. The right breast fillets (n=18 per group) were removed from the carcass and put in a polypropylene bag, hung for 24 h at 4 ± 2°C and then blotted dry and weighed again to measure drip loss. The remaining right (n=9 per group) breast fillets were vacuum packed and stored at -20°C for four days. They were then defrosted for 24h at 5°C, blotted dry and weighed in order to determine thawing loss. After the thawing loss was determined, the fillets were cooked in a warm water bath (80°C) for 30 min. Afterwards they were blotted dry and weighed to record cooking loss. Drip loss, thawing loss and cooking loss was used to evaluate the water holding capacity.

Myofibrillar and sarcoplasmic protein solubility. Protein solubility was determined based on the difference in extractability of proteins in solutions at different ionic strength. Sarcoplasmic protein solubility was determined by homogenizing (Ultraturrax, T25 basic, New Brunswick NJ) 3 g of minced meat in 80 mL of extraction medium (150 mM sodium chloride, 0.01 mM iodo acetic acid). Supernatant was centrifuged (3000 g, 10 min) and filtered (Schleicher & Schuell nr. 597½) and protein concentration of this supernatant was determined with the biuret method (Layne, 1957). To determine myofibrillar protein solubility, the remaining pellet was suspended in 45 mL of extraction buffer (0.1 M citric acid, 1 mM EDTA, 0.4 M sodium chloride, 0.01 mM iodo acetic acid). The suspension was incubated at room temperature for 2h. The supernatant was centrifuged (5000 g, 20 min) and filtered (Schleicher & Schuell nr. 597½) and protein concentration of this supernatant was determined with the biuret method (Layne, 1957).

Marinade uptake. In order to assess marinade performances, meat was cut in order to obtain parallel cut samples (8x4x2 cm, weighing about 80 g), which were individually labelled and marinated by the addition of 20% marinade solution (6% sodium chloride and 1.8% sodium tripolyphosphate) using a small-scale vacuum tumbler (model MGH-20, Vakona Qualitat, Lienen, Germany). Tumbling time was 40 min under vacuum (-0.95 bar) (two working cycles of 20 min/cycle and one pause cycle of 5 min). After tumbling, samples were weighed again and the difference in weight was used to determine marinade uptake.

Statistical Analysis

Statistical analysis was performed in R for Windows (version 3.5.1). All data were checked for outliers and normality of the residuals. Slaughter yield and meat quality were analysed by a General Linear Model (GLM) with ‘Zinc source’ and ‘Vitamin E level’ as fixed factors and block as a random factor (factorial analysis). In the two-factorial analyses, when there was no significant interaction or no trend, only the main effects were taken into account. In case of a

significant interaction a Tukey test was performed in order to compare the treatments. In the tables, data with different superscripts differ significantly ($P < 0.05$).

RESULTS

Slaughter Yield and Meat Quality

There were no interactions observed between dietary zinc source and vitamin E level for total slaughter yield and the different meat quality parameters (Table 1). Live body weight of broilers supplemented with ZnAA showed a tendency ($P=0.052$) to be higher than for broilers supplemented with $ZnSO_4$. Only a main effect of the zinc source on breast yield and certain meat quality parameters was observed, whereas no main effect of vitamin E level was observed. The zinc source significantly affected breast yield and water holding capacity of the breast meat. A higher breast meat yield was observed for birds fed a diet supplemented with ZnAA as compared to birds fed a diet supplemented with $ZnSO_4$. Breast meat of birds fed a diet supplemented with ZnAA were characterized by a significantly lower drip and thawing loss compared to birds fed a diet supplemented with $ZnSO_4$. Dietary treatment did not significantly influence protein solubility and marinade uptake in breast meat (Table 2).

Table 1: Effect of supplemental zinc (Zn) source and vitamin E level on carcass composition of broilers at slaughter age (day 37)

Zn source	**Vit E level (IU/kg)**	**Live weight (g)**	**Breast weight (g)**	**Carcass yield (%)**	**Breast (%)**	**Drumstick (%)**	**Thigh (%)**	**Wing (%)**
$ZnSO_4$	50	2821	343.3	71.66	32.05	12.78	22.44	10.04
ZnAA	50	2965	364.2	72.10	33.60	12.52	21.96	9.80
$ZnSO_4$	100	2890	340.5	71.02	32.45	12.88	22.82	9.82
ZnAA	100	2935	340.9	71.28	33.01	12.69	21.96	9.85
SEM		48.33	14.03	0.48	0.37	0.21	0.95	0.13
$ZnSO_4$		2843	341.9	71.34	32.25	12.83	22.63	9.93
ZnAA		2950	352.5	71.69	33.31	12.60	21.96	9.82
	50	2893	353.8	71.88	32.83	12.65	22.20	9.92
	100	2913	340.7	71.15	32.73	12.79	22.39	9.84
Source x level		0.292	0.309	0.916	0.208	0.823	0.952	0.352
Zn source		**0.052**	**0.032**	0.346	**0.005**	0.224	0.494	0.383
Vit E level		0.677	0.807	0.098	0.768	0.425	0.750	0.572

ZnAA (zinc amino acid complex); Vit E (Vitamin E); SEM (standard error of the mean)

Table 2: Quality characteristics and functional properties of breast meat of broilers at slaughter age (day 37)

Zn source	Vit E level (IU/kg)	pH	L*	a*	b*	Drip loss (%)	Thawing loss (%)	Cooking loss (%)	Marinade uptake (%)	Myofibrillar protein solubility (mg/ml)	Sarcoplasmic protein solubility (mg/ml)
$ZnSO_4$	50	6.10	58.42	7.27	15.39	5.29	10.92	21.08	10.4	7.15	19.08
ZnAA	50	6.16	58.66	8.48	16.74	3.88	7.77	20.88	9.6	7.44	19.85
$ZnSO_4$	100	6.23	57.69	7.63	15.21	5.58	10.94	20.82	7.3	6.72	17.91
ZnAA	100	6.13	57.65	7.69	15.31	4.30	8.37	21.47	9.2	7.07	18.85
SEM		0.05	1.01	0.62	0.64	0.05	0.01	0.01	0.37	0.33	0.87
$ZnSO_4$		6.17	57.95	7.45	15.30	5.44	10.93	20.95	8.9	6.94	18.50
ZnAA		6.14	58.16	8.09	16.03	4.09	8.07	21.18	9.4	7.25	19.35
50			6.13	58.54	7.88	4.59	9.35	20.98	10.0	7.30	18.50
100			6.18	57.67	7.66	4.94	9.66	21.15	8.3	6.90	18.38
Zn source		0.968	0.623	0.104	0.300	**0.027**	**0.026**	0.401	0.066	0.353	0.347
Vit E level		0.685	0.858	0.782	0.353	0.582	0.819	0.765	0.052	0.244	0.236
Source x level		0.427	0.864	0.742	0.524	0.818	0.425	0.452	0.924	0.924	0.924

ZnAA (zinc amino acid complex); Vit E (Vitamin E); SEM (standard error of the mean); L* (Lightness), b* (yellowness), a* (redness)

DISCUSSION

Broilers reared under high temperatures often show lower meat yield and impaired meat quality (Zaboli et al., 2018). Breast meat of broilers exposed to chronic heat stress results, in pale meat color (Petracci et al., 2004, Zhang et al., 2012, Wang et al., 2017), decreased WHC (Wang et al., 2009) and increased cook and drip losses (Wang et al., 2017, Woelfel et al., 2002) and is characterized by an increased denaturation of sarcoplasmic or myofibrillar proteins and a lower WHC (Zaboli et al., 2018).

In the present study, it was observed that replacing $ZnSO_4$ by ZnAA, increased breast meat yield, whereas total carcass yield was not affected. This could mainly be attributed to difference in live body weight of broilers, which tended to be higher for broilers fed a diet supplemented with ZnAA. These findings are in contrast with the results reported in Chapter 2 on live body weight at slaughter age, were an interaction effect was found. Due to practical reasons the selection of broilers was prone to be biased. Furthermore, , the number of birds for slaughter yield was less extensive than for performance, which lower in a lower statistical power.

Moreover, modern broilers are selected for increased yield of Pectoralis major muscles (Zuidhof et al., 2014). It has been shown that ZnAA are more bioavailable than zinc from inorganic sources (Star et al., 2012). In chapter 1 was shown that ZnAA can improve performance in thermoneutral conditions. As zinc plays an important role in normal development and growth, it is possible that zinc supplements with an increased bioavailability, may better support growth under heat stress conditions. Although, this could not be concluded from this study, as no thermoneutral control group was included.

Higher breast meat yields are often associated with lower meat quality, with a higher drip and cooking loss and lower marinade uptake (Petracci et al., 2015, Wang et al., 2017). Losses of juice during refrigerated storage (drip loss) and defrosting (thaw loss) are amongst the major quality deterioration factors in the meat industry (Jensen et al., 1998, Leygonie et al., 2012). Although it has been acknowledged that vitamin E has a positive effect on meat quality by protecting membranes against lipid oxidation and thus reducing drip loss in meat (Estevez, 2015; Pompeu et al., 2018), in the present study no effects could be observed when dietary vitamin E level was increased. Interestingly, it was observed that replacing $ZnSO_4$ by ZnAA, resulted in breast meat with a lower drip and thawing loss. No differences in cooking loss were observed in the present study, this is probably due to the fact that fillets used to asses for thawing loss were also used to asses cooking loss. Under thermoneutral conditions, no effect of zinc source on carcass yield and meat quality was observed when comparing the supplementation of three different zinc sources ($ZnSO_4$, a zinc amino acid complex and zinc proteinate) (kakhi et al., 2011). Bowker and Zhuang (2015) suggested that a difference in sarcoplasmic protein solubility is correlated with water holding capacity in breast meat, however, in the present study no difference in both sarcoplasmic or myofibrillar protein solubility were observed.

It has already been demonstrated in previous studies, that zinc supplementation as compared to a non-supplemented control group, can positively affect meat quality, by decreasing drip loss under thermoneutral conditions (Yang et al., 2011, Liu et al., 2011). Moreover, increasing dietary Zn supplementation can improve water holding capacity of the meat (Yang et al., 2011). Trace minerals help to sustain the production in animals, improve nutrient utilization and at the same time effectively neutralize the oxidant stress and enhance the compromised immune system of heat stressed birds. As the requirements for trace minerals increase during heat stress, inclusion of a more readily available zinc source might be more efficient in

reducing the adverse effects of heat stress on meat quality (Chand et al., 2014, Farag and Alagawany, 2018, Sahin et al., 2009). In addition, Liu et al. (2015) reported that increased dietary supplementation of Zn can upregulate the expression of Zn-containing superoxide dismutase. The negative impact of high ambient temperatures on meat quality, is mainly caused by oxidative damage to the skeletal muscle (Zaboli et al., 2018). The improved quality traits when replacing $ZnSO_4$ by ZnAA, could be ascribed to improved support of the antioxidant defense system (Azad et al., 2013; Liu et al., 2014). In chapter 1 was shown that ZnAA could decrease the activity of the glutathione peroxidase in plasma on day 36, while malondialdehyde levels did not differ, indicating that ZnAA might better support oxidative status.

Overall, it can be argued that an organic form of Zn which is characterized by an improved bioavailability, might be able to better mitigate lipid and protein oxidation in post-rigor breast muscles and increase both water holding capacity and water binding ability. Further research should be performed in order to elucidate how a Zn source improves meat quality.

In conclusion, replacing $ZnSO_4$ with more readily available ZnAA, might improve breast meat yield and increase water holding capacity in broilers exposed to chronic cyclic heat stress at the end of the production cycle. Moreover, the beneficial effects of ZnAA on breast meat yield and quality seem to be independent of the vitamin E level.

GENERAL DISCUSSION

GENERAL DISCUSSION

1.1 Introduction

As mentioned in the introduction of this thesis, zinc is an essential trace element for all forms of life and plays an important role in several biological processes. Providing insufficient zinc in broilers may lead to a reduced feed intake and growth, delayed feather development, and to various bone abnormalities, (Kidd et al., 1996). Due to the absence of a specialized storage system, a daily intake of zinc is necessary to maintain and support the numerous functions of zinc, and thus preventing the negative effects of insufficient zinc supply (Bonaventura et al., 2015b). As feedstuffs (such as, grains and legumes) used in poultry rations, generally contain insufficient bioavailable zinc, it has to be provided to animals by in-feed supplementation (Ranaldi et al., 2013a). As described in the introduction (5.1) the recommended level of zinc in poultry diets is between 40 mg/kg and 75 mg/kg (Council, 1994b).

In the last years, organic zinc sources have been used increasingly, due to their increased bioavailability. Although there is a general consensus that organic zinc sources are characterized by a higher bioavailability, studies investigating the effects on performance and health parameters show inconsistent results. One of the reasons may be the lack of detailed scientific data regarding effects of the different zinc sources on host functions at the cellular and molecular level. Furthermore, there are also few studies directly comparing the effects of supplying zinc either as an inorganic or organic source at the same inclusion level. Due to this lack of knowledge, experiments were performed to evaluate the effect of supplying zinc either as zinc amino acid complex (ZnAA) or as $ZnSO_4$. In the first study (chapter 1), zinc was supplied either as $ZnSO_4$ or as a ZnAA, and compared for effects on digestibility, performance, intestinal health, microbiota composition and oxidative status. In the second

study (chapter 2 and 3), these effects of zinc source were evaluated when the broilers were subjected to chronic cyclic heat stress in the finisher period.

1.2 How does the supplemented zinc source affect broiler growth and health?

1.2.1 Critical episodes in the life of the birds

In this doctoral thesis, a positive effect of replacing $ZnSO_4$ by ZnAA on performance was confirmed in both performance studies. In the first performance study (chapter 1), a decreased feed conversion ratio (FCR) and an increased body weight were observed in the starter period. The positive effect on performance of young broilers was confirmed in the second performance study (chapter 2). This pronounced effect on performance in the starter period, might be caused by the stressfulness of this period for the young animals, in which they need to overcome many challenges. In this period, they switch from the protein-rich yolk to a complex carbohydrate- rich feed, they need to establish their own thermoregulation and immune system, and a balanced microbiota needs to be developed. The development of the immune system is initiated during the embryotic phase and continues in the early weeks following hatch (Ratcliffe et al., 1996). At hatch the components of the immune system of the broiler are already present but not completely developed. The innate immune system does not depend on long periods of induction before coming active and no memory will be created (Korver, 2012). The acquired immune system of broilers is not fully developed at hatch, and therefore maternal antibodies will protect the hatchling. Approximately around the age of 5 days the hatchling will begin to produce own immunoglobulins, independent from the maternal immunoglobulins (Grindstaff et al., 2003). The maternal antibodies will be catabolized within a period of 14 days post hatch and by the end of this period the broiler chick needs to rely on its own immune system. This period of dependency on passive immunity while the own adaptive immune system gradually develops has been proposed as a critical window in immune development (Butler and Šinkora, 2007). As zinc has a very

important function in the immune system, providing a more readily available source might be able to support the development more efficiently and in that way more energy could be invested in growth. However, this hypothesis should be further investigated.

In the second study (chapter 2), in the finisher period, when a temperature challenge was applied, a decreased FCR and an increased weight gain was observed when $ZnSO_4$ was replaced by ZnAA. One would expect an effect of zinc source in the grower period, because this is the period in which gut health issues mainly arise. However, the experimental studies were performed at research facilities under ideal conditions, in which gut health issues are not likely to arise. Taken together, the data from these studies makes us hypothesize that providing an organic zinc source mainly exerts its positive effects when the needs increase.

In our second study (chapter 2 and 3), the effect of two different zinc sources and two vitamin E levels was evaluated, and a significant interaction between zinc source and vitamin E level was observed. The positive effects of ZnAA on performance were only observed at a level of 50 IU vitamin E per kg. An additional level of vitamin E was added to both of the zinc sources, but did not result in an additional improvement. Our results suggest that the vitamin E level interacts with the effect of the zinc source for performance parameters. This is probably attributed to their shared relation with the action of the Cu/Zn SOD. Therefore, it would be interesting to determine the activity of this enzyme in future research. The information obtained in our study is useful to optimize the inclusion level of vitamin E, in order to maximize gains, because vitamin E is one of the most expensive vitamins provided in commercial supplements for poultry. However, further research should be invested in this topic in order to further elucidate whether providing zinc as a ZnAA might give rise to possibilities to reduce vitamin E supplementation.

1.2.2 Effects on meat quality and meat yield

In chapter 3, a positive effect on breast meat yield was observed when replacing $ZnSO_4$ by ZnAA. An increased breast meat yield is often associated with decreased meat quality (Petracci et al., 2019). Moreover, decreased meat quality is often reported in birds exposed to high temperatures (Zhang et al., 2012). However, at slaughter age breast meat of broilers supplemented with ZnAA, was characterized by an increased water holding capacity in comparison with breast meat of broilers fed a diet supplemented with $ZnSO_4$. Broilers exposed to high environmental temperatures, tend to show an increased incidence and severity of breast myopathies (Lu et al., 2017, Zaboli et al., 2019). Broilers with high feed efficiency show a shift towards a more oxidative metabolism in breast muscles as compared to broilers with low feed efficiency (Abasht et al., 2016, Pampouille et al., 2018). Because zinc is an important antioxidant, a more readily available zinc source such as ZnAA might play a role in decreasing oxidative damage and regeneration in this type of tissue. However, no further research was invested to confirm this hypothesis.

1.2.3 Effects on intestinal health

Intestinal health is a key determinant for efficient absorption of nutrients in production animals, and thus a good intestinal health is essential for performance (Ducatelle et al., 2018). Impaired villus morphology, characterized by a decreased villus length, an increased crypt depth and a decreased villus/crypt ratio, is often observed in case of intestinal inflammation. Microscopic evaluation of these parameters still remains the gold standard to evaluate intestinal health, and has been used in many studies evaluating the effectiveness of dietary interventions (Aguirre et al., 2019, Teirlynck et al., 2009, De Maesschalck et al., 2015b, Onrust et al., 2018). In our studies (chapter 1 and 2), evaluation of villus morphology showed an increased villus length and villus/crypt ratio in duodenum sections of broilers fed a diet supplemented with ZnAA compared to $ZnSO_4$. Infiltration of CD3 positive T-lymphocytes in

duodenum tissue was evaluated as an indication of intestinal inflammation. In both performance studies (chapter 1 and 2), no differences in the T-lymphocyte infiltration were observed at the end of the starter and the grower period. However, when broilers were exposed to heat stress in the finisher period (chapter 2), a decreased infiltration of CD3 positive T-lymphocytes could be observed at slaughter age in duodenum tissue of broilers fed a diet supplemented with ZnAA. Additionally, a decreased ovotransferrin leakage in ileum content was observed in broilers supplemented with ZnAA after heat stress was applied. This positive effect of ZnAA on villus morphology, stress induced inflammation and gut leakage might partly explain the positive effects on performance. Analysis of the effect of zinc source on microbiota composition and the accompanied predicted metabolic functions of the microbial communities, revealed that there was no difference in bacterial diversity or richness in the ileum content, but there was a trend towards a changing microbial composition. Single genera belonging to the phylum Firmicutes were reduced when supplementing ZnAA. Several genera belonging to the phylum of the Proteobacteria were less abundant in the ileum content of the group supplemented with ZnAA compared to the $ZnSO_4$ group, and overall relative abundance of this phylum was reduced. Analysis of the metabolic function prediction of the microbial communities showed an enrichment of the predicted microbial metabolic pathways involved in oxidative reactions in the group of $ZnSO_4$ supplemented broilers. Additionally, a higher activity of the glutathione peroxidase (GPx) in the plasma of broilers fed a diet supplemented with $ZnSO_4$. Expansion of the Proteobacteria has been proposed as a microbial signature of intestinal dysbiosis and epithelial dysfunction (Litvak et al., 2017, Weiss and Hennet, 2017b). In turn, intestinal dysbiosis and inflammation are associated with oxidative stress (Lauridsen, 2019), and might explain why pathways associated with oxidative stress are enriched in the microbiota from $ZnSO_4$ supplemented broilers. These observations suggest

that ionic zinc might cause (some) oxidative stress in bacterial cells. Alternatively, the microbiota might experience indirect oxidative effects from the host.

1.3 Zinc retention

1.3.1 Zinc retention versus zinc bioavailability

Based on the results of the digestibility trial discussed in chapter 1, nutrient digestibility was not significantly different between the two groups of broilers receiving a different zinc source, with the notable exception of the apparent zinc digestibility. The increased apparent zinc retention for ZnAA is in line with the improved growth observed in broilers supplemented with ZnAA. Due this improved growth it is possible that more zinc was deposited in body tissues. Alternatively, it is also possible that zinc is locally used by the intestinal epithelium or microbiota. Bioavailability is defined as the proportion of an ingested nutrient that is absorbed in a form that can be utilized in the metabolism by an animal in a normal health state. So this definition, contains more than just the digestibility of an element. To confirm whether ZnAA are characterized by an improved bioavailability, an additional experiment in which tibia zinc content is monitored, should be performed. It would also be interesting to determine whole body zinc and zinc deposition in different types of tissues.

1.3.2 How can a difference in bioavailability play a role when zinc requirements are fulfilled?

The definition of bioavailability stresses that the mineral must be available, not only at the dietary level but also at the tissue level. Bioavailability is thus the result of successive phases: accessibility in the intestinal lumen, absorption through the intestinal mucosa, retention, and incorporation in a functional form (e.g., cofactor of an enzyme) (Ammerman et al., 1995). Additionally, the bioavailability is also influenced by factors influencing the health status of the animals (Nockels et al., 1993). Thus, bioavailability strongly depends on the presence of interacting components in the feed, the intestinal health status and the overall health of the

animal. The nutritional requirements of minerals and vitamins increase in stressed animals. Depending on the type of stressor, this might be attributed to differences at the intestinal level. High environmental temperatures decrease the concentration of vitamins and minerals in the serum and increase their excretion (Khan et al., 2012). Heat stress reduces intestinal barrier integrity (Pearce et al., 2013), and therefore the increased need for vitamins and minerals under heat stress conditions can be explained by increased losses and repair and maintenance processes in the intestinal mucosa (Lambert, 2009). Finally, the chemical form in which zinc is supplied, might interact with the way it is used in the cell metabolism of the animal.

It has been shown that several dietary components influence the bioavailability of zinc, and the interaction of zinc with these components strongly defines its digestibility or bioavailability (Lönnerdal, 2000, Schlegel et al., 2013). Phytates are considered as the most important dietary factor limiting zinc bioavailability in non-ruminants, due to formation of insoluble phytate zinc- complexes. Linares et al. (2007) showed that zinc, at a similar concentration (23-24 mg/kg), was more available to broilers in low-phytate barley, than it was in conventional barley, as the retention coefficient of zinc increased from 42 to 63%, respectively. The stability of the phytate zinc-complex is pH-dependent, and shows moderate solubility at low pH and poor solubility at pH 7 (Tang and Skibsted, 2017). Hence, zinc ions do not even have to be complexed by phytate in the feed, because, at an intestinal pH (luminal pH is 6.0–7.4), phytate binds the cation effectively and forms stable complexes with low solubility (Lonnerdal et al., 1999, Fallingborg, 1999, Khouzam et al., 2011). Consequentially, zinc complexed with phytate is not available for absorption, and is excreted with the faeces .The bioavailability of zinc is also affected by competition with other minerals (Lönnerdal, 2000, Sauer et al., 2017). Dietary zinc levels and zinc status affect the absorption of iron by inhibiting the divalent metal transporter-1, which is the principal pathway for uptake of non-haem iron (Gunshin et al., 1997). Additionally, free iron can be absorbed across zinc

channels, in competition with zinc (Jeong and Eide, 2013). The interaction between zinc and copper is possibly even more important. Copper inhibits zinc uptake and zinc also inhibits copper absorption (Hogstrand, 2011). The levels of both elements need to be well balanced in order to prevent copper or zinc deficiency. It has also been described that divalent ions might interact with free fatty acids and bile acids, leading to the formation of poorly soluble soaps and salts, rendering divalent minerals less bioavailable (Corte-Real and Bohn, 2018, Collett, 2012a). Therefore it may be advantageous to supply a zinc source, which is taken up by a route that is not inhibited or saturated by zinc and other trace metals. It has been shown that ZnAA complexes are taken up by amino acid transporters as opposed to zinc salts (e.g., $ZnSO_4$ and $ZnCl_2$), which are taken up by zinc transporters (Sauer et al., 2017, Gao et al., 2014). The uptake of zinc via zinc transporters can be inhibited by zinc uptake antagonists (Lönnerdal, 2000). This alternative supply route of zinc might explain the higher bioavailability and increased bio-efficiency of ZnAA as opposed to $ZnSO_4$ or $ZnCl_2$.

Fibers may also impair zinc bioavailability through the formation of insoluble chelates (Knudsen et al., 1996). It is sometimes difficult to evaluate the direct effect of fibers on zinc availability, because some fibrous feedstuffs are also rich in phytates. Additionally, they may also increase endogenous losses of zinc by increasing cell sloughing in the intestine. Young broilers are particularly sensitive to increased intestinal viscosity (Classen, 1996). High viscosity leads to the reduction in digestive enzyme activities and absorption efficiency (Smits and Annison, 1996). These changes elicit a decrease in the digestibility of various nutrients, such as cholesterol, tri-glycerides, vitamins and minerals (Smits and Annison, 1996). According to Mohanna et al. (1999), zinc bioavailability in poultry may be depressed when intestinal viscosity is increased because of the presence of water-soluble NSP in the diet. In our experiments (chapter 1, 2, 3), a wheat-based diet was used, without the addition of NSP enzymes, in order to create a challenge at the intestinal level. The increased villus length and

villus/crypt ratio observed in these experiments, might indicate that providing zinc as ZnAA might be of interest to overcome the decreased absorption efficiency in case of increased intestinal viscosity.

When taking the definition of bioavailability into account, one should consider that bioavailability is not only determined through absorption but also through utilization of the trace mineral in a specific function or tissue. It is difficult to quantitatively assess the actual utilization of trace minerals with a response criterion that is sensitive enough to determine statistical differences in a small group of animals. In our experiments (chapter 1 and 2), positive effects on growth, intestinal health and oxidative status were observed, when zinc was provided as ZnAA as compared to $ZnSO_4$. These effects may be attributed to differences in absorption; and that once absorbed, differences in zinc utilization may exists which eventually lead to improved performance and overall health.

Finally, bioavailability is also determined by the health status of the animal. Dietary, environmental or infectious stressors might impair the animals health status, thereby increasing the demand for certain nutrients. Intensive broiler farming has led to increased pressure on the animals' health status, and intestinal health or breast myopathy issues are frequently reported. Therefore, it is presumable that providing a zinc source which is more readily available, might benefit these animals, certainly under stressful dietary conditions.

1.4 Zinc competition between the intestinal microbiota and the host

There is a clear relation between animal performance and intestinal health, and the latter is also associated with microbiota composition (Kers et al., 2018, Diaz Carrasco et al., 2019). Johnson et al. (2018) even found an association between certain bacterial groups and animal performance. Although these recent published studies show clearly that microbiota composition has an effect on performance, conclusions are not as straight forward and remain subject for discussion. The importance of zinc for human and animal health has been proven, however limited information is available on whether zinc solely benefits the host, or whether there is also some effect on the gut microbiota (Lopez and Skaar, 2018). Therefore, the effect of zinc sources on microbiota composition in the ileum and cecum, was evaluated in Chapter 1.

Facultative pathogenic bacteria express proteins with a high affinity for zinc, which favors these bacteria under zinc limiting conditions. In humans, zinc deficiency is considered to be a risk factor for childhood diarrhea (Walker et al., 2013). However, excess zinc could also disrupt the microbial balance; this was shown in mice by Zackular et al. (2016). Reed et al. (2015) performed a study to characterize changes in the cecal bacterial composition between zinc-deficient and zinc-replete broilers. They concluded that chronic zinc deficiency significantly decreases species richness (evaluated by Chao1) and species diversity (total observed OTU). Thus, a zinc-depleted environment might lead to a less diverse bacterial community, preferentially composed of bacterial species that are viable under zinc limited conditions. These results indicate that zinc bioavailability may significantly impact microbiota composition. There is very little information in literature, regarding the influence of different zinc sources on the bacterial community. Ishaq et al. (2019b) performed a study in yearling rams on the effect on rumen bacterial communities of supplying zinc either as ZnAA or as $ZnSO_4$, and found that the zinc source could alter bacterial communities.

Therefore, it is likely that dietary changes in zinc intake or changes in zinc bioavailability, shape the intestinal microbial population. It has already been shown that low intestinal zinc concentrations in the intestinal content may shift the microbiota composition away from beneficial bacteria, and may favor the expansion of pathogenic bacteria (Reed et al., 2015, Starke et al., 2014, Lopez and Skaar, 2018). In our experiment as already described in section 2.3 of the general discussion, the microbial composition in the ileum did not differ in bacterial diversity or richness, but did show a trend towards a changing microbial composition, whereas microbial composition in the cecum was not affected at all. Single genera belonging to the phylum Firmicutes, were reduced when supplementing ZnAA, without affecting the overall relative abundance of the phylum. Conversely, several genera belonging to the phylum of the Proteobacteria, were less abundant in the ileum content of the group supplemented with ZnAA compared to the $ZnSO_4$ group, and overall relative abundance was also reduced. In the present thesis (chapter 1), it was confirmed that apparent zinc retention of ZnAA was superior to that of $ZnSO_4$. This indicates that more zinc is absorbed by the host in the proximal small intestine (Lonnerdal et al., 1988, Steel and Cousins, 1985), leaving less zinc available for the bacteria present in the lower intestinal lumen. Therefore, direct competition for zinc in caeca is less likely, which might explain the lack of an effect of cecal microbiota composition. One possible explanation could be that the genera, which were reduced in abundance in the ZnAA group, would be more susceptible to reduced zinc bioavailability, but this was not further investigated.

FUTURE PERSPECTIVES FOR FURTHER RESEARCH

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Zinc amino acid complexes are characterized by an increased bioavailability. Yet, no difference could be observed in serum zinc levels. This might either be explained by the strong homeostatic regulation and the fact that zinc serum levels are not a good biomarker to asses zinc status. Alternatively, the improved bioavailability can lead to a decreased excretion of zinc, as was observed in the digestibility study (chapter 1), and might indicate an increased utilization in tissues or biological processes. Based on our results, it is clear that the zinc source significantly impacts intestinal health and performance in male broilers. However, at present, there is still insufficient knowledge available on what happens at the cellular level, and how several tissues can be influenced. Zinc is absorbed and transported into the blood at the level of the intestinal epithelial cells. ZnAA follow another route of absorption as compared to other organic and inorganic zinc sources (Gao et al., 2014, Sauer et al., 2017). However, the question is whether ZnAA are also differently metabolized or influence cellular metabolism in another way. To find out how exactly ZnAA can improve intestinal health, oxidative status and performance needs to be further elucidated by *in vivo* and *in vitro* experiments.

At present, there is no poultry intestinal epithelial cell line available to perform the *in vitro* experiments. Therefore, another suitable cell line, such as Caco-2 cells, have been used extensively as a model of the intestinal barrier, because these cells spontaneously differentiate in culture and develop in a monolayer of cells that exhibit morphological and functional characteristics of the mature enterocyte (Sambuy et al., 2005). Another possibility would be to work with primary cells derived from broiler intestinal tissue, however, these have a limited lifetime. Moreover, transfer of this type of cells from the *in vivo* tissue to an *in vitro* cell culture, often leads to loss of the initial structure and functionality of the cells Recently, progress has been made in the use of the metabolomics platform to evaluate the effect of

additives on colon tissue and cell lines (Rombouts et al., 2019)It would be interesting to culture Caco-2 cells in media supplied with different zinc sources at different concentration levels. After cultivation, the cells could be extracted and analysed with a targeted or untargeted metabolomics approach, in order to evaluate whether there are differences in the metabolic profile. Moreover, highly sensitive fluorescent probes for bio-imaging in living cells are now available, which could be used to elucidate how much zinc is absorbed and distributed in the cells (Lu et al., 2018).

An additional *in vivo* experiment could be performed to evaluate bioavailability by collecting blood from the portal vein. This would give an accurate image of how much zinc is absorbed in the blood, before it is transported with the blood stream to other tissues. In this experiment, tissue samples could be collected to analyse with the metabolomics or transcriptomic approach, in order to elucidate how ZnAA affects cell signalling and metabolism, and what the mode of action might be. Analysis of the transcription would allow to gain more insight on how different zinc sources affect the expression of zinc or amino acid transporter and metallothionein. The metabolomics approach would give more information on how the cellular metabolism is affected, and which metabolic pathways are activated.

CONCLUSIONS

CONCLUSIONS

Zinc is an important trace mineral and is essential to support broilers' health and growth. In the past, inorganic zinc sources were preferentially used in broiler production. However, ZnAA, which are more readily available, might be more efficient in supporting the high growth rates in broilers. Results from our experiments indicate that ZnAA is characterized by an apparent zinc retention as compared to $ZnSO_4$, which supports previous reports on improved bioavailability. A positive effect on villus morphology, microbiota composition, oxidative status and early performance, was observed when zinc was supplied as ZnAA in comparison with $ZnSO_4$. By applying an additional stressor (e.g., heat stress), a positive effect on performance and breast meat yield and quality was observed when zinc was supplied as ZnAA. However, the positive effects on performance seem to diminish when an increased level of vitamin E was supplied. Positive effects on performance and villus morphology might be attributed to an improved oxidative status and better support of immune system. In general, the results of our experiments show that ZnAA have an increased efficiency in supporting broilers' performance and health as compared to the inorganic $ZnSO_4$. However, further research needs to be conducted to elucidate the mechanism of improvement in comparison with other organic and inorganic zinc sources.

SUMMARY

SUMMARY

The last two decades, broiler production has evolved enormously. Broilers have been selected for increased weight gain and decreased feed conversion ratio. This in order to support the high demand for broiler meat, and in turn to increase profits in the broiler industry. Genetic selection of fast-growing hybrids had led to increased susceptibility to infections and nutritional or environmental stressors. This combined with the constantly increasing environmental temperatures, as a consequence of global warming, can have a negative impact on growth, intestinal health and meat quality in broiler production. Together with an increased mortality, this leads to high losses in poultry production. Additionally, the ban on antimicrobial growth promotors increased concerns on gut health issues. Maintenance of intestinal health is of key importance to support growth and to meet the high demands for weight gain and feed conversion. The maintenance of a healthy gut relies on a delicate balance between dietary components, intestinal microflora and the mucosa of the intestinal wall. Zinc is an important dietary component, as it is an essential trace element which supports numerous biological processes. Zinc needs to be provided to animals by in-feed supplementation. Zinc can be supplied as an oxide or sulphate, or as an organic complexed form. Although there is an overall consensus that organic zinc sources have a higher bioavailability than inorganic zinc sources, there is no consensus on the effects on performance and overall health parameters.

The aim of this thesis was to evaluate the difference between a specific inorganic ($ZnSO_4$) and organic (zinc amino acid complex, ZnAA) zinc source, supplemented at the recommended physiological levels in broilers. Effects on performance, intestinal health, microbiota composition, oxidative stress and meat quality were monitored.

Quite some studies have been conducted in order to evaluate the bioavailability of different zinc sources; however, few studies have been conducted to evaluate the effect of zinc source on performance and other health parameters. A first *in vivo* study was conducted in order to compare the effect of supplementation with $ZnSO_4$ vs. ZnAA, included at the same level in the feed. In this study a digestibility trial and performance trial were performed in parallel.

For nutrient digestibility, only an effect on zinc digestibility was observed. Zinc digestibility increased if broilers were fed a diet supplemented wih ZnAA instead of $ZnSO_4$, suggesting an increased absorption of zinc from ZnAA. Furthermore, supplying zinc as ZnAA instead of $ZnSO_4$, significantly improved feed conversion ratio and decreased plasma malondialdehyde (MDA) levels in the starter period. Moreover, villus length and villus/crypt ratio were increased at the end of both the starter and grower period. At the end of the finisher period, a decreased glutathione peroxidase activity (GPx) was observed in the plasma of broilers supplemented with ZnAA, while MDA level was not affected by zinc source. Analysis of microbiota composition showed no effect of zinc source on bacterial diversity or richness, but did show a trend towards a changing microbial composition in the ileum, whereas microbial composition in the cecum was not affected at all. Single genera belonging to the phylum Firmicutes, were reduced when supplementing ZnAA, without affecting the overall relative abundance of the phylum. Conversely, several genera belonging to the phylum of the Proteobacteria were less abundant in the ileum content of the group supplemented with ZnAA compared to the $ZnSO_4$ group, and overall relative abundance was also reduced. Analysis of the metabolic function prediction of the microbial communities, showed enrichment of predicted microbial metabolic pathways involved in oxidative reactions in the group of $ZnSO_4$ supplemented broilers. The observation of metabolic pathways responding to oxidative stress in the intestinal microbiota, is in line with the observations of the plasma MDA and GPx levels.

In a second *in vivo* study, the effects on performance, intestinal health and meat quality of replacing ZnAA by $ZnSO_4$, were monitored when broilers were subjected to chronic cyclic heat stress in the finisher period. The positive effect of supplying zinc as ZnAA on performance and villus morphology observed in the first trial, were confirmed for the starter period. Additionally, when broilers were subjected to chronic cyclic heat stress in the finisher period, ZnAA also showed beneficial effects on performance, intestinal health and meat quality. A decreased infiltration of CD3 positive T-lymphocytes in duodenum and a decreased leakage of ovortransferrine in the ileum were observed in broilers supplemented with ZnAA. This indicates a protective effect of ZnAA against oxidative stress induced inflammation. Determination of slaughter yield showed no effect on total yield, but did show an increased breast meat yield when providing zinc as ZnAA. It's generally accepted that more breast meat often yields a decreased meat quality, but this was not the case in our experiment. In contrast, a significant decreased drip and thawing loss even indicated an improved meat quality, observed for the breast meat of broilers supplemented with ZnAA, as compared to breast meat of broilers supplemented with $ZnSO_4$.

One of the most important conclusions based on these trials, is that providing zinc as ZnAA can improve broilers' performance, intestinal health and meat quality. These effects are most probably attributed to the increased bioavailability, which allows a better support of cellular responses on oxidative stress and inflammation by a more readily available zinc source. However, further research needs to be invested to further elucidate the mechanism of improvement.

SAMENVATTING

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De laatste twee decennia kende de vleeskippen industrie een enorme evolutie, doordat vleeskippen hoofdzakelijk geselecteerd werden op een hogere gewichtstoename en een lagere voederconversie. Deze selectie vond plaats om enerzijds te kunnen beantwoorden aan de alsmaar toenemende vraag naar gevogeltevlees, en anderzijds om de winstmarge in de vleeskuikensector te vergroten. De genetische selectie naar snelgroeiende hybriden leidde echter tot een verhoogde vatbaarheid voor infecties en voeder- of omgevingsstressoren. Daarnaast wordt de pluimveesector de laatste jaren frequenter geconfronteerd met toenemende omgevingstemperaturen. Deze kunnen niet enkel een negatieve invloed hebben op de groei, darmgezondheid en vleeskwaliteit van de vleeskuikens, maar kunnen ook het sterftecijfer doen toenemen. Dit leidt uiteraard tot grote verliezen. Bovendien heeft ook het verbod op het gebruik van antimicrobiële groeibevorderaars, de bezorgdheden omtrent de darmgezondheid van de vleeskuikens doen toenemen. Een optimale darmgezondheid is immers van cruciaal belang om te kunnen voldoen aan de hoge eisen voor gewichtstoename en voederconversie, en zo de groei te ondersteunen. Het behoud van een gezonde darm is afhankelijk van een delicaat evenwicht tussen voedercomponenten en darmmicrobiota. Zink is een zeer belangrijke component in het vleeskuikenvoeder, aangezien het een essentieel sporenelement is dat vele biologische processen ondersteunt. Omdat er voor zowel mensen als dieren geen gespecialiseerd opslagsysteem bestaat, moet zink gesupplementeerd worden via het voeder. Zink wordt gewoonlijk in het voeder toegevoegd als zinkoxide (ZnO), zinksulfaat ($ZnSO_4$) of als een organisch complex. Dit laatste bestaat uit zink gebonden aan een eiwit, peptide of een aminozuur. Algemeen wordt aangenomen dat organische zinkbronnen een hogere biologische beschikbaarheid hebben in vergelijking met de anorganische bronnen (cfr. ZnO en $ZnSO_4$). Tot op heden zijn vrij veel studies uitgevoerd om de biologische beschikbaarheid van diverse zinkbronnen te evalueren, maar er zijn slechts weinig studies

uitgevoerd om het effect van de zinkbron op de prestaties en gezondheidsparameters bij vleeskuikens te evalueren.

Het doel van dit doctoraat was om de effecten van een specifieke anorganische ($ZnSO_4$) en organische (zink-aminozuurcomplex, ZnAA) zinkbron te evalueren bij vleeskuikens. Effecten op de technische prestaties, villusmorfologie, samenstelling van de microbiota, oxidatieve stress en vleeskwaliteit werden geëvalueerd.

Een eerste *in vivo* studie werd uitgevoerd om het effect van de toegevoegde anorganische zinkbron ($ZnSO_4$) versus zink-aminozuurcomplexen (ZnAA) te bepalen. Een verterings- en prestatieproef werden hiertoe parallel uitgevoerd. Uit de verteringsproef bleek enkel de verteerbaarheid van de zinkbron beïnvloed te worden, waarbij de hoogste verteringscoëfficiënt werd bekomen door toevoegen van ZnAA. De prestatieproef toonde een betere voederconversie en verlaagd gehalte aan plasma malondialdehyde (merker voor oxidatieve stress) aan, en dit tijdens de starterperiode na supplementatie met ZnAA. Bovendien werd zowel aan het einde van de starter- als de groeierperiode, een verhoogde villuslengte, alsook een verhoging in de villuslengte/cryptediepte ratio waargenomen. Op slachtleeftijd werd een verminderde plasma glutathionperoxidase-activiteit (GPx) waargenomen bij vleeskuikens die een voeder kregen gesupplementeerd met ZnAA. De malondialdehyde concentratie werd op dat moment niet beïnvloed door de zinkbron. Daaruit blijkt dat er een verminderde activiteit van een enzym met antioxidante werking nodig was om een gelijkaardige oxidatieve status te bekomen. De analyse van de microbiota-samenstelling toonde geen effect van zinkbron op de bacteriële diversiteit of rijkdom, maar toonde wel een trend naar een veranderende microbiële samenstelling in het ileum. In het ileum van vleeskippen gesupplementeerd met ZnAA, werd immers een verlaagde abundatie waargenomen van enkele genera die tot het phylum Firmicutes behoren, hoewel de totale abundantie van het phylum niet werd beïnvloed. Dit in tegenstelling tot de genera die tot het

phylum Proteobacteria behoren, waarbij naast een aantal genera ook de totale abundantie van het phylum Proteobacteria verlaagd was. De metabole functie predicties van de microbiële gemeenschappen tonen een verrijking van de microbiële metabole routes, betrokken bij oxidatieve reacties in de groep die werd gesupplementeerd met $ZnSO_4$. De waarneming van verhoogde metabole routes die reageren op oxidatieve stress in de darmmicrobiota, is in lijn met de waarnemingen van MDA en GPx in het plasma.

In een tweede *in vivo* studie werden de effecten van een supplementatie van het vleeskuikenvoeder met ZnAA, vergeleken met een supplementatie van het voeder met $ZnSO_4$, en dit op gebied van technische prestaties, darmgezondheid en vleeskwaliteit. Bijkomend werden de vleeskuikens in deze studie in de finisherperiode onderworpen aan een chronische, cyclische hittestress.

Het positief effect van de zinksupplementatie onder de vorm van ZnAA op prestaties en villusmorfologie, werd bevestigd in de starterperiode. Bovendien werden deze positieve effecten ook waargenomen op slachtleeftijd, nadat de vleeskuikens in de finisherperiode blootgesteld werden aan chronische, cyclische hittestress. Op slachtleeftijd werd een verminderde infiltratie van CD3-positieve T-lymfocyten in het duodenum en een neiging tot verminderde lekkage van ovotransferrine in het ileum waargenomen bij vleeskuikens gesupplementeerd met ZnAA. Dit wijst op een beschermend effect van ZnAA tegen ontstekinggeïnduceerde oxidatieve stress. Het slachtrendement werd in deze studie niet beïnvloed door het type zinkbron, maar er werd wel een verhoogde borstvleesopbrengst waargenomen bij het aanleveren van zink onder de vorm van ZnAA. Algemeen wordt aangenomen dat een hoger percentage borstvlees vaak een verminderde vleeskwaliteit kent. Echter, in dit experiment werd bij deze groep een verminderd drip- en dooiverlies waargenomen, wat wijst op een verbeterde vleeskwaliteit voor borstvlees afkomstig van deze vleeskuikens.

Eén van de belangrijkste conclusies uit dit doctoraat is dat het verstrekken van zink als ZnAA via het vleeskuikenvoeder de prestaties van vleeskuikens, hun darmgezondheid en vleeskwaliteit kan verbeteren. Deze effecten kunnen hoogstwaarschijnlijk worden toegeschreven aan een verhoogde biologische beschikbaarheid, die een betere ondersteuning biedt van de cellulaire respons op oxidatieve stress en ontsteking.

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LIST OF ABBREVIATIONS

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ADF	acid-detergent fibre
ADL	acid-detergent lignin
DNA	Deoxyribonucleic acid
CD	Crypt depth
CD3	Cluster of differentiation 3
CTAB	Cetyl trimethylammonium bromide
Cu	Copper
CuAA	Copper amino acid complex
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FA	Fatty acids
FFA	Free fatty acids
FDR	False Discovery Rate
Fe	Iron
GLM	General Linear Model
GPx	Glutathione peroxidase
ICP-MS	Inductively coupled plasma mass spectrometry
IU	Unit
KEGG	Kyoto Encyclopedia of Genes and Genomes
Kg	Kilogram
KO	KEGG Orthology
LPS	Lipopolysaccharide
MDA	Malondialdehyde

NADPH	Nicotinamide adenine dinucleotide phosphate
NDF	neutral-detergent fibre
NDO	Non digestible oligosaccharides
NRC	Nutrition Research Council
NSP	Non-starch polysaccharide
OTU	Operational taxonomic unit
RH	Relative Humidity
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
SEM	Standard
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
VIT E	Vitamin E
VL	Villus length
WHC	Water holding capacity
ZIP	Zrt-/Irt-like protein
Zn	Zinc
ZnAA	Zinc amino acid complex
$ZnCl_2$	Zinc chloride
ZnO	Zinc oxide
$ZnSO_4$	Zinc sulphate

CURRICULUM VITAE

ABOUT THE AUTHOR

Annatachja De Grande werd geboren op 20 januari 1991 te Brugge. Na het behalen van haar diploma secundair onderwijs in de richting Wetenschappen-Wiskunde, starte ze met de studies Biomedische Laboratoriumtechnologie aan de Hogeschool Gent. Vervolgens starte ze het schakelprogramma tot Industrieel Ingenieur aan de Universiteit Gent. In 2015 behaalde zij het diploma van Industrieel ingenieur (optie biochemie). Aansluitend hierop ging ze aan de slag als wetenschappelijk onderzoeker aan de vakgroep Levensmiddelentechnologie, Voedselveiligheid en Gezondheid van de Faculteit Bio-Ingenieurswetenschappen.

In 2016 startte zij bij de vakgroep Pathologie, Bacteriologie en Pluimveeziekten van de Faculteit Diergeneeskunde aan de Universiteit Gent haar doctoraatsonderzoek aan, waarin zij effect van verschillende zinkbronnen op de prestaties, darmgezondheid en vleeskwaliteit van vleeskippen bestudeerde. Verder begeleidde zij verschillende studenten in het behalen van hun bachelor- of masterproef en vervolledige zij het trainingsprogramma van de Dotoral Schools of Life Science and Medicine van de Universiteit Gent.

Annatachja is auteur en co-auteur van verschillende wetenschappelijke publicaties in internationale tijdschriften en gaf verschillende presentaties op meerdere nationale en internationale congressen.

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EXPERIENCE

Research associate (Jan 2020 - Current)

- Research Institute for Agriculture, Fisheries and Food (ILVO), Animal Sciences Unit
- Extending the laying cycle of aged laying hens. (VLAIO project)
- Alternatieve beheersingsmethoden ter preventie en bestrijding van worminfecties bij biologische leghennen (CCBT, Bioforum Vlaanderen, ILVO)

PhD Research (2016-2019)

- Ghent University, Faculty of Veterinary Medicine, Department of Pathology, bacteriology and avian diseases, Laboratory for Bacteriology and Mycology

Scientific researcher (2015)

- IWT/TETRA project on quality screening of cooked ham (“Kookham doorgelicht”)
  (Ghent University, Faculty of Bioscience Engineering, Department of Food Technology, Safety and Health)

Master of Science in Industrial Biological Sciences, Ghent University (2013-2015)

- Biofilms in the food industry: chemical characterisation and biofilm production by lactic acid bacteria
- Faculty of Bioscience Engineering, Department of Food Technology, Safety and Health

Bachelor of Science in Biomedical laboratory Technology (2011-2013)

- Evaluation of different cryopreservation techniques and agents for human semen
- University College Ghent, Faculty of Education, Health and Social Work

TRAINING AND CERTIFICATES

Doctoral Schools (1/2016-12/2019)

Transferable Skills

- Introduction Day for PhD students
- Advanced Academic English: Conference skills - effective slide design
- Job Market for Young Researchers
- Applying for a postdoctoral job

Specialist Courses

- Diagnostic Veterinary Bacteriology and Mycology
- Laboratory Animal Science (online course, 80h)
- qPCR course: qPCR tips and tricks and primer design

Additional courses and workshops

- Humanitarian Food Science and Technology (September 2014, University of Lille and Ghent University)
- Workshop on R (October 2018, ILVO, animal unit)
- Working with amphibians, reptiles and birds as laboratory animals (May 2018, Ghent University)
- Pain recognition, euthanasia and analgesia in laboratory animals (May 2018, Ghent University)
- Improve animal Welfare (May 2018, Ghent University)
- Training euthanasia with ANOXIA (ILVO and Agrologic)

Accreditation program

- Good laboratory practices and analysis (in compliance with the ISO 17025)

STUDENTS

Tutor or supervisor of different dissertations

- Kelly Willems (Bachelor in Biomedical laboratory technology): "The effect of different zinc sources on intestinal health in broilers"
- Marjolein Brack (Master of Science in Veterinary Medicine): "Oxidative stress associated with the form of zinc present in the feed of broilers"
- Anaïs Elewaut (Master of Science in Biochemistry and Biotechnology): "The effect of inorganic and organic zinc sources on intestinal health in broilers"
- Tilemachos D. Mantzios (Master in Veterinary Medicine): "Training in laboratory techniques to evaluate intestinal health"

Member of reading and examination committee

- Vrydaghs Tom (Master of Veterinary Medicine): Coronary dominance in humans and animals, prevalence, variations and clinical importance"
- Ledeganck Liesbeth (Master of Veterinary Medicine): "The Marshall ligament
- Carette Alain (Master of Veterinary medicine):" The urban contamination with gastrointesitnal parasites in dogs "
- Creve Rhea (Master of Veterinay medicine): "Diagnosis and impact of parasitic infections in poultry"
- Elisa Ceyssens (Master of Veterinary Medicine):"Identification of biomarkers for kidney damage in birds, development of a nefrotoxic model in pigeons.
- Elise Verkindt (Master of Science in Biochemical Egineering Technology): "Influence of food characteristics on the antimicrobial effect of citral"
- Larissa Quak (Bachelor in Biomedical Laboratory technology):"Evaluation of the Cobas 8000: Sex Hormone Binding Globuline (SHBG) and Cancer antigen 15-3.
- Arzu Aksoy, Celine De Sterck, Liebet Demaegd (Bachelor in Biomedical Laboratory Technology):"Valdidation of the Sysmex XN 9000 body-fluid-mode to count and differentiate cells from body fluids.

PUBLICATIONS

Journal articles (A1)

De Grande A, Ducatelle R, Leleu S, Torres C, Rapp C, Petracci M, De Smet S, Michiels J, Haesebrouck F, Van Immerseel F, Delezie E (2020). Effects of dietary zinc source and vitamin E level on carcass yield and meat quality in male broilers reared under chronic cyclic heat stress conditions.
In preparation

De Grande A, Dietary zinc source affects performance and intestinal health parameters in male broilers reared under high temperatures. De Grande A., R. Ducatelle., Delezie E., Rapp C., De Smet S., Michiels J.., Haesebrouck F., Van Immerseel F., Leleu S. Submitted to Journal of Animal Physiology and Animal Nutrition

De Grande A, Leleu S, Delezie E, Rapp C, De Smet S, Goossens E, Haesebrouck F, Van Immerseel F, Ducatelle R. "Dietary zinc source impacts intestinal morphology and oxidative stress in young broilers". Poultry Science (2020) Vol 99 (1), http://dx.doi.org/10.3382/ps/pez525
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Impact factor: 2.216, category Agriculture, Dairy & Animal Science, rank: 5/60

Steen L, Neyrinck ., De Mey E, **De Grande A**, Telleir D, Raes K, Paelinck H, Fraeye I. "Impact of raw ham quality and tumbling time on the technological properties of polyphosphate-free cooked ham." Meat Science (2020) Vol 164, https://doi.org/10.1016/j.meatsci.2020.108093.
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Proceedings (conferences & symposia)

De Grande A, Leleu S, Whalström A, Ducatelle R, Van Immerseel F. « Zinc amino acid complex is associated with improved intestinal health in broilers." (2017) In proceedings of the 21st European Symposium on Poultry Nutrition.

De Grande A, Delezie E, Rapp C, Ducatelle R, Van Immerseel F, Leleu S. « Un complexe d'acides aminées de zinc améliore la morphologie des villosités intestinales chez les poulets ». (2019) In proceedings e-book 13e JRA Journées de la Recherche Avicole et Palmèdes à foie gras, pages 262-267

Abstracts (conferences &symposia)

Van de Vel E, Sampers I, **De Grande A**, Nguyen S, Raes K. "Interference of the polymeric material of swabs with the quantification of extracellular polymeric substances in biofilm samples". (2015) In 20th Conference of Food Microbiology, Abstract, Belgian Society of Food Microbiology.

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De Grande A, Delezie E, Rapp C, Ducatelle R, Van Immerseel F, Leleu S. "ut" (2019) In 22nd European Symposium on Poultry Nutrition

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DANKWOORD

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