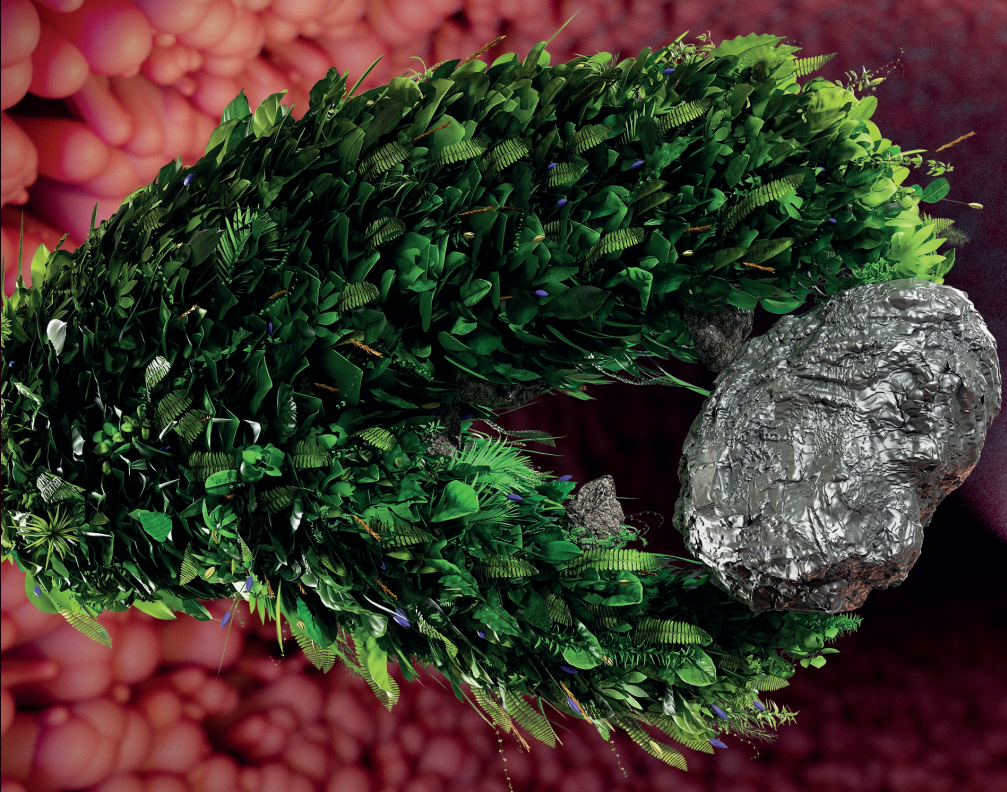
Trace mineral chelation for sustainable animal nutrition

Enhancing zinc availability with L-glutamic acid, N,N-diacetic acid



Gavin M. Boerboom

Propositions

1. Understanding physiological regulation of trace minerals is more important than estimating dietary requirements.

   (this thesis)

2. Estimating efficacy of trace mineral solutions on the basis of performance is wrong.

   (this thesis)

3. Confidence intervals should become the new measure of significance instead of p-values.
4. Impact of research will increase when limitations on abstracts are removed.
5. Successful innovative companies have to cannibalize part of their market.
6. Expertise is more commonly defined by social media, rather than education.
7. Civic duties, such as tax declaration, should be made mandatory during education.

Propositions belonging to the thesis, entitled

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Gavin Boerboom

Wageningen, 15 October 2021

TRACE MINERAL CHELATION FOR SUSTAINABLE ANIMAL NUTRITION

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TRACE MINERAL CHELATION FOR SUSTAINABLE ANIMAL NUTRITION

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Thesis

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Chapter 1

General introduction

1.1 Background

Trace minerals, such as zinc (Zn) and copper (Cu) are essential to ensure an organism's health and productivity (Goff, 2018; Miller et al., 2007). All organisms contain widely varying amounts and proportions of mineral elements. The first indications for nutritional significance in humans were found in 1791 by Fordyce. One of the first persons to report the need for trace minerals in livestock was Boussingault in 1847, who showed that cattle needed common salts in their diet. It was not until the beginning of the 20th century that people started to notice the essentiality of some trace minerals for proper health. By 1981, 22 minerals were identified to be essential for production animal health of which 15 were trace elements: iron, iodine, zinc, copper, manganese, cobalt, molybdenum, selenium, chromium, tin, vanadium, fluorine, silicon, nickel and arsenic (Rink, 2011; Underwood, 1981; Underwood, 1999). The uncertainty in the bioavailability of minerals when included in diets of livestock in commercial practice is compensated by calculating gross from net requirements using a worst-case bioavailability. Consequently, many minerals are supplemented in diets in amounts many folds higher than the quantity retained by the animals, resulting in excessive excretion (Brugger and Windisch, 2019). The high dietary content of trace minerals, especially trace metals such as Cu and Zn, is seen as an environmental burden and hence further improving bioavailability of trace minerals is essential for future sustainable animal food production (Additives and Feed, 2014; Burrell et al., 2004; Dozier III et al., 2003; Moore et al., 1995).

Understanding of the complexity of trace minerals has gradually increased over the last decade as a result of the use of molecular biology techniques and insights in recent years. Especially the latter has enabled visualization of complex mechanisms by which minerals are safely transported across cell membranes. Besides the complexity of absorption, trace mineral complexity is further increased by the multiplicity of functions that can be performed by the same element. Zinc for example serves as a cofactor for over 300 enzymes and 2000 transcription factors (Bao and Choct, 2009; Maret, 2019; Suttle, 2010). Trace minerals can be characterized to perform four broad types of functions as related to animal nutrition:

1. Structural: minerals such as copper, calcium and zinc can form structural dietary components., e.g. grit is poultry diets.
2. Physiological: minerals are present in tissue to control physiological processes such as membrane permeability, acid-base balance and maintenance of osmotic pressure.
3. Catalytic: minerals can act as catalysts in enzyme and endocrine systems in both an anabolic as well as a catabolic manner.
4. Regulatory: minerals can regulate cell replication and differentiation.

Trace minerals can be both beneficial as harmful, in accordance with a quote by Paracelsus in the 16th century: "All substances are poisons; there is none which is not poison. The right dose differentiates a poison from a remedy". A high level of free metal ions can lead to the formation of free radicals like superoxide. Free radicals are highly reactive and unstable and can lead to a

chain reaction in which the radical is passed on to other structures such as DNA and cell membranes (Balaban et al., 2005; Olechnowicz et al., 2018; Schieber and Chandel, 2014; Vergauwen et al., 2017). The body tries to minimize the presence of free, unbound minerals by producing proteins that form reversable bonds with the minerals to prevent them from binding electrons from other molecules. The body however also takes advantage of this tendency of minerals to donate or take up electrons by incorporating them into specialized antioxidant enzymes such as superoxide dismutase and glutathione peroxidase (Valko et al., 2007). In this case the enzyme controls which molecules will be donating an electron, or, which will be accepting an electron. This principle shows that it is important to find the proper balance between mineral supply and demand. One of the most important aspects to consider is trace mineral metabolism.

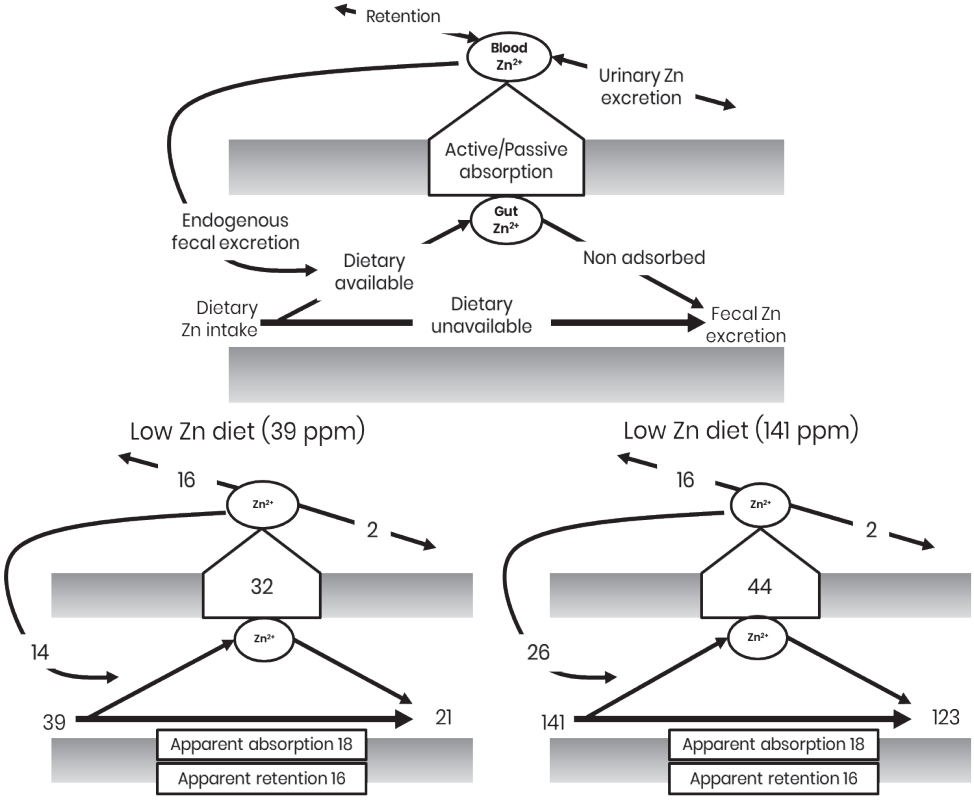

Figure 1.1 Schematic representation of the homeostatic regulation of Zn absorption and retention in rats fed diets high (141 mg/kg) and low (39 mg/kg) in Zn. Adapted from Weigand and Kirchgessner (1980).

1.2 Trace mineral metabolism

Given the importance of minerals in a multitude of biological functions, it is vital that the concentrations of minerals in cells are well controlled. For example, trials investigating Zn absorption and retention in rats showed a tightly regulated homeostatic mechanism (Weigand and Kirchgessner, 1980). The apparent absorption and retention levels were similar, regardless of the amount of Zn that was present in the feed, showing that the tightly regulated system is aimed at controlling Zn to an extreme extent. When high levels of Zn are fed, the body "compensates" by increasing endogenous faecal excretion of Zn, keeping the apparent absorption and retention levels equal. The more minerals are fed, the lower the apparent efficiency by which they are absorbed (Figure 1.1) (Brugger and Windisch, 2015; Brugger and Windisch, 2017).

In order to understand how this mechanism works, a deeper understanding of trace mineral uptake and excretion is required. Minerals can be absorbed from any section of the gastrointestinal (GI) tract but are mainly absorbed by the small intestine (Eide, 2011; Krebs, 2000; Lichten and Cousins, 2009; Mondal et al., 2010; Svihus, 2014). The small intestine is lined by a single layer of epithelial cells that are joined together by tight junction proteins. These epithelial cells have microvilli on their apical surface, which are many tiny membrane folds that increase the total surface area. Absorptive cells in the GI tract have a specialized 3-step transcellular transport mechanism that allows for efficient uptake of minerals, even when their concentration is low. During step 1, the mineral is absorbed over the apical membrane surface. In order to be absorbed, minerals need to be present in the lumen in a free Zn^{2+} form or loosely bound to water-soluble molecules. Minerals rely mainly on active transporters for transportation into the cell. Some minerals have a specific transporter, but the different minerals can also compete for the same transporter with zinc and copper being the most well-known example (Richards et al., 2010; Zhao et al., 2010). Step 2 requires the mineral to move from the apical membrane towards the basolateral membrane. Some minerals do this by simple diffusion; however, for most minerals this requires a transporter protein (chaperones). This is done to ensure they do not affect cell function due to acting as a second messenger or by oxidizing other components. Each mineral has a specific transport vesicle which has a high affinity for that mineral, allowing for tight control of uptake. Homeostatic regulation can influence this step in the mineral absorption too. Metallothionein can be expressed to bind minerals in the case of high minerals instead of the specific transport vesicle. In the case of high metallothionein release, the minerals are more likely to be bound to metallothionein than by their respective transporter. Metallothioneins are less efficient in their release of minerals. Considering that the lifetime of enterocytes is +/- 2 days this can be regarded as a mechanism of mineral regulation, as enterocytes that die are excreted in the faeces, along with these cellular contained minerals (Goff, 2018; Hijova, 2004). Step 3 requires the mineral to be moved from the cytosol of the epithelial cell to the interstitial space below the tight junctions. This is mostly done by pumps that require ATP. The benefit of the active transcellular transport is that the efficiency of these transporters can be upregulated if there is an increased need of a certain mineral and it can be downregulated when sufficient levels of a

mineral are present (Goff, 2018; Windisch, 2002). Even though all these controlled mechanisms are present, minerals can still cross the barrier in a process called paracellular absorption (Figure 1.2). The tight junctions that are present within the GI tract are not completely solid and the openings are small, but large enough for minerals to easily penetrate. In addition, an electric potential difference is present which offers protection to the uncontrolled absorption of cations. For paracellular transport to occur there needs to be a higher concentration of minerals in a freely ionized state at the luminal side of the tight junction in comparison to ionized concentration of that same mineral in the interstitial space on the other side of the tight junction. The point at which this occurs is also driven by the size and electrical charge of the ionized mineral. Paracellular absorption is a process that is unsaturable.

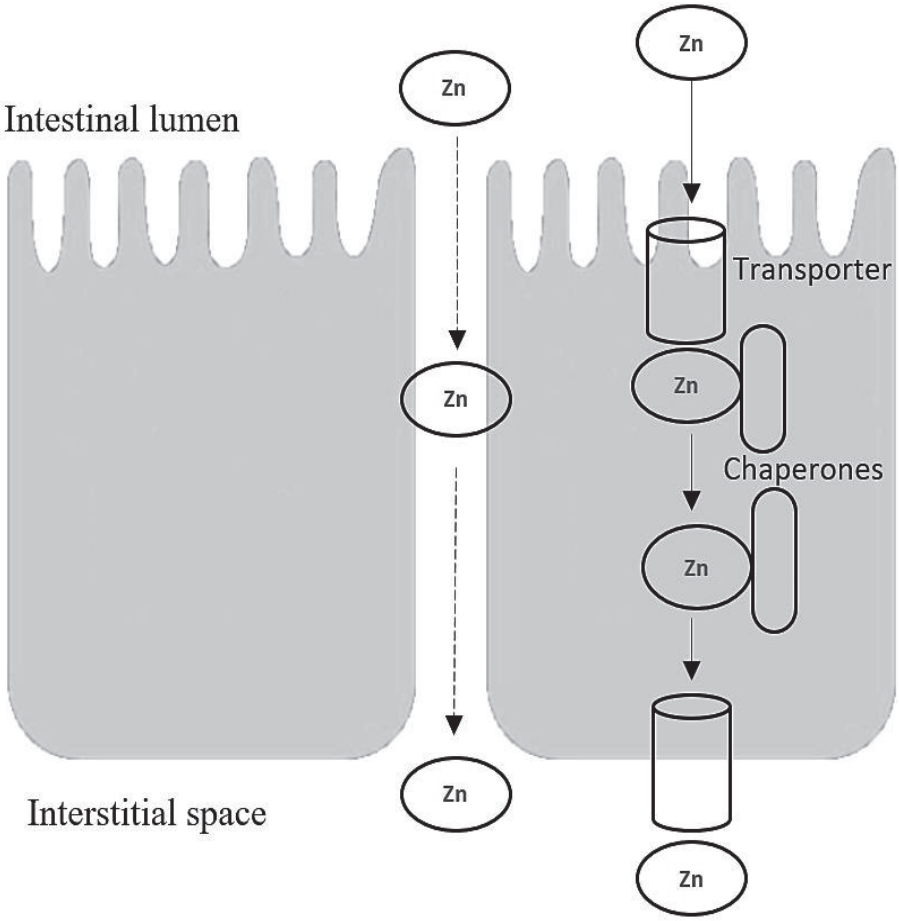

Figure 1.2 Schematic overview of tight junctions between two epithelial cells with the two methods of transport for minerals. Paracellular absorption is designated by a dashed line. Transcellular absorption is designated by a fixed line.

Underneath the epithelial cells described above there is a rich vascular and lymphatic network to be able to transport the minerals to the liver. From the liver they are then transported by the peripheral bloodstream towards different organs and tissues (Skrypnik and Suliburska, 2018). The efficiency of the uptake system described above relies for a large part on the availability of the mineral within the gastrointestinal tract.

1.3 Main factors influencing trace mineral uptake

The uptake of trace minerals is affected by many factors, with some already discussed above (Salim et al., 2008; Schlegel et al., 2010; Schlegel et al., 2013). Within the GI tract there is competition for transporters between different minerals. Unspecific transporters can be used by all minerals, but even the specific transporters are able to transport several minerals, if the concentration is sufficiently high (Lichten and Cousins, 2009; Richards et al., 2010; Wang and Zhou, 2010). The concentration of other minerals will, therefore, influence the availability of a mineral as well. Trace minerals can bind with other dietary components, with phytic acid being one of the most prominent components (Brugger and Windisch, 2017; Brugger and Windisch, 2019). Phytic acid serves as the principle storage for phosphorus in plants and is a six-fold dihydrogen phosphate ester of inositol, also called inositol hexakisphosphate (IP6) (Liang, 2007). Catabolites of phytic acid can also occur, which contain less than six phosphates (IP5, IP4, IP3). Phytic acid dissociates at low pH and re-arranges at a higher pH. During this re-arrangement it has the capability to bind divalent cations, such as Zn and Cu, creating insoluble complexes (Humer et al., 2015). When feed has been ingested, it is first exposed to a low pH in the stomach, leading to dissociation. If a trace mineral is present in a soluble form, the chances of complex formation with phytic acid at elevated pH in the small intestine are high, making the mineral unavailable for uptake. The complex formation capabilities of cations and phytic acid is (from strong to weak) Cu > Zn > Co > Mn > Fe > Ca and the stability of complexes is (from strong to weak) Zn > Cu > Ni > Co > Mn > Ca. Thus, Zn is a mineral which is affected the most by phytic acid (Yu et al., 2017; Yu et al., 2008; Yu et al., 2010). The chemical form in which a trace mineral is consumed directly affects its bioavailability, with a stable form increasing bioavailability (Thompson and Fowler, 1990).

1.4 Trace mineral sources

Inorganic forms of trace minerals are relatively cheap but suffer from high rates of loss due to dietary antagonism. Minerals bound to sulphates for example rapidly go into solution and dissociate, which increases the formation of insoluble complexes, thereby lowering the availability for uptake at the gut barrier (Bao and Choct, 2009; Brugger and Windisch, 2017). Chelation can be used to create more stable mineral complexes and chelation involves the formation or presence of two or more separate bonds between a ligand and a single central atom (McNaught and Wilkinson, 1997). The word chelation is derived from Greek χηλή, chēlē, meaning claw; the ligands lie around the central atom like the claws of a lobster. Organic trace minerals contain minerals that are linked by chelation to organic ligands (e.g. peptides, amino acids), which provides

more stability of the complex in the upper gastrointestinal tract compared to inorganic forms. This minimizes mineral losses to antagonists and allows the complex to be delivered to the epithelium of the small intestine for mineral uptake (Burrell et al., 2004; Dozier III et al., 2003; Manangi et al., 2012; Star et al., 2012). These organic trace minerals are formed by a chemical reaction between the ligand and the inorganic trace mineral. There are different organic trace minerals and not all are as potent in increasing the bioavailability of trace minerals (Aksu et al., 2010; Ao et al., 2007; Ao et al., 2009; Cao et al., 2002). Their efficacy is for a large part dependent on the strength of the bond between the mineral and the ligand and can be quantified using the formation quotient value (Qf) (Holwerda et al., 1995). The latter provides a measure of the strength of the bond by determining the electrical charge needed to break the bond between the ligand and the trace mineral. The stronger the binding strength, the better the availability in the animal (Holwerda et al., 1995; Yu et al., 2010). The strength of the bond becomes increasingly important in the case of higher phytate levels as depicted in Figure 1.3.

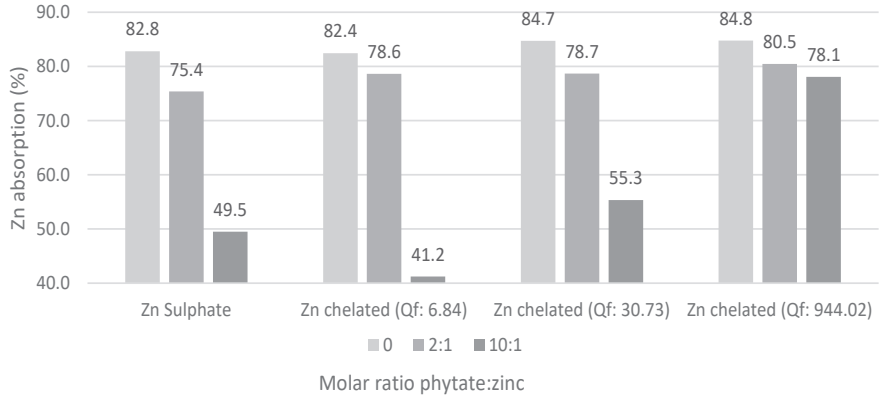

Figure 1.3 Zinc uptake in duodenum in broilers with increasing phytate levels using $ZnSO_4$, a weak chelator (Qf: 6.84), moderate chelator (Qf: 30.73) and a strong chelator (Qf: 944.02). Adapted from data from Yu et al. (2010).

Single strong chelating agents can also be used to increase mineral bioavailability. Chelating agents are comprised of molecules with a high affinity to bind trace elements and can potentially provide stability of the complex in the upper gastrointestinal tract, which minimizes the formation of insoluble complexes (Krezel and Maret, 2016; Vohra and Kratzer, 1964). The binding strength of these molecules is exponentially higher than the binding strength of organic ligands. The strength of this interaction is usually defined by the stability constant (log K). Literature of the 1960's and 70's determined the efficacy of these single strong chelators (Davis et al., 1962; Kratzer and Starcher, 1963; Vohra and Kratzer, 1964; Vohra and Kratzer, 1968). Vohra and Kratzer compared the effect on growth of different single strong chelators, classified on stability constant of Zn, allowing for determination of the optimal chelation strength for growth (Figure 1.4) (Vohra and Kratzer, 1964).

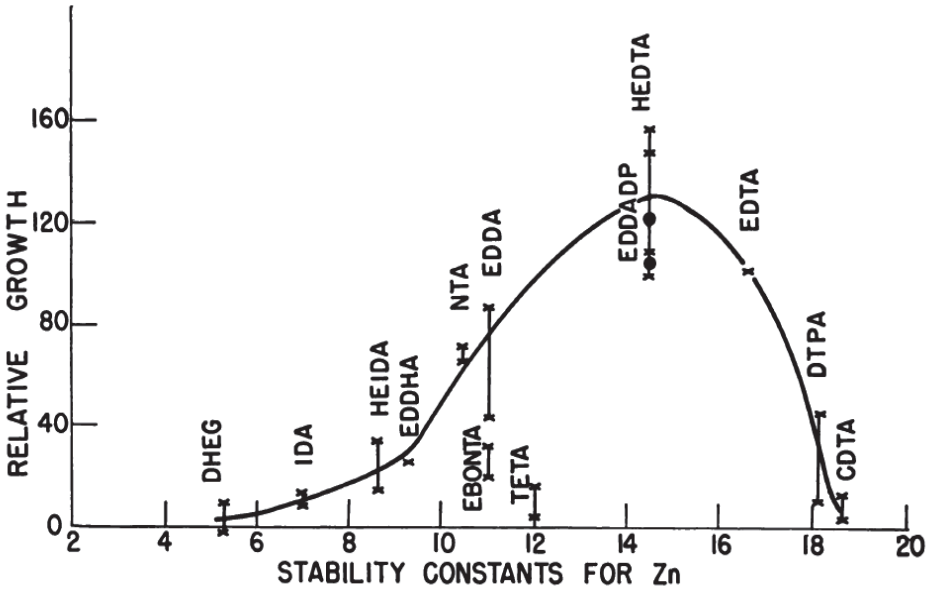

Figure 1.4. Relation of stability constant for Zn of several chelating agents to their growth promoting effects in turkey fed a Zn-deficient diet (Vohra and Kratzer, 1964).

Chelators such as ethylenediaminetetraacetic acid (EDTA) showed benefits in both human and animal applications to increase bioavailability of minerals (Heimbach et al., 2000; Hurrell, 1997; MacPhail et al., 1994). Iron EDTA has been shown to have significant beneficial effects on iron status by increasing iron bioavailability in human diets, allowing for application in cereals (Heimbach et al., 2000). It is also used in the treatment of excessive mineral presence such as lead poisoning or Wilson's disease and its potential as a treatment for cardiovascular disease is being investigated (Ferrero, 2016; Hauptman et al., 2017). Ethylenediaminetetraacetic acid is also widely used in everyday life in formulations such as shampoos, shower gels or cleaning agents, as well as in industry such as the textile industry and the pulp and paper industry (Münz, 2017). While EDTA serves many positive functions, the longevity of EDTA can pose serious issues in the environment. It has limited biodegradability and can accumulate in soil and surface water, thereby contaminating the environment (Hu et al., 2014; Wu et al., 2015). Considering the increased need of more sustainable farming it is important to find an alternative chelating agent with a low ecological footprint that can increase mineral bioavailability, allowing for more precise mineral feeding.

1.5 L-glutamic acid N,N-diacetic acid (GLDA)

A novel chelator, L-glutamic acid N,N-diacetic acid (GLDA) can be regarded as a sustainable alternative to EDTA, with less negative impact on the environment. GLDA is a molecule that has four carboxylate groups and combined with a centralized nitrogen atom these carboxylate groups

provide strong multiple bonds with di- and trivalent metal ions (Figure 1.5) (Wang et al., 2019). It belongs to the group of amino polycarboxylates.

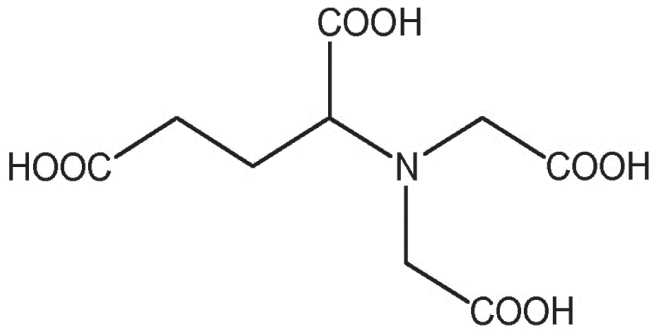

Figure 1.5 Structure of GLDA.

The molecule is produced from corn sugar-fermented monosodium glutamate, making it primarily bio-based, a chelator with 'green' carbon atoms. The production process itself is very efficient, with the only by-product being ammonia, which can be collected and re-used for other industrial processes. This makes the ecological footprint of GLDA significantly lower than other chelators such as EDTA (Wang et al., 2019). Toxicity tests have shown that GLDA has a low toxicity level and it has a relatively high application potential (Borowiec et al., 2009; Kołodyńska, 2013). The biodegradation tests of the L-form of GLDA have shown that it degrades much faster than for example EDTA, with more than 60% being degraded within 28 days (Tang et al., 2017; Wang et al., 2018). From an application point of view the greatest benefit of GLDA is its solubility at low and high pH values, being active in terms of Zn chelation between a pH of 3 to 12 (Seetz and Stafford, 2007; Seetz and Stanitzek, 2008). The stability constants, a measure for the strength of the interaction between the reagents that form the complex, are in the same range as those for EDTA (Table 1.1) (Bretti et al., 2016; Smith et al., 2004). Theoretically, 5 ppm of GLDA is capable of binding 1 ppm of Zn.

Table 1.1 Stability constants (log K values) of EDTA and GLDA complexes (Bretti et al., 2016; Smith et al., 2004).

Metal ion	EDTA	GLDA	Metal ion	EDTA	GLDA
Al^{3+}	16.4	12.2	Hg^{2+}	21.5	14.3
Ba^{2+}	7.9	3.5	Mg^{2+}	8.7	5.5
Ca^{2+}	10.6	6.4	Mn^{2+}	13.9	7.6
Cd^{2+}	16.5	9.1	Ni^{2+}	18.4	10.9
Co^{2+}	16.5	10.0	Pb^{2+}	18.0	10.5
Cu^{2+}	18.8	13.1	Sr^{2+}	8.7	4.1
Fe^{2+}	14.3	8.7	Zn^{2+}	16.5	10.0
Fe^{3+}	25.1	11.7			

1.6 Thesis aim and outline

The literature described above shows potential for GLDA to be used as an additive in feed to better control mineral supply to farm animals, allowing for a reduction in mineral usage, thereby reducing the excretion of mineral in the manure. This can have a positive effect on sustainable livestock production. To date however, there are no data available to support these claims. This thesis aims to improve our understanding and determine the potential of using GLDA to increase the availability of minerals in livestock production. To determine the potential of GLDA, a direct comparison to EDTA is made in the research described in this thesis, as the effects of EDTA are well known in the scientific literature. This comparison is made by feeding EDTA and GLDA in equimolar amounts to Zn at 5, 10 and 20 mg/kg in broilers and will be discussed in Chapter 2. In addition to this, the effects of GLDA on the availability of Zn from a basal feed is described in that same chapter. Feed formulators often make use of supplemental minerals, most commonly fed as sulphates (Ao et al., 2009). The total amount of minerals is often fed above requirements, reducing the efficiency of absorption (Brugger and Windisch, 2017). Being able to reduce the total amount of minerals fed will therefore not only improve the efficiency by which absorption takes place, it will also allow for a reduction in minerals in manure. In Chapter 3 I aim to determine what reduction in supplemental Zn is possible without affecting Zn status of the animal. A dose response study in broilers with fixed dosage of GLDA and incremental levels of Zn is described to determine the level of Zn reduction. Chapter 4 establishes the safety and tolerance of GLDA for broilers and provides a discussion in relation to consumer safety. Chelators are known to have negative side-effects when fed at high levels, for example due to chelation of minerals within the cell walls, leading to cell wall disruption (Heimbach et al., 2000; Prachayasittikul et al., 2007). In order to establish potential negative side-effects, GLDA was fed at levels of 0, 100, 300, 1000, 3000 and 10000 mg/kg with performance and blood parameters investigated to establish its effect in broilers. To be able to determine consumer safety the residue levels of GLDA in edible tissues was also determined.

The previous chapters involved studies in broilers but considering Zn regulation is to a large extent similar between monogastric species, the potential benefit of GLDA would also be applicable for piglets. Chapter 5 aimed to determine the potential of GLDA in piglets. Finally, in Chapter 6 the findings of all the 4 research chapters is combined and discussed in relation to the overall aim of this thesis, and recommendations for the use of GLDA in livestock production and future research are provided.

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Chapter 2

Efficacy of l-glutamic acid N,N-diacetic acid to improve the dietary trace mineral bioavailability in broilers

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Abstract

Trace minerals are commonly supplemented in the diets of farmed animals in levels exceeding biological requirements, resulting in extensive faecal excretion and environmental losses. Chelation of trace metal supplements with ethylenediaminetetraacetic acid (EDTA) can mitigate effects of dietary antagonists by preserving the solubility of trace minerals. Lack of EDTA biodegradability, however, is of environmental concern. L-glutamic acid N,N-diacetic-acid (GLDA) is a readily biodegradable chelating agent that could be used as a suitable alternative to EDTA. The latter was tested in sequential dose response experiments in broiler chickens. Experiment 1 compared the effect of EDTA and GLDA in broilers on supplemental zinc availability at three levels of added zinc (5, 10 and 20 mg/kg) fed alone or in combination with molar amounts of GLDA or EDTA equivalent to chelate the added zinc, including negative (no supplemental zinc) and positive (80 mg/kg added zinc) control treatments. Experiment 2 quantified the effect of GLDA on the availability of native trace mineral feed content in a basal diet containing no supplemental minerals and supplemented with three levels of GLDA (54, 108 and 216 mg/kg). In experiment 1, serum and tibia Zn clearly responded to the increasing doses of dietary zinc with a significant response to the presence of EDTA and GLDA ($P<0.05$). These results are also indicative of the equivalent nutritional properties between GLDA and EDTA. In experiment 2, zinc levels in serum and tibia were also increased with the addition of GLDA to a basal diet lacking supplemental trace mineral, where serum zinc levels were 60% higher at the 216 mg/kg inclusion level. Similar to the reported effects of EDTA, these studies demonstrate that dietary GLDA may have enhanced zinc solubility in the gastrointestinal tract and, subsequently enhanced availability for absorption, resulting in improved nutritional zinc status in zinc deficient diets. As such, GLDA can be an effective nutritional tool to reduce supplemental zinc levels in broiler diets thereby maintaining health and performance while reducing the environmental footprint of food producing animals.

2.1 Introduction

Trace minerals, in particular trace metals such as zinc (Zn), copper (Cu), manganese (Mn) and iron (Fe) are essential to ensure health and performance in highly productive farm animals. To fulfil the biological requirement for these trace minerals, animals should receive sufficient levels of a bioavailable source (Brugger and Windisch, 2017; Brugger and Windisch, 2019; Goff, 2018; Skrypnik and Suliburska, 2018). In commercial poultry diets, it is common to supply trace minerals as inorganic sources (i.e. sulphates and oxides). The most common inorganic sources typically undergo hydrolysis into the metal ion form during digestion, leaving them susceptible to precipitation with dietary antagonists like phytate, which, reduces their nutritional availability. Consequently, nutritionists formulate diets where mineral inclusion is in amounts many folds higher than the quantity retained by the animals, resulting in excessive excretion (Brugger and Windisch, 2015). As such, the fate of dietary trace minerals, particularly Cu and Zn, can be an environmental burden and improving the bioavailability of trace minerals is an important step

towards more sustainable animal food production (Burrell et al., 2004; Dozier III et al., 2003; Moore et al., 1995).

Organic trace minerals, in which the mineral links by chelation to organic ligands, such as amino acids or organic acids, are also used in animal nutrition. Organic complexation maintains the solubility of trace minerals within the digestive tract, thereby preserving their bioavailability (Ao et al., 2009; Richards et al., 2010; Star et al., 2012). Although not commonly used in nutritional formulation, strong chelating agents can also increase the bioavailability of trace metals by maintaining the solubility of these elements during the process of digestion (Vohra and Kratzer, 1964; Vohra and Kratzer, 1968). Strong chelating agents, such as ethylenediaminetetraacetic acid (EDTA), are molecules with a high affinity to form strong complexes with trace metals and maintain stability of the mineral complex in the upper gastrointestinal tract, which minimizes the formation of insoluble molecules (Vohra and Kratzer, 1964; Vohra and Kratzer, 1968; Whittaker and Vanderveen, 1990). The binding strength of these chelators is many exponents greater than that of small organic ligands such as amino acids or organic acids. Strong chelating agents represents an opportunity to both lower inclusion levels of trace minerals while reducing fecal losses to the environment. Ethylenediaminetetraacetic acid has been proven to enhance the nutritional availability for trace metals (Davis et al., 1962; Forbes, 1961; Vohra and Kratzer, 1964; Vohra and Kratzer, 1968), however, it may not be a suitable solution to reduce environmental losses because of its limited biodegradability and accumulation in soils and surface waters (Bucheli-Witschel and Egli, 2001). L-glutamic acid N,N-diacetic-acid (GLDA) is a readily biodegradable alternative to EDTA. This molecule could be considered as an environmental friendlier alternative to EDTA, with a relatively high chelation affinity for relevant trace metal nutrients and with a much lower environmental persistency, with more than 60% being degraded within 28 days (Borowiec et al., 2009; Kołodyńska, 2013; Wu et al., 2015). To date, little information is available on the efficacy of GLDA towards enhancing dietary trace mineral availability in animals (ECHA, 2010). The suitability of this application may be affected by the animal species in question, supplemental levels of the trace elements and of the chelating agent, gastrointestinal conditions, and the chemical affinity of the ligand for the different trace elements and for the other much more abundant metals such as Ca. This manuscript aimed to investigate the effect of GLDA on trace mineral availability in diets high in Ca and phytate, which are relatively well understood antagonists. Two experiments ran simultaneously with different objectives. The first experiment investigated the GLDA effect on Zn sulphate when added in molar amounts with equal chelation capacity to the added Zn and compared the effect with EDTA, also added in equal chelation capacity to Zn sulphate. The second experiment investigated the effect of GLDA on trace mineral availability of basal feed minerals (without supplemented Zn, Cu, Fe and Mn). It was hypothesized that GLDA would improve trace mineral availability.

2.2 Materials and Methods

2.2.1 Animals

The experiments ran simultaneously and were designed and carried out in full compliance with Spanish legislation for the welfare of experimental animals. A total of 1728, one-day-old, Ross 308 male broilers (Ross 308, Aviagen, Huntsville, AL, USA) were sourced from a commercial hatchery (SADA, Cazalegas, Toledo, Spain) where birds had been vaccinated against coccidiosis, infectious bronchitis and Marek's disease. Upon arrival at the research centre (Trouw Nutrition Poultry Research Centre, Casarrubios del Monte, Toledo, Spain), birds were randomly distributed and assigned to 96 pens with 18 animals per pen (1.25 m^2). The pens were located in two rooms with similar characteristics and pine wood shavings as litter. Pens were blocked by proximity and similarity in three groups of 16 pens per room with treatments randomly assigned within each block. Experiment 1 consisted of 72 pens, having 6 pens per treatment, with 12 pens assigned to the negative control for better baseline estimation. 24 pens were assigned to experiment 2 with 6 pens per treatment. All treatments were equally distributed over block and room.

2.2.2 Diets

In the first six days of the trial, all chicks received a standard mineral-adequate starter diet, formulated to fulfil all nutrient requirements (NRC, 1994). On day seven, birds received the experimental diets until day 21 of age. Diets (feeders) and water (nipples) were provided *ad libitum*. Treatments consisted of 15 differently formulated diets. The 11 diets used for the first study contained a basal premixture formulated to meet or exceed all nutritional requirements with the exception of Zn (NRC, 1994). Diets included a negative control without added Zn and a positive control with 80 mg/kg of supplemental Zn. Three levels of supplemental Zn sulphate (5, 10 and 20 mg/kg of Zn) were fed alone or in combination with molar GLDA or EDTA equivalents to chelate 5, 10 and 20 mg/kg of Zn (27, 54 and 108 mg/kg feed; Trouw Nutrition, Amersfoort, The Netherlands, 26, 51 and 103 mg/kg feed; Sigma-Aldrich, St Louis, MO, USA) making 9 different diets. The four diets of study 2 included a negative control and three incremental levels of GLDA (54, 108 and 216 mg/kg feed). These levels are the molar GLDA equivalents to chelate 10, 20 and 40 mg/kg of Zn, based on an *in vitro* assessment in which the amount of soluble Zn was measured after 6h incubation with a chelator and feed (Trouw Nutrition, unpublished). A basal meal was formulated for these four diets to fulfil or exceed all nutritional requirements, except for Zn, Cu, Mn and Fe, which were not supplemented (NRC, 1994).

The basal feed for all the diets used in both studies was a combination of corn (15%), wheat (30%), soybean meal (29%) and soy oil (6%). To challenge trace mineral availability, an elevated level of total Ca was applied (9.8 g/kg), as well as 15% rice bran inclusion, which increased phytic acid level to 11.3 g/kg. In order to reduce endogenous phytase activity from the feedstuff, the

basal meal was pelleted at an elevated temperature (75°C) (Brugger et al., 2014). Representative samples of the diets were taken after production to determine moisture (EC regulation 152/2009, appendix III A), ash, ether extract (EC regulation 152/2009, appendix III H method A), starch, fibre fractions (ISO 6865:2000) and crude protein content (ISO 16634-1:2008). Calcium, Zn, Cu, Mn and Fe content was analysed in duplicate using inductively couple plasma mass spectrometry (ICP-MS) after calcination and HCl-extraction according to method NEN-EN 15510 (Bikker et al., 2017). GLDA content was analysed in duplicate by liquid chromatography-mass spectrometry (LC-MS) (Masterlab B.V., Boxmeer, The Netherlands). Phosphorus was analysed by spectrophotometry (AOAC, method 4.8.14). Phytic acid was analysed by the colorimetric AOAC method number 965.17, based on reaction of vanadomolybdate on inorganic phosphate produced by action of 6-phytase on phytic acid-containing substrate (Novo et al., 2018).

2.2.3. Measurements

General performance including bodyweight, bodyweight gain, feed intake, daily weight gain and feed conversion ratio were determined between d7 and d21. At the end of the study (d21), blood samples were taken from the wing vein of three randomly selected birds from each pen. An aliquot of blood was centrifuged for 30 min, the serum collected and divided in two aliquots of 1 mL per bird in labelled 2.5 mL cryotubes. Serum and whole blood samples were stored at -20°C until further analysis. Serum Zn, Cu, Mn and Fe were analysed by the Scottish Trace Elements and Micronutrient Reference Laboratory (Glasgow, U.K.) using inductively coupled plasma mass spectrometry (Agilent series 7500ce). The samples were diluted 20-fold in a solution of 2% butanol, 0.1% ethylenediamine tetra-acetic-acid, 0.2% triammonium citrate, 0.1% triton-X-100, 2% ammonia, with 50 ug/L germanium as internal standard. Haemoglobin in fresh blood was measured with a HemoCue® Hb 201+ (HemoCue Diagnostics BV, Waalre, the Netherlands). After blood collection, the birds were anaesthetized by intramuscular injection of a solution made of 50 ml sedamun and 30 ml ketamine (1 ml/kg bodyweight) and 20 minutes later euthanized by an intravenous injection of T61 (an aqueous solution containing 200 mg embutramide, 50 mg mebezoniumiodide, and 5 mg tetracainehydrochloride per mL). Left and right tibias were dissected out and stored at 4°C until further processing. Tibias were cleaned from soft tissue after boiling in water and analysed for Zn and Mn content at the Ainia Centro Tecnológico (Paterna, Spain) using microwave digestion, followed by inductively coupled plasma atomic emission spectrometer analysis (Horiba Jobin Yvon, Ultima model). Tibia Zn and Mn values were then pooled by pen.

2.2.4. Statistical analysis

Experiment 1

Data were analysed using SAS Studio (SAS institute Inc., Cary, NC). Performance data, serum Zn and total and concentration of Zn in tibias were analysed using the MIXED procedure with diet as a fixed factor and block as a random effect. Significantly different means were identified with a

Tukey test (P<0.05). The linear and quadratic effects of sulphate, GLDA and EDTA were also determined using the MIXED procedure. Regression analysis on serum and tibia Zn response was performed using the NLMIXED procedure. Since Zn absorption is primarily a saturable, carrier-mediated process, it is non-linear and using non-linear regression over data transformation and linear regression is preferred (Miller et al., 2007). The model used for the analysis of Zn availability using EDTA or GLDA was selected based on the best fit and biological meaning (Archontoulis and Miguez, 2015). Parameters used for determining best fit were the Akaike information criterion (AICC), root mean squared error (RMSE) and concordance correlation coefficient (CCC). The negative control treatment containing no chelator and no added Zn was used in all three lines as the starting point and the high Zn treatment was used in all three lines to define an assumed homeostatic plateau. The following model was used:

$$Y = Asymptote * \exp\left(-\exp\left(-(kSul + kEDTA + kGLDA) * (Zn\ dose - T)\right)\right)$$

in which:

Y = response parameter, serum and tibia Zn content,

Asymptote = asymptote, representing the maximum response in the Y variable,

k = rate parameter determining the steepness of the curve,

T = inflection point at which the response rate is maximized,

Sul = factor representing only sulphate inclusion (0,1),

GLDA = factor representing dietary GLDA inclusion on top of sulphate (0,1),

EDTA = factor representing dietary EDTA inclusion on top of sulphate (0,1), and

Zn dose = the amount of Zn sulphate added.

Significance (P<0.05) between the three fitted models (Sulphate, EDTA and GLDA) was determined using NLMIXED and the optimal model was used to determine the Zn supplementation required to reach a response of 95% of the asymptotic value for serum Zn and Zn concentration in tibia ash. This value was considered as criterion for estimating the bioavailability of the Zn in the diet (Huang et al., 2013).

Experiment 2

Data were analysed using SAS Studio (SAS institute Inc., Cary, NC). Performance parameters, serum minerals, haemoglobin and bone mineral concentrations were analysed using the MIXED procedure, with GLDA inclusion level as a fixed factor and block as a random effect. Significant different GLDA means were identified with a Tukey test (P<0.05). The linear and quadratic effects of GLDA were also determined using the MIXED procedure. Animal performance data also

included initial weight as a covariate in cases where this effect was significant. Zinc levels in serum and tibia were subsequently analysed using the NLMIXED procedure using the following model:

$$Y = Asymptote * \exp\left(-\exp\left(-k * \left(GLDA\ dose - T\right)\right)\right)$$

Y = response parameter, serum and tibia Zn content,

Asymptote = asymptote, representing the maximum response in the Y variable,

k = rate parameter determining the steepness of the curve,

T = inflection point at which the response rate is maximized, and

GLDA dose = the amount of GLDA added.

2.3 Results

Experiment 1

Analyses of the feed confirmed the high levels of Ca and phytic acid intended by design and the required Zn, EDTA and GLDA levels (Table 2.1). The MIXED and NLMIXED procedures therefore used the anticipated Zn, GLDA and EDTA levels as a dose-response continuous variable. No significant differences were detected in daily weight gain and FCR between treatments in study 1 (diets 1-11). Significant differences were observed for feed intake between the treatments at the 5 mg/kg supplementation with birds fed the EDTA having a higher intake compared to the birds fed the GLDA. No difference was present at any of the other dosages (Table 2.2).

Table 2.1 Calculated and chemically analysed (between brackets) nutrient composition of the starter feed and experimental feed.

Nutrient composition	Unit	Starter feed (0-7 days)		Experimental diet (7-21 days)	
Dry matter	g/kg	890	(881.4)	896	(894.0)
Crude protein	g/kg	220	(206.9)	220	(212.2)
Ash	g/kg	61.8	(54.0)	71.1	(61.1)
Crude fiber	g/kg	28.3	(26.0)	34.0	(34.5)
Non digestible fiber	g/kg	107	(107.6)	123	(127.2)
Acid detergent fiber	g/kg	*n.a.*	(36.2)	*n.a.*	(40.9)
Acid detergent lignin	g/kg	*n.a.*	(7.0)	*n.a.*	(10.3)
Ether extract	g/kg	72	(59)	100	(96)
Starch	g/kg	389	(396)	334	(337)
Ca	g/kg	9.2	(*n.d.*)	9.2	(9.8)
P	g/kg	7.5	(*n.d.*)	10.3	(9.7)
Phytic acid	g/kg	2.6	(*n.d.*)	10.6	(11.3)

n.a.: not available, nd: not determined

Table 2.2 Least square mean performance values of broilers receiving non-chelator (None), ethylenediaminetetraacetic-acid (EDTA) and L-glutamic acid N,N-diacetic-acid (GLDA) containing diets with increasing levels of Zn from d7-21.

Parameter	Chelator	Zn inclusion level, mg/kg					Model		SEM
		0	5	10	20	80	Linear	Quadratic	
Body weight, d7									
	None	204	205	202	207	202	0.55	0.43	
	EDTA	-	205	206	201	-	0.39	0.25	0.7
	GLDA	-	203	203	208	-	0.32	0.15	
Body weight, d21									
	None	1087	1081	1074	1086	1061	0.95	0.6	
	EDTA	-	1099	1098	1089	-	0.07	0.09	3.7
	GLDA	-	1066	1091	1100	-	0.24	0.08	
Daily weight gain, d7-21									
	None	63.0	62.6	62.2	62.9	61.3	0.78	0.90	
	EDTA	-	63.9	63.8	63.4	-	0.13	0.22	0.2
	GLDA	-	61.6	63.4	63.9	-	0.48	0.28	
Daily feed intake, d7-21									
	None	90.9	90.8[ab]	88.7	89.0	88.5	0.02	0.05	
	EDTA	-	91.6[a]	90.8	89.9	-	0.51	0.36	0.3
	GLDA	-	87.3[b]	89.4	90.9	-	0.001	0.001	
Feed conversion rate, d7-21									
	None	1.44	1.45	1.43	1.42	1.45	0.001	<.0001	
	EDTA	-	1.43	1.42	1.42	-	0.06	0.36	0.002
	GLDA	-	1.42	1.41	1.43	-	<.0001	0.001	

[ab]Values with different superscripts within column are significantly different (P<0.05).

Serum and tibia Zn content clearly responded to increasing doses of dietary Zn and this response was strongly significantly affected by the supply of equimolar amounts of EDTA or GLDA in the diets (Table 2.3).

Table 2.3 Least square mean of serum and tibia Zn concentration of broilers receiving non-chelator (None), ethylenediaminetetraacetic acid (EDTA) and L-glutamic acid N,N-diacetic-acid (GLDA) containing diets with increasing levels of Zn from d7-21.

Parameter	Chelator	Zn inclusion level, mg/kg					Model		SEM
		0	5	10	20	80	Linear	Quadratic	
Serum Zn (µg/L)									
	None	801	961	1184	1382[b]	1662	<.01	<.01	
	EDTA	-	1110	1353	1594[a]	-	<.01	<0.01	46.5
	GLDA	-	1007	1216	1669[a]	-	<.01	0.65	
Tibia Zn (mg/kg)									
	None	33.8	39.3	42.0[a]	57.0	69.2	<.01	<.01	
	EDTA	-	41.2	53.0[b]	63.0	-	<.01	0.05	1.69
	GLDA	-	41.1	52.2[b]	64.2	-	<.01	0.19	
Total tibia Zn (µg)									
	None	234	269	277[a]	396	481	<.01	<.01	
	EDTA	-	278	372[b]	428	-	<.01	0.06	14.4
	GLDA	-	277	355[b]	433	-	<.01	0.34	

[ab]Values with different superscripts within column are significantly different (P<0.05).

Table 2.4 Parameter of a non-linear model* describing the response of serum and tibia Zn concentration in broilers to dietary Zn supplementation with Zn sulphate (Sul), ethylenediaminetetraacetic-acid (EDTA) and L-glutamic acid N,N-diacetic-acid (GLDA) and estimates of Zn requirements (95% of asymptote).

Parameter	Serum Zn, µg/L			Tibia Zn content, mg/kg			Total tibia Zn, µg		
	Estimate	SE	P-value	Estimate	SE	P-value	Estimate	SE	P-value
A	1730	41.5	<.0001	72	2.0	<.0001	500	18.6	<.0001
kSul	0.069[a]	0.008	<.0001	0.055[a]	0.007	<.0001	0.052[a]	0.009	<.0001
kEDTA	0.034[b]	0.008	<.0001	0.022[b]	0.006	<.0001	0.022[b]	0.007	0.0003
kGLDA	0.021[b]	0.007	0.0032	0.022[b]	0.006	<.0001	0.019[b]	0.007	0.0002
T	-3.4	0.6	<.0001	-4.4	0.8	<.0001	-4.4	1.0	<.0001
s2e[+]	12783	1845	<.0001	22.1	3.2	<.0001	1796	259	<.0001
AICC[#]	1193			583			1005		
RMSE[α]	113			4.7			274		
CCC[β]	0.94			0.93			0.88		
Supplementary dietary Zn sulphate level (mg/kg) to reach 95% of asymptote									
Sulphate	39.9			50.0			52.4		
EDTA	25.6			34.4			35.7		
GLDA	29.9			34.2			37.2		

[ab]Values with different superscripts within row are significantly different (P<0.05).

* Y=A×exp(-exp(-(kSul+kEDTA+kGLDA)×(Zn dose-T))) where Y=dependent variable (serum Zn, tibia Zn concentration or total tibia Zn content), A=asymptote, k(Sul, EDTA, GLDA)=rate parameter determining the steepness for sulphate, EDTA and GLDA, respectively, Zn dose=dietary Zn sulphate supplementation, T=inflection point at which k is maximized.

[+,#,α,β]AICC=Akaike information criterion, CCC=Concordance correlation, RMSE=root mean squared error, s2e=variance, SE=standard error.

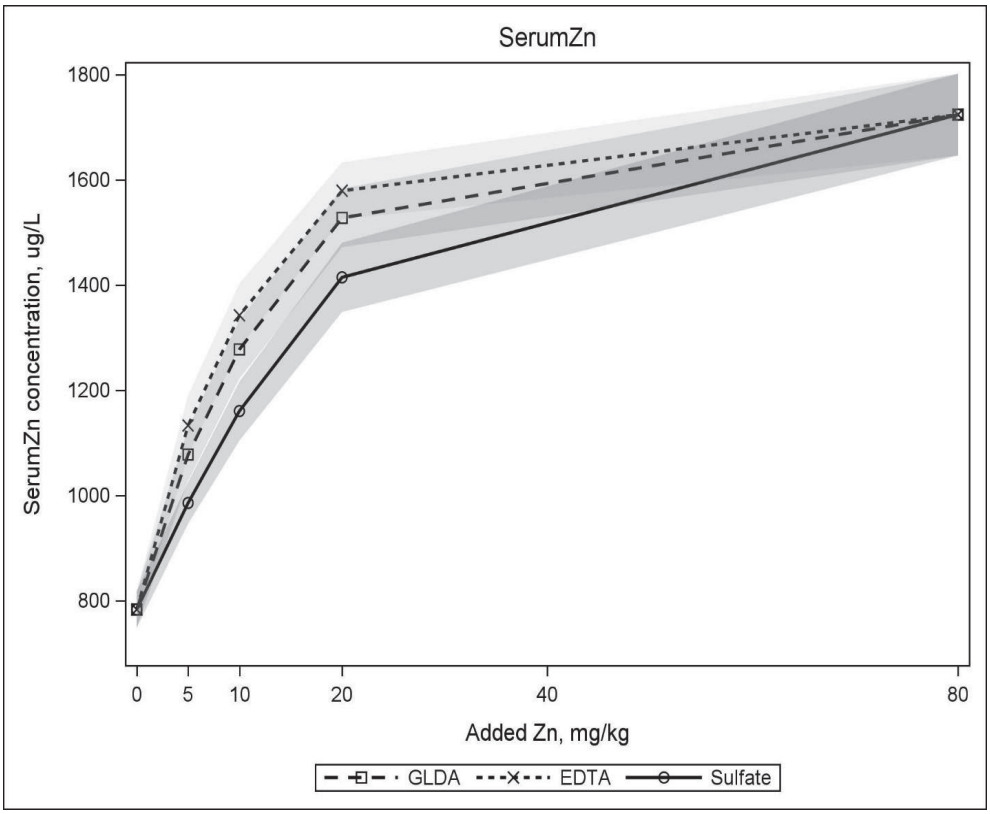

Figure 2.1 Response of serum Zn levels in broilers when fed dietary Zn supplementation with Zn sulphate, ethylenediaminetetraacetic-acid (EDTA) and L-glutamic acid N,N-diacetic-acid (GLDA). Grey area indicates 95% confidence interval.

The results of the non-linear regression analysis showed a clear dose-response effect for Zn on serum Zn, tibia Zn and total tibia Zn values (Table 2.4, Figure 2.1). The tibia and serum Zn data showed a similar response for both EDTA and GLDA. Significant differences between the two chelators and sulphate were observed, but not between EDTA and GLDA (Table 2.4). The estimated dietary Zn level to reach 95% of the model asymptote determined from serum and tibia Zn concentration when EDTA and GLDA were included in the diet were, on average, 69.5 and 68.6% of the estimate when Zn sulphate was used, respectively (Table 2.4). Also, when total tibia Zn amount was used as a response criterion, the estimated dietary Zn level was 71.0% (EDTA) and 69.6% (GLDA) of the Zn sulphate estimate.

Experiment 2

None of the treatments were significantly different from the control with regards to FCR (Table 2.5). Furthermore, GLDA supplementation showed significant linear and quadratic effects on daily weight gain (DWG) ($P<0.05$) and a trend for daily feed intake ($P<0.10$).

Table 2.5 Least square mean performance parameters of broilers receiving a basal diet with increasing levels of L-glutamic acid N,N-diacetic-acid (GLDA) from d7-21.

Parameter	GLDA inclusion levels, mg/kg				P-value		SEM
	0	54	108	216	Linear	Quadratic	
Bodyweight, d7 (g)	206	203	203	204	0.35	0.38	1.4
Bodyweight, d21 (g)	1051	1069	1080	1073	0.02	0.05	0.6
Daily weight gain (g)	60.5	61.8	62.6	62.1	0.02	0.05	0.5
Daily feed intake (g)	88.1	90.5	89.8	88.9	0.06	0.06	0.8
Feed conversion ratio (g/g)	1.46^{ab}	1.47^{b}	1.44^{a}	1.43^{a}	0.35	0.89	0.006

^{ab}Values with different superscripts within row are significantly different (P<0.05).

A linear and quadratic response was observed on serum Zn with increasing dose of GLDA, while no differences were observed for the other three trace minerals in serum nor for haemoglobin (Table 2.6).

Table 2.6 Least square mean mineral concentration in serum and tibia, haemoglobin levels in serum and tibia weight of broilers receiving a basal diet with increasing levels of L-glutamic acid N,N-diacetic-acid (GLDA) from d7-21.

Parameter	GLDA inclusion levels, mg/kg				Model		SEM
	0	54	108	216	Linear	Quadratic	
Serum Zn (µg/L)	737^{a}	952^{b}	1110^{bc}	1183^{c}	<.0001	<.0001	39.1
Serum Cu (µg/L)	121	121	116	123	*n.s.*	*n.s.*	4.4
Serum Mn (µg/L)	5.9	6.6	7.7	5.9	*n.s.*	*n.s.*	0.68
Serum Fe (µg/L)	2338	2379	2114	1855	*n.s.*	*n.s.*	200.9
Haemoglobin (mmol/L)	5.80	5.81	5.82	5.84	*n.s.*	*n.s.*	0.088
Tibia weight (g)	6.78	6.74	6.86	6.65	*n.s.*	*n.s.*	0.179
Bone Zn (mg/kg)	31.0^{a}	39.5^{b}	45.2^{bc}	47.2^{c}	<.01	<.01	1.9
Bone Zn (mg)	211^{a}	266^{b}	310^{b}	313^{b}	<.01	<.01	14
Bone Mn (mg/kg)	1.42	1.41	1.51	1.44	*n.s.*	*n.s.*	0.087
Bone Mn (µg)	9.7	9.5	10.4	9.6	*n.s.*	*n.s.*	0.69

n.s.: not significant

None of the other trace minerals were significantly affected by the addition of GLDA. Bone weight was also unaffected by the dietary treatments. Bone Zn expressed as Zn concentration in tibia as well as total tibia Zn increased in a similar fashion as serum Zn, increasing with an increasing GLDA dose (Table 2.6). No differences were observed for Mn levels in tibia. The results of the non-linear regression showed a clear dose-response effect with increasing levels of GLDA in both bone and serum Zn markers (Table 2.7). Furthermore, variation in serum Zn appeared to be reduced with increasing GLDA levels (Figure 2.2).

Table 2.7 Estimates of a non-linear model* describing the response of serum and tibia Zn in broilers to dietary L-glutamic acid N,N-diacetic-acid (GLDA) inclusion in a basal diet.

Parameter	Serum Zn, µg/L		Tibia Zn			
			Concentration, mg/kg		Total content, µg	
	Full model	SE	Full model	SE	Full model	SE
A	1214.4	6.05	48.03	2.08	319.7	4.67
k	0.08	0.003	0.09	0.029	0.098	0.012
T	-8.7	0.28	-9.3	3.07	-8.7	1.07
s2e[+]	6551	1891	15.4	4.4	1070.6	309.1
AICC[#]	289.1		143.8		245.6	
RMSE[α]	80.9		3.92		32.72	
CCC[β]	0.89		0.84		0.76	

*Y=A×exp(-exp(-k×(GLDA dose-T))) where Y=dependent variable (serum Zn, tibia Zn concentration or total tibia Zn content), A=asymptote, k=rate parameter determining the steepness, GLDA dose=dietary GLDA supplementation, T=inflection point at which k is maximized.

[+,#,α,β]AICC=Akaike information criterion, CCC=Concordance correlation, RMSE=Root mean squared error, s2e=variance, SE=standard error.

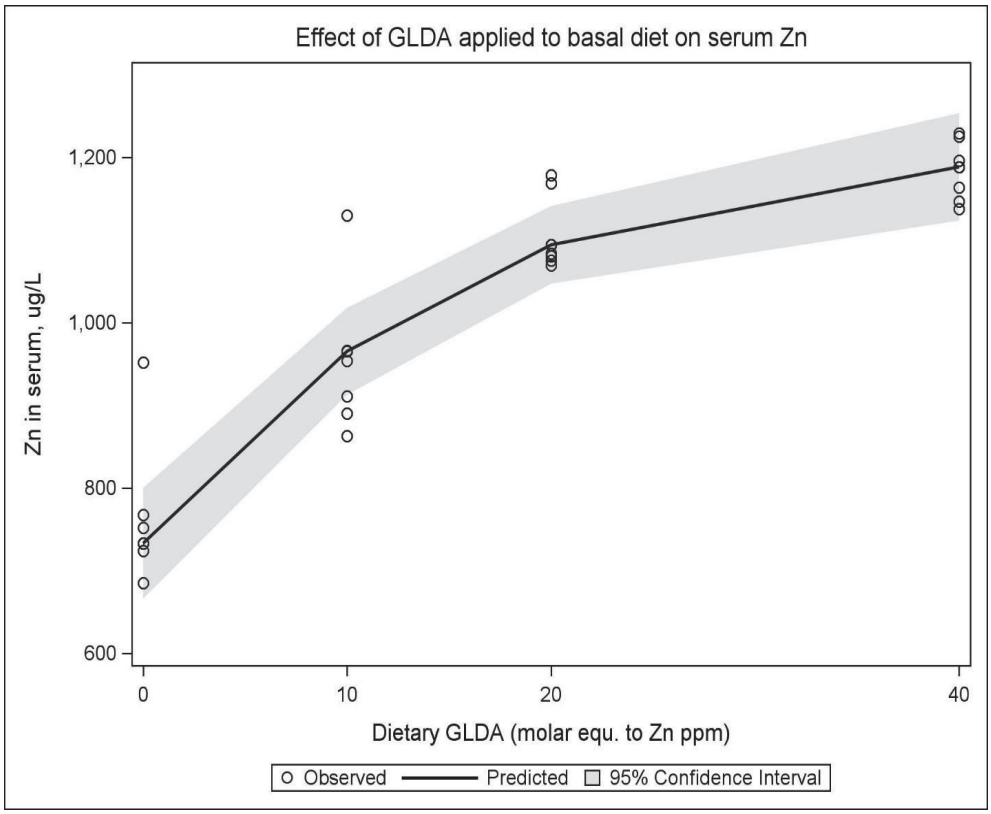

Figure 2.2. Response of serum Zn levels in broilers when fed increasing dietary L-glutamic acid N,N-diacetic-acid (GLDA) levels in basal diet. Grey area indicates 95% confidence interval.

2.4 Discussion

In the first study, a greater Zn absorption was observed, as indicated by the higher levels of serum and tibia Zn, with increasing doses of Zn in the presence of the two chelating agents tested. These results confirm that both GLDA and as EDTA, improve Zn status of broiler chickens. The mode of action, although not studied here, can be expected to be identical to EDTA namely improving Zn solubility in the gastrointestinal tract allowing for a greater Zn uptake. The second experiment demonstrated that GLDA is capable of increasing the nutritional availability of native Zn from the basal meal containing no phytase and high phytate. No effects of GLDA on Cu, Mn or Fe status were observed.

Serum and bone Zn contents are considered a valid indirect indication of Zn absorption (Wedekind and Baker, 1990; Wedekind et al., 1992). Serum Zn can be regarded as a short term marker for Zn status, whereas, bone zinc content can be considered as a responsive criterion for Zn bioavailability in chickens, regardless of low or high dietary trace mineral content (Ammerman et al., 1995; Cao et al., 2002; Huang et al., 2009; Huang et al., 2009; Wedekind et al., 1992).

It is unclear which expression of bone Zn provides the best assessment of overall Zn status. Dietary Zn influences both total bone Zn and Zn concentration in bone, while total bone Zn can be considered a long-term marker for Zn status of an animal. However, Zn status affects overall growth, which may dilute Zn concentration in tissues such as bone. In general, it is important to include the assessment of both serum as well as bone Zn to address the shortcomings of both (Wedekind et al., 1992). We therefore analysed both in this study.

The incremental response to supplemental Zn was lowest for Zn sulphate when fed alone. The response that was achieved with the addition of EDTA or GLDA can be explained by a chemical inhibition of gastrointestinal precipitation of Zn with other dietary factors, thereby improving Zn solubility. The prevention of Zn binding to phytic acid in poultry by EDTA has been known for some time (Likuski and Forbes, 1964), but data here seem to indicate that GLDA can have a similar effect. The second study also indicated that the availability of the native Zn fraction present in the raw materials is improved by addition of GLDA, with a greater amount of dietary Zn reaching tissues by incremental doses of GLDA. Relative differences in tissue Zn are not only determined by Zn supply and availability, but also by the Zn status of the animal (Batal et al., 2001; Brugger and Windisch, 2017). Intestinal absorption plays a key role in Zn homeostasis. For this reason, Zn availability is evaluated by rate of response to incremental doses and quantified in relative terms to a reference inorganic source, mainly Zn sulphate, and in this case also EDTA (Edwards III and Baker, 1999). Regression analyses estimated that the effect of GLDA on Zn availability is comparable to that of EDTA for all tissues sampled in this trial. This indicates equivalent nutritional properties between these two amino polycarboxylates. EDTA has been extensively studied for its ability to increase the nutritional availability of trace metals, with emphasis on Fe in humans. These properties are also well demonstrated for Zn and other trace metals in poultry (Davis et al., 1962). This nutritional property is common to many strong chelating agents with stability constants (logK) for Zn between 5 and 20, and the affinity for Zn is quadratically related to increasing dietary Zn concentrations (Vohra and Kratzer, 1964; Vohra and Kratzer, 1968). The stability constant of the GLDA Zn complex was determined at 10.0 (Kołodyńska, 2011), representing an affinity level that justifies the observed nutritional property described here. EDTA has a stability constant for Zn of 16.5 and considering the GLDA stability constant for Zn it is surprising to see that the two chelators have a similar response in this study (Vohra and Kratzer, 1964). It may be the case that the chelation strength required to reduce precipitation of Zn (by preventing binding to phytate) can already be achieved by using GLDA, giving EDTA no advantage even though its chelation strength is higher. The asymptotes determined in the NLMIXED procedure were estimated to be higher than those measured in serum and tibia. The measured concentrations however were still within the confidence limit of the regression. Having a higher number of birds sampled or more levels tested would have most likely increased this estimation.

Regression analysis for GLDA conducted in the second study indicated that the response lowered near the maximum level of GLDA tested. Typically, a decrease in the response to an increasing

availability of Zn is an indication of regulation of absorption as the nutritional requirements are met. The data from study 2 indicates otherwise, as the asymptotes are much lower than in the first study as well as in those found in the literature (Mondal et al., 2010; Uyanik et al., 2002). The observed lower plateau with GLDA may be interpreted as a saturation effect of GLDA in the solubilisation of the basal dietary Zn content or it may indicate that there was insufficient Zn present in the basal diet to reach a similar plateau as in the first experiment. Considering that Zn retention of broilers as a fraction of their feed intake is close to 20 mg/kg (Batal et al., 2001; Dewar and Downie, 1984), and that basal Zn was 32 mg/kg, it can be speculated that the digestive process was not able to make all dietary Zn available and hence the asymptote could not be reached.

Incremental doses of GLDA on native Cu showed no effect on serum Cu, indicating that nutritional status was already adequate. Serum levels of Mn also remained unchanged by incremental doses of GLDA. Iron status was studied by serum Fe and blood haemoglobin and serum Fe was found to be in the range of that described for adequately fed animals at 20 days (Mondal et al., 2010). Therefore, regulation of absorption may have overshadowed any difference in Fe availability created by GLDA. This conclusion is also supported by the blood haemoglobin data, which were already similar in the negative control diet to that described for healthy broiler chickens at 21 days of age (5.83±0.12 mmol/L) (Martinez and Diaz, 1996). Haemoglobin remained unchanged with increasing doses of GLDA. A highly regulated factor such as blood haemoglobin would only respond to an increased dietary availability of Fe in conditions of deficiency. The present data cannot confirm or reject the hypothesis that GLDA has a positive effect on Fe availability as described for EDTA (being similar in effect) in humans (Hurrell et al., 2000; Viteri and Garcia Inbanez, 1978; Viteri et al., 1978) and rats (Whittaker and Vanderveen, 1990). This effect has not been investigated in broiler chickens, most likely because it is difficult to induce Fe deficiency in the animal model (Davis et al., 1962).

Chelation is a promising tool to reduce faecal output of Zn in broiler chicken production by allowing the safe reduction of Zn inclusion in the feed. The results from the regression analysis indicate that a potential reduction of 15-20 mg/kg in dietary Zn supplementation would not compromise the Zn supply of the broilers and the physiological status of the birds with dietary supplementation of EDTA or GLDA. However, because amino polycarboxylates go unabsorbed through the gastrointestinal tract (Zhu et al., 2006), EDTA would be excreted via the faeces. Additionally, EDTA is considered a potential pollutant with a low level of biodegradability (Bucheli-Witschel and Egli, 2001). In contrast, the biodegradability of GLDA makes it a more environmentally friendly feed component alternative to reduce dietary Zn supply, indirectly reducing Zn output in broiler production systems (Kołodyńska, 2013).

2.5 Conclusion

The results of this study indicate that GLDA significantly increased the nutritional dietary availability of supplemental Zn in a manner and magnitude like that of EDTA in diets high in phytate and without phytase. GLDA has a positive effect on nutritional availability of Zn present in the dietary ingredients. This study demonstrates that GLDA may be used as an effective supplement to increase Zn bioavailability and to reduce the use of supplemental Zn levels in broiler diets. The effect of GLDA on the availability of native Cu, Mn and Fe requires further study using a deficiency model for these nutrients.

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Chapter 3

Effect of L-glutamic acid N,N-diacetic acid on the availability of dietary zinc in broiler chickens

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Abstract

Chelating agents can be used to improve the nutritional availability of trace minerals within the gastrointestinal tract. This study was conducted to determine the effect of a novel chelating agents, L-glutamic acid N,N-diacetic acid (GLDA), a biodegradable alternative to ethylenediaminetetraacetic acid on the nutritional bioavailability of zinc in broilers. Twelve dietary treatments were allocated to 96 pens in a randomized block design. Pens contained 10 Ross 308 male broilers in a factorial design with six incremental zinc levels (40, 45, 50, 60, 80 and 120 mg/kg of total Zn), with and without inclusion of GLDA (0 and 100 mg/kg) as respective factors. Experimental diets were supplied from day 7 to 21/22 and serum, liver and tibia Zn content were determined in three birds per pen. Growth performance and liver characteristics were not affected by dietary treatments, but both supplemental Zn and GLDA enhanced tibia and serum zinc concentration. The positive effect of GLDA was observed at all levels of the dietary Zn addition. The amount of zinc needed to reach 95% of the asymptotic Zn response was determined using non-linear regression. When GLDA was included in the diet, based on tibia Zn, the same Zn status was achieved with a 19 mg/kg smaller Zn dose while based on serum Zn this was 27 mg/kg less Zn. Dietary GLDA reduces supplemental Zn needs to fulfil nutritional demands as defined by tibia Zn and serum Zn response. Considering the positive effect on the nutritional availability of Zn in broilers, GLDA presents an opportunity as biodegradable additive, to reduce Zn supplementation to livestock and thereby reducing Zn excretion into the environment, while fulfilling the nutrition Zn needs of farmed animals.

3.1 Introduction

Zinc (Zn) is a component of almost every metabolic pathway, making it a critical nutrient to farm animal health and productivity. To fulfil their biological demands for zinc, animals need adequate levels of bioavailable zinc (free and loosely bound Zn^{2+} ions) in their diet/ration (Brugger and Windisch, 2017; Goff, 2018; Skrypnik and Suliburska, 2018). The availability of zinc is the result of efficient digestion as well as the interaction with other dietary components. Commercial farm animal diets are generally supplemented with high levels of Zn to guarantee the fulfilment of the nutritional requirements. However, Zn supplementation above the requirement decreases the relative efficiency of Zn absorption, which increases faecal Zn excretion (Weigand and Kirchgessner, 1980). Higher inclusion of Zn in diets will therefore lead to an increase in environmental pollution (Brugger and Windisch, 2015; Brugger and Windisch, 2019). A reduction of excessive supplemental levels is however not as straightforward as it may seem. These levels of supply are justified by unpredictability of trace mineral availability in the diet. Bioavailability might be compromised interactions and antagonisms with other dietary components in the digestive tract (Bao et al., 2010; Underwood, 1999). Ionizable supplemental forms such as Zn sulphates rapidly solubilize and dissociate in a solution, which enables other dietary components to interact with the zinc ion, with phytic acid being the best described antagonist (Humer et al., 2015; Windisch, 2002). Under mild acidic to neutral conditions (pH=5-7) phytic acid develops

sustainable bonds with divalent cations yielding insoluble phytate complexes (Humer et al., 2015; Linares et al., 2007). There is indication that poultry have inadequate intrinsic phytase activity to utilize the phytate bound minerals and as such cannot absorb the complete mineral pool present in plant biomass (Brugger and Windisch, 2017). Dietary solutions that lower the effect of antagonists and thereby increase zinc bioavailability for production animals are required to realize an adequate mineral supply and to reduce the environmental burden (EFSA Panel on Dietetic Products and Allergies, 2011). Apart from supplements of exogenous phytase, single strong chelating agents can be used to minimize the effect of antagonisms and thereby increase mineral bioavailability. Chelating agents comprise of molecules with a high affinity to bind to trace elements and can potentially provide stability of the soluble complex in the upper gastrointestinal tract, which minimizes the formation of insoluble complexes. The binding strength of these chelating agents is exponentially higher than binding strength of most naturally occurring organic ligands (Goli et al., 2012; Krezel and Maret, 2016). Ethylenediaminetetraacetic acid (EDTA) is an example of a strong chelating agent, with well described effects on nutritional trace mineral availability (Maenz et al., 1999; O'Dell et al., 1964; Oberleas et al., 1966; Vohra and Kratzer, 1964). EDTA was found to decrease supplemental Zn requirements when isolated soybean protein served as the source of dietary protein. The addition of 100 mg/kg of EDTA to the diets chelated about 8 mg/kg of Zn, which was assumed to be the amount of Zn bound by the soybean proteins (Kratzer and Starcher, 1963). EDTA however has limited biodegradability and can accumulate in soil and surface water, thereby contaminating the environment (Wu et al., 2015). A novel chelator, L-glutamic acid N,N-diacetic acid (GLDA) can be regarded as a more environmentally friendly alternative to EDTA, with a smaller impact on surface waters and soils because of its faster biodegradability. It exhibits good chelating capacity towards a plethora of metal ions, including Zn, while it has a high biodegradability, with more than 60% being degraded within 28 days (Kołodyńska, 2013; Wu et al., 2015). Its production is based on the flavour enhancer monosodium glutamate from fermentation of readily available corn sugars (Seetz and Stanitzek, 2008).

Previous work from this group indicated that the effect on Zn availability for GLDA and EDTA are similar (Boerboom et al., 2020). The effect of GLDA at a fixed level with increasing amounts of supplemental dietary Zn has not been described before. Confirmation and quantification of its potential effect on Zn bioavailability in the diet would offer an opportunity for reduction of Zn supplementation in complete feeds, while safeguarding the Zn status of the animal, thereby mitigating this aspect of environmental impact of animal production. A dose-response study was designed to test the hypothesis that GLDA improves the Zn availability in broiler diets, thereby allowing for a reduction in Zn supplementation. Furthermore, upon confirmation of the hypothesis, the potential effect of GLDA on Zn supplemental needs was quantified.

3.2 Materials & Methods

3.2.1 Animals

This experiment was performed in accordance with the Dutch legislation and regulations for animal experiments and approved by the Committee for Animal Experiments of Wageningen University and Research, The Netherlands (AVD401002015196, IvD code 2016057.a).

This study was conducted at the facilities of Wageningen Bioveterinary Research, Building 161, Lelystad, The Netherlands using 1100 one-day-old Ross 308 male broilers. During the first week, broilers were housed as one group in a floor pen bedded with white wood shavings (2kg/m^2). All birds received a standard starter diet formulated to adequately comply with all nutritional requirements of the broilers including 80 mg/kg of supplemental Zn derived from Zn sulphate monohydrate ($ZnSO_4.H_2O$). After one week, 960 healthy animals were selected and equally distributed according to a weight class system (10 animals/pen) over 96 floor pens with a flexible slatted plastic floor. From this moment on until the end of the study, the birds received treatment specific diets. The 12 dietary treatments were randomly distributed over eight blocks of 12 pens. Treatments were randomly allocated to pens within blocks aiming to create a balanced distribution of treatments within blocks and over the experimental room taking into account location of blocks within the barn. Four birds within each pen were marked with marker sprays at day 7, three animals were marked blue and one green. This was done to ensure a random selection of birds was later used for blood and tibia sampling at completion of the study. The birds marked in blue were sampled and the bird marked in green served as a backup in case a blue-sprayed bird died during the study.

3.2.2 Diets

The dietary treatments comprised six incremental levels of supplemented Zn (0, 5, 10, 20, 40 and 80 mg/kg Zn from $ZnSO_4.H_2O$, leading to 40, 45, 50, 60, 80, 120 mg/kg of Total Zn) and presence or absence of supplemental GLDA (0 and 100 mg/kg GLDA, i.e., 0 and 333 mg/kg GLDA-silica premix (30%)) (Trouw Nutrition, Amersfoort, The Netherlands). A basal diet was formulated to meet or exceed all nutritional requirements other than from zinc. The basal meal was formulated with maize, wheat, soybean meal, wheat bran, soybean oil and rice bran, the latter to provide a higher presence of phytate in the diet (Table 3.1). With the intention to additionally challenge trace mineral availability a surplus of calcium was added (10 g/kg in the form of feed grade limestone). In order to inactivate endogenous phytase from the feedstuff, the basal was pelleted at elevated temperature (80°C) and subsequently milled through a 4.0 mm screen in a hammer mill. The basal meal contained approximately 40 mg of native Zn from feed ingredients per kg. Treatment specific premixtures were used to prepare the experimental diets and an amount of hydrated silica, equal to the inclusion level of GLDA, was added to the non-GLDA supplemented diets as a control. All diets were pelleted through a 3.2 mm die with the addition of steam (75°C).

The experimental feeds were formulated and produced by Research Diet Services B.V. (Wijk bij Duurstede, the Netherlands).

Table 3.1 Composition starter and grower phase feeds.

Ingredients	Starter, g/kg	Grower, g/kg
Wheat	396	299
Corn	200	200
SBM	315	234
Potato protein	-	15.0
Wheat bran	-	85.0
Rice bran	-	65.0
Soybean oil (veg.)	42.0	57.3
L-Lysine	2.3	2.6
DL-Methionine	2.4	2.3
L-Threonine	0.4	0.5
Limestone	16.5	19.2
Mono-calcium phosphate	16.5	11.0
Salt	2.0	1.8
NaHCO3	2.4	2.3
Zn excluded Premix	5.0	5.0
Total	1000	1000

3.2.3 Diet analysis

Representative samples of the diets were taken and ground to 0.5 mm to determine Zn, Cu and GLDA content. Zinc and Cu content were measured in duplicate by using inductively couple plasma mass spectrometry (ICP-MS, ThermoFisher, Waltham, United States) after ashing and HCl-extraction according to method NEN-EN 15510 (Bikker et al., 2017). GLDA content was measured in duplicate by using liquid chromatography-mass spectrometry (LC-MS, ThermoFisher, Waltham, United States) (Masterlab B.V., Boxmeer, The Netherlands). GLDA was quantitatively water extracted from ground feed samples. The extracted GLDA and extracted components from the experimental diets were separated applying reversed phase liquid chromatography, using an Alltima C18 AQ 3 µm column as stationary phase and 0.2 % tri-fluoroacetic acid in water as mobile phase. GLDA presence was detected at m/z 264.070 using a Triple Quadrupole LC-MS mass spectrometer. GLDA levels in feed samples were calculated using a GLDA standard curve based on a calibrated GLDA standard.

3.2.4 Measurements

Performance parameters, including bodyweight (BW), feed intake (FI), body weight gain (BWG) and feed conversion ratio (FCR), were determined at 7, 14 and 21/22 days of age. At the end of the study (at Day 21 (Pen 1-48) and Day 22 (Pen 49-96)) blood samples were taken from the three blue-marked birds in each pen to determine Zn concentration in serum. The blood samples were taken from the wing vein in Zn-free serum tubes (VACUETTE® TUBE 6 ml Z No Additive). In total 288 (96 pens x 3 animals) blood samples were taken. After collection the blood was centrifuged for approximately 30 minutes. Subsequently, the harvested serum was divided in two aliquots of approximately 1 mL per bird in coded 2.5 mL cryotubes. The serum samples were stored at -20°C until further analysis. Serum samples were analysed for Zn content with inductively coupled plasma mass spectrometry (ICP-MS) after hydrochloric acid destruction at Masterlab B.V. (Boxmeer, The Netherlands) (Olukosi et al., 2018). After blood collection, the birds were anaesthetized by intramuscular injection of a solution made of 50 ml sedamun and 30 ml ketamine (1 ml/kg bodyweight) and 20 minutes later euthanized by an intravenous injection of T61 (an aqueous solution containing 200 mg embutramide, 50 mg mebezoniumiodide, and 5 mg tetracainehydrochloride per mL) and the left and right tibia bones and the liver were collected. The tibia bones were pooled per animal and stored at 4°C until further processing. Tibia bones were cleaned from flesh after soaking in hot water and incinerated overnight at 500°C to determine ash content, followed by hydrochloric acid destruction and ICP-MS analysis to determine the Zn content in tibia ash at Masterlab B.V. (Boxmeer, The Netherlands) (Olukosi et al., 2018). The liver samples were stored at -20°C until further processing. Each liver sample was incinerated at 500°C to determine ash content, followed by hydrochloric acid destruction and ICP-MS analysis to determine the Zn content at Masterlab B.V. (Boxmeer, The Netherlands) (Olukosi et al., 2018).

3.2.5 Statistical analysis

The data were analysed using SAS Studio (SAS Institute Inc., Cary, NC). Outliers were identified using the influence statement within the procedures. Growth performance data, and liver, serum and tibia characteristics were analysed using the MIXED procedure. Treatments, GLDA and Zn dose were analysed as main effects with block as a random effect and time (if applicable) as a repeated effect. Pen was used as experimental unit. Zinc levels in serum and tibia were subsequently analysed using non-linear regression (NLMIXED). Since Zn absorption is primarily a homeostatically regulated, saturable, carrier-mediated process, the response to dietary Zn is non-linear and using non-linear regression was preferred over data transformation and linear regression (Miller et al., 2007). The non-linear regression model used for the analysis of serum and tibia Zn content in this study was selected based on statistical fit and biological meaning (Archontoulis and Miguez, 2015).

Y=A*exp(-exp(-k*(dietary Zn level-T)))

In which:

Y=response parameter, serum or tibia Zn content,

Dietary Zn level=Total dietary Zn level (mg/kg),

A=Asymptote, representing the maximum response in the Y variable,

k=Rate parameter controlling the steepness of the curve, and

T=inflection point at which the response rate is maximized.

In addition, the effect of GLDA inclusion on the regression parameters was determined by including a factor representing GLDA inclusion and parameters A1, K1 and T1 representing the difference between the control and GLDA supplemented diets. The full model is described below.

Y = (A+A1*GLDA)*exp(-exp(-(k+k1*GLDA)*(Dietary Zn level-(T+T1*GLDA))))

In which:

Y=response parameter, serum or tibia Zn content,

A, A1=Asymptote, representing the maximum response in the Y variable,

k, k1=Rate parameter controlling the steepness of the curve,

T,T1= inflection point at which the response rate is maximized,

GLDA= factor representing dietary GLDA inclusion (0,1), and

Dietary Zn level=Total dietary Zn level (mg/kg).

A reduced model was created including only significant GLDA effects after exclusion of all non-significant GLDA effects on parameters using a backward elimination procedure. Finally, this reduced model was used to determine the dietary Zn level required to reach a response of 95% of the asymptotic value for serum Zn and Zn concentration in tibia ash. This value was considered as criterion for estimating the bioavailability of the Zn in the diet (Huang et al., 2013), but most importantly to describe the potential minimum supplemental dose of Zn for fulfilment of nutritional requirements.

3.3 Results

In Table 3.2, the intended and analysed concentration of Zn and GLDA in the experimental diets is included. The results indicate that the analysed content of Zn and GLDA was aligned with the intended Zn and GLDA levels and in accordance with the experimental design. The MIXED and NLMIXED procedures therefore were based on the intended dietary Zn levels. Table 3.3 indicated the analysed nutrient contents of both the starter diets as well as the basal meal.

Table 3.2 Intended and analysed moisture, zinc and L-glutamic acid N,N-diacetic acid (GLDA) content in experimental diets used to determine the effect of GLDA in broilers. Supplemental Zn is calculated as analysed total Zn content in each diet minus 41 mg/kg from basal meal.

Treatment	Analysed moisture, g/kg	Analysed total Zn, mg/kg	Intended supplemental Zn, mg/kg	Analysed supplemental Zn, mg/kg	Intended GLDA, mg/kg	Analysed GLDA, mg/kg
1	118	41	0	-	-	0
2	117	45	5	4	-	0
3	115	49	10	8	-	0
4	114	56	20	15	-	0
5	113	74	40	33	-	0
6	113	116	80	75	-	0
7	112	40	0	-	100	100
8	113	46	5	5	100	102
9	115	51	10	10	100	99
10	113	61	20	20	100	103
11	112	78	40	37	100	105
12	113	112	80	71	100	103

Table 3.3 Intended and analysed nutrient contents of the starter diet (0-7 days) and the basal meal used for production of the treatment diets (7-21/22 days) to determine the effect of L-glutamic acid N,N-diacetic acid (GLDA) in broilers.

Component		Starter diet		Basal diet	
		Intended	Analysed	Intended	Analysed
Dry Matter	g/kg	*n.a*	897	*n.a.*	891
Moisture	g/kg	*n.a.*	103	*n.a.*	109
Ash	g/kg	65	59	69	65
Starch	g/kg	378	375	385	322
Crude Protein (CP)	g/kg	216	215	202	198
Fat (EE)	g/kg	58	67	79	90
Crude Fibre (CF)	g/kg	24	23	32	33
Phosphorus (P)	g/kg	7.4	7.4	8.2	7.0
Calcium (Ca)	g/kg	10.0	10.9	10.0	11.3
Zinc (Zn)	mg/kg	113	110	36	43
Copper (Cu)	mg/kg	22	21	21	23
Iron (Fe)	mg/kg	200	342	186	273
Manganese (Mn)	mg/kg	115	121	124	146

n.a. = not available

Zinc dose and the inclusion of GLDA did not affect bird performance and there no interactions were detected between them (Table 3.4). Growth performance observed in this study was in line with Ross 308 guidelines (Aviagen, 2014).

Table 3.4 Effect of dietary L-glutamic acid N,N-diacetic acid (GLDA) and Zn levels on growth performance of broilers from Day 7 to 21/22.

GLDA, mg/kg	Total Zn, mg/kg						P-value			
	40	45	50	60	80	120	SE	GLDA	Zn	GLDA*Zn
Bodyweight d7, g										
0	137	134	135	136	135	134	0.2	0.29	0.81	0.28
100	134	134	134	134	135	136				
Bodyweight d14, g										
0	437	432	438	435	434	436	1.4	0.86	0.75	0.84
100	433	438	436	430	435	443				
Bodyweight d21/22, g										
0	942	923	918	915	911	919	4.9	0.61	0.94	0.66
100	914	919	927	907	917	917				
Bodyweight gain 7-14, g										
0	300	298	303	300	299	302	1.3	0.71	0.69	0.87
100	299	304	302	295	300	307				
Bodyweight gain 7-21/22, g										
0	805	789	782	780	770	785	4.9	0.46	0.58	0.70
100	780	784	789	772	782	781				
Bodyweight gain 14-21/22, g										
0	505	491	479	480	477	483	4.3	0.52	0.92	0.53
100	481	481	490	477	482	474				
Feed conversion ratio 7-14										
0	1.32	1.30	1.32	1.29	1.30	1.30	0.003	0.51	0.35	0.06
100	1.31	1.28	1.29	1.31	1.32	1.29				
Feed conversion ratio 14-21/22										
0	1.48	1.51	1.49	1.48	1.49	1.48	0.004	0.68	0.91	0.89
100	1.51	1.49	1.48	1.49	1.5	1.49				
Feed conversion ratio 7-21/22										
0	1.42	1.43	1.42	1.41	1.42	1.42	0.003	0.98	0.62	0.59
100	1.43	1.41	1.41	1.42	1.43	1.41				
Feed intake 7-14, g										
0	397	387	399	386	387	393	1.5	0.96	0.48	0.77
100	390	390	391	387	395	395				
Feed intake 14-21/22, g										
0	749	741	715	711	712	719	7.2	0.64	0.90	0.38
100	724	718	727	710	724	706				
Feed intake 7-21/22, g										
0	1146	1128	1114	1097	1090	1112	7.7	0.45	0.29	0.42
100	1115	1108	1112	1097	1119	1101				

Dietary inclusion of Zn or GLDA had no significant effect on the liver measurements nor on tibia weight or tibia ash content (Table 3.5). Zinc content in tibia and serum increased significantly with increasing levels of dietary Zn $P<0.001$). In addition, GLDA significantly enhanced the Zn content in serum and tibia across all levels of supplemental Zn including the zero. The effect of GLDA inclusion depended on the amount of Zn that was added to the diet as shown by the significant interaction ($P<0.001$).

Table 3.5 Effect of dietary L-glutamic acid N,N-diacetic acid (GLDA) inclusion and Zn levels in broilers on liver, tibia and serum characteristics when fed from d7-21.

	Total Zn, mg/kg							P-value		
GLDA, mg/kg	40	45	50	60	80	120	SE	GLDA	Zn	GLDA*Zn
Liver										
Weight, g										
0	37.3	37.0	39.1	36.4	33.9	37.6	0.46	0.46	0.38	0.59
100	38.5	37.8	37.7	38.2	36.7	36.5				
Ash, g/kg										
0	1.23	1.22	1.22	1.21	1.24	1.22	0.005	0.63	0.83	0.90
100	1.24	1.23	1.23	1.22	1.21	1.24				
Zn in Fresh, mg/kg										
0	18.9	18.2	17.5	17.6	18.8	20.1	0.34	0.84	0.72	0.95
100	18.4	17.7	18.5	18.7	19.4	18.9				
Total Zn, mg										
0	0.73	0.69	0.69	0.64	0.64	0.77	0.02	0.77	0.91	0.95
100	0.72	0.68	0.70	0.73	0.71	0.69				
Ratio Liver/BW, %										
0	3.96	3.98	4.17	3.92	3.79	3.96	0.04	0.44	0.61	0.75
100	4.04	3.95	3.88	3.97	3.87	3.75				
Tibia										
Weight, g										
0	4.77	4.44	4.72	4.71	4.49	4.81	0.05	0.24	0.13	0.38
100	4.87	4.79	4.70	4.80	4.49	4.95				
Ash, %										
0	39.4	40.4	39.7	38.2	38.9	39.3	0.19	0.18	0.65	0.47
100	38.8	38.8	38.9	39.2	38.9	38.6				
Zn, mg/kg ash										
0	200^{A}	219^{AB}	241^{C}	268^{D}	287^{D}	314^{E}	2.53	<.001	<.001	<.001
100	232^{BC}	248^{C}	276^{D}	282^{D}	311^{E}	313^{E}				
Serum										
Zn, mg/L										
0	0.97^{A}	1.07^{AB}	1.31^{CD}	1.43^{CDE}	1.63^{EF}	1.75^{F}	0.02	<.001	<.001	<.001
100	1.22^{BC}	1.31^{CD}	1.52^{DE}	1.65^{EF}	1.79^{F}	1.84^{F}				

^{A,B} Values with different superscripts within a parameter differ significantly (P<0.05).

The results of the regression analysis with the non-linear model for serum Zn are described in Table 3.6 and illustrated in Figure 3.1 and 3.2. The reduced model indicated that no significant effects of GLDA were present for the maximum response in serum Zn concentration (asymptote) and inflection point (T) in serum Zn response. The similar Akaike information criterion (AICC), root mean squared error (RMSE) and concordance correlation coefficient (CCC) of the two models confirmed that these parameters did not contribute to the prediction of the serum Zn response. GLDA significantly affected the steepness of the response in serum Zn to increasing dietary Zn levels (parameter k), indicating an increased rate in serum Zn response. GLDA enhanced the Zn concentration in serum when added to the diet of the birds. In presence of GLDA, a substantially lower dietary Zn level was adequate to reach 95% of the maximum serum Zn concentration. GLDA inclusion in the diets reduced the required level of dietary Zn by 22 mg/kg in the full model and by 27 mg/kg in the reduced model.

Table 3.6 Parameter estimates of a non-linear model describing the response of serum Zn to total dietary Zn content and L-glutamic acid N,N-diacetic acid (GLDA) including an estimate of Zn requirements (95% of asymptote).

Parameters	Full Model	Standard error	Reduced model	Standard error
A	1.75	0.05	1.81	0.035
A1	0.09	0.07	n.s.	
k	0.056	0.01	0.046	0.007
k1	0.010	0.19	0.033	0.008
T	-9.10	2.40	-10.99	2.01
T1	-3.65	4.05	n.s.	
s2e[+]	0.055	0.004	0.055	0.005
AICC[#]	-4.8		-4.8	
RMSE[α]	0.24		0.24	
CCC[β]	0.73		0.73	
Total Zn (mg/kg) to reach 95% of asymptote A				
Control	83.5		94.1	
GLDA	61.2		67.0	

** model: Y = (A+A1*GLDA)*exp(-exp(-(k+k1*GLDA)*(Dietary Zn level-(T+T1*GLDA))))*

+,#,α,βAICC=Akaike information criterion, CCC=Concordance correlation, RMSE=Root mean squared error, s2e=variance, SE=standard error. n.s.: not significant.

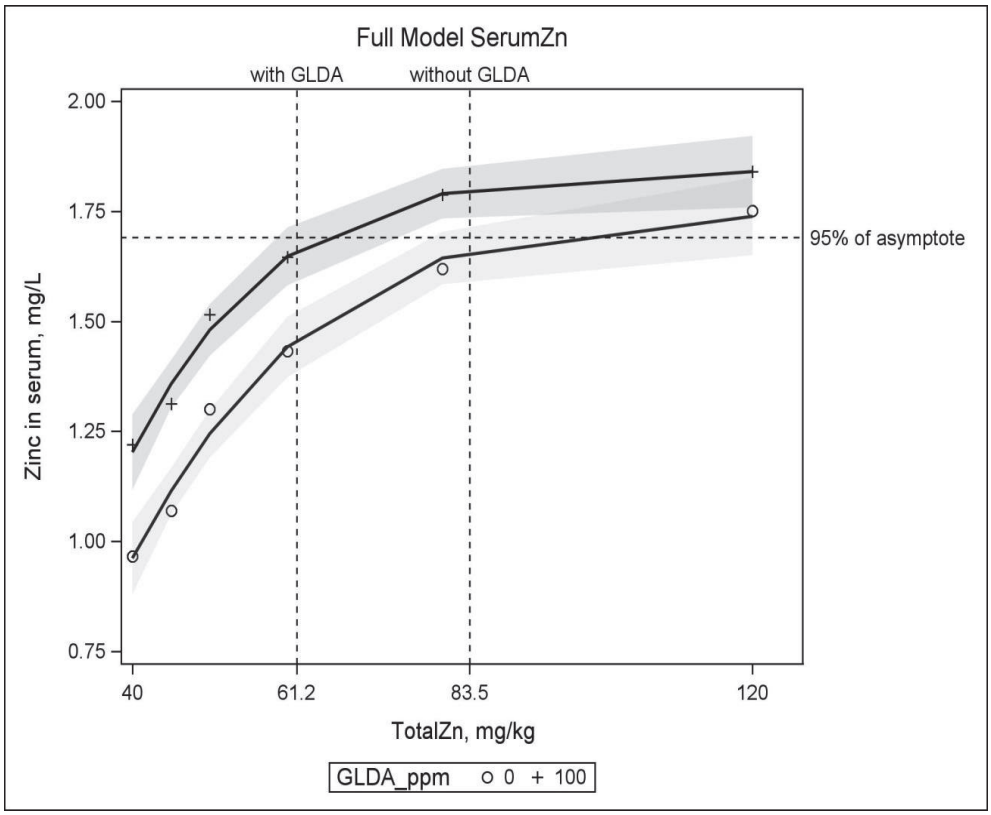

Figure 3.1 Effect of dietary L-glutamic acid N,N-diacetic acid (GLDA) inclusion and total dietary Zn content on the Zn concentration in serum using a non-linear full model. Dotted lines represent the value for Zn at which the serum Zn level is equal to 95% of the asymptote.

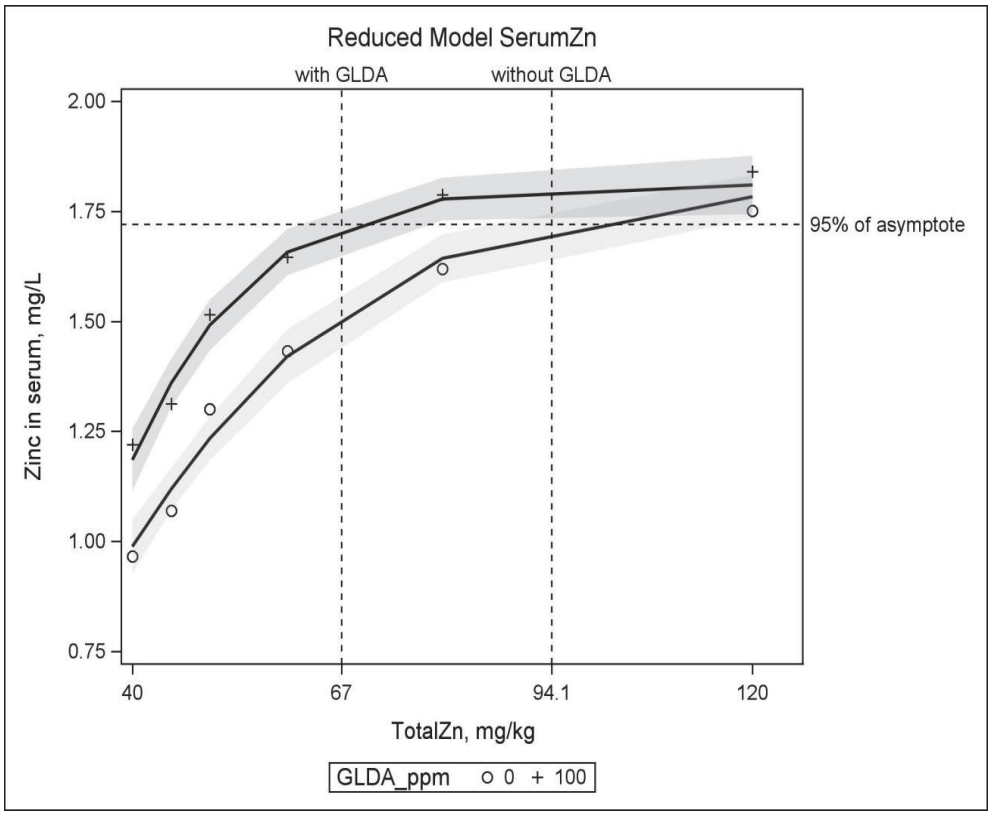

Figure 3.2 Effect of dietary L-glutamic acid N,N-diacetic acid (GLDA) inclusion and total dietary Zn content on the Zn concentration in serum using a non-linear reduced model. Dotted lines represent the value for total Zn at which the serum Zn level is equal to 95% of the asymptote.

The results for Zn in tibia ash using a non-linear model are described in Table 3.7 and illustrated in Figure 3.3. The reduced model indicated that no significant effects of GLDA were present for the maximum response in tibia Zn (asymptote) and the inflection point (T) in tibia Zn response. The similar RMSE and CCC of the two models confirmed that these parameters did not contribute to the description of the Zn response in tibia ash. The AICC improved slightly when the reduced model was used in comparison to the full model indicating an improved model performance. GLDA significantly affected the steepness in response of Zn in tibia ash to an increase in dietary Zn increase (parameter k), indicating an increased rate in response of Zn in tibia ash. GLDA enhanced Zn concentration in tibia when added to the diet of the birds. In presence of GLDA, a lower dietary Zn level was adequate to reach 95% of the maximum Zn content in tibia. GLDA inclusion in the diets reduced the required level of dietary Zn by 17 mg/kg in the full model and by 19 mg/kg in the reduced model.

Table 3.7 Parameter estimates of a non-linear model describing the response of tibia Zn to total dietary Zn content and L-glutamic acid N,N-diacetic acid (GLDA) including an estimate of Zn requirements (95% of asymptote).

Parameters	Full Model	Standard error	Reduced model	Standard error
A	314.4	5.44	314.5	3.25
AI	0.53	6.83	*n.s.*	
k	0.048	0.007	0.046	0.0045
KI	0.015	0.012	0.020	0.003
T	-16.77	2.41	-17.54	1.88
TI	-1.97	3.94	*n.s.*	
s2e^{+}	454.4	38.47	454.9	38.51
AICC$^{\#}$	2513		2510	
RMSE$^{\alpha}$	21.3		21.3	
CCC$^{\beta}$	0.85		0.85	
Total dietary Zn (mg/kg) to reach 95% of asymptote A				
Control	85.3		86.5	
GLDA	67.9		67.1	

** model: Y = (A+AI*GLDA)*exp(-exp(-(k+kI*GLDA)*(Dietary Zn level-(T+TI*GLDA))))*

$^{+,\#,\alpha,\beta}$AICC=Akaike information criterion, CCC=Concordance correlation, RMSE=Root mean squared error, s2e=variance, SE=standard error.

n.s.: not significant.

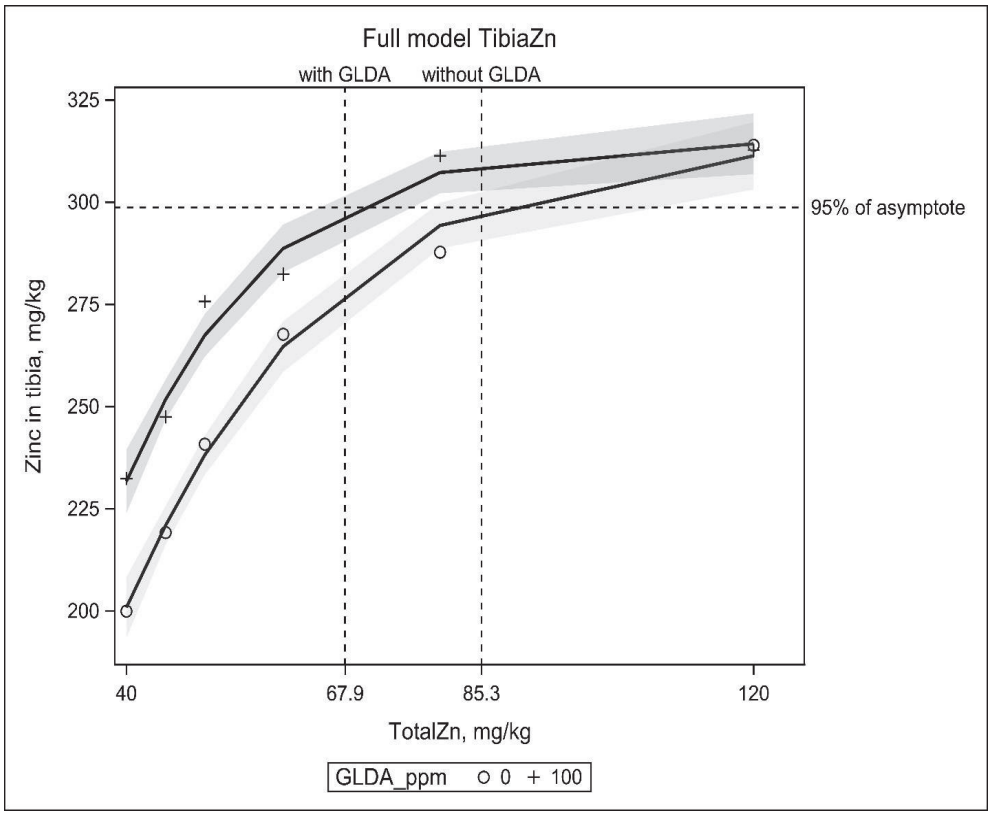

Figure 3.3 Effect of dietary L-glutamic acid N,N-diacetic acid (GLDA) inclusion and total dietary Zn content on the Zn concentration in tibia using a non-linear full model. Dotted lines represent the value for Zn at which the tibia Zn level is equal to 95% of the asymptote.

3.4 Discussion

Zinc is an essential trace mineral nutrient in broiler diets. The dietary Zn content from most combinations of feed ingredients is assumed to be below the requirements. Furthermore, Zn availability may be compromised by dietary antagonists of variable and uncertain presence including phytate and fibre (Windisch, 2002; Yu et al., 2010). Therefore, broilers are generally supplemented with Zn in their diets to assure adequate supply to sustain performance and health and to avoid Zn deficiency. In practice, broiler diets are generally formulated with Zn levels above the reference recommendations and even close to the maximum allowed legal limits (120 mg Zn/kg in the European Union) (Additives and Feed, 2014). However, Zn utilization of broilers is rather low with only a small portion of dietary Zn retained in the body (Brugger and Windisch, 2017). This results in a high excretion of non-utilized Zn in the faeces or excreta, contributing to accumulation of Zn in the environment, especially in soil and surface water. A novel chelator with low environmental persistency and a high chelation strength, GLDA, was tested in this study.

Dietary inclusion of this compound was hypothesized to increase the availability of Zn in the digestive tract of broilers, which would allow a reduction in Zn supplementation of diets.

No significant effects of dietary inclusion of Zn and GLDA were detected for any of the performance parameters analysed during the experimental period of 0-21/22 days. This is in accordance with the expectations since 41 mg/kg Zn was present in the basal diet and literature indicates that a dietary Zn content around 40 mg/kg is adequate for normal growth (Mohanna and Nys, 1999a; Schlegel et al., 2010). In addition, literature suggests that intrinsic phytase activity in broilers may be increased in the case of high phytate levels, which might also have contributed to the absence of an effect on performance (Zeller et al., 2015). Growth performance is generally regarded as an insensitive criterion for mineral availability (Huang et al., 2013; Jongbloed et al., 2002). Bone and serum zinc content, on the other hand, are considered to be more sensitive criteria for Zn bioavailability in broiler chickens, regardless of the low or high supplementation of Zn in the diet (Cao et al., 2002; Huang et al., 2013). Indeed, the results of the present study confirm a substantial and significant response of serum and tibia Zn content to Zn supplementation of the diet.

Results in this study demonstrated a significant effect of GLDA on tibia and serum Zn content, both in diets containing supplemental Zn as well as in the diets containing no supplemental Zn. This shows that GLDA can make the inherent Zn from common feed ingredients in diets more bioavailable. Strong chelators such as EDTA ensure protection against precipitation of minerals, which was thought to improve mineral solubility in the gastrointestinal tract (Maenz et al., 1999; O'Dell et al., 1964; Oberleas et al., 1966). This improved solubility appears to coincide well with the observed effect of GLDA, allowing for better uptake of Zn in the current study. These results are in line with previous research of our group in which the effect of Zn availability in broilers between GLDA and EDTA were compared and equivalent nutritional properties between the two were observed (Boerboom et al., 2020). Improvement of nutritional availability by the use of chelators has been well demonstrated for strong chelators with stability constants (logK) for Zn between 5 and 20 (Davis et al., 1962; Vohra and Kratzer, 1968; Vohra and Kratzer, 1964). GLDA has a stability constant of 10, which indicates that the observed changes in Zn availability are justified (Kołodyńska, 2011). The results also indicate that even though GLDA has a high chelation strength, the transporters of the intestinal tract are strong enough to ensure sufficient uptake of Zn. This was not the case for some other chelating agents that were tested before (Vohra and Kratzer, 1964). The effect of GLDA on Zn retention was constant at lower levels of dietary Zn (<60 mg/kg) and this effect gradually decreased with increasing levels of Zn, without significant differences at higher inclusion levels of Zn. The lack of significance does not indicate that GLDA has no effect at higher Zn levels; it indicates that there is a strict regulation in Zn homeostasis. In the case of abundant Zn, the expression of Zn transporters and related proteins is decreased to downregulate the absorption of Zn. The strict regulation of Zn homeostasis prevents a physiological response after the asymptote has been reached (Ao et al., 2007; Schlegel et al., 2013). The results in this study indicate that GLDA does not compromise the Zn control

mechanisms that are in place and as such supports the broilers in maintaining a proper Zn status. The lack of response at high dietary Zn also indicates that the GLDA Zn complex appears to exert its effect only in the gastrointestinal tract, with limited passive absorption taking place. The increased solubility of the complex would in the case of passive absorption also lead to an increase in Zn levels when higher levels of Zn are fed with GLDA. To estimate the amount of Zn that is made bioavailable by the addition of GLDA, the results of serum and tibia Zn were analyzed applying nonlinear regression. The higher regression coefficient k in presence of GLDA indicated an improvement in the bioavailability of Zn, in accordance with the results discussed previously. Hence, using concentrations of Zn in serum and Zn in tibia ash as response criteria, the inclusion of GLDA would allow for a reduction in dietary Zn supplementation of 20 mg/kg, without compromising the Zn supply of the broilers and the physiological Zn status of the birds. This would allow for a similar reduction of Zn in the excreta and thereby into the environment. Adopting a Zn supplementation of 80 mg/kg, as often used in commercial diets, and a total dietary Zn content close to 120 mg/kg, the estimate of 20 mg/kg as discussed above would allow for a reduction of approximately 20% in total dietary Zn content and Zn excretion.

3.5 Conclusion

The results of this study demonstrate that dietary inclusion of GLDA improved the bioavailability of Zn in broiler diets. GLDA exerts its effect in the gastro-intestinal tract with both diets containing only inherent Zn from common feed ingredients as well as diets containing supplemental Zn. This demonstrates that GLDA can be used as a dietary ingredient to safely reduce Zn supplementation in complete feed without compromising the Zn status of the animals. GLDA inclusion can substantially contribute to a reduction of Zn excretion into the environment if Zn supplementation in the diets is reduced.

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Chapter 4

L-glutamic acid N,N-diacetic acid has a high tolerance level for broilers and poses no risk for consumer food safety

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To be submitted

Abstract

The novel chelator, L-glutamic acid, N,N-diacetic acid (GLDA) can be used as a dietary ingredient to safely reduce Zn supplementation in complete feed, without compromising the Zn status of farm animals. The objective of this study was to study dietary tolerance, bioaccumulation, and evaluate the safety of GLDA when supplemented in broiler diets at 0, 100, 300, 1000, 3000 and 10000 mg/kg. A total of 480 one-day-old Ross 308 male broilers were randomly allocated to 48 pens and fed one of the six experimental diets. Production performance was used to assess tolerance to the additive. At trial end, toxicity was evaluated using haematology, plasma biochemistry (N=144) and gross necropsy (N=48). Residue levels of GLDA were assessed in liver, kidney and breast tissue of birds used for necropsy. Performance results showed a positive significant effect ($P<0.05$) on body weight for GLDA inclusion at 300 mg/kg. A negative effect on the measured performance parameters was found for the 10000 mg/kg GLDA inclusion level ($P<0.05$). The additive is added as a tetra-sodium salt, leading to sodium levels being 2.5 times higher in this treatment compared to the control diet which could have led to impaired intestinal barrier function. Mortality showed no difference between treatments. Residue levels for GLDA at the highest inclusion indicates that 0.0005% of total GLDA consumption is accumulated in breast tissue. Marginally higher values of GLDA were found in kidney and liver at the highest inclusion level, potentially confirming that the small fraction of GLDA absorbed is readily excreted by the animal. At 100 and 300 mg/kg GLDA inclusion there were negligible amounts of GLDA present in tissue. The present experiment proved a high dietary tolerance to GLDA in broilers and indicated that GLDA poses no significant risk to food safety when supplemented below 3000 mg/kg.

4.1 Introduction

Trace minerals, such as zinc (Zn) and copper (Cu), are vital nutrients to ensure human and animal health (Richards et al., 2010; Rink, 2011). Zinc for example serves as a cofactor for over 300 enzymes and 2000 transcription factors. Minerals can be absorbed from any portion of the gastrointestinal tract but are mainly absorbed in the small intestines (Rink, 2011; Svihus, 2014; Yu et al., 2017). Many factors may influence the absorption of minerals, such as dietary antagonists (phytic acid), dietary levels of the minerals, interactions between different minerals within the gastrointestinal-tract and even the interaction of minerals with the microbiota (Brugger and Windisch, 2017; Brugger and Windisch, 2019; Humer et al., 2015). The chemical form in which Zn is supplemented to diets also directly defines its bioavailability in a complete diet. In animal systems, Zn is often supplemented above the requirements to compensate for the uncertain bioavailability defined by the factors described above. This practice results in a low relative efficiency of Zn utilization, while producing a higher Zn excretion into animal manures (Weigand and Kirchgessner, 1980). This higher excretion of Zn and other excessively supplemented trace minerals is a factor of environmental pollution due to animal production (Brugger and Windisch, 2015; Brugger and Windisch, 2019).

Single strong chelating agents in diets are able to increase mineral bioavailability. Chelating agents comprise of molecules with a high affinity to bind trace elements and keep them in solution. During digestion, the formation of this stable complex in the upper gastrointestinal tract minimizes the formation of insoluble complexes, resulting in a preservation of nutritional bioavailability (Krezel and Maret, 2016). Chelators such as ethylenediaminetetraacetic acid (EDTA) have been used in both human and animal diets to increase bioavailability of minerals (Heimbach et al., 2000; Hurrell et al., 2000; MacPhail et al., 1994). In humans, iron EDTA has shown to have a beneficial effect on iron status (Davidsson et al., 1994; Heimbach et al., 2000). A novel chelator, L-glutamic acid N,N-diacetic acid (GLDA) has shown to increase the nutritional availability of Zn in broilers. In this way, it can be used as a dietary ingredient to safely reduce Zn supplementation in complete feed without compromising the Zn status of the animal, contributing to the reduction of environmental trace element pollution of animal production (Boerboom et al., 2020; Boerboom et al., 2021; Kołodyńska, 2011; Wu et al., 2015). In contrast with other chelators such as EDTA, GLDA is readily biodegradable in the environment presenting a low persistence in soils and surface waters. EDTA has shown adverse effects at higher concentrations, showing induction of oxidative stress, tissue injury and disruption of tight junction and membrane integrity (Prachayasittikul et al., 2007). It is understood that the high chelation strength of EDTA affects the metal ions in the outer membrane, resulting in lipopolysaccharide and protein dissociation. Whether GLDA, being a strong chelator as well, would present such adverse effects and at which dietary concentration remains unclear. The current experiment aims to evaluate tolerance and safety of the use of GLDA by inclusion in broiler diets at incremental levels up to a dose of 100-fold of the lowest recommended dosed as used in the previous study (Boerboom et al., 2021). In addition, the bioaccumulation of GLDA in different edible tissues was determined to address potential food safety. The hypothesis was that no negative effects on broiler performance or increase in bioaccumulation of GLDA would be observed within the range of levels studied.

4.2 Material and Methods

The implementation of the trial and experimental design was defined in accordance with the technical guidance, as stipulated by European Feed Safety Association Panel on Additives and Products or Substances used in Animal Feed (Additives and Feed, 2011). The experiment was performed in compliance with the Dutch legislation for animal experiments, and the protocol was approved by the Committee for Animal Experiments of Wageningen University and Research, The Netherlands.

4.2.1 Animals

This study was conducted in the facilities of Wageningen Bioveterinary Research, Building 161, Lelystad, The Netherlands, using 480 one-day-old Ross 308 male broilers. A total of 48 pens (0.75m^2) bedded with wood shavings were used in the experiment. Each pen contained one feeder bin and a drinking line with two drink cups providing water and feed ad libitum. Each pen had 10

broilers from day 0 to 35. Six dietary treatments were randomly allocated to the pens within blocks to include environmental factors into a statistical block factor. At day 10, three birds of each pen were randomly selected to be used for representative sampling of blood and tissues at study end.

4.2.2 Diets

Dietary treatments consisted of six incremental levels of GLDA (0, 100, 300, 1000, 3000, 10000 mg/kg of GLDA, i.e. 0, 333, 999, 3330, 9990 and 33300 mg/kg of GLDA-silica premix (30% GLDA) (Trouw Nutrition, Amersfoort, The Netherlands) and a fixed dose of supplemental Zn (100 mg/kg of Zn from $ZnSO_4.H_2O$). The experimental diets were fed from day 0 to 35 of age. A corn-soybean meal-based diet was formulated to fulfil all standard nutritional requirements (NRC, 1994) (Table 4.1). One basal meal was prepared and subdivided for the production of the treatments specific diet. All diets were supplemented with 100 mg/kg Zn (from $ZnSO_4.H_2O$), 10 mg/kg of Cu (from $CuSO_4.H_2O$), 100 mg/kg of manganese (from MnO) and 120 mg/kg of iron (Fe) (from $FeSO_4.H_2O$). The diet was subdivided in two feeding phases, the starter from day 0 to 10 and the grower from day 11 to day 35 of age. All experimental diets were formulated and produced by Research Diet Services B.V., Wijk bij Duurstede, the Netherlands.

Table 4.1. Ingredient composition of the basal mixture for the experimental diets.

Ingredients, g/kg	Starter	Grower
Corn	579	605
Soybean meal	345	310
Oil (veg.)	36.5	52.5
L-Lysine	0.7	1.4
DL-Methionine	1.9	2.0
L-Threonine	0.0	0.3
Limestone	14.0	11.0
Mono-calcium phosphate	13.0	8.5
Salt	2.5	2.5
$NaHCO_3$	2.5	2.1
Premixture[1]	5.0	5.0
Total	1000	1000
Metabolizable energy broilers (CVB) (MJ/kg)	11.9	12.6
Crude protein (g/kg)	217	203
Fat (g/kg)	61	78
CF (g/kg)	25	24
Starch (g/kg)	382	396
Ca (g/kg)	9.0	7.0
Phosphorus tot (g/kg)	6.3	5.2
Phosphorus dig. (g/kg)	3.6	2.7
Magnesium (g/kg)	1.7	1.5
Potassium (g/kg)	9.7	9.0
Sodium (g/kg)	1.7	1.6
Chlorine (g/kg)	2.0	2.2
Lysine (g/kg)	10.5	10.2
Methionine (g/kg)	4.8	4.7
Methionine+Cysteine (g/kg)	7.7	7.4
Threonine (g/kg)	6.8	6.6
Tryptophan (g/kg)	2.2	2.0
D-arginine (g/kg)	13.0	12.0

[1]Supplemented vitamin and mineral levels per kg feed based on a 0.5% inclusion level of premixture: 12000 IU vitamin A, 2400 IU vitamin D3, 50 mg vitamin E, 1.5 mg vitamin K3, 2 mg vitamin B1, 7.5 mg vitamin B2, 35 mg niacin amide (vitamin B3), 12 mg d-pantothenic acid (vitamin B5), 3.5 mg vitamin B6, 0.2 mg biotin, 20 µg vitamin B12, 1 mg folic acid, 460 mg choline chloride, 0.4 mg Co (as $CoSO_4.7H_2O$), 0.8 mg I (as KI), 0.15 mg Se (as Na_2SeO_3) and 125 mg anti-oxidant. 120 mg Fe (as $FeSO_4.H_2O$), 10 mg Cu (as $CuSO_4.5H_2O$), 100 mg Zn (as $ZnSO_4.H_2O$) and 100 mg Mn (as MnO) were added.

4.2.3 Diet analysis

Samples of all the basal meals were analysed for moisture (EC No 152/2009), ash (EC No 152/2009), protein (ISO 5983-2-2009), crude fat (EC No 152/2009 method B), crude fibre (KWAWRC equivalent to NEN-EN ISO 6865), and trace minerals (Zn, Cu, Mn, Fe) (AAS in following NEN-EN ISO 6869) (Pre Mervo, Utrecht, The Netherlands). The experimental diets were tested for dry matter, Zn and GLDA. GLDA content was measured in duplicate by using liquid chromatography-mass spectrometry (LC-MS) (Masterlab B.V., Boxmeer, The Netherlands). GLDA was quantitatively water extracted from ground feed samples. The extracted GLDA and extracted components from the experimental diets were separated applying a reversed phase liquid chromatography, using an Alltima C18 AQ 3 µm column as stationary phase and 0.2 % tri-fluoroacetic acid in water as mobile phase. The GLDA molecule was detected at m/z 264.070 using a Triple Quadrupole LC-MS mass spectrometer. GLDA levels in feed samples were calculated using a GLDA standard curve based on a calibrated GLDA standard.

4.2.4 Measurements

This tolerance study measured performance parameters, including body weight (BW), feed intake (FI), body weight gain (BWG), daily weight gain (DWG) and feed conversion ratio (FCR), at 0, 10 and 35 days of age. In the case of mortality, the cause of death was determined by necropsy. At the end of the study, at day 35 of age, the three marked birds were used to collect five blood samples from each bird, taken from the wing veins: 2x serum tube, 1 EDTA tube, 1 NaF tube and 1x Zn-free tube. The Zn-free tube containing blood was centrifuged immediately after sampling and aliquoted in coded cryotubes (Centrifuge Centra CL3r, serial number 37560759, serum tubes 3000 rpm/1800 rcf 8 minutes, 18°C, heparin blood tubes 3000 rpm/1800 rcf 4 minutes 4°C). The cryotubes were stored frozen at -80°C until shipment. Serum samples were sent frozen in a cool box (with dry ice) to the Glasgow Royal Infirmary, Glasgow, United Kingdom, and analysed for Zn, Cu, Mn and Fe content. Samples were centrifuged initially because precipitate was visually evident in several of the samples. Then samples were diluted 1:10 with 0.1% EDTA, 0.1% Triton X, 2% butan-1-ol, 1% ammonia and 50 µg/L germanium & scandium as internal standards. Analysis was done by inductively couple plasma mass spectrometry using a helium reaction cell. The remaining blood samples were analysed by the Animal Health Service (GD), Deventer, The Netherlands, for general haematology assessment and routine blood chemistry including: white blood cells, red blood cells, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, differential leucocyte count (haematology analyser), albumin, gamma-glutamyl transferase, aspartate aminotransferase, alanine aminotransferase, urea, total protein, glucose, alkaline phosphatase, lactate dehydrogenase, albumin/globulin, creatinine, calcium, phosphorus, total bilirubin (Analyser−UV/VIS), chlorine, sodium and potassium (ISE) (Additives and Feed, 2011). On day 35, one bird from each pen was randomly selected for gross necropsy. Birds were humanely slaughtered by T61 (an aqueous solution containing (in mg per ml) embutramide, 200 mg/ml; mebezoniumiodide, 50 mg/ml; tetracainehydrochloride, 5 mg/ml) via

wing vein injection. General necropsy reports had special focus on kidney and liver abnormalities. Samples of breast muscle, liver and kidney were taken for GLDA content analysis (from one bird from each pen receiving 100, 300 and 10000 mg/kg GLDA). These samples were frozen at -18^{o}C until further analysis. The frozen organ tissue was cut into three equal pieces using a surgical blade knife. From each three sub-samples approximately 0.5 - 1.0 g tissue was dissected and cut into smaller pieces. 0.5g of tissue (weight was recorded to the nearest 0.01 g) was then transferred into a 2 ml volume micro-centrifuge tube containing ± 0.5 g ceramic beads (1.4 mm) and 1.0 ml of dimethyl sulfoxide. The mixture was vigorously agitated using a Magna Lyser for 3 x 20 seconds at 6000 rpm velocity. After cell disruption the mixture was centrifuged for 10 minutes at 17000 rpm/26810 rcf. The supernatants of the three sub-samples were collected and pooled to obtain one cell-free extract per tissue sample. For analysis of GLDA in a cell-free extract, GLDA was separated applying reversed phase liquid chromatography, using a Synnergy Polar C18 4 µm column as stationary phase and 0.2 % trifluoroacetic acid in water as mobile phase. The GLDA molecules were detected at m/z 264.07 using a TSQ Endura Triple Quadrupole Mass Spectrometer with turbo pump and rotary pre-vacuum pump, fitted with a Heated Electro Spray Ionisation probe. GLDA levels were calculated against standard curves of GLDA, prepared from calibrated GLDA standards. The limit of detection and limit of quantification values for GLDA were determined at 0.5/1.0 µg/kg respectively. The developed LC-MS method was therefore considered sufficiently sensitive to measure relevant levels of GLDA.

4.2.5 Statistical analysis

Data were analysed using SAS Studio (SAS institute Inc., Cary, NC). Outliers were checked using the influence statement within the procedures applied. Performance data, serum minerals, GLDA residues in body tissues and blood characteristics were analysed using the MIXED procedure. GLDA dose was the main effect with block as random effect and time (if applicable) as a repeated effect. Pen was considered the statistical unit.

$$Y = \mu + GLDA + Block + Error$$

Y = Response parameter,

µ = General mean,

GLDA = effect of GLDA,

Block = Effect of block and,

Error = Error term

Main effects with $P<0.05$ were considered to be statistically significant and a P-value between 0.05 and 0.10 was considered a trend. Linear and quadratic effects of GLDA were also determined using the MIXED procedure.

4.3 Results

GLDA recoveries in the starter diets for 100, 300, 1000, 3000 and 10000 mg/kg were slightly below dosing, representing 87, 94, 95, 92 and 89% of the dose respectively. Similarly, GLDA recoveries in the grower diets for 100, 300, 1000, 3000 and 10000 mg/kg was 91, 95, 91, 92 and 98% respectively. The results of the proximate analysis of the starter and grower diet met the calculated contents (90-110% of expected values). Analysed Zn content was lower than calculated, but in a consistent way across treatments (about 70% of calculated values).

Performance results showed that GLDA presence in feed resulted in effects on bodyweight, growth, feed efficiency, feed intake and daily weight gain in the total period and in the grower period ($P<0.05$) (Table 4.2). Feed intake in the starter period (0-10 days) showed a trend towards an effect of GLDA ($P<0.10$). Quadratic responses were present for the grower and total period for all parameters but feed intake ($P<0.05$). GLDA inclusion at 1000 and 3000 mg/kg showed an improvement in FCR over the entire period compared to control, with GLDA at 1000 mg/kg showing an improvement in FCR in the starter phase as well ($P<0.05$). Inclusion of GLDA at the highest dose (10000 mg/kg) led to a negative effect on all measured performance parameters aside from FCR in the starter phase ($P<0.05$).

Table 4.2 Effect of dietary L-glutamic acid N,N-diacetic acid (GLDA) on growth performance of broilers from Day 0 to 35.

GLDA, mg/kg	0	100	300	1000	3000	10000	SEM	GLDA	Linear	Quadratic
Bodyweight, d0	44.1	44.3	44.0	44.2	44.2	44.1	0.04	0.36	0.26	0.25
Bodyweight, d10	291	293	289	290	287	277*	1.7	0.11	0.47	0.95
Bodyweight, d35	2338	2348	2439*	2377	2374	2044*	23	<.0001	0.32	0.01
Bodyweight gain, 0-10d	246	248	245	246	242	233*	2.0	0.11	0.46	0.93
Bodyweight gain, 10-35d	2048	2056	2150*	2086	2087	1767*	22	<.0001	0.26	0.01
Bodyweight gain, 0-35d	2294	2304	2395*	2333	2330	2000*	23	<.0001	0.32	0.01
Feed conversion ratio, 0-10d	1.13	1.11	1.10*	1.10*	1.11#	1.11	0.004	0.27	0.34	0.33
Feed conversion ratio, 10-35d	1.48	1.49	1.47	1.46	1.45*	1.53*	0.005	<.0001	<0.01	<.0001
Feed conversion ratio, 0-35d	1.44	1.45	1.43	1.42*	1.41*	1.48*	0.004	<.0001	<0.01	<.0001
Feed intake, 0-10d	278	275	269	270	268	259*	2.0	0.09	0.23	0.53
Feed intake, 10-35d	3026	3064	3151#	3046	3021	2702*	27	<.0001	0.74	0.23
Feed intake, 0-35d	3304	3339	3420#	3316	3289	2961*	28	<.0001	0.66	0.27
Daily weight gain, 0-10d	24.6	24.8	24.5	24.6	24.2	23.3*	0.17	0.11	0.46	0.93
Daily weight gain, 10-35d	81.9	82.2	86.0*	83.5	83.5	70.7*	0.88	<.0001	0.26	0.01
Daily weight gain, 0-35d	65.5	65.8	68.4*	66.7	66.6	57.1*	0.66	<.0001	0.32	0.01

* indicates significant difference compared to the control (P<0.05)
\# indicates trend compared to control (P0.05-0.10)

Serum mineral levels showed that GLDA presence in feed resulted in an effect on Zn levels only (P<0.05) (Table 4.3). GLDA inclusion at 10000 mg/kg tended to increase serum Cu (P<0.10), without a main effect of GLDA or linear/quadratic effects. Serum Fe levels showed a trend to behave quadratically (P<0.10), showing a numerical decrease in the intermediate level.

Table 4.3 Least square mean of serum mineral concentration of broilers receiving L-glutamic acid N,N-diacetic-acid (GLDA) at increasing levels from day 0 to 35

GLDA, mg/kg	0	100	300	1000	3000	10000	SEM	GLDA	Linear	Quadratic
Serum Zn	1391	1511	1512	1565	1620	1926*	36	<.0001	0.09	0.63
Serum Cu	96	100	106	104	87	135#	6.1	0.31	0.38	0.19
Serum Fe	1.31	1.38	1.29	1.21	1.24	1.37	0.03	0.29	0.11	0.08
Serum Mn	16.0	14.7	14.0	11.8	18.0	15.0	1.3	0.85	0.54	0.56

* indicates significant difference compared to the control (P<0.05)
# indicates trend compared to control (P0.05-0.10)

Haematology assessment indicated an effect of GLDA presence on erythrocytes, haemoglobin, mean corpuscular haemoglobin, haematocrit, bilirubin, alkaline phosphatase, γ-glutamyl transferase, total protein, albumin and albumin/globulin ratio (P<0.05) (Table 4.4). The response was quadratic for erythrocytes, mean corpuscular haemoglobin, bilirubin, lymphocytes, sodium, alanine aminotransferase, lactate dehydrogenase, total protein and glucose. Sodium and chlorine levels were elevated in broilers receiving the experimental diet containing GLDA at 10000 mg/kg compared to broilers receiving the control diets. Alkaline phosphatase activity was reduced in broilers receiving the experimental diets containing GLDA at levels above 300 mg/kg compared to the broilers receiving the control diet. No differences were present in total mortality and the cause of death showed no specific pattern related to the GLDA dosages administered (Table 4.5).

Table 4.4 Effect of dietary L-glutamic acid N,N-diacetic acid (GLDA) inclusion at incremental levels on general haematology assessment and blood chemistry

GLDA, mg/kg	reference value[1]	0	100	300	1000	3000	10000	SEM	GLDA	Linear	Quadratic
Haematology											
Erythrocytes count, 10^12/L	2.3-3.0	2.5	2.6	2.5	2.4	2.5	3.0*	0.04	<0.001	0.38	0.05
Haemoglobin, mmol/L	2.0-5.7	4.5	4.7	4.5	4.5	4.7	5.2*	0.06	0.001	0.50	0.71
Mean corpuscular haemoglobin, fmol	1.7-3.4	1.8	1.8	1.8	1.8	1.9*	1.7	0.01	0.006	0.001	0.0003
Mean corpuscular volume, fL	120-130	123	125	125	125	125	125	0.3	0.17	0.40	0.40
Haematocrit, L/L	0.31-0.55	0.31	0.32	0.31	0.31	0.31	0.37*	0.005	0.001	0.44	0.07
Bilirubin, µmol/L	4.5-9.2	2.3	2.5#	2.2	2.2	2.1	2.3	0.03	0.02	0.02	0.02
Leucocyte count, 10^9/L	20.0-24.0	26.7	24.5	27.4	26.2	23.4	30.6	0.76	0.11	0.15	0.06
Heterophil granulocytes, 10^9/L	6.3-6.5	11.9	11.1	11.8	12.3	10.6	14.7*	0.42	0.10	0.33	0.12
Lymphocytes, 10^9/L	13.9-16.1	13.9	13.0	14.1	13.2	11.5	15.5	0.51	0.31	0.09	0.05
Monocytes, 10^9/L	1.2-1.4	0.88	0.83	1.22	1.18	1.30	1.87*	0.12	0.17	0.35	0.71
Electrolytes											
Chlorine, mmol/L	100-112	111	111	110	110	110	113*	0.33	0.07	0.63	0.24
Calcium, mmol/L	2.0-4.5	2.6	2.6	2.5	2.5	2.5	2.6	0.02	0.69	0.36	0.32
Phosphate, mmol/L	0.65-1.45	3.2	3.0	3.1	2.9#	3.0	3.2	0.04	0.35	0.32	0.20
Potassium, mmol/L	3-5	11.4	10.4	11.6	9.9#	10.7	11.0	0.26	0.39	0.40	0.38
Sodium, mmol/L	140-160	149	151	149	149	150	156*	0.5	<.0001	0.40	0.01
Enzymes											
ALP, IU/L	1884-5822	12700	10776	9669*	7993*	9566*	6724*	486	0.004	0.16	0.43
ALT, IU/L	6-7	5.8	5.6	5.6	6.0	6.4	5.7	0.13	0.51	0.05	0.05
AAT, IU/L	179-330	418	387	431	391	456	371	15.5	0.60	0.29	0.21
GGT, IU,L	16-46	20.4	21.2	18.4	19.5	17.8#	23.6*	0.47	0.001	0.02	0.003
LDH, IU/L	378-416	4575	2892	3333	2806	7761#	3614	530	0.05	0.01	0.01
Metabolites											
Total protein, g/L	27-36	33.3	35.8#	33.6	32.8	33.0	38.7*	0.46	<.0001	0.08	0.007
Albumin, g/L	14-20	15.3	16.4*	15.5	15.2	15.7	17.9*	0.21	<.0001	0.54	0.10
Albumin/Globulin Ratio	0.78-0.92	0.85	0.85	0.87	0.87	0.91*	0.87	0.005	0.002	<.0001	<.0001
Urea (mmol/L)	0.3-2.5	<2	<2	<2	<2	<2	<2	*n.a.*	*n.a.*	*n.a.*	*n.a.*
Glucose, mmol/L	11.1-25.0	29.4	29.8	28.8	29.0	32.4	25.8	0.72	0.22	0.11	0.05
Creatinine, µmol/L	22-57	23.8	17.5#	17.4#	20.1	25.3	21.9	1.19	0.15	0.11	0.14

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AAT, aspartate aminotransferase; GGT, γ-glutamyl transferase; LDH, lactate dehydrogenase. * indicates significance compared to the control (P<0.05) # indicates trend compared to control (P0.05-0.10) [1] (Al-Hussary and Kudair, 2010; Andretta et al., 2012; Igene et al., 2012; Kalmar et al., 2012; Piotrowska et al., 2011; Rezende et al., 2017; Silva et al., 2007; Talebi et al., 2005)
n.a.: not available.

Table 4.5. Causes of mortality (number of animals per disease)

GLDA, mg/kg	0	100	300	1000	3000	10000
Yolk sac inflammation	-	-	1	1	-	-
Broken femur head	-	-	1	1	-	-
Enlarged liver and liver rupture	-	-	1	-	-	-
Proventricular dilatation, intestinal disorder	-	1	1	-	1	-
Intestinal disorder	4	1	1	1	2	-
Polyserositis	3	2	-	-	-	1
Sudden Death Syndrome	1	-	-	-	-	-
Pericarditis	-	-	-	-	1	1
No abnormalities detected	-	-	2	2	1	2
Autolyse, no diagnosis could be determined	-	-	-	1	-	-
Total	8	4	7	6	5	4

The necropsy results indicated a decrease in live weight of the necropsied broilers receiving GLDA at a concentration of 10000 mg/kg compared to birds receiving the control diets (Table 4.6). Kidney weight, expressed as a percentage of liveweight, was higher in birds receiving GLDA at 10000 mg/kg compared to the control.

Table 4.6. Least square mean of tissues of broilers receiving L-glutamic acid N,N-diacetic-acid (GLDA) at increasing levels from day 0 to 35

GLDA, mg/kg	0	100	300	1000	3000	10000	SEM	GLDA	Linear	Quadratic
Body weight d35, g	2214	2316	2407#	2366	2272	1988*	33.4	0.002	0.82	0.27
Liver, g	48.9	51.9	49.8	52.3	48.5	44.9	0.87	0.15	0.73	0.86
Liver, % of body weight	2.21	2.24	2.07	2.23	2.13	2.28	0.04	0.64	0.58	0.46
Kidney, g	15.2	15.0	16.5	16.7#	16.2	15.6	0.26	0.30	0.21	0.19
Kidney, % of body weight	0.68	0.65	0.69	0.70	0.70	0.79*	0.01	0.005	0.17	0.59

* indicates significance compared to the control (P<0.05)
\# indicates trend compared to control (P=0.05-0.10)

An increase in GLDA residues could be observed in tissues of birds receiving the experimental diets containing 10000 mg/kg of GLDA compared to the tissues of birds receiving 100 and 300 mg/kg of GLDA (Table 4.7). GLDA residue levels observed in the tissue of birds receiving 100 and 300 mg/kg of GLDA were low (<0.14 mg/kg).

Table 4.7. Effect of dietary L-glutamic acid N,N-diacetic acid (GLDA) inclusion at incremental levels on GLDA residues in broiler tissues at 35 days of age.

Tissue	GLDA inclusion, mg/kg	Average GLDA residue level, mg/kg
Breast meat	100	$0.008^{A} \pm 0.003$
	300	$0.021^{A} \pm 0.008$
	10000	$0.378^{B} \pm 0.102$
Liver	100	$0.010^{A} \pm 0.002$
	300	$0.018^{A} \pm 0.004$
	10000	$1.35^{B} \pm 0.12$
Kidney	100	$0.100^{A} \pm 0.019$
	300	$0.131^{A} \pm 0.018$
	10000	$3.99^{B} \pm 0.37$

Superscripts indicate significant differences ($P<0.05$)

4.4 Discussion

Analysed Zn levels of the different diets showed a lower Zn level than what was calculated, being around 70% of expected values. The different diets did not show large differences between Zn levels which indicates that the Zn levels within the premix were likely lower than dosed. Since the bias in Zn content was consistent across all diets, it does not impair the contrast of the hypothesis.

Dietary supplementation of GLDA dosed up to a level of 3000 mg/kg showed no negative effects on any of the performance parameters measured. Growth performance of the birds in this study was above the Ross 308 guidelines (Aviagen, 2014). Dietary supplementation of 300 mg/kg of GLDA indicated an improved final body weight (Day 35, $P<0.05$). This was unexpected as trace mineral levels in the feed were higher than the levels required for adequate growth, even though they were lower than calculated (Mohanna and Nys, 1999; Schlegel et al., 2010). It indicates that GLDA inclusion in a diet may improve performance of broilers even when minerals supply is assumed as adequate. In a similar way, the current experiment showed an improved FCR with GLDA inclusion of 1000 and 3000 mg/kg ($P<0.05$).

On the other hand, dietary supplementation of 10000 mg/kg of GLDA reduced performance in the grower period as compared to the negative control and below the Ross 308 performance objectives (BW, DWG and FCR) (Table 4.2) (Aviagen, 2014). This may unequivocally be the result of intolerance to dietary GLDA supply. The product was included in the diet as a tetra-sodium salt and the subtotal mass percentage of sodium within GLDA-Na_4 is 26.19%. Feeding the GLDA molecule at 10000 mg/kg led to an additional 2619 mg/kg of sodium in this dietary treatment i.e. a level 2.5 times higher level compared to the control. This resulted in higher levels of sodium in blood at the highest inclusion level compared to the control (Table 4.4).

In addition, a higher amount of Zn in serum was detected in birds receiving the 10000 mg/kg of GLDA. Increased trace metal permeability in the presence of a chelator has been associated to loss of gut barrier function. In this light, higher serum Zn could be indicative of impaired intestinal barrier function, also potentially resulting from the elevated dietary sodium (Baloš et al., 2016; Tanaka and Itoh, 2019). As an alternative explanation, increased gut permeability could be explained by the chelating agent itself. Strong chelators, such as EDTA, have been described to destabilize membranes when present at high concentrations (Banin et al., 2006; Prachayasittikul et al., 2007). This seems to happen by intercalation between EDTA and the phospholipid molecules through salt bridge formation, by reaction with the polar head of phospholipids. This can mechanically stress the bilayer phospholipid organization leading to membrane disruption and leakage (Prachayasittikul et al., 2007). Considering the much higher chelation strength towards Zn of EDTA compared to GLDA (log $K_{16.5}$ vs log K_{10}) it is more plausible that the increased permeability results from increased osmolality due to high dietary sodium. Mortality numbers show no increase in broilers receiving the experimental diets containing 10000 mg/kg GLDA, indicating that while this level was inadequate for health or performance, it still was a tolerable dose.

Inclusion of GLDA did not affect serum levels of Mn and Fe, in accordance with previous findings (Boerboom et al., 2020; Boerboom et al., 2021; Kołodyńska, 2011; Wu et al., 2015). Copper levels when GLDA was fed at 10000 mg/kg showed an elevated trend compared to the control, which can be explained by the gut integrity effects described above, considering the high affinity of GLDA for Cu (13.1) (Kołodyńska, 2013). The absence of effects at serum level for these minerals is in line with expectations, because all diets contained nutritionally adequate levels for all minerals and as such, chelation is not expected to result in differences in absorption despite of any difference in availability due to down-regulation of absorption (Mondal et al., 2010).

Surprisingly, dietary inclusion of GLDA at 3000 mg/kg increased the albumin to globulin ratio in comparison to the control broilers, while still being within the reference values (Rezende et al., 2017). Total protein and albumin itself were not affected by this treatment and therefore it can be concluded that the difference in albumin to globulin ratio was not the result of increased inflammatory processes (Rezende et al., 2017). Alkaline phosphatase activity was lower when GLDA was included at all levels higher than 300 mg/kg. This is inconsistent with the increased serum levels of Zn observed at higher levels of GLDA, because alkaline phosphatase has been described to correlate with serum Zn levels (Al-Daraji and Amen, 2011; Amen and Al-Daraji, 2011). Across treatments, potassium levels were higher than reference values and this might indicate pseudo hyperkalaemia. This is typically caused by haemolysis during venepuncture and it is a laboratory artefact rather than a biological abnormality (Sevastos et al., 2006). Heterophil granulocyte levels were elevated compared to reference values in all broilers and elevated when feeding GLDA at 10000 mg/kg (Andretta et al., 2012). Heterophil granulocytes are part of the innate immune system and are the first line of defence against pathogenic infections, which is in line with the hypothesis of increased gut permeability (Bojesen et al., 2004). The mortality

numbers and performance results however do not show any indication of decreased performance or disease pressure.

Necropsy detected no hepatic changes. However, kidney weight, expressed as a percentage of live weight, showed an increase in birds receiving 10000 mg/kg of GLDA at 10000 mg/kg ($P<0.05$). This effect could also result from the levels of sodium in this diet due to GLDA being supplied as a tetrasodium salt. Increases in kidney size has been reported in literature in association to excessive dietary levels of sodium (Mushtaq et al., 2014). As a reference, the absorption of EDTA in human subjects is described to be around 5% and the pharmacokinetics are similar in experimental animals as compared to humans (Heimbach et al., 2000). Studies in rats have shown that most of the ingested EDTA was not absorbed and the fraction that was absorbed was to a large extent excreted through the urine (Foreman et al., 1953; Heimbach et al., 2000). The total lifetime consumption of GLDA in the current study, when included at 100, 300 or 10000 mg/kg, would be 352, 1056 and 35200 mg of GLDA respectively. The fraction of GLDA found in breast tissue is estimated to be 0.01% of total GLDA assumed to be absorbed and is as low as 0.0005% of the bird's total GLDA consumption during the trial period. Taking into account the low toxicity profile of GLDA (Braun et al., 2012), these values are considered as very low and do not pose any safety risk. The higher deposition of GLDA in kidney compared to liver and muscle reflects the renal pattern of excretion, indicating that very likely the small fraction of GLDA that is absorbed is actively excreted by the animal, as shown with EDTA (Foreman et al., 1953; Heimbach et al., 2000). The limited absorption of GLDA indicates that the role of GLDA affecting Zn availability takes place within the gastrointestinal tract of the animal, by sustaining solubility during digestive processes as described for other chelating agents (Krezel and Maret, 2016). The complex of GLDA with a mineral is not expected to be absorbed, it only mediates in the availability of the mineral ion for absorption, relying on active and controlled uptake. Chelation of GLDA is expected to support trace mineral homeostasis of the animal, as active and controlled uptake is downregulated when sufficient minerals are present (Richards et al., 2010; Windisch, 2002).

The lowest no-observed-adverse-effect-level found in animal studies with GLDA is 300 mg/kg body weight per day (ECHA, 2010). When an overall uncertainty factor of 100 is applied, one can derive an acceptable daily intake (ADI) of 3 mg GLDA/kg bodyweight. The exposure to GLDA of consumers of edible chicken tissue derived from birds receiving GLDA was calculated using the theoretical daily human consumption of tissues from birds of the standard food basket i.e. 300 g of breast meat, 100 g of liver and 10 g of kidney and the residues of GLDA (mean value plus 3×SD) detected in edible tissue at 10000 mg/kg GLDA inclusion (Additives and Feed, 2012). This results in a theoretical intake of GLDA of 0.007 mg/kg body weight per day in a 60-kg adult which is only 0.24% of the ADI (3 mg GLDA/kg bodyweight). The consumption of edible tissue from chickens fed GLDA at 100 times the lowest recommended level of 100 mg GLDA/kg complete feed would result in an exposure to GLDA 416-fold lower than the ADI and therefore, in daily practice at much lower dosing levels, it is not likely to pose a safety concern for the consumer.

4.5 Conclusion

In conclusion the data indicate that dietary GLDA inclusion up to 3000 mg/kg did not result in any adverse effects on performance, haematology and plasma chemistry. The inclusion of GLDA at 10000 mg/kg resulted in a reduction in performance, most likely due to compromised barrier function, potentially explained by a combination of direct effects of the chelator and the high sodium load present in the salt form administered. Necropsy results however did not show any pathological changes or indications of severe adverse health effects in any of the inclusion levels. Moreover, GLDA residue levels showed low levels (<0.01%) in breast tissue even when dosed at 100 times the (lowest) recommended dose, e.g. 10000 mg/kg. The residue levels in liver and kidney indicated that the small fraction of GLDA that is absorbed is actively excreted. The residue results indicate that GLDA supports the active transcellular transport system of trace minerals, thereby supporting trace mineral homeostasis. The present study reveals a high level of tolerance and safety for GLDA in the broilers and demonstrates no consumer risk of inadvertently increased GLDA intake through consumption of chicken meat or liver from poultry supplemented at a dose of up to 3000 mg/kg of GLDA/kg feed.

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Chapter 5

Dietary L-glutamic acid N,N-diacetic acid affects short term zinc homeostasis in weaned piglets at different levels of dietary zinc

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To be submitted

Abstract

Subclinical zinc deficiency is common in malnourished humans and can occur in farm animals. A novel chelator L-glutamic acid N,N-diacetic acid (GLDA) can be used as a dietary component to improve zinc availability in cereal-based diets. The present study aimed at testing the hypothesis that GLDA is able to increase the Zn retention from phytate-rich-diets and, consequently, allows a reduction of the necessary supplementation with Zn sulphate to complete feed. A total of 96 piglets were divided over two runs, with 16 dietary treatments. Animals were fed a zinc adequate diet during a 2-week acclimatization period. During a total experimental period of 8 days, all piglets were fed restrictively (450 grams/day) a diet containing added zinc at 0, 5, 10, 15, 20, 25, 45, 75 mg/kg with and without 200 mg/kg of GLDA. Faecal, blood, liver and bone samples were collected. No animals showed signs of clinical Zn deficiency and no phenotypical differences were observed between feeding groups. Broken line analysis of the response to dose, indicated that the gross Zn requirement threshold was around 55 mg/kg of zinc. Supplementation of zinc above this threshold lead to a saturation of the response in apparently digested feed zinc and an increase in liver zinc. Bone and serum zinc responded to the dose in a linear fashion, most likely due to the timeframe of zinc homeostatic adaptation. Inclusion of GLDA into the diets yielded higher intercepts in the respective zinc response patterns. Responses to GLDA inclusion at the lowest zinc dose indicated the ability of GLDA to protect a significant fraction of soluble luminal zinc from being lost to phytic acid complexation. Liver zinc accumulation was higher for piglets receiving GLDA, indicating a higher zinc influx. Taken together, GLDA can be used to mitigate negative effects of phytate content in plant-based diets, by sustaining Zn solubility, thereby improving nutritional Zn availability.

5.1 Introduction

Zinc (Zn) is an essential nutrient for humans and animals due to its involvement in structural, catalytic, and regulatory processes (López-Alonso, 2012; Nielsen, 2012; Richards et al., 2010). To fulfil the biological zinc requirements, humans and animals need to consume a certain level of bioavailable zinc with their diet. The amount of absorbable zinc, consisting of free and loosely bound Zn^{2+} ions, is the result of efficient digestion as well as the interaction with other dietary components (i.e. phytic acid, Ca) (Goff, 2018; Rink, 2011; Suttle, 2010). Zinc deficiency is prevalent in developing regions, especially in countries in which cereals are a large fraction of the diet (Hambidge and Krebs, 2007; Prasad, 2009). It has been estimated that Zn deficiency contributes to 800,000 excess deaths or 28 million daily adjusted life years annually among children under 5 years (Caulfield et al., 2004). Most of the documented Zn deficiency is considered relatively mild, with no clinical signs. The same accounts to pigs and especially weaned piglets. The fact that most pig diets are usually generously supplied with Zn salts makes the occurrence of clinical events of Zn deficiency rather rare (NRC, 2012). However, short-term fluctuations in dietary Zn intake, e.g. within the first days post-weaning, have been associated with subclinical Zn deficiency, which presumably occurs regularly under practical rearing conditions

(Davin et al., 2013). Chelators such as ethylenediaminetetraacetic acid (EDTA) increase bioavailability of minerals in both human and animal diets (Hurrell, 1997; Hurrell et al., 2000; MacPhail et al., 1994). Chelators are organic molecules with a high affinity to form highly stable complexes with a transition metal or metalloid. In this way, the complex sustains the solubility of the metal alongside the upper gastrointestinal tract, by minimizing the formation of insoluble complexes of the associated metal with for example phytic acid (Vohra and Kratzer, 1964; Vohra and Kratzer, 1968; Whittaker and Vanderveen, 1990). This sustained solubility results in the chelator complex delivering the mineral to the site of uptake within the gastrointestinal tract, where metal transporters, having a higher chelation strength than the chelator, are then able to absorb the mineral. A novel dietary chelator, L-glutamic acid N,N-diacetic acid (GLDA) has been shown to increase the nutritional availability of Zn in broilers and can be used as a dietary ingredient to improve the availability of Zn in the gastrointestinal tract, similarly to EDTA (Boerboom et al., 2020; Boerboom et al., 2021). The benefits of GLDA compared to EDTA are its higher biodegradability and its relatively lower chelation strength (10^10 for GLDA versus 10^16.5 for EDTA) (Kołodyńska, 2011; Kołodyńska, 2013). Metal binding proteins such as metallothionein have a stability constant around 11-13, indicating that EDTA, with a stability constant of 16.5, can influence the release of Zn (Kimura and Kambe, 2016; Kochańczyk et al., 2015). This was also observed in studies performed in turkeys (Vohra and Kratzer, 1964).

Weaned piglets are susceptible to dietary Zn deficiency (Suttle, 2010). This is due to their inability to deal with dietary phytate levels without the support of exogenous phytase supplements, a fate they share with humans (Hurrell, 1997; Hurrell et al., 2000; Lopez et al., 2002). During its transfer through the gastrointestinal tract, dietary phytate from cereal-based diets dissociates under the acidic conditions in the stomach. Subsequently, under the neutral conditions in the small intestinal lumen, phytic acid chelates divalent cations forming insoluble and at times almost crystalline complexes. These precipitated forms are not digestible by swine or human endogenous phosphatases. In particular, some complexes, e.g. Ca-Zn-phytates, are especially resistant to phytase digestion. Hence, supplying weaned pigs and monogastric mammals with diets rich in phytic acid, without addition of Zn salts and/or phytase supplements, impedes Zn absorption from the gut. This affects both dietary Zn as well as endogenous secreted Zn. In fact, dietary phytic acid concentrations of ≥ 8 g/kg were associated with zero true Zn absorption in ^{65}Zn-labelled rats and a negative apparent Zn absorption in weaned piglets (Brugger et al., 2014; Windisch and Kirchgessner, 1999). Hence, the weaned piglet is an excellent model for dietary intervention studies in monogastric mammals including humans targeting the improvement of Zn supply to the organism. Brugger et al. have recently developed an experimental model of subclinical Zn deficiency in weaned piglets to facilitate basic and applied research in this very relevant phenotype of Zn malnutrition in humans and animals (Brugger et al., 2014). The model is based on a classic dose-response setup, in which weaned piglets with adequate full body Zn stores are subject to eight days of varying dietary Zn supply, spanning the range from deficient dosages to mild oversupply of a corn-soybean based diet with an average native phytic acid

concentration of 9 g/kg. We demonstrated the model induces no visual signs of Zn deficiency but, at the same time, promotes changes on the metabolic and subcellular level (e.g. decreased pancreatic digestive capacity, reduced cardiac redox capacity, redistribution of Zn pools within the organism) (Brugger and Windisch, 2016; Brugger and Windisch, 2017; Brugger and Windisch, 2019a). Therefore, this approach provides a dietary phenotype suitable to investigate the efficacy of dietary intervention for the mitigation of the effects of subclinical Zn deficiency in monogastric mammals. We applied the model of Brugger et al. to precisely dissect the effects of GLAD on the Zn status of weaned piglets challenged with finely graded differences in dietary Zn supply (Brugger et al., 2014). The present study tested the hypothesis that GLDA is able to increase the Zn retention from phytate-rich-diets and, consequently, allowing a reduction of the necessary supplementation with Zn to complete feeds. Given the high similarity of pigs and humans concerning their nutritional physiology, the present dataset aims to generate information applicable to both species.

5.2 Materials and Methods

This animal study was reviewed and approved by the responsible animal welfare officer of the TUM School of Life Sciences, Technical University of Munich, as well as registered and approved by the responsible animal welfare authorities (district government of Upper Bavaria, federal state of Bavaria (Germany) (case number. 55.22-1-54-2532.3-63-11)). The study was conducted at the experimental metabolic pig unit of the Chair of Animal Nutrition, TUM School of Life Sciences, Technical University of Munich.

5.2.1 Animals and diets

The experimental approach of the present study is an adaption of the subclinical zinc deficiency model originally suggested by Brugger et al (2014). Forty-eight weaned piglets (hybrids of (German Large White x German Landrace) x Piétrain) from 12 litters (50% male-castrated, 50% female, initial average body weight 7.96 ± 1.06 kg, 4 weeks of age) were purchased from a commercial pig operation (Christian Hilgers, Freising (Germany)). All animals were housed in individual pens (equipped with individual feeders and nipple drinkers) during the complete study period and had access to drinking water (tap water) ad libitum. The water supply was regularly checked for the Zn concentration to ensure constant negligible background levels. At d1 of the acclimatization phase, room temperature was set to 30°C and gradually decreased by 1°C per week until the end of the experimental phase. The humidity fluctuated between 45 and 55%. Room temperature and humidity were screened in real-time applying a thermohygrograph drum recorder (Type 252; Lamprecht Meterological Instruments). The light cycle consisted of 12h daylight and 12h crepuscular light during night-time. Since the piglet stable provided space for only half of the experimental animals, the study was conducted in two identical subsequent runs (48 piglets per run). Within each experimental run, all 16 dietary treatments were represented in 3 blocks, following a randomized complete block design approach. Within each block, the treatments were randomly distributed over pens. Within and between experimental runs and

blocks, animals were allocated according to a balanced distribution of live weight, littermates and sex. Within each block, randomization took place within the GLDA factor in sub-block pairs according to sex and litter.

In our present study as well as the earlier work of Brugger et al. (2014) all animals were fed at the highest applied Zn dose during the 14d acclimatization period after which, dietary Zn levels were gradually reduced for the different feeding groups (Brugger et al., 2014). In the beginning of each individual run, all animals were fed a basal diet *ad libitum,* consisting mainly of corn and soybean meal with an adequate dietary Zn supplementation level. Zinc was added as $ZnSO_4 \cdot H_2O$ (analytical grade, 96495, Sigma-Aldrich; added amount of dietary Zn: 75 mg/kg, analysed final concentration of total dietary Zn: 103 mg/kg) during a two-week acclimatization phase, to ensure full body Zn stores at d1 of the experimental phase. During this phase, the diet also contained 200 mg hydrated silica/kg diet, which was the carrier substrate for the test substance (GLDA) and served as a placebo in the control diets during the experimental phase (see details below). Subsequently, animals were assigned to the 16 diets according to the above described complete randomized block design. During a total experimental period of 8 d, all piglets were fed restrictively (450 g/d) the same basal diet as during the acclimatization phase. These feeds contained varying supplementation levels of $ZnSO_4 \cdot H_2O$ (added amount of diet Zn: 0, 5, 10, 15, 20, 25, 45, 75 mg/kg; resulting in analysed final concentrations of dietary Zn: 30.9, 35.7, 40.5, 45.3, 50.8, 55.6, 74.2, 103 mg/kg). These Zn levels were combined with the addition of 200 mg GLDA/kg or 200 mg hydrated silica/kg diet as placebo, respectively; resulting in average analysed final concentrations of GLDA in GLDA supplemented and non-supplemented groups of 198 ± 3.16 mg/kg and <1 mg/kg, respectively.

The highest Zn supplied group (103 mg Zn/kg) without additional GLDA served as positive control because it represented the initial feeding situation for all animals during acclimatization from which the variations of Zn and GLDA levels of all other groups were changed during the 8d experimental phase. The basal diet contained all nutrients according to published feeding recommendations for piglets except for Zn (Table 5.1) (NRC, 2012) including TiO_2 (3 g/kg diet) as indigestible marker for the estimation of apparent total tract digestion of feed Zn.

Table 5.1. Composition, metabolizable energy and crude nutrient contents of the basal diet

Ingredients	Contents, %	Chemical composition	Contents, kg/diet
Corn	42.3	Analysed values	
Soybean meal (40% crude protein)	26.7	Dry matter, g	893
Potato protein	10.3	Crude protein, g	233
Wheat bran	5.14	Crude fat, g	41.1
Sugar beet pulp	3.08	Crude fibre, g	46.4
Premix[1]	5.00	Crude ash, g	65.2
Feeding sugar	2.06	Estimated values[2]	
Soybean oil	1.53	Metabolizable Energy, MJ	13.0
$Ca(H_2PO_4)_2$	1.64	Lysine, g	14.1
$CaCO_3$	1.44	Methionine, g	4.09
NaCl	0.51	Threonine, g	10.4
TiO_2	0.30	Tryptophan, g	2.99

[1] Premix composition: 2.80% MgO; 0.08% CuSO4·5H2O; 2.00% FeSO4·7H2O; 0.20% MnSO4·H2O; 0.002% Na2SeO3 ·5·H2O; 0.002% KI; 0.05% retinyl propionate; 0.007% cholecalciferol; 0.20% all-rac-α-tocopherol; 0.002% menadione; 0.01% thiamin; 0.03% riboflavin; 0.10% nicotinic acid; 0.02% pantothenic acid; 0.02% pyridoxine; 0.15% hydroxocobalamin; 0.03% biotin; 0.002% folic acid; 6.70% choline; 77.6% corn meal.

[2] The contents of metabolizable energy and essential amino acids were estimated according to feed table information (http://datenbank.futtermittel.net/). Vitamin and trace element contents (except zinc) met the requirements according to NRC (NRC, 2012). The corn meal content mixed into the premix as a carrier was a fraction of the 42.3% total corn meal in the basal diet.

All experimental diets were isoenergetic and isonitrogenous and differed only in their total concentrations of Zn, GLDA and hydrated silica, respectively. Each experimental diet was sampled in triplicate and samples were stored in air-tight polyethylene bottles at -20°C and milled through a 0.5 mm screen prior to chemical analysis.

5.2.2 Measurements

Animal individual faecal grab samples were pooled from the last three experimental days, freeze-dried and stored at -20°C. All animals were killed by bleeding under anaesthesia (combination of Azaperone and Ketamine) without fasting after eight experimental days, and blood in Li-Heparin monovettes, as well as liver (*Lobus hepatis sinister lateralis*) and bone (left femoral head) samples were taken. Blood plasma was collected by centrifugation at 1100 g for 10 minutes at 4°C and stored at -20°C until further usage. Liver samples for gene expression analysis were incubated in RNAlater® according to manufacturer instructions (Thermo Scientific) and subsequently stored at -80°C. Bone samples were ashed (470°C) overnight prior to Zn analysis. The workflow and methods applied by (Brugger et al., 2014) were implemented, regarding the chemical analyses in diets, faeces, bone, blood plasma and liver tissue. Dietary parameters included dry matter, crude nutrients, Zn and Titanium. Faecal parameters comprised DM, Zn and TiO2. Bone, blood plasma and liver were subject to the analyses of total Zn. Analyses of DM and crude nutrients followed the standard procedures of the Association of German Agricultural Analytic and Research Institutes (Methods 3.1, 4.1.1, 5.1, 6.1.1, 8.1; (VDLUFA, 2012)). TiO_2 was analysed according to Brandt and Allam (1987). All Zinc concentrations were measured by atomic absorption spectrometry (NovAA 350, Analytik Jena AG) after microwave wet digestion (Ethos 1, MLS GmbH).

Alkaline phosphatase activity in blood plasma was assessed by a commercial kit according to manufacturer instructions. The percentage zinc binding capacity, which represents the percentage free Zn binding sites in blood plasma, was assessed according to Roth and Kirchgessner (Roth and Kirchgessner, 1980). QPCR assay quality control and chemical procedures (total ribonucleic acid (RNA) extraction, reverse transcription (RT), quantitative polymerase chain reaction (qPCR)) were performed according to Brugger et al. (2014). Quantity and purity of the extracts from wet liver tissue were measured on the NanoDrop 2000 (Thermo Scientific) (total RNA quantity: 1436 ± 574 ng/µL, ratio of optical density (OD): OD_{260nm}/OD_{280nm}: 2.04 ± 0.05). Total RNA integrity was assayed by automated capillary gel electrophoresis (Experion, Biorad) (RNA quality index: 6.44 ±0.7). Primer pairs (supplier: Eurofins Scientific) were designed for the potential reference transcripts *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *beta-glucuronidase* (*GUSB*), *beta-actin* (*ACTB*), *β_2 microglobulin* (*B2M*), *histone H3* (*H3*), *hypoxanthine-guanine phosphoribosyl transferase* (*HPRT1*), *lactate dehydrogenase A* (*LDHA*), *transferrin receptor protein 1* (*TFRC*), and *ubiquitin C* (*UBC*) as well as the target transcript *metallothionein 1A* (*MT1A*), using published porcine sequence information (O'Leary et al., 2016) (Primer specifications shown by Brugger et al. (2014). All oligonucleotides bind to homologous regions of respective transcripts to amplify potential transcript variants (O´Leary et al., 2016). We applied the whole Ct dataset (target and potential reference gene measurements) to the online tool RefFinder (Xie et al., 2012), which uses geNorm, Normfinder, BestKeeper and the comparative Delta-Ct method to to compare and rank the tested genes. In this way, we identified *GUSB* and *HPRT1* as suitable reference genes for data normalization. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was used

to normalize the gene expression data because determination of the amplification efficiency revealed comparable values between 95-100% of applied RT-qPCR assays (see Brugger et al., 2014) for details on the amplification efficiency estimation).

5.2.3 Statistical analysis

Data were analysed using SAS 9.4 (SAS Institute Inc., Cary, NC). Zootechnical data was analysed by multifactorial ANOVA (Zn, GLDA, block, Zn*GLDA) and subsequent Student-Newman-Keul's test to identify significantly different means between groups of animals receiving different concentrations of Zn and GLDA in the diet, respectively. Linear broken-line regression models were calculated using NLMIXED for blood, bone and liver Zn status parameters. The broken-line regression approach represents an iterative procedure to estimate a potential dietary threshold (breakpoint) within non-linear data sets above and below which, respectively, a significant difference in the response behaviour of a certain parameter to the dietary treatment is evident (Robbins et al., 2006). Testing non-linear broken line models instead yielded no increase in the goodness-of-fit, when applying the workflow of McDonald (2009) (McDonald, 2009). If no significant broken-line model could be fitted to a respective data set, a linear regression model was tested instead (procedure REG, $y = a + bx$; this comprised all blood and bone Zn status parameters). The fitting of regression models was carried out independently for GLDA supplemented and non-supplemented animals. Curve parameters (intercepts, slopes) of respective broken-line and linear regression models of GLDA and non-GLDA supplemented animals were statistically compared by two-sided T-Tests. Only significant regression models were used for data presentation and interpretation. A threshold of $P \leq 0.05$ was considered to be significant for all statistical procedures.

The necessary minimum number of animals to identify diet Zn effects under the present experimental conditions was based on estimation of effect size and statistical power by applying SAS procedure Power the dataset of Brugger et al. (2014). The goal was to reach a minimum power of $1 - \beta = 0.8$ as suggested by McDonald (2009) (McDonald, 2009). The present data sets exceeded in any case the minimum statistical power of 0.8. These were analysed using broken-line and linear regression models to compare the response to different concentrations of diet Zn, the underlying effect sizes of presented models as well as t tests (within the procedures REG and NLMIXED) on the significance of certain statistical measures within and between respective models (slopes, breakpoints, intercepts).

5.3 Results

Figure 5.1 highlight the results of statistical analyses on diet quality with respect to the supplementation of Zn and GLDA. Linear regression of supplemented amounts of dietary Zn against average analysed dietary Zn concentrations indicated a high degree of homogeneity of the final feed mixtures as expressed by a significant slope ($P<0.0001$) of 0.97 ($R^2 = 1.0$), which points towards high analytical recovery close to 100%. Finally, linear regression of zinc contents in batches without versus with addition of GLDA indicated a highly significant ($P<0.0001$) slope of

1.01 (R^2 = 1,0). This highlights that Zn loads at respective Zn dose levels were identical between the batches containing or missing the test substance, respectively.

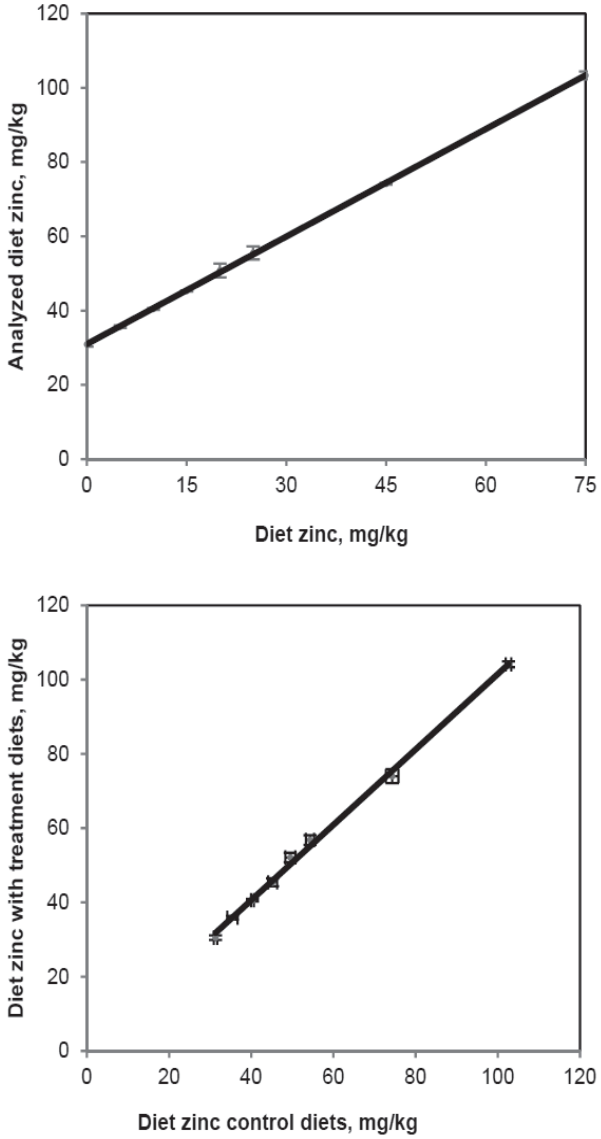

Figure 5.1. Effects of varying supplementation of Zn from $ZnSO_4 * H_2O$ on the average total analysed diet Zn concentration in experimental diet batches as well as the correlation of total analysed diet Zn concentrations in control vs. treatment experimental dietary batches.

Values are arithmetic means ± SDs, n = 8. Error bars represent respective standard deviation from mean values. Diet zinc, dietary zinc; GLDA, L-glutamic acid N,N-diacetic acid, tetrasodium salt; Zn, Zn, zinc.

Table 5.2 shows the average analysed GLDA concentrations within the 16 batches of experimental diets. In contrast, GLDA-supplemented dietary batches showed an average

concentration of 198 ± 3.16 mg/kg, which represents an analytical recovery rate of 99% with respect to the supplemented level of 200 mg/kg diet.

Table 5.2. Average analysed concentrations of GLDA and Zn in non-supplemented and GLDA supplemented dietary batches receiving varying supplementation of Zn from $ZnSO_4*H_2O$[1].

Zn supplementation level, mg/kg	GLDA supplementation level, mg/kg	Analysed diet Zn, mg/kg	Analysed diet GLDA, mg/kg
0	0	31.3	<1
5	0	35.4	<1
10	0	40.3	<1
15	0	45.2	<1
20	0	49.5	<1
25	0	54.3	<1
45	0	74.3	<1
75	0	102.6	<1
0	200	30.5	197
5	200	35.9	200
10	200	40.6	200
15	200	45.4	203
20	200	52.1	194
25	200	56.8	194
45	200	74.0	199
75	200	104.1	197

[1]Each result represents the average value determined in 3 independent feed samples, analysed in duplicate weighing; GLDA, L-glutamic acid N,N-diacetic acid, tetrasodium salt; Zn, zinc.

No animals showed signs of clinical Zn deficiency (e.g. growth depression, anorexia, impaired organ development, tissue necrosis etc. (Tucker and Salmon, 1955)) or any other signs of pathological events throughout the whole feeding trial, based on continuous veterinary surveillance. No significant differences were observed between feeding groups regarding their average daily weight gain, average daily feed intake or feed:gain ratio (Table 5.3).

Table 5.3. Zootechnical performance of animals in response to varying dietary Zn and dietary GLDA supplementation.

	Dietary Zn supplementation (mg/kg)								SEM[3]	Treatment effects[2] (p-value)		
	0 (30.9)[1]	5 (35.7)	10 (40.4)	15 (45.3)	20 (50.8)	25 (55.6)	45 (74.1)	75 (103.3)		Zn	GLDA-Na_4	Zn*GLDA-Na_4
Body weight (kg)												
Start acclimatization period	7.7	8.1	7.9	8.0	8.2	7.8	8.1	7.8	0.34	0.96	0.84	1.00
[4]E1	12.4	12.7	12.8	12.7	13.0	12.7	13.2	12.7	0.50	0.97	0.68	1.00
[5]E8	15.6	16.2	16.3	16.1	16.4	16.1	16.8	16.2	0.46	0.75	0.39	0.94
Daily weight gain (kg)	0.403	0.444	0.435	0.417	0.428	0.423	0.455	0.433	0.019	0.63	0.24	0.38
Daily feed intake (kg)	0.438	0.445	0.448	0.448	0.448	0.448	0.448	0.444	0.003	0.13	0.73	0.70
Feed:gain	1.10	1.03	1.08	1.14	1.08	1.07	1.00	1.04	0.06	0.78	0.26	0.37

[1]Between brackets, the average analysed total Zn concentrations determined in the different experimental feeds. [2]Treatment effects with $p \leq 0.05$ were considered statistically significant; [3]SEM = standard error of means (pooled standard error of the linear model); [4]E1 = 1[st]experimental day; [5]E8 = 8[th] (last) experimental day; GLDA, L-glutamic acid N,N-diacetic acid, tetrasodium salt; Zn, zinc.

Figure 5.2 presents broken-line models on the response of apparent Zn digestion to varying dietary Zn levels in the presence or absence of GLDA. The statistical parameters of the respective regression curves and results of T-statistics for the comparison of curve parameters of GLDA supplemented vs. control animals are provided supplementary Table S5.1 and S5.2 Apparent Zn digestion exhibited a non-linear behaviour in response to changes in dietary Zn concentrations, independently of GLDA inclusion. Break points were identified for curves reflecting the response of control and GLDA supplemented animals, respectively ($P<0.01$ in all cases), at a similar dietary Zn level. Also, the slopes over dietary Zn doses below the respective breakpoint were numerically identical between both curves, whereas the slopes reflecting the response to diet Zn above the breakpoints was numerically lower for the GLDA curve ($P>0.05$). Y-intercepts for linear curve sections above and below the respective dietary thresholds were in any case higher for the GLDA-curve but this difference was only significant for the curve section above the breakpoint ($P<0.0001$).

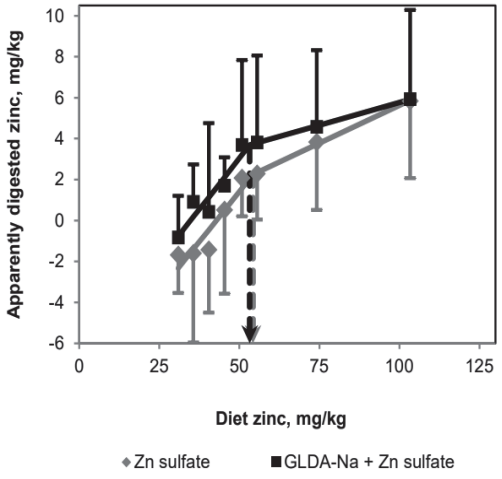

Figure 5.2. Response of [1]apparently digested diet zinc (mg/kg diet intake) in weaned piglets fed control and treatment diets for 8d.

Values are arithmetic means ± SDs, n = 18. Error bars represent respective standard deviation of mean values of control and treatment diets, respectively. Dashed grey and black errors highlight statistical breakpoints in parameter response of control and treatment diets, respectively, to varying dietary Zn supply. Diet zinc, dietary zinc; GLDA-Na_4, L-glutamic acid N,N-diacetic acid, tetrasodium salt; Zn, zinc.

[1]Apparently digested feed Zn was calculated on the basis of respective ratios of Zn and TiO_2 concentrations in feed and faeces, respectively, and is expressed as mg/kg feed intake.

Figure 5.3 highlights the response of Zn status parameters in bone and plasma to varying dietary Zn in the presence or absence of GLDA, respectively. Respective statistical regression parameters for these curves and T-statistics on the comparison between the curve parameters in the presence of GLDA vs. control are shown in supplementary Table S5.2. and S5.3.

Zn concentrations in bone and plasma as well as plasma zinc binding capacity and plasma APA concentration followed a straight linear response over the whole dose range for GLDA and control animals, respectively. Individual slopes were in any case significant ($P<0.001$). This was also the case for the individual Y-intercepts ($P\leq0.02$) with the exception of plasma Zn in response to diet Zn with and without GLDA, respectively. Slopes were parallel for plasma Zn and zinc binding capacity curves, respectively, whereas slopes of control curves were in any case numerically higher for plasma APA and bone Zn ($P>0.05$). All intercepts of GLDA curves increased compared to respective control curves with the exception of plasma zinc binding capacity for which it was decreased. However, these differences were only significant for bone Zn and plasma Zn binding capacity ($P\leq0.05$).

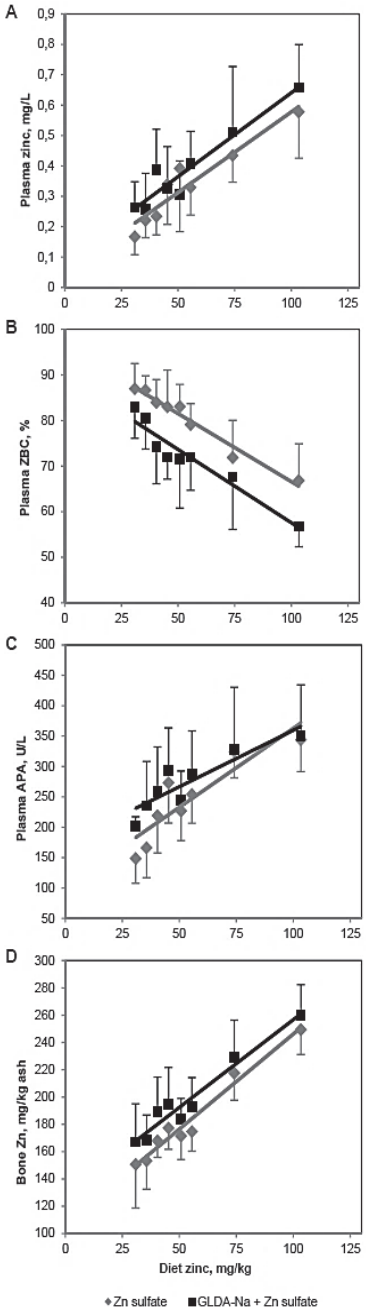

Figure 5.3. Response zinc (A), relative zinc binding capacity (B) and alkaline phosphatase activity (C) in blood plasma as well as bone zinc (D) in weaned piglets fed control and treatment diets.

Values are arithmetic means ± SDs, n = 8. Error bars in figures represent respective standard deviation of mean values of control and treatment diets, respectively. Dashed grey and black errors highlight statistical breakpoints in parameter response of control and treatment diets, respectively, to varying dietary Zn supply. APA, alkaline phosphatase activity; Diet zinc, dietary zinc; GLDA-Na_4, L-glutamic acid N,N-diacetic acid, tetrasodium salt; ZBC, relative zinc binding capacity; Zn, zinc.

The response in liver parameters to diet Zn in the presence and without GLDA is shown in Figure 5.4. The respective statistical parameters of the curves and T-statistics on comparisons between respective GLDA and control curves can be found in supplementary Table S5.2 and S5.4. Both, liver Zn and hepatic MT1A gene expression responded in a non-linear broken line fashion. Significant dietary thresholds were estimated at 70.4 and 55.6 mg/kg diet as well as 61.3 and 45.3 mg Zn/kg diet for liver Zn as well as MT1A gene expression (control and GLDA curves, respectively) ($P \leq 0.004$). Breakpoints of GLDA curves were numerically lower than in control curves but this difference was only statistically significant for MT1A gene expression ($P < 0.0001$). GLDA curves however showed steeper slopes than control curves over dietary Zn doses above and below the respective dietary Zn threshold, however, this difference was only significant for MT1A gene expression ($P \leq 0.05$). None of the Y-intercepts was significantly different between curves ($P > 0.05$) but the values were numerically higher for liver Zn in the presence of GLDA and MT1A gene expression without GLDA, respectively.

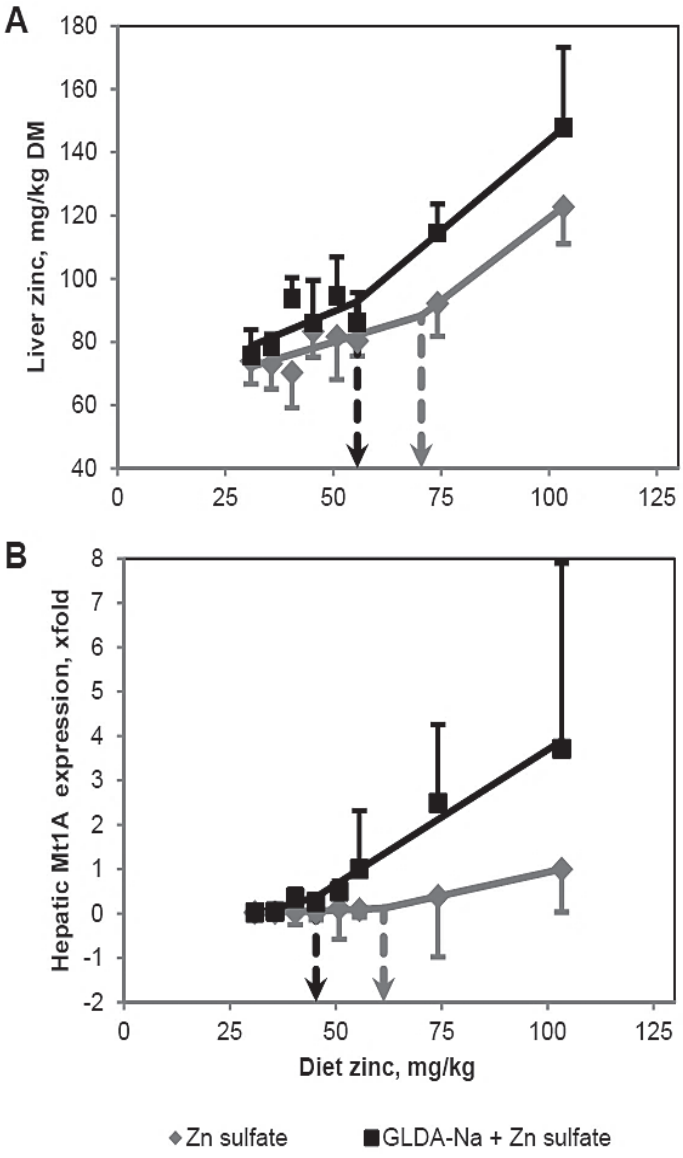

Figure 5.4. Response of liver zinc and relative hepatic *metallothionein 1A* gene expression in weaned piglets fed control and treatment diets for 8d[1].

[1]xfold differences in relative hepatic *MT1A* gene expression response in treatment and control groups were calculated relative to a gene expression response of 1.0 (not regulated) in the highest Zn supplied control group (103 mg/kg diet without GLDA-Na_4 addition) using the relative gene expression value according to Livak and Schmittgen (Livak and Schmittgen, 2001). Values are arithmetic means ± SDs, n = 18. Error bars in figures represent respective standard deviation of mean values of control and treatment diets, respectively. Dashed grey and black errors highlight statistical breakpoints in parameter response of control and treatment diets, respectively, to varying dietary Zn supply. Diet zinc, dietary zinc; DM, dry matter; GLDA-Na_4, L-glutamic acid N,N-diacetic acid, tetrasodium salt; *Mt1A*, *metallothionein 1A*; Zn, zinc.

5.4 Discussion

The present study aimed to test the value of GLDA to improve feed Zn utilization in a phytate rich diet supplemented with decreasing levels of Zn in the form of $ZnSO_4*H_2O$. The experimental design was based on the approach developed by Brugger et al (Brugger et al., 2014) in which finely graded differences in Zn status are induced ranging from different grades of subclinical Zn deficiency to mild oversupply above the estimated gross Zn requirement (~60 mg Zn/kg diet). This double dose response approach allows a comparative regression analysis where each GLDA supplemented group at given Zn dosage is compared to a negative control receiving the same amount of Zn without the test substance.

Classic experiments in rodents have demonstrated the basic principles of mammal Zn homeostatic regulation in light of varying Zn supply levels. Under the conditions of deficient dietary Zn supply, the organism attempts to stabilize its endogenous Zn levels by increasing the expression and presentation of SLC39A4 at the apical gut mucosal layer, which has been shown in mice and pigs (Brugger et al., 2021; Weaver et al., 2007). This explains the increase in the relative efficiency of true Zn absorption, which has been shown in classical studies on ^{65}Zn-labelled rats (Weigand and Kirchgessner, 1980; Windisch, 2003). In parallel, endogenous losses are reduced to an inevitable minimum mostly by reducing pancreatic losses into the gastrointestinal tract, through modulation of ZIP and ZnT transporters (Brugger and Windisch, 2016; Weigand and Kirchgessner, 1980). Studies in mice suggest this is due to down- and upregulation of SLC39A5 and SLC30A1, respectively, at the basolateral membrane of pancreatic acinar cells and reduced SLC30A2-dependent Zn loading into zymogen granules within these cells (Dufner-Beattie et al., 2004; Guo et al., 2010; Liuzzi et al., 2004; Liuzzi et al., 2009). On the other hand, the described adaptation of Zn absorption capacity and endogenous losses reversed under conditions of satiated Zn requirements to avoid excess accumulation and the associated detrimental consequences of excessive Zn retention (Liuzzi et al., 2004; Weaver et al., 2007; Weigand and Kirchgessner, 1980). These principles have recently been translated to pigs under conditions of subclinical Zn deficiency (Brugger et al., 2014). The authors demonstrated a non-linear adaption of apparent Zn digestion and liver Zn while plasma and bone parameters responded in a straight linear fashion. The data from the present study directly correspond to these findings irrespective of the GLDA supply level. Again, we were able to map the gross Zn requirement threshold by applying the apparently digested amount of feed Zn under given dietary conditions in a comparable range as Brugger et al. (2014) (55 vs. 58 mg/kg diet in the present dataset vs. the earlier dataset) (Brugger et al., 2014). Supplementation above this threshold lead to a satiation response of apparently digested feed Zn and a linear accumulation of liver Zn. The latter was accompanied by a linear increase in MT1A gene expression. This reflects the well-described phenomenon of unregulated passive influx of Zn, which is less efficient than the regulated active route (Martin et al., 2013). Interestingly, plasma and bone parameters again responded in a straight linear fashion despite the non-linear response of Zn absorption and liver Zn. This phenomenon seems to reflect the short experimental period since long-term studies

e.g. in chickens also point towards a non-linear response of these parameters after >2 weeks (Wedekind et al., 1992). This discrepancy can be explained by the timeframe of Zn homeostatic adaption. After changing dietary supply conditions, the organism needs about 3-5 days to adapt its absorption capacity and endogenous losses, which has been shown earlier in rats. Hence, during this time frame the organism still loses disproportionally more Zn into the gastrointestinal tract which is compensated by increased bone Zn mobilization (Windisch and Kirchgessner, 1994). In our present study as well as the earlier work of Brugger et al. (2014) all animals were fed at the highest applied Zn dose during the 14d acclimatization period after which, dietary Zn levels were gradually reduced for the different feeding groups (Brugger et al., 2014). The reduction from the highest dose down to the gross Zn requirement threshold appeared to promote bone Zn losses, which highlights the detrimental effect of fluctuations in nutritionally available Zn in time. This occurs even if the ranges within which it occurs do not show apparent phenotypical effects and are expected to be sufficient to meet mid-term Zn requirements.

Although, the general response pattern to diminishing dietary Zn supply of GLDA-supplemented animals was not different to that of the control animals, there was an overall numerical improvement as compared to non-supplemented animals. This was indicated by higher intercepts of GLDA curves, despite the slopes being in most cases parallel to the control curves. This was different for liver parameters, indicating that most of the Zn absorbed was quickly released to the circulation for transportation to soft tissues. Overall, this suggests that GLDA holds more Zn absorbable in the presence of high phytate concentrations within the gastrointestinal lumen, which is in line with earlier data on GLDA effects in broiler chickens (Boerboom et al., 2020; Boerboom et al., 2021). Comparing the X-axis intercepts of the regression curves of apparently digested feed Zn in GLDA-supplemented vs. control animals yields a numerical difference of 8 mg Zn/kg diet. In other terms, dietary Zn could be reduced by 8 mg/kg to result in the same parameter response. Based on own in vitro observations, GLDA at 200 mg/kg is able to bind around 40 mg/kg of Zn under neutral aqueous conditions (data not shown), making the difference observed here lower than expected, as well as lower than observed in previously published work (Boerboom et al., 2020; Boerboom et al., 2021). This discrepancy can be explained by the present experimental design, since the exposure to experimental feeds was limited to 8 days. Especially interesting is the finding of increased parameter response at the lowest Zn dose, which received just the native Zn from raw feed components. At the lowest supply level, the response in apparent Zn digestion of control animals is negative (~2 mg Zn/kg feed intake), which means these animals on average lost more Zn with the faeces than they consumed. Native Zn in these diets ranged around ~30 mg/kg diet, which is the usual level for corn-soybean based diets. In gross terms, this content would be sufficient for piglets, as they are estimated to have a net Zn requirement of 15-20 mg/kg diet, when being fed semi-purified feed components mostly absent of antagonistic substances like phytate (Shanklin et al., 1968; Smith et al., 1958). The fact that it was necessary to supplement ~25 mg Zn /kg on top of the basal feed to satiate Zn requirements at ~55 mg/kg indicates that the native Zn fraction has been completely associated to phytic acid during the

transfer through the gastrointestinal tract. However, in the presence of GLDA less native Zn appeared to be associated to phytic acid, which is evident by the higher apparently absorbed amount of Zn in the GLDA supplemented group receiving the lowest dietary Zn supply (^-0.5 mg Zn/kg feed intake). The association of Zn to phytic acid happens during different stages of digestion, when the phytic acid dissociates under the acidic conditions in the stomach and, subsequently, is able to chelate divalent cations within the neutral pH of the small intestinal lumen (Humer et al., 2015). Hence, GLDA appeared to protect a significant fraction of soluble luminal Zn from precipitation by phytic acid and sustained Zn solubility for transport mechanisms in the gut mucosa. This promoted higher Zn retention in GLDA-supplemented animals. This led to lower necessity for mobilization of body Zn stores in subclinically deficient piglets (+16.6 mg/kg bone ash in GLDA supplemented animals at 30.9 mg Zn/kg diet) to compensate for endogenous losses in the presence of GLDA.

All animals fed above the gross Zn requirement threshold experienced a mild excess absorption as indicated by a linear increase in liver Zn. This phenomenon of unregulated passive Zn uptake has been described earlier for example in pigs (Martin et al., 2013). GLDA-supplemented animals experienced a higher Zn accumulation in liver than control animals, suggesting that GLDA increased the non-regulated Zn influx, likely through uptake by other metal transporters. The reduced slope of apparent Zn digestion over Zn doses above the gross Zn requirement in the presence of GLDA suggests that the organism responded to this influx by a more efficient excretion of excess amounts via pancreatic and hepatic routes in combination with a stronger down regulation of active absorptive processes (mainly by SLC39A4) at the apical mucosal membrane. However, this has to be confirmed in future studies applying techniques that allow for a discrimination of unabsorbed feed Zn and endogenously secreted Zn, as well as by quantification of Zn transporter expression in the mucosa. The effect of GLDA on the influx of other divalent trace elements in piglets should also be determined in future studies. No effect of GLDA on retention of other trace elements was observed in poultry in previously published work (Boerboom et al., 2020; Boerboom et al., 2021).

5.5 Conclusion

In conclusion, 200 mg GLDA/kg diet increased Zn retention in weaned piglets over the whole range of applied dietary Zn dosages (30.9-103 mg/kg diet). The reduced depletion of bone and liver Zn at the lowest dietary supply level in the presence of GLDA indicates that the test substance is capable of reducing Zn precipitation by phytate within the gastrointestinal lumen. Most importantly, our data suggests that in the presence of GLDA the necessary dietary Zn supplementation to cereal-legume-based diets can be reduced by at least ~8 mg Zn/kg diet based on the parameter response of the apparently digested Zn in groups that were fed below the gross Zn requirement. Additionally, accumulation of Zn in the liver was increased when feeding GLDA to animals supplied at the gross Zn requirement threshold or above. In response to the more stable bioavailability of feed Zn in the presence of GLDA, the liver can store and subsequently

distribute more Zn to the rest of the organism allowing the system to remain much more stable under the impact of short-term subclinical Zn deficiency. Altogether, GLDA can mitigate negative effects of high-phytate levels in plant-based diets, by sustaining Zn solubility, thereby improving Zn availability. This reduces the chance of subclinical or clinical Zn deficiency. In farming animals, this could lead to a reduction in overall necessity for Zn fortification and the associated Zn emissions into the environment. In current practice, the uncertainty in the availability of zinc as affected by dietary and digestive factors is compensated by calculating gross requirements from net requirements using a worse-case availability factor in the conversion (Brugger and Windisch, 2019b; Underwood, 1999). Consequently, the higher levels of Zn inclusion lead to a reduction in relative efficiency of uptake, as levels fed are higher than Zn requirements (Weigand and Kirchgessner, 1980). Ultimately, the result of this is an increase in zinc manure, which can result in high Zn levels in soil when this manure is used (Brugger and Windisch, 2015; Jondreville et al., 2003; Monteiro et al., 2010). Such pollution has been accompanied by declined crop yields in maize, sorghum and bush beans (Abd El-Hack et al., 2017). The effect on the environment is largely dependent on the animal species. Investigations into Zn load in manure of pigs and cattle in Central Europe indicated that the Zn content in manure of pig farms exceeded that of cattle by a factor of 5 (1500 mg/kg dry matter vs 300 mg/kg) (Hölzel et al., 2012; Kickinger et al., 2008; Kickinger et al., 2010). In this context, the high biodegradability of GLDA together with the lower Zn inclusion levels allows for more sustainable farming practices. We are confident that these results can also be directly translated from pigs to humans given the high similarities in nutrition physiology between both species.

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Supplementary Data

Table S5.1. Broken-line regression analyses of the response of apparently digested diet zinc[1] (mg/kg diet intake) in weaned piglets fed control and treatment diets for 8d.

	Regression models[2]	Parameter estimates	*P* values	R^2
Control	$y = a_1 + b_1x$ for $x \leq X_B$	X_B, 54.3 ± 4.93	<0.0001	0.97
	$y = a_2 + b_2X$ for $x > X_B$	Y_B, 2.25 ± 0.71	0.01	
		a_1, -8.40 ± 1.40	0.004	
		a_2, -1.75 ± 0.18	0.01	
		b_1, 0.20 ± 0.03	0.0001	
		b_2, 0.07 ± 0.01	0.0006	
GLDA-Na_4	$y = a_1 + b_1x$ for $x \leq X_B$	X_B, 53.2 ± 3.57	<0.0001	0.96
	$y = a_2 + b_2X$ for $x > X_B$	Y_B, 3.69 ± 0.52	<0.0001	
		a_1, -6.94 ± 1.35	0.007	
		a_2, 1.33* ± 0.12	0.06	
		b_1, 0.20 ± 0.03	<0.0001	
		b_2, 0.04 ± 0.01	0.008	

[1]Apparently digested feed Zn was calculated on the basis of respective ratios of Zn and TiO_2 concentrations in feed and faeces, respectively, and is expressed as mg/kg feed intake; [2]Broken-line regression models were estimated on the basis of independent arithmetic group means relative to dietary zinc concentration (n = 8). Parameter estimates are presented as means ± SEs to indicate the precision of estimation. $P \leq 0.05$ was considered to be significant. b_1, slope of the broken-line regression curves over dietary zinc doses ≤ X_B; b_2, slope of the broken-line regression curves over dietary zinc doses >X_B; GLDA-Na_4, L-glutamic acid N,N- diacetic acid, tetrasodium salt; X_B, *X* intercept of the breakpoint in the parameter response; Y_B, *Y* intercept of the breakpoint in the parameter response; Zn, zinc.

Table S5.2. T-Statistics of curve parameters from regression models describing the response of different Zn status parameters in control versus treatment animals receiving different dietary Zn supply.

Broken-line models						
Apparently digested diet Zn	X_B	Y_B	a_1	a_2	b_1	b_2
Control	54.3	2.25	-8.40	-1.75[b]	-0.2	0.07
GLDA	53.2	3.69	-6.94	1.33[a]	-0.2	0.04
P-value	*n.s.*	*n.s.*	*n.s.*	< 0.0001	*n.s.*	*n.s.*
Liver Zn	X_B	Y_B	a_1	a_2	b_1	b_2
Control	70.4	88.3	59.55	14.6	-0.41	1.05
GLDA	55.6	92.8	61.1	28.8	-0.57	1.15
P-value	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Hepatic MT1A gene expression	X_B	Y_B	a_1	a_2	b_1	b_2
Control	61.3[a]	0.12[b]	-0.10	-1.17	-0.004[b]	0.02[b]
GLDA	45.3[b]	0.37[a]	-0.83	-2.38	-0.03[a]	0.06[a]
P-value	< 0.0001	< 0.0001	*n.s.*	*n.s.*	≤ 0.05	≤ 0.05
Linear models						
Plasma Zn	a	b		Plasma APA	a	b
Control	0.05	0.005		Control	102	2.61
GLDA	0.09	0.005		GLDA	173	1.87
P-value	n.s.	n.s.		P-value	*n.s.*	*n.s.*
Plasma ZBC	a	b		Bone Zn	a	b
Control	96.6[a]	-0.3		Control	106[b]	1.40
GLDA	89.8[b]	-0.32		GLDA	128[a]	1.29
P-value	≤ 0.05	n.s.		P-value	≤ 0.05	*n.s.*

P ≤ 0.05 was considered to indicate a significant difference between respective curve parameters; GLDA, L-glutamic acid N,N-diacetic acid, tetrasodium salt; n.s., not significant; Zn, zinc. *n.s.*: not significant.

Table S5.3. Linear regression of the response of zinc (mg/L), relative zinc-binding capacity[1] (%) and alkaline phosphatase activity (U/L) in blood plasma as well as bone zinc (mg/kg ash) in weaned piglets fed control and treatment diets for 8d.

		Regression models[2]	Parameter estimates	*P* values	R^2
Plasma Zn	Control	y = a + bx	*a*, 0.05 ± 0.04	0.30	0.90
			b, 0.005 ± 0.0007	0.0003	
	GLDA-Na_4	y = a + bx	*a*, 0.09 ± 0.04	0.07	0.92
			b, 0.005 ± 0.0007	0.0002	
Plasma ZBC	Control	y = a + bx	*a*, 96.6 ± 1.32	<0.0001	0.97
			b, -0.30 ± 0.02	<0.0001	
	GLDA-Na_4	y = a + bx	*a*, 89.8 ± 2.37	<0.0001	0.91
			b, -0.32 ± 0.04	0.0002	
Plasma APA	Control	y = a + bx	*a*, 102 ± 31.1	0 02	0.80
			b, 2.61 ± 0.53	0.003	
	GLDA-Na_4	y = a + bx	*a*, 173 ± 22.2	0.0002	0.80
			b, 1.87 ± 0.38	0.003	
Bone Zn	Control	y = a + bx	*a*, 106 ± 6.30	<0.0001	0.97
			b, 1.40 ± 0.11	<0.0001	
	GLDA-Na_4	y = a + bx	*a*, 128 ± 7.12	<0.0001	0.95
			b, 1.29 ± 0.12	<0.0001	

[1]Relative zinc binding capacity describes the percentage amount of free zinc binding sites in blood plasma; [2]Bone samples included the left femoral head of every animal; [3]Linear regression models were estimated on the basis of independent arithmetic group means relative to dietary zinc concentration (n = 8). Parameter estimates are presented as means ± SEs to indicate the precision of estimation. $P \leq 0.05$ was considered to be significant. a, Y-intercept of the respective linear regression curve; APA, alkaline phosphatase activity; b, slope of the respective linear regression curve; GLDA-Na_4, L-glutamic acid N,N- diacetic acid, tetrasodium salt; ZBC, relative zinc binding capacity; Zn, zinc.

Table S.5.4. Broken-line regression analyses of the response of liver zinc (mg/kg DM) and relative hepatic *metallothionein 1A* gene expression (xfold)[1] in weaned piglets fed control and treatment diets for 8d.

		Regression models[2]	Parameter estimates	*P* values	R^2
Liver Zn	Control	$y = a_1 + b_1x$ for $x \leq X_B$	X_B, 70.4 ± 8.30	<0.0001	0.96
		$y = a_2 + b_2X$ for $x > X_B$	Y_B, 88.3 ± 7.04	<0.0001	
			a_1, 59.55 ± 5.59	0.0001	
			a_2, 14.6 ± 0.00	<0.0001	
			b_1, 0.41 ± 0.14	0.02	
			b_2, 1.05 ± 0.14	<0.0001	
	GLDA-Na_4	$y = a_1 + b_1x$ for $x \leq X_B$	X_B, 55.6 ± 14.2	0.004	0.96
		$y = a_2 + b_2X$ for $x > X_B$	Y_B, 92.8 ± 9.62	<0.0001	
			a_1, 61.1 ± 14.0	0.01	
			a_2, 28.8 ± 8.21	0.28	
			b_1, 0.57 ± 0.23	0.04	
			b_2, 1.15 ± 0.22	0.0008	
Liver *MT1A*	Control	$y = a_1 + b_1x$ for $x \leq X_B$	X_B, 61.3 ± 1.96	<0.0001	0.99
		$y = a_2 + b_2X$ for $x > X_B$	Y_B, 0.12 ± 0.02	0.001	
			a_1, -0.10 ± 0.04	0.05	
			a_2, -1.17 ± 0.00006	<0.0001	
			b_1, 0.004 ± 0.0009	0.004	
			b_2, 0.02 ± 0.0009	<0.0001	
	GLDA-Na_4	$y = a_1 + b_1x$ for $x \leq X_B$	X_B, 45.3 ± 0.05	<0.0001	0.98
		$y = a_2 + b_2X$ for $x > X_B$	Y_B, 0.37 ± 0.10	0.008	
			a_1, -0.83 ± 0.42	0.28	
			a_2, -2.38 ± 0.58	0.06	
			b_1, 0.03 ± 0.01	0.11	
			b_2, 0.06 ± 0.004	<0.0001	

[1]xfold differences in relative hepatic *MT1A* gene expression response in treatment and control groups were calculated relative to a gene expression response of 1.0 (not regulated) in the highest Zn supplied control group (103 mg/kg diet without GLDA-Na_4 addition) using the relative gene expression value according to Livak and Schmittgen (Livak and Schmittgen, 2001); [2]Broken-line regression models were estimated on the basis of independent arithmetic group means relative to dietary zinc concentration (n = 8). Parameter estimates are presented as means ± SEs to indicate the precision of estimation. $P \leq 0.05$ was considered to be significant. b_1, slope of the broken-line regression curves over dietary zinc doses ≤ X_B; b_2, slope of the broken-line regression curves over dietary zinc doses >X_B; DM, dry matter; GLDA-Na_4, L-glutamic acid N,N- diacetic acid, tetrasodium salt; *Mt1A*, *metallothionein 1A*; X_B, *X* intercept of the breakpoint in the parameter response; Y_B, *Y* intercept of the breakpoint in the parameter response; Zn, zinc.

Chapter 6

General discussion

6.1 Introduction

It is well established that certain trace minerals (Fe, Zn, Mn, Cu, Co, I and Se) are essential nutrients to maintain normal functioning of all biochemical processes in the body (López-Alonso, 2012; Nielsen, 2012; Richards et al., 2010). The most important functions in which trace minerals are involved within the body are structural, catalytic, and regulatory in nature (Goff, 2018). The importance of Zn is evident by the presence of Zn in proteins containing Zn-binding domains, which represent approximately 10% of the total human proteome and considering the high degree of conservation of these genes, the same applies to food production animals (Andreini et al., 2006). Zinc serves as a cofactor for many transcription factors and enzymes as well, such as superoxide dismutase and carbonic anhydrase (Marreiro et al., 2017). It is important that the dietary supply meets the endogenous requirements for trace minerals. Over the last decades, trace minerals in animal nutrition have been provided at levels higher than requirements to ensure they were not limiting growth due to still unknown fluctuations in daily demands and dietary homogeneity, and the low costs associated with their use made this an inexpensive insurance (Brugger and Windisch, 2015; Brugger and Windisch, 2017). In recent years however, fuelled by the concerns over human health and environmental issues related to intensive farming, more restrictions are implemented on the levels of trace minerals to be used in animal feed (Additives and Feed, 2014; Burrell et al., 2004; Dozier III et al., 2003).

A novel chelator, L-glutamic acid N,N-diacetic acid (GLDA) can play an important role in enabling a more sustainable use of trace minerals by increasing their availability (Chapter 1). This thesis aimed to establish the potential of GLDA as a stabilizing agent to improve availability of Zn, allowing for a reduction in Zn levels in complete feeds. In Chapter 2, the effects of GLDA were compared with the reference chelating agent ethylenediaminetetraacetic acid (EDTA) in broilers, in order to compare efficacy to GLDA. The effects of GLDA on availability of basal feed trace minerals were also established. In Chapter 3, a dose response study with GLDA in broilers is presented, which aimed to quantify the proper dosage, as well as estimating the tolerance of chickens to excessive levels of GLDA in complete feed. Additional samples of edible tissues were also taken to establish potential consumer safety issues. In Chapter 4, the effects of a fixed dosage of GLDA (100 mg/kg) with increasing levels of dietary Zn in broilers was investigated to allow estimation of the reduction potential of GLDA towards Zn. In Chapter 5, the effect of GLDA at a fixed dose (200 mg/kg) was tested in piglets in the presence of finely graded differences in dietary Zn supply to determine its efficacy in the most important non-ruminating farm animal species. Pigs have a larger environmental impact compared to broilers, and they are considered translational models for human nutrition physiology. In the present chapter, the impact of GLDA on Zn retention and bioavailability in farm animals will be discussed in context of the available literature and aforementioned novel datasets. Initially, it discusses the importance of study design when assessing trace mineral availability as well as the consequences of GLDA supplementation on Zn retention and availability. The second part will

address the impact of GLDA on the health of the animal and the environment. The chapter closes by presenting the main conclusions and the impact of the work performed.

6.2 Optimizing study design for assessment of trace mineral availability

Many studies published each year aimed at evaluating the efficacy of various trace mineral supplements. Some of the studies performed thus far investigating organically complexed trace minerals have shown that, in some cases, different trace mineral sources allow for better retention and performance (Aksu et al., 2010; Manangi et al., 2012). Many studies in literature investigated the efficacy of Zn feeding strategies or products using animals in a state of clinical Zn deficiency (Brugger and Windisch, 2017). Under these conditions visible symptoms of Zn deficiency occur such as a reduction in feed intake, leading to secondary metabolic events due to deficiencies of other nutrients, which could further affect normal homeostatic regulation of Zn (Brugger and Windisch, 2017; Erdman Jr et al., 2012; Nielsen, 2012; Richards et al., 2010; Suttle, 2010). Upon re-supplementation, clinically Zn deficient animals must not only meet basal Zn need, but also replenish their depleted body Zn stores and compensate for degenerative processes. As such, this can lead to high expression of active Zn transporters, leading to an overestimation of Zn utilization and an overestimation of addition potential (Brugger and Windisch, 2017; Erdman Jr et al., 2012). The magnitude of this overestimation is conditioned by the length of the period of deficiency, the associated depletion of body Zn stores (mainly bone) and its pathological impact. Experiments looking at the effect of internal Zn stores (liver and the intestine) on the onset of Zn deficiency symptoms indicated that while there are Zn stores available, they respond after 4-5 days and the magnitude of the Zn released is based on the Zn levels fed prior to the deficiency period (Emmert and Baker, 1995). Bone Zn is an exception as recent work in rats using labelled Zn indicated that a small part of bone Zn storage is mobilizable (Brugger et al., 2018). An additional limitation of clinical Zn deficiency models is that a prolonged Zn deficiency in practical feeding is highly unlikely because of the high Zn levels being fed, which limits extrapolation of results from these trials into commercial settings (Erdman Jr et al., 2012).

Subclinical Zn deficiency could be defined as a condition in which the Zn status of the animal is challenged, but with the absence of visible symptoms such as growth reduction. This condition could allow for better determination of the nutritional bioavailability of Zn because the response of Zn absorptive/excretive mechanisms occur within basal ranges (Brugger and Windisch, 2017), making it a more suitable model to study Zn based nutritional interventions, such as dose or source. Subclinical Zn deficiency is also more likely to occur in commercial settings in case of increased requirements or decreased uptake (for example in disease state). Furthermore, to properly investigate trace mineral availability, it is important to define the dose range in such a way that it allows for discrimination between Zn deficient and Zn adequate animals, while avoiding clinical Zn deficiency (Brugger and Windisch, 2019).

The results from the broiler studies performed in Chapter 2 and 3 indicated indeed that the controls, receiving no additional Zn and no uptake or availability supporting additives, had no reduction in feed intake or growth or any observable signs of health problems, indicating the apparent absence of clinical Zn deficiency. The non-linear response in serum and tibia Zn observed in Chapter 2 and 3, however, indicates that the animals had subclinical Zn deficiency, as an increase in added Zn leads to an increase in uptake of Zn in tibia (long term Zn status) and serum (short term Zn status). Chapter 5 indicates the same for piglets, but this model was already validated for this use in literature and the results obtained in that study were in agreement with the previously published study in piglets (Brugger et al., 2014). Quite differently, the experimental design of Chapter 4 did not intend to describe differences in mineral availability, but instead was designed to define the tolerance levels of broilers to GLDA, and as such, there was no state of subclinical or clinical Zn deficiency.

6.3 Trace mineral availability and GLDA

As explained in the introduction, Zn homeostasis is tightly regulated with the uptake and excretion efficiency of Zn being driven by the current Zn requirement of the animal (Wedekind et al., 1992; Weigand and Kirchgessner, 1980). Literature describes that for piglets the gross dietary Zn requirements are approximately 60 mg Zn/kg of feed and for broilers approximately 40 mg Zn/kg of feed (Brugger et al., 2014; Council, 2012; Mohanna and Nys, 1999; Schlegel et al., 2010). Experiments performed by Weigand et al. in the 80's observed a strong regulation of Zn in rat studies (Figure 6.1). Their data clearly shows that around 40 mg/kg of dietary Zn the apparent absorption reaches a plateau. Apparent absorption is defined as the element intake minus total faecal excretion (faecal excretion of dietary origin + faecal excretion of endogenous origin) whereas true absorption being defined as apparent absorption + total faecal excretion of endogenous origin. True absorption is higher as the uptake of Zn partly takes place in an unregulated way, leading to active excretion of the excessive Zn intake through the intestine or partly through renal excretion. The point at which apparent absorption reaches the threshold can be identified as the amount of Zn that is required to meet Zn requirements. As such, this is the point at which the efficiency of uptake is reduced and the efficiency of excretion is increased (the action of both mechanisms culminates in the measurement of apparent absorption), of which the magnitude is determined by the level of Zn present in the diet (Brugger and Windisch, 2015; Brugger and Windisch, 2017; Goff, 2018; Weigand and Kirchgessner, 1980).

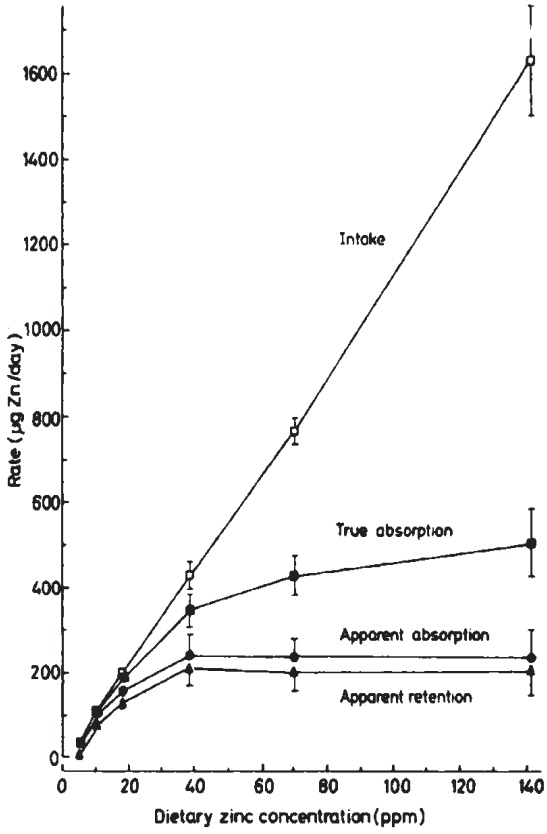

Figure 6.1. Rate of intake, apparent and true absorption and apparent retention of zinc supply from dietary zinc sulphate in rats (Weigand and Kirchgessner, 1980).

The present data shows that GLDA fed in combination with low levels of Zn increases relative Zn absorption in broilers and piglets, thereby increasing the relative efficiency of uptake. When higher levels of Zn are added along with GLDA, the relative efficiency of uptake decreases, indicating that GLDA does not overrule homeostatic regulation of Zn (Chapter 2, 3 and 5). Further supportive evidence is provided in Chapter 4. Birds in this trial were fed a diet containing 140 mg/kg of total Zn, leading to Zn requirements of birds (defined to be around 40 mg/kg) being met already in the control group. Additional inclusion of GLDA up to 3000 mg/kg led to a non-significant response in Zn retention, indicating that GLDA does not affect Zn utilization when Zn requirements are met. GLDA residue analysis further confirms this, by the limited GLDA uptake when fed up to 3000 mg/kg. The absence of relevant levels of GLDA absorption suggests that the chelator promotes ionic Zn absorption through uptake of transporters capable of transporting Zn by influencing gastrointestinal Zn speciation. As such, the data indicates that GLDA supports Zn homeostasis by allowing the absorptive system to operate more efficiently in case requirements are unmet and less efficient when Zn

requirements are met. In other terms, GLDA holds more Zn absorbable in the presence of high phytic acid levels and transfers the Zn^{2+} ion to the relevant ion transport mechanism. In contrast, inclusion of 10000 mg/kg GLDA increased Zn retention in broilers (when Zn was fed at 120 mg/kg), which is more likely due to potential membrane destabilization induced by the high sodium content (>2600 mg/kg) or by intercalation between GLDA and the phospholipids molecules present in the membrane through salt bridge formation (Banin et al., 2006; Prachayasittikul et al., 2007).

During the introduction we touched upon work of Vohra and Kratzer from the 1970's, a period in which a plethora of research was published on the application of chelating agents in farm animal feeding (Kratzer et al., 1959; Nielsen et al., 1966; O'Dell et al., 1964; Oberleas et al., 1966; Vohra et al., 1968; Vohra and Kratzer, 1964; Vohra and Kratzer, 1968). Most of these trials focused on determining the effect of minerals and chelators on growth when feeding Zn deficient diets. In one of the studies, multiple single strong chelators were tested in turkeys when fed a Zn deficient diet and the results were presented based on the stability constant/binding strength for Zn of the respective chelator (Figure 6.2). Turkeys in the experiment of Vohra and Kratzer (1964) were fed a purified, Zn deficient diet for 5 days followed by 20 days of feeding experimental feed containing 15 mg/kg Zn or 200 mg/kg EDTA, or other chelating agents at levels equimolar with EDTA. As the 5 days of feeding a Zn deficient diet led to Zn deficient turkeys, they were able to determine a growth effect by the dietary inclusion of different chelators. Chelators were included in equimolar amounts in order to distinguish them based on chelation strength. The resulting curve showed that a stability constant for Zn higher than 8 is needed to be achieved in order to improve the availability of Zn from soybean protein (Figure 6.2). The optimum is a stability constant of 13-14 for Zn. Chelating agents with a stability constant higher than 14.5 have a lesser capability of improving Zn availability, presumably as these agents may release less Zn to the metal transporters at the gut mucosa. On top, these chelating agents might interfere with metallo-enzymes present within the gastrointestinal tract (Vohra and Kratzer, 1964). Metal binding proteins such as metallothionein have a stability constant around 11-13 for Zn, indicating that EDTA, with a stability constant of 16.5, can indeed influence the release of Zn from intracellular pools (Kimura and Kambe, 2016; Kochańczyk et al., 2015). At higher levels of dietary EDTA inclusion (2000 mg/kg), negative effects of EDTA were observed, which could be due to its high stability constant (Ebrahimnezhad et al., 2008). Overall, it can be concluded that a chelating agent needs to have a stability constant that is sufficiently high to allow sequestration of Zn from the feed materials, while being sufficiently low to allow for efficient uptake. Figure 6.2 provides an indication of where GLDA would fit, based on the stability constant it has towards Zn (Kołodyńska, 2011; Smith et al., 2004).

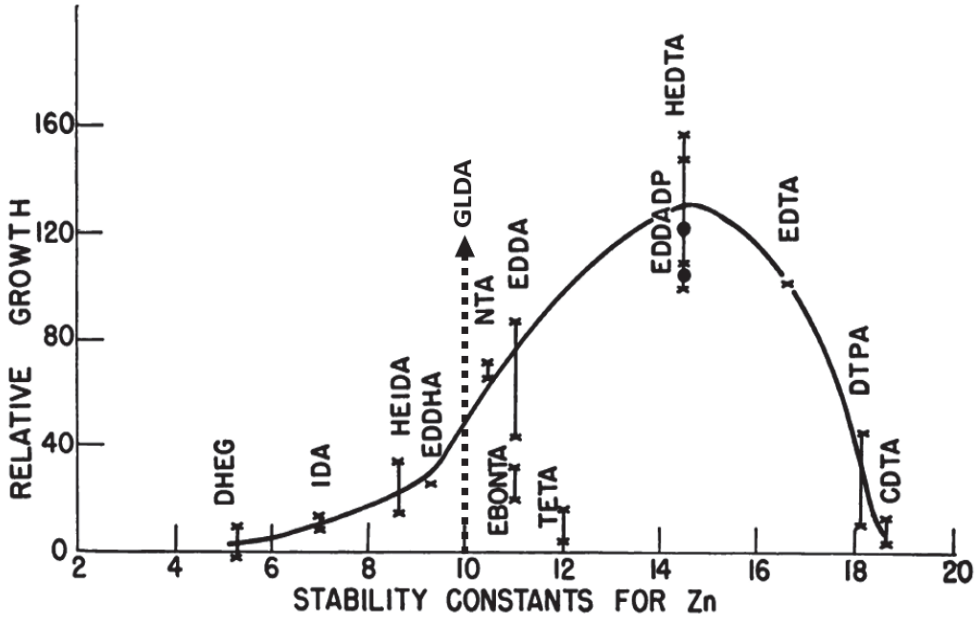

Figure 6.2. Relation of stability constant for zinc (Zn) of several chelating agents to their growth promoting effects in turkey fed a Zn-deficient diet, adapted from Vohra and Kratzer (Vohra and Kratzer, 1964). A hypothetical position of GLDA has been added based on its stability constant for Zn (dashed line).

Chapter 2 showed that the difference in Zn availability between GLDA and EDTA was not significant, with the increase in retention being identical, when feeding a corn, wheat, soybean meal-based diet with high rice bran. This differs from the isolated soybean protein Vohra and Kratzer (1964) used. The latter authors also used animal performance in a clinically deficient state as a marker of chelating agent performance, leading to a potential overestimation of chelating agent performance due to reasons discussed earlier in this chapter. The stability constant of GLDA is closer to the metal binding proteins present, potentially allowing for easier uptake of the mineral from the chelated mineral complex (Kołodyńska, 2011; Kołodyńska, 2013). Taking into account its similar binding strength to metal binding proteins, the probability for GLDA of capturing protein associated Zn inside the organism is reduced, hence, it can be considered a safer alternative in trace mineral availability improvement strategies than EDTA. This is further substantiated by the limited uptake of GLDA as pointed out earlier.

6.4 GLDA and phytate

Many plant foods contain phytate, myo-inositol hexaphosphate, the salt of myo-inositol hexaphosphoric acid (IP6) (Persson et al., 1998). In corn, phytate is primarily contained in the germ in a water-soluble form, while in legumes phytate has been shown to be associated with protein (Oberleas et al., 1966).

Phytate has effects on the availability of Zn and other minerals which are dependent on the form of phytate, the amount of phytate, as well as the concentration of other minerals such as calcium (Ca) (Ao et al., 2007; Crea et al., 2008; Fordyce et al., 1987; Persson et al., 1998). Phytate complexes with divalent metal ions have a low solubility at physiological pH and as such the constituents of the complex suffer from reduced bioavailability. As explained in the introduction, phytic acids dissociate at low pH and re-arranges at higher pH, during which it has the capability to bind divalent cations. Elevated levels of phytic acid, Ca and rice bran were added in the experiments performed here aiming to challenge Zn availability, based primarily on the work in rats (Fordyce et al., 1987). In their studies, the authors estimated the effects of different phytate×Ca/Zn molar ratio and indicated that a ratio higher than 3.5 led to growth depression effects and reduced Zn availability (Fordyce et al., 1987). The diets applied in Chapter 3 for example had a ratio of phytate×Ca/Zn of 4.88 for the control diet, to ensure Zn availability was challenged and subclinical Zn deficiency was achieved, allowing to describe the effect of GLDA on Zn bioavailability. The form of phytic acid is, as mentioned above, important for complex formation as well. Hydrolysis of IP6 results in the formation of a large number of isomers from IP5 to IP1, potentially having different mineral binding capacities (Carlsson et al., 2001; Pontoppidan et al., 2007). The mineral binding capacity is a function of the number of phosphate groups present, leading to IP6 having the largest metal binding capacity (Persson et al., 1998). Feed materials high in IP6, such as rice bran and sunflower meal are, therefore, more impactful than feed materials such as maize (Carlsson et al., 2001; Pontoppidan et al., 2007). The higher the phytate levels and corresponding IP6 levels, the stronger the effect of a high chelating agent will be (Yu et al., 2010). The stability constant of phytic acid for Zn is between 7-11, dependent on the form of phytate as well as the pH (Torres et al., 2005). As GLDA has a stability constant of ~10, it prevents Zn from binding to much of the phytate whereby the formation of the insoluble Zn/Ca/phytate complex is reduced and allows a larger fraction of Zn to remain in soluble form, hence making Zn more nutritionally available. My experimental diets contained a high level of rice bran, and it is important for future studies to show the effects in diets containing different feed ingredients. If phytic acid alone was responsible for the low bioavailability of minerals, one could argue that increasing the levels of phytase would make the application of GLDA obsolete. However, while phytase is able to break down phytate, it will not keep the released Zn soluble/available. The Zn fraction released from phytate by phytase is capable of binding to other feed materials and as such can be unavailable for uptake. The benefit of GLDA is that it allows minerals to be taken up by trace mineral transporters if needed, but otherwise keeping them unreactive/soluble. Aside from this, if the breakdown speed of IP6 into lower molecular weight variants is reduced, GLDA will still ensure sufficient soluble and therefore available Zn is present for utilization by the animal.

6.5 GLDA and the implications on health

Chapters 2 to 5 indicate that GLDA allows for an increase in relative Zn availability in the case of unmet Zn requirements and a decrease in relative Zn availability in the case Zn requirements are met. In order to sustain health and performance, it is important that Zn supply is matching Zn requirements, thereby keeping Zn levels within the homeostatic window (Brugger and Windisch, 2019; Maret, 2019; Rink, 2011). An excessive supply of minerals can lead to secondary reactions due to their intrinsic reactivity, which could potentially have negative effects (Monaghan et al., 2009; Vergauwen et al., 2017). These negative effects can occur within the GI-tract (e.g. interaction with phytate leading to insoluble complexes, oxidation of dietary components such as vitamins) as well as within the cells (Zn acting promoting oxidative stress at higher levels) (Maret, 2019). An overflow of mineral uptake by the animal due to unregulated uptake can be compensated to large extent, as explained in the introduction, by increased (active) excretion. The higher mineral level often present in feed in these scenarios will however affect the availability and uptake of other feed components (e.g. high levels of Zn will lower Cu uptake) (Goff, 2018). Care should therefore be taken as to not overfeed minerals.

Zinc is an important mineral in the immune response and oxidative stress response. The requirements for Zn in the case of an immune response stimulation are higher than in non-stimulated scenarios (Monaghan et al., 2009; Valko et al., 2007). As such, it is important to talk about trace mineral regulation and function, rather than talking about trace mineral supply only. In general, health and animal performance as influenced by trace minerals could be visualized as a normal distribution, with suboptimal supply, either too high or too low, negatively affecting health and performance. Considering the effects described for GLDA above, its inclusion in diets would lead to a greater probability to fall within the homeostatic window of trace mineral regulation, as it will improve mineral retention in the case of unmet needs and will reduce mineral retention in the case of met needs (Figure 6.3).

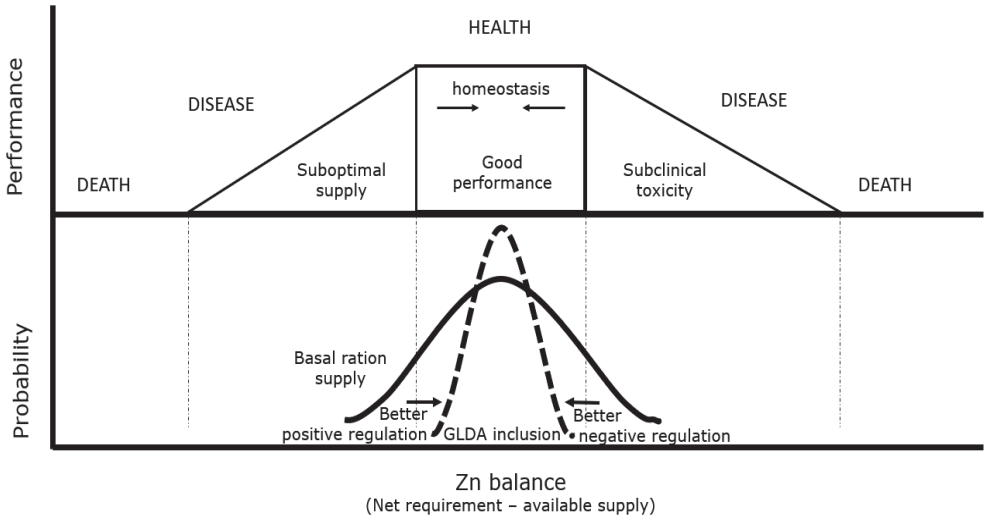

Figure 6.3. Health and animal performance as influenced by Zn balance. Probability curves indicate the chance to be within a certain range. Courtesy of Dr. Javier Martin-Tereso.

The studies reported in this thesis research work did not cover the effects of GLDA on subclinical Zn toxicity. Previous authors, however, did test the effects of EDTA inclusion in the case of elevated Zn feeding, with their data indicating that EDTA lowered the negative effects of high Zn feeding (Vohra et al., 1968; Vohra and Kratzer, 1968). Freely available Zn will be absorbed through the tight junctions in the gastrointestinal tract or by uptake through other divalent transport proteins (such as DMT-1) in an uncontrolled manner. Zinc bound to EDTA or any other chelating agent will hardly pass through these tight junctions and as such limited the negative effects of high mineral intake in these studies. Since these effects were determined by the use of EDTA and our data indicates that GLDA is hardly absorbed as well, the assumption can be made that GLDA will have a similar effect as EDTA in the case of subclinical toxicity. An in vivo trial using high levels of Zn (240 mg/kg total Zn) and three dosages of GLDA (300, 600, 1200 mg/kg feed) indeed indicated that such an effect would be possible, however, levels fed during this study were not high enough to induce subclinical toxicity (see **text box 1**). The results indicate that implementation of GLDA in trace mineral nutrition will, therefore, increase the probability of being within the homeostatic window/optimal conditions.

Text box 1. Estimation of GLDA effect on Zn retention when feeding high levels of Zn

The effect of GLDA on Zn retention in broilers fed 240 mg/kg of feed Zn was studied. Ross 308 broilers were fed a diet formulated to adequately comply with all nutritional requirements for seven days to ensure proper development. From day 7 to day 24, birds received treatment specific diets, containing 240 mg/kg feed Zn and higher levels of calcium (9 g/kg feed). Four different levels of GLDA were fed (0, 300, 600, and 1200 mg/kg feed). At study end, birds were sampled for tibia to determine Zn retention. Tibias were processed and analyzed according to the methods described in the previous chapters of this thesis. Results showed that inclusion of GLDA led to a reduction in Zn retention in tibia, giving first indications of a protective effect of GLDA when feeding Zn at levels above requirements.

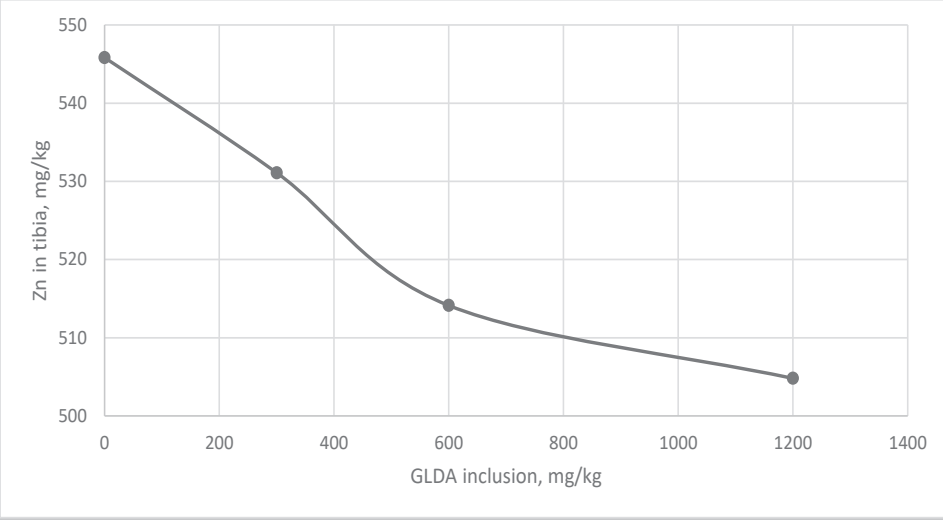

Figure 6.4. Effect of dietary L-glutamic acid N,N-diacetic acid (GLDA) inclusion on the Zn concentration in tibia in broilers fed a diet containing 240 mg/kg Zn.

6.6 Environmental potential

In the previous parts of this discussion, the focus was on the effects of GLDA on absorptive Zn homeostasis in the animal. When Zn is fed at much higher levels than requirements, absorption efficiency decreases, driven by homeostatic control mechanisms. Surplus absorption still takes place, but the relative retention compared to what is currently fed results in very inefficient Zn use in animal nutrition. In broilers, gross Zn requirements are around 20-40 mg/kg, while practical feeds contain around 120 mg/kg of total Zn (40 mg/kg in raw materials and 80 mg/kg of supplemental Zn) (Brugger and Windisch, 2015; NRC, 1994). These generous safety margins

applied in Zn supplementation are included to ensure sufficient supply of Zn with respect to the high uncertainty of dietary Zn availability, fluctuating Zn demands and varying mixing homogeneity of diets. However, the consequence of this is that a large fraction of Zn fed being excreted in the manure, which can result in high Zn levels in soil when manures are used as fertilizer. Most environmental concerns nowadays revolve around phosphorus and nitrogen pollution of soils from animal manure applications. However, in specific regions with high animal densities, soils are also vulnerable to Zn pollution. The consequences of such pollution can be a decline in crop yields, as has been shown for maize, sorghum and bush beans (Abd El-Hack et al., 2017). The degree of environmental effects from Zn emissions are to a large extent dependent on the animal species and region. If pigs are fed according to the NRC (2012) requirements, the Zn level in manure dry matter will be between 500-700 mg/kg (Brugger et al., 2014; Council, 2012; Kickinger et al., 2010). Cattle farms in Europe had a Zn content in manure of around 300 mg/kg dry matter (Brugger and Windisch, 2015; Kickinger et al., 2008; Kickinger et al., 2010). This seems to indicate that the pig production sector is the predominant user of dietary Zn (Brugger and Windisch, 2017). The models applied in this thesis indicate that there is potential for a further reduction in dietary Zn concentration without promoting the risk of Zn deficiency and associated health issues. Results reported in Chapter 2 and 3 indicate that the amount of Zn required to reach 95% of the asymptote was reduced by 15-20 mg/kg when GLDA was included in the diet. The level at which the requirements were met in these trials were around 60-70 mg/kg of total Zn. Based on this, implementation of GLDA into the feed formulation would allow reduction of added Zn to 20 mg/kg (total feed Zn being 60 mg/kg), allowing for sufficient uptake to reach requirements. Considering that the retention of Zn in broilers can expected to be approximately 20 mg/kg as shown by Weigand et al. (1980) in rats, this leads to 40 mg/kg of Zn from feed being present in the excreta (Weigand and Kirchgessner, 1980). The Zn content in raw materials is around 40 mg/kg and as such Zn retention can mirror Zn feeding, leading to endogenous Zn losses in excreta being equal to the level of Zn in the raw materials. The levels of Zn in manure will be substantially higher than this number. Zn accumulates in excreta, with the Zn present being the product of multiple meals as well as endogenous secretion. On top, dry matter digestibility of feed is around 70-80%, leading to a much higher concentration of Zn in excreta than that of the diet. However, a substantial reduction in faecal Zn can still be achieved with the application of GLDA.

In Chapter 5, piglets showed a lower Zn reduction potentially based on Zn digestibility (8 mg Zn/kg diet) with a higher dose of GLDA (200 mg/kg) compared to broilers. While this could be attributed to a difference in Zn binding capacity of GLDA in piglets, it more likely has to do with the difference in study design compared to the broilers. Piglets were fed an experimental diet for only eight days. Considering a lag-time in adaptation of absorption capacity and endogenous losses of 3-5 days, this means that the homeostatic baseline level, that was observed in the broiler studies reported here was not yet met. Additionally, this estimation on Zn reduction potential was made on basis of apparent digestibility of Zn, as the bone and serum

level of Zn did not yet reach the asymptotic phase of the response that we observed in broilers. The reduction of Zn in animal feed leads to lower levels of Zn present in excreta, but the data of this thesis does indicate the accumulation of GLDA in excreta as GLDA is poorly digested or fermented up when fed up to 3000 mg/kg (Chapter 4). As such, it is important to determine the impact of GLDA on the environment. Data in literature indicate that GLDA is degraded by more than 60% within 28 days in the environment (Kołodyńska, 2011; Kołodyńska, 2013). Compared to other chelators such as EDTA, diethylenetriaminepentaacetic acid (DTPA) and nitrilotriacetic acid (NTA) the biodegradability of GLDA is much higher. EDTA for example is resistant to most bacterial biodegradation and data in the literature published in the 1980's indicates that after 45 weeks 70% was degraded in soil (Means et al., 1980; Oviedo and Rodríguez, 2003). This leads to persistence and slow transformation in the environment, remobilization of toxic metal ions from sediments and soils as well as implications of eutrophication (overgrow of algae) of natural water systems (Kołodyńska, 2011; Kołodyńska, 2013; Oviedo and Rodríguez, 2003; Xie et al., 2007). These effects led to a risk assessment report from the European Food Safety Authority (EFSA), indicating that there is a need to limit the risk of the use of these aminocarboxylates from an environmental perspective (Munn et al., 2004). Therefore, GLDA can play an important role in more sustainable farming practices.

6.7 Impact and implications of the findings

6.7.1 Zinc feeding strategies

The results described in this thesis indicated that GLDA can be used as a feed additive for pigs and poultry to increase the nutritional availability of Zn in the presence of relevant concentrations of phytic acid. This application could significantly change the way in which Zn supplementation is applied in practice. The main impact of GLDA would be to allow for a further reduction of maximum Zn inclusion limits in the feed, reducing the safety margin provided nowadays and lowering the high levels of Zn feeding. In addition, GLDA will provide a higher and more predictable nutritional availability of dietary Zn. More research, however, is required to determine the effect of GLDA under high Zn requirement conditions, such as infection models.

Although the work reported here investigated the influence of GLDA on Zn utilization by broilers and piglets, the use of GLDA in ruminants, layers, and fish could also be beneficial. Laying hen diets contain a higher level of Ca compared to broilers, to ensure sufficient Ca is available for eggshell quality (~40 g/kg) (Bar et al., 2002; Lichovníková and Zeman, 2008). This amount of Ca, along with the presence of phytic acid and Zn can increase the likelihood of complexation of Ca, phytic acid and Zn, decreasing the solubility of the complex. In aquaculture nutrition, the use of fish meal and fish oil has been reduced over the past 10-15 years leading to an increased dietary incorporation of plant protein sources. This change has led to the increased presence of phytic acid in these diets with all its negative effects on mineral bioavailability. These effects are especially present in salmon where most commercial diets contain >70% of terrestrial plant ingredients (Silva et al., 2019; Vera et al., 2020). Some authors indicated that due to this transition the NRC requirements established in 2011 should be changed for micronutrients such as trace elements to ensure sufficient minerals are available for growth (Vera et al., 2020). In this context, having a chelating agent present that increases the availability of minerals would be a solution to the problem, without having to increase the total trace mineral content of the diets (Wreesmann et al., 2012).

6.7.2 Registration

In order to make GLDA available for practical application in animal feed, it would have to be registered as a feed additive by the appropriate regulatory bodies. Within Europe this is controlled by the rules as set in regulation No 1831/2003 of the European Parliament and of the council. The rules specify the formation of categories in which feed additives can be positioned and specifies the important role EFSA plays in the assessment of the dossiers filed (Additives and Feed, 2012). The categories currently in place are technological additives, sensory additives, nutritional additives, zootechnical additives and coccidiostats and histomonostats. Zootechnical additives are further divided into four functional groups: Digestibility enhancers, gut flora stabilizers, substances which favourably affect the environment

and other zootechnical additives (Additives and Feed, 2012). When considering GLDA and the data described in our work, one could argue the groups in which GLDA would fit best are favourably affecting the environment or the group of other zootechnical additives. Technological additives could be seen as group to consider for GLDA as well, but the guidelines state that technological additives are intended to affect characteristics of the feed, but generally have no biological effect on animal production (Additives and Feed, 2012). The data of the studies indicated that these characteristics don't fit GLDA and as such this group is not a good fit for GLDA registration. The requirements of the other zootechnical additive group specify that performance benefits need to be observed. Performance benefits are defined as feed efficiency, average daily gain, milk or egg production, carcass composition, herd performance or reproduction parameters (Additives and Feed, 2012). In the case of GLDA, the registration dossier as zootechnical additive requires three separate *in vivo* studies demonstrating a significant effect on performance. As explained in the earlier parts of this discussion, an effect on performance will only be observed in animals that are clinically deficient in Zn, as Zn homeostasis is tightly controlled in animals. Animals that are clinically deficient in Zn do not express normal functioning homeostatic Zn regulation systems which leads not only to an overestimation of the GLDA effect, but it would also require a study design that is unwanted from an ethical point of view or with respect to practical relevance. Clinically Zn deficient animals are suffering due to secondary metabolic changes taking place (Brugger and Windisch, 2017; Brugger and Windisch, 2019). Considering the above, the requirements of this group of zootechnical additives, in light of trace mineral nutrition, are unwanted from both a biological, as well as an ethical point of view.

The requirements of a substance that favourably affects the environment indicate that a direct effect is required, without making a change to the feed formulation (Additives and Feed, 2012). As mentioned above, Zn homeostasis is tightly controlled by the animal, regardless of the amount of Zn being fed. In this way, the direct effect on Zn digestibility/retention without changing the diet will not be easily measurable. However, as my data show, an indirect environmental effect can be obtained through the use of GLDA as a reduction in supplemental Zn levels without compromising the Zn status and performance of the animal can be achieved. This would then lead to lower Zn levels being present in the excreta/faeces and a reduced Zn emission into the environment. The goal of GLDA is not to affect animal performance or health directly, but to aid the Zn homeostatic regulation of Zn allowing for changes in feed formulation. The fact that GLDA itself is quickly biodegradable further contributes to its environmental benefit compared to other chelating agents.

While it is important to have regulation in place to control the use and safety of feed additives within different markets, the registration issues of a molecule like GLDA shows how the system is lacking flexibility for disruptive innovations. Sustainability is high on the agenda of the European Union and they have committed to be a frontrunner in implementing the 17 sustainability development goals for 2030 adopted by the United Nations, in which a healthy

planet is one of the concrete objectives for the next 15 years. Livestock farming plays an important role in the sustainability discussion, especially in the last decade (Brugger and Windisch, 2015; Jondreville et al., 2003; Monteiro et al., 2010). The framework currently implemented by the European Union with feed additive registration, however, does not allow for rapid application of potential solutions like GLDA. It is, therefore, important for governmental bodies to address these issues and to lobby for an improved framework to allow innovative products outside of the current framework to more easily receive registration.

6.7.3 Human nutrition

Chelators such as EDTA are used in human nutrition, especially in supplements for children who are at risk of iron deficiency (Davidsson et al., 1994; Hurrell et al., 2000; Wreesmann, 2014). The joint FAO/WHO Expert Committee on Food Additives (JECFA) classifies iron EDTA as 'a suitable source of iron for food fortification' (Additives, 2007). Chelators such as EDTA can also be added to foods and beverages as preservative agents and they are used to treat lead poisoning (Benjelloun et al., 2007). The data generated in piglets, a useful animal model for humans, does indicate potential for the use of GLDA in cereal-based human nutrition as common especially in developing countries (Rink, 2011). Future studies are required to demonstrate the real potential of GLDA in this field. The implementation of GLDA instead of chelators such as EDTA in human nutrition will improve sustainability due to the above mentioned lower environmental persistency and more sustainable production methods. In addition, the binding strength of GLDA could also increase the safety of the use of a chelator, since the binding strength of GLDA (10) is lower than that of metal transporters (11-13) and especially lower than that of EDTA (16.5) (Kimura and Kambe, 2016; Kochańczyk et al., 2015; Kołodyńska, 2011).

6.8 Main conclusions

- Dietary GLDA inclusion can improve Zn availability in broilers and piglets by sustaining Zn solubility during gastrointestinal speciation, allowing for improved mineral homeostasis
- Inclusion in broiler diets up to 3000 mg/kg shows no adverse effects of GLDA while its effectivity on Zn availability is demonstrated at much lower levels (100-300 mg/kg)
- GLDA can be a biodegradable solution in Zn reduction strategies to improve sustainable trace mineral feeding in farm animals
- Broilers can be brought into subclinical Zn deficiency using high levels of dietary calcium and phytate, thereby providing a suitable model for evaluating trace mineral availability
- Trace mineral availability determination is best studied under subclinical trace mineral deficiency to ensure proper estimation of effect

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Summary

In current farm animal practice, the uncertainty in the availability of zinc (Zn), as affected by dietary and digestive factors, is compensated by calculating gross requirements from net requirements using a worse-case availability factor in the conversion. Consequently, the higher levels of Zn inclusion lead to a reduction in relative efficiency of uptake, as levels fed are higher than Zn requirements. Ultimately, the result of this is an increase in Zn manure which can result in high Zn levels in soil when this manure is used, increasing the environmental impact of farm animals. A novel chelator, L-glutamic acid N,N-diacetic acid (GLDA), is a chelating agent, capable of binding di- and trivalent metal ions. By binding to these metal ions, it potentially provides stability of the complex in the upper gastrointestinal tract, which minimizes the formation of insoluble complexes, thereby improving nutritional bioavailability. This thesis aims to improve our understanding on trace mineral nutrition and determine the potential of using GLDA to increase the availability of minerals in livestock production.

In **Chapter 2** the impact of GLDA was compared to the well-established chelating agent ethylenediaminetetraacetic acid (EDTA). Previous work in literature showed effects of EDTA on trace mineral retention, but EDTA suffers from low biodegradability and its high chelation strength can be considered to be too high compared to metal transporters in the body. In experiment 1 broilers were fed Zn sulphate with GLDA or EDTA in molar amounts equivalent to chelate the level of Zn added. In experiment 2 the effect of GLDA on a basal diet containing no additional minerals was established. Serum and tibia Zn clearly responded to the increasing doses of dietary zinc with a significant response to the presence of EDTA and GLDA. These results are also indicative of the equivalent nutritional properties between GLDA and EDTA. In experiment 2, zinc levels in serum and tibia were also increased with the addition of GLDA to a basal diet lacking supplemental trace mineral, where serum zinc levels were 60% higher at the 216 mg/kg inclusion level. The study demonstrated that dietary GLDA enhanced availability of Zn.
The aim of **Chapter 3** was to quantify the reduction of dietary Zn that could be achieved in broilers to obtain the same Zn status. Broiler were fed Zn in a dose response manner with and without GLDA. The results indicated that when GLDA was included in the diet, based on tibia Zn, the same Zn status was achieved with a 19 mg/kg smaller Zn dose while based on serum Zn this was 27 mg/kg less Zn.

Chelators are known to have negative side-effects when fed at high levels, for example due to chelation of minerals within the cell walls, leading to cell wall disruption. In order to determine the effects of high GLDA inclusion a dose response with GLDA up to 10000 mg/kg was performed in **Chapter 4**. The results of this study indicated that there are no negative side effects of GLDA inclusion up to 3000 mg/kg of GLDA/kg feed. The GLDA residue levels in breast tissue indicated that 0.01% of total GLDA absorption is stored in breast tissue. Higher values are found in kidney and liver for the highest inclusion level, indicating that the fraction of GLDA that is absorbed is actively excreted by the animal. The limited absorption of GLDA

indicates that the role of GLDA affecting Zn availability takes place within the gastrointestinal tract of the animal, by sustaining solubility during digestive processes.

As the Zn load in manure of pigs is greater than that of broilers, the effect of GLDA in piglets was determined in **Chapter 5**. On top, pigs are a better model for potential human implications than broilers, which is important considering the high prevalence of Zn deficiency in the developing world. GLDA appeared to protect a significant fraction of soluble luminal Zn from being captured by phytic acid and mediated it towards the Zn transport mechanisms in the gut mucosa, thereby promoting higher Zn retention in GLDA-supplemented animals compared to control animals. This led to lower necessity for mobilization of body Zn stores to compensate for endogenous losses in the presence of GLDA, lowering the chance of subclinical or clinical Zn deficiency.

In **Chapter 6** we discussed the impact of GLDA on Zn availability and retention in farm animals by combining the results of previous chapters with existing literature. The importance of study design when assessing trace mineral availability is discussed. The impact of GLDA on trace mineral availability is discussed by showing the increased retention of Zn when using adding GLDA to diets of animals. The potential impact on the environment by implementing GLDA is discussed and put into context regarding sustainable farming, emphasizing the key role GLDA can play.

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About the author

Curriculum Vitae

Gavin Boerboom was born on January 16, 1992 in Waalwijk, the Netherlands. After finishing secondary school (Dr. Mollercollege, Waalwijk, The Netherlands) in 2010, he followed a BSc program in Health Sciences at the University of Maastricht for one year. In 2014 he obtained a BSc degree in Biomedical Sciences at the University of Maastricht with a major in Biological Health Sciences and minors in Neuroscience & Toxicology and Physical Exercise. During his BSc internship he worked on NADPH oxidase-targeted therapy. In 2014 he continued his studies by following the MSc Health Food Innovation Management at the University of Maastricht. During his MSc internship at Trouw Nutrition he looked into meat quality issues in broilers using metabolomics. After graduating for his MSc degree in Health Food Innovation Management he took up the position as Researcher at Trouw Nutrition (Amersfoort, The Netherlands). While being employed by Trouw Nutrition, Gavin became a PhD scholar at the Animal Nutrition Group of Wageningen University, of which the results are presented in this thesis. Gavin continuous to work at the R&D department of Trouw Nutrition as a Feed Additives Researcher within the Ingredient Research Centre of Trouw Nutrition.

List of publications

Boerboom, G., van Kempen, T., Navarro-Villa, A., & Pérez-Bonilla, A. (2018). Unraveling the cause of white striping in broilers using metabolomics. Poultry Science, 97(11), 3977-3986.

Boerboom, G. M., Busink, R., Smits, C. H., Hendriks, W. H., & Martín-Tereso, J. (2020). Efficacy of L-glutamic acid, N, N-diacetic acid to improve the dietary trace mineral bioavailability in broilers. Journal of Animal Science, 98(12), skaa369.

Boerboom, G., Busink, R., Smits, C., van Harn, J., & Bikker, P. (2021). Effect of L-glutamic acid N, N-diacetic acid on the availability of dietary zinc in broiler chickens. Poultry Science, 100(3), 100913.

Boerboom, G., Martín-Tereso, J., Veldkamp, T., van Harn, J., & Bikker, P., Busink, R. (2021). Tolerance and safety evaluation of L-glutamic acid, N,N-diacetic acid as a feed additive in broiler diets. *Manuscript submitted for publication.*

Boerboom, G., Ganslmaier, E., Oeckl, J., Busink, R., Martín-Tereso, J., Windisch, W.M., Brugger, R. Dietary L-glutamic acid N,N-diacetic acid, tetrasodium salt affects short term zinc homeostasis in weaned piglets at different levels of dietary zinc. *Manuscript submitted for publication.*

Training and supervision plan[1]

	Year
The Basic Package (2 ECTS[2])	
WIAS Introduction Course, Wageningen, the Netherlands	2018
Philosophy and Ethics of Science Course, Wageningen, the Netherlands	2018
Disciplinary Competences (15 ECTS)	
Multivariate statistics for understanding complex data, SAS institutes	2017
Writing PhD Research Proposal	2017
Speciation and bioavailability of metals, organics and nanoparticles	2019
Course on Mixed Models	2018
Course on Glimmix	2018
Course on Power Calculations	2019
Course on Design of Experiments	2020
Course on Meta-Analysis	2020
Course Laboratory Animal Sciences	2020
Swine Specific Animal Experimental Work	2020
Course on Survival Analysis	2020
Professional Competences (6 ECTS)	
Project management course	2017
Intercultural management training	2017
Essentials of scientific writing and presenting	2018
Scientific writing	2019
Project & Time management	2021
Critical thinking & argumentation	2021
Presentation Skills (4 ECTS)	
Egg Meat conference, Edinburgh, Schotland (Oral presentation)	2017
International Congress of Meat Science and Technology, Berlin, Germany (Invited speaker)	2019
Webinar on Meat Science (Oral presentation)	2020
Trouw Nutrition Germany Meeting (Oral presentation)	2020
International Conference of Trace Elements and Minerals, Aachen, Germany (Oral presentation)	2021
International Conference of Trace Elements and Minerals, Aachen, Germany (Oral presentation)	2021
Teaching Competences (4 ECTS)	
Lecture on Data Visualization	2017
Lecture on Non-Linear Regression	2020
Lecture on Trace Mineral Nutrition	2020
Supervising MSc students	2021

Total 31 ECTS

[1]Completed in fulfilment of the requirements for the education certificate of the Wageningen Institute of Animal Sciences (WIAS)
[2]One ECTS credit equals a study load of 28 hours

Colophon

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