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 fecal 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. Study 1 compared the effect of EDTA and GLDA in broilers on supplemental zinc availability at three levels of added zinc (5, 10 and 20 ppm) 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 ppm added zinc) control treatments. Study 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 ppm). In study 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 study 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 ppm 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.

Key words: Broiler, L-glutamic-acid-N-N-diacetic-acid, trace mineral, zinc
ABBREVIATIONS

Zn=Zinc

Copper=Cu

Manganese=Mn

Iron=Fe

EDTA= ethylenediaminetetraacetic acid

GLDA= L-glutamic acid, N,N-diacetic-acid

ICPMS= inductively couple plasma mass spectrometry

LC-MS= liquid chromatography-mass spectrometry

AICC= Akaike information criterion

RMSE=root mean squared error

CCC=concordance correlation coefficient
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 fulfill the biological requirement for these trace minerals, animals should receive sufficient levels of a bioavailable source (Brugger and Windisch, 2017; Goff, 2018; Skrypnik and Suliburska, 2018; Brugger and Windisch, 2019). In commercial poultry diets, it is common to supply trace minerals as inorganic sources (i.e. sulfates 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 fold 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 (Moore et al., 1995; Dozier III et al., 2003; Burrell et al., 2004).

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 maintain 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, 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, 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 (Forbes, 1961; Davis et al., 1962; Vohra and Kratzer, 1964, 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 studies ran simultaneously with different objectives. The first study 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 study 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.

**MATERIALS AND METHODS**

**Animals**

The studies 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 m²). 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. Study 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 study 2 with 6 pens per treatment. All treatments were equally distributed over block and room.

**Diets**

In the first six days of the trial, all chicks received a standard mineral-adequate starter diet, formulated to fulfill 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 ppm of supplemental Zn. Three levels of supplemental Zn sulfate (5, 10 and 20 ppm of Zn) were fed alone or in combination with molar GLDA or EDTA equivalents to chelate 5, 10 and 20 ppm 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 ppm 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 fulfill or exceed all nutritional requirements, with the exception of 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 feedstuffs, 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, fiber fractions (ISO 6865:2000) and crude protein content (ISO 16634-1:2008). Calcium, Zn, Cu, Mn and Fe content was analyzed 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).

**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 labeled 2.5 mL cryotubes. Serum and whole blood samples were stored at -20°C until further analysis. Serum Zn, Cu, Mn and Fe were analyzed 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 analyzed 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.

**Statistical analysis**

**Study 1.** Data were analyzed using SAS Studio (SAS institute Inc., Cary, NC). Performance data, serum Zn and total and concentration of Zn in tibias were analyzed 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:

\[
Y = \text{Asymptote} \times \exp(-\exp(-kSul + kEDTA + kGLDA) \times (Zn \ \text{dose} - T))
\]

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,

$S_{ul} =$ factor representing only sulfate inclusion (0,1),

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

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

$Zn$ dose = the amount of $Zn$ sulfate added

Significance ($P<0.05$) between the three fitted models (Sulfate, 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).

**Study 2.** Data were analyzed using SAS Studio (SAS institute Inc., Cary, NC). Performance parameters, serum minerals, haemoglobin and bone mineral concentrations were analyzed 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 analyzed using the NLMIXED procedure using the following model:

$$ Y = Asymptote \times \exp(- \exp(-k \times (GLDA\ dose - T))) $$

$Y =$ 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,

GLDA dose = the amount of GLDA added

RESULTS

Study 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 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).

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 3). 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 4, Figure 1). The tibia and serum Zn data showed a similar response for both EDTA and GLDA. Significant differences between the two chelators and sulfate were observed, but not between EDTA and GLDA (Table 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 sulfate was used, respectively (Table 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.

**Study 2**

None of the treatments were significantly different from the control with regards to FCR (Table 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).

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 6). Furthermore, variation in serum Zn appeared to be reduced with increasing GLDA levels (Figure 2). 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 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 7).

**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 able to increase 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 (Wedekind et al., 1992; Ammerman et al., 1995; Cao et al., 2002; Huang et al.,
2009b, a). 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 analyzed both in this study.

The incremental response to supplemental Zn was lowest for Zn sulfate 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 sulfate, 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 aminopolycarboxylates. 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, 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 actually 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 (Uyanik et al., 2002; Mondal et al., 2010). 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 ppm (Dewar and Downie, 1984; Batal et al., 2001), and that basal Zn was 32 ppm, 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 (Viteri and Garcia Inbanez, 1978; Viteri et al., 1978; Hurrell et al., 2000) 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 aminopolycarboxylates go unabsorbed through the gastrointestinal tract (Zhu et al., 2006), EDTA would be excreted via the feces. 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).

**CONCLUSION**

The results of this study indicate that GLDA significantly increased the nutritional dietary availability of supplemental Zn in a manner and magnitude similar to 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.

**DISCLOSURE**

The authors, except WHH, are employed by Trouw Nutrition, a company that has commercial interests in mineral nutrition of food producing animals. Trouw Nutrition R&D adheres to the principles of the European Code of Conduct for Research Integrity (Drenth, 2012).
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Figure 1 Response of serum Zn levels in broilers when fed dietary Zn supplementation with Zn sulfate, ethylenediaminetetraacetic-acid (EDTA) and L-glutamic acid, N,N-diacetic-acid (GLDA). Gray area indicates 95% confidence interval.

Figure 2 Response of serum Zn levels in broilers when fed increasing dietary L-glutamic acid, N,N-diacetic-acid levels in basal diet. Gray area indicates 95% confidence interval.
Table 1 Calculated and chemically analyzed (between brackets) nutrient composition of the starter feed and experimental feed.

| Nutrient composition | Starter feed (0-7 days) | Experimental diet (7-21 days) |
|----------------------|-------------------------|------------------------------|
| DM                   | g/kg                    | 889.7 (881.4) 895.7 (894.0) |
| CP                   | g/kg                    | 220 (206.9) 220.0 (212.2)   |
| Ash                  | g/kg                    | 61.8 (54.0) 71.13 (61.1)    |
| CF                   | g/kg                    | 28.3 (26.0) 34.0 (34.5)     |
| NDF                  | g/kg                    | 107.1 (107.6) 123.2 (127.2) |
| ADF                  | g/kg, n.a.              | (36.2) n.a. (40.9)          |
| ADL                  | g/kg, n.a.              | (7.0) n.a. (10.3)           |
| EE                   | g/kg                    | 72.2 (59.0) 100.0 (95.7)    |
| Starch               | g/kg                    | 389.2 (396.0) 334.0 (337.0) |
| Ca                   | g/kg, (nd)              | 9.2 (nd) 9.2 (9.8)          |
| P                    | g/kg, (nd)              | 7.5 (nd) 10.3 (9.7)         |
| Phytic acid          | g/kg, (nd)              | 2.6 (nd) 10.6 (11.3)        |
Table 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 | 0.7 |
|                         | EDTA     | -  | 205| 206| 201| -  | 0.39 | 0.25 |     |
|                         | GLDA     | -  | 203| 203| 208| -  | 0.32 | 0.15 |     |
| Body weight, d21        | None     | 1087| 1081| 1074| 1086| 1061| 0.95 | 0.6  | 3.7 |
|                         | EDTA     | -  | 1099| 1098| 1089| -  | 0.07 | 0.09 |     |
|                         | 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 | 0.2 |
|                         | EDTA     | -  | 63.9| 63.8| 63.4| -  | 0.13 | 0.22 |     |
|                         | GLDA     | -  | 61.6| 63.4| 63.9| -  | 0.48 | 0.28 |     |
| Daily feed intake, d7-21| None     | 90.9| 90.8| 88.7| 89.0| 88.5| 0.02 | 0.05 | 0.3 |
|                         | EDTA     | -  | 91.6| 90.8| 89.9| -  | 0.51 | 0.36 |     |
|      |      |      |      |      |      |
|------|------|------|------|------|------|
| GLDA | 87.3 | 89.4 | 90.9 |      |      |
|      |      |      |      | 0.001| 0.001|

Feed conversion rate, d7-21

|      |      |      |      |      |      |
|------|------|------|------|------|------|
| None | 1.44 | 1.45 | 1.43 | 1.42 | 1.45 |
| EDTA | 1.43 | 1.42 | 1.42 |      |      |
| GLDA | 1.42 | 1.41 | 1.43 |      |      |
|      |      |      |      | 0.001| 0.001|

^a^Values with different superscripts within column are significantly different (P<0.05)
Table 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 |        |        |
|------------|----------|--------------------------|-------|-----|--------|--------|
|            |          |                          | Linear|     |       |        |
| Serum Zn (μg/L) |          |                          | SEM   |     |       |        |
| None       |          |                          | <.01  | <.01|       |        |
| EDTA       |          |                          | <.01  | <0.01| 46.5  |        |
| GLDA       |          |                          | <.01  | 0.65|       |        |
| Tibia Zn (mg/kg) |          |                          | <.01  | <.01|       |        |
| None       |          |                          | <.01  | 0.05| 1.69  |        |
| EDTA       |          |                          | <.01  | 0.19|       |        |
| GLDA       |          |                          | <.01  | 0.34|       |        |
| Total tibia Zn (μg) |          |                          | <.01  | <.01|       |        |
| None       |          |                          | <.01  | 0.06| 14.4  |        |
| EDTA       |          |                          | <.01  | 0.34|       |        |
| GLDA       |          |                          | <.01  | 0.34|       |        |

Values with different superscripts within column are significantly different (P<0.05).
Table 4 Parameter of a non-linear model* describing the response of serum and tibia Zn concentration in broilers to dietary Zn supplementation with Zn sulfate (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 | <.00 | 72   | 2   | <.00 | 500     | 18.6 | <.0001 |
| kSul      | 0.069    | 0.00 | <.00 | 0.055 | 0.00 | <.00 | 0.052   | 0.00 | <.0001 |
| kEDT      | 0.034    | 0.00 | <.00 | 0.022 | 0.00 | <.00 | 0.022   | 0.00 | 0.0003 |
| A         | b 8 01   | b 6 01 | b 7 | 32   | 6 01 | b 7 | 0.019   | 0.00 | 0.0002 |
| kGLD      | 0.021    | 0.00 | 0.00 | 0.022 | 0.00 | <.00 | 0.019   | 0.00 | 0.0002 |
| T         | -3.4     | 0.6  | 01   | -4.39 | 0.8  | 01  | -4.4    | 1    | <.0001 |
| s2e⁺      | 1278     | 184  | <.00 | 22.1  | 3.2  | 01  | 1796    | 259  | <.0001 |
| AICC      | # 1193   | 583  | 1005 |
| RMS       | 113      | 4.7  | 274  |
| E⁺        | 0.94     | 0.93 | 0.88 |
Supplementary dietary Zn sulphate level (mg/kg) to reach 95% of asymptote

|       | Sulphate | EDTA | GLD A |
|-------|----------|------|-------|
| Value | 39.9     | 25.6 | 29.9  |
|       | 50       | 34.4 | 34.2  |
|       | 52.4     | 35.7 | 37.2  |

* 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.

AICC=Akaike information criterion, CCC=Concordance correlation, RMSE=root mean squared error, s2e=variance, SE=standard error.
Table 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 | Quadrati | SEM |
|---------------------|-----------------------------|---------|----------|-----|
|                     | 0  | 54  | 108 | 216 | Linear | c |
| 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 | 0.78 |
| Feed conversion ratio (g/g) | 1.46<sup>a</sup> | 1.47<sup>b</sup> | 1.44<sup>a</sup> | 1.43<sup>a</sup> | 0.35 | 0.89 | 0.006 |

<sup>ab</sup>Values with different superscripts within row are significantly different (P<0.05).
Table 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, s

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

<sup>ab</sup>Values with different superscripts within row are significantly different (P<0.05).
Table 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.

| Parameters | Serum Zn, μg/L | Tibia Zn |
|------------|----------------|----------|
|            | 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  |
| $s^2e^+$   | 6551           | 1891     | 15.4       | 4.4   | 1070.6     | 309.1 |
| AICC#      | 289.1          |          | 143.8      |       | 245.6      |       |
| RMSEAα     | 80.9           |          | 3.92       |       | 32.72      |       |
| CCCβ       | 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.

AICC=Akaike information criterion, CCC=Concordance correlation, RMSE=Root mean squared error, $s^2e=$variance, SE=standard error.
Figure 1

SerumZn concentration (ug/L) vs. Added Zn (mg/kg).

- GLDA
- EDTA
- Sulfate
Figure 2

Effect of GLDA applied to basal diet on serum Zn

Dietary GLDA (molar equiv. to Zn ppm)

Zn in serum, μg/L

○ Observed  —  Predicted  —  95% Confidence Interval