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 6 incremental zinc levels (40, 45, 50, 60, 80, and 120 ppm of total Zn), with and without inclusion of GLDA (0 and 100 ppm) as respective factors. Experimental diets were supplied from day 7 to 21/22 and serum, liver and tibia Zn content were determined in 3 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 nonlinear regression. When GLDA was included in the diet, based on tibia Zn, the same Zn status was achieved with a 19 ppm smaller Zn dose while based on serum Zn this was 27 ppm less Zn. Dietary GLDA reduces supplemental Zn needs to fulfill 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.

Key words: GLDA, bioavailability, chelator, broiler, zinc

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INTRODUCTION

Zinc (**Zn**) is a component of almost every metabolic pathway, making it a critical nutrient to farm animal health and productivity. To fulfill 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 fulfillment of the nutritional requirements. However, Zn supplementation above the requirement decreases the relative efficiency of Zn absorption, which increases fecal 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, 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 (Underwood 2004; Bao et al., 2010). Ionizable supplemental forms such as Zn sulfates 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 (Windisch, 2002; Humer et al., 2015). Under mild acidic to neutral conditions (pH = 5-7) phytic acid develops sustainable bonds with divalent cations yielding insoluble phytate complexes (Linares et al., 2007; Humer et al., 2015). There are indications that poultry have inadequate intrinsic phytase activity to utilize the

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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 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 (O'Dell et al., 1964; Vohra and Kratzer, 1964; Oberleas et al., 1966; Maenz et al., 1999). Ethylenediaminetetraacetic acid was found to decrease supplemental Zn requirements when isolated soybean protein served as the source of dietary protein. The addition of 100 ppm of EDTA to the diets chelated about 8 ppm of Zn, which was assumed to be the amount of Zn bound by the soybean proteins (Kratzer and Starcher, 1963). Ethylenediaminetetraacetic acid 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 toward a plethora of metal ions, including Zn, whereas it has a high biodegradability, with more than 60% being degraded within 28 d (Kołodyńska, 2013; Wu et al., 2015). Its production is based on the flavor 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, whereas 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, on confirmation of the hypothesis, the potential effect of GLDA on Zn supplemental needs was quantified.

MATERIALS AND METHODS

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 (2 kg/m^2). All birds received a standard starter diet formulated to adequately comply with all nutritional requirements of the broilers including 80 ppm of supplemental Zn derived from Zn sulfate monohydrate ($ZnSO_4.H_2O$). After 1 wk, 960 healthy animals were selected and equally distributed as per 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 8 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, 3 animals were marked blue and one green. This was performed 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.

Diets

The dietary treatments comprised 6 incremental levels of supplemented Zn (0, 5, 10, 20, 40, and 80 ppm Zn from $ZnSO_4.H_2O$, leading to 40, 45, 50, 60, 80, 120 ppm of total Zn) and presence or absence of supplemental GLDA (0 and 100 ppm GLDA, i.e., 0 and 333 ppm 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. 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). 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).

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, Thermo Fisher, Waltham, MA) after ashing and HCl extraction in accordance with method NEN-EN 15510 (Bikker et al., 2017). L-glutamic acid N,N-diacetic acid content was measured in duplicate by using liquid chromatography–mass spectrometry (LC-MS, Thermo Fisher, Waltham, MA) (MasterLab B.V., Boxmeer, The Netherlands). L-glutamic acid N,N-diacetic acid 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% trifluoroacetic acid in water as mobile phase. L-glutamic acid N,N-diacetic acid presence was detected at m/z 264.070 using a Triple Quadrupole LC-MS mass spectrometer. L-glutamic acid N,N-diacetic acid levels in feed samples were calculated using a GLDA standard curve based on a calibrated GLDA standard.

Measurements

Performance parameters, including body weight (**BW**), feed intake, body weight gain, and feed conversion ratio, were determined at 7, 14, and 21/22 d 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 3 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 × 3 animals) blood samples were taken. After collection the blood was centrifuged for approximately 30 min. Subsequently, the harvested serum was divided in 2 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 analyzed 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 BW) and 20 min later euthanized by an intravenous injection of T61 (an aqueous solution containing 200 mg embutramide, 50 mg mebezonium iodide, and 5 mg tetracaine hydrochloride 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. The 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).

Statistical Analysis

The data were analyzed 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 analyzed using the MIXED procedure. Treatments, GLDA and Zn dose were analyzed as main effects with block as a random effect and time (if applicable) as a repeated effect. Pen was used as the experimental unit.

Zinc levels in serum and tibia were subsequently analyzed using nonlinear regression (NLmixed). Because Zn absorption is primarily a homeostatically regulated, saturable, carrier-mediated process, the response to dietary Zn is nonlinear and using nonlinear regression was preferred over data transformation and linear regression (Miller et al., 2007).

The nonlinear 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).

$$Y = A * \exp(-\exp(-k * (\text{dietary Zn level} - T)))$$

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;
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.

$$Y = (A + A1 * GLDA) * \exp(-\exp(-(k + k1 * GLDA) * (\text{Dietary Zn level} - (T + T1 * GLDA))))$$

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);
Dietary Zn level = total dietary Zn level (mg/kg).

A reduced model was created including only significant GLDA effects after exclusion of all nonsignificant 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 fulfillment of nutritional requirements.

RESULTS

In Table 1, the intended and analyzed concentration of Zn and GLDA in the experimental diets is included. The results indicate that the analyzed content of Zn and GLDA was aligned with the intended Zn and GLDA levels and in accordance with the experimental design (Table 1). The MIXED and NLMIXED procedures therefore were based on the intended dietary Zn levels. Table 2 shows the diet composition, with Table 3 showing analyzed nutrient contents of both the starter diets as well as the basal meal.

Zinc dose and the inclusion of GLDA did not affect bird performance and there no interactions were detected between them (Table 4). Growth performance observed in this study was in line with Ross 308 guidelines (Aviagen, 2014).

Dietary inclusion of Zn or GLDA had no significant effect on the liver measurements or on tibia weight or tibia ash content (Table 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 ($P < 0.001$).

The results of the regression analysis with the nonlinear model for serum Zn are described in Table 6 and illustrated in Figures 1 and 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, root mean squared error, and concordance correlation coefficient of the 2 models confirmed that these parameters did not contribute to the prediction of the serum Zn response. L-glutamic acid N,N-diacetic acid 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. L-glutamic acid N,N-diacetic acid 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. L-glutamic acid N,N-diacetic acid inclusion in the diets reduced the required level of dietary Zn by 22 ppm in the full model and by 27 ppm in the reduced model.

The results for Zn in tibia ash using a nonlinear model are described in Table 7 and illustrated in Figure 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 root mean squared error and concordance correlation coefficient of the 2 models confirmed that these parameters did not contribute to the description of the Zn response in tibia ash. The Akaike information criterion improved slightly when the reduced model was used in comparison with using the full model indicating an improved model performance. L-glutamic acid N,N-diacetic acid 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. L-glutamic acid N,N-diacetic acid 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. L-glutamic acid N,N-diacetic acid inclusion in the diets reduced the required level of dietary Zn by 17 ppm in the full model and by 19 ppm in the reduced model.

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 fiber (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

Table 1. Intended and analyzed moisture, zinc and L-glutamic acid N,N-diacetic acid (GLDA) content in experimental diets used to determine the effect of GLDA in broilers.

Treatment	Analyzed moisture, g/kg	Analyzed total Zn, mg/kg	Intended supplemental Zn, mg/kg	Analyzed supplemental Zn, mg/kg	Intended GLDA, mg/kg	Analyzed 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

Supplemental Zn is calculated as analyzed total Zn content in each diet minus 41 mg/kg from basal meal.

high excretion of nonutilized Zn in the feces 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 analyzed during the experimental period of 0-21/22 d. This is in accordance with the expectations because 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, 1999; 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 (Jongbloed et al., 2002; Huang et al., 2013). 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 (O'Dell et al., 1964; Oberleas et al., 1966; Maenz et al., 1999). This improved solubility appears to coincide well with the observed effect of GLDA, allowing for better uptake of Zn in the present 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 2 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, 1964, 1968). L-glutamic acid N,N-diacetic acid 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 ppm) 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

Table 2. Composition starter and grower phase feeds.

Ingredients	Starter, g/kg	Grower, g/kg
Wheat	395.5	299.0
Corn	200.0	200.0
SBM	315.0	234.0
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	1,000.0	1,000.0

Table 3. Intended and analyzed nutrient contents of the starter diet (0-7 d) and the basal meal used for production of the treatment diets (7-21/22 d) to determine the effect of L-glutamic acid N,N-diacetic acid (GLDA) in broilers.

		Starter diet		Basal diet	
Feed Material		Intended	Analyzed	Intended	Analyzed
Dry matter (DM)	g/kg	n.a.[1]	897	n.a.[1]	891
Moisture	g/kg	n.a.[1]	103	n.a.[1]	109
Ash (g/kg)	g/kg	65	59	69	65
Starch (g/kg)	g/kg	378	375	385	322
Crude protein (CP)	g/kg	216	215	202	198
Fat (EE)	g/kg	58	67	79	90
Crude fiber (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

[1]n.a. = Not available.

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

Table 4. Effect of dietary L-glutamic acid N,N-diacetic acid (GLDA) and Zn levels on growth performance of broilers from d 7 to 21/22.

		Total Zn, mg/kg							*P*-value		
Parameter	GLDA, mg/kg	40	45	50	60	80	120	SE	GLDA	Zn	GLDA*Zn
BW d7, g											
	0	137	134	135	136	135	134	0.2	0.29	0.81	0.28
	100	134	134	134	134	135	136				
BW d14, g											
	0	437	432	438	435	434	436	1.4	0.86	0.75	0.84
	100	433	438	436	430	435	443				
BW 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				
BWG 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				
BWG 7-21/22, g											
	0	805	789	782	780	770	785	4.9	0.46	0.58	0.7
	100	780	784	789	772	782	781				
BWG 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				
FCR 7-14											
	0	1.32	1.3	1.32	1.29	1.3	1.3	0.003	0.51	0.35	0.06
	100	1.31	1.28	1.29	1.31	1.32	1.29				
FCR 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				
FCR 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				
FI 7-14											
	0	397	387	399	386	387	393	1.5	0.96	0.48	0.77
	100	390	390	391	387	395	395				
FI 14-21/22											
	0	749	741	715	711	712	719	7.2	0.64	0.9	0.38
	100	724	718	727	710	724	706				
FI 7-21/22											
	0	1,146	1,128	1,114	1,097	1,090	1,112	7.7	0.45	0.29	0.42
	100	1,115	1,108	1,112	1,097	1,119	1,101				

Table 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 to d21.

GLDA,	Total Zn, mg/kg							*P*-value		
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}	$219^{A,B}$	241^{C}	268^{D}	287^{D}	314^{E}	2.53	<0.001	<0.001	<0.001
100	$232.2^{B,C}$	247.5^{C}	276.0^{D}	282.4^{D}	311.4^{E}	312.7^{E}				
Serum										
Zn, mg/L										
0	0.97^{A}	$1.07^{A,B}$	$1.31^{C,D}$	$1.43^{C,D,E}$	$1.63^{E,F}$	1.75^{F}	0.02	<0.001	<0.001	<0.001
100	$1.22^{B,C}$	$1.31^{C,D}$	$1.52^{D,E}$	$1.65^{E,F}$	1.79^{F}	1.84^{F}				

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

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

Table 6. Parameter estimates of a nonlinear 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	SE	Reduced model	SE
A	1.75	0.05	1.81	0.035
A1	0.09	0.07	NS	
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	NS	
s2e	0.055	0.004	0.055	0.005
AICC	−4.8		−4.8	
RMSE	0.24		0.24	
CCC	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)))).

Abbreviations: AICC, Akaike information criterion; CCC, Concordance correlation; RMSE, Root mean squared error.

s2e, variance.

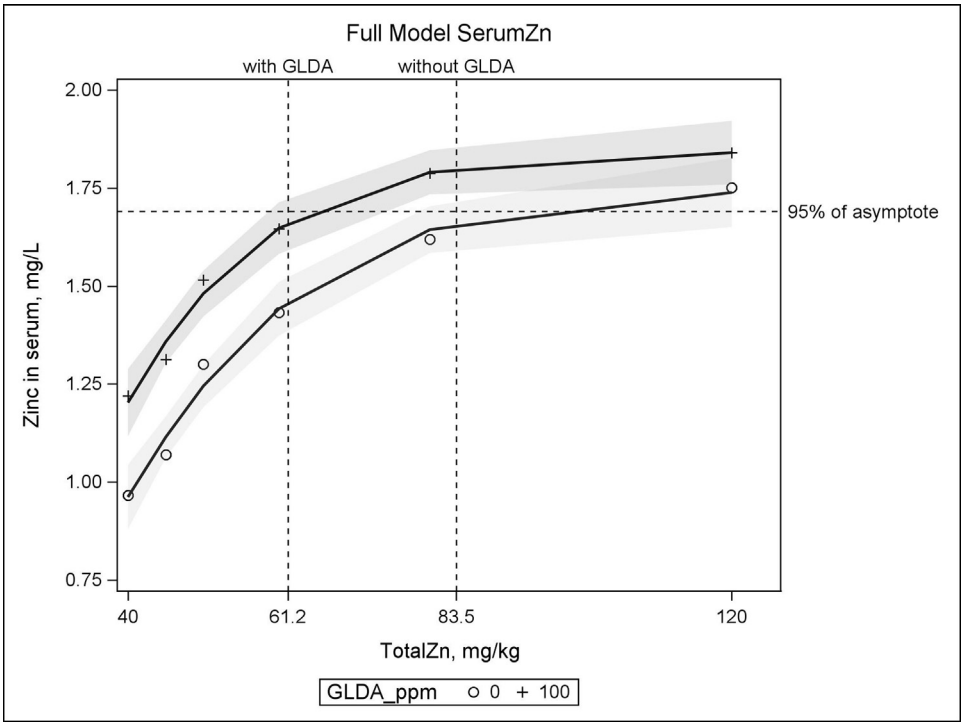

Figure 1. Effect of dietary L-glutamic acid N,N-diacetic acid (GLDA) inclusion and total dietary Zn content on the Zn concentration in serum using a nonlinear full model. Dotted lines represent the value for Zn at which the serum Zn level is equal to 95% of the asymptote.

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

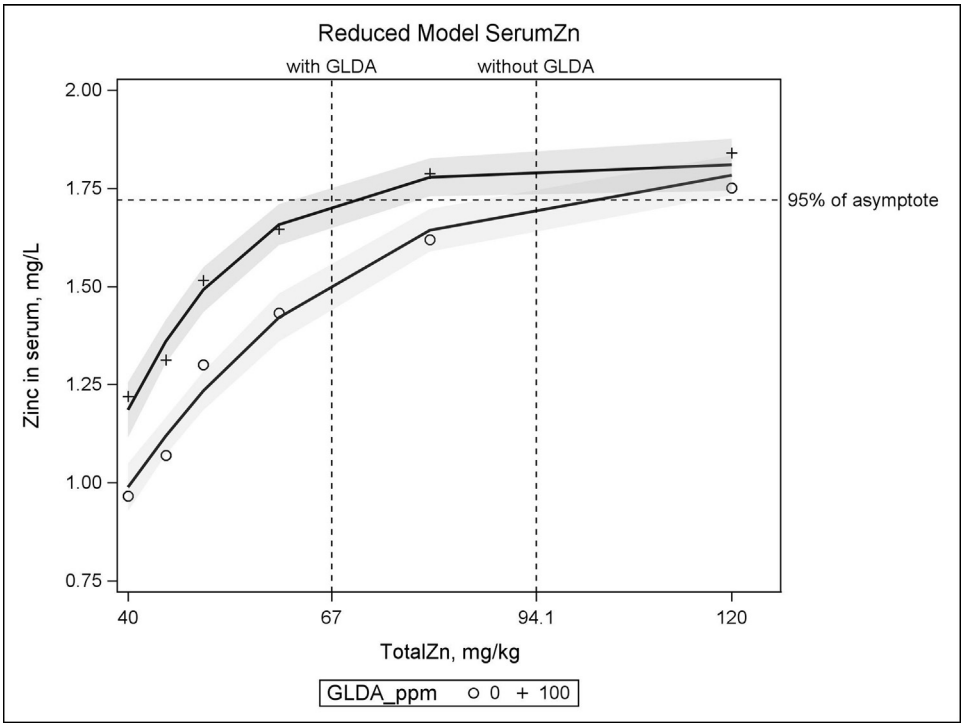

Figure 2. Effect of dietary L-glutamic acid N,N-diacetic acid (GLDA) inclusion and total dietary Zn content on the Zn concentration in serum using a nonlinear reduced model. Dotted lines represent the value for total Zn at which the serum Zn level is equal to 95% of the asymptote.

Table 7. Parameter estimates of a nonlinear 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	SE	Reduced model	SE
A	314.4	5.44	314.5	3.25
A1	0.53	6.83	NS	
k	0.048	0.007	0.046	0.0045
K1	0.015	0.012	0.020	0.003
T	−16.77	2.41	-17.54	1.88
T1	−1.97	3.94	NS	
s2e	454.4	38.47	454.9	38.51
AICC	2,513		2,510	
RMSE	21.3		21.3	
CCC	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 + A1 × GLDA) × exp(-exp(-(k + k1 × GLDA) × (dietary Zn level-(T + T1 ×GLDA)))).

Abbreviations: AICC, Akaike information criterion; CCC, Concordance correlation; RMSE, Root mean squared error; s2e, variance.

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.

CONCLUSION

The results of this study demonstrate that dietary inclusion of GLDA improved the bioavailability of Zn in broiler diets. L-glutamic acid N,N-diacetic acid exerts its effect in the gastrointestinal 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. L-glutamic acid N,N-diacetic acid inclusion can substantially contribute to a reduction of Zn excretion into the environment if Zn supplementation in the diets is reduced.

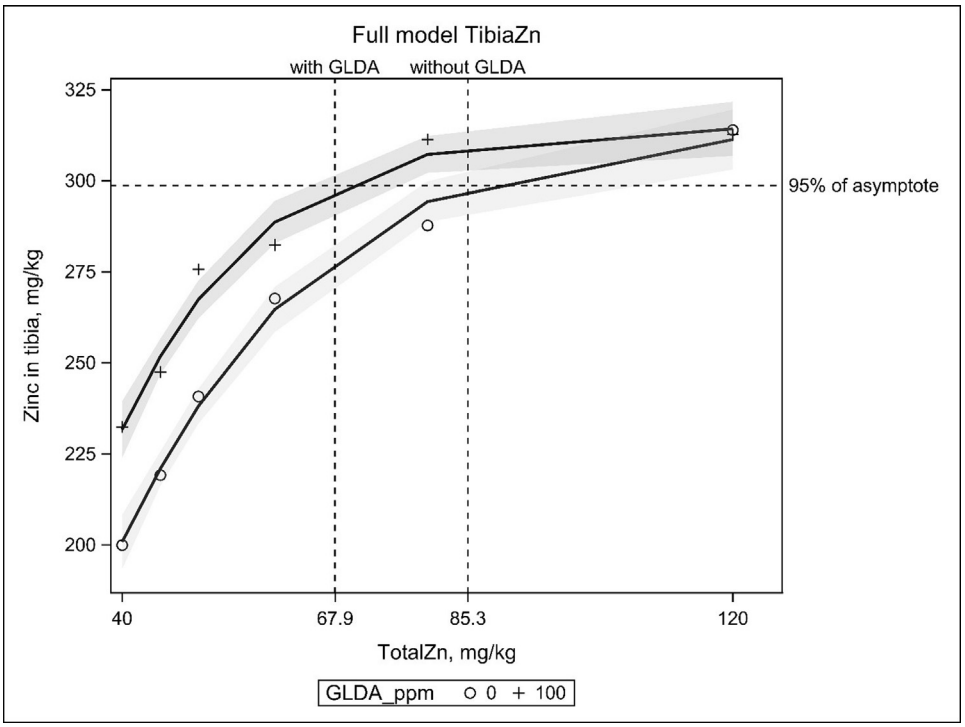

Figure 3. Effect of dietary L-glutamic acid N,N-diacetic acid (GLDA) inclusion and total dietary Zn content on the Zn concentration in tibia using a nonlinear full model. Dotted lines represent the value for Zn at which the tibia Zn level is equal to 95% of the asymptote.

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DISCLOSURES

The authors, except Jan van Harn and Paul Bikker, 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). All other authors declare no real or perceived conflicts of interest.

REFERENCES

Additives, E. P. O., and P. O. S. U. I. A. Feed. 2014. Scientific Opinion on the potential reduction of the currently authorised maximum zinc content in complete feed. Efsa J. 12:3668.

Ao, T., J. Pierce, A. Pescatore, A. Cantor, K. Dawson, M. Ford, and B. Shafer. 2007. Effects of organic zinc and phytase supplementation in a maize–soybean meal diet on the performance and tissue zinc content of broiler chicks. Br. Poult. Sci. 48:690–695.

Archontoulis, S. V., and F. E. Miguez. 2015. Nonlinear regression models and applications in agricultural research. Agron. J. 107:786–798.

Aviagen, P. O. 2014. Ross 308 Broiler Performance objectivesAviagen Limited Newbridge. Midlothian EH28 8SZ, Scotland, UK.

Bao, Y., M. Choct, P. Iji, and K. Bruerton. 2010. Trace mineral interactions in broiler chicken diets. Br. Poult. Sci. 51:109–117.

Bikker, P., N. ten Tije, and A. Tijkorte. 2017. Fosforbenutting Bij Biologisch Gehouden vleesvarkens. No. 1069. Wageningen Livestock Research.

Boerboom, G., R. Busink, C. Smits, W. Hendriks, and J. Martín-Tereso. 2020. Efficacy of L-glutamic acid, N, N-diacetic acid to improve the dietary trace mineral bioavailability in broilers. J. Anim. Sci. 98:skaa369.

Brugger, D., and W. M. Windisch. 2015. Environmental responsibilities of livestock feeding using trace mineral supplements. Anim. Nutr. 1:113–118.

Brugger, D., and W. M. Windisch. 2017. Strategies and challenges to increase the precision in feeding zinc to monogastric livestock. Anim. Nutr. 3:103–108.

Brugger, D., and W. M. Windisch. 2019. Zn metabolism of monogastric species and consequences for the definition of feeding requirements and the estimation of feed Zn bioavailability. J. Zhejiang Univ. Sci. B. 20:617–627.

Cao, J., P. Henry, S. Davis, R. Cousins, R. Miles, R. Littell, and C. Ammerman. 2002. Relative bioavailability of organic zinc sources based on tissue zinc and metallothionein in chicks fed conventional dietary zinc concentrations. Anim. Feed Sci. Tech. 101:161–170.

Davis, P. N., L. Norris, and F. Kratzer. 1962. Interference of soybean proteins with the utilization of trace minerals. J. Nutr. 77:217–223.

Drenth, P. J. 2012. A European code of conduct for research integrity. Pages 161–168 in Promoting Research Integrity in a Global Environment. T. Mayer and N. Steneck, eds. World Scientific Publishing, Singapore.

EFSA Panel on Dietetic Products, N., and Allergies. 2011. Guidance on the scientific requirements for health claims related to antioxidants, oxidative damage and cardiovascular health. EFSA J. 9:2474.

Goff, J. P. 2018. Invited review: mineral absorption mechanisms, mineral interactions that affect acid–base and antioxidant status, and diet considerations to improve mineral status. J. Dairy Res. 101:2763–2813.

Goli, M. B., M. Pande, and N. Bellaloui. 2012. Effects of chelating agents on protein, oil, fatty acids, and minerals in soybean seed. Agric. Sci. 3:517.

Huang, Y., L. Lu, J. Xie, S. Li, X. Li, S. Liu, L. Zhang, L. Xi, and X. Luo. 2013. Relative bioavailabilities of organic zinc sources with different chelation strengths for broilers fed diets with low or high phytate content. Anim. Feed Sci. Tech. 179:144–148.

Humer, E., C. Schwarz, and K. Schedle. 2015. Phytate in pig and poultry nutrition. J. Anim. Physiol. Anim. Nutr. 99:605–625.

Jongbloed, A., P. Kemme, G. De Groote, M. Lippens, and F. Meschy. 2002. Bioavailability of Major and Trace Minerals. EMFEMA, International Association of the European Manufacturers of Major, Trace and Specific Feed Mineral Materials, Brussels, Belgium.

Kołodyńska, D. 2011. Cu (II), Zn (II), Co (II) and Pb (II) removal in the presence of the complexing agent of a new generation. Desalination 267:175–183.

Kołodyńska, D. 2013. Application of a new generation of complexing agents in removal of heavy metal ions from different wastes. Environ. Sci. Pollut. R. 20:5939–5949.

Kratzer, F., and B. Starcher. 1963. Quantitative relation of EDTA to availability of zinc for Turkey poults. Proc. Soc. Exp. Biol. Med. 113:424–426.

Krezel, A., and W. Maret. 2016. The biological inorganic chemistry of zinc ions. Arch. Biochem. Biophys. 611:3–19.

Linares, L., J. Broomhead, E. Guaiume, D. Ledoux, T. Veum, and V. Raboy. 2007. Effects of low phytate barley (Hordeum vulgare L.) on zinc utilization in young broiler chicks. Poult. Sci. 86:299–308.

Maenz, D. D., C. M. Engele-Schaan, R. W. Newkirk, and H. L. Classen. 1999. The effect of minerals and mineral chelators on the formation of phytase-resistant and phytase-susceptible forms of phytic acid in solution and in a slurry of canola meal. Anim. Feed Sci. Tech. 81:177–192.

Miller, L. V., N. F. Krebs, and K. M. Hambidge. 2007. A mathematical model of zinc absorption in humans as a function of dietary zinc and phytate. J. Nutr. 137:135–141.

Mohanna, C., and Y. Nys. 1999. Effect of dietary zinc content and sources on the growth, body zinc deposition and retention, zinc excretion and immune response in chickens. Br. Poult. Sci. 40:108–114.

O'Dell, B., J. Yohe, and J. Savage. 1964. Zinc availability in the chick as affected by phytate, calcium and ethylenediaminetetraacetate. Poult. Sci. 43:415–419.

Oberleas, D., M. E. Muhrer, and B. L. O'Dell. 1966. Dietary metal-complexing agents and zinc availability in the rat. J. Nutr. 90:56–62.

Olukosi, O. A., S. van Kuijk, and Y. Han. 2018. Copper and zinc sources and levels of zinc inclusion influence growth performance, tissue trace mineral content, and carcass yield of broiler chickens. Poult. Sci. 97:3891–3898.

Schlegel, P., Y. Nys, and C. Jondreville. 2010. Zinc availability and digestive zinc solubility in piglets and broilers fed diets varying in their phytate contents, phytase activity and supplemented zinc source. Animal 4:200–209.

Schlegel, P., D. Sauvant, and C. Jondreville. 2013. Bioavailability of zinc sources and their interaction with phytates in broilers and piglets. Animal 7:47–59.

Seetz, J., and T. Stanitzek. 2012. GLDA: the new green chelating agent for detergents and cosmetics. Proc. SEPAWA Congress European Detergents Conf. Proc Vol. 1, 2008:17e22.

Skrypnik, K., and J. Suliburska. 2018. Association between the gut microbiota and mineral metabolism. J. Sci. Food Agr. 98:2449–2460.

Underwood, E. J., and N. F. Suttle. 2004. The Mineral Nutrition of Livestock. 3rd ed. CABI Publishing, Cambridge, UK.

Vohra, P., and H. Kratzer. 1964. Influence of various chelating agents on the Avaliability of zinc. J. Nutr. 82:249–256.

Vohra, P., and F. Kratzer. 1968. Zinc, copper and manganese toxicities in Turkey poults and their alleviation by EDTA. Poult. Sci. 47:699–704.

Weigand, E., and M. Kirchgessner. 1980. Total true efficiency of zinc utilization: determination and homeostatic dependence upon the zinc supply status in young rats. J. Nutr. 110:469–480.

Windisch, W. 2002. Interaction of chemical species with biological regulation of the metabolism of essential trace elements. Anal. Bioanalytical Chemistry 372:421–425.

Wu, Q., Y. Cui, Q. Li, and J. Sun. 2015. Effective removal of heavy metals from industrial sludge with the aid of a biodegradable chelating ligand GLDA. J. Hazard Mater. 283:748–754.

Yu, Y., L. Lu, R. Wang, L. Xi, X. Luo, and B. Liu. 2010. Effects of zinc source and phytate on zinc absorption by in situ ligated intestinal loops of broilers. Poult. Sci. 89:2157–2165.

Zeller, E., M. Schollenberger, I. Kühn, and M. Rodehutscord. 2015. Hydrolysis of phytate and formation of inositol phosphate isomers without or with supplemented phytases in different segments of the digestive tract of broilers. J. Nutr. Sci. 4.