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Effects of hot-melt extruded nano-copper on the Cu bioavailability and growth of broiler chickens

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No potential conflict of interest relevant

Abstract

This study was aimed to investigate the Cu bioavailability, growth response, digestibility of nutrients, and blood metabolites of broiler chicks fed $CuSO_4$ in nano or common forms. A total of 720 broiler chickens were distributed between eight treatments according to a completely randomized design. There were 8 treatments and 6 replicates in each treatment with 15 birds/replicate. The treatments were divided into common copper sulfate at the doses of 16 ppm, 40 ppm, 80 ppm, and 120 ppm (INO) and hot-melt extruded copper sulfate at the doses of 16 ppm, 40 ppm, 80 ppm, and 120 ppm (HME-Cu). The experiment was operated for 35 days in 2 phases (phase 1, d 0 to 14; and phase 2, d 15 to 35). No significant differences were shown in growth performance, feed intake, FCR, and nutrient digestibility among the treatments. The concentration of Cu in the serum was increased in the HME-Cu broilers compared with the INO broilers at phase 2. A linear increase was observed in the concentration of Cu in the liver in broilers fed INO diets, however, no significant differences were observed by the supplementation of HME-Cu levels. The linear increase was detected in the content of Cu in excreta in the INO and HME-Cu treatments by increasing the dietary Cu content. The HME-Cu treatments showed a lower Cu concentration in the excreta compared with the INO treatments. The higher bioavailability of Cu in HME form can decrease the recommended dose of Cu in broiler diets.

Keywords: Nano copper, Bioavailability, Chickens, Liver, Serum, Excreta

INTRODUCTION

Copper (Cu) is essential as a component of various metalloenzymes that are associated with bone mineralization, collagen formation, immunity, and red blood cell production by assisting in iron absorption and metabolism [1,2]. Copper has been known as an indispensable mineral that commonly supplemented with either inorganic (sulfates) or organic (bioplexes and chelates) sources due to a deficiency of Cu in diets. The bioavailability of Cu is dependent on the source of Cu where the organic

to this article was reported.

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Availability of data and material
Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions
Conceptualization: Hosseindoust A, Chae B.
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Ethics approval and consent to participate
The project underwent proper ethical standards and the experiments (KW-170519-1) were approved by the Institutional Animal Care and Use Committee of Kangwon National University, Chuncheon, Korea.

sources mostly show a greater bioavailability compared with inorganic salts [3,4]. The inorganic minerals have to break down to smaller particles in the digestion process, and this process hinders their absorption [5]. It has been reported that a pharmacological dose of Cu improves performance and feed efficiency in broilers [1,4,6,7]. The antimicrobial properties of high levels of $CuSO_4$ have also well documented [4,8,9]. However, continuous use of trace elements in high doses as growth stimulators causes environmental issues concerning the high mineral salts content in poultry wastes [2,10]. There are ongoing studies aimed at finding proper options to maximize the growth performance, particularly that the growth performance of broiler chickens has been dramatically increased because of the genetically improved species.

In recent years, the researchers have focused on the development of highly bioavailable mineral sources such as nano-sized particles in the animal feed industry, due to their novel properties [11]. The hot-melt extrusion (HME) procedure is among the top-down methods to reduce particle size was introduced before. This technique was used to produce the nanoparticle size of trace elements as colloidal status [11]. Through the HME process, inorganic Cu ($CuSO_4$) was spread out, mixed with insolvent, and prepared for the present study. As known in the previous study, the HME technique already used in the drug industry via solid dispersion formulation even product as low level in water-soluble drugs [12,13]. Moreover, the soluplus as the main polymer matrix was applied in the HME process to optimize the solubility and bioavailability due to high amphiphilic characteristics [11]. The soluplus-based HME manufacturing process can be considered as a promising method in the pharmaceutical industry and animal feed industry. In addition, environmental pollution is likely to be reduced by improved absorption and utilization of trace minerals. Therefore, the present study has been designed to focus on the bioavailability of Cu from $CuSO_4$ and HME-Cu sources in broilers fed a basal diet. Furthermore, this study evaluates the effects of dietary supplemented Cu concentration and source on body weight (BW) gain, concentration of Cu in tissue or excreta, and nutrients digestibility.

MATERIALS AND METHODS

The protocol for this study was approved by the Institutional Animal Care and Use Committee of Kangwon National University, Korea.

Process of HME-Cu production

Copper sulfate, Tween 80, Span 80, and PEG 6000 were blended at 12:20:4:64 ratios to initiate the processing. The process was started by moving the mixture to the feed hopper by a speed of 45 g/min. The used STS-25HS twin-screw extruder (Hankook E.M., Pyeongtaek, Korea) was incorporated with a round-shaped die (1 mm diameter) to produce the extruded items [11]. A temperature of 45℃ and 50℃ were considered for the barrel and die, respectively. A speed of 150 rpm was considered for hot-melt extruder screw during the HME process. The samples were extruded through the die after passing the conveyer and kneader sections in the barrel. The milling process of extruded substances was performed by a grinder (HBL-3500S, Samyang Electronics, Gunpo, Korea). The dynamic light scattering and laser doppler methods (ELS-Z1000, Otsuka Electronics, Tokyo, Japan) were applied to measure the polydispersity index, hydrodynamic size, and zeta potential of $CuSO_4$ nanoparticles.

Cu concentration

Cu concentrations in the diets, feces, serums, and livers were analyzed on the dissolved ashes produced by inductively coupled plasma (ICP) emission spectroscopy according to AOAC [14].

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The Cu determination samples were measured in triplicates and one g of ground diets and feces samples were dry ashed for one h using a muffle furnace at the temperature of 600℃. Then, after cooling the ashed samples, a 10 mL of 50 % HCl (v/v) was used to dissolve the samples and keep covered overnight. The filtration was performed by using Whatman filter paper in a 100 mL volumetric flask by three times washing the crucibles. Then, the deionized distilled water was used to dilute the samples to determine the Cu concentrations by ICP. For serum sample, one mL sample was used in porcelain crucibles, then oven-dried (4 h at 105℃) and ashed (1 h at 600℃) in a muffle furnace. The liver sample was dried (24 h at 105℃) and ground by a steel blade grinder. One g of liver sample was selected and dry ashed (600℃ for 1 h) in a muffle furnace. Then, the concentration of Cu in the liver and serum samples were determined by the same procedure as the diet and feces samples. The copper content of basal diet was 8.61 mg/kg diet.

Animals and experimental design

A total of 720 broilers (Ross 308 - day old) with an average BW of 41.62 ± 0.8 g were randomly divided between the treatments as a randomized complete block (RCB) design. The study was performed in the Research Center of Animal Life Sciences at Kangwon National University. There were 8 treatments and 6 replicates in each treatment with 15 birds/replicate. The treatments were divided into common copper sulfate at the doses of 16 ppm, 40 ppm, 80 ppm, and 120 ppm (INO) and hot-melt extruded copper sulfate at the doses of 16 ppm, 40 ppm, 80 ppm, and 120 ppm (HME-Cu). Two forms of diets were provided for this experiment for 35 days in 2 phases (phase 1, d 0 to 14; and phase 2, d 15 to 35). The diets formula was according to nutrient requirements in Ross 308 catalog [15] to maintain all the nutrients (Table 1).

Experimental procedure and sampling

Growth performance

The growth performance, feed consumption (FI), and feed conversion ratio (FCR) were measured in groups (15 chicks/pen) and calculated by recording the birds performance weekly. The FCR was calculated as FCR = total feed intake /total bogy weight gain.

Blood, slaughtering, and tissues sampling

At day14 and 35, five birds per replicate around the average BW were selected for the blood collection. The collection of blood samples was performed via the jugular vein after 2-hr feed withdrawal and ethilen dianmin acetic acid tube and serum separate tube were used for blood collection. Then, the centrifuge at the speed of 1,000 g was applied for 15 min at 4℃ to isolate the serum. The collected serum was immediately transferred and frozen at −20℃ for further analyses. Natt-Herrick solution was applied to count the number of red blood cells (RBC) and white blood cells (WBC). The hematocrit (HCT) and hemoglobin (Hb) analysis were performed by microhaematocrit and cyanmethaemoglobin methods, respectively. At day 35 of the feeding trial, two birds around the average weight per replicate were selected and slaughtered for tissue sampling. The harvested liver samples were directly frozen in liquid nitrogen to be kept at −80℃ to await analysis.

Digestibility

The excreta samples were collected to evaluate the digestibility of dry matter (DM), crude protein (CP), and gross energy (GE) at the end of each phase. At the last 6 days in each phase, 2 birds per replicate were transferred to individual cages (two bird/cage) for excreta samples collection. During

Table 1. Ingredients and chemical composition of basal diet (as-fed basis)

Item	Phase 1	Phase 2
Ingredient (%)	100.00	100.00
Corn	52.88	58.93
Soybean meal	38.8	31.56
Corn gluten meal	2.5	3.6
Animal fat	2.2	2.4
Limestone	0.61	0.57
Mono-di calcium phosphate	1.67	1.56
Salt	0.2	0.2
Vitamin premix[1)]	0.15	0.15
Mineral premix[2)]	0.15	0.15
$_{L}$-Lysine (55%)	0.24	0.20
$_{L}$-Threonine (78%)	0.24	0.38
$_{DL}$-Methionine (84%)	0.28	0.22
Choline chloride (42%)	0.08	0.08
Calculated chemical composition		
Metabolizable energy (kcal/kg)	3,000	3,100
Crude protein (%)	23.00	21.50
Calcium (%)	0.96	0.87
Available phosphorus (%)	0.48	0.44
SID lysine (%)	1.28	1.15
SID methionine (%)	0.58	0.52

[1)]Supplied per kg diet: 12,000 U vitamin A, 5,000 U vitamin D3, 80 mg vitamin E, 3.2 mg vitamin B1, 8.6 mg vitamin B2, 5.4 mg vitamin B6, 0.017 mg vitamin B12, 3.2 mg vitamin K3, 18 mg pantothenic acid, 60 mg niacin, 0.30 mg biotin, 2.2 mg folic acid, 18 mg ethoxyquin.

[2)]Supplied per kg diet: 20 mg Fe, 110 mg Zn, 120 mg Mn, 1.25 mg I, 0.3 mg Se. The copper content was depend on the treatments.

SID, standardized ileal digestiblity.

the trial, 2.5 g/kg indigestible index (chromium oxide) was added to the diet. A hundred-gram excreta sample was collected daily per cage at phase 1 (9–11 d) and phase 2 (30–33 d). A forced air-drying oven was applied to dry the samples at 60℃ temperature for 72 h and minced with Wiley laboratory mill (Thomas Model 4 Wiley®Mill, Thomas scientific, Swedesboro, NJ, USA) by 1 mm screen.

The total nutrient utilization used this formula as:

$$\text{Total nutrient utilization (\%)} = 100 - \left[100 \times \left(\frac{\text{Cr in feed (\%)}}{\text{Cr in excreta (\%)}} \times \frac{\text{Nutrient in excreta (\%)}}{\text{Nutrient in feed (\%)}}\right)\right]$$

Statistical analysis

The experimental data were analyzed by using the GLM procedure of SAS (SAS Institute, Cary, NC, USA) in a randomized complete block design. For doses comparison (16 ppm, 40 ppm, 80 ppm, and 120 ppm), the linear and quadratic contrasts were applied to evaluate the influences of increasing dietary Cu sources. Also, the polynomial contrast was used to compare the HME and INO treatments by the SAS program (SAS Institute). Pen replicate was the experimental unit for growth performance and individual broiler chick was considered as an experimental unit for blood parameters, digestibility of nutrients, and Cu concentrations in the organs. Significance level was set at $p < 0.05$ and values $0.05 \leq p \leq 0.1$ were considered as tendency.

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RESULTS

Nanoparticle properties

An average size of 41 nm (11–67 nm) was detected for HME-Cu nanoparticles. The average zeta potential of the nanoparticles solution was −18.7 mV, showing a medium stability of colloids.

Growth performance, feed intake, and feed conversion ratio

The influence of Cu source and concentration on growth responses is presented in Table 2. During phases 1, 2, and overall, two main factors (Cu source and concentration) did not show any significant differences in growth performance, feed intake, and FCR among the treatments.

Cu concentration in the serum, liver, and excreta

The influence of Cu source and concentration on blood compositions is presented in Table 3. In phase 1, there was no difference in the serum Cu, however, the concentration of Cu in the serum was increased ($p < 0.01$) in the HME-Cu broilers compared with the INO broilers at phase 2. A linear increase ($p < 0.01$) was observed in the concentration of Cu in the liver in broilers fed INO diets, however, no significant differences were observed by the supplementation of HME-Cu levels. The linear increase was detected in the content of Cu in excreta ($p < 0.01$) in the INO and HME-Cu treatments by increasing the dietary Cu content. The HME-Cu treatments showed a lower ($p < 0.05$) Cu concentration in the excreta compared with the INO treatments.

Table 2. Effects of dietary Cu concentrations and sources on growth performance in broiler chickens

		Weight gain (g/bird)			Feed intake (g/bird)			FCR		
		0 to 14 d	15 to 35 d	0 to 35 d	0 to 14 d	15 to 35 d	0 to 35 d	0 to 14 d	15 to 35 d	0 to 35 d
Cu source × Concentration										
INO (ppm)	16	317	1,366	1,683	480	2,213	2,693	1.52	1.62	1.60
	40	324	1,372	1,696	492	2,214	2,706	1.52	1.61	1.60
	80	322	1,377	1,699	492	2,172	2,665	1.53	1.58	1.57
	120	323	1,397	1,719	492	2,225	2,717	1.53	1.59	1.58
HME (ppm)	16	320	1,366	1,686	486	2,185	2,671	1.52	1.60	1.58
	40	323	1,409	1,731	493	2,225	2,718	1.53	1.58	1.57
	80	333	1,379	1,711	501	2,172	2,673	1.51	1.58	1.56
	120	329	1,377	1,706	500	2,202	2,703	1.52	1.60	1.58
Pooled SEM		1.72	5.69	5.63	2.29	14.41	14.78	0.01	0.01	0.01
Contrast										
HME vs INO		0.171	0.216	0.717	0.688	0.762	0.547	0.418	0.908	0.519
Effect of INO level										
SEM		2.29	9.04	8.72	2.93	23.54	24.05	0.01	0.01	0.01
Linear		0.517	0.253	0.173	0.209	0.978	0.902	0.524	0.385	0.460
Quadratic		0.512	0.715	0.833	0.296	0.608	0.704	0.775	0.757	0.798
Effect of HME level										
SEM		2.54	7.08	7.17	3.49	17.10	17.74	0.01	0.01	0.01
Linear		0.088	0.988	0.516	0.107	0.987	0.771	0.835	0.974	0.873
Quadratic		0.529	0.116	0.077	0.570	0.890	0.813	0.966	0.343	0.376

FCR, feed conversion ratio; INO, copper sulfate; HME, hot melt extrusion copper sulfate.

Table 3. Effects of dietary Cu concentrations and sources on Cu concentration in liver, serum, and excreta in broiler chickens

		Serum (ng/dL)		Liver (mg/kg)	Excreta (mg/kg)
		14 d	35 d		
Cu source × Concentration					
INO (ppm)	16	7.6	9.5	1.77	34.20
	40	9.8	9.9	1.78	64.50
	80	10.8	9.4	1.87	140.30
	120	9.5	10.3	2.14	162.97
HME (ppm)	16	12.0	12.4	1.93	26.93
	40	11.6	12.3	2.01	52.22
	80	11.5	10.4	1.84	123.57
	120	9.2	13.3	1.73	141.63
Pooled SEM		0.50	0.42	0.04	11.46
Contrast					
INO vs HME		0.137	0.007	0.201	0.038
Effect of INO level					
Linear		0.743	0.586	0.008	< 0.001
Quadratic		0.981	0.789	0.169	0.399
Effect of HME level					
Linear		0.230	0.911	0.184	< 0.001
Quadratic		0.539	0.225	0.435	0.067

INO, copper sulfate; HME, hot melt extrusion copper sulfate.

Nutrient digestibility

The influence of Cu source and concentration on nutrient digestibility is presented in Table 4. The two main factors (different Cu source and concentration) in this experiment did not change the digestibility of DM, GE, and CP at phase 1 and phase 2.

Blood compositions

The influence of Cu source and concentration on hematological parameters is presented in Table 5. The two main factors (different Cu source and concentration) in this experiment did not change the blood content of WBC, RBC, Hb, and HCT at phase 1 and phase 2. However, a tendency ($p = 0.069$) was observed for the Hb of broiler chickens to be influenced by the HME treatments compared the INO treatments.

DISCUSSION

To maintain the Cu requirement in chicks diet, 16 mg Cu per kg of feed is recommended [15]. However, in recent years, the supplementation of Cu at much higher levels than the recommendation is a routine work to improve growth performance [1,3,4]. Wang et al. [16] reported that the growth performance and feed intake of broilers received 50, 100, or 150 mg/kg of copper chitosan nanoparticles were increased during 0 to 42 d, in comparison with the control treatment without Cu supplementation. The copper sulfate is the most common Cu form used in poultry diets, then in this study was selected as a control treatment. There were no differences in the weight gain, feed intake, and FCR, and it may show that the levels of dietary Cu were much higher than the requirement. In agreement, Liu et al. [17] reported no improvement in growth performance and

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Table 4. Effects of dietary Cu concentrations and sources on nutrients digestibility in broiler chickens

		Dry matter		Gross energy		Crude protein	
		7 to 14 d	28 to 35 d	7 to 14 d	28 to 35 d	7 to 14 d	28 to 35 d
Cu source × Concentration							
INO (ppm)	16	69.49	68.59	70.04	69.42	61.22	60.93
	40	71.82	69.32	72.00	70.03	62.18	61.42
	80	72.07	73.51	73.19	71.39	62.46	61.87
	120	71.27	70.63	71.60	71.26	61.51	61.25
HME (ppm)	16	71.30	70.58	71.55	69.30	61.38	61.03
	40	71.02	70.14	71.18	70.01	60.92	60.73
	80	71.92	71.19	70.82	69.49	61.10	60.92
	120	71.81	70.77	72.35	71.52	62.25	61.76
Pooled SEM		0.29	0.70	0.28	0.36	0.28	0.33
Contrast							
INO vs HME		0.559	0.662	0.507	0.921	0.583	0.743
Effect of INO level							
SEM		0.45	1.36	0.46	0.63	0.30	0.48
Linear		0.149	0.449	0.109	0.290	0.686	0.777
Quadratic		0.080	0.552	0.040	0.791	0.155	0.627
Effect of HME level							
SEM		0.36	0.48	0.34	0.38	0.48	0.47
Linear		0.527	0.748	0.527	0.066	0.575	0.631
Quadratic		0.921	0.994	0.201	0.334	0.469	0.610

INO, copper sulfate; HME, hot melt extrusion copper sulfate.

feed intake in broiler chickens by supplementating 125 or 250 mg/kg copper sulfate, whereas the concentration of Cu in the plasma and liver were linealy increased. However, in ovo administration of 50 mg/kg copper sulfate or Cu nanoparticles [18], 50 mg/kg in ovo Cu nanoparticles plus 20 mg/kg Cu nanoparticles in drinking water [7] increased chicken growth performance and FCR. The trace elements particles can be agglomerated in basic or acidic environment [17,18], as showed for copper sulfate nano particles [19]. The insignificant growth performance result shows that the pharmacological doses of Cu are not recommended for broiler chickens, particularly for the recent genetically modified species.

In the present experiment, the excretion of Cu was linearly increased in both the INO and HME treatments. It may be because of the interactive effects of Fe and Zn with Cu absorption [3,6], particularly when the dietary Cu level is high. Moreover, the excretion of Cu was lower in the HME treatments compared with the INO treatments, indicating that the supplementation of nanoparticles can decrease the amount of Cu excretion when the same level of Cu is supplemented either as HME-Cu or $CuSO_4$. Among the possible reasons, the particle size of Cu is a determinant factor for absorption, as the smaller particle sizes cross the intestinal mucus layer at a higher rate [2]. The nanoparticles can also be absorbed by an active transport system through enterocytes [20]. After absorption in the GIT, the nanoparticle size of Cu enters the bloodstream and retains in the relative organs or it independently flows in the blood circulation [21]. Thus, the smaller particle size and higher uniformity of Cu nanoparticles may be associated with their improved absorption and decreased excretion. The increase of liver Cu in this experiment was in agreement with previous findings that supplementation of 150 mg/kg Cu increased the concentration of Cu in the liver compared with broiler chickens fed 50 mg/kg Cu [22]. In addition, the higher concentration of

Table 5. Effects of dietary Cu concentrations and sources on haematological parameters in broiler chickens

Item		WBC (10^3/μL)		RBC (10^6/μL)		Hb (g/dL)		HCT (%)	
		14 d	35 d	14 d	35 d	14 d	35 d	14 d	35 d
Cu source × Concentration									
INO (ppm)	16	28.09	22.01	1.93	2.21	9.80	6.73	22.00	28.00
	40	26.55	24.41	1.87	2.28	8.90	7.06	21.53	29.04
	80	22.25	25.15	1.89	2.32	9.23	7.28	21.07	29.30
	120	26.80	25.07	1.81	2.22	8.40	7.25	20.67	28.20
HME (ppm)	16	23.66	22.91	1.84	2.44	9.47	7.48	21.33	30.70
	40	26.75	23.50	1.98	2.20	8.70	7.13	22.37	29.03
	80	28.00	24.32	1.95	2.18	8.97	7.44	22.03	27.86
	120	20.75	25.76	1.88	2.29	9.43	7.36	21.83	28.92
Pooled SEM		0.74	0.64	0.02	0.04	0.19	0.12	0.20	0.50
Contrast									
INO vs HME		0.387	0.665	0.272	0.626	0.885	0.069	0.144	0.460
Effect of INO									
SEM		0.89	1.06	0.07	0.04	0.34	0.15	1.01	0.54
Linear		0.192	0.379	0.309	0.879	0.183	0.261	0.159	0.880
Quadratic		0.670	0.601	0.881	0.346	0.957	0.591	0.960	0.385
Effect of HME level									
SEM		0.90	0.80	0.07	0.07	0.33	0.19	0.95	0.81
Linear		0.411	0.217	0.710	0.450	0.947	0.121	0.630	0.396
Quadratic		0.998	0.801	0.947	0.226	0.286	0.145	0.270	0.435

WBC, white blood cell; RBC, red blood cell; Hb, hemoglobin; HCT, hematocrit; INO, copper sulfate; HME, hot melt extrusion copper sulfate.

Cu in the serum of the HME-Cu treatment may confirm the geater bioavailability compared with birds fed common $CuSO_4$.

A previous study [23] showed that the counts of WBC, lymphocytes, and eosinophils in chicks was higher in common $CuSO_4$ compared to nanoparticle Cu. However, in this study, HME-Cu showed no change in the count of leucocytes compared with the INO treatment. In agreement, Samantha et al. [1] reported no change in the number of heterophils, eosinophil, basophil, lymphocytes, and monocytes in broiler chicks fed a range of 0 to 250 mg Cu/kg diet, however, they reported a higher Hb in chicks fed 250 mg Cu compared with chicks fed 0 or 75 mg Cu per kg diet. In the current study, no significant change was observed among the treatments, however, there was a tendency (p = 0.069) for increased number of Hb in the HME-Cu treatments compared with the INO treatments. The increased concentration of Hb allows the chickens to maintain oxygen transport efficiency at a high level [23]. It has been known that Cu stimulates the synthesis of erythrocyte, which increase the iron absorption and consequently increasing Hb production [2]. The result of this study may emphasize the homeopathic function of Cu in increasing Hb levels.

The Cu salts obtain a low digestibility of around 20% [24], excreting through feces, and producing environmental issues [25]. Several studies have confirmed the effective influences of nano Cu supplementation in poultry diets [2,7], however, the processing method and nano technique may affect the bioavailability of Cu. In this experiment, we used HME-Cu processed Cu as a feed additive delivered. However, this experiment did not show significant differences in growth performance but the higher bioavailability of HME-Cu may emphasize the role of the

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nano process on the efficiency of Cu utilization.

CONCLUSION

In conclusion, the present study showed no significant differences in the final growth performance of chickens, showing that the pharmacological dose of Cu (125 mg/kg) is not essentially necessary to be used in broiler diets. However, the lower excretion of Cu through excreta in the HME-Cu treatments shows the higher bioavailability and may allow decreasing the dietary dose of Cu in maintaining the requirement of broiler chickens compared with the common $CuSO_4$.

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