Sources and levels of copper and manganese supplementation influence performance, carcass traits, meat quality, tissue mineral content, and ileal absorption of broiler chickens

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ABSTRACT This study was conducted to evaluate the effects of different levels and sources of Cu and Mn (sulfate or hydroxychloride - H) on growth performance, carcass traits, meat and skin quality, footpad dermatitis severity, litter quality, liver and plasma mineral content, and ileal mineral absorption. A total of 1,920 one-day-old male Cobb 500 broiler chicks were assigned randomly to one of 2 × 3+2 factorially arranged treatments: CuH (15 and 150 ppm) × MnH (40, 80, 120 ppm) + 15 ppm Cu Sulfate with 80 ppm Mn Sulfate (control 1) or 150 ppm Cu Sulfate with 120 ppm Mn sulfate (control 2) for 42 d. Each treatment consisted of 8 replicates of 30 birds. At 42-day-old were slaughtered for carcass yield and meat quality analyses. At 43-day-old, it was determined the apparent ileal absorption of minerals and the concentration of Cu and Mn in the liver and plasma. The resistance and elasticity of the skin, and footpad dermatitis severity were also evaluated. The level 150 ppm CuH improved the FCR compared to the 15 ppm CuH and 15 ppm Cu Sulfate level. Broilers fed diets containing 150 ppm CuH showed higher breast yield compared to those fed diets containing 15 ppm. Breast yield was positively influenced by the inclusion of 40 ppm MnH. There was an interaction between the CuH and the MnH for skin elasticity, and the highest elasticity was found when the supplementation levels were 150 ppm CuH and 40 ppm MnH. High levels of copper decreased the incidence of footpad dermatitis. The hydroxychloride source determined a higher mineral concentration in the liver and plasma and greater apparent ileal absorption of Cu and Mn. In conclusion, dietary supplementation with 150 ppm CuH and 40 or 80 ppm MnH enhance breast yield and improves skin resistance. The inclusion of 150 ppm CuH has the possibility to improve the FCR and decrease leg injuries. Furthermore, the hydroxychloride source seems to be more bioavailable than the sulfate source.

Key words: bioavailability, inorganic source, hydroxychloride, poultry nutrition, trace minerals

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INTRODUCTION

Trace minerals, such as copper and manganese, are particularly important for maintaining health and productivity of broiler chickens, due to their effects on several physiological processes (Richards et al., 2010). Copper (**Cu**) is a structural component of macromolecules and a cofactor for several essential oxides and monooxidases (cuproenzymes) (McDowell, 2003; Kaneko et al., 2008). The lysyl oxidase, an enzyme that cross-links the collagen subunits in mature protein forms to increase its resistance, is copper-dependent (Rucker et al., 1998) and many other important functions in animal metabolism and health, as well as it is a growth promoter (Pesti and Bakalli, 1996; Paik et al., 1999; Morais et al., 2001). Manganese (**Mn**) is an important nutrient in poultry nutrition and feeding due to its role in the development of bones and eggshell, enzymes component and nutrient metabolism (Tufarelli and Laudadio, 2017). There are several manganese-dependent enzymes called metalloenzymes, including arginase, phosphoenolpyruvate decarboxylase, glutamine synthetase, as well as Mn-superoxide dismutase (**Mn-SOD**) (McDowell, 2003).

Mineral supplementation in chicken feed normally occurs from inorganic sources (sulfates, carbonates, chlorides, and oxides) and fewer from organic sources (metal ions linked to organic molecules). The inorganic

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source is known to have low bioavailability while the organic source is controversial in the literature about its real bioavailability. To increase stability and bioavailability of these trace minerals, the industry is now focusing at the hydroxychlorides (Perez et al., 2017; Olukosi et al., 2018; Nguyen et al., 2020).The hydroxychlorides of copper and manganese in broiler nutrition (Intellibond C - CuH and Intellibond M - MnH, respectively) have emerged as an alternative source of mineral supplementation and are known for their great strength in chemical bonding resulting in more stability in the diet and in gastrointestinal tract (Cohen and Steward, 2012; Villagómez-Estrada et al., 2020).

Although studies have been carried out with hydroxychlorides, in isolation or the association of copper with zinc, to our knowledge there are no studies on the interaction of copper and manganese hydroxychloride for broilers chickens. Therefore, it is important to understand how these hydroxychloride minerals acting in comparing to the traditional inorganic sources, and if the use of different levels of Cu and Mn influences chicken growth performance. Thus, the objective of the present study was to evaluate the effect of different sources and levels of copper and manganese (sulfate and hydroxychloride) on growth performance, carcass characteristics, meat quality, footpad and litter condition, skin resistance, liver and plasma mineral content, and apparent ileal absorption of Cu and Mn.

MATERIALS AND METHODS

All procedures described below were previously approved by Ethics Committee on Animal Use from the São Paulo State University (Unesp), School of Veterinary Medicine and Animal Science, Botucatu, under protocol number 0191/2018.

Experimental Design and Diets

The experiment was conducted in the poultry laboratory at São Paulo State University (**UNESP**), College of Veterinary Medicine and Animal Science, Botucatu. A total of 1,920 one-day-old male Cobb broiler chicks were allocated to 8 dietary treatments in a fully randomized design with a 2 × 3+2 factorial arrangement. The factors were 2 levels of Cu hydroxychloride (15 and 150 ppm) and 3 levels of Mn hydroxychloride (40, 80, 120 ppm) and 2 additional treatments that were the controls with sulfate source (15 ppm Cu sulfate with 80 ppm Mn sulfate and 150 ppm Cu sulfate with 120 ppm Mn sulfate). Each of the eight treatments had 8 replicates and 30 birds per replicate, randomly assigned to 64 pens. The levels of mineral supplementation were determined according to the levels of nutritional requirement proposed by Rostagno et al. (2011), using recommended levels, above or below the requirement.

Table 1. Ingredients (%) and calculated nutritional levels of the basal diet.

Ingredients, %	Pre-starter (1−7 d)	Starter (8−21 d)	Grower (22−35 d)	Finisher (36−42 d)
Corn, 8%	59.06	61.58	64.44	68.75
Soybean meal, 46%	36.64	33.81	30.31	26.48
Soybean oil	0.50	1.32	2.31	2.21
Dicalcium phosphate	1.22	0.87	0.64	0.41
Limestone*	0.95	0.98	0.94	0.87
Salt	0.52	0.48	0.46	0.44
DL-Methionine, 99%	0.340	0.290	0.270	0.250
L-Lysine HCl, 99%	0.320	0.270	0.270	0.300
Premix[1]	0.200	0.180	0.160	0.120
L-Threonine 98,5%	0.120	0.090	0.080	0.080
Choline Chloride, 60%	0.072	0.063	0.058	0.038
Salinomycin	0.055	0.055	0.055	-
Phytase 500FTU[3]	0.005	0.005	0.005	0.005
Calculated levels				
AME[2], Kcal/kg	2,964	3,050	3,150	3,200
Crude protein, %	22.39	21.20	19.80	18.40
Calcium, %	0.93	0.84	0.76	0.66
Available phosphorus, %	0.47	0.40	0.35	0.31
Sodium, %	0.23	0.21	0.20	0.20
Potassium, %	0.87	0.83	0.77	0.71
Chlorine, %	0.42	0.39	0.37	0.37
Lysine dig, %	1.32	1.21	1.13	1.06
Methionine dig, %	0.66	0.60	0.56	0.53
Met+Cys dig, %	0.95	0.88	0.83	0.77
Threonine dig, %	0.86	0.79	0.74	0.69

[1]Trace mineral and vitamim premix supplied the following per kg of diet: vitamin A, 13,500 UI; vitamin D3, 3,750 UI; vitamin E, 30 UI; vitamin K3, 3.75 mg; vitamin B1, 3 mg; vitamin B2, 9 mg; pantothenic acid, 18 mg; vitamin B6, 4.5 mg; vitamin, B12 22.5 μg; niacin, 52.5 mg; folic acid, 2.25 mg; Biotin, 0.15 mg; Se, 0.375 mg; Fe, 50 mg; Co, 1 mg; I, 1 mg; Zn Hydroxychloride, 80 mg.

[2]Apparent metabolizable energy.

[3]Used of the nutritional matrix.

*Each level and source of supplementation of Cu and Mn were included in the basal diet through the replacement of limestone.

The basal diet was formulated based on corn and soybean meal following the recommendations by Rostagno et al. (2011) in four phases: pre-starter (d 1−7), starter (d 8−21), grower (d 22−35), and finisher (d 36−42) (Table 1). The premix was Cu and Mn free. Each level and source of supplementation of Cu and Mn were included in the basal diet (**BD**) through the replacement of limestone.

To achieve the required dietary levels of Cu in control group (15 and 150 ppm) copper sulfate monohydrate (**CSM**) 35% was added at the rates of 43 or 429 g/ton, respectively. To achieve the required dietary levels of Cu hydroxychloride (15 and 150 ppm) was added 28 or 278 g/ton, respectively of CuH 54%. To achieve the required dietary levels of Mn in control group (80 or 120 ppm) sulfate monohydrate (**MSM**) 31% was added at the rates 258 or 387 g/ton, respectively. To achieve the required dietary levels of Mn hydroxychloride (40, 80 and 120 ppm) was added 89, 179, or 268 g/ton, respectively of MnH 44%.

Feed samples from each treatment and period were analyzed to determine Cu and Mn content (ppm) using inductively coupled plasma-mass spectroscopy (**ICP-MS**) (Table 2).

Table 2. Supplementary levels of trace minerals fed to broilers and analyzed levels of Copper and Manganese (ppm) in the diet by treatment and period.

Treatments			Analyzed levels in the diet							
			Pre-starter		Starter		Grower		Finisher	
Mineral source	Cu, ppm	Mn, ppm	Cu, ppm	Mn, ppm	Cu, ppm	Mn, ppm	Cu, ppm	Mn, ppm	Cu, ppm	Mn, ppm
Sulfate[1]	15	80	13.0	80.6	14.2	87.9	19.0	101.5	18.0	95.0
Sulfate	150	120	142.1	125.9	142.5	137.1	132.4	127.5	134.1	132.8
hydroxychloride[2]	15	40	16.7	53.5	15.2	52.4	17.4	44.6	20.2	59.1
hydroxychloride	15	80	22.0	82.1	18.0	94.9	16.4	86.7	19.7	85.2
hydroxychloride	15	120	18.7	134.8	10.0	137.5	23.0	156.1	18.3	118.0
hydroxychloride	150	40	148.9	62.0	160.0	49.1	198.4	50.9	142.7	57.2
hydroxychloride	150	80	140.7	86.8	151.5	82.1	158.7	96.8	146.4	96.3
hydroxychloride	150	120	145.2	131.1	142.2	135.2	176.1	137.4	147.6	126.3

[1]Analytical grade copper sulfate monohydrate was used, 35% - CSM ($CuSO_4 \cdot H_20$) and manganese sulfate monohydrate, 31% – MSM ($MnSO_4 \cdot H_20$).
[2]Were used Cu Hydroxychloride, (CuH, Selko IntelliBond C, Trouw Nutrition) and Mn hydroxychloride (MnH, Selko IntelliBond M, Trouw Nutrition).

Feeding and Management

The experiment was conducted in a poultry house from 1 to 42 d of age. The chicks were housed in 2 m^2 pens on reused wood shavings. Each pen was equipped with a tubular feeder and a nipple drinking. The initial heating was performed using infrared lamps (250 watts) and at the end of the 14th d of the experiment, they were removed. The poultry house was equipped with automatic control of exhaust fans and nebulizers. The temperature and lighting programs were set following the Cobb500 Management Guide (Cobb-Vantress, 2018). Daily, the values of minimum, maximum and instantaneous temperature, and relative humidity were measured using a digital thermo hygrometer (HOBO). Mash feed and water were available ad libitum.

Productive Performance

At d 21 and 42 growth performance parameters (body weight, feed intake, and feed conversion ratio) were determined. Mortality was recorded daily.

Carcass and Cuts Yield

On d 42 of age in experiment, 192 birds (24 birds per treatment) were weighed, and humanely slaughtered via electrical stunning. Afterward, they were scalded and feathers, head, viscera, and feet were removed. Carcass yield and abdominal fat were calculated in relation to body weight before slaughtering. Parts yield (breast, thighs, wings, and back, all including skin and bones) were obtained in relation to the weight of the eviscerated carcass.

Meat Quality

The breast (*Pectoralis major*) muscle of 16 broilers per treatment was stripped and stored immediately at 4°C for subsequent determination of meat quality. The muscle pH values were measured in triplicate 24 h postmortem using an electronic pH meter (HI8314, HANNA Instrument Science and Technology Co., Ltd., Beijing, China) which was calibrated with pH 4.6 and 7.0 buffers equilibrated at 37°C. Measurement was directly conducted by inserting the probe into the right side of the muscle samples. The meat color of pectoralis muscle was assessed 24 h postmortem in triplicate using a handheld colorimeter CR-400 (Minolta CR-400, Konica Minolta Sensing, Osaka, Japan) which was previously calibrated against a white tile according to the manufacturer's manual. The color was reported in the CIELAB trichromatic system as L* (lightness), a* (redness), and b* (yellowness).

In the water-holding capacity, 1g of muscle was collected, identified, wrapped in filter paper, and centrifuged 1,500 rpm for 4 min. After centrifugation, the samples were weighed and placed in the oven at 70°C for 12 h and after the fragments were weighed again. Holding water capacity (%) was calculated as follows: 100 × [(weight after centrifugation - weight after drying)/initial sample weight] (Nakamura and Katok, 1985). For cook loss the left breast muscles were weighed and cooked in a water bath in plastic bags to an internal temperature of 80°C. Then the muscles were allowed to cool to room temperature and, the mass changes were used to calculate the loss of cooking based on the initial muscle weight. Cooking loss (%) was calculated as follows: 100 × [(initial weight - cooked final weight)/initial weight] (Honikel, 1998). Then, the cooked samples were used for shear force measurement using a Texture Analyzer (TAXT-Plus, Stable Micro Systems, Surrey, UK) equipped with Warner-Bratzler device, calibrated with a normal weight range of 5 kg and device speed was 10 mm/s, evaluating the shear force (N) according to the procedure described by Cavitt et al. (2004). The samples were cut into 5 rectangles (1.0 × 1.0 × 2.0 cm) on the cranial surface and the fibers oriented perpendicular to the Razor Blade.

Skin Resistance, Footpad Scoring, and Litter Quality

Samples of the drumstick skin (3 cm) from 16 chickens per treatment were used to determine the breaking strength (kg) and elasticity (mm). The texture analyzer (Model TA-XT2i, Stable Mycro Systems LTDA., Godalming, UK) was used for this test. The samples were submitted to the flexion test at the constant strain rate for elastic material. The speed of the device was 1 mm/s, the firing force was 10 g and the tension was 15 mm (Ribeiro, 2016).

Footpad scoring was done on d 42 on 5 randomly selected birds per pen by visual inspection and three scores were assigned. The score 1 − Foot pad without apparent injuries; score 2 − Foot pad with mild injuries, and score 3 − Foot pad with severe injuries, according to Zhao et al. (2010). Total footpad score (**TFS**) was calculated using the following formula (Olukosi et al., 2018):

$$\mathrm{TFS} = \frac{(1 \times \mathrm{n}) + (2 \times \mathrm{n}) + (3 \times \mathrm{n})}{Total\ number\ of\ birds\ scored}$$

Where the **numbers 1 to 3** indicate the score assigned to each bird and **n** indicates the number of birds assigned the particular score in each pen. A lower score is associated with better footpad quality.

Dry matter content of litter and quality from each pen was evaluated at the end of the grower phase. To litter dry matter (**LDM**), about 10 g of litter was collected from 4 points per pen and placed to dry for 24 h at 105° C. Through the difference between the initial weight and the final weight, the LDM content was obtained (%). To litter quality, scores from 1 to 5 were attributed based on the welfare quality assessment protocol for poultry (Botreau et al., 2009). Score 1, if the litter was friable with no capping or compaction, to score 5, if litter was wet and dough-like. The total litter score (**TLS**) was calculated as follows:

$$\mathrm{TLS} = \frac{(1 \times \%) + (2 \times \%) + (3 \times \%) + (4 \times \%) + (5 \times \%)}{100}$$

Where the **numbers** 1 to 5 were the scores as described previously and the **%** was the percentage of the pen area corresponding to each score in each pen. A lower score is associated with better litter quality. The litter was stirred weekly until the 28th day of age.

Mineral Content in Plasma and Liver

To mineral concentration in plasma, about 5 mL of blood were collected via the jugular vein and placed in tubes with sodium heparin. Then, the blood was centrifuged for 15 min at 2000 rpm to obtain plasma and stored in plastic microtubules at −20°C. Fragments of the right lobe liver were collected and frozen at −20° until further analysis. For extraction of the metal nutrients in plasma 30 μL de plasma was transferred to 10 mL glass flask, to which were added 1.0 mL of nitric acid 14 mol/L and 0.3 μL of hydrogen peroxide 30% m/m and subsequently the samples were submitted to heating. For extraction of the metal nutrients in liver, approximately 100 mg of sample were transferred directly to the microwave oven's Teflon flasks, to which were added 3.0 mL of nitric acid 14 mol/L and 2.0 mL of hydrogen peroxide 30% m/m. For heating it was used a microwave oven (Provecto Analítica model DGT-100 Plus, Campinas, Brazil) (Rosa et al., 2002). Copper and manganese were determined using a SHIMADZU AA-6800 atomic absorption spectrometer (Neves et al., 2009).

Apparent Ileal Mineral Absorption

A total of 24 broilers with 42-day-old were randomly selected and separated according on the treatment. These birds received for 24 h the experimental diets containing 0.3% of chromium oxide (marker). After, the animals were euthanized and ileum digesta were collected from taking the distal two-thirds part of the ileum after dissection from Meckel's diverticulum to the ileocecocolic junction. The samples were stored at −20°C until analysis. Approximately 50 mg of sample and 10 mL of 0.10 mol/L hydrochloric acid solution were transferred to 50 mL Teflon flasks. The ileum digesta was then subjected to ultrasonic shaking to extract the metal analytes according to Neves et al. (2009) and digestion using nitric and perchloric acids was used to extract chromium (Kimura and Miller, 1957). The concentration of Cu, Mn, and Cr in the feed and in the ileum digesta were analyzed using inductively coupled plasma-mass spectroscopy (ICP-MS).

Apparent ileal absorption (**AIA**) of minerals was calculated using the following formula (Hafeez et al., 2014):

$$AIA(\%) = 100 - [(MF - MI) \times (NI/NF) \times 100]$$

MF = concentration (%) of marker in feed; **MI** = concentration (%) of marker in digesta; **NI** = concentration (%) of nutrient in digesta; **NF** = concentration (%) of nutrient in feed.

Statistical Analysis

The data were submitted to normally test, and after the data were analyzed by ANOVA at the $P < 0.05$ level. Significant differences of the factorial were determined by Tukey test at 5% of probability. In order to compare the factorial dietary treatment means with the additional treatments, the Dunnett test was performed ($P < 0.05$). The data were analyzed with Minitab 18 (Minitab 18 Statistical software, 2017).

RESULTS

There was no Cu × Mn interaction ($P > 0.05$) on growth performance response. When assessing the main factors, there was an effect for the FCR at 42 d of age for copper. Birds that were fed diets supplemented with 150 ppm CuH had improved the FCR than birds that fed 15 ppm CuH. In addition, the supplementation of 150 ppm CuH + 40 ppm MnH improved the FCR than 15 ppm CSM + 80 ppm MSM (Table 3).

Broilers fed diets supplemented with 150 ppm CuH showed higher breast yield compared to those fed diets containing 15 ppm. Breast yield was positively

Table 3. Growth performance of broilers supplemented with different sources and levels of copper and manganese at 1 to 21 and 1 to 42 d of age.

Treatments		1 to 21 d-old			1 to 42 d-old		
		BW, g	FI, g	FCR	BW, g	FI, g	FCR
CuH 15ppm		997.1	1,312.3	1.374	3,046.0	4,858.3	1.616[b]
CuH 150ppm		998.8	1,296.7	1.357	3,055.9	4,810.2	1.597[a]
	MnH 40ppm	998.9	1,304.8	1.378	3,061.7	4,853.7	1.606
	MnH 80ppm	996.2	1,295.7	1.360	3,060.6	4,821.6	1.604
	MnH 120ppm	999.0	1,313.0	1.358	3,030.6	4,827.3	1.613
CuH 15ppm	MnH 40ppm	1,001.8	1,322.0	1.394	3,064.2	4,899.8	1.620
CuH 15ppm	MnH 80ppm	992.8	1,297.1	1.366	3,041.2	4,803.2	1.601
CuH 15ppm	MnH 120ppm	996.8	1,318.0	1.361	3,032.6	4,871.8	1.627
CuH 150ppm	MnH 40ppm	995.9	1,287.6	1.363	3,059.2	4,807.6	1.592[X]
CuH 150ppm	MnH 80ppm	999.6	1,294.4	1.354	3,080.0	4,840.0	1.600
CuH 150ppm	MnH 120ppm	1,001.3	1,308.1	1.354	3,028.6	4,782.8	1.599
Control 1		985.5	1,317.1	1.391	3,010.5	4,852.8	1.634
Control 2		1,006.3	1,333.3	1.384	3,059.3	4,901.7	1.623
		P - value					
CuH		0.784	0.176	0.171	0.743	0.360	0.046
MnH		0.924	0.451	0.294	0.634	0.865	0.597
CuH vs. MnH		0.701	0.508	0.691	0.796	0.526	0.116
Control 1 vs. all		0.760	0.508	0.251	0.899	0.767	0.074
Control 2 vs. all		0.902	0.259	0.397	0.899	0.767	0.044
Control 1 vs. control 2		0.311	0.443	0.814	0.311	0.443	0.215
CV. %		2.04	3.06	3.04	3.22	3.34	3.03

BW, body weight; CuH, Cu Hydroxychloride; CV, coefficient of variation; FI, feed intake; FCR, feed conversion ratio; MnH, Mn Hydroxychloride. Control 1= 15 ppm Cu sulfate + 80 ppm Mn sulfate; Control 2= 150 ppm Cu sulfate + 120 ppm Mn sulfate.

[a,b]Means of factors followed by different lowercase letters differ by Tukey's test.

[X]Controls were tested by Dunnett's test and means followed by the letter "X" differ from control 1.

influenced by the inclusion of 40 ppm Mn hydroxychloride, comparing with 120 ppm, but back and wing yield were higher at 120 ppm than 80 ppm and 40 ppm, respectively (Table 4).

For the color of breast meat, higher levels of CuH showed higher value of L* and b*. Meanwhile, 40 ppm of MnH determined higher L* values compared to the 120 ppm level. Water-holding capacity, cooking loss and shear force showed no difference among treatments. Comparing the sulfate source were the controls groups to the hydroxychloride source, the pH of the control 2 (150 ppm Cu sulfate + 120 ppm Mn sulfate) determined lower values than the other treatments. Meanwhile, broilers of the control 1 determined a higher value of L* and b* compared to 15 ppm CuH and 120 ppm MnH. Control 2 determined lower values for L* compared to150 ppm CuH and 40 or 80 ppm MnH (Table 5).

There was no difference ($P > 0.05$) among different supplementation sources. It was observed an interaction between the different supplementation sources and

Table 4. Parameters of carcass and parts yield of broilers at 42 d of age supplemented with different sources and levels of copper and manganese.

Treatments		CY, %	Breast, %	Thigh, %	Back, %	Wings, %	AF, %
CuH 15ppm		74.42	39.71[b]	29.20	18.92	11.83	1.14
CuH 150ppm		74.82	40.38[a]	28.95	18.69	11.88	1.09
	MnH 40ppm	74.52	40.48[a]	28.83	18.87[ab]	11.66[b]	1.14
	MnH 80ppm	74.71	40.25[ab]	29.18	18.41[b]	11.86[ab]	1.04
	MnH 120ppm	74.64	39.41[b]	29.21	19.14[a]	12.06[a]	1.09
CuH 15ppm	MnH 40ppm	74.18	40.05	28.77	19.25	11.67	1.23
CuH 15ppm	MnH 80ppm	74.55	40.17	29.11	18.42	11.78	1.10
CuH 15ppm	MnH 120ppm	74.54	38.90	29.71	19.10	12.05	1.08
CuH 150ppm	MnH 40ppm	74.86	40.91	28.88	18.50	11.65	1.06
CuH 150ppm	MnH 80ppm	74.86	40.32	29.26	18.40	11.93	0.97
CuH 150ppm	MnH 120ppm	74.74	39.92	28.72	19.17	12.06	1.11
Control 1		73.88	40.31	28.68	19.11	11.83	1.26
Control 2		74.65	40.10	28.99	18.68	11.84	1.09
P - value							
CuH		0.241	0.049	0.289	0.213	0.683	0.183
MnH		0.686	0.030	0.310	0.006	0.032	0.418
CuH vs. MnH		0.909	0.543	0.076	0.140	0.840	0.485
Control 1 vs. all		0.418	0.053	0.145	0.056	0.273	0.207
Control 2 vs. all		0.876	0.061	0.170	0.060	0.278	0.552
Control 1 vs. control 2		0.099	0.670	0.459	0.082	0.972	0.163
CV. %		2.08	4.98	4.85	5.70	6.13	2.20

Abbreviations: AF, Abdominal fat; CuH, Cu Hydroxychloride; CY, Carcass yield; CV, coefficient of variation; MnH, Mn Hydroxychloride. Control 1= 15 ppm Cu sulfate + 80 ppm Mn sulfate; Control 2= 150 ppm Cu sulfate + 120 ppm Mn sulfate.

[a,b]Means of factors followed by different lowercase letters differ by Tukey's test. Controls were tested by Dunnett's test.

Table 5. pH, lightness (L*), redness (a*), yellowness (b*), water-holding capacity (HWC), cooking loss, and shear force in chicken breasts at 42 d of age supplemented with different sources and levels of copper and manganese.

Treatments		pH	a*	b*	L*	WHC, %	Cook loss, g	Shear force, N
CuH 15ppm		6.26	4.24	6.10[b]	54.64[b]	60.42	19.39	14.78
CuH 150ppm		6.26	4.56	6.81[a]	56.16[a]	59.78	19.77	14.14
	MnH 40ppm	6.28	4.25	6.91	56.01[a]	60.24	20.11	15.71
	MnH 80ppm	6.28	4.45	6.35	55.55[ab]	59.73	18.98	14.26
	MnH 120ppm	6.23	4.51	6.09	54.64[b]	60.35	19.65	13.42
CuH 15ppm	MnH 40ppm	6.28	3.84	6.64	55.58	60.23	20.10	15.18
CuH 15ppm	MnH 80ppm	6.25	4.43	6.33	54.87	59.94	18.88	15.45
CuH 15ppm	MnH 120ppm	6.26	4.46	5.33[X]	53.49[X]	61.10	19.19	13.73
CuH 150ppm	MnH 40ppm	6.29[Y]	4.66	7.19[X]	56.45[Y]	60.24	20.11	16.24
CuH 150ppm	MnH 80ppm	6.30[Y]	4.47	6.38	56.24[Y]	59.51	19.08	13.07
CuH 150ppm	MnH 120ppm	6.21	4.57	6.85	55.80	59.60	20.11	13.12
Control 1		6.27[a]	4.30	6.20	55.24	60.92	19.05	13.94
Control 2		6.18[b]	5.03	6.52	54.85	60.18	17.78	14.22
P - value								
CuH		0.930	0.867	0.030	0.001	0.361	0.719	0.428
MnH		0.202	0.282	0.106	0.036	0.743	0.676	0.074
CuH vs. MnH		0.171	0.256	0.168	0.389	0.660	0.932	0.221
Control 1 vs. all		0.339	0.353	0.049	0.003	0.752	0.972	0.146
Control 2 vs. all		0.021	0.379	0.059	0.002	0.884	0.811	0.175
Control 1 vs. control 2		0.025	0.158	0.598	0.610	0.414	0.331	0.827
CV. %		1.73	4.03	24.46	25.61	5.19	24.42	26.81

Abbreviations: CuH, Cu Hydroxychloride; CV, coefficient of variation; MnH, Mn Hydroxychloride; WHC, water-holding capacity; L*= lightness; a*= redness; b*= yellowness. Control 1= 15 ppm Cu sulfate + 80 ppm Mn sulfate; Control 2 = 150 ppm Cu sulfate + 120 ppm Mn sulfate.

[a,b]Means of factors followed by different lowercase letters differ by Tukey's test.

[X,Y]Controls were tested by Dunnett's test and means followed by letter "X" differ from control 1 and letter "Y" differ from control 2.

levels of minerals for skin elasticity. The interaction shows that the level 150 ppm of Cu + 40 ppm of Mn determined higher elasticity than level of 150 ppm of Cu + 80 ppm of Mn. For TFS, higher levels of copper improved footpad scores and lower litter humidity value ($P < 0.05$). The MnH did not alter the TFS or litter quality ($P > 0.05$; Table 6).

Broiler fed diets supplemented with higher level of Cu and Mn determined higher ($P < 0.05$) plasma and liver Cu concentration. The levels of 80 and 120 ppm of MnH determined higher Mn plasma contents and the level 120 ppm of MnH determined higher concentration of Mn in the liver. There was an interaction ($P < 0.05$) between CuH and MnH for liver Mn contents. Mn liver content tended to be lower when higher level of Cu was supplemented in the diet along with 80 ppm of Mn, and lower Mn levels decrease Mn liver concentration. Comparing the sulfate source with the hydroxychloride, it can be to observed that the use of hydroxychloride, in most cases, determined higher concentrations of Cu and Mn in the liver and plasma (Table 7).

The apparent ileal absorption (AIA%) of Cu and Mn had a significant effect on broilers supplemented with the different levels and mineral source ($P < 0.05$). The 150 ppm Cu level increased the AIA of Cu when compared to 15 ppm. By supplying 80 or 120 ppm of Mn increased the AIA of this mineral than 40 ppm. When 15 ppm of CSM was supplemented, the AIA of Cu is lower than diets supplemented with hydroxychlorides, except for the diet supplemented with 15 ppm CuH + 40 ppm MnH. Finally, when 120 ppm MnH + 40 ppm CuH was used, the AIA of Cu was greater than the sulfate source with 120 ppm Mn and 150 Cu.

DISCUSSION

The improve feed conversion of broilers supplemented with high levels of CuH may be related to the performance improvement effect of Cu. Studies have shown an improved performance of broilers fed diets containing Cu levels above 125 ppm (Pesti and Bakalli, 1996; Paik et al., 1999; Morais et al., 2001; Santos et al., 2021). However, this effect was only found when the hydroxychloride source was used, demonstrating that it is necessary to increase the inclusion levels in sulfate sources to obtain similar results as they are less bioavailable. However, it is important to note that very high levels of copper sulphate, above 220 ppm (Yang et al., 2011), can have toxic effects and may interact with phytate; at levels above 300 ppm, they affect animal performance (Hamdi et al., 2018). The authors of a previous study reported the performance-enhancing effect at the 150-ppm level (Pesti and Bakalli, 1996). Thus, hydroxychlorides are more bioavailable and, therefore, a promising alternative source of mineral supplementation to improve production gain (Miles et al., 1998; Lu et al., 2010; Cohen and Steward, 2012; M'Sadeq et al., 2018). Nguyen et al. (2020) also determined that the copper hydroxychloride source, when compared to the sulfate source, improved the productive performance of broilers at 35 d of age. Santos et al. (2021)determined that the 150-ppm level of Cu from the hydroxychloride source improved the feed conversion of broilers at 42 days of age compared to 15 ppm of copper.

Yang et al. (2011) and Philpot et al. (2020) did not observe an improved performance in broilers supplemented with high levels of Cu. The mechanism of action of Cu as a performance enhancer includes its

Table 6. Thigh skin quality, footpad (TFS) and litter (TLS) score and, litter dry matter (LDM) of broilers at 42 d of age supplemented with different sources and levels of copper and manganese.

Treatments		Skin quality				
		BS, N	Elasticity, mm	TFS	LDM, %	TLS
CuH 15ppm		56.62	8.61	1.610^{a}	50.81^{b}	3.063
CuH 150ppm		58.44	8.59	1.350^{b}	54.19^{a}	2.992
	MnH 40ppm	60.94	8.90	1.440	53.35	3.000
	MnH 80ppm	56.42	8.45	1.513	51.38	3.046
	MnH 120ppm	55.23	8.45	1.488	52.76	3.047
CuH 15ppm	MnH 40ppm	57.17	8.50^{Aa}	1.530	51.09	3.063
CuH 15ppm	MnH 80ppm	57.59	8.99^{Aa}	1.650	51.67	3.031
CuH 15ppm	MnH 120ppm	55.12	8.34^{Aa}	1.650	49.66	3.094
CuH 150ppm	MnH 40ppm	64.71	9.30^{Aa}	1.350	55.61	2.938
CuH 150ppm	MnH 80ppm	55.25	7.91^{Bb}	1.375	51.10	3.061
CuH 150ppm	MnH 120ppm	55.35	8.56^{Aab}	1.325	55.86	3.000
Control 1		50.16	8.62	1.425	52.64	2.969
Control 2		50.64	8.55	1.400	50.42	3.094
P - value						
CuH		0.558	0.934	0.001	0.043	0.394
MnH		0.282	0.291	0.689	0.942	0.836
CuH vs. MnH		0.401	0.017	0.689	0.893	0.660
Control 1 vs. all		0.232	0.141	0.053	0.559	0.897
Control 2 vs. all		0.246	0.101	0.054	0.437	0.849
Control 1 vs. control 2		0.915	0.895	0.823	0.227	0.312
CV. %		26.28	16.29	17.72	13.03	7.97

Abbreviations:CuH, Cu Hydroxychloride; MnH, Mn Hydroxychloride; BS, breaking strength; TFS, Total footpad score; LDM, Litter dry matter; TLS, total litter score; CV, coefficient of variation. Control 1= 15 ppm Cu sulfate + 80 ppm Mn sulfate; Control 2= 150 ppm Cu sulfate + 120 ppm Mn sulfate.

[a,b]Means of factors followed by different lowercase letters differ by Tukey's test. In factorial, capital letters ([AB]) differ from each other within the MnH factor and lowercase letters differ from each other within the CuH factor. Controls were tested by Dunnett's test.

bacteriostatic activity against pathogenic bacteria (Pesti and Bakalli, 1996; Puig and Thiele, 2002; Pang and Applegate, 2007). Thus, this mineral favors the proliferation of beneficial microorganisms in the gastrointestinal tract, altering the microbiota (Silva, 2014). Therefore, when broilers are reared with a low degree of health challenge, this effect may be absent. In addition to aspects related to the modulation of the intestinal microbiota, lower copper supplementation can impair cellular and humoral immunity (Prohaska and Failla, 1993), superoxide dismutase activity (Jones and Suttle, 1981, Stabel and Spears, 1990, Gengelbach et al., 1997), T cell proliferation, cytokine production, and antibody production (Stabel and Spears, 1990). Due to the action of the copper- and zinc-dependent enzyme superoxide dismutase, acting on the removal of free radicals

Table 7. Mineral contents in liver and plasma and apparent ileal absorption (AIA) of Cu and Mn of broilers at 42 d of age supplemented with different sources and levels of copper and manganese.

Treatments		Plasma Cu, ppm	Plasma Mn, ppm	Liver Cu, ppm	Liver Mn, ppm	AIA of Cu, %	AIA of Mn, %
CuH 15ppm		0.454^{b}	0.456^{b}	14.28^{b}	21.24^{b}	65.70^{b}	82.04
CuH 150ppm		0.579^{a}	0.503^{a}	15.43^{a}	22.85^{a}	77.84^{a}	78.83
	MnH 40ppm	0.516	0.470^{b}	14.87	21.45^{b}	70.37	74.18^{b}
	MnH 80ppm	0.497	0.512^{a}	15.69	21.00^{b}	71.96	82.87^{a}
	MnH 120ppm	0.549	0.510^{a}	15.72	23.70^{a}	73.00	84.24^{a}
CuH 15ppm	MnH 40ppm	0.428	0.438	14.29^{X}	20.55$^{XY\ Ab}$	63.15	74.70^{X}
CuH 15ppm	MnH 80ppm	0.417	0.511^{XY}	13.82^{X}	23.55$^{XY\ Aab}$	66.67	84.82
CuH 15ppm	MnH 120ppm	0.517^{X}	0.475	14.73^{X}	24.44$^{XY\ Aa}$	67.29	86.60^{y}
CuH 150ppm	MnH 40ppm	0.605^{XY}	0.503	15.46^{X}	22.32$^{XY\ Aa}$	77.59^{X}	73.68^{X}
CuH 150ppm	MnH 80ppm	0.577^{X}	0.513^{XY}	17.56^{XY}	18.45 ^{Bb}	77.25^{X}	80.92
CuH 150ppm	MnH 120ppm	0.581^{X}	0.545^{XY}	16.70^{XY}	22.96$^{XY\ Aa}$	78.70^{X}	81.88
Control 1		0.389^{b}	0.442	11.27^{a}	18.21	55.89^{b}	82.53
Control 2		0.491^{a}	0.441	13.92^{b}	17.63	76.05^{a}	79.11
P - value							
CuH		<0.001	0.002	<0.001	<0.001	0.002	0.207
MnH		0.124	0.031	0.263	<0.001	0.848	0.005
CuH vs. MnH		0.066	0.095	0.081	<0.001	0.905	0.822
Control 1 vs. all		<0.001	<0.001	<0.001	<0.001	0.005	0.023
Control 2 vs. all		<0.001	<0.001	<0.001	<0.001	0.097	0.029
Control 1 vs. control 2		0.028	0.974	0.006	0.481	0.015	0.274
CV. %		37.47	25.77	29.59	20.04	20.58	10.73

Abbreviations: CuH, Cu Hydroxychloride; CV, coefficient of variation; MnH, Mn Hydroxychloride. Control 1= 15 ppm Cu sulfate + 80 ppm Mn sulfate; Control 2= 150 ppm Cu sulfate + 120 ppm Mn sulfate.

[a,b]Means of factors followed by different lowercase letters differ by Tukey's test. In factorial, capital letters ([AB]) differ from each other within the MnH factor and lowercase letters differ from each other within the CuH factor.

[X,Y]Controls were tested by Dunnett's test and means followed by letter "X" differ from control 1 and letter "Y" differ from control 2.

produced during phagocytosis, this causes the immune system defense cells to be less susceptible to free radical attack, thus improving the immunity of broilers (Harris, 1997).

Although copper had no effect on broiler body weight, high levels of CuH improved broiler breast yield; therefore, the improved performance was also visible through this variable. Copper acts on collagen and elastin synthesis as it is a cofactor of lysyl oxidase, an enzyme involved in collagen cross-linking (Rucker et al., 1998). Therefore, copper indirectly improves the quality of muscles, skin, bones, and other tissues that change size as a result of filling, such as intestines and blood vessels (Richards et al., 2010). Philpot et al. (2020) found no difference in the weight gain of broilers supplemented with high levels of copper; however, they noted that the breast yield of broilers supplemented with higher levels was higher at 54 d.

According to Gajula et al. (2011), Mn supplementation at the level of 60 ppm would be sufficient for the performance of broilers. In contrast, Cupertino et al. (2005) state values between 30 and 40 ppm. However, these studies reported the optimal values to meet nutritional requirements and ensure better results in productive performance, discarding responses that improve health and welfare parameters in addition to the performance of the birds.

Manganese is an important micromineral in bone formation, and high levels of Mn can positively influence the development of this tissue (Suttle, 2010). As the wings and back have a high proportion of bone tissue in relation to the amount of meat, the 120-ppm level was more suitable for the yield of this cut due to its positive influence on bone mineralization. Jasek et al. (2019) reported that a level of 160 ppm of MnH improved the quality and shear force of broiler bones. The level of 40 ppm of MnH increased breast yield as this cut has a lower proportion of bone tissue in relation to the amount of meat.

According to Lu et al. (2006), increasing the Mn inclusion level can decrease meat pH without altering other quality parameters. The pH is correlated with the water-holding capacity of the muscle; if the pH is above 6.2, the various cuts of meats may retain more water than under normal conditions (Barbut, 1997; Le Bihan-Duval et al., 1999). However, the water-holding capacity of the meats was within the expected values (Droval et al., 2012; Jayasena et al., 2013) and did not differ among treatments. Thus, pH assessment alone is not sufficient to state that there was a change in the quality of the meat. High meat pH values were found in several studies, without changes in other quality parameters. According to Alnahhas et al. (2016), 24-h postmortem pH values above 6.10 can be found in genetically improved lines such as those used in this study to obtain higher breast yield, such as pH+ lines.

The lightness of the meat is related to whether the meat is dark or light; the higher the L* value, the paler the meat. According to Le Bihan Duval et al. (1999), increasing live weight and breast yield can increase meat pallidness. This information corroborates this study since the highest values of breast yield obtained were at the level of 150 ppm of CuH, with 40 ppm of MnH, and showed higher values of lightness. Although the L* content was in the range of 54, which indicates a PSE (pale, soft, and exudative) meat, in this study, the pH values 24 h after slaughter were between 6.2; to be considered PSE meat, the value should be below 5.7 (Droval et al., 2012). Furthermore, in the evaluation of PSE meats, Droval et al. (2012) found an L* value of 59.26. Moreover, when using 150 ppm CuH with 40 ppm MnH, it is possible to increase breast yield without compromising the quality of the meat. Water-holding capacity, shear force, and cooking loss were not altered when the hydroxychloride source was used, indicating that this source can be used in broilers fed without damaging the meat quality.

In the analysis of skin elasticity, there was an interaction between the factors CuH and MnH. The skin elasticity of the broilers was improved when using a high level of CuH with a low level of MnH. Copper is an important micromineral for the maintenance of tissues such as the skin as it is the main cofactor of the enzyme lysyl oxidase. This enzyme participates in the formation of cross-links in collagen and elastin by adding a hydroxyl group to lysine residues, providing rigidity and elasticity to connective tissue proteins (Rucker et al., 1998; Richards et al., 2010). High levels of copper are associated with greater activation and action of this enzyme and, therefore, allow greater elasticity to tissues (Werman et al., 1995; Zhao et al., 2010). The greatest breaking strength (BB) can reduce skin lesions such as dermatitis and cellulite, resulting in lower economic losses (Oliveira et al., 2016).

Birds supplemented with a high level of CuH showed a reduction of approximately 16% in footpad dermatitis. As already mentioned, copper has several benefits for broilers, and one of them is the increase in tissue resistance and elasticity (Rucker et al., 1998; Richards et al., 2010). Richards et al. (2010) and Zhao et al. (2010) reported a reduction in footpad lesions when higher values of Zn and Cu were used as these minerals play a key role in tissue remodeling, both physiological and pathological. The evaluation of footpads is a relevant parameter to measure the welfare of birds. Footpad dermatitis is a condition that causes necrotic lesions on the paws (paw burns), causing pain and, consequently, reducing productivity. Therefore, it is important to prevent this pathological condition to generate productive gains in addition to preserving welfare.

The litter score did not change with the different levels and sources of Cu and Mn studied. With higher amounts of CuH supplementation, the litter became drier, which also explains the better quality of the footpads of the birds that received 150 ppm CuH. Since copper is beneficial to bacteria in the gastrointestinal tract (Silva, 2014) and has a bacteriostatic action against pathogenic microorganisms (Pesti and Bakalli, 1996; Puig and Thiele, 2002), it improves gastrointestinal transit, reducing the diarrhea incidence and other infectious intestinal disorders that increase the moisture of excreta and, consequently, of the litter.

In general, when the same levels of mineral supplementation were compared, broilers that received the hydroxychloride source had higher concentrations of Cu and Mn in the plasma and liver than those that received the sulfate source. This result confirms the principle that hydroxychlorides have a greater relative bioavailability than sulfates. Similarly, Olukosi et al. (2018) evaluated the effect of supplementing chicken feed with copper and zinc hydroxychloride compared to the sulfate source and found a higher concentration of copper in the liver of birds that received the hydroxychloride source. Lu et al. (2010) also found greater hepatic copper deposition in broilers that were supplemented with the hydroxychloride source compared to the sulfate source, thus improving the relative bioavailability of the hydroxymineral source.

Hydroxychlorides have covalent bonds that are stronger than the ionic bonds of sulfates and therefore improve the stability of feed components and the bioavailability of minerals by avoiding antagonistic interactions with other nutrients in the diet (Cohen and Steward, 2012; Villagómez-Estrada et al., 2020). The great advantage of this source is the reduction in manufacturing costs, in addition to the decrease in mineral inclusion; as they are absorbed more easily, the environmental impact is lower (Cohen and Steward, 2012; M'Sadeq et al., 2018). Miles et al. (1998) and Lu et al. (2010) found that hydroxychlorides were less reactive in the feed and more bioavailable than other inorganic forms.

In view of the higher values of Cu and Mn supplemented in the diet, there was an increase in the contents of these minerals in the liver and plasma of the chickens. According to Suttle (2010) and Yang et al. (2011), the liver is the target organ for copper and manganese deposition. Copper, when in excess, accumulates mainly in lysosomes and in the cytoplasm of hepatocytes, and liver toxicity has been reported for Cu levels above 220 ppm, with an impairment of the mitochondrial function of hepatocytes (Yang et al., 2011). Excess Mn is sequestered into mitochondria, consequently interfering with cellular respiration, with an increased release of reactive oxygen species (**ROS**) (Cupertino et al., 2005; Tufarelli and Laudadio, 2017).

There was an interaction for Cu and Mn in the manganese content in the chicken liver. In general, when 120 ppm of MnH was used with 15 or 150 ppm of CuH, there was a greater deposition of Mn in this organ. Thus, higher levels of supplemented MnH increased the content of this mineral in the liver.

Measurements of the absorption of trace minerals such as Cu, Mn, and Zn are difficult to perform due to the complexity of endogenous mineral excretion (M'Sadeq et al., 2018). These elements are absorbed in almost the entire gastrointestinal tract and excreted in the intestine via bile. When this occurs, the unabsorbed mineral and the nutrient absorbed and excreted in the bile can mix in the intestine, hindering the exact calculation of both (Finley et al., 1997). In this study, the apparent ileal absorption of CuH was higher compared to that of sulfate, and when the ration was supplemented with 15 ppm of MSM, copper mineral absorption was lowest, showing that the sulfate source has a lower bioavailability. Values close to 60% of the apparent ileal absorption for Cu are found when the sulfate source is used at the level of 15 ppm (Hafeez et al., 2014).

Regarding Mn, when 120 ppm MnH was used compared to 120 ppm MSM, the hydroxymineral source again showed a higher AIA than the inorganic source. M'Sadeq et al. (2018) found no difference in the AIA of copper and manganese, compared to the sulfate source, for 38-day-old broilers; however, the levels of hydroxychlorides used showed a reduced inclusion compared to the sulfate source. Thus, even receiving supplementation with lower levels, the hydroxychloride source corresponded to the sulfate source for this parameter.

In conclusion, the hydroxychloride source was more bioavailable than the sulfate source due to the higher apparent ileal absorption of minerals and the higher concentration of minerals in the liver and plasma. Thus, as hydroxychlorides are more bioavailable to broilers, it is possible to reduce the inclusion of this source in relation to sulfate without compromising productive performance. In addition, because of the decreased mineral excretion, environmental pollution is reduced. However, this aspect will need to be further evaluated to support this hypothesis. High levels of Cu hydroxychloride can improve FCR, breast yield, footpad quality, and skin elasticity. Low levels of Mn hydroxychloride increase breast yield, whereas high levels of Mn hydroxychloride can increase back and wing yield. Overall, hydroxychlorides can be used as a substitute for the sulfate source without compromising the quality of broiler chicken meat. Therefore, 150 ppm of CuH + 80 ppm of MnH are suitable sources for supplementing broiler diets to increase productivity.

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DISCLOSURES

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