DOI: http://dx.doi.org/10.1590/1678-992X-2018-0071

ISSN 1678-992X

SCIENTIA AGRICOLA

Research Article

Organic trace minerals and calcium levels in broilers' diets to 21 days old

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Edited by: Antonio Faciola

Received March 09, 2018
Accepted August 01, 2018

ABSTRACT: This study was undertaken to evaluate the effects of dietary calcium levels and supplementation with organic trace minerals selenium, copper, iron, zinc and manganese on performance, tissue deposition and litter mineral concentration. A total of 2,496 one-day-old male Cobb 500 broilers were randomly assigned to a 3 × 4 factorial experimental design with three levels of dietary Ca [8, 10 and 12 g kg^{-1}, while maintaining the same Ca:nPP (non-phytate phosphorus) ratio (2:1)] and four levels of micromineral supplementation (0.62, 0.72, 0.82 and 0.92 g kg^{-1}). There was a total of 12 treatments, with eight replicates of 26 birds per pen. Micromineral supplementation (MS) was achieved by adding different levels of the product Bioplex TR Se® and Ca supplementation was achieved by adding increasing levels of limestone and dicalcium phosphate. An interaction between Ca and MS levels was observed ($p < 0.05$) for the parameters of performance, liver Cu concentration, breast Se and Cu concentrations and litter Se, Mn and Zn concentrations. No interactions were observed ($p > 0.05$) for Ca, P or ash concentrations in the tibia, which were influenced only by dietary Ca levels ($p < 0.05$). The Ca level of 10 g kg^{-1} promoted higher Ca and P concentration in the tibia and lower micromineral excretion in the litter. The combination of MS level of 0.82 g kg^{-1} with Ca level of 10 g kg^{-1} led to the best BWG response. The supplementation conditions that led to higher micromineral levels in the liver and breast varied for each mineral.

Keywords: chicken, micromineral, tibia, breast muscle

Introduction

Inadequate mineral supplementation during the growth phase of birds results in an imbalance in mineral homeostasis and improper development of bones, i.e., abnormal bone calcification. However, excess calcium (Ca) may act as an antagonist, making it difficult to absorb trace minerals such as iron (Fe), copper (Cu), zinc (Zn) and other minerals such as magnesium (Mg), sodium (Na) and potassium (K) (Smith and Kabaija, 1985; Waldroup, 1996). High Ca levels in broiler chicken feed increase the need for phosphorus (P) because Ca interferes with phosphorus absorption. Ca and P form complexes in the intestine, making P less available hindering the absorption of phytin phosphorus by the bird (Wise, 1983).

Trace minerals such as selenium (Se), Cu, Fe, manganese (Mn) and Zn are essential to chicken development because they are active in several metabolic pathways. These minerals are involved in physiological functions that are essential to the maintenance of life, including reproduction, growth, immune system function, bone formation and energy metabolism (Dieck et al., 2003; Bao et al., 2007; Dibner et al., 2007). They are usually supplemented in the form of inorganic salts, such as sulphates, oxides and carbonates, to ensure healthy development and greater productivity. However, these inorganic forms are thought to interact with other minerals, such as Ca. This negative effect can be minimized by supplementing these minerals in organic form.

The aim of the present study was to evaluate the effects of dietary Ca levels (while maintaining the same Ca:P ratio) and different supplementation levels of the organic trace minerals Se, Cu, Fe, Zn and Mn on the performance, tissue deposition and mineral excretion in the litter.

Materials and Methods

The study was approved by the Ethics Committee for the Use of Production Animals (CEUAP) of the Universidade Federal de Viçosa (UFV; Minas Gerais, Brazil), with number 100/2014.

The experiment was conducted in Viçosa (20°45′14″ S, 42°52′53″ W and 648.74 m of altitude), in the state of Minas Gerais, Brazil.

A total of 2,496 one-day-old male Cobb 500 broiler chicks with a mean initial weight of 44 g were housed up to 21 days of age in 96 pens (experimental units) with $3m^2$ each, which were lined with wood shavings and located within a masonry shed.

The experimental design was fully randomized, with 12 treatments (Table 1) in a 3 × 4 factorial design that consisted of three levels of dietary Ca [8, 10 and 12 g kg^{-1}, with a constant Ca:nPP (non-phytate phosphorus) ratio of 2:1] and four levels of micromineral supplementation (0.62, 0.72, 0.82 and 0.92 g kg^{-1}). Each treatment consisted of eight replicates of 26 birds.

The corn-soybean meal basal diets (Table 2) were formulated so as to be adequate in all nutrients according to Rostagno et al. (2011) with the exception of the microminerals (Mn, Zn, Fe, Cu and Se) and Ca levels, which were added based on respective treatments. The diets were supplemented with a *Escherichia coli* - derived

Table 1 – Experimental treatments and organic trace minerals levels[1].

Treatment	Ca	nPP	MS	Mn	Zn	Fe	Cu	Se
	g kg^{-1}			ppm				
T1	8	4	0.62	31	24.8	18.6	3.72	0.112
T2			0.72	36	28.8	21.6	4.32	0.130
T3			0.82	41	32.8	24.6	4.92	0.148
T4			0.92	46	36.8	27.6	5.52	0.166
T5	10	5	0.62	31	24.8	18.6	3.72	0.112
T6			0.72	36	28.8	21.6	4.32	0.130
T7			0.82	41	32.8	24.6	4.92	0.148
T8			0.92	46	36.8	27.6	5.52	0.166
T9	12	6	0.62	31	24.8	18.6	3.72	0.112
T10			0.72	36	28.8	21.6	4.32	0.130
T11			0.82	41	32.8	24.6	4.92	0.148
T12			0.92	46	36.8	27.6	5.52	0.166

[1]The minerals were supplied by the micromineral supplementation (MS), which contained 50 g kg^{-1} manganese (Mn), 40 g kg^{-1} zinc (Zn), 30 g kg^{-1}, iron (Fe), 6 g kg^{-1} copper (Cu), 180 mg kg^{-1} selenium (Se) and 2 g kg^{-1} iodine (I). Ca supplementation was achieved by adding increasing levels of limestone (377 g of Ca per kg of limestone) and dicalcium phosphate (245 g of Ca and 185 g of P available per kg of dicalcium phosphate).

phytase (5,000 FTU g^{-1} of phytase, providing 1000 FTU per kg of diet) to simulate a commom comercial diet.

Trace mineral supplementation was achieved by adding different levels of a commercial micromineral supplement (MS), which contains 50 g Mn, 40 g Zn, 30 g Fe and 6 g Cu (as proteinates), 180 mg Se (derived from yeast enrichment) and 2 g I (as inorganic source) per kilogram of the product.

Ca supplementation was performed by adding increasing levels of limestone (377 g of Ca per kg of limestone). The Ca:nPP ratio was maintained in the different treatments by adding different levels of dicalcium phosphate ($CaHPO_{4;}$ with 245 g of Ca and 185 g of P available per kg of dicalcium phosphate), limestone and an inert material (washed sand).

Each experimental unit was equipped with a nipple and feeder to provide water and feed *ad libitum*. The feed was given according to the consumption of the animals in each experimental unit. The birds and leftover feed were weighed at the beginning of the experiment

Table 2 – Ingredients and nutrient composition of the basal diets.

	Ca (g kg^{-1})		
Ingredient	8	10	12
Corn	537.40	537.40	537.40
Soybean meal 46	379.10	379.10	379.10
Soy oil	35.00	35.00	35.00
Washed sand*	15.93 / 15.83 / 15.73 / 15.63[2]	8.72 / 8.62 / 8.52 / 8.42[3]	1.53 / 1.43 / 1.33 / 1.23[4]
Dicalcium phosphate	9.97	15.37	20.78
Limestone	9.25	11.06	12.84
NaCl	4.80	4.80	4.80
DL-methionine	2.85	2.85	2.85
L-lysine HCl	1.57	1.57	1.57
L-threonine	0.41	0.41	0.41
Choline chloride, 60 %	1.00	1.00	1.00
Vitamin supplement[1]	1.25	1.25	1.25
Mineral supplement*	0.62 / 0.72 / 0.82 / 0.92[2]	0.62 / 0.72 / 0.82 / 0.92[3]	0.62 / 0.72 / 0.82 / 0.92[4]
Anticoccidial salinomycin 12 %	0.55	0.55	0.55
Antioxidant[5]	0.10	0.10	0.10
Phytase[6]	0.20	0.20	0.20
Calculated nutrient content			
Crude protein	217.13	217.13	217.13
ME (kcal kg^{-1})[7]	3,000	3,000	3,000
Calcium	8.0	10.0	12.0
Non-phytate phosphorus	4	5	6
Na	2.09	2.09	2.09
Dig. lysine	12.00	12.00	12.00
Dig. methionine	5.73	5.73	5.73
Dig. methionine + cysteine	8.68	8.68	8.68
Dig. threonine	7.80	7.80	7.80
Dig. tryptophan	2.47	2.47	2.47
Dig. arginine	13.86	13.86	13.86
Dig. valine	9.24	9.24	9.24

[1]Supplied per kilogram of diet: vitamin A, 8250 IU; vitamin D_3, 2090 IU; vitamin E, 31 IU; vitamin B_1, 2.20 mg; vitamin B_2, 5.50 mg; vitamin B_6, 3.08 mg; vitamin B_{12}, 0.013 mg; pantothenic acid, 11.0 g; biotin, 0.077 mg; vitamin K3, 1.65 mg; folic acid, 0.77 mg and nicotinic acid, 33 mg. *Supplementation levels of Bioplex TR Se microminerals and amount of washed sand (inert) in the respective treatments: [2]T1, T2, T3 and T4; [3]T5, T6, T7 and T8; [4]T9, T10, T11 and T12; [5]Butylated hydroxyl toluene 99 %; [6]Concentration: 5,000 FTU g^{-1} phytase; [7]Calculated Metabolizable Energy.

and at 21 days of age to determine body weight gain (BWG, kg), feed intake (FI, kg) and feed-to-gain ratio (F:G, kg kg^{-1}). The number of birds dying throughout the experimental period was quantified to calculate viability. F:G was determined considering the correction of feed intake by birds alive in each unit. For this each feeder unit was weighed after a dead bird was detected. Temperature was measured daily. In the first week, the mean minimum and maximum temperatures were 25 °C and 32 °C, respectively. These temperatures were 21 °C and 31 °C from day 8 to 21.

At 21 days of age, one bird per pen (eight birds per treatment) was selected for slaughter based on the mean weight of its experimental unit. The birds were slaughtered using the cervical displacement method followed by exsanguination.

To collect the liver, an incision was made in the abdominal cavity of the bird to expose the viscera.

After plucking and cleaning, a single sample of breast muscle weighing approximately 30 g was collected from each experimental unit.

Litter samples were collected from a previously established site at the centre of each experimental unit (pen) to determine the mineral (Mn, Zn, Fe, Cu and Se) concentration in the litter. All of the material contained within a 90-cm^2 piece of plastic lining was collected.

Samples of the right tibia, liver, and right portion of the breast musculature from each bird and litter of each experimental unit were collected and placed separately in plastic bags labelled with the corresponding treatments and stored in a freezer at –20 °C.

The tibia were washed, cleaned of all residual tissue and dried at 60 °C for 72 h. Subsequently, pre-degreasing was performed for 4 h with petroleum ether in a glass vessel. The tibia were then ground, and a sample of each was removed (eight samples per treatment, totalling 96 samples), dried for 12 h at 105 °C to determine dry mass and then ashed in a muffle furnace (600 °C for 4 h) to determine ash content. Calcium and P were determined after wet-ash digestion with nitric and perchloric acids according to the 935.13 method (AOAC, 2000). Calcium in wet-ashed samples was determined by the atomic absorption spectrophotometric method 968.08 (AOAC, 2000) using an atomic absorption spectrometer (AAnalyst 300). Phosphorus concentration was determined using a colorimetric assay (Fiske and Subbarow, 1925). Acid molybdate and Fiske's SubbaRow reducer solution were added to wet-ash samples to perform a phosphor-molybdenum complex. Color intensity was proportional to P concentration and was determined with a spectrophotometer using absorbance at 620 nm (SpectraCount, Model #AS1000). The analyses were performed at the Animal Nutrition Laboratory in Viçosa (20°45′14″ S, 42°52′53″ W and 648.74 m of altitude), in the state of Minas Gerais, Brazil.

Samples of diets, liver, breast and litter were sent to a laboratory (54′23″ S, 47°3′42″ W and 760 m of altitude, Campinas, in the state of São Paulo, Brazil) to determine the concentrations of Mn, Zn, Fe, Cu and Se. For this the frozen samples of liver and breast were packed with ice in a Styrofoam box to maintain their physiochemical characteristics. The litter samples were dried for 72 h in a ventilated oven at 60 °C and were then ground (0.5 mm).

Zinc, Mn, Fe, Cu and Se concentrations in liver, breast, litter and diet samples were determined following the methods described by AOAC (1984). An aliquot of 0.5 g of diets, tissues and litter samples were weighed on the analytical balance and added 5 ml of 4:1 nitroperchloric acid solution (4 parts nitric acid and 1 part perchloric acid). The samples were heated and digested at 200 °C in the digester block and the residue was filtrated through quantitative paper (7.5 µm pore) and completed for 50 mL with distilled water. This solution was analyzed in an atomic absorption spectrophotometer (AAS) to obtain the mineral concentrations (Mn, Fe, Zn and Cu) and for Se concentrations the graphite furnace atomic absorption spectroscopy (GFAAS) was used.

Data were analysed using the GLM procedure (SAS v. 9.3). Pens containing 26 birds were considered as the experimental units. The model included the main effects of Ca level and micromineral supplementation and their interaction, as described below:

$$y_{ijk} = \mu + Ca_i + MS_j + (Ca \times MS)_{ij} + \varepsilon_{ijk}\ i = 1,\ldots, a;\ j = 1,\ldots,b;\ k = 1,\ldots,n,$$

where: y_{ijk} = observation k in level i of factor Ca and level j of factor MS; µ = the overall mean; Ca_i = the effect of level i of the factor *Ca level*, MS_j = the effect of level j of the factor *Mineral supplementation*; $(Ca \times MS)_{ij}$ = the effect of the interaction of level i of factor Ca with level j of factor MS; ε_{ijk} = residual random error; a = the number of levels of factor Ca; b = the number of levels of factor MS; n = the number of observations for each $Ca \times MS$ combination.

The isolated effects of mineral supplementation were analysed by orthogonal polynomial contrasts, and the isolated effects of Ca level were compared using Tukey's means test. The effects were considered significant at $p < 0.05$.

Results

The interaction between MS and Ca level was significant ($p < 0.05$) for BWG, FI and F:G (Table 3). At the Ca level of 10 g kg^{-1}, BWG showed a quadratic response to MS, with $Y = 0.435 + 1.141X - 0.622X^2$ ($R^2 = 0.86$). The greatest BWG was observed at the MS level of 0.917 g kg^{-1}. MS did not influence BWG at Ca levels of 8 and 12 g kg^{-1}.

At a Ca level of 8 g kg^{-1}, FI showed a quadratic response to MS, with $Y = 2.617 - 3.708X + 2.481X^2$ ($R^2 = 0.89$). The lowest feed intake was observed at the MS level of 0.747 g kg^{-1}. However, at the Ca level of 10

g kg^{-1}, FI showed a linear relationship with MS, represented by the equation Y = 1.024 + 0.293X (R^2 = 0.84). At the MS level of 0.92 g kg^{-1}, FI was lower at dietary Ca levels of 10 and 12 g kg^{-1}.

At the Ca level of 8 g kg^{-1}, F:G showed a quadratic response to MS, according to the equation Y = 2.429 – 2.959X + 1.981X^2 (R^2 = 0.97). The lowest feed-to-gain ratio was observed at the MS level of 0.747 g kg^{-1}. In the same way, at the MS level of 0.92 g kg^{-1}, F:G was lower at dietary Ca levels of 10 and 12 g kg^{-1}.

Viability did not interact with and was not independently affected by the factors analysed (p > 0.05).

Additionally, MS had no independent or interactive effect on the percentages of Ca, P and ash in the tibia (p > 0.05). However, increasing the dietary Ca level resulted in increased Ca, P and ash concentrations in this bone (p < 0.05) (Table 4).

The MS and Ca independently influenced the concentrations of Mn and Zn in the liver and MS levels influenced the Se liver concentration (p < 0.05) (Table 5). The MS level was positively and linearly correlated with Se and Mn concentrations (p < 0.05) in the liver, while a quadratic relationship was observed for the Zn concentration (p < 0.05). These three relationships can be represented by the equations Y = 0.189 + 0.912X (R^2 = 0.99), Y = 5.284 + 6.020X (R^2 = 0.94) and Y = 38.937 + 162.288X – 78.00X^2 (R^2 = 0.83), respectively. According to this equation, the highest Zn deposition in the liver occurs with 1.04 g kg^{-1} of MS. However, as this level is outside the MS range tested here, this equation is not representative of high Zn deposition in the liver. Higher Mn concentrations were observed at Ca levels of 8 and 10 g kg^{-1}.

The concentrations of Cu and Fe were affected by interaction between the MS and Ca levels (p < 0.05; Table 5). At Ca levels of 8 and 10 g kg^{-1}, MS produced a linear increase in liver Cu concentration, according to the equations Y = -65.497 + 137.007X (R^2 = 0.96) and Y = -43.278 + 116.803X (R^2 = 0.95), respectively. At the lowest MS level (0.62 g kg^{-1}), the highest liver Cu concentration was observed at a Ca level of 12 g kg^{-1}.

For Fe in liver, at a Ca level of 12 g kg^{-1}, MS had a quadratic effect on the liver Fe concentration, with Y = 2124.465 – 4544.524X + 3106.109X^2 (R^2 = 0.92), with the lowest Fe deposition observed at 0.73 g kg^{-1} MS. At a 0.72 g kg^{-1} MS, the highest liver Fe deposition was observed at 8 g kg^{-1} Ca.

Table 3 – Influence of dietary Ca and MS on body weight gain (BWG), feed intake (FI), feed-to-gain ratio (F:G), viability (Viab.)[1].

Treatments		Variables			
Ca	MS	BWG	FI	F:G	Viab.
g kg^{-1}		kg		intake/gain	%
8 [3, 5]	0.62	0.936	1.268	1.354	97.59
	0.72	0.938	1.247	1.330	99.04
	0.82	0.926^{z}	1.232	1.330	97.48
	0.92	0.946	1.310^{w}	1.385^{w}	97.60
10 [2, 4]	0.62	0.907	1.212	1.336	98.56
	0.72	0.922	1.215	1.317	99.04
	0.82	0.964^{w}	1.282	1.331	97.12
	0.92	0.955	1.286^{zw}	1.348^{zw}	98.56
12	0.62	0.934	1.256	1.346	97.67
	0.72	0.929	1.235	1.330	99.04
	0.82	0.928^{z}	1.226	1.320	98.56
	0.92	0.935	1.233^{z}	1.320^{z}	98.08
MS	0.62	0.926	1.245	1.345	97.94
	0.72	0.930	1.232	1.326	99.04
	0.82	0.940	1.247	1.327	97.72
	0.92	0.945	1.277	1.351	98.08
Ca	8	0.937	1.264	1.350	97.93
	10	0.937	1.249	1.333	98.32
	12	0.931	1.238	1.329	98.33
ANOVA	MS	0.129	0.005	0.004	0.337
	Ca	0.727	0.051	0.012	0.754
	MS × Ca	0.045	< 0.001	0.032	0.932
	CV%*	3.352	3.431	2.157	2.807

[1]Means represent eight experimental units with 26 birds per experimental unit; [2]BWG_{Ca10} = 0.435 + 1.141X – 0.622X^2(R^2 = 0.86); [3]FI_{Ca8} = 2.617 – 3.708 + 2.481 X^2 (R^2 = 0.89); [4]FI_{Ca10} =1.024 + 0.293X (R^2 = 0.84); [5]$F:G_{Ca8}$ = 2.429 – 2.959X + 1.981X^2 (R^2 = 0.97); [z and w]means represents by different letters in the same column are different from each other (p < 0.05) consider the effect of Ca levels in MS levels; *Coefficient of variation.

Table 4 – Dry basis percentages of calcium (Ca), phosphorus (P) and ash in the tibia of birds under different treatments[1].

Treatments		Variables		
Ca	MS	Ca	P	Ash
g kg^{-1}		%		
8	0.62	16.61	9.48	51.61
	0.72	16.67	9.55	51.83
	0.82	16.51	9.85	52.10
	0.92	16.14	9.47	51.77
10	0.62	16.19	10.08	52.54
	0.72	16.61	10.13	52.53
	0.82	17.17	9.89	52.23
	0.92	17.30	10.11	52.34
12	0.62	17.05	9.65	52.31
	0.72	17.07	9.76	52.84
	0.82	17.08	10.06	52.55
	0.92	17.17	9.84	53.29
MS	0.62	16.64	9.74	52.15
	0.72	16.78	9.81	52.40
	0.82	16.92	9.93	52.29
	0.92	16.87	9.81	52.47
Ca	8	16.37^{b}	9.59^{c}	51.83^{b}
	10	16.82^{a}	10.05^{a}	52.41^{a}
	12	17.09^{a}	9.83^{b}	52.75^{a}
ANOVA	MS	0.194	0.326	0.296
	Ca	0.002	< 0.001	< 0.001
	MS × Ca	0.242	0.153	0.066
	CV%*	4.703	3.740	1.143

[1]Means represent eight experimental units with 26 birds per experimental unit; [a, b, c]Means within the same column that have different letters are significantly different from each other (p < 0.05); *Coefficient of variation.

Table 5 – Treatment effects on the dry basis concentrations of the micronutrients selenium (Se), copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn) in broiler livers[1].

Treatments		Minerals				
Ca	MS	Se	Cu	Fe	Mn	Zn
g kg^{-1}		ppm				
8^{2}	0.62	0.743	22.176	517.785	9.094	111.480
	0.72	0.818	27.972	555.503	10.120	108.015
	0.82	0.979	49.017	474.627	10.521	115.102
	0.92	0.983	60.830	492.504	10.665	123.090
10^{3}	0.62	0.779	30.545	502.226	9.208	108.332
	0.72	0.829	41.050	436.407	9.998	110.564
	0.82	0.919	47.828	525.005	10.569	115.685
	0.92	1.031	67.220	542.522	10.855	118.898
12^{4}	0.62	0.717	56.151	506.252	8.310	111.773
	0.72	0.928	40.976	446.404	9.075	118.865
	0.82	0.920	45.765	502.715	10.207	136.517
	0.92	1.054	48.944	567.110	10.412	121.812
MS	0.62	0.746	36.291	508.754	8.871	110.529
	0.72	0.858	36.666	479.438	9.731	112.481
	0.82	0.939	47.537	500.782	10.432	122.435
	0.92	1.023	58.998	534.046	10.644	121.267
Ca	8	0.881	39.999	510.105	10.100^{a}	114.422^{a}
	10	0.889	46.661	501.540	10.158^{a}	113.370^{a}
	12	0.905	47.959	505.620	9.501^{b}	122.242^{a}
ANOVA	MS	< 0.001^{L1}	< 0.001	0.170	< 0.001^{L2}	0.011^{Q}
	Ca	0.832	0.142	0.921	0.008	0.040
	MS × Ca	0.669	0.004	0.034	0.878	0.349
	CV%*	17.99	38.09	16.67	9.22	12.82

[1]Means represent eight experimental units with 26 birds per experimental unit; ^{L1}Linear effect (Y = 0.189 + 0.912X); ^{L2}Linear effect (Y = 5.284 + 6.020X); ^{Q}Quadratic effect (Y = 38.937 + 162.288X – 78.00X^{2}); ^{a,b}Means within the same row that have different letters are significantly different from each other; [2]Cu liver $_{Ca8}$ = -65.497 + 137.007X (R^2 = 0.96); [3]Cu liver $_{Ca10}$ = -43.278 + 116.803X (R^2 = 0.95); [4]Fe liver $_{Ca12}$ = 2124.465 – 4544.524X + 3106.109X^{2} (R^2 = 0.92); *Coefficient of variation.

Table 6 – Treatment effects on the dry basis concentrations of the micronutrients selenium (Se), copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn) in broiler breasts[1].

Treatments		Minerals (ppm)				
Ca	MS	Se	Cu	Fe	Mn	Zn
g kg^{-1}		ppm				
8^{2}	0.62	0.219	8.633	25.798	0.693	31.356
	0.72	0.233	9.332^{t}	28.158	0.727	31.010
	0.82	0.307	6.936^{f}	29.149	0.834	31.145
	0.92	0.255^{z}	8.875^{h}	26.678	0.793	31.928
10^{3}	0.62	0.204	10.893	25.776	0.668	31.730
	0.72	0.258	5.821^{t}	23.795	0.677	29.474
	0.82	0.263	7.557^{f}	22.710	0.699	32.993
	0.92	0.287^{w}	8.564^{h}	22.113	0.639	32.303
$12^{4,5}$	0.62	0.233	9.011	21.285	0.650	30.186
	0.72	0.255	17.703^{s}	25.743	0.729	31.474
	0.82	0.280	17.994^{e}	26.587	0.830	31.997
	0.92	0.329^{w}	17.233^{g}	26.051	0.803	34.459
MS	0.62	0.219	9.512	24.286	0.670	31.091
	0.72	0.249	10.952	25.899	0.711	30.653
	0.82	0.283	10.829	26.149	0.788	32.045
	0.92	0.291	11.557	24.947	0.745	32.896
Ca	8	0.253	8.444	27.449^{a}	0.762^{a}	31.360
	10	0.253	8.209	23.599^{b}	0.671^{b}	31.625
	12	0.274	15.485	24.916^{a}	0.753^{a}	32.029
ANOVA	MS	< 0.001	0.594	0.633	0.001^{Q}	0.027^{L}
	Ca	0.087	< 0.001	0.023	0.001	0.618
	MS × Ca	0.036	0.008	0.365	0.222	0.216
	CV%*	16.59	49.39	21.99	14.11	8.65

[1]Means represent eight experimental units with 26 birds per experimental unit; ^{Q}Quadratic effect (Y = - 0.723 + 3.536X - 2.10X^{2}); ^{L}Linear effect (Y = 26.430 + 6.807X); ^{a,b}Means within the same column that have different letters are significantly different from each other ($p < 0.05$); [2]Se breast $_{Ca8}$ = -0.831 + 2.666X – 1.625X^{2} (R^2 = 0.62); [3]Se breast $_{Ca10}$ = Y = 0.057 + 0.254X (R^2 = 0.88); [4]Se breast $_{Ca12}$ = 0.032 + 0.315X (R^2 = 0.96); [5]Cu breast $_{Ca12}$ = -140.908 + 388.932X – 236.347X^{2} (R^2 = 0.95); $^{z \text{ and } w, s \text{ and } t, g \text{ and } h, e \text{ and } f}$means represents by different letters in the same column are different from each other ($p < 0.05$) consider the effect of Ca levels in MS levels; *Coefficient of variation.

The MS levels independently influenced the Mn and Zn concentrations in the breast muscle ($p < 0.05$) (Table 6). The Mn concentration in the breast showed a quadratic response to MS, where Y = -0.723 + 3.536X – 2.10X^{2} (R^2= 0.84). The highest Mn deposition was observed at 0.84 g kg^{-1} MS. Moreover, the concentration of Zn in the breast increased linearly with MS, with Y = 26.430 + 6.807X (R^2 = 0.77). The dietary Ca level influenced independently the Fe and Mn concentration in the breast, with the lowest levels observed at 10 g kg^{-1} Ca.

The Se and Cu concentrations in the breast were affected by the interaction between MS and Ca levels ($p < 0.05$; Table 6). At 8 g kg^{-1} Ca, the Se concentration in the breast showed a quadratic response to MS, with Y = -0.831 + 2.666X – 1.625X^{2} (R^2 = 0.62). The highest concentration was observed at 0.83 g kg^{-1} MS. At the Ca levels of 10 and 12 g kg^{-1}, MS produced a linear increase in breast Se concentrations, according to the equations Y = 0.057 + 0.254X (R^2 = 0.88) and Y = 0.032 + 0.315X (R^2 = 0.96), respectively.

At 12 g kg^{-1} Ca, the breast Cu concentration showed a quadratic response to MS, according to the equation Y = -140.908 + 388.932X – 236.347X^{2} (R^2 = 0.95). The highest Cu concentration was observed at 0.82 g kg^{-1} MS.

At the MS level of 0.92 g kg^{-1}, the highest breast Se concentrations were observed at 10 and 12 g kg^{-1} Ca. At the MS levels of 0.72, 0.82 and 0.92 g kg^{-1}, the highest breast Cu concentrations were found at 12 g kg^{-1} Ca.

The Cu and Fe concentrations in the litter increased linearly with MS ($p < 0.05$) according to the equations Y = 20.851 + 15.915X (R^2 = 0.08) and Y = 964.071 + 768.073X (R^2 = 0.18), respectively (Table 7).

The Ca level affected the Fe concentration in the litter ($p < 0.05$), with the lowest value observed at 10 g kg^{-1} Ca (Table 7).

The Se, Mn and Zn concentrations in the litter showed interactive effects with MS and the Ca level ($p < 0.05$) (Table 7).

For Se, at 10 g kg^{-1} Ca, MS showed a quadratic effect, Y = -1.245 + 3.678X – 2.219X^{2} (R^2 = 0.99), with the highest concentration found at 0.829 g kg^{-1} MS. At

Table 7 – Treatment effects on the dry mass concentrations of the micronutrients selenium (Se), copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn) excreted in litter[1].

Treatments		Minerals (ppm)				
Ca	MS	Se	Cu	Fe	Mn	Zn
g kg^{-1}		ppm				
$8^{4,7}$	0.62	0.234^{w}	28.938	1562.19	196.886^{w}	132.331
	0.72	0.249	29.455	1547.61	213.264^{t}	148.234^{w}
	0.82	0.260	36.155	1599.65	219.481^{h}	160.574
	0.92	0.276^{t}	34.100	1704.48	207.558^{q}	162.304
$10^{2,5,8}$	0.62	0.184^{z}	29.630	1304.23	167.561^{z}	116.114
	0.72	0.248	29.940	1413.41	182.594^{s}	121.984^{z}
	0.82	0.284	35.715	1512.30	201.659^{h}	144.023
	0.92	0.259^{s}	37.034	1538.34	244.118^{p}	158.399
$12^{3,6,9}$	0.62	0.239^{w}	35.530	1521.66	222.910^{w}	126.316
	0.72	0.244	33.684	1540.56	231.845^{t}	136.503^{z}
	0.82	0.229	31.716	1567.33	251.186^{g}	146.951
	0.92	0.310^{t}	35.376	1854.10	258.015^{p}	171.720
MS	0.62	0.219	31.366	1462.69	195.786	124.920
	0.72	0.247	31.026	1500.53	209.234	135.573
	0.82	0.258	34.529	1559.76	224.109	150.516
	0.92	0.282	35.503	1698.97	236.563	164.141
Ca	8	0.255	32.162	1603.48^{a}	209.30	150.86
	10	0.243	33.080	1442.07^{b}	198.98	135.13
	12	0.255	34.077	1620.91^{a}	240.99	145.37
ANOVA	MS	<0.001	0.024^{L1}	<0.001^{L2}	<0.001	<0.001
	Ca	0.083	0.451	<0.001	<0.001	<0.001
	MS × Ca	<0.001	0.137	0.273	<0.001	0.018
	CV%*	11.88	18.24	10.54	7.35	7.22

[1]Means represent eight experimental units with 26 birds per experimental unit; ^{L1}Linear effect (Y = 20.851 + 15.915X); ^{L2}Linear effect (Y = 964.071 + 768.073X); ^{a,b}Means within the same column that have different letters are significantly different from each other ($p < 0.05$); [2]Se litter $_{Ca10}$= -1.245 + 3.678X – 2.219X^2 (R^2 = 0.99); [3]Se litter $_{Ca12}$= 0.102 + 0.19X (R^2 = 0.48); [4]Mn litter $_{Ca8}$= -230.792 + 1127.829X – 707.531X^2 (R^2 = 0.99); [5]Mn litter $_{Ca10}$=7.458 + 248.734X (R^2 = 0.94); [6]Mn litter $_{Ca12}$= 145.004 + 124.656X (R^2 = 0.97); [7]Zn litter $_{Ca8}$= 72.122 + 102.258X (R^2 = 0.91); [8]Zn litter $_{Ca10}$= 20.482 + 148.894X (R^2 = 0.96); [9]Zn litter $_{Ca12}$= 32.444 + 146.660X (R^2 = 0.94); ^{z} and w, ^{s} and t, ^{g} and h, ^{p} and ^{q}Means represents by different letters in the same column are different from each other ($p < 0.05$) consider the effect of Ca levels in MS levels; *Coefficient of variation.

12 g kg^{-1} Ca, increasing MS produced a linear increase in litter Se concentration, according to the equation Y = 0.102 + 0.19X (R^2 = 0.48).

With respect to the Mn concentration in the litter, MS had a quadratic effect at 8 g kg^{-1} Ca, represented by Y = -230.792 + 1127.829X – 707.531X^2 (R^2 = 0.99). The highest litter Mn concentration was observed at 0.797 g kg^{-1} MS. At 10 and 12 g kg^{-1} Ca, MS had a linear effect on litter Mn concentration, according to the equations Y = 7.458 + 248.734X (R^2 = 0.94) and Y = 145.004 + 124.656X (R^2 = 0.97), respectively.

At all Ca levels (8, 10 and 12 g kg^{-1}), the litter Zn concentration increased linearly with MS, according to the equations Y = 72.122 + 102.258X (R^2 = 0.91), Y = 20.482 + 148.894X (R^2 = 0.96) and Y = 32.444 + 146.660X (R^2 = 0.94), respectively.

At 0.62 and 0.92 g kg^{-1} MS, the lowest litter Se concentration was observed at 10 g kg^{-1} Ca.

The lowest litter Mn concentrations at 0.62 and 0.72 g kg^{-1} MS were also observed at 10 g kg^{-1} Ca. At 0.82 g kg^{-1} MS, the lowest Mn concentrations were found at 8 and 10 g kg^{-1} Ca. At 0.92 g kg^{-1} MS, the lowest litter Mn concentration was found at 8 g kg^{-1} Ca.

At 0.72 g kg^{-1} MS alone, the lowest Zn concentration was found at 10 and 12 g kg^{-1} Ca.

Discussion

At all MS levels in the diets used in this study, the micromineral concentrations per kilogram of feed were lower than those recommended by the NRC, (1994) and Rostagno et al., (2011).

Research into minerals from inorganic sources has demonstrated an antagonistic relationship between the macromineral Ca and certain trace microminerals, such as Mn, Fe and Zn. However, many studies have reported that this antagonistic relationship may be reduced by supplementing these microminerals using organic trace minerals sources at levels much lower than those recommended.

In the present study, the observed responses of BWG, FI and F:G confirm the hypothesis of an interaction between the levels of dietary Ca and trace micromineral supplementation during the starter phase of broiler production.

When 10 g Ca per kilogram of diet was used, a mineral supplementation level of 0.82 g per kg of diet produced the largest BWG. At Ca levels above or below the recommendation, BWG was not affected, indicating the importance of adequately defining Ca levels as well as supplementing microminerals from organic sources.

The observed FI responses were distinct and also dependent on the levels of Ca and MS. At the lowest Ca concentrations (8 and 10 g kg^{-1}), the highest MS level (0.92 g kg^{-1}) promoted greater FI, which showed a quadratic response and a linear increase with MS at these levels. At the highest Ca concentration (12 g kg^{-1}), this effect was not observed, possibly resulting from excess dietary Ca.

These observed responses of BWG and FI are consistent with those observed for F:G. At the lowest Ca level, the highest MS level stimulated FI, thus decreasing F:G. The observed performance effects confirm the influence of Ca on F:G via the birds' FI.

These results are supported by Bao et al. (2007), who observed better performance in the group of broilers whose diet contained moderate levels of organic mineral, close to those used in the present study and lower than those recommended by the NRC, (1994). Corroborating these results, Peric et al. (2007) and Nollet et al. (2008) found that supplementation with organic minerals souces at levels lower than those currently used with inorganics does not affect the performance of broiler chickens. Both studies also verified that Ca levels lower than 10 and 12 g kg^{-1} and greater than 8 g kg^{-1} yielded favorable performances in broiler chickens when used in combination with organic mineral sources.

When evaluating the effect of increasing dietary Ca levels, Qian et al. (1997) and Alves et al. (2002) observed decreased consumption and weight gain as the level of this mineral in the feed increased. This reduction in consumption with increasing dietary Ca may be related to elevated ionic Ca in the blood, which inhibits the birds' appetite (Lobaugh et al., 1981). Reduced consumption results in lower nutrient availability and utilization, which Shafey et al. (1991) attributed to a decrease in nitrogen digestion. However, this lower nutrient utilization can be attributed to an increased intestinal microflora population, causing increasing irritation in the intestinal mucosa. In contrast, Salter (1973) reported an increase in mucosal thickness, which would impede effective intestinal absorption and diminish dietary transit.

The responses of bone tissue development was dependent on the dietary Ca level, with Ca and ash deposition being positively affected and P deposition negatively affected by higher Ca levels. These results are consistent with the fact that an intermediate Ca level (10 g kg^{-1}) is currently recommended, following requirements suggested by Rostagno et al. (2011) that the Ca levels need to be 0.92 g kg^{-1} (one-to seven days old) and 0.84 g kg^{-1} (eight to 21d old), and support the higher Ca level (12 g kg^{-1}) and impaired P deposition in the bird tibia.

High dietary Ca levels may impair the efficiency of phytase, which was used in all experimental diets in this study, and interfere with the absorption of Ca, P, Zn and Mn. Very low Ca levels impair P absorption, consequently reducing the concentrations of these minerals in the tibia (Schoulten et al., 2002).

Shafey et al. (1991) found that a high Ca concentration (greater than 15.3 g kg^{-1}) in the feed elevated the pH of the crop and ileum contents but did not affect the pH of the other segments of the chicken's gastrointestinal tract. This result suggests that the elevation of intestinal pH as a function of increasing dietary Ca levels reduces the soluble fraction of minerals and their availability for absorption. According to the NRC, (1994), the Ca requirement for one- to 21-day-old broilers is 1 % for diets of 3,200 kcal kg^{-1}. However, Karunajeewa (1976) concluded that 6 g kg^{-1} Ca and 5.7 g kg^{-1} phosphorus are sufficient for adequate growth and bone formation when the minerals are present in highly available forms.

In the present study, the interaction between Ca and MS levels in tissue concentration suggests that although higher Ca levels may affect micromineral utilization, these effects may have been minimized by the use of organic mineral sources with high bioavailability. Consequently, this apparently increased the liver and breast Se and Mn concentrations without reducing the Fe and Zn concentrations. The organic mineral sources of Se and Mn effectively permitted the deposition of these minerals in the tissues. Notably, several studies using inorganic selenium salts have found no increase in the concentration of this mineral in the breast. This micromineral is of interest to the industry because producers are currently seeking to increase its concentration in human food and to advance the productive characteristics of broilers. Thus, Se supplementation is an important strategy to enrich the final product.

Bao et al. (2007) found that trace mineral concentrations (Cu, Fe, Mn and Zn) in the livers of broilers fed a control diet without MS were higher than those in supplemented birds (three levels of organic minerals and one inorganic). Similar results have been attributed to a dilution effect as a result of rapid growth of adequate diets and slow growth in the control diet (Roth, 2003).

Ao et al. (2009) evaluated the effects of different forms of Zn and Cu on broiler performance and tissue mineral content and observed that liver Cu concentration was reduced by Zn supplementation. The Zn content in the duodenal mucosa increased with the addition of organic Zn in the diet, and the Cu content in the duodenal mucosa also increased with the addition of both organic Zn and Cu.

Schoulten et al. (2002) found that increasing the Ca level decreased Mn absorption, while Sebastian et al. (1996) found no effect on the relative retention of Mn in one- to 21-week-old broiler chickens when dietary Ca was increased from 6 to 12.5 g kg^{-1}.

Li et al. (2005) evaluated Mn bioavailability in diets with a high Ca level (18.5 g kg^{-1}) and a normal Ca level (11.7 g kg^{-1}) and found that organic Mn with moderate or strong chelation strength could partially or completely resist the antagonistic effect of increased dietary Ca during digestion and exhibited higher relative bioavailability than under normal dietary Ca conditions.

The organic sources in higher levels of MS (0.82 and 0.92 g kg^{-1}) were responsible for an increase in the mineral concentration of Zn, Mn and Se in tissues. An MS interaction with Ca influenced Fe, Cu and Se. These levels are lower than the current levels practiced for better growth or a better feed-to-gain ratio. These results show the importance of using the sources and levels effectivily to permit higher tissue concentration and are perharps reflected in health or meat quality. The impact of Ca levels needs to be observed in the production system because low levels impaired better growth performance and higher levels interact more with the concentration of microminerals in tissues.

The observed Cu and Fe micromineral concentrations in the litter reflected only the increase in MS, showing higher concentrations with increasing MS in the diet. The interaction between Ca and MS levels confirmed that Ca can interfere with micromineral absorption and, consequently, excretion. Excretion is reduced when the Ca level recommended by Rostagno et al. (2011), i.e., 10 g kg^{-1}, is used, as shown by the results for excreted Se, Mn and Zn.

Bao et al. (2007) also detected a linear increase in Cu, Mn, Zn and Fe excretion with increasing consumption of these microminerals. Thus, birds that were supplemented with 0.82 g kg^{-1} MS and 10 g kg^{-1} Ca and

assured the best performance had lower excretion than those in treatments with a higher MS level. Bao et al. (2010) utilized microminerals from organic sources and evaluated the interaction between them, finding in one experiment that adding Cu, Fe, Mn and Zn in combination not only affected the concentrations of the trace minerals but also influenced Ca and P excretion. These authors also showed that Mn supplementation alone increased only Mn excretion and not the excretion of the other minerals. Compared to Mn supplementation, combined supplementation with Zn, Mn, Cu and Fe had no additional effect on Mn excretion but showed decreased Ca excretion and increased Cu and Fe excretion.

Using 50 % of the NRC, (1994) recommendation for the microminerals Zn, Mn and Cu in organic form and 10.7 or 9 g kg^{-1} Ca in broiler diets, El-Husseiny et al. (2012) found improved performance and carcass yield and lower excretion of these minerals compared to birds whose diet contained 100 % of the recommendation in inorganic form. Manangi et al. (2012) compared low Zn, Cu and Mn levels from organic sources to the industry-standard supplementation levels of the same minerals from inorganic sources. These authors found that the concentration of these microminerals was reduced in the litter of birds supplemented with Zn, Cu and Mn from organic sources by 40, 74 and 35 %, respectively. In contrast, there was no difference in Zn, Cu, Ca and P concentrations in the tibiae of animals that received high dietary supplementation levels from inorganic sources.

The present study demonstrates that micromineral supplementation from organic sources decreases antagonistic interactions with macromineral Ca and reduces binding with nutrients and non-nutritive components of the digesta resulting in less negative interaction in absorption and, consequently, better utilization of these minerals by poultry, favouring adequate tissue deposition (breast and liver) and lower excretion.

Conclusion

The Ca level of 10 g kg^{-1} combined with an MS level of 0.82 g kg^{-1} results in greater weight gain, higher deposition of the macrominerals Ca and P in bone tissue and of the microminerals Mn, Zn, Fe, Cu and Se in the liver and breast and lower excretion of these microminerals in the litter.

Acknowledgments

The authors would like to thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Institutos Nacionais de Ciência e Tecnologia (INCT) and Universidade Federal de Viçosa for important financial support.

Authors' Contributions

Conceptualization: Faria, B.D.; Hannas, M.I.; Rostagno, H.S.; Albino, L.F.T.; Ferreira, A.H.N. Data acquisition: Faria, B.D.; Silva, L.M. Data analysis: Faria, B.D.; Hannas, M.I.; Rostagno, H.S.; Albino, L.F.T. Design of methodology: Faria, B.D.; Hannas, M.I.; Rostagno, H.S.; Albino, L.F.T. Software development: Faria, B.D.; Ribeiro Junior, V. Writing editing: Faria, B.D.; Hannas, M.I.; Ribeiro Junior, V.

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