UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA
CÂMPUS DE BOTUCATU

EFEITOS DE DIFERENTES FONTES DE COBRE E ZINCO NA ALIMENTAÇÃO DE FRANGOS DE CORTE SOBRE O DESEMPENHO, CARACTERÍSTICAS DE CARCAÇA E QUALIDADE ÓSSEA

TATIANE SOUZA DOS SANTOS

Tese apresentada ao Programa de Pós-graduação em Zootecnia como parte das exigências para obtenção do título de Doutor em Zootecnia

BOTUCATU - SP
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S237e

Santos, Tatiane Souza dos
Efeitos de diferentes fontes de cobre e zinco na alimentação de frangos de corte sobre o desempenho, características de carcaça e qualidade óssea / Tatiane Souza dos Santos. -- Botucatu, 2020
121 p. : il., tabs.

Tese (doutorado) - Universidade Estadual Paulista (Unesp), Faculdade de Medicina Veterinária e Zootecnia, Botucatu
Orientador: José Roberto Sartori

1. biodisponibilidade. 2. cobre. 3. frangos de corte. 4. hidroximineral. 5. zinco. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca da Faculdade de Medicina Veterinária e Zootecnia, Botucatu. Dados fornecidos pelo autor(a).

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Tatiane Souza dos Santos - nascida em 19 de maio de 1991, na cidade de Tatuí/SP, filha de José Carlos dos Santos e Marta de Souza dos Santos, ingressou no curso de Zootecnia da Universidade Estadual Paulista "Júlio de Mesquita Filho" – Unesp - Faculdade de Ciências Agrárias e Tecnológicas, Câmpus de Dracena, em julho de 2009 e graduou-se em julho de 2014. Durante a graduação foi bolsista pela Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), processos: 2011/18449-9 e 2013/08637-8, e realizou estágio de conclusão de curso na Universidade Estadual da Carolina do Norte (EUA), onde recebeu a Bolsa Estágio de Pesquisa no Exterior (BEPE - FAPESP), processo: 2013/21763-2. Em agosto de 2014 iniciou o curso de Mestrado em Zootecnia da Unesp - Faculdade de Medicina Veterinária e Zootecnia - Câmpus de Botucatu, onde foi bolsista pelo Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), e pela Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), processo: 2014/27175-8, e graduou-se em junho de 2016. Em agosto de 2016 iniciou o curso de Doutorado em Zootecnia da Unesp - Faculdade de Medicina Veterinária e Zootecnia - Câmpus de Botucatu, onde foi bolsista pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e pela Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), processo: 2017/00338-3. Durante o curso de Doutorado realizou intercâmbio na Universidade da Geórgia (EUA) pelo período de seis meses, com auxílio da Bolsa Estágio de Pesquisa no Exterior (BEPE - FAPESP), processo: 2018/09422-9. Durante a graduação e pós-graduação atuou na área de nutrição de frangos de corte.

Dedico esta conquista aos meus pais ***José Carlos dos Santos*** (*in memoriam*) e ***Marta de Souza dos Santos*** (*in memoriam*). E mesmo estando em outro plano, vocês nunca deixaram de cuidar dos meus passos. A realização deste sonho me traz a certeza que consegui alcançar um pedacinho daquilo que vocês planejaram para minha vida.

E também dedico à minha “tia-mãe” ***Eli Delfino de Souza*** a qual assumiu o papel de mãe em um momento tão doloroso. Você é um exemplo de vida, mãe, amiga e pessoa.

Dedicatória

Ao meu orientador ***Prof. Dr. José Roberto Sartori*** pela excelente orientação durante a minha formação nos cursos de Mestrado e Doutorado. Obrigada pelos ensinamentos, oportunidades, e principalmente por confiar no meu trabalho.

À ***Dra. Juliana Célia Denadai*** a qual sempre me acompanhou e que muito me ajudou na realização deste trabalho. Agradeço pela sua amizade e ensinamentos.

Agradecimento especial

Agradecimentos

À ***Deus***, por nunca deixar de guiar os meus passos.

Aos meus tios, ***Eli Delfino de Souza*** e ***Joaquim Amado Quevedo****,* os quais foram fundamentais para que eu pudesse chegar até aqui.

Aos meus irmãos ***Fábio*** e ***Juliano****,* minha sobrinha ***Júlia*** e minha cunhada ***Caroline****,* por todo apoio e por acreditarem no meu sonho.

Ao ***Leone****,* pelo amor, companheirismo, e por toda força na finalização do meu Doutorado. Obrigada por tornar essa caminhada mais leve.

Ao ***Programa de Pós-graduação em Zootecnia***, em especial a ***Claúdia***, ***Seila*** e ***Ellen*** por sempre estarem prontas a nos ajudar.

À ***Universidade Estadual Paulista "Júlio de Mesquita Filho"*** câmpus de Dracena e câmpus de Botucatu.

À empresa ***Trouw Nutrition***, pela parceria e financiamento da pesquisa.

À ***Fundação de Amparo à Pesquisa do Estado de São Paulo*** pela concessão da bolsa de Doutorado, processo: 2017/00338-2, e pela concessão da Bolsa Estágio de Pesquisa no exterior, processo: 2018/09422-9.

O presente trabalho foi realizado com apoio da **Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES)** - Código de Financiamento 001.

Aos amigos e integrantes da equipe do LabAves, entre novos e antigos (***Mônica, Mayara Rodrigues, Jú Denadai, Jú Rezende, Paola, Natani, Amanda, Fabiana, Nathália, Mariana***); (***Armando, Lívia, Smigo, Léo, Netto, Dani, Calvina, Robert, Fernanda, Jú Batistioli, Priscila, Pequi, Cálcio, Érica, Giovana e Andrey***), e a todos estagiários que muito nos ajudaram. Além de companheiros de trabalho, vocês se tornaram grandes amigos. Sou muita grata por todo apoio que sempre me deram, com certeza essa conquista também é mérito de vocês!

Ao ***Wanderley Thiago da Silva***, Técnico do LabAves, que muito contribuiu para a execução deste trabalho, além de todo apoio para nossa equipe.

Aos funcionários da Fábrica de Ração da Unesp de Botucatu, ***Adriano, Adilson, Alexandre e Sandro****.*

À ***Profa. Dra. Valquíria Cação Cruz-Polycarpo*** Unesp-Dracena, pelos ensinamentos, oportunidades e principalmente pelo direcionamento na minha trajetória acadêmica. Obrigada pela excelente orientação durante a minha IC.

À ***Profa. Dra. Maria Márcia Pereira Sartori*** do Dpto. de Produção e Melhoramento Vegetal–FCA, pela sua grande contribuição neste trabalho com a realização das análises estatísticas.

Ao ***Prof. Dr. Luiz Carlos Vulcano*** do Dpto. de Reprodução Animal e Radiologia Veterinária, pela grande colaboração com a análise de densitometria óssea.

Ao ***Prof. Dr. Adriano Wagner Ballarin*** do Dpto. de Engenharia Agronômica –FCA, por ter contribuído com o uso de seu laboratório e ao **Sr. Ailton** por ter me auxiliado nas análises de resistência óssea.

Ao ***Prof. Dr. Roberto de Oliveira Roça*** por ceder seu laboratório para realização das análises de qualidade de carne. Agradeço também ***Carolina* e *Nataly*** por toda ajuda.

Ao ***Prof. Dr. Paulo Roberto de Lima Meirelles*** do Dpto. de Melhoramento e Nutrição Animal–FMVZ por ter contribuído com parte das análises bromatológicas no Laboratório de Bromatologia FMVZ-Botucatu e, especialmente, a ***Gisele Setznagl*.**

Ao ***Prof. Dr. Pedro de Magalhães Padilha*** do Dpto. de Química e Bioquímica –IBB, por ter contribuído com a realização das análises de minerais. Agradeço também ao ***José*** pela paciência e toda ajuda.

À ***Profa. Dra. Regina Kiomi Takahira*** do Dpto. de Clínica Veterinária-FMVZ e seus orientados, ***Vitor e Roberta*** pela ajuda com a realização dos hemogramas.

À ***Profa. Dra. Maria Claúdia Araripe Sucupira*** do Dpto. de Clínica Médica – FMVZ/USP, pela ajuda com a realização da análise enzimática da ceruloplasmina.

Ao ***Dr. Woo Kyun Kim*** pela orientação durante a realização do Doutorado Sanduíche (Universidade da Geórgia), e toda sua equipe: ***Fernanda Castro**, **Sudhir Yadav**, **Po-Yun Teng**, **Sean**, **Dima**, **Dorsey*** e ***Jinquan*.**

À ***Karolina Von Zuben Augusto***, pela parceria durante a realização deste trabalho, e principalmente por todo apoio e incentivo na busca de novos desafios.

Aos professores, ***José Roberto Sartori, Margarida Maria Barros e Antonio Celso Pezzato*** por todas contribuições no Exame Geral de Qualificação.

À banca examinadora da minha defesa de Tese do Doutorado, ***Prof. Dr. José Roberto Sartori, Prof. Dr. Antonio Celso Pezzato, Profa. Dra. Valquíria Cação Cruz-Polycarpo, Dra. Carolina Toledo Santos e Dra. Karolina Von Zuben Augusto.***

Às amigas ***Viviane, Mariane, Camila, Pâmela e Laura***, por todo carinho e conselhos

À querida ***República Karkará*** da Unesp-Dracena.

Aos meus companheiros voadores, ***Teca, Teco, Pipoca, Pintinha e Piu Piu***.

E a todos aqueles que de alguma forma contribuíram para a realização deste trabalho.

MUITO OBRIGADA!

Epígrafe

Não fui eu que lhe ordenei?
Seja forte e corajoso!
Não se apavore, nem se desanime, pois o Senhor, o seu Deus,
estará com você por onde você andar".

Josué 1:9

RESUMO GERAL

O experimento foi conduzido para investigar os efeitos da suplementação de cobre e zinco nas dietas de frangos de corte, utilizando duas fontes minerais (hidroximineral ou sulfato) no desempenho, rendimento de carcaça e partes, qualidade de carne, concentração mineral nos tecidos e absorção aparente ileal de cobre e zinco, características do tecido ósseo e pele, e indicadores do status de saúde. O total de 1.792 pintainhos machos de um dia da linhagem Cobb 500 foram distribuídos aleatoriamente em oito tratamentos com oito repetições por tratamento. As fontes de hidroximineral utilizadas foram: dois níveis de hidroxicloreto de Cu (CHC) (baixo e alto), combinados com três níveis de hidroxicloreto de Zn (ZHC) (baixo, médio e alto); e dois tratamentos adicionais contendo sulfato, sendo o sulfato de Cu monohidratado (SCM) (baixo e alto) combinado com alto nível de sulfato de Zn monohidratado (SZM). Os tratamentos foram: 15 mg/kg de SCM + 120 mg/kg de SZM; 150 mg/kg de SCM + 120 mg/kg de SZM; 15 mg/kg de CHC + 80 mg/kg de ZHC; 15 mg/kg de CHC + 100 mg/kg de ZHC; 15 mg/kg de CHC + 120 mg/kg de ZHC; 150 mg/kg de CHC + 80 mg/kg de ZHC; 150 mg/kg de CHC + 100 mg/kg de ZHC; 150 mg/kg de CHC + 120 mg/kg de ZHC. As médias foram submetidas à ANOVA, e quando significativas foram comparadas pelos testes de *Tukey* e *Dunne*t com 5% de significância. Aos 21 dias de idade, frangos alimentados com dietas contendo alto-CHC apresentaram melhores resultados de peso corporal, ganho de peso, conversão alimentar em comparação aos que receberam baixo-CHC. Ao final do período experimental, frangos pertencentes ao grupo alto- CHC combinado com baixo ou médio-ZHC, apresentaram melhor conversão alimentar em comparação aos suplementados com baixo-CHC. Na qualidade de carne, a maior incidência de vermelho no músculo do peito foi observada em frangos suplementados com alto-CHC em comparação aos suplementados com baixo-CHC. A suplementação com nível baixo-ZHC proporcionou maior rendimento de carcaça, resistência de pele, em comparação ao médio ou alto-ZHC. Na qualidade óssea, a densidade mineral óssea da epífise proximal, o teor de cinzas, e o conteúdo mineral da tíbia foram influenciados positivamente com a suplementação de baixo-CHC combinado com med-ZHC em comparação ao alto-CHC. Portanto, nós concluímos que a fonte de hidroximineral apresentou resultados satisfatórios, podendo ser considerada como alternativa para a substituição do sulfato nas dietas de frangos de corte. Além disso, entre os níveis de cobre e zinco suplementados, o nível alto-CHC foi eficiente para os melhores resultados de desempenho, enquanto o nível médio-ZHC demonstrou melhorar o desenvolvimento ósseo, a integridade de pele, e paramêtros de rendimento de carcaça. Sendo assim, sugerimos que a suplementação de cobre e zinco pode ser utilizada como estratégia nutricional para garantir o crescimento de aves saudáveis e prevenir a incidência de problemas ósseos.

Palavras-chave: biodisponibilidade, cobre, frangos de corte, hidroximineral, zinco

ABSTRACT

The experiment was carried out to investigate the effects of copper and zinc supplementation on broiler diets, using two mineral sources (hydroxymineral or sulfate) on performance, carcass and parts yield, meat quality, mineral concentration in tissues and apparent ileal absorption of copper and zinc, bone characteristics and skin tissue, and indicators of health status. The total of 1,792 day-old male chicks of the Cobb 500 strain were randomly assigned to eight treatments with eight replicates per treatment. The hydroximineral sources used were: two levels of Cu hydroxychloride (CHC) (low and high), combined with three levels of Zn hydroxychloride (ZHC) (low, medium and high); and two additional treatments containing sulfate, with Cu sulfate monohydrate (SCM) (low and high) combined with a high level of Zn sulfate monohydrate (SZM). The treatments were: 15 mg/kg of SCM + 120 mg/kg of SZM; 150 mg/kg SCM + 120 mg/kg SZM; 15 mg/kg of CHC + 80 mg/kg of ZHC; 15 mg/kg of CHC + 100 mg/kg of ZHC; 15 mg/kg of CHC + 120 mg/kg of ZHC; 150 mg/kg of CHC + 80 mg/kg of ZHC; 150 mg/kg of CHC + 100 mg/kg of ZHC; 150 mg/kg of CHC + 120 mg/kg of ZHC. The means were submitted to ANOVA, and when significant, they were compared by *Tukey's* and *Dunnet's* tests with 5% significance. At 21 days of age, chickens fed diets containing high-CHC showed better results for body weight, weight gain, feed conversion as compared to those receiving low-CHC. At the end of the experimental period, broilers in the group high-CHC combined with low or med-ZHC, showed better feed conversion as compared to those supplemented with low-CHC. In meat quality, the highest incidence of red in the breast muscle was observed in broilers supplemented with high-CHC as compared to those supplemented with low-CHC. Supplementation with low-ZHC level provided higher carcass yield and skin strenght as compared to med or high-ZHC. In bone quality, bone mineral density of the proximal epiphysis, the ash content, and the mineral contente of the tibia were positively influenced with low-CHC supplementation combined with med-ZHC as compared to high-CHC. Therefore, we conclude that the hydroxymineral source presented satisfactory results, which can be considered as an alternative to replace sulfate in broiler diets. In addition, among supplemented copper and zinc levels, the high-CHC level was effictive for the best performance results, while the med-ZHC level was shown to improve bone development, skin integrity, and carcass yield parameters. Therefore, we suggest that copper and zinc supplementation can be used as a nutritional strategy to ensure the growth of healthy birds and prevent the incidence of bone problems.

Keywords: bioavailability, copper, broilers, hydroxymineral, zinc

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LISTA DE ABREVIATURAS

a*	Redness
b*	Yellowness
BA	Bone ash
BS	Bone strength
BW	Body weight
BWG	Body weight gain
CHC	Copper hydroxychloride
CL	Cooking loss
Cp	Ceruloplasmin
Cr	Cromo/ Chromium
CSM	Copper sulfate monohydrate
Cu	Cobre/ copper
DE	Distal epiphysis
DL	Drip loss
FCR	Feed conversion ratio
EPEF	European production efficiency factor
FI	Feed intake
L*	Lightness
ME	Metabolizable energy
P-value	Probability
PE	Proximal epiphysis
pH	Hidrogenionic potential
ppm	Part per million
SEM	Standard error of the mean
SF	Shear force
SOD	Superoxide dismutase
VB	Viability
ZHC	Zinc hydroxychloride
ZSM	Zinc sulfate monohydrate
Zn	Zinco/ zinc

SUMÁRIO

CAPÍTULO 1

Considerações iniciais

A avicultura de corte brasileira tem demostrando amplo crescimento nas últimas décadas, isso porque o número de pesquisas nas áreas de nutrição, genética, manejo, bem-estar e ambiência tem gerado cada vez mais conhecimento de como proporcionar condições ideais para a criação das aves. Em 2015 o Brasil deixou de ocupar a terceira posição na categoria de produção mundial de carne de frango, e passou para segundo lugar, sendo precedido apenas pelos Estados Unidos. No último relatório publicado pela Associação Brasileira de Proteína Animal (ABPA, 2019), o Brasil ocupou a posição de primeiro exportador mundial (4.101 mil toneladas/ano) e segundo produtor mundial de carne de frangos de corte (12.86 mil toneladas/ano), onde o crescimento no setor avícola pode ser confirmado com os respectivos números citados.

Dentro desse contexto, pesquisas relacionadas com a nutrição animal objetivam investigar estratégias nutricionais eficientes para atender as exigências, manter um bom estado de saúde animal, além de reduzir o impacto ambiental gerado pelo sistema de produção intensivo. Pesquisas realizadas na área da nutrição de aves têm indicado que a suplementação de microminerais com níveis acima do recomendado pelos manuais da linhagem tem mostrado diversos benefícios para a saúde e para os índices de desempenho (LEESON, 2009; ABD EL-HACK, 2017). Os minerais são essenciais para diversos processos biológicos, já que desempenham papéis fisiológicos, estruturais, regulatórios e catalíticos (SUTTLE, 2010), onde os microminerais cobre (Cu) e zinco (Zn) têm sido associados com as melhores respostas frente aos desafios no sistema de produção de aves (LEESON, 2009; ABD EL-HACK, 2017).

O Cu teve sua essencialidade descoberta por volta de 1920 quando Hart et al. (1928) observaram que a deficiência deste elemento desencadeou o processo de anemia em ratos. De acordo com Leeson (2009) esse micromineral é essencial como cofator enzimático, constituinte de proteínas no sangue, e como melhorador de desempenho quando suplementado com doses elevadas nas dietas de frangos de corte. O Zn foi descoberto por volta de 1930, e sua essencialidade está associada com diversos processos biológicos, como crescimento e desenvolvimento do tecido ósseo, além de ser cofator de mais de 300 enzimas no metabolismo (COHEN e STEWARD, 2014; ABD EL-HACK, 2017).

As fontes inorgânicas foram as primeiras a serem utilizadas para a suplementação mineral na dieta, sendo utilizadas nas formas de sulfatos, óxidos ou carbonatos. De acordo com Leeson (2009), a fonte mais utilizada para a suplementação de Cu na dieta das aves é o sulfato de cobre pentahidratado; enquanto que para o Zn, o óxido e sulfato monohidratado são as fontes mais utilizadas (LEESON e SUMMERS, 1997). No entanto, embora a suplementação com níveis acima do recomendado de determinados minerais seja benéfica para o desempenho de frangos de corte, o excesso de mineral eliminado pode ser prejudicial e causar impactos ambientais. Pesti e Bakalli (1998) suplementaram galinhas poedeiras com 250 mg/kg de sulfato de cobre pentahidratado e encontram 940 mg/kg de Cu nas excretas, contra 35 mg/kg de Cu daquelas que receberam 5 mg/kg de Cu. A razão para esse efeito negativo pode ser explicada pelas propriedades químicas das fontes inorgânicas, as quais são formadas por ligações iônicas e que facilmente se dissociam nas primeiras porções do trato gastrintestinal da ave (no papo, por exemplo), possibilitando a formação de quelatos ao longo do trato gastrintestinal entre mineral-mineral ou mineral e outros nutrientes (aminoácidos e proteína), e por fim, prejudicando o processo de absorção (COHEN e STEWARD, 2014).

Em função dos aspectos negativos causados pelo uso das fontes mencionadas anteriormente, estudos realizados na década de 90 desenvolveram outra fonte inorgânica chamada de hidroximineral. Este composto contém ligações covalentes entre o mineral e grupos OH, o que proporciona maior resistência às condições do trato gastrintestinal, em comparação as fontes convencionais como sulfatos, por exemplo (Figura 1). Dessa forma, espera-se que mais mineral possa atingir o local alvo de absorção, sem que ocorram interações negativas (COHEN e STEWARD, 2014).

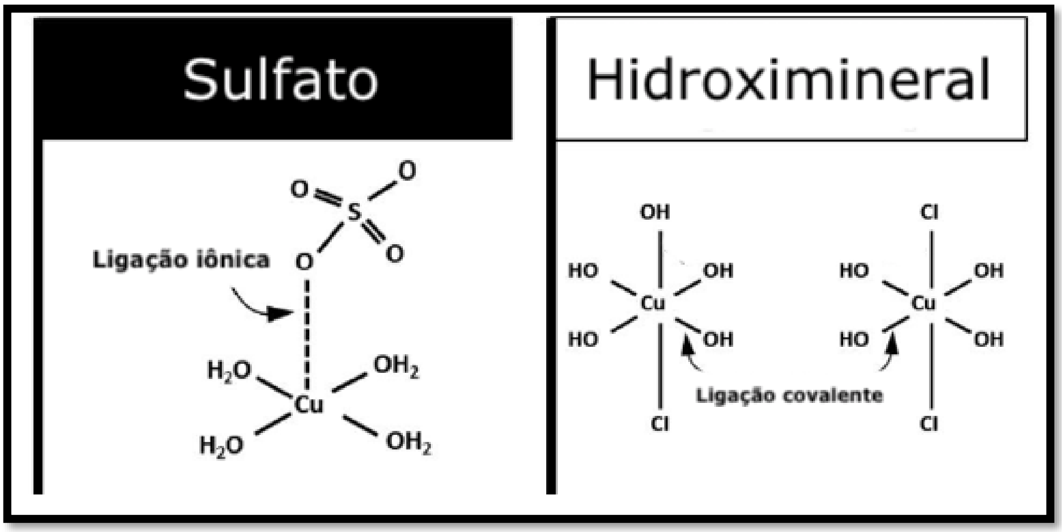

Figura 1 Tipos de ligações
Fonte: Adaptado de KVIDERA, 2019

Devido a necessidade de se estabelecer níveis ideais para suplementação mineral, e do uso de fontes que causem menor impacto ambiental, novos estudos são necessários para reduzir os questionamentos gerados durante a formulação de dietas para aves. O excesso e a deficiência de um determinado mineral são prejudiciais ao desenvolvimento animal, principalmente pela questão do antagonismo (SUTTLE, 2010). No que diz respeito ao impacto ambiental, o acúmulo de Cu e Zn no solo pode ser tóxico para plantas e microorganismos (JONDREVILLE et al. 2003).

Visto a importância que ambos microminerais representam para o desenvolvimento adequado de frangos de corte, este estudo objetivou avaliar se a combinação de Cu e Zn utilizando diferentes níveis de inclusão e diferentes fontes na dieta seria capaz de proporcionar melhorias no desempenho, características de carne e parâmetros ósseos.

1. REVISÃO DE LITERATURA

1.1. Minerais: definição e importância

Os minerais estão entre os nutrientes imprescindíveis para o desenvolvimento e manutenção do organismo, participando de várias reações metabólicas e, diferente dos outros nutrientes, estes não são sintetizados pelo organismo (BETERCHINNI, 2004). Nas aves, os minerais representam cerca de 2,8 a 3,2% do peso vivo e podem ser divididos de acordo com a função biológica nos tecidos. Os minerais são classificados em macrominerais como: cálcio, cloro, magnésio, fósforo, potássio, sódio e enxofre; e como microminerais: cobre, cobalto, iodo, ferro, zinco, manganês, selênio, cromo, molibdênio, boro e flúor (BETERCHINNI, 2004).

A importância desses nutrientes passou a ser investigada após estudos realizados com ratos que receberam dietas purificadas, e os resultados revelaram que o cobre, manganês e zinco foram limitantes para o crescimento (UNDERWOOD, 1977). Diversas funções têm sido atribuídas aos minerais, sendo elas: fisiológicas, estruturais, catalíticas e regulatórias (SUTTLE, 2010).

Em dietas para frangos de corte formuladas a base de milho e farelo de soja os macrominerais suplementados em maior quantidade são cálcio (Ca) e fósforo (P), utilizando normalmente o calcário calcitico e fosfato bicálcico, respectivamente. Esses macrominerais estão presentes em sua maior parte nos ossos na forma de hidroxiapatita, conferindo rigidez e resistência ao tecido (FIELD, 2000; JUNQUEIRA e CARNEIRO, 2008). No caso de microminerais, estes devem ser suplementados na dieta sob forma de uma pré-mistura. O excesso e a deficiência de um determinado mineral são prejudiciais ao desenvolvimento animal, principalmente pela questão do antagonismo (SUTTLE, 2010). Exemplos clássicos de interação negativa entre o cobre com outros minerais como zinco, ferro e molibdênio são descritos na literatura. Sendo assim, é de grande importância respeitar os limites de suplementação e evitar o desperdício (LEESON, 2009).

Entre as várias opções disponíveis para suplementação mineral nas dietas das aves, estas podem ser divididas em duas formas: inorgânicas e orgânicas. As fontes inorgânicas foram as primeiras a serem estudadas, e o baixo custo de obtenção possibilitou o seu uso nas dietas. No entanto, o principal fator negativo atribuído para as fontes inorgânicas é a rápida dissociação nas primeiras porções do trato gastrintestinal da ave, levando a formação de quelatos os quais tornam os minerais indisponíveis para absorção (GOMES et al., 2009; AKSU et al., 2012; PACHECO et al., 2017). Os minerais apresentam elevada capacidade de interação química entre si, e com outros nutrientes, desde o momento do preparo da dieta, até chegar no intestino da ave, que é o principal local de absorção (COHEN e STEWARD, 2014). Mais tarde, na década de 70 foram introduzidas no mercado as fontes orgânicas, as quais compreendem a ligação do mineral com um composto orgânico, utilizando ligação covalente. Entretanto, o elevado custo dessa fonte é apresentado como fator decisivo para sua escolha (COHEN e STEWARD, 2014).

Nesse sentido, estudos realizados na década de 90 desenvolveram outra fonte inorgânica de baixo custo, chamada de hidroximineral. A principal diferença entre sulfato e o hidroximineral é o tipo de ligação na estrutura química, onde o primeiro é formado por ligação iônica, e o segundo por ligação covalente. O hidroximineral apresenta uma rede cristalina tridimensional de alta estabilidade, tornando as ligações covalentes fortes o suficiente para limitar reações parasitarias, e fracas o suficiente para liberar o metal no intestino (COHEN e STEWARD, 2014). Dessa forma, devido a presença de ligações covalentes nos hidroximinerais, é provável que a absorção do mineral ocorra com maior eficiência, uma vez que essas ligações são mais resistentes às condições do trato gastrintestinal quando comparada com as ligações iônicas. Portanto, o ponto chave para garantir a absorção no local desejado são as forças de ligação da fonte utilizada (COHEN e STEWARD, 2014).

Os hidroximinerais encontram-se disponíveis para a suplementação de cobre, zinco ou manganês nas dietas das aves, podendo ser utilizado nos limites da exigência nutricional e também com níveis superiores aos recomendados. Na área da nutrição de aves, a suplementação de microminerais com níveis acima do recomendado tem mostrado diversos benefícios para a saúde e para os índices de desempenho, e no caso do cobre (Cu) e do zinco (Zn), estes têm sido associados com as melhores respostas frente aos desafios do sistema de produção de aves (LEESON, 2009; ABD EL-HACK, 2017).

Uma comparação entre as fontes de sulfato e hidroximineral de cobre foi realizada por Klasing et al. (2010), visando avaliar a capacidade de dissociação do cobre ao longo do trato gastrintestinal de frangos de corte. Estes autores observaram maior quantidade de cobre indisponível ao longo do trato gastrintestinal de frangos que receberam a fonte de sulfato em comparação aos que receberam hidroximineral, e confirmaram que esta fonte apresenta maior biodisponibilidade em relação ao sulfato. Olukosi et al. (2018) avaliaram a inclusão de duas fontes de cobre e zinco (sulfato e hidroximineral) e dois níveis de inclusão de zinco (80 e 20 mg/kg) na dieta de frangos de corte, e observaram melhores resultados de eficiência alimentar e rendimento de peito aos 35 dias de idade, em frangos que receberam suplementação com hidroximineral de cobre e zinco em comparação a fonte de sulfato.

A suplementação de cobre na dieta de frangos de corte é realizada normalmente com a utilização de sulfato de cobre pentahidratado, embora existam outras fontes disponíveis, como os óxidos, carbonatos e citratos (LEESON, 2009). A ausência de suplementação de minerais em dietas a base de milho e soja, pode proporcionar deficiência mineral, já que as condições de manejo do solo também podem influenciar diretamente a qualidade dos ingredientes utilizados para o preparo da dieta (LEESON, 2009). Estudos anteriores relataram os benefícios da suplementação de cobre na dieta de frangos de corte, como é o caso de Pesti e Bakalli (1996) e Ewing et al. (1998), os quais afirmaram que o citrato de cobre é mais eficiente que o sulfato de cobre para frangos de corte. Por outro lado, o cloreto tribásico de cobre (hidroximineral) tem sido considerado como alternativa ao uso de sulfato de cobre nas dietas de frangos de corte e poedeiras, pois em estudo realizado por Pang e Applegate (2007) foi constatado que a solubilidade do sulfato de cobre na água foi de 99%, enquanto que o cloreto de cobre tribásico foi insolúvel em pH neutro e sob condições ácidas apresentou solubilidade de 80%. Luo et al. (2005) sugeriram que o cloreto de cobre tribásico foi menos destrutivo para vitaminas, uma vez que os níveis de vitamina E no plasma e fígado foram maiores em frangos alimentados com essa fonte quando comparados com dietas contendo sulfato.

Com relação ao zinco, as fontes inorgânicas mais utilizadas são óxido de zinco (ZnO: 72% Zn) e sulfato de zinco monohidratado ($ZnSO_4$-H_2O: 36% Zn) (LEESON e SUMMERS, 2005). O óxido de zinco apresenta menor biodisponibilidade quando comparado com o sulfato para as aves (SANDOVAL et al., 1997). No entanto, o sulfato apresenta maior solubilidade na água, o que permite a formação de íons livres, os quais tem a capacidade de apresentar interação negativa com vitaminas e reduzir o valor nutritivo da dieta (ABD EL-HACK et al., 2017). Por outro lado, estudos utilizando fontes orgânicas de zinco têm reportado efeitos benéficos após a sua suplementação na dieta das aves. Ao et al. (2006) observaram que a suplementação da dieta de frangos de corte com Zn-proteína (5 a 40 mg/kg) melhorou o ganho de peso e consumo de ração. No estudo de Feng et al. (2010), os frangos receberam dietas suplementadas com níveis entre 90 a 120 mg/kg de Zn-glicina e nenhuma diferença significativa foi encontrada entre as dietas com nível de 120 mg/kg de Zn orgânico, comparado com aves do tratamento suplementado com Zn inorgânico. Os autores concluíram que a fonte de zinco pode ter pouca influência no desempenho, e que o estabelecimento do nível ótimo de suplementação é mais importante para a obtenção de melhores resultados (ABD EL-HACK et al., 2017).

1.2. Cobre e zinco: metabolismo

Cobre

A primeira evidência da importância de cobre para o organismo foi encontrada por Hart et al. (1928) quando observaram quadro de anemia em ratos que receberam dietas com deficiência de cobre. A interação entre o cobre e o ferro ocorre pela presença do cobre na estrutura de proteínas plasmáticas (enzima ferroxidase) as quais estão envolvidas na conversão do ferro^{2+} para ferro^{3+} para ser utilizado na formação da hemoglobina (LEESON, 2009). O cobre é cofator de diversas enzimas, como a citocromo oxidase, lisil oxidase, ceruloplasmina, superóxido dismutase e dopamina beta-hidroxilase (LIM e PAIK, 2006). Além disso, é componente de proteínas sanguíneas como a eritrocupreína (eritrócitos), estando envolvido no metabolismo do oxigênio, e também é essencial para mecanismos relacionados a reprodução e formação óssea (LEESON, 2009; SUTTLE, 2010). Desta forma, os sinais da deficiência de cobre são anemia, inadequada formação da cartilagem, redução no crescimento, fibrose do miocárdio e diarreia (LEESON, 2009).

O cobre é absorvido ligado a albumina plasmática, sendo transportado até o fígado, local onde é incorporado com a ceruloplasmina e depois liberado para a circulação. A ceruloplasmina contém entre 80 a 97% do cobre plasmático em todas a espécies (BUKLEY, 2000). Nas aves, a absorção do cobre ocorre no proventrículo e principalmente no duodeno, e dependendo da concentração deste mineral na dieta, diferentes mecanismos de absorção são utilizados, sendo que altas concentrações do mineral na dieta proporcionam absorção pela difusão simples, e em baixas concentrações, o transporte ativo é utilizado com o auxílio de transportadores de membrana (MASSUQUETTO et al., 2017). O mecanismo de transporte ativo do cobre é mediado pela proteína transportadora de cobre (CRT1) o qual também pode realizar o transporte do ferro e zinco para dentro da célula intestinal (MASSUQUETTO et al., 2017). Em função da possibilidade de outros minerais se ligarem ao mesmo carreador do cobre, o excesso de outro mineral como o zinco por exemplo, pode induzir a deficiência de cobre no organismo (LEESON, 2009).

Após a absorção, diferentes rotas são utilizadas pelo cobre e, de acordo com McDonald et al. (2010), este mineral pode permanecer estocado temporariamente para as trocas sanguíneas e excreção pela bile no fígado, e pode ser incorporado com a ceruloplasmina ou permanecer armazenado durante um longo período.

A concentração de cobre encontrada no fígado dos monogástricos varia em torno de 8 ou 9% (BUKLEY, 2000). Com relação as diferentes espécies de animais (ruminantes e não ruminantes) há grande diferença na homeostase do cobre no fígado, pois a concentração de 150 mg/kg de cobre na dieta de ovelhas é altamente tóxica, enquanto que para frangos de corte, este nível é eficiente para melhorar o desempenho (BUKLEY, 2000).

A maior parte do cobre encontra-se armazenada no fígado, e a bile é a sua rota de excreção pelo processo de exocitose por meio das lisozimas hepatocelulares (LEESON, 2009). A reciclagem do cobre é eficiente nos túbulos renais (DAVIDSON et al., 1974) e pouco eficiente pela secreção biliar (AYOAGI e BAKER, 1995), desta forma, o excedente de cobre é eliminado pela bile quando as reservas do fígado são maximizadas (LEESON, 2009).

Zinco

Em estudo realizado por Todd et al. (1934) foi descoberta uma dentre muitas funções que o zinco desempenha no organismo. Os autores deste estudo observaram que o zinco estava presente na anidrase carbônica, a qual atua no transporte do dióxido de carbono dos tecidos para o pulmão. A partir disso, diversas funcionalidades foram atribuídas ao zinco, sendo que mais de 300 enzimas presentes no organismo são dependentes deste mineral, além de atuar no metabolismo energético de carboidratos, de ácidos nucleicos, das proteínas, e também de alguns hormônios como o glucagon, insulina, hormônios sexuais e de crescimento (VALLE e FALCHUK, 1993; NAZ et al., 2016). As metaloenzimas dependentes de zinco são divididas em seis classes, sendo elas: oxidoredutase (catalisa oxirredução entre dois substratos), transferase (catalisa a transferência de um grupo que não seja hidrogênio), hidrolase (catalisa a hidrólise dos ésteres, éter, peptídeo, glicosil, anidrido ácido, ligações C-C, C-halogeneto ou P-N), liase (catalisa a remoção de grupos a partir de substratos por outros mecanismos de hidrólise, deixando duplas ligações), isomerase (catalisa a interconversão de isômeros ópticos, geométricos, ou posicionais), e ligases (catalisa a ligação de dois componentes acoplados à quebra de uma ligação pirofosfato em ATP ou um composto similar) (O'DELL, 1992).

O zinco é um micromineral que, assim como o cobre, é requerido para diversos processos biológicos, por exemplo, durante o crescimento e mantença, desenvolvimento ósseo, empenamento e regulação do apetite (BATAL et al., 2001; OLGUN e YIDIZ, 2016). O zinco é indispensável para ação do sistema antioxidante, pois durante os processos metabólicos há a produção de compostos altamente reativos conhecidos como *espécies reativas ao oxigênio*

(EROs), e que por sua vez são capazes de induzir a doenças crônicas, danos na membrana e DNA da célula (RAHMAN et al., 2014). A suplementação de zinco na dieta pode proporcionar dois mecanismos contra os efeitos deletérios do estresse oxidativo, sendo agudo ou crônico. O mecanismo agudo compreende o antagonismo ativo-redox para metais de transição e pela proteção de proteínas sulfidrilas; o mecanismo crônico envolve a proteção indireta a partir de pró-oxidantes a partir da indução de outras substancias antioxidantes, como a metalotioneina por exemplo, a qual tem a capacidade de combater os EROs (OTEIZA et al. 1996).

Nas aves, a absorção do zinco ocorre principalmente no duodeno e nas primeiras porções do jejuno com a utilização do transporte ativo ou difusão passiva, e sua entrada no enterócito é mediada pela transportador de cátion bivalente 1 (DCT1), o qual também auxilia na absorção de outros minerais como o manganês, cobre e níquel (COMINETTI e COZZOLINO, 2009; MASSUQUETTO et al., 2017). Após a entrada no enterócito, o zinco pode se ligar a proteína intestinal rica em cisteína (CRIP) ou metalotioneína (MT) (MASSUQUETTO et al., 2017).

Após a absorção, o zinco é transportado das células intestinais para outros tecidos com o auxílio de transportadores específicos, os quais são divididos em *Zinc Transporters* (ZnT) e *Zinc and Irt-like Proteins* (ZIPs) (SALGUEIRO et al., 2000; LIUZZI e COUSINS, 2004; COUSINS et al., 2006; COMINETTI e COZZOLINO, 2009). Na classe dos transportadores ZnT, existem 10 tipos que diferem de acordo com o tecido alvo, por exemplo o ZnT1 é expresso em diversos tecidos, enquanto o ZnT2 é expresso apenas no intestino, rins e testículo; no caso dos transportadores ZIPs, são divididos em 14, e estes levam o zinco dos fluídos extracelulares ou de vesículas intracelulares para dentro do citoplasma das células (SALGUEIRO et al., 2000; LIUZZI e COUSINS, 2004; COUSINS et al., 2006; COMINETTI e COZZOLINO, 2009).

Os tecidos considerados como *pools* de zinco são os ossos e músculos, enquanto o fígado representa menos que 5% do total deste mineral no corpo, e quando transportado no plasma, o zinco encontra-se ligado a albumina, e além disso o zinco plasmático é pouco representativo, estando em torno de 0,5% em relação ao zinco corporal (BUCKLEY, 2000).

O processo de absorção do zinco ocorre conforme a necessidade para os processos em que este mineral é utilizado, via transporte ativo (SUTTLE, 2010). Em estudo realizado por Jonhson et al. (1988) observou-se que houve redução substancial no coeficiente de absorção do zinco, quando ratos receberam dietas com maiores concentrações deste mineral.

1.3. Cobre e zinco na saúde e desenvolvimento

Os microminerais Cu e Zn são essenciais para o crescimento, além de beneficiar a saúde dos animais. As exigências nutricionais desses microminerais de acordo com Rostagno et al.

(2017) são: 11,68, 10,54, 8,31, 6,79 e 6,08 mg/kg para o cobre; e de 76,15, 68,72, 54,21, 44,29 e 39,67 mg/kg para o zinco durante as fases pré-inicial, inicial, crescimento 1, crescimento 2 e final de frangos de corte, respectivamente. O fornecimento desses minerais nos níveis recomendados é indispensável para garantir o adequado desenvolvimento das aves. Por outro lado, o uso de doses elevadas (também chamadas de farmacológicas), variando entre 125-250 mg/kg de cobre nas dietas de frangos de corte, tem sido relacionada como alternativa eficiente para melhorar o desempenho, devido ao efeito bacteriostático deste mineral, promovendo melhor saúde intestinal (PESTI e BAKALLI, 1996; EWING et al., 1998).

A suplementação de cobre na dieta de frangos de corte com níveis acima do recomendado além de beneficiar a saúde intestinal, também é capaz reduzir o colesterol plasmático, em função do cobre estar envolvido no metabolismo lipídico quando suplementado em dose elevada, regulando a biossíntese do colesterol plasmático por meio da redução do fluxo de carbonos para a via mevalonato (KIM et al., 1992; BAKALLI e PESTI, 1995). Em estudo realizado por Samanta et al. (2011), os autores utilizaram níveis de 75, 150 e 250 mg/kg de sulfato de cobre pentahidratado na dieta de frangos de corte, e os mesmos concluíram que a inclusão de 150 mg/kg de sulfato de cobre proporcionou melhor ganho de peso, conversão alimentar além de menores níveis de colesterol plasmático em comparação aos outros níveis utilizados. Por outro lado, Persia et al. (2004) sugeriram ocorrência de toxicidade quando realizou a suplementação de 640 ppm de cloreto de cobre, além da redução no ganho de peso de frangos de corte. No caso de animais monogástricos, a tolerância para elevados níveis de cobre na dieta é alta, e quando fornecido em excesso, o cobre é inicialmente armazenado no fígado, e ao atingir 20 a 25x a mais que o nível normal, o cobre é liberado na corrente sanguínea, podendo provocar hemólise (AOYAGI e BAKER, 1993; LESSON, 2009).

De acordo com estudo realizado por Jensen e Maurice (1979), a suplementação de cobre na dieta de frangos de corte com níveis entre 500-700 mg/kg provocou redução no crescimento das aves. Além disso, foi observado em outro estudo realizado por Wideman et al. (1996) que a suplementação de 200 mg/kg de cobre na dieta provocou proventriculite. Em aves infectadas por *Eimeria,* a suplementação com 500 ppm de cobre proporcionou acúmulo de 60 x mais de cobre no fígado, enquanto que as aves do tratamento controle apresentaram acúmulo de 20 x de cobre no fígado (FOX et al., 1987).

Alguns estudos têm evidenciado a relação entre a suplementação com elevados níveis de cobre com a redução do colesterol no ovo e na carne. Pesti e Bakalli (1998) observaram redução de 11,7 mg/kg para 8,6 mg/kg de colesterol no ovo após a suplementação com 125 ppm de cobre. No estudo de Lien et al. (2004), a suplementação com 250 ppm de cobre na dieta proporcionou redução de colesterol no ovo em 20% e redução de 10% no soro. Em frangos de corte, foi observado redução de 20% do colesterol na carne do peito após a suplementação com 250 ppm de cobre na dieta (BAKALLI e PESTI, 1995).

A suplementação de zinco na dieta de frangos de corte deve ser realizada devido à escassez deste mineral nos ingredientes (BAO et al., 2007). Os benefícios da inclusão de zinco na dieta foram reportados por diversos autores. El-Hussey et al. (2008) encontraram melhor ganho de peso, produção de ovos e qualidade de ovos após a suplementação de poedeiras com oxido de zinco. Chand et al. (2014) observaram maior ganho de peso em frangos suplementados com 60 mg/kg de zinco inorgânico, submetidos ao estresse pelo calor.

A deficiência de zinco nas aves é associada principalmente com a redução do crescimento, já que a maior parte deste mineral está armazenado nos ossos, e uma forma de manter os níveis adequados para as funções dependentes deste mineral é por meio do mecanismo de redistribuição, que visa a retirada do zinco dos tecidos com maior concentração, transportando para tecidos com maior necessidade (BUCKLEY, 2000).

Alguns fatores como nível de suplementação de zinco, presença de outros minerais no lúmen intestinal, disponibilidade do zinco proveniente dos quelatos e a síntese de moléculas carreadoras nas células da mucosa do intestino delgado, são responsáveis por influenciar ~~a~~ absorção deste mineral (SONG, 1987).

Além de desempenhar as funções mencionadas anteriormente, juntos o Cu e o Zn atuam na defesa do sistema antioxidante, uma vez que são cofatores da enzima superóxido dismutase. Durante o estresse oxidativo, são produzidas substâncias conhecidas como radicais livres, os quais danificam as membranas celulares. As enzimas do sistema antioxidante são responsáveis por combater os efeitos deletérios dos radicais livres, sendo elas: superóxido dismutase, catalase, glutationa peroxidase e glutationa redutase (BARBOSA et al., 2010). Portanto, a adequada suplementação mineral também pode ser uma ferramenta para reduzir os efeitos negativos do estresse em aves.

1.4. Cobre e zinco nos parâmetros de carcaça a saúde óssea

O zinco participa ativamente no processo da síntese de proteínas estruturais, como a queratina e o colágeno e, portanto, a suplementação deste mineral é responsável por beneficiar a resistência de pele e redução de pododermatite (LESSON e SUMMERS, 2001). Dessa forma, a integridade de pele é essencial para reduzir a entrada de patógenos que possam comprometer a saúde da ave, e também de diminuir a condenação de carcaças devido a incidência de arranhões (ROSSI et al., 2007).

Diversos estudos têm associado a melhora nos parâmetros de carcaça após a suplementação das dietas com zinco. Jahanian et al. (2008) observaram aumento linear no rendimento do peito após a suplementação com zinco orgânico na dieta (40, 80 ou 120 mg/kg). Em estudo realizado com frangos de corte recebendo dietas suplementadas com Zn-metionina foi observado maior rendimento de carcaça e redução da gordura abdominal em comparação aqueles que receberam dietas suplementadas com Zn inorgânico, e os autores afirmaram que essa melhora está associada com a maior biodisponibilidade do Zn de fonte orgânica comparado à inorgânica, o que resultou em maior quantidade de mineral para ser utilizado na biossíntese de proteínas e formação do músculo, principalmente do peito (JAHANIAN e RASOULI, 2015).

No estudo de Olukosi et al. (2018) foi encontrado maior rendimento de peito e carcaça após a suplementação da dieta de frangos de corte com hidroxicloreto de zinco (80 mg/kg). Mohanna e Nys (1999) encontraram aumento linear na concentração de zinco no plasma e tíbia de frangos de corte, até atingir o platô de 75 mg/kg, indicando possível saturação dos canais de absorção. Dessa forma, é importante estabelecer nível ótimo de suplementação para evitar o desperdício, uma vez que o excesso de zinco não é utilizado pela ave.

No que diz respeito ao tecido ósseo, o cobre é cofator da enzima lisil-oxidase, a qual atua na formação de ligações cruzadas e que são responsáveis pela resistência mecânica e elasticidade à fibra do colágeno e da elastina presentes nos ossos (PIZZAURO JUNIOR et al., 2017). O Zn é responsável por ativar os osteoblastos durante a formação óssea, e por inibir a reabsorção óssea com a sinalização dos osteoclastos. Além disso, as enzimas colagenase e fosfatase alcalina são dependentes deste elemento durante o desenvolvimento ósseo (PIZZAURO JUNIOR et al., 2017). Portanto, as deformidades ósseas provocadas durante a deficiência mineral, são os resultados da necessidade de cobre e zinco para a atuação nos processos de formação óssea (YAMAGUCHI, 1998; KWIECIEŃ et al., 2014).

1.5. Impacto ambiental gerado pela excreção de minerais

A escolha da fonte e do nível para suplementação mineral (orgânica ou inorgânica) pode influenciar a quantidade de mineral a ser excretada pela ave. No caso de fontes inorgânicas, as quais apresentam ligações iônicas, é possível que ocorram perdas elevadas nas primeiras partes do trato gastrintestinal, impossibilitando a absorção. Pesti e Bakalli (1998) observaram aumento de 35 ppm para 940 ppm de cobre na excreta de galinhas poedeiras após a suplementação com 250 ppm de cobre na dieta. Em contrapartida, Kim e Patterson (2003) observaram efeito positivo da elevada concentração de cobre na excreta de frangos de corte, que foi a redução de produção de amônia pela consequente redução da atividade da uricase, enzima responsável pela hidrólise do ácido úrico para produção de NH_3.

A incorreta suplementação de cobre e zinco na dieta das aves pode proporcionar a excreção destes minerais devido ao não aproveitamento pela ave, e dependendo da forma como este produto é tratado, pode provocar poluição ambiental. O foco principal é na excreção do excesso de fósforo, que também é responsável por causar poluição, no entanto já existem algumas áreas em que os microminerais em excesso são prejudicais para o solo (BURREL et al., 2004).

De acordo com ABD EL-HACK et al. (2017) outra questão importante é que microminerais como o cobre e o zinco são capazes de ligar-se ao fitato, devido a estrutura química, formando complexos que não são absorvidos e, consequentemente, são excretados para o ambiente. Os mesmos autores observaram que após suplementação de fitase variando entre 600-850 FTU foi possível poupar de 6 a 8 mg/kg de Zn, para as fontes orgânicas e inorgânicas, e com consequente redução na excreção para o ambiente.

Portanto, além da adequada suplementação mineral, respeitando os níveis indicados pelos manuais, é importante conhecer o tipo de fonte de suplementação, para que se possa evitar o excesso de mineral excretado, além de evitar a formação de complexos com outros nutrientes da dieta.

2. JUSTIFICATIVA E OBJETIVO

A maior parte dos estudos envolvendo a suplementação de minerais nas dietas de frangos de corte têm mostrado resultados benéficos quanto ao uso de fontes orgânicas comparadas as inorgânicas. No entanto, alguns aspectos com relação a interação dessas fontes (orgânicas ou inorgânicas) com outros nutrientes da dieta, além do elevado custo das fontes orgânicas, levou ao desenvolvimento de outra fonte inorgânica, os chamados hidroximinerais. Os hidroximinerais são formados por ligações covalentes, e diferente dos sulfatos, parecem ser mais resistentes as condições do trato gastrintestinal da ave. No que diz respeito aos níveis de suplementação mineral ainda há muita divergência na literatura, o que pode estar relacionado com o tipo de fonte mineral e ingredientes da dieta. Nesse sentido, devemos saber quais são os benefícios e malefícios da suplementação mineral com níveis acima do recomendado para evitar possíveis desperdícios. Portanto, já que o Cu e o Zn são essenciais para diversos processos biológicos relacionados com o desenvolvimento da ave, este estudo teve como objetivo investigar os efeitos da suplementação combinada desses microminerais na forma de hidroximineral na dieta de frangos de corte.

O Capítulo II, foi intitulado **“Copper and Zinc supplementation in broiler diets enhance the growth performance, carcass traits and apparent ileal mineral absorption”** foi adequado de acordo com as normas estabelecidas pelo periódico **Poultry Science**, sob responsabilidade editorial do Poultry Science. O objetivo do presente estudo foi avaliar os efeitos de diferentes níveis e fontes de suplementação de cobre e zinco para frangos de corte sobre o desempenho, parâmetros de carne, e absorção aparente ileal do cobre e do zinco.

O Capítulo III, foi intitulado **“Effects of copper and zinc supplementation in broiler diets on bone development, reduction of footpad dermatitis, hock burn and skin scratches incidences”** foi adequado de acordo com as normas estabelecidas pelo periódico **Poultry Science**, sob responsabilidade editorial do Poultry Science. O objetivo do presente estudo foi avaliar os efeitos de diferentes fontes e níveis de suplementação de cobre e zinco sobre o desenvolvimento ósseo, redução das incidências de lesão de pata, lesão de jarrete e arranhões de pele.

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CAPÍTULO 2

“Copper and Zinc supplementation in broiler diets enhance the growth performance, carcass traits and apparent ileal mineral absorption”

Copper and Zinc supplementation in broiler diets enhance the growth performance, carcass traits and apparent ileal mineral absorption

This study aimed to investigate the effects of hydroxy trace minerals (HTM) compared to sulfate trace minerals (STM) supplementation on growth performance, carcass parameters and mineral retention in broilers. A total of 1,792 male Cobb 500 d-old were allocated in a completely randomized design among eight dietary treatments with eight replicates per treatment. The HTM sources used were two levels of Cu hydroxychloride (CHC) (low and high), combined with three levels of Zn hydroxychloride (ZHC) (low, med and high) and two additional treatments STM, being Cu sulfate monohydrate (CSM) (low and high) combined with high Zn sulfate monohydrate (ZSM). At 21 and 42 d-old growth performance was evaluated. Additionally, at 42 d-old the carcass traits, meat quality, apparent ileal absorption and activity of antioxidant enzymes were accessed. A main effect on level of Cu showed that broilers receiving high-CHC had higher body weight, body weight gain and better feed conversion ratio as compared to low-CHC at 21 d-old. On day 42, feed conversion ratio was improved for birds supplemented with high-CHC in diets containing med-ZHC, as compared to low-CHC. Dietary Cu increased the redness of breast by the level of high-CHC as compared to low-CHC. Greater results were observed on carcass traits at the level med-ZHC as compared to low-ZHC or high-ZHC. The ceruloplasmin activity in serum increased after supplementation of high-CSM in diets containing high-ZSM as compared to low-CSM. The AIA of Cu was higher in broilers supplemented with high-CHC containing med-ZHC as compared to low-CHC. Otherwise, the AIA of Zn increased in broilers fed with low-CHC containing low-ZHC or med-ZHC as compared to high-CHC. We recommend the mineral trace supplementation of broilers at the levels of high-CHC and low-ZHC as a good alternative to replace sulfate sources in diets.

Keywords: bond strength, carcass yield, Cu, feed conversion ratio, mineral source, Zn

INTRODUCTION

Minerals are responsible in performing several roles in the body as a physiological, structural, catalytic and regulatory functions. These nutrients are present in cereals used in poultry diets formulations and can also be obtained commercially from inorganic and organic sources. The requirement of trace mineral for poultry is low, but their deficiency may affect the growth performance and reflect on losses in the production system (Suttle, 2010).

Trace minerals as copper (Cu) and zinc (Zn) present low concentration in feedstuffs normally used in poultry diets, as corn and soybean meal, for instance. In this way, since 1920 the mineral supplementation has been an alternative to attend the poultry requirements, and inorganic sources (sulfates, carbonates and oxalates) were used as first option, but negative characteristics have been related, such as low availability in the gastrointestinal tract as a function of the ability to react with other minerals and also makes them unavailable (Gomes et al., 2009; Pacheco et al., 2017). Minerals present high ability of chemical interaction with other compounds, since the moment of the mixture of the diet, until to reach the gut of the bird (Suttle, 2010; Silva and Pascoal, 2014).

Studies in the 90s revealed a new salt able of react with an alkali and produce a hydrolyzed inorganic complex (Cohen and Steward, 2012). Chemically this compound is formed by three-dimensional lattice of high stability, making the covalent bonds strong enough to limit parasitic reactions, and weak enough to release the metal in the intestine (Cohen and Steward, 2012). Hydroxychloride forms can be find as acatamite (Cu), simonkolleite (Zn) and hibbingite (Fe) (Palache et al., 1944). The use of hydroxychloride has already been tested and the authors observed greater bioavailability of minerals, as Batal et al. (2001) who observed that zinc hydroxychloride was 122% more available when compared to sulfate. Olukosi et al. (2018) evaluated the inclusion of two sources of copper and zinc (sulfate and hydroxychloride) and two levels of zinc (80 and 20 mg/kg) in diet of broiler chickens, and observed better results of feed conversion and breast yield at 35 days of age for broiler chickens supplemented with hydroxychloride compared to sulfate.

Copper had your essentiality discovered in the past by Hart et al. (1928) in a study with rats receiving low-copper diets, and an anemia was observed once this mineral is important for synthesis of hemoglobin with iron. Many other functions have been attributed to Cu, for instance, as a cofactor of cytochrome oxidase, lysyl oxidase, ceruloplasmin, superoxide dismutase and dopamine beta-hydroxylase (Lim and Paik, 2006). In broiler diets, the National Research Council (NRC, 1994) recommends 8 mg/kg of Cu throughout the breeding period, while Rostagno et al. (2017) recommends 11.68; 10.54; 8.31; 6,79 and 6,08 mg/kg during growing phases. On the other hand, the use of high doses between 125-250 mg/kg has already been elucidated through studies with broilers as an efficient alternative for growth performance. As a result of the restriction of inclusion of antibiotics as growth promoters in the broilers diet, the inclusion of high doses of Cu has gained more attention, since this mineral has a bacteriostatic property (Pesti and Bakalli, 1996; Ewing et al., 1998). In a study of Samanta et al. (2011) broilers were supplemented with 150 mg/kg of copper sulfate pentahydrate and a better weight gain and feed conversion were observed as compared those birds supplemented with lowest levels.

Zinc is a trace mineral required for several biological processes, during growth and maintenance, bone development, feathering and appetite regulation (Batal et al., 2001; Olgun and Yidiz, 2017). Zinc is indispensable for the antioxidant system, once during the metabolic processes occurs the production of highly reactive compounds known as "reactive oxygen species" (ROS), which in turn are capable of inducing chronic diseases, membrane and cell DNA damage (Rahman et al., 2014). Zinc is a component of more than 300 enzymes involved in energy metabolism of carbohydrates, nucleic acids and proteins; and also of some hormones such as glucagon, insulin, sex and growth hormones (Naz et al., 2016). The National Research

Council (NRC, 1994) recommends 40 mg/kg of Zn in broiler diets, and according to Rostagno et al. (2017) the recommendations are 76.15; 68.72; 54.21; 44.29 and 39.67 mg/kg in the different phases. The benefits of zinc supplementation in the diet have been reported by several authors in the literature, such as El-Husseiny et al. (2008) which found better weight gain, egg production and egg quality in layers after supplementation with zinc oxide. Chand et al. (2014) observed greater weight gain in broiler chickens supplemented with 60 mg/kg of inorganic zinc and, submitted to heat stress.

Considering the essentiality of trace minerals in poultry development and the need of alternatives to replace the conventional mineral sources ensuring a good absorption, this study aimed to investigate the effects of different levels and sources of copper and zinc supplementation in broiler diets.

MATERIALS AND METHODS

All procedures performed in this study were approved by the Ethics Committee on Animal Use - CEUA/FMVZ, process Nº 0008/2017.

Birds, Diets and Treatments

A total of 1,792 d-old male Cobb 500 broiler chickens were reared in concrete floor pens with 2.0 m^2 with 28 birds per pen (14 birds/m^2), that contained wood shavings as bedding during the period of 1 to 42 days of age. Each pen was equipped with a tubular feeder and nipple drinkers (five nipples/pen). Birds were allocated to eight dietary treatments with eight replicates in a completely randomized design. The hydroxy trace minerals (HTM) sources used were two levels of Cu hydroxychloride (CHC) (low and high), combined with three levels of Zn hydroxychloride (ZHC) (low, med and high) and two additional treatments sulfate trace minerals (STM), being Cu sulfate monohydrate (CSM) (low and high) combined with high Zn sulfate monohydrate. The corn-soybean meal-based diets were supplemented with trace mineral and vitamin premix without copper and zinc. Both CHC (IntelliBond Cu) and ZHC (IntelliBond Zn), and the trace mineral and vitamin premix were provided by Trouw Nutrition, Brazil. The supplemental levels of CHC, ZHC, CSM and ZSM were added replacing to limestone in all diets. Levels of trace mineral supplementation are present in Table 1. Diets isoenergetic and isoproteic were provided in mash form and they were formulated according recommendations of Rostagno et al. (2011) (Table 2). Diets and water were provided *ad libitum* throughout the entire study. The results of Cu and Zn analyzed in all experimental diets are shown in Table 3.

Table 1 Levels of supplementation (mg/kg)* of copper and zinc sources for the dietary treatments.

Diets	CSM	ZSM	CHC	ZHC
Low-CSM/ High-ZSM	15 (42.85)	120 (342.86)	-	-
High-CSM/ High-ZSM	150 (428.57)	120 (342.86)	-	-
Low-CHC/ Low-ZHC	-	-	15 (27.27)	80 (148.15)
Low-CHC/ Med-ZHC	-	-	15 (27.27)	100 (185.18)
Low-CHC/ High-ZHC	-	-	15 (27.27)	120 (222.22)
High-CHC/ Low-ZHC	-	-	150 (272.72)	80 (148.15)
High-CHC/ Med-ZHC	-	-	150 (272.72)	100 (185.18)
High-CHC/ High-ZHC	-	-	150 (272.72)	120 (222.22)

*Values in parentheses are the added Cu and Zn sources and values out parentheses are the inclusion level of mineral in diets.

Low-CSM:15 mg/kg; High-CSM: 150 mg/kg; High-ZSM: 120 mg/kg; Low-CHC: 15 mg/kg; High-CHC: 150 mg/kg; Low-ZHC: 80 mg/kg; Med-ZHC: 100 mg/kg; High-ZHC: 120 mg/kg.

CSM - Copper sulfate monohydrate

ZSM - Zinc sulfate monohydrate

CHC – Intellibond hydroxychloride Cu

ZHC – Intellinbond hydroxychloride Zn.

Table 2 Ingredient (g/kg) and composition of the experimental diets.

	Pre-starter	Starter	Grower	Finisher
Corn	590.11	615.38	644.00	687.47
Soybean meal	366.41	338.12	303.10	264.80
Soy oil	5.00	13.23	23.14	22.07
Dicalcium phosphate	12.20	8.65	6.37	4.15
Limestone	9.60	9.90	9.48	8.76
Salt (NaCl)	5.18	4.80	4.56	4.44
DL-Methionine	3.40	2.90	2.70	2.50
L-Lysine HCl	3.20	2.70	2.70	3.00
Trace mineral and vitamin premix [1,2]	2.00	1.80	1.60	1.20
L-threonine	1.20	0.90	0.80	0.80
Choline chloride 60%	0.72	0.63	0.58	0.38
Anticoccidian	0.55	0.55	0.55	-
Phytase 500 FTU	0.05	0.05	0.05	0.05
Sulfate of zinc, 35%	0.34	0.34	0.34	0.34
Sulfate of copper, 35%	0.04	0.04	0.04	0.04
Intellibond Zn, 55%	-	-	-	-
Intellibond Cu, 54%	-	-	-	-
Calculated nutrients				
ME (kcal/kg)	2964	3050	3149	3199
Crude protein %	22.40	21.20	19.80	18.40
Calcium, %	0.92	0.84	0.76	0.66
Total phosphorus, %	0.71	0.63	0.57	0.52
Available phosphorus, %	0.47	0.40	0.35	0.31
Copper, mg	0.017	0.017	0.017	0.017
Zinc, mg	0.131	0.131	0.132	0.132
Lisyne, %	1.32	1.21	1.13	1.06
Methionine, %	0.66	0.60	0.56	0.53
Met+Cis, %	0.95	0.88	0.83	0.77
Threonine, %	0.86	0.79	0.73	0.69

[1]Trace mineral premix (levels per kg of feed): Iron 62.5 mg; Iodine 1.25 mg; Manganese 88 mg; Selenium 0.375 mg.

[2] Vitamin premix (levels per kg of feed): Vitamin A 9,375 UI; Vitamin D3 2,375 UI; Vitamin E 35 UI; Vitamin B12 0.015 mg; Vitamin B7 0.088 mg; Vitamin B9 0.875 mg; Vitamin K3 1.88 mg; Vitamin B1 2.5 mg; Vitamin B6 3.5 mg; Vitamin B2 6.25 mg; Vitamin B5 12.5 mg; Vitamin B3 37.5 mg; B.H.T 15 ppm.

Table 3 Analyzed copper and zinc composition (mg/kg) in all experimental diet.

Diets	Pre starter		Starter		Grower		Finisher	
	Cu	Zn	Cu	Zn	Cu	Zn	Cu	Zn
Low-CSM/ High-ZSM	20.23	159.80	29	94	16.28	132.70	15.26	132.70
High-CSM/ High-ZSM	130.50	154.20	129.70	133	88.68	128.47	98.56	126.47
Low-CHC/ Low-ZHC	23.77	138.20	32.50	104.80	18.95	108.88	18.95	103.83
Low-CHC/ Med-ZHC	21.96	139.10	26.63	116.30	14.98	108.98	14.93	108.63
Low-CHC/ High-ZHC	17.92	154.80	28.75	136.20	16.88	120.20	15.88	120.20
High-CHC/ Low-ZHC	150.30	136.40	130.10	98.10	91.34	87.66	91.34	87.55
High-CHC/ Med-ZHC	162.60	153.70	177.90	129.50	100.81	111.16	100.61	111.15
High-CHC/ High-ZHC	149.80	176.50	108.30	133.20	101.38	131.80	101.36	131.30

Low-CSM:15 mg/kg; High-CSM: 150 mg/kg; High-ZSM: 120 mg/kg; Low-CHC: 15 mg/kg; High-CHC: 150 mg/kg; Low-ZHC: 80 mg/kg; Med-ZHC: 100 mg/kg; High-ZHC: 120 mg/kg.
CSM - Copper sulfate monohydrate
ZSM - Zinc sulfate monohydrate
CHC – Intellibond hydroxychloride Cu
ZHC – Intellibond hydroxychloride Zn.

Growth performance

Growth performance was evaluated at 21 and 42 d-old by measuring body weight (BW), body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR) and, viability (VB) determination. In addition, European Broiler Index (EBI) at 42 days of age was calculated by:

$$\text{EBI} = [(\text{BWG (g)} \times \text{Viability (\%)}) \div \text{FCR}] \div 10$$

Sampling and analytical methods for carcass evaluation and meat quality

At the end, three birds from each pen were selected according to the average body weight and fasted overnight. Then. broilers were weighed individually and slaughtered according to the welfare of animal slaughter regulations of Brazil. Carcass weight measurements were done after bleeding, defeathering and evisceration. Thus, heads, necks and feet were removed, then carcasses and abdominal fat (leaf fat surrounding the cloaca and abdominal fat surrounding the gizzard) were weighted to access the percentage relative to live weight.

Breast, thigh, drumstick, back and wings were removed and weighed to determine the percentage relative to carcass weight. The *Pectoralis major* muscle was divided in two parts and in the left side were measured pH, color (L*, b* and a*) and drip loss, and in the right side the cook loss and shear force were measured. All parameters of meat quality were conducted 24 h after slaughter.

The pH was measured at a depth of 2.5 cm below the surface using a pH meter (Hanna® Instruments Brasil, model HI99163), previously calibrated with pH 4.0 and 7.0 buffer solutions (Merck) at room temperature.

Color was determined by the colorimetric method according to accepted standards with a CR-400 Minolta Chroma Meter (Konica Minolta, modelo CR-400 New Jersey, USA). Results were presented in CIELab scale as L*, a*, b*. Values L*, a*, and b*, corresponding to lightness, redness, and yellowness, respectively (Honikel, 1998).

Drip loss was determined according the method described by Intruction manual for EZ-DripLoss (Danish Meat Research Institute, 2018). Duplicate samples of *Pectoralis major* muscle were weighed before being placed into EZ-DripLoss tubes and were stored at 4 °C for 24 h. Then muscles were removed from the tubes and reweighed in order to measure drip loss that was expressed as a percentage relative to the initial weight.

Cooking loss was expressed as percentage, based on weights before and after cooking, according to Honikel (1998). The *Pectoralis major* muscle was placed in plastic bag and was cooked in temperature-controlled water bath at 85°C until the temperature of the meat center reached 75°C. When the endpoint temperature has been attained, samples were removed and cooled at room temperature. The meat was taken from the bag, dried with paper towel that was changed when in excessive humidity and then weighed.

Shear force was measured in five points using a TAXT-Plus (Stable Micro Systems, Surrey, UK) calibrated with normal weight range of 5 kg evaluating energy (N/mm) and shear strength (N) according to procedures proposed by Cavitt et al. (2004) oriented perpendicular to the *Razor Blade*, with device velocity of 10 mm/s.

Chemical Analysis

Cu and Zn concentration in diet, plasma and liver: mineral concentration was determined in all diets *in natura*. One bird per pen was randomly selected to a blood and liver collect. Plasma was obtained after centrifuge process at 3,000g for 10 min. The entire liver was also obtained from the same bird selected for blood analyze. Cu and Zn concentration was performed in all samples using the atomic absorption spectrometry method (FAAS - Flame Atomic Absorption Spectrometry), as described by Neves et al. (2009).

Apparent ileal mineral absorption: Cu and Zn absorption were evaluated by apparent ileal absorption (AIA) method and chromium oxide was used in the finisher diet as indigestible marker (Chromium Oxide - Cr) in proportion of 0.1%. At 42 d-old, three birds with average weight from each experimental unit were slaughtered and ileal digesta was collected taking the distal two-thirds part of the ileum after dissection from Meckel's diverticulum to the ileo-cecocolic junction. Thus, pool of digesta (three birds) was homogenized and samples were freeze-dried at a temperature of - 40 ° C, for 72 hours. The marker concentration in diet and ileum digesta was determined by atomic absorption spectrometry, according to Williams et al. (1962). Cu and Zn concentration in diet and ileum digesta was determined by atomic absorption spectrometry (FAAS - Flame Atomic Absorption Spectrometry), as described by Neves et al. (2009).

The apparent ileal absorption of Cu and Zn was calculated using the following formula: apparent ileal absorption (%) = 100 - [(concentration of marker in diet/ concentration of marker in ileum) × (concentration of nutrient in the ileum/ concentration of nutrient in diet) × 100] (Hafeez et al., 2014).

Enzymes activities

The blood of one bird per pen was collected at 42 d-old to evaluate the superoxide dismutase, glutathione peroxidase and ceruloplasmin activities. Samples were centrifuged at 4 ºC for 10 min at 1,315 × *g* and was stored at -80ºC. Superoxide dismutase (SOD) was determined using commercial kit (Ransod, Randox Laboratories, UK) by inhibition of a formazan-producing colorimetric reaction (Suttle and McMurray, 1983). Glutathione peroxidase (GPx) was determined according Paglia and Valentine (1967), where the GPx catalyzes the oxidation of GSH reduced by a hydroperoxide in the enzymatic process using glutathione reductase (G3664 Sigma-Aldrich). Ceruloplasmin (Cp) was measured using the substrate o-dianisidine dihydrochloride (D3252 Sigma-Aldrich) in the end-point method, as described by Schoslnsky et al. (1974).

Statistical Analysis

Data were analyzed in a (2x3)+2 factorial arrangement and pen was the experimental unit. This model had two levels of copper (low and high) and three levels of zinc (low, med and high) plus two additional treatments that sulfates sources of Cu and Zn were used. Initially data were submitted to the normality test and then to analysis of variance using the PROC GLM of Minitab® 16 (2009). Mean of factorial arrangement and mean of additional treatments were compared using Tukey test. Furthermore, a comparison between sulfate with hydroxychloride treatments was performed using Dunett’s test. Statistical significance was set at $P < 0.05$.

RESULTS

Growth Performance

There was no significant interaction between levels of Cu and Zn on growth performance during 1 to 21 days of age (Table 4). On the other hand, main effect of Cu (P <

0.05) indicated that broilers fed diets with high-CHC showed better body weight (BW), body weight gain (BWG) and feed conversion ratio (FCR) as compared to low-CHC. Sulfate versus hydroxychloride results indicated that broilers fed diets with low-CHC combined with med-ZHC presented higher FCR as compared to S2 (150 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate), and broilers fed diets with high-CHC combined with med-ZHC presented better FCR as compared to S1 (15 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate).

A significant interaction between Cu and Zn levels in overall period (Table 5) on FCR was observed. The interaction shows that birds in the group low-ZHC or med-ZHC had better FCR when high-CHC was used as compared to low-CHC. Birds in the group low-CHC had better FCR when high-ZHC was included. On the other hand, birds in the group high-CHC had better FCR when low-ZHC or med-ZHC was used. A main effect of Cu level ($P < 0.05$) shows that high-CHC improved the EBI as compared to low-CHC (Table 5).

In addition, sulfate versus hydroxychloride sources results (Table 5) shows that diet containing low-CHC combined with low-ZHC affected negatively the FCR when compared to diets S1 or S2.

Table 4 Growth performance of broilers supplemented with different sources and levels of copper and zinc at 1 to 21 days of age.

Cu	Low			High						*P*-value				
Zn	Low	Med	High	Low	Med	High	S1	S2	SEM	Cu	Zn	S1	S2	S1×S2
BW, g	797.4b	790.0b	789.0b	818.1a	813.4a	805.9a	805.5	814.7	2.90	**0.006**	0.484	0.062	0.062	0.187
BWG, g	751.3b	743.7b	743.0b	771.6a	767.6a	760.0a	759.7	768.5	2.90	**0.006**	0.514	0.065	0.065	0.213
FI, g	1104.7	1098.9	1072.6	1108.8	1097.8	1094.9	1124.5	1100.4	5.72	0.564	0.429	0.600	0.600	0.157
FCR, g g^{-1}	1.477b	1.484b +	1.448b	1.443a	1.432a*	1.439a	1.481b	1.435a	0.005	**0.013**	0.478	**0.027**	**0.027**	**0.010**
VB, %	98.9	98.8	99.0	98.8	99.1	99.1	99.1	99.0	0.075	0.536	0.568	0.881	0.881	0.554

BW, body weight; BWG, body weight gain; FI, feed intake; FCR, feed conversion ratio; VB, viability; S1: 15 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate. S2: 150 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate.

[a, b] means within a row with different superscripts are significantly different by Tukey test, at 5% probability.

Means follow of the * differ from S1 and means follow of the + differ from S2 by Dunnett's test, at 5% probability.

SEM: Standard error of the mean.

Table 5 Growth performance of broilers supplemented with different sources and levels of copper and zinc at 1 to 42 days of age.

Cu	Zn			S1	S2	SEM	*P*-value					
	Low	Med	High				Cu	Zn	Cu×Zn	S1	S2	S1×S2
	BW,g											
Low	2517.2	2579.4	2545.7	2589.1	2602.3	15.56	0.060	0.654	0.434	0.418	0.418	0.791
High	2660.3	2626.9	2575.7									
	BWG, g											
Low	2471.1	2533.0	2499.8	2543.3	2556.0	15.57	0.060	0.658	0.439	0.423	0.423	0.799
High	2613.8	2581.1	2529.8									
	FI, g											
Low	4471.4	4527.7	4430.2	4468.5	4486.0	26.27	0.293	0.873	0.672	0.879	0.879	0.801
High	4588.8	4513.9	4546.8									
	FCR, g g^{-1}											
Low	1.816bB*+	1.805abB	1.770aA	1.786	1.769	0.004	0.025	0.986	**0.0001**	**0.002**	**0.002**	0.308
High	1.760aA	1.768abA	1.803bB									
	VB, %											
Low	98.5	98.3	98.7	98.4	98.5	0.106	0.432	0.404	0.984	0.891	0.891	0.734
High	98.8	98.5	98.9									
	EBI											
Low	319.3y	328.7y	332.1y	333.7	339.3	2.47	**0.015**	0.792	0.074	0.084	0.080	0.535
High	349.5x	342.6x	330.2x									

BW, body weight; BWG, body weight gain; FI, feed intake; FCR, feed conversion ratio; VB, viability; EBI, European broiler index. S1: 15 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate. S2: 150 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate.
[A,B] means within a column with different superscripts are significantly different for Cu by Tukey test, at 5% probability.
[a,b,c] means within a row with different superscripts are significantly different for Zn by Tukey test, at 5% probability.
[x,y] means within a column with different superscripts are significantly different for Cu by Tukey test, at 5% probability.
Means follow of the * differ from S1 and means follow of the + differ from S2 by Dunnett's test, at 5% probability.
SEM: Standard error of the mean.

Meat quality

The effects of Cu and Zn supplementation on parameters of meat quality are shown in Table 6. There was no significant interaction between the factors on parameters of meat quality. There was a main effect ($P < 0.05$) of level of Cu on redness and drip loss (Table 6). Broilers fed diets with high-CHC had higher redness in the breast muscle as compared to low-CHC. Furthermore, high-CHC increased drip loss as compared to low-CHC.

Sulfate versus hydroxychloride comparison showed a significant effect on drip loss, and diets containing high-CHC combined with med-ZHC or high-ZHC increased drip loss relative to S1 or S2 (Table 6).

Table 6 Effect of different sources and levels of copper and zinc on parameters of pH, lightness (L*), redness (a*), yellowness (b*), drip loss (DL), cooking loss (CL) and shear force (SF) in chicken breast fillets at 42 days of age.

Cu	Zn			S1	S2	SEM	*P*-value					
	Low	Med	High				Cu	Zn	Cu×Zn	S1	S2	S1×S2
	pH											
Low	5.97	5.94	5.94	5.94	6.02	0.012	0.111	0.992	0.720	0.551	0.551	0.102
High	5.98	6.00	6.00									
	L*											
Low	54.75	54.32	54.65	56.36	54.97	0.262	0.191	0.609	0.836	0.410	0.410	0.247
High	55.93	55.02	55.01									
	a*											
Low	2.75y	3.20y	2.99y	3.47	3.67	0.105	**0.028**	0.951	0.131	0.399	0.399	0.683
High	3.87x	3.14x	3.47x									
	b*											
Low	5.64	5.62	5.95	6.25	5.68	0.181	0.424	0.521	0.645	0.588	0.588	0.236
High	6.35	5.65	6.25									
	DL											
Low	0.53y	0.62y	0.40y	0.52	0.37	0.003	**0.007**	0.619	0.430	**0.010**	**0.010**	0.066
High	0.70x	0.79x+	0.83x*+									
	CL, %											
Low	23.53	22.92	21.69	22.47	22.34	0.962	0.062	0.821	0.272	0.452	0.452	0.910
High	21.14	21.12	21.92									
	SF, N											
Low	6.93	8.30	8.22	6.99	7.25	0.210	0.075	0.283	0.338	0.290	0.290	0.742
High	6.86	6.47	7.48									

S1: 15 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate. S2: 150 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate.
[x,y] means within a column with different superscripts are significantly different for Cu by Tukey test, at 5% probability.
Means follow of the * differ from S1 and means follow of the + differ from S2 by Dunnett's test, at 5% probability.
SEM: Standard error of the mean.

Carcass evaluation

The carcass yield of chickens fed diets with different levels of Cu and Zn are shown in Table 7. The significant interaction shows that broilers in the group low-ZHC had higher abdominal fat and thigh yield when low-CHC was used as compared to high-CHC (Table 7). Sulfate versus hydroxychloride sources of Cu and Zn indicated that diets containing low-CHC combined with low-ZHC or diets containing high-CHC combined with high-ZHC increased abdominal fat when compared to S1 or S2. There were no significant interactions between Cu and Zn levels on carcass, breast, drumstick, back and wings yields. There was a main effect of Zn and a higher ($P < 0.05$) breast and lower drumstick yields were found using med-ZHC. Furthermore, high-ZHC increased wing and back yields as compared to low-ZHC, while low-ZHC improved carcass yield as compared to high-ZHC (Table 7). Furthermore, in sulfate versus hydroxychloride sources, a reduction in breast yield was observed in broilers fed with diet containing low-CHC combined with low-ZHC as compared to S1 or S2 (Table 7).

Table 7 Effect of different sources and levels of copper and zinc on parameters of carcass and parts yield of broilers at 42 days of age.

Cu	Zn			S1	S2	SEM	*P*-value					
	Low	Med	High				Cu	Zn	Cu×Zn	S1	S2	S1×S2
	Carcass, %											
Low	75.46x	75.84xy	74.48y	75.01	75.31	0.149	0.578	**0.011**	0.163	0.076	0.076	0.627
High	75.94x	74.65xy	74.62y									
	Breast, %											
Low	40.96y*+	42.75x	41.74y	42.99	42.51	0.183	0.138	**0.010**	0.218	**0.016**	**0.016**	0.498
High	42.48y	43.25x	41.49y									
	Thigh, %											
Low	13.32A	12.98	12.84	13.11	12.86	0.065	0.541	0.571	**0.032**	0.218	0.218	0.353
High	12.82B	12.81	13.26									
	Drumstick, %											
Low	16.75x	15.90y	16.09xy	15.92	16.01	0.133	0.485	**0.011**	0.090	0.053	0.053	0.798
High	16.18x	15.86y	16.38xy									
	Back,%											
Low	17.73y	18.00xy	18.19x	17.65	17.57	0.086	0.997	**0.022**	0.411	0.070	0.070	0.836
High	17.75y	17.68xy	18.53x									
	Wings, %											
Low	10.54y	10.64xy	10.78x	10.59	10.70	0.051	0.339	**0.013**	0.565	0.520	0.520	0.552
High	10.53y	10.74xy	11.01x									
	Abdominal fat, %											
Low	2.15aA*+	1.69b	1.92ab	1.64	1.52	0.038	0.036	0.002	**0.019**	**0.0001**	**0.0001**	0.509
High	1.67bB	1.53b	2.05a*+									

S1: 15 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate. S2: 150 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate.
[A,B] means within a column with different superscripts are significantly different for Cu by Tukey test, at 5% probability.
[a,b,c] means within a row with different superscripts are significantly different for Zn by Tukey test, at 5% probability.
[x, y] means within a row with different superscripts are significantly different for Zn by Tukey test, at 5% probability.
Means follow of the * differ from S1 and means follow of the + differ from S2 by Dunnett's test, at 5% probability.
SEM: Standard error of the mean.

Blood parameters and mineral retention

Plasma mineral concentrations were not influenced by different levels of Cu and Zn in the diets (Table 8). There was significant interaction among the factors on Cu concentration in the liver ($P < 0.05$), and broilers in the group high-CHC with inclusion of any three levels of ZHC (low, med or high) had higher Cu concentration as compared to group low-CHC (Table 8). In addition, broilers supplemented with the S2 diet also showed a higher concentration of Cu in the liver as compared to S1. Sulfate versus hydroxychoride results showed that broilers fed diets with low-CHC combined with low-ZHC or med-ZHC had lower Cu concentration as compared to S1 and S2.

A main effect of Zn concentration ($P < 0.001$) was observed in the liver (Table 8), and broilers fed diets with med-ZHC or high-ZHC showed higher Zn concentration as compared to group low-ZHC. The Zn concentration in the liver in sulfate versus hydroxychloride treatments indicated that diets low-CHC combined with high-ZHC had lower concentration as compared to S2, and diet containing high-CHC combined with med-ZHC showed higher Zn concentration as compared to S1.

Results of enzymatic activities are present in Table 9. Different dietary levels of Cu and Zn did not affect superoxide dismutase activity and glutathione peroxidase. However, broilers supplemented with S2 diet had higher ($P < 0.05$) ceruloplasmin activity as compared to S1 diet.

There was a significant interaction in AIA of Cu and Zn (Table 10). In the interactions, results indicated that broilers in the group med-ZHC combined with low-CHC had lower Cu absorption as compared to high-CHC. Besides that, broilers in the group low-CHC combined with high-ZHC had higher Cu absorption as compared to med-ZHC. In sulfate versus hydroxychloride comparisons, broilers in the group high-CHC combined with any Zn inclusion (low, med or high) presented higher Cu absorption as compared to S1. A higher Cu absorption was observed for S2 treatment versus S1 ($P = 0.003$).

Likewise, Zn absorption was affected by dietary treatments (Table 10). Broilers in the low-CHC group with inclusion of low-ZHC showed greater absorption of Zn compared to med-ZHC, and the latter in turn, were better than those with inclusion of high-ZHC. On the other hand, broilers from the high-CHC group with inclusion of med-ZHC or high-ZHC obtained better results of Zn absorption as compared to low-ZHC. The Zn absorption was higher for broilers in the low-ZHC and med-ZHC groups with low-CHC inclusion as compared to broilers in the group with high-CHC inclusion. Sulfate versus hydroxychloride results indicated that the highest values of Zn absorption were found in broilers that received the diets with low-CHC combined with low-ZHC, and diets with low-CHC combined with med-ZHC, as they presented values higher as compared to S1 and S2. A higher Zn absorption was observed for S1 as compared to S2 (P = 0.0001).

Table 8 Mineral concentration (ppm) in plasma and liver of broilers supplemented with different sources and levels of copper and zinc at 42 days of age.

Cu	Zn Low	Zn Med	Zn High	S1	S2	SEM	*P*-value Cu	*P*-value Zn	*P*-value Cu×Zn	*P*-value S1	*P*-value S2	*P*-value S1×S2
	Cu plasma, ppm											
Low	0.025	0.023	0.021	0.024	0.027	0.017	0.817	0.538	0.740	0.834	0.834	0.550
High	0.023	0.026	0.021									
	Zn plasma, ppm											
Low	0.406	0.433	0.459	0.436	0.433	0.412	0.846	0.926	0.916	1	1	0.981
High	0.418	0.431	0.416									
	Cu liver, ppm											
Low	16.00bB*+	12.56bB*+	24.68aB+	28.28	40.50	1.68	<0.0001	0.019	**0.008**	**<0.0001**	**<0.0001**	**0.034**
High	47.98A*	52.45A+	50.28A*									
	Zn liver, ppm											
Low	134.76y	157.28x	149.64x+	146.21	154.18	3.45	0.592	**<0.001**	0.101	**0.002**	**0.002**	0.456
High	130.97y	153.19x*	166.69x									

S1: 15 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate. S2: 150 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate.
[A,B] means within a column with different superscripts are significantly different for Cu by Tukey test, at 5% probability.
[a,b,c] means within a row with different superscripts are significantly different for Zn by Tukey test, at 5% probability.
[x, y] means within a row with different superscripts are significantly different for Zn by Tukey test, at 5% probability.
Means follow of the * differ from S1 and means follow of the + differ from S2 by Dunnett's test, at 5% probability.
SEM: Standard error of the mean.

Table 9 Activity of superoxide dismutase (SOD, U/ml), glutathione peroxidase (GPx, U/ml) and ceruloplasmin (Cp, U/l) in serum of broilers supplemented with different sources and levels of copper and zinc.

Cu		Low			High						*P*-value				
	Zn	Low	Med	High	Low	Med	High	S1	S2	SEM	Cu	Zn	S1	S2	S1×S2
SOD		13.79	15.38	14.63	12.09	11.00	13.61	12.67	9.83	0.50	0.088	0.685	0.141	0.141	0.108
GPx		6.07	6.14	6.82	6.59	6.23	6.03	6.78	6.09	0.12	0.836	0.807	0.533	0.533	0.183
Cp		2.37	1.81	1.33	1.67	1.36	1.70	0.81a	2.13b	0.16	0.544	0.590	0.458	0.458	**0.010**

S1: 15 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate. S2: 150 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate.
[a, b] means within a row with different superscripts are significantly different by Tukey test, at 5% probability.
Means follow of the * differ from S1 and means follow of the + differ from S2 by Dunnett's test, at 5% probability.
SEM: Standard error of the mean.

Table 10 Apparent ileal absorption of Cu and Zn of broiler at 42 days of age supplemented with different sources and levels of trace minerals.

	Zn						*P*-value					
Cu	Low	Med	High	S1	S2	SEM	Cu	Zn	Cu×Zn	S1	S2	S1×S2
		Cu, %										
Low	38.54ab	30.82bB+	41.82a	32.25b	42.20a	1.11	0.0001	0.596	**0.009**	**0.0001**	**0.0001**	**0.003**
High	45.36*	48.72A*	42.74*									
		Zn, %										
Low	88.76aA*+	88.06bA*+	82.86c*	85.04a	82.67b	0.32	0.0001	0.0001	**0.0001**	**0.0001**	**0.0001**	**0.0001**
High	81.88bB*	82.55aB*	82.77a*									

S1: 15 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate. S2: 150 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate.
[A,B] means within a column with different superscripts are significantly different for Cu by Tukey test, at 5% probability.
[a,b,c] means within a row with different superscripts are significantly different for Zn by Tukey test, at 5% probability.
Means follow of the * differ from S1 and means follow of the + differ from S2 by Dunnett's test, at 5% probability.
SEM: Standard error of the mean.

DISCUSSION

This study hypothesized that the replacement of STM by HTM in poultry diets could provide better performance and benefit the bird development. Pang and Applegate (2007) showed that HTM were less soluble in gastrointestinal tract conditions, allowing greater mineral bioavailability, and compared to STM the HTM was insoluble in water and showed around 81% of solubility under acidic conditions (pH<4). HTM have an OH group and are formed by covalent bonds that are more resistant to conditions of the gastrointestinal tract compared to conventional sources such as sulfates, which have ionic bonds. The presence of covalent bonds allows the mineral to reach the target absorption site without negative interactions with other nutrients in the gastrointestinal tract (Cohen and Steward, 2012).

The improvement observed on FCR of broilers supplemented with different sources and levels of Cu and Zn can be attributed to a role that these minerals develop in the body. Independent of source, the high level of Cu (150 mg/kg) promoted better results as compared to low level (15 mg/kg). Furthermore, high level of Cu combined with low level of Zn (80 mg/kg) seems to have been better. Cu and Zn can use the same transporter (CRT1 – copper transporter) to be absorbed in the enterocyte, and the excess of one these trace mineral can affect the absorption from the other, and that mechanism can be one reason for the diet high-CHC combined with high-ZHC have not provided good results (Massuquetto et al., 2017). Previously studies indicated that the inclusion of copper above recommended is beneficial for performance (Fisher, 1973). Some mechanisms were proposed to explain how copper provides better performance, being: 1) alteration of the microbiota with the presence of copper; 2) increased mitogenic activity in serum; 3) increased expression of growth hormone by the pituitary gland; 4) post-translational modification of regulatory peptides (LaBella et al., 1973; Eipper and Mains, 1988; Zhou et al., 1994).

After antibiotics restriction in some countries as a growth promoter in broiler diets, the use of "pharmacological" doses of Cu has been evaluated as an alternative, due to this mineral has a bacteriostatic property which is beneficial for the intestinal microbiota (Bunch et al., 1961; Pang and Applegate, 2007).

In several studies using levels of 150-200 mg/kg of Cu, broilers showed better growth performance, by the reduction of pathogenic organisms in the gut through bacteriostatic activity and feed intake stimulation (Puig and Thiele, 2002; Xia et al., 2004; Samanta et al., 2011). However, some contradictory results may vary according to the copper source and level of supplementation. Pesti and Bakalli (1996) supplemented diets of broilers with 250 mg/kg and 375 mg/kg of copper sulfate pentahydrate and found no effect on growth performance. On the other hand, Lu et al. (2010) observed that broiler chickens supplemented with 200 mg/kg of CHC showed higher daily weight gain than those supplemented with levels of 150 mg/kg of CHC or 200 mg/kg of copper sulfate.

During the overall period in the current study, diets supplemented with high-CHC resulted in the best European broiler index (EBI) as compared to low-CHC. The EBI is a conventional measurement that involves variables as body weight gain, viability, and FCR, as indicated that birds in treatments supplemented with high-CHC were benefited. Likewise, Kim et al. (2011) evaluated different levels of dietary organic copper (Cu-soy proteinate) to broiler and observed an improvement in the EBI with supplementation of 100 mg/kg.

The high level of copper appeared to protect the gut against the action of pathogenic microorganisms, and thus allowed nutrient absorption occurred normally improving the growth performance, and when combined with low level of zinc was effective to protect the immune system against the action of reactive oxygen species and produced better results for performance.

Mineral supplementation did not influence the pH, shear force and cooking loss in breast fillets in our research. According to Ristic and Damme (2013) values of pH ranging from 5.9 to 6.2 in broiler meat are an indicative that conditions of standard meat properties were obtained, and the average of our pH results was 5.97. The measurement of shear force was performed by Razor Blade method developed by Cavitt et al. (2004) and the values observed in the present study were around 7.30 N, showing higher tenderness acceptance (Petracci and Berri, 2017). Cooking loss of breast fillets showed average values around 22.14% and is in agreement with Bowker and Zhuang (2019) that evaluated cooking loss in breast fillets of broilers and found value of 22.80% in normal conditions. Even no effect of the trace mineral supplementation was observed, the results indicated a good meat quality and it imply that this supplementation can be used without impair the parameters of meat.

Meat color and drip loss were affected by copper supplementation ($P<0.05$). Some parameters of meat quality determine the acceptance of the product by the consumer, and meat color is one of the most important attributes (Aksu et al., 2011). Furthermore, several factors can influence the meat quality, as handling, slaughtering, processing and packaging (Fletcher, 1989; Petracci and Fletcher, 2002; Aksu et al., 2011). Few studies have reported some color meat change after mineral supplementation and seems like the composition of the diet is more related. Yang et al. (2011) supplemented broilers with organic source containing copper, zinc, iron and manganese, and found effect on lightness and yellowness on the broilers breast. Curiously in study of Buckiuniene et al. (2016) no effect was found on meat color of broilers supplemented with organic or inorganic iron, once this mineral participate on hemoglobin production. Higher incidence of redness in broiler breast was observed by Akiba et al (2000) who provided diets containing 'non-synthetic astaxanthin. Supplements that contain some type of pigment in their composition may alter meat color parameters. Toyomizu et al. (2001) provided diets supplemented with different levels of spirulina for broiler chickens and observed a higher redness in broilers breast with 40 g/kg of spirulina. The authors hypothesized that the increase in the incidence of redness may have occurred due to the increase in the concentration of myoglobin in the breast of chickens, being related to the high concentration of iron in spirulina.

As the same detected on meat color parameter, few studies have reported effect of mineral supplementation on drip loss. Although the trace mineral Cu be recognized for its benefits on growth performance, some studies have suggested that the excess of Cu can increase lipid oxidation by Fenton and Haber-Weiss reactions and decrease stability of the muscle. In this way, high level of Cu free can prejudice the meat quality (Halliwell and Gutteridge, 1989).

According to our results, the carcass, breast, back and wing yields were benefited by the use of ZHC source and was observed that supplementation of 100 mg/kg ZHC promoted the best results (Table 7). Once zinc is involved in the synthesis of structural proteins such as collagen and keratin, the improvement can be attributed to a role that this mineral participates and the greater mineral bioavailability of Zn supported the development (Vallee 1983; Linder 1991; Valle and Falchuck 1993; Underwood and Suttle, 1999; Batal et al., 2001). Keratin is the main structural protein of the hull, horn, feathers, skin, beaks and nails, while collagen is the main protein in the extracellular matrix and connects the internal tissues such as bone and cartilage (O'Dell et al., 1958; Young et al., 1958). Some studies have reported a positive effect on carcass traits of broilers supplemented with zinc, as in study of Tronina et al. (2007) that they reported increase of 4.15% in carcass yield of broiler chickens that received Zn-glycine (79.71, 71.03, 76.40 mg/kg) as compared those that received an inorganic source. On the other hand, higher yields of *Pectorallis major* and *Pectorallis minor* were found in the study of Qudsieh et al. (2018) by supplementing broiler chickens with 120 and 240 mg/kg of ZSM. In a study of Olukosi et al. (2018), the authors used two sources of copper and zinc (sulfate and hydroxychloride) and two zinc levels (20 and 80 mg/kg) in diet of broiler chickens and they reported that the source of hydroxychloride and the lowest (20 mg/kg) level of zinc promoted higher yield of carcass and breast when compared to sulfate source and higher zinc level.

The higher level of zinc used in the current study (120 mg/kg) was not beneficial for the evaluated parameters, which indicates that there is a maximal limit of efficiency. According to Coppen and Davis (1987) the proportion of zinc absorbed decreases with the increase of zinc in the intestinal lumen, possibly due to the change in the location where zinc is absorbed in the digestive tract (Yu et al., 2008).

A reduction on abdominal fat observed in this study was also observed by Skrivan et al. (2002) after supplementation of copper in diets of broilers. Due de participation of copper in the regulation of several lipid metabolism enzymes, our hypothesis is that copper can contribute to abdominal fat reduction, as observed in broiler chickens that received diet high-CHC combined with low-ZHC, but more studies are needed to elucidate this mechanism.

Copper and zinc plasma concentrations were not affected by different sources and levels of minerals in the diets, being an indicative of normal status of mineral in the body. According Silva and Pascoal (2014) during the mineral deficiency changes can be observed on bloodstream.

Copper concentrations on liver of broiler chickens increased with the inclusion of high-CHC (150 mg/kg). Liver is the first organ in which copper is stored, and this organ has the function of regulating the distribution of mineral in the body. When the maximum copper level is reached in the liver, this mineral is released into the bloodstream and accumulated in other organs. Furthermore, poultry has a high tolerance for copper intake, different from sheep and cattle that they have different mechanism for copper absorption (Sutlle, 2010, Hamdi et al., 2018).

Zinc concentration on liver was increased in broiler chickens supplemented with different levels of ZHC, and this data corroborates with Liu et al. (2015) who also found a linear increase of zinc (60, 120, 180 mg/kg) on liver after the supplementation with different (Zn sulfate, Zn amino acid chelate and Zn proteinate) sources of zinc in broilers diets. Likewise, for copper, liver is the first site as the storage for this element, and is a site for synthesis of proteins involving Zn (Tang et al. 2015). The adequate results observed on plasma Zn agree with results of Zn on liver, being an indicative of greater bioavailability with the sources used in this study.

The superoxide dismutase (SOD) and glutathione peroxidase (GPx) participates in the immune system defense of the body and a greater amount of mineral supplemented in this study appears contributed to maintain a good health of birds. According Surai (2016) the SOD intracellular and extracellular are the isoforms Cu-Zn dependents while the mitochondrial isoform is manganese dependent. The GPx is involved in regulating the production of glutathione (GSH) and glutathione disulfide (GSSG), which indicate the oxidative stress status. In this way, a high production of GSH can be induced by a high amount of hydrogen peroxide as a result of oxidative stress (Barbosa et al., 2010). The SOD and GPx mechanism of regulation is related to a production of free radicals during a stress oxidative appropriate and availability of trace minerals. Cao and Chen (1991) reported that copper deficiency reduced CuZn-dependent superoxide dismutase activity and increased the activity of Mn-dependent superoxide in the liver, while zinc deficiency resulted in increased free radical production. Results in the literature indicated that activity of the antioxidant enzymes can be changed when birds are subjected to some chemical or thermal stress, as in study of Perez et al. (2017) that submitted birds to a lipopolysaccharide challenge and observed that SOD activity was compromised 24 hours post challenge. The same authors when provided higher doses of hydroxychloride of zinc and manganese in the diet, observed greater activity of the enzyme, due to the higher bioavailability these minerals.

Ceruloplasmin (Cp) is a serum alpha-globulin containing 90-95% of copper, and carry this element to extrahepatic tissues is one of its functionalities (Cousins, 1985, McDonald et al., 2002). In addition, ceruloplasmin is an acute phase protein that is related to inflammation generated in response to stress (Chamanza et al., 1999). Koh et al. (1996) reported that ceruloplasmin activity increased when used higher levels of copper in the diet of broiler chickens during stress. On the other hand, Berwanger et al. (2018) supplemented copper sulfate (0.0, 3.5, 7.0, 10.5, 14 and 17.5 mg/kg) broiler breeder diets and found no difference in serum ceruloplasmin activity, and it seems no relationship among copper supplementation and activity of ceruloplasmin. According to our results, broilers supplemented with high-CSM combined with high-ZSM showed high activity of ceruloplasmin in serum and maybe some inconsistence in the data may have occurred, since the ceruloplasmin is low in poultry and no challenge was used during the trial. More studies are need to elucidate the mechanism of ceruloplasmin after supplementation with high level of Cu.

The AIA of Cu showed an increase around 10% (37.06 and 45.61%) after supplementation of 15 mg/kg and 150 mg/kg respectively, and this imply that birds were able to absorb a greater amount of this nutrient even when high levels of Cu were used. The AIA of Zn showed low percentage variation (85.32, 85.30 and 82.82%) from the levels supplemented (80, 100 and 120 mg/kg), respectively. These minerals in question have different mechanisms of absorption and while Cu can be storage in the liver after a high supplementation, Zn has a plateau, indicating that high levels of supplementation are not used (Suttle, 2010). Mohanna and Nys (1999) also observed that the linear increase of supplementation with Zn (40, 50, 65, 85, 170 mg / kg) from inorganic source in diet of broilers showed that 75 mg/kg was the plateau trough Zn in plasma and tibia concentration, and concluded that this may be related with absorption saturation.

The use of mineral source with greater bioavailability can provide better utilization of the mineral by the bird, decreasing the excretion to the environment and consequently, reduce the environmental impact. The assessment of apparent ileal absorption (AIA) is a way to identify the efficiency of the sources used, once this method is defined by the portion of the nutrient in the food provided that “disappears” in the digestive tract (Costa et al., 2008).

In conclusion, the replacement of STM by HTM sources of Cu and Zn was efficacious, and the different levels of supplementation tested in the current experiment showed that high level (150 mg/kg) of Cu seems be more beneficial instead low level (15mg/kg) of inclusion for performance and AIA of Cu. In the case of Zn, low level (80 mg/kg) of supplementation was enough to reach good results of carcass traits. These two trace minerals were indispensable to ensure the health of the birds and the hydroxychloride source can be a potential alternative to replace conventional mineral sources in broiler diets.

ACKNOWLEDGMENTS

This study was financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001; by São Paulo Research Foundation (FAPESP Process: 2017/00338-2) and by Trouw Nutrition. The authors gratefully acknowledge for all institution they sponsored this research.

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CAPÍTULO 3

“Effects of copper and zinc supplementation in broiler diets on bone development, reduction of footpad dermatitis, hock burn and skin scratches incidences”

Effects of copper and zinc supplementation in broiler diets on bone development, reduction of footpad dermatitis, hock burn and skin scratches incidences

A study was carried out to evaluate the effects of supplementing different levels of copper (Cu) and zinc (Zn) and two sources of mineral, being sulfate mineral trace (STM) or hydroxy mineral trace (HTM) on bone characteristics, skin strength, footpad dermatitis and hock burn incidence in broilers at 42 days of age. A total of 1,792 d-old male Cobb 500 broiler chickens were randomly distributed among eight dietary treatments with eight replicate per treatment. The HTM sources used were two levels of Cu hydroxychloride (CHC) (low and high), combined with three levels of Zn hydroxychloride (ZHC) (low, med and high) and two additional treatments STM, being Cu sulfate monohydrate (CSM) (low and high) combined with high Zn sulfate monohydrate (ZSM). On 42d, blood samples from jugular vein were collected of one bird/pen for hematological parameters analysis. The incidence of footpad dermatitis and hock were recorded at 42 days of age in five birds/pen, then, two birds/pen were slaughtered, tibia and femur were collected to access the parameters of bone and skin quality. The means were subjected to ANOVA and, when significant, compared by Tukey's test ($P < 0.05$) or Dunnet's ($P < 0.05$) test. The hematological parameters and footpad dermatitis were not influenced by mineral supplementation. However, the inclusion of low-ZHC enhanced the skin strength as compared to high-ZHC. Furthermore, the bone mineral density of tibia proximal epiphysis, tibia ash and tibia mineral content were positively improved with supplementation of low-CHC combined with med-ZHC as compared to high-CHC combined with med-ZHC. This study showed that HTM sources is a potential alternative to replace the STM sources in broilers diets. Moreover, among the Cu and Zn levels, the low-CHC (15 mg/kg) and med-ZHC (100 mg/kg) demonstrated improve the bone development and skin integrity, suggesting that can be a nutritional strategy to prevent incidence of leg disorders.

Keywords: bone health, copper, hydroxychloride, skin integrity, zinc.

INTRODUCTION

Bone tissue composition comprises approximately 70% of mineral (macromineral and trace mineral), 22% of protein and 8% of water, and the main function is to support and protect the body (Burwell, 1986; Lawrence and Fowler, 1997). The damages during the bone formation are related to growth rate, nutrition, genetic and stocking density factors (Cook, 2000). In addition, bone problems and skin scratch incidences are strongly associated to the condemnation of carcasses leading to economic losses (Waldenstedt, 2006).

The improvements on genetic selection of broiler strains has shown a positive impact on performance in order to improve the indexes, however, strains of fast-growing are inclined to develop bone abnormalities, due to fast weight gain in a short period of time. Thus, the appropriate supply of nutrients from the beginning of life is essential for development, and the minerals are totally related to the bone tissue formation in broilers, and also in the formation of eggs in laying hens (Suttle, 2010).

Diets based on corn and soybean meal normally has the inclusion of limestone and dicalcium phosphate as inorganic sources for the nutritional requirement of calcium (Ca) and phosphorus (P), respectively. In the body, these macrominerals are stored in the bones as hydroxyapatite form, giving rigidity and resistance to the tissue (Field, 2000). Some trace minerals are essential for bone formation, being copper (Cu) and zinc (Zn). Cu is a cofactor of the enzyme lysyl oxidase that is responsible on formation of crossed links and for the mechanical and elastic resistance to the collagen and elastin fiber present in the bones (Massuquetto et al. 2017). Zn is essential to bone cells, being linked to the osteoblasts (activation) during bone formation and the osteoclasts (inhibition) during bone resorption. Additionaly, collagenase and alkaline phosphatase enzymes are Zn-dependent during bone development (Pizzauro Junior et al. 2017). The status of zinc can change the synthesis of several proteins, as is the case of structural proteins as keratin and collagen (Suttle, 2010). Therefore, Zn deficiency can lead abnormality on bone and skin development (Lesson and Summers, 2001).

The main concern regarding to the trace minerals supplementation in the diet is due to the choice of source, once inorganic sources (sulfates, oxides and carbonates) are formed by ionic bonds, which are quickly dissociated in the gastrointestinal tract and impair the mineral absorption. Although the nutritional requirement of trace minerals for broilers is low, the incorrect supplementation can lead to several problems related to growth and consequent poor performance (Suttle, 2010).

Studies in the 90s revealed a new salt able of react with an alkali and produce a hydrolyzed inorganic complex, known as hydroxychloride. Chemically this compound is formed by three-dimensional lattice of high stability, making the covalent bonds strong enough to limit parasitic reactions, and weak enough to release the metal in the gut (Cohen and Steward, 2012). Hydroxychloride forms can be find as acatamite (Cu), simonkolleite (Zn) and hibbingite (Fe) (Palache et al., 1944). According to Pang and Applegate (2007) the hydroxychloride source showed low solubility in basic pH conditions when compared to sulfate, in an in vitro test. It means that the hydroxychloride can be more effective in the first sites of the gastrointestinal tract, avoiding negative interactions with other nutrients and faciliting the appropriate absorption in the small intestine.

The copper supplementation in broiler diets using levels above the normal requirement showed a positive result on performance and health, as in study of Pesti and Bakalli (1996) who observed that copper sulfate supplementation (125-150 mg/kg) improved the performance of broilers and the authors attributed this result to bacteriostatic property of copper. On the other hand, some studies reported that Zn supplementation with levels above the requirement showed different effects as compared to copper, once these elements have different mechanisms of absorption and retention (Mohanna and Nys, 1999).

Due the lack of establishment regarding the appropriate levels of mineral supplementation for the different kinds of sources available (inorganic or organic) more studies are needed, in the way to solve the questions during the feed formulation to provide the essential nutrients and reduces the environmental impact.

Thus, with the need to seek new alternatives to replace conventional sources of minerals, this study aimed to investigate the effects of two sources (sulfate and hydroxychloride), in addition the supplementation of Cu and Zn with levels above the recommended, combined in the diet of broilers on bone quality parameters.

MATERIALS AND METHODS

All procedures performed in this study were approved by the Ethics Committee on Animal Use - CEUA/FMVZ, process Nº 0008/2017.

Birds, diets and treatments

A total of 1,792 d-old male Cobb 500 broiler chickens were housed in 2.0 m^2 (14 birds/m^2) floor pens with 28 birds per replicate, that contained wood shavings as bedding during the period of 1 to 42 days old. Each pen was equipped with a tubular feeder and nipple drinkers (five nipples/pen). Birds were allocated to eight diet treatments with eight replicates, in a completely randomized design. The hydroxy trace minerals (HTM) sources used were two levels of Cu hydroxychloride (CHC) (low and high), combined with three levels of Zn hydroxychloride (ZHC) (low, med and high) and two additional treatments sulfate trace minerals (STM), being Cu sulfate monohydrate (CSM) (low and high) combined with high Zn sulfate monohydrate. The corn-soybean meal-based diets were supplemented with trace mineral premix without copper and zinc. Both CHC (IntelliBond Cu) and ZHC (IntelliBond Zn) were provided by Trouw Nutrition, Brazil. The supplemental levels of CHC, ZHC, CSM and ZSM were added replacing to limestone in all diets. Levels of trace mineral supplementation are present in Table 1. Diets isoenergetic and isoproteic were provided in mash form and they were formulated according recommendations of Rostagno et al. (2011) (Table 2). Diets and water were provided ad libitum throughout the entire study. The results of Cu and Zn analyzed in all experimental diets are shown in Table 3.

Table 1 Levels of supplementation (mg/kg)* of copper and zinc sources for the dietary treatments.

Diets	CSM	ZSM	CHC	ZHC
Low-CSM/ High-ZSM	15 (42.85)	120 (342.86)	-	-
High-CSM/ High-ZSM	150 (428.57)	120 (342.86)	-	-
Low-CHC/ Low-ZHC	-	-	15 (27.27)	80 (148.15)
Low-CHC/ Med-ZHC	-	-	15 (27.27)	100 (185.18)
Low-CHC/ High-ZHC	-	-	15 (27.27)	120 (222.22)
High-CHC/ Low-ZHC	-	-	150 (272.72)	80 (148.15)
High-CHC/ Med-ZHC	-	-	150 (272.72)	100 (185.18)
High-CHC/ High-ZHC	-	-	150 (272.72)	120 (222.22)

*Values in parentheses are the added Cu and Zn sources and values out parentheses are the inclusion level of mineral in diets.
Low-CSM:15 mg/kg; High-CSM: 150 mg/kg; High-ZSM: 120 mg/kg; Low-CHC: 15 mg/kg; High-CHC: 150 mg/kg; Low-ZHC: 80 mg/kg; Med-ZHC: 100 mg/kg; High-ZHC: 120 mg/kg.
CSM - Copper sulfate monohydrate
ZSM - Zinc sulfate monohydrate
CHC - Intellibond hydroxychloride Cu
ZHC - Intellinbond hydroxychloride Zn.

Table 2 Ingredient (g/kg) and composition of the experimental diets.

	Pre-starter	Starter	Grower	Finisher
Corn	590.11	615.38	644.00	687.47
Soybean meal	366.41	338.12	303.10	264.80
Soy oil	5.00	13.23	23.14	22.07
Dicalcium phosphate	12.20	8.65	6.37	4.15
Limestone	9.60	9.90	9.48	8.76
Salt (NaCl)	5.18	4.80	4.56	4.44
DL-Methionine	3.40	2.90	2.70	2.50
L-Lysine HCl	3.20	2.70	2.70	3.00
Trace mineral and vitamin premix [1.2]	2.00	1.80	1.60	1.20
L-threonine	1.20	0.90	0.80	0.80
Choline chloride 60%	0.72	0.63	0.58	0.38
Anticoccidian	0.55	0.55	0.55	-
Phytase 500 FTU	0.05	0.05	0.05	0.05
Sulfate of zinc 35%	0.34	0.34	0.34	0.34
Sulfate of copper 35%	0.04	0.04	0.04	0.04
Intellibond Zn 55%	-	-	-	-
Intellibond Cu 54%	-	-	-	-
Calculated nutrients				
ME (kcal/kg)	2964	3050	3149	3199
Crude protein, %	22.40	21.20	19.80	18.40
Calcium, %	0.92	0.84	0.76	0.66
Total phosphorus, %	0.71	0.63	0.57	0.52
Available phosphorus, %	0.47	0.40	0.35	0.31
Copper, mg	0.017	0.017	0.017	0.017
Zinc. mg	0.131	0.131	0.132	0.132
Lisyne, %	1.32	1.21	1.13	1.06
Methionine, %	0.66	0.60	0.56	0.53
Met+Cis, %	0.95	0.88	0.83	0.77
Threonine, %	0.86	0.79	0.73	0.69

[1]Trace mineral premix (levels per kg of feed): iron 62.5 mg; iodine 1.25 mg; manganese 88 mg; selenium 0.375 mg.

[2] Vitamin premix (levels per kg of feed): vitamin A 9,375 UI; vitamin D3 2,375 UI; vitamin E 35 UI; vitamin B12 0.015 mg; vitamin B7 0.088 mg; vitamin B9 0.875 mg; vitamin K3 1.88 mg; vitamin B1 2.5 mg; vitamin B6 3.5 mg; vitamin B2 6.25 mg; vitamin B5 12.5 mg; vitamin B3 37.5 mg; B.H.T 15 ppm.

Table 3 Analyzed copper and zinc composition (mg/kg) in all experimental diet.

Diets	Pre starter		Starter		Grower		Finisher	
	Cu	Zn	Cu	Zn	Cu	Zn	Cu	Zn
Low-CSM/ High-ZSM	20.23	159.80	29.00	94.00	16.28	132.70	15.26	132.70
High-CSM/ High-ZSM	130.50	154.20	129.70	133.00	88.68	128.47	98.56	126.47
Low-CHC/ Low-ZHC	23.77	138.20	32.50	104.80	18.95	108.88	18.95	103.83
Low-CHC/ Med-ZHC	21.96	139.10	26.63	116.30	14.98	108.98	14.93	108.63
Low-CHC/ High-ZHC	17.92	154.80	28.75	136.20	16.88	120.20	15.88	120.20
High-CHC/ Low-ZHC	150.30	136.40	130.10	98.10	91.34	87.66	91.34	87.55
High-CHC/ Med-ZHC	162.60	153.70	177.90	129.50	100.81	111.16	100.61	111.15
High-CHC/ High-ZHC	149.80	176.50	108.30	133.20	101.38	131.80	101.36	131.30

Low-CSM:15 mg/kg; High-CSM: 150 mg/kg; High-ZSM: 120 mg/kg; Low-CHC: 15 mg/kg; High-CHC: 150 mg/kg; Low-ZHC: 80 mg/kg; Med-ZHC: 100 mg/kg; High-ZHC: 120 mg/kg.

CSM - Copper sulfate monohydrate

ZSM - Zinc sulfate monohydrate

CHC - Intellibond hydroxychloride Cu

ZHC - Intellibond hydroxychloride Zn.

Footpad dermatitis and hock burn incidence

The incidences of footpad dermatitis and hock burn were evaluated at 42 days of age in five birds/pen. The footpad dermatitis was measured according to five scores: score 1: no injury; score 2: hypertrophic and hyperkeratotic scales, covered by yellowish to brownish exudates, size <50%; score 3: hypertrophic and hyperkeratotic scales, covered by yellowish to brownish exudates, size >50%; score 4: depressed injury, loss of substance (= ulceration), with or without thick adhrent crust, size <50%; score 5: depressed injury, loss of substance (= ulceration), with or without thick thick adhrent crust, size >50% (Michel et al., 2015).

The hock burn incidence was evaluated in three scores: score 0: no evidence; score 1: minimal evidence; score 2: evidence of lesion. (Welfare Quality®, 2009).

Skin strength

Skin strenght and elasticity were measured using skin samples from the drumsticks of 16 birds per treatment, which were slaughtered at 42 days of age. The standard for measurement was adopted and all samples were cut around 3 cm (height, width and length). The samples were submitted to the flexion test at the constant strain rate for visco-elastic material using a texturometer (Model TA-XT2i, Stable Mycro Systems LTDA., Goldalming, UK). The parameters used were: speed of 1 mm/s, firing force of 10 g and tension of 15 mm (Ribeiro, 2016).

Hematological parameters

At 42 days of age blood samples of one bird/pen from jugular vein were collected using syringes without anticoagulant and placed in EDTA 5% tubes. Hematological values determined were: red blood cells (RBC) count, hemoglobin rate (Hb), pocked cell volume (PCV), total and differential leukocyte, and thrombocyte count. The determination of the PCV was performed using the microhematocrit method, using a capillary tube centrifuged at 1,200g for five minutes, the result was expressed as a percentage. The Hb (g/dl) was measured using the cyanomethaemoglobin technique, using kit (BIOCLIN®). The count of red blood cells and leukocytes, was performed in a Neubauer chamber using a Natt-Herrick's solution, in a dilution of 1:200. The differential leukocyte count was measured in blood film slides. The following Wintrobe hematimetric indexes were calculated from the data obtained in the blood count, being the mean corpuscular hemoglobin concentration (MCHC), expressed in (%); mean corpuscular volume (MCV), expressed in phentoliters (fl). The total protein was evaluated by refractometer.

Chemical Analysis

Cu and Zn concentration in diet: mineral concentration was determined in all diets *in natura* using the atomic absorption spectrometry method (FAAS - Flame Atomic Absorption Spectrometry), as described by Neves et al. (2009).

Parameters of bone quality

Two birds from each experimental unit were slaughtered at 42 d of age by cervical dislocation for collecting the right legs, which were immediately frozen at −20 °C for later analysis.

Bone mineral density (BMD): thigh and the drumstick with bones, muscle, and skin on the right side of each bird were thawed and then radiographed at the Veterinary Hospital of FMVZ — UNESP Botucatu. The radiographic technique was performed with an X-ray apparatus placed at a 90 cm focus-film distance and calibrated for 50 kVp and 5.0 mAs. The (phantom) aluminum ladder was placed on the tape 3.0 cm away from the region that was X-rayed to be used as the reference standard. The densitometry readings were performed in the proximal epiphysis and distal epiphysis of the tibia and the femur using the software CROMOX® developed by company ATHENA (Advanced Smart System, São José dos Campos, SP, Brazil) and were expressed in millimeters of aluminum (mm Al) (Louzada et al., 1998).

Breaking strenght (BS): after BMD measurements, the adherent tissues were removed with a scalpel. The EMIC DL 300 kN test set (Instron, Brazil) was used to perform the breaking strenght tests, and it was adjusted to allow the diaphysis free span to be 3.0 cm. This value was the maximum spacing achieved for the smallest bone found, thus it was fixed to the other bones. The values found (kgf) can be compared only with the fixation of a free span for the evaluations of resistance (Almeida Paz et al., 2006).

Bone ash: the analysis of ash and mineral concentration were performed in the same bones used for the BS analysis. The tibia and femur were dried for 24 h at 105 °C in a forced circulation oven. Afterwards, they were defatted in ethyl ether using the Soxhlet extractor for 5 h and then taken back to the oven at 105 °C for 12 h. For evaluating the ash content, the dried and defatted bones were muffled for 4 h at 600 °C. Bone ash was determined based on defatted dry matter and expressed as ash percentage (method 972.15, AOAC 2000).

Mineral concentration: calcium (Ca), copper (Cu), magnesium (Mg), phosphorus (P) and zinc (Zn) contents were determined from bone ash, where 100 milligrams of sample were weighed, which was digested with 3 milliliters of nitric acid (65% PA) and 2 milliliters of hydrogen peroxide (35% PA) using closed fluoropolymer tubes (PFA) in a Microwave Oven (Speedwave model SW-4). Ca, Cu, Mg and Zn content was obtained by the SHIMADZUAA-6800 atomic absorption spectrophotometer according to Neves et al. (2009) and the P content by the spectrophotometric method of phosphomolybdic acid according to Moraes et al. (2009).

Statistical Analysis

Data was analyzed in a (2x3)+2 factorial arrangement and this model had two levels of copper (low and high) and three levels of zinc (low, med and high) plus two additional treatments that sulfates sources of Cu and Zn were used. Initially data were submitted to the normality test and then to analysis of variance using the PROC GLM of Minitab® 16 (2009). The Kruskal-Wallis test was applied to variables that did not meet the normality test. Mean of factorial arrangement and mean of additional treatments were compared using Tukey test. Furthermore, a comparison between sulfate with hydroxichloride treatments was performed using Dunett's test. Statistical significance was set at $P < 0.05$.

RESULTS

There was no significant interaction between the factors ($P > 0.05$) for footpad dermatitis (Table 4) and hock burn (Table 5) incidences. A main effect of Cu was observed in incidence of hock burn score, and a higher frequency of broilers with score 0 was observed in the group low-CHC as compared to high-CHC, while a lower frequency of broilers with score 2 was observed in the group low-CHC as compared to high-CHC.

Table 4 Footpad dermatitis incidence (%) in broilers supplemented with different sources and levels of Cu and Zn at 42 days of age.

Score	Cu	Low			High						*P*-value				
	Zn	Low	Med	High	Low	Med	High	S1	S2	SEM	Cu	Zn	S1	S2	S1×S2
1		27.50	17.50	20	30	15	10	22.50	17.50	0.022	0.515	0.064	0.352	0.352	0.559
2		30	30	27.50	15	30	42.50	20	15	0.038	0.818	0.324	0.292	0.292	0.609
3		10	22.50	2.50	20	12.50	12.50	12.50	5	0.025	0.610	0.335	0.360	0.360	0.245
4		25	25	50	27.50	37.50	30	35	52.50	0.035	0.601	0.166	0.061	0.061	0.069
5		7.50	5	0	7.5	5	5	10	10	0.016	0.637	0.534	0.810	0.810	0.987

S1: 15 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate, S2: 150 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate.
Means follow of the * differ from S1 and means follow of the + differ from S2 by Dunnett's test, at 5% probability.
SEM: Standard error of the mean.
Score 1: no injury; score 2: hypertrophic and hyperkeratotic scales, covered by yellowish to brownish exudates, size <50%; score 3: hypertrophic and hyperkeratotic scales, covered by yellowish to brownish exudates, size >50%; score 4: depressed injury, loss of substance (= ulceration), with or without thick adhrent crust, size <50%; score 5: depressed injury, loss of substance (= ulceration), with or without thick thick adhrent crust, size >50%.

Table 5 Hock burn incidence (%) in broilers supplemented with different sources and levels of Cu and Zn at 42 days of age.

Score	Cu	Low			High						*P*-value				
	Zn	Low	Med	High	Low	Med	High	S1	S2	SEM	Cu	Zn	S1	S2	S1×S2
0		57.50a	65.83a	52.50a	42.50b	52.50b	27.50b	50	48.13	0.055	**0.040**	0.142	0.380	0.380	0.717
1		42.50	34.17	47.50	47.50	40	67.50	42.50	51.88	0.052	0.277	0.113	0.303	0.303	0.583
2		0b	0b	0b	10a	5a	5a	7.50	0	0.014	**0.047**	0.748	0.406	0.406	0.177

S1: 15 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate, S2: 150 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate.

[a, b] means within a row with different superscripts are significantly different by Tukey test, at 5% probability.

Means follow of the * differ from S1 and means follow of the + differ from S2 by Dunnett's test, at 5% probability.

SEM: Standard error of the mean.

Score 0: no evidence; score 1: minimal evidence; score 2: evidence of lesion.

Results of skin strength and elasticity are present in Table 6. There was no significant interaction between the factors. The main effect of Zn ($P < 0.05$) on skin strength shows that broilers in group low-ZHC presented higher skin strength as compared to high-ZHC irrespective when using low-CHC or high-CHC.

Table 6 Parameters of strength and elasticity of drumstick skin of broilers supplemented with different sources and levels of Cu and Zn at 42 days of age.

Item	Low			High						*P*-value				
	Low	Med	High	Low	Med	High	S1	S2	SEM	Cu	Zn	S1	S2	S1×S2
Strength, g	6187.9a	5568.4ab	4736.6b	5965.9a	5697.3ab	5522.0b	5597.1	6019.9	0.134	0.454	**0.046**	0.208	0.208	0.431
Elasticity, mm	9.6	8.8	9	9.1	9.1	8.9	8.7	9.2	0.108	0.549	0.362	0.548	0.548	0.248

S1: 15 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate, S2: 150 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate.
[a. b] means within a row with different superscripts are significantly different by Tukey test, at 5% probability.
Means follow of the * differ from S1 and means follow of the + differ from S2 by Dunnett's test, at 5% probability.
SEM: Standard error of the mean.

No significant statistical differences were observed in hematological parameters (Table 7) of broilers supplemented with different sources and levels of Cu and Zn.

Table 7 Hematological parameters of broilers supplemented with different sources and levels of Cu and Zn[1].

	Low			High						
Item[2]	Low	Med	High	Low	Med	High	S1	S2	P-value	SEM
RBC (x10^6/ul)	2.38	2.44	2.21	2.26	2.41	2.21	2.40	2.54	0.4376	0.048
HB (g/dl)	10.25	11.70	10.40	10.95	10.80	10.70	10.60	11.05	0.2215	0.193
PCV (%)	27.50	29.50	28	29.50	29	28.50	29.50	30	0.1940	0.414
MCV (fl)	126.20	122.95	125.80	128.25	124.50	126.65	125.30	119.5	0.9046	3.038
MCHC (%)	36	39.05	36.55	37.65	37.40	38.50	35.65	38.70	0.2799	0.362
TP (g/dl)	3.20	3.20	3.10	3.00	3.20	3.20	3.30	2.90	0.2371	0.040
Trombocytes (/ul)	18050	19100	20750	15650	25050	21000	20750	20600	0.8372	1254.41
Leukocytes (/ul)	12300	12300	17800	13300	14400	12300	14350	12100	0.8056	986
Heterophils (%)	41.50	45.50	39.50	39.50	45.50	38.50	47.50	42	0.2531	1.380
Lymphocytes (%)	54	51	56.50	56.50	47	55	50.50	50.50	0.6037	1.263
Monocytes (%)	4	4.5	5	4.5	4	6	3	6.5	0.2362	0.377

[1] values of medians evaluated by Kruskal-Wallis test, by 5% of probability.
[2] RBC, red blood cells; Hb, haemoglobin; PCV, packed cell volume; MCV, mean corpuscular volume; MCHC, mean corpuscular haemoglobin concentration; TP, total protein.
S1: 15 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate, S2: 150 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate.
SEM: standard error of the mean.

The bone mineral density (BMD) results are shown in Table 8, and a significant interaction was observed for PE of tibia (*P* < 0.05). In the interactions, birds in the group low CHC had higher BMD when low-ZHC was used as compared to high-ZHC. Furthermore, birds in the group med-ZHC presented highest BMD when low-CHC was used as compared to high CHC. Results of sulfates versus hydroxychlorides sources showed that birds in the treatment high-CHC combined with low-ZHC had higher BMD as compared to S1, and broilers in the treatment low-CHC combined with high-ZHC presented lower BMD as compared to S2.

Table 8 Bone mineral density of proximal epiphysis (PE) and distal epiphysis (DE) of tibia and femur of broilers supplemented with different sources and levels of Cu and Zn.

CHC	ZHC			S1	S2	SEM	*P*-value					
	Low	Med	High				Cu	Zn	Cu×Zn	S1	S2	S1×S2
	PE, tibia											
Low	2.65a	2.58abA	2.26b+	2.40	2.45	0.028	0.720	0.121	**0.004**	**0.009**	**0.009**	0.549
High	2.61*	2.28B	2.48									
	DE, tibia											
Low	2.69	2.74	2.83	2.68	2.82	0.026	0.890	0.391	0.396	0.577	0.577	0.155
High	2.68	2.83	2.72									
	PE, femur											
Low	2.38	2.49	2.36	2.27	2.69	0.051	0.612	0.786	0.579	0.568	0.568	0.065
High	2.39	2.43	2.59									
	DE, femur											
Low	2.26	2.16	2.12	2.20	2.33	0.035	0.255	0.825	0.338	0.723	0.723	0.456
High	2.23	2.22	2.36									

CHC: copper hydroxychloride, ZHC: zinc hydroxychloride, S1: 15 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate, S2: 150 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate.

[A.B] means within a column with different superscripts are significantly different for Cu by Tukey test, at 5% probability.

[a.b.c] means within a row with different superscripts are significantly different for Zn by Tukey test, at 5% probability.

Means follow of the * differ from S1 and means follow of the + differ from S2 by Dunnett's test, at 5% probability.

SEM: Standard error of the mean.

Breaking strenght (BS) and bone ash (BA) of tibia (Table 9) were significantly improved by different levels of Cu and Zn supplemented in the diet. Broilers in the group high CHC presented higher tibia BS when low-ZHC was used as compared to med-ZHC. Moreover, broilers in the group low-ZHC presented higher tibia BS when high-CHC was used, as compared to low-CHC.

The tibia BA of broilers in the group low-CHC increased when med-ZHC was included as compared to high-ZHC. Besides that, an increase on tibia BA was observed in broilers in the group high-ZHC combined with high-CHC, as compared to low-CHC. In sulfates versus hydroxychlorides sources, birds in the group high-CHC combined with high ZHC had higher BA as compared to S1, and broilers in the group low-CHC combined with high-ZHC had lower BA as compared to S2.

Table 9 Breaking strength, kgf (BS) and bone ash, % (BA) of tibia and femur of broilers supplemented with different sources and levels of Cu and Zn.

CHC	ZHC			S1	S2	SEM	*P*-value					
	Low	Med	High				Cu	Zn	Cu×Zn	S1	S2	S1×S2
	BS, tibia											
Low	36.38 B	38.94	37.71	36.62	39.31	0.769	0.234	0.399	**0.053**	0.176	0.176	0.347
High	44.59aA	36.62b	38.25ab									
	BA, tibia											
Low	50.97ab	51.16a	49.88bB+	50.18b	51.70a	0.131	0.406	0.389	**0.027**	**0.007**	**0.007**	**0.001**
High	51.11	50.46	51.19A*									
	BS, femur											
Low	32.27	29.18	33.28	31.62	34.79	0.609	0.572	0.749	0.263	0.512	0.512	0.149
High	32.26	33.26	31.69									
	BA, femur											
Low	49.79	49.73	50.01	50.13	49.72	0.121	0.780	0.353	0.530	0.790	0.790	0.469
High	50.26	49.48	50.01									

CHC: copper hydroxychloride, ZHC: zinc hydroxychloride, S1: 15 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate, S2: 150 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate.

[A.B] means within a column with different superscripts are significantly different for Cu by Tukey test, at 5% probability.

[a.b.c] means within a row with different superscripts are significantly different for Zn by Tukey test, at 5% probability.

Means follow of the * differ from S1 and means follow of the + differ from S2 by Dunnett's test, at 5% probability.

SEM: Standard error of the mean.

The mineral content in tibia and femur in response to dietary treatments are shown in Table 10 and Table 11, respectively. Several interactions ($P < 0.05$) were observed among the different levels of Cu and Zn in the diet, once bone is the main place for mineral storage.

The use of high-ZHC combined with low-CHC or high-CHC in the diets reduced the Ca, P, and Mg in tibia (Table 10). Besides that, higher concentrations Ca 43.49 %, P-24.17 %, Mg-6.77 % and Zn- 319.37 ppm were observed with the use of med-ZHC combined with low-CHC, as compared to high-CHC. Cu concentration increased in the group low-CHC with inclusion of low-ZHC (9.86 %), as compared to med-ZHC.

In sulfate versus hydroxychloride sources for tibia (Table 10), broilers in the group low CHC combined with med-ZHC showed higher Ca and Mg concentration as compared to S1. Higher P concentration was observed when diet containing high-CHC combined with low-ZHC was used as compared to S1.

The significant interactions in femur mineral concentration (Table 11) showed that broilers in the group high-ZHC presented high Ca and Cu concentrations when high-CHC was used as compared to low-CHC.

In sulfate versus hydroxychloride sources for femur (Table 11), higher Ca and Cu concentrations were observed in the group high-CHC combined with med-ZHC as compared to S2. For P and Mg concentrations, broilers in the group high-CHC combined with high-ZHC showed higher concentrations as compared to S1. Zn concentration in femur was not affected by dietary treatments, as follow Table 11.

Table 10 Mineral concentration of calcium (Ca), phosphorus (P), magnesium (Mg), copper (Cu) and zinc (Zn) in tibia of broilers supplemented with different sources and levels of Cu and Zn.

CHC	ZHC Low	ZHC Med	ZHC High	S1	S2	SEM	P-value Cu	P-value Zn	P-value Cu×Zn	P-value S1	P-value S2	P-value S1×S2
		Ca, %										
Low	29.30b+	43.39Aa*+	20.21Bc*	30.80a	21.39b	0.907	0.019	0.0001	**0.001**	**0.0001**	**0.0001**	**0.010**
High	25.91b	33.20Ba+	23.72Ab									
		P, %										
Low	20.98Bab	24.17Aa	17.28Bb	19.97	20.68	0.480	0.147	0.010	**0.002**	**0.001**	**0.001**	0.726
High	24.90Aa*+	20.42Bb	21.49Aab									
		Mg, %										
Low	6.73*	6.77A*	5.49B*	3.38b	5.96a	0.198	0.0001	0.0001	**0.0001**	**0.0001**	**0.0001**	**0.002**
High	7.06a*	1.77Bc*+	6.09Ab*									
		Cu, ppm										
Low	9.86Aa	5.59Bb	7.30ab	7.63	8.34	0.384	0.818	0.192	**0.0001**	**0.0001**	**0.0001**	0.621
High	3.19Bb*+	10.75Aa	8.28a									
		Zn, ppm										
Low	335.76+	319.37A	304.81	277.50	265.44	7.288	0.0001	0.001	**0.0387**	**0.0001**	**0.0001**	0.649
High	311.38a	192.25Bb*+	295.16a									

CHC: copper hydroxychloride, ZHC: zinc hydroxychloride, S1: 15 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate, S2: 150 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate.

[A.B] means within a column with different superscripts are significantly different for Cu by Tukey test, at 5% probability.

[a.b.c] means within a row with different superscripts are significantly different for Zn by Tukey test, at 5% probability.

Means follow of the * differ from S1 and means follow of the + differ from S2 by Dunnett's test, at 5% probability.

SEM: Standard error of the mean.

Table 11 Mineral concentration of calcium (Ca), phosphorus (P), magnesium (Mg), copper (Cu) and zinc (Zn) in femur of broilers supplemented with different sources and levels of Cu and Zn.

	ZHC						*P*-value					
CHC	Low	Med	High	S1	S2	SEM	Cu	Zn	Cu×Zn	S1	S2	S1×S2
		Ca, %										
Low	22.44ab*	32.73a+	14.52Bb*+	36.86a	26.15b	0.859	0.0001	0.0001	**0.0001**	**0.0001**	**0.0001**	**0.0001**
High	24.89b*	36.66a+	33.83Aab+									
		P, %										
Low	19.16+	22.62	20.25	19.95b	22.72a	0.379	0.382	0.051	0.066	**0.016**	**0.016**	**0.009**
High	19.84	20.84	23.73*									
		Mg, %										
Low	6.02Bab*	6.30Aa*	4.92b*+	2.18b	6.61a	0.174	0.001	0.0001	**0.0001**	**0.0001**	**0.0001**	**0.0001**
High	7.08Aa*	2.58Bb+	5.51a*+									
		Cu, ppm										
Low	5.32	3.96B	4.21B	6.11	4.70	0.326	0.0001	0.013	**0.0001**	**0.0001**	**0.0001**	0.239
High	4.83	10.18A*+	9.41A*+									
		Zn, ppm										
Low	353.95	329.35	284.02	315.12	316.33	6.165	0.916	0.087	0.130	0.227	0.277	0.962
High	329.86	309.87	323.12									

CHC: copper hydroxychloride, ZHC: zinc hydroxychloride, S1: 15 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate, S2: 150 mg/kg of copper sulfate and 120 mg/kg of zinc sulfate.
[A.B] means within a column with different superscripts are significantly different for Cu by Tukey test, at 5% probability.
[a.b.c] means within a row with different superscripts are significantly different for Zn by Tukey test, at 5% probability.
Means follow of the * differ from S1 and means follow of the + differ from S2 by Dunnett's test, at 5% probability.
SEM: Standard error of the mean.

DISCUSSION

The use of nutritional strategies during the breeding period of broiler chickens have been studied as alternative to prevent incidence of leg disorders. These bone abnormalities started after a long period of genetic development for fast growth together with intensive husbandry conditions, and the fast weight gain in a short time is one of the main factors (Almeida Paz and Martins, 2014). The main concerns regarding to leg problems are the impacts generated on welfare of broilers due the pain during the locomotion for essential activities, as feed and water intake, reflecting in a poor growth performance. Thus, the appropriate mineral nutrition is important to avoid leg problems, improve the skin integrity and reduce the footpad dermatitis, once the incidence of these disorders may serve a point for entry of pathogenic bacteria in the body. In this way, the use of mineral sources that ensure better bioavailabity can be an alternative to reduce problems during the bird development.

As observed in the current study, there was not incidence of footpad dermatitis, being an indicative that good litter conditions were provided. In the hock burn evaluation, our results showed an increase of birds with score 2 in the group high-CHC as compared to low-CHC. On the other hand, the hock burn incidence is much more related to a litter quality, therefore, is not possible affirm that high level of Cu can increase that problem, once birds with score 2 showed an incidence of only 6.67%. According Jacob et al. (2016) the lesions on feet are related to litter reutilization, quality of substrate for litter and inappropriate litter management. These authors observed in the study with broilers that the use of new litter showed a higher risk of footpad dermatitis and hock burn incidences as compared to reused litter, due the quality of the substrate used the experiment.

The skin integrity is an important factor to avoid the contact with pathogens organisms, besides that, the high incidence of scratch can lead to condemnation of carcasses, generating economic losses. In addition, the element zinc has been associated to reduction of skin scratchs due the role on synthesis of structural proteins, as the keratin and collagen, which are responsible by the skin integrity (Underwood et al. 2001). Among the zinc levels used in the current study, the low-ZHC (80 mg/kg) showed higher skin strenght as compared to high-ZHC (120 mg/kg) and one possible explanation could be the fact of excess of zinc is not used by the bird. Zinc has a mechanism of saturation in the absorption channels, therefore, when reach the plateau the absorption is ceased (Mohanna and Nys, 1999). In a study of Rossi et al. (2007) broilers were supplemented with organic zinc (15, 30, 45 and 60 mg/kg) and the higher collagen content and skin strenght were observed in the highest level of supplementation.

The hematological parameters of broilers were not affected by dietary treatments, and our results for all variables evaluated corroborate with Talebi et al. (2005) for Cobb broiler strain reared in normal conditions. Previous studies have reported that the trace mineral copper is involved on hemoglobin production with the iron (Hart et al., 1928) and as expected, one possible change could have occurred on hemoglobin rate, once 10 times of Cu was supplemented in the diet, but our results indicated normal rate. Samanta et al. (2011) supplemeted broilers with high levels of Cu in the diet (75, 150 and 250 mg/kg) and observed reduction in hemoglobin rate in the group with 250 mg/kg as compared to other treatments, and postulated that the excess dietary of Cu can reduce the hemoglobin in the blood due the accumulation of Cu in the liver. Our results support the hypothesis that good conditions during breeding period benefited the good profile of heath.

Leg disorders in poultry production is a problem that has been faced for a long a time, being caused by different factors as management, genetic or dietary imbalance. Nowadays, several techniques are available to access the bone quality of broilers, and the bone mineral density (BMD) is a noninvasive method with low cost that measure the bone formation and can indicate disorders in the tissue. According Onyango et al. (2003) the BMD has a high correlation with bone ash and bone mineral content, predicting the bone mineralization, and it means that is possible follow the bone development of the bird during the life. In the current study, the BMD evaluation showed a little change on proximal epiphysis of tibia, and broilers in the group of med-ZHC had higher BMD when low-CHC was used as compared to high-CHC. Nutritional changes with different mineral supplements usually promote effects on the proximal epiphysis, which is the region where the growth zone is present (Orban et al., 1999; Oliveira et al., 2008). Therefore, our results indicate an appropriate bone formation, once the mineral deficiency is the main factor for the delay in the process of mineralization, as observed by Santos et al. (2019) in broilers fed with diets with low levels of available phosphorus and showed low BMD values on tibia and femur of broilers.

The tibia breaking strength (BS) was affected by different levels of Cu and Zn supplemented in the diet. Broilers in the group low-ZHC had higher BS when high-CHC (44.59, kg) was used as compared to low-CHC (36.38, kg). Several factors can influence the BS parameter, as methods of storing, handling and crosshead speed (Onyango et al., 2003). In the current experiment, the bone samples were evaluated *in natura* as a way to avoid the maximum of handling, once that Kim at al. (2004) observed that this method gives better response, as compared to dry or fat free dry preparation methods. These results indicate a possible mineral synergism during bone formation, once Cu is cofactor of lysyl oxidase, which gives more resistance to crossed links, and Zn plays a role on bone formation, being essential to osteoblasts and osteoclasts (Pizzauro Junior et al. 2017). In addidtion, Zn is a cofactor of alkaline phophatase which hydrolyzes pyrophosphate and releases inorganic phosphate to form hydroxyapatite (Pizzauro Junior et al. 2017). Phosphorus (P) and calcium (Ca) forms the hydroxyapatite in the inorganic bone matrix being responsible to give rigidity and compressive strength to the tissue (Field, 2000). Based on our results, we suppose that the tibia BS was benefited by Cu and Zn supplementation in the diet, once these trace minerals works together with Ca and P during bone formation.

Bone ash (BA) parameter represents the inorganic material content and indicates the status of bone mineralization (Kim et al. 2004). According the current study, appears that the different sources and levels of Cu and Zn supplemented in the diet showed a low influence on tibia BA, and the values were around 50%. Olukosi et al. (2018) found no effect of Zn supplementation (20 or 80 mg/kg) in hydroxychloride or sulfate source in broiler diets, on tibia bone ash.

The tissue mineral content is indicator of mineral status and body storage (Wang et al., 2007). On basis in our results of bone mineralization, it is possible confirm that the BMD parameter has relationship with bone mineral content, as in study of Onyango et al. (2003). Table 8 shows that broilers in the group med-ZHC had higher tibia BMD when low-CHC was used, as compared to high-CHC. In addition, this same group had a greater tibia Ca (43.39 %), P (24.17 %) and Mg (6.77 %) concentrations, as follow Table 10. This suggests that low CHC (15 mg/kg) combined with med-ZHC (100 mg/kg) supplementation can be enough to support normal bone growth. Mineral supplementation in poultry diets should be performed carefully, due the interactions among the elements during the absorption process in the gut. The most of Ca, P, Mg and Zn in the body are storaged in the bone tissue, and have been demonstrated that these elements can have interaction with each other (synergistic or antagonistic) (Suttle, 2010; Silva and Pascoal, 2014). The elements Ca and P are essential to ensure the bone formation, therefore, it is interesting to avoid the excess of elements that can have possible negative interaction. In the same way, P contains “P phytic” that can form quelates with bilavent minerals (Ca^{2+}, Cu^{2+} and Zn^{2+}), reducing mineral absorption (Kornegay, 2001).

The Cu concentration in tibia and femur of group med-ZHC reduced when low-CHC was used as compared to high-CHC, as follow Table 10 and Table 11. However, the results of Cu concentration in bone tissue are in agreement with the normal level, being around 10 ppm. The most Cu in the body is storaged in the liver (Suttle, 2010). Curiously the Zn concentration was not affected by dietary treatments, and appears that the levels fixed of Zn supplementation in the current experiment (80, 100 or 120 mg/kg) were enough to attend the requirement, showing that excess of Zn is not essential for the bird. On the other hand, Olukosi et al. (2018) supplemented broilers with two levels of Zn (20 and 80 mg/kg) in the diet, using different sources, and found an increase on Zn deposition in tibia in the highest level of supplementation. Therefore, this imply that is important respect the level of supplementation to ensure the appropriate utilization.

The trace minerals Cu and Zn plays different roles in the biological pathways in the body, and based in this statement, we expected with the present study an improvement on bone and skin characteristics, as a way to reduce some problems faced by broiler chicken production. We conclude that the hydroxychloride showed potential results, being an alternative to replace the sulfate sources in broiler diets. Moreover, among the Cu and Zn levels, the low-CHC (15 mg/kg) and med-ZHC (100 mg/kg) demonstrated improve the bone development and skin integrity, suggesting that can be a nutritional strategy to prevent incidence of leg disorders.

AKNOWLEDGMENTS

This study was financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001; by São Paulo Research Foundation (FAPESP Process: 2017/00338-2) and by Trouw Nutrition. The authors gratefully acknowledge for all institution they sponsored this research.

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CAPÍTULO 4

1

IMPLICAÇÕES

Atualmente estão disponíveis no mercado diversas opções de fontes minerais para suplementação nas dietas das aves, no entanto, a falta de estabelecimento de níveis de adequados para as diferentes fontes disponíveis, tem gerado questionamentos no momento da formulação de dietas que forneçam os nutrientes necessários para a ave, e que seja capaz de causar menor impacto ambiental.

No presente estudo, alguns dos parâmetros avaliados não foram responsivos ou precisos para mostrar os efeitos do uso de diferentes fontes e níveis de minerais na dieta das aves. Com relação a avaliação da atividade enzimática da ceruloplasmina, era esperado que com o aumento da quantidade suplementada de cobre na dieta (15 vs. 150 mg/kg), a atividade enzimática também apresentasse alguma alteração. No entanto, os resultados obtidos indicaram que mesmo com o aumento de 10 x no nível de suplementação na dieta, a atividade da enzima no soro não foi alterada. As aves por sua vez, apresentam baixa atividade desta enzima, fator que também dificulta a avaliação. Portanto, não se recomenda a avaliação deste parâmetro como resposta às alterações dietéticas de cobre.

Entre os métodos empregados para avaliar os efeitos da suplementação mineral, utilizou-se o método da absorção mineral ileal aparente. Para obtenção de resultados mais condizentes de digestibilidade de minerais, é obrigatório o uso de gaiolas livres de qualquer material que contenha mineral durante o ensaio, evitando interações entre o mineral presente na dieta com o mineral da estrutura das gaiolas. Deste modo, para a avaliação da absorção do cobre e zinco, optamos pelo método da avaliação da absorção mineral ileal aparente, em função da estrutura experimental disponível.

A literatura afirma que a suplementação com níveis elevados de Cu é benéfica para o desempenho das aves devido ao papel bacteriostático que o Cu exerce no intesino, e nossos resultados confirmaram esse efeito após a suplementação com 150 mg/kg. Por outro lado, o elevado nível de Cu parece não ter influencido os outros parâmetros avaliados. O desenvolvimento ósseo, integridade da pele, e paramêtros de rendimento de carcaça foram beneficiados pela inclusão de Zn. Sendo assim, no momento da escolha suplementação, é ideal levar em consideração a função específica de cada mineral, além da exigência nutricional da ave.