UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE AGRONOMIA

PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOTECNIA


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**Exigência e disponibilidade de ferro para frangos de corte**


Porto Alegre (RS), Brasil

Dezembro de 2023

Julmar da Costa Feijó

**Exigência e disponibilidade de ferro para frangos de corte**

Tese apresentada como um dos requisitos à obtenção do grau de Doutor em Zootecnia na área de concentração em nutrição e metabolismo animal

Porto Alegre (RS), Brasil

Dezembro de 2023

CIP - Catalogação na Publicação

Documento assinado digitalmente
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Submetida como parte dos requisitos

para obtenção do Grau de

**DOUTOR EM ZOOTECNIA**

Programa de Pós-Graduação em Zootecnia

Faculdade de Agronomia

Universidade Federal do Rio Grande do Sul

Porto Alegre (RS), Brasil

Aprovada em: 20.12.2023
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"A maior recompensa para o trabalho não é o que se recebe por ele, mas o que alguém se torna através dele".

John Ruskin

## AGRADECIMENTOS

À Deus, criador de tudo e todas as coisas, por ter guiado-me até aqui.

À minha família, em especial ao meu pai Jorge e minha mãe Carmen, pela formação do meu caráter, pela educação maravilhosa, pelo amor, por todos os incentivos durante a minha jornada, que nunca deixaram de acreditar no meu potencial, que sempre me apoiaram em tudo e pelo orgulho que tem de mim.

Aos meus irmãos, Joilson, Juliana, Carmen e Carlos, pela confiança, amizade, motivação e apoio.

Ao Professor Sergio Luiz Vieira, que me acolheu no Aviário de Ensino e Pesquisa, pelo apoio e confiança, proporcionando-me aprendizado, experiência profissional e todas as oportunidades para ascender intelectualmente.

Aos amigos, colegas e funcionários do Aviário de Ensino e Pesquisa, pela ajuda, parceria nos momentos difíceis. Todos foram importantes durante o doutorado, à todos eu sou muito grato.

À todas as pessoas que contribuíram para que eu chegasse até aqui nesta jornada, por todo o apoio, conselhos, amizade e companheirismo.

Ao Conselho Nacional de Desenvolvimento Ciêntífico e Tecnológico pela concessão da bolsa a nível de Doutorado.

À Universidade Federal do Rio Grande do Sul e aos professores do Programa de Pós-Graduação em Zootecnia pela possibilidade de realização do curso de Doutorado em Zootecnia.

# Exigência e disponibilidade ferro para frangos de corte[1]

Autor: Julmar da Costa Feijó
Orientador: Sergio Luiz Vieira

**RESUMO –** Esta tese foi conduzida para avaliar exigência de Fe em frangos de corte suplementados com fitase, assim como avaliar o uso do calcário e fósfato bicálcico como fonte de ferro. Dois experimentos (Exp. 1 e 2) foram conduzidos utilizando um total de 1856 frangos de corte, machos Cobb 500. No Exp. 1, as aves foram distribuídas em um arranjo fatorial 2 x 5 (suplementação com fitase x 5 suplementações de Fe) em 80 gaiolas, sendo 8 repetições de 8 pintinhos cada. O experimento foi repetido uma vez. Os pintinhos foram alimentados com uma dieta deficiente em Fe sem fitase (Fe analisado = 31,30 ± 3,79 mg/kg) desde o alojamento até o sétimo dia e depois distribuídos aleatoriamente em gaiolas com tratamentos dietéticos correspondentes com ou sem fitase e aumentos graduais de Fe na ração. As rações foram formuladas com milho e farelo de soja, carbonato de cálcio de qualidade laboratorial e ácido fosfórico, sendo a maioria do Fe da dieta proveniente das fontes vegetais (a ração analisada tinha 53,3 ± 1,41 mg/kg de Fe). A fitase foi adicionada em excesso (4.452 ± 487 FTU/kg). Sulfato ferroso hepta-hidratado ($FeSO_4 7H_2O$) foi suplementado para a obtenção dos níveis crescentes e o Fe analisado nas rações foi: 53,3 ± 1,41, 65,5 ± 0,59, 77,2 ± 1,97, 87,6 ± 1,72, 97,7 ± 1,33 mg/kg. No Exp. 2, com 8 dias de idade as aves foram distribuídas em 6 tratamentos em 72 gaiolas, sendo 12 repetições de 8 pintinhos cada no momento do alojamento. As rações foram formuladas com milho, farelo de soja, carbonato de cálcio de qualidade laboratorial e ácido fosfórico (contendo traços de Fe). Os tratamentos tinham aumentos de Fe proveniente de calcário calcítico e fosfato bicálcico (Fe analisado 7.218 e 4.783 mg/kg, respectivamente). O Fe analisado nas rações foi 57,6 ± 2,1, 92,0 ± 2,3, 124,1 ± 2,7, 159,3 ± 3,1, 187,2 ± 3,2, 223,7 ± 3,6 mg/kg, respectivamente). Não foram observadas interações entre a fitase e aumentos de Fe no Exp. 1. O desempenho produtivo dos frangos de corte não foi afetado em ambos os experimentos com os aumentos de Fe. As rações suplementadas com fitase resultaram em melhor desempenho, bem como maior energia digestível ileal e digestibilidade ileal de Fe (P < 0,05) no Exp. 1. No Exp. 2, o aumento do Fe na dieta a partir de calcário e fosfato bicálcico levou a uma redução linear (P < 0,05) na porcentagem de Fe digestível ileal. O aumento do Fe na dieta levou a aumentos lineares na retenção e excreção de Fe, no conteúdo de Fe no fígado no Exp. 1 e 2 (P < 0.05). No Exp. 1, respostas quadráticas (P < 0,05) foram observadas para hemoglobina aos 21 dias, ferritina sérica nos dias 14, 21 e 28, com respostas máximas de 83,3, 104,0, 91,9 e 88,3 mg/kg Fe, respectivamente. Resultados destes experimentos mostraram que a suplementação de fitase melhora a digestibilidade de Fe. O desempenho não foi afetado pelo aumento de Fe na dieta. Os parâmetros sanguíneos são afetados pelo aumento de Fe retido. A taxa de retenção de Fe do calcário e do fosfato bicálcico é baixa, em torno de 1,90%. Frangos alimentados com rações a base de milho e farelo de soja não necessitam de suplementação de Fe em pré-misturas.

Palavras-chave: Frango de corte, ferro, fitase, digestibilidade, desempenho.

# Iron requirement and availability for broiler chicken[2]

Author: Julmar da Costa Feijó
Advisor: Sergio Luiz Vieira

**ABSTRACT -** This thesis was conducted to evaluate the Fe requirement in broiler chickens supplemented with phytase, as well as to assess the use of limestone and dicalcium phosphate as a source of Fe. Two experiments (Exp. 1 and 2) were conducted using a total of 1856 broiler chickens, Cobb 500 males. In Exp. 1, birds were distributed in a 2 x 5 factorial arrangement (phytase supplementation x 5 Fe supplements) in 80 cages, with 8 replicates of 8 chicks each. The experiment was replicated once. Chicks were fed an iron-deficient diet without phytase (analyzed Fe = 31.30 ± 3.79 mg/kg) from housing until the seventh day and then randomly distributed into cages with corresponding dietary treatments with or without phytase and gradual increases of Fe in the feed. Feeds were formulated with corn and soybean meal, laboratory-grade calcium carbonate, and phosphoric acid, with the majority of Fe in the diet originating from plant sources (analyzed diet had 53.3 ± 1.41 mg/kg Fe). Phytase was added in excess (4,452 ± 487 FYT/kg). Fe supplementation was from ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) and analyzed Fe in supplemented diets was: 53.3 ± 1.41, 65.5 ± 0.59, 77.2 ± 1.97, 87.6 ± 1.72, 97.7 ± 1.33 mg/kg. In Exp. 2, birds were distributed into 6 treatments in 72 cages, with 12 replicates of 8 chicks each at the time of housing. Feeds were formulated with corn, soybean meal, laboratory-grade calcium carbonate, and phosphoric acid (containing traces of iron). At 8 days, birds were allocated to dietary treatments. Treatments had increases in Fe from commercial limestone and dicalcium phosphate (analyzed iron 7,218 and 4,783 mg/kg, respectively) progressively replacing calcium carbonate and phosphoric acid (analyzed Fe in diets was 57.6 ± 2.1, 92.0 ± 2.3, 124.1 ± 2.7, 159.3 ± 3.1, 187.2 ± 3.2, 223.7 ± 3.6 mg/kg, respectively). No interactions were observed between phytase and Fe increments in Exp. 1. Live performance of broiler chickens was not affected in both experiments with increases in Fe. Feeds supplemented with phytase showed better live performance, as well as higher ileal digestible energy and digestibility Fe ($P < 0.05$) in Exp. 1. In Exp. 2, increasing Fe in the diet from limestone and dicalcium phosphate led to a linear reduction in the percentage of ileal digestible Fe. Increasing Fe in the diet resulted in linear increases ($P < 0.05$) in Fe retention and excretion, Fe content in the liver in Exp. 1 and 2 ($P < 0.05$). In Exp. 1, quadratic responses ($P < 0.05$) were observed for hemoglobin at 21 days, serum ferritin on days 14, 21, and 28, with maximum responses of 83.3, 104.0, 91.9, and 88.3 mg/kg Fe, respectively. Results from these experiments showed that phytase supplementation improves Fe digestibility. Live performance is not affected by increased Fe in the diet. However, blood parameters are affected by increased retained Fe. The retention rate of Fe from limestone and dicalcium phosphate is low, around 1.90%. Broilers fed corn and soybean meal-based diets do not require Fe supplemental in premixes.

Key words: broiler chickens, iron, phytase, digestibility, performance.

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# RELAÇÃO DE ABREVIATURAS

| | |
|---|---|
| ATP | Adenosina trifosfato |
| BWG | Body weight gain |
| Ca | Cálcio |
| DcytB | Redutase citocromo b duodenal |
| DNA | Ácido desoxirribonucleico |
| DMT | Transportadora de metal divalente |
| Fe | Ferro |
| $Fe^{3+}$ | Íon férrico |
| $Fe^{2+}$ | Íon ferroso |
| $FeSO_4 7H_2O$ | Sulfato ferroso heptahidratado |
| FCR | Feed conversion ratio |
| FI | Feed intake |
| GLM | General lineal model |
| HCP | Proteína carreadora de heme |
| NADH | Dinucleotídeo de adenine nicotinamida |
| $O_2$ | Oxigênio |
| P | Fósforo |

**CAPÍTULO I**

# INTRODUÇÃO

Os minerais são nutrientes importantes para o crescimento e desenvolvimento dos organismos vivos, uma que vez que estão envolvidos em inúmeros processos bioquímicos no corpo (WEYH et al., 2022). O ferro (Fe) é um micromineral essencial envolvido em vários processos metabólicos, sendo o transporte de oxigênio, transporte de elétrons, síntese e reparação do DNA os mais relevantes (THEIL & GOSS, 2009; CARTER, et al., 2022). Esse micromineral é suplementado em rações de frangos de corte com a finalidade de prevenir deficiências que podem interferir no crescimento, assim como também levar a um quadro de anemia (WORWOOD, 1990; MANOR et al., 2017).

O Fe é um dos minerais mais abundantes no mundo (NRC, 1994). É encontrado nos ingredientes das rações nas formas heme e não-heme, sendo a heme exclusiva dos ingredientes de origem animal e não-heme encontrada nos vegetais, porém pode estar complexado com o fitato o que impede o aproveitamento pelo trato gastrointestinal (ALLEN & PEERSON, 2009; AKTER et al., 2015). Outras formas de Fe não-heme estão na forma salina em premixes, assim como em calcários e fosfatos suplementados nas rações, os quais nesses últimos acredita-se contenham Fe férrico ($Fe^{3+}$) e, portanto, de uma disponibilidade potencialmente menor para aves quando comparado ao Fe heme e Fe ferroso ($Fe^{2+}$) (HUNT, 2003; PARK et al., 2004; SUN et al., 2022).

As formas moleculares de como o Fe se apresenta nos ingredientes utilizados em rações de aves, possuem diferentes mecanismos de absorção. A absorção e o transporte do Fe dietético através da mucosa intestinal, dependem do status de Fe no organismo e ocorrem tanto a partir de formas heme quanto a não-heme, chamadas inorgânicas. O Fe heme tem uma via de absorção intestinal preferencial, associada a proteína transportadora de heme 1 (HCP1), presente no enterócito (TAKO & GLAHN, 2011; CONRAD & UMBREIT, 2002). Por outro lado, o Fe inorgânico, nas formas $Fe^{3+}$ e $Fe^{2+}$, possuem mecanismos diferentes de aborção. Entretanto, a entrada de Fe para o interior do enterócito ocorre apenas via $Fe^{2+}$, sendo mediado pelo transportador de metal divalente 1 (DMT1) (OKAZAKI et al., 2012). Todavia, pode ocorrer uma redução de $Fe^{3+}$ para $Fe^{2+}$ mediada pela redutase citocromo b duodenal (DcytB) presente na borda em escova dos enterócitos duodenais, a partir disso permitindo a absorção de $Fe^{2+}$ (CONRAD et al., 2000).

As recomendações comuns para a suplementação de Fe em rações para frangos de corte podem ser encontradas nos manuais de manejo das linhagens, sendo altamente variáveis o que se denota poucos estudos na área. Por exemplo, as recomendações do NRC (1994); Rostagno et al. (2017), Cobb (2018) e Aviagen (2022) são 80, 52,8, 40 e 20 mg/kg Fe, respectivamente.

Milho, farelo de soja, calcário e fosfatos são comumente utilizados nas rações para as aves, sendo assim, as rações acabam tendo a presença de diferentes formas de Fe se avaliarmos apenas esses ingredientes. A presente tese foi conduzida para avaliar as exigências de Fe de frangos de corte, os efeitos da suplementação de fitase sobre a aproveitamento de ferro e energia, assim como a disponibilidade do Fe presente no calcário e fosfato em dietas a base de milho e farelo de soja a partir de respostas oriundas do desempenho vivo das aves, parâmetros sanguíneos e digestibilidade.

# REVISÃO BIBLIOGRÁFICA

## Funções do Ferro

O Fe é um micromineral que está presente em muitas enzimas responsáveis pelo transporte de elétrons, como por exemplo, as citocromos. Esse micromineral está associado as enzimas oxidases e oxigenases, em processos que envolvem a ativação do oxigênio ($O_2$), como também diretamente associado ao transporte de oxigênio através da hemoglobina e mioglobina, sendo o Fe heme presente nessas proteínas (ABBASPOUR et al., 2014; DUTT et al, 2022).

O sistema citocromo consiste em uma série de reações nas quais oxidações ocorrem com a produção de adenosina trifosfato (ATP) e formação de água. O Fe participa de atividades como oxidação, redução e transporte de elétrons, ativando sítios de enzimas óxido redutoras e proteínas ligadas ao oxigênio (WILLIAMS et al., 1976).

Hemoglobina e mioglobina, possuem similaridade em suas estruturas, ambas possuem ferro heme complexado a porfirina, um componente essencial do grupamento heme carreador de oxigênio. Todavia, enquanto a primeira contém quatro grupamentos heme a outra contém apenas um ligado ao $O_2$ (LEESON & SUMMERS, 2001; MARENGO-ROWE, 2006). Em torno de 70% do total de Fe corpóreo é encontrado nessas proteínas, sendo o restante presente na ferritina, como forma de estoque corporal (HAMBIDGE et al., 1986; OBERLEAS et al., 1999).

As porfirinas também são encontradas em hemoproteínas como citocromos, catalases, peroxidases, os quais cumprem funções na formação de ligações moleculares entre o $O_2$ e o grupamento heme, assim como na transferência de elétrons nos citocromos e na clivagem de peróxidos estruturais das reações de catalases e peroxidases (FINZEL et al., 1984; VIDOSSICH et al., 2012).

Os citocromos, de forma geral, são proteínas que mediam as cadeias transportadoras de elétrons das cristas das mitocôndrias em todas as células aeróbicas, são cruciais na fosforilação oxidativa para a produção de ATP (GROTTO, 2008). O citocromo c é uma proteína abundante no músculo cardíaco, ligado à cadeia de globulina, isolada com um grupo heme e um átomo de Fe (YU et al., 1972). O citocromo P-450 é encontrado dentro das membranas dos microssomos nas células hepáticas e da mucosa intestinal, atua na degradação oxidativa (GUENGERICH, 2018). Já as catalases atuam na quebra do peróxido de hidrogênio em água e oxigênio

molecular (HECH et al., 2010).

Existem outras atividades biológicas desempenhadas pelo Fe, como por exemplo, na síntese e reparação do DNA, produção de energia e imunidade. Nas funções fundamentais do metabolismo do DNA, o Fe é essencial em múltiplas enzimas das quais participam da integridade e do transporte de carga do DNA (PUIG et al., 2017). As enzimas necessárias para a síntese e reparo de DNA que abrigam ferro funcionalmente relevante incluem a DNA polimerases, DNA helicases, nucleases, glicosilases e desmetilases, bem como como ribonucleotídeo redutases (ZHANG, 2014).

No metabolismo energético, a aconitase, uma metalproteína que possui Fe em sua estrutura, cumpre função espacial apropriada entre os grupos hidroxilas e carbono, converte citrato em isocitrato no ciclo de Krebs (LUSHCHAK et al., 2014). No mesmo caminho, a desidrogenase succínica que contém Fe heme converte o succinato em fumarato (KIM & WINGE, 2013). Certas enzimas que possuem Fe em suas estruturas como as gliceraldeído-3-fosfato desidrogenases, são enzimas encontradas no citoplasma e na mitocôndria e usam NADH (dinucleotídeo de adenina nicotinamida) como co-enzima e reduz dihidróxiacetona fosfato em L-α-glicerolfosfato, composto necessário para a biossíntese dos triglicerídeos (LAZAREV et al., 2020). As flavoproteínas são enzimas transportadoras de íons, na mitocôndria auxiliam na transdução de energia (HENRIQUES et al., 2021).

**Absorção e metabolismo do ferro**

A absorção do Fe depende, inicialmente, do status de Fe no organismo, de suas formas heme e não-heme, do seu estado oxidativo, podendo ser $Fe^{2+}$ ou $Fe^{3+}$. Três estágios são reconhecidos no mecanismo de absorção de Fe no duodeno e jejuno: passagem pela borda em escova, trânsito ou armazenamento nos enterócitos e liberação no sangue (MACKENZIE & GARRICK, 2005)

Na mucosa o Fe heme e não heme são processados e regulados diferentemente. A absorção do Fe heme é mediada pela HCP1, posicionada na membrana apical das células duodenais, por sua vez, o heme liga-se à membrana da borda em escova dos enterócitos e a HCP1 atravessa intacta a membrana plasmática, importando o heme extracelular (CONRAD & UMBREIT, 2002). No interior da célula, o Fe é liberado da porfirina pela heme oxigenase passando a fazer parte do mesmo pool de Fe não heme, podendo fazer parte da ferritina ou seguir para corrente

sanguínea (SHAYEGHI et al., 2005). A HCP1 também é expressa em outros locais, como o fígado e baço. A regulação é feita de acordo com o nível de Fe intracelular, em caso de deficiência, a HCP1 posiciona-se do citoplasma para a membrana plasmática das células duodenais, em caso de excesso de Fe, o posicionamento ocorre a partir da borda em escova da célula para o seu citoplasma. Por outro lado, a síntese de HCP1 é facilitada na hipóxia (LATUNDE-DADA, 2006). Esse mecanismo regulado permite que o Fe  heme da dieta possa ser aproveitado sem ser eliminado pelo peristaltismo intestinal, assim como evita a captação desnecessária de Fe e o seu provável acúmulo.

A absorção da forma não-heme é regulada, em parte, pelas concentrações intracelulares de Fe nos enterócitos. Íons de $Fe^{3+}$ podem ser reduzidos pela DcytB para $Fe^{2+}$ na borda em escova da membrana duodenal, uma vez convertido em $Fe^{2+}$ pode ser internalizado no enterócito pela DMT1 (LATUNDE-DADA et al., 2008). Uma vez no meio intracelular do enterócito, o Fe pode ser armazenado como ferritina ou transportado para a membrana basolateral por ação da ferroportina. Conrad et al. (2000) em um ensaio in vitro demonstrou que o $Fe^{3+}$ pode ser internalizado na célula via β3-integrina e mobilferrina, porém não esclareceu detalhes sobre a importância desse processo.

Na corrente sanguínea o Fe é conduzido até os tecidos pela transferrina e para isso o mesmo precisa ser oxidado, uma vez que a transferrina tem afinidade pela forma $Fe^{3+}$, por sua vez, essa oxidação é mediada pela hefaestina (SHARP & SRAI, 2007). A transferrina se liga a 2 átomos de $Fe^{3+}$, e é então catalisada por uma ferroxidase que, no sangue, existe sob a forma de uma ou mais proteínas como a ceruloplasmina, ferroxidase I e ferroxidase II. A transferrina libera para o metabolismo, somente um dos dois átomos de $Fe^{3+}$ que, então, se reduz a $Fe^{2+}$ para as reações de formação de mioglobina, hemoglobina, enzimas de heme e para excreção na bile (PONKA et al., 1998).

O Fe é absorvido de acordo com as demandas fisiológicas, sendo afetadas pela idade, pelas reservas corpóreas, suplementação de ácido ascórbico, fontes de Fe (FEATHEESTON et al., 1968; MISKI & KRATZER, 1976; TAKO et al., 2010). A comunicação sistêmica entre as reservas, demandas, locais de absorção e utilização é feita pela hepcidina, hormônio peptídeo circulante (Figura 1) (LUDWICZEK et al., 2004; GALY et al., 2013).
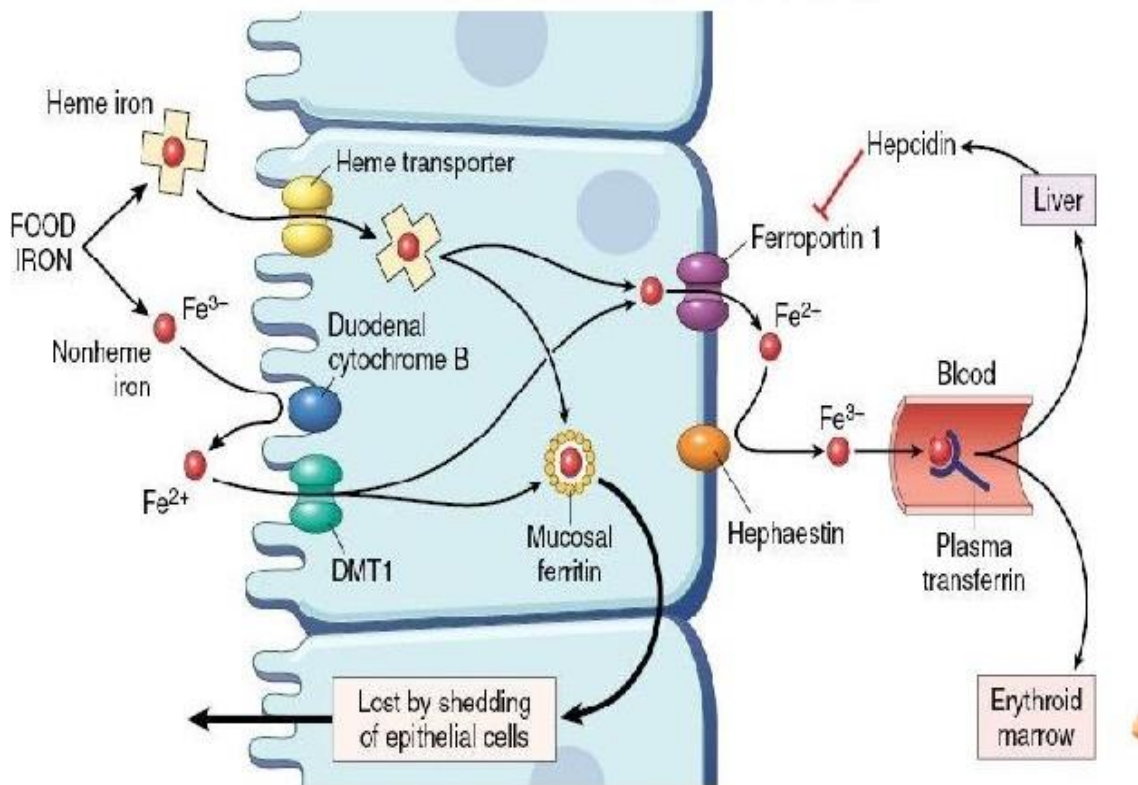
Figura 1. O enterócito, proteínas e sistemas envolvidas na absorção do Fe. Dcytb: Duodenal Cytochrome B/ferroredutase; DMT1: transportador de metal divalente; proteína transportadora de heme; ferroportina; hefaestina; hepcidina; transferrina.

## Distribuição do ferro no organismo

O Fe fica estocado nas células reticuloendoteliais do fígado, baço e medula óssea na forma de ferritina e hemossiderina. A ferritina é uma apoferritina contendo um núcleo férrico, sendo esta a forma solúvel de armazenamento. Deste modo, a ferritina contém e mantém os átomos de ferro que poderiam formar agregados de precipitados tóxicos, sendo principal estrutura responsável pelo armazenamento de Fe no organismo e possui a capacidade de mobilizar rapidamente grandes quantidades do metal quando necessário (WALTERS et al., 1973; THEIL, 2004). A hemossiderina corresponde à forma degradada da ferritina, sendo esta a forma insolúvel de armazenamento. Isto ocorre, quando a quantidade total de ferro no organismo é superior a que pode ser acomodada no reservatório de depósito de ferritina (BONKOVSKY, 1991).

Dois terços do ferro total do corpo estão na forma de hemoglobina. Por este motivo a fagocitose e degradação de hemácias senescentes representam uma fonte importante de ferro (LUTZ & BOGDANOVA, 2013). A quantidade de Fe reciclada é o suficiente para manter a eritropoiese. As hemácias circulam pelo sistema circulatório

por 120 dias, em média, antes de serem destruídas. Embora estas células sejam privadas de núcleos, com exceção das hemácias das aves, possuem uma variedade de enzimas no interior do citoplasma.

O Fe livre é encontrado ainda nas mitocôndrias, com isso esta organela tem papel crucial para o metabolismo do Fe, uma vez que é o único local onde ocorre a síntese do heme e do cluster Fe-S, sendo este último envolvido em processos de transferência de elétrons no processo de respiração celular (WARD & CLOONAN, 2019). O mecanismo da entrada do Fe na mitocôndria ainda não se encontra bem esclarecido, todavia, se sabe que ao ser transportado para o meio intramitocondrial, uma proteína denominada frataxina, regula a utilização do ferro mitocondrial, destinando este à síntese do heme ou a gênese dos clusters Fe-S. A frataxina tem como principal função formar um complexo com o Fe prevenindo a formação de radicais livres na mitocôndria (BENCZE et al., 2006).

A cadeia respiratória mitocondrial, além de estar envolvida no transporte de elétrons, tem como papel fundamental a conversão do ferro férrico em ferroso, única forma química aceita pela ferroquelatase para então catalisar a etapa terminal da biossíntese do heme, a inserção de ferro ferroso na protoporfirina IX para produzir heme (OBI et al., 2022). Após essa etapa, inicia-se o transporte de heme para o citosol, em seguida é incorporado nas proteínas que contém heme (YEN & PERFETTO, 2022). Os clusters Fe-S podem ser transportados para o citosol da célula mediado pelo transportador ABCB7 (PEARSON & COWAN, 2021).

**Fontes de ferro**

A ingesta do Fe se dá pela dieta. As principais fontes de ferro são as proteínas de origem animal, esse micromineral está presente na hemoglobina e mioglobina, as quais possuem o ferro heme. O Fe não-heme é encontrado em vegetais, assim como presentes em óxidos de ferro, sulfatos de ferro, que estão presentes em premixes das rações e em matérias oriundos de rochas, como por exemplo o calcário e fosfatos. Todavia, o Fe na forma não heme tem uma biodisponibilidade menor que o Fe heme e, por isso, é menos absorvido pelo intestino.

Milho e farelo de soja são os principais ingredientes para fabricação de rações, por sua vez, apresentam quantidade variadas de Fe. Milho contém aproximadamente 23,5 a 45 mg/kg de Fe, enquanto que o farelo de soja de 150 a 170 mg/kg de Fe (NRC, 1994; ROSTAGNO et al., 2017). Calcário e fosfato bicálcico, são

usualmente utilizados em rações como fontes de cálcio (Ca) e fósforo (P), todavia ambos possuem altas quantidade de Fe. Calcário possui em torno de 2000 mg/kg de Fe, enquanto que o fosfato bicálcico pode ter uma variação de 20 a 11000 mg/kg (NRC, 1994, LIMA et al., 1995, ROSTAGNO et al., 2017). Outros ingredientes tais como sorgo, quirera de arroz, farelo de arroz e trigo, são boas fontes desse micromineral, contendo cerca de 45 a 59,7, 10 a 15,6, 115.4 a 190, 170 a 205,3 mg/kg de Fe, respectivamente (NRC, 1994; ROSTAGNO et al., 2017). Para os produtos de origem animal, o conteúdo de Fe nas farinhas de carne de ossos varia de 248 a 816 mg/kg, na farinha de sangue varia de 1664 a 2080 mg/kg e na farinha de pena varia de 76 a 568 mg/kg (NRC, 1994; ROSTAGNO et al., 2017).

Outras fonte de Fe a serem utilizados como suplementos em rações avícolas são o Óxido ferroso (FeO), carbonato de ferro ($FeCO_3$), sulfato ferroso monohidratado ($FeSO_4H_2O$), sulfato ferroso heptahidratado ($FeSO_47H_2O$), Óxido ferroso (FeO) que contem em torno de 77.8%, 43%, 30% e 20% de Fe, respectivamente (ROSTAGNO et al., 2017). Existem também as fontes comerciais de Fe, chamadas "orgânicas", como por exemplo, proteinato de ferro, complexo ferro aminoácido, complexo ferro metionina, complexo ferro lisina, gluconatos de ferro, dentre outras fontes com altas biodisponibilidades, uma vez que o Fe está quelatado com uma substância que possui carbono, oxigênio e hidrogênio em sua estrutura (Rostagno et al., 1994; FEDNA, 2018, EBBING et al., 2019).

**Exigência de ferro para frangos de corte**

Os estudos conduzidos para estimar as exigências de Fe para frangos de corte envolveram uma série de avaliações que foram mensurados a partir de parâmetros de desempenho vivo, parâmetros sanguíneos, conteúdo de Fe nos órgãos, rendimentos de carcaça e cortes, assim como coloração da carne, ou seja, as pesquisas levaram em conta o papel do Fe nas funções fisiológicas, seja manutenção ou construção de tecidos. Por sua vez, as investigações conduzidas até hoje deram suporte para elaboração de manuais e tabelas de exigências nutricionais de Fe em frangos de corte.

As exigências são identificadas principalmente por curvas de dose-resposta. Entretanto, devido aos inúmeros fatores que impactam no resultado de experimentos, como por exemplo, critérios utilizados para avaliar os resultados, a composição da dieta e das linhagens utilizadas, pode-se encontrar alguns resultados

de exigência variáveis, mesmo para uma dada espécie e linhagem (SAKOMURA & ROSTAGNO, 2007).

As recomendações de suplementação de Fe para frangos de corte são encontradas nos manuais das linhagens Cobb 500 (Cobb, 2018) e Ross (Aviagen, 2022), essas possuem sugestões de suplementação de 40 e 20 mg/kg de Fe para frangos de corte para todas fases de criação, respectivamente. As tabelas brasileiras para aves e suínos recomendam uma suplementação de Fe inorgânico de 52.8 mg/kg de Fe para frangos de corte de 8 a 21 dias. Por outro lado, o NRC (1994) fornece uma recomendação de 80 mg/kg de Fe a ser suplementado. Ressaltando que os valores de recomendação de suplementação e valores de exigência são diferentes, visto que a suplementação não considera os níveis já presentes nos ingredientes da dieta.

## Fitase

As fitases (mio-inositol hexafosfato fosfohidrolase) são hidrolases capazes de catalisar a hidrólise gradual de mio-inositol hexafosfato (ácido fítico; IP6). As fitases relevantes para a alimentação animal podem ser divididas em 2 subclasses, 3-fitases ou 6-fitases, dependentes de qual fosfato inicia a catálise no núcleo mio-inositol (ADEOLA & COWIESON, 2011).

A fitase é comumente adicionada às dietas de aves para reduzir os efeitos antinutricionais do fitato, liberando quantidades significativas de P do ácido fítico, assim como a liberação de outros minerais e demais nutrientes, consequentemente, melhorando o desempenho dos frangos de corte, contribuindo para menor excreção de P no ambiente (COWIESON et al., 2013; NAVES et al., 2016).

O fitato está presente nos alimentos de origem vegetal, como por exemplo, milho e farelo soja contém 0,19% e 0,34%, respectivamente (ROSTAGNO et al., 2017). Esta molécula não pode ser eficientemente hidrolisada por enzimas endógenas em aves e aproximadamente dois terços do P acabam ficando indisponíveis para a absorção animal, podendo dessa forma alterar o *turnover* das células intestinais e pode causar irritação da mucosa, aumentando a produção de mucinas e, consequentemente, a perda de nitrogênio endógeno (COWIESON et al., 2009).

Existe uma variação no que rege a liberação de P ligado ao fitato em rações a base de milho e farelo de soja, podendo variar de 50 a 100%, isso depende principalmente da dose suplementada (SIMONS et al., 1990; Waldroup et al., 2000;

SOMMERFELD et al., 2019). O uso de fitase tem demonstrado efeito positivo na deposição de Ca e P na tíbia de frangos de corte, principalmente quando se tem redução nos níveis de fósforo disponível (VIEIRA et al., 2015; BROCH et al., 2020).

Recentes investigações demonstraram que a fitase pode melhorar a digestibilidade de microminerais como zinco e cobre, uma vez que esses e outros microminerais podem estar complexados com o fitato (MOSS et al., 2018; SOSTER et al., 2023). A digestibilidade de aminoácidos e energia podem ser melhorados com o uso de fitase em função da liberação das moléculas ligadas ao inositol hexafosfato, assim como também pela redução das perdas endógenas (COWIESON et al., 2006; BASSI et al., 2021).

# HIPÓTESES E OBJETIVOS

## Hipóteses

Níveis crescente de Fe em rações a base de milho e soja para frangos de corte afetam o desempenho, parâmetros sanguíneos, conteúdo de Fe no fígado em frangos de corte.

A suplementação de fitase pode melhorar a digestibilidade e retenção de energia e Fe em frangos de corte.

O Fe oriundo do calcário e fosfato bicálcico está em uma forma de baixa digestibilidade.

## Objetivos

Determinar a exigência de ferro para frangos de corte.

Avaliar os efeitos de níveis de crescentes de Fe em dietas para frangos de corte de 8 a 28 d sobre o desempenho, parâmetros sanguíneos e conteúdo de Fe no fígado.

Avaliar os efeitos da suplementação de fitase sobre a digestibilidade e retenção de energia e Fe em frangos de corte.

Avaliar a interação da suplementação de fitase e Fe em dietas para frangos de corte.

Avaliar a digestibilidade e retenção de Fe oriundo do calcário e fosfato bicálcico em frangos de corte.

# CAPÍTULO II[1]

1   Iron requirements of broiler chickens as affected by supplemental phytase

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19 **Lay summary**

20 Iron is routinely supplemented in broiler feeds to prevent dietary deficiencies. We carried out

21 an experiment with the objective of evaluating the Fe requirements of broilers fed with the

22 exogenous enzyme phytase. From the eighth day, a total of 1,280 male broilers were distributed

23 in a combination of feeds supplemented with phytase or not and 5 graded increases in dietary

24 Fe. Diets were formulated with corn and soybean meal, laboratory grade calcium carbonate

25 and phosphoric acid. Phytase was added in excess (4,452 ± 487 FYT/kg analyzed) to facilitate

26 complete degradation of dietary phytate. Laboratory-grade ferrous sulfate heptahydrate was

27 increasingly added to feeds to provide Fe.  Iron in the experimental diets was present at 53.3 ±

28 1.41, 65.5 ± 0.59, 77.2 ± 1.97, 87.6 ± 1.72, 97.7 ± 1.33 mg/kg). Supplementing diets with

29 phytase resulted in enhanced live performance, along with increased digestibility of ileal

30 energy and Fe. Linear increases in Fe retention and excretion, hepatic Fe contents, and  were

31 observed with the progressive increase in dietary Fe. The supplementation of a total of 97.7

32 mg/kg of Fe in diets was found to have no significant impact on live performance traits.

33 However, the Fe-related blood parameters reached their maximum levels at a dietary Fe level

34 of 91.9 mg/kg. Phytase supplementation provided a significant increase in the digestibility of

35 Fe and other nutrients evaluated.

36

37 **Teaser Text**

38 Nutritionally balanced feeds are those providing adequate amounts of nutrients that

39 simultaneously meet maintenance and production needs. In the context that broiler

40 performance is consistently improved over time, feeding programs and diet formulations are

41 continuously changing. The study clarified Fe requirements of broiler chickens, as well as the

42 improvement of Fe digestibility with the use of phytase.

**Abstract**

Iron is routinely supplemented in broiler feeds intending to prevent dietary deficiencies. The present research was conducted with the objective of assessing Fe requirements of broilers when fed supplemental phytase. A total of 1,280 1-d-old male Cobb x Cobb 500 were distributed in a 2 X 5 factorial arrangement (phytase-supplemented feeds x 5 graded increases of supplemental Fe) in 80 battery cages, eight replications of eight chicks each. The trial was replicated once. Chicks were fed a Fe-deficient diet without phytase (Fe analyzed at $31.30 \pm 3.79$ mg/kg) from placement to 7 d and then randomly distributed into battery cages with corresponding feeding treatments with or without phytase and graded increases of supplemental Fe. Feeds were formulated with corn and soybean meal (SBM), laboratory grade calcium carbonate and phosphoric acid; therefore, the vast majority of dietary Fe originated from corn and SBM (analyzed feed had $53.3 \pm 1.41$ mg/kg Fe). Phytase was added in excess to the producer recommendation of 1,000 FYT ($4,452 \pm 487$ FYT/kg analyzed) such that phytate degradation was expected to be maximized. Supplemental Fe was from laboratory grade ferrous sulfate heptahydrate ($FeSO_47H_20$) which was increasingly added to the feeds (analyzed Fe in the supplemented feeds were: $53.3 \pm 1.41$, $65.5 \pm 0.59$, $77.2 \pm 1.97$, $87.6 \pm 1.72$, $97.7 \pm 1.33$ mg/kg). There were no interactions between phytase and dietary Fe for any response throughout the study (P > 0.05). Supplementing phytase had not effects on Fe intake or Fe excretion, as well as on hematocrit (Ht), hemoglobin (Hb), ferritin, Fe contents in the liver or thigh muscle color (P > 0.05). However, phytase supplemented feeds produced better live performance as well as higher ileal energy and Fe digestibility (P<0.05). No effects were found for dietary Fe in live performance at d 28 (P > 0.05). On the other hand, increasing dietary Fe led to linear increases in Fe retention and excretion, Fe contents in livers, as well as Ht and Hb at 14 d (P < 0.05). Quadratic responses (P < 0.05) were observed for Hb at 21d, serum ferritin on d 14, 21 and 28 (maximum responses were 83.3, 104.0, 91.9 and 88.3 mg/kg

68    Fe, respectively). In conclusion, supplementing Fe adding to a total of 97.7 mg/kg dietary Fe

69    did not affect live performance traits. However, the average of Fe related blood parameters was

70    maximized at 91.9 mg/kg dietary Fe. Supplementing phytase provided a significant increase in

71    Fe digestibility.

72

73    **Key words:** broilers, iron, phytase.

74 **Abbreviations**: AME, apparent metabolizable energy; BWG, body weight gain; CP, crude

75 protein; DM, dry matter; FCR, feed conversion ratio; Fe, iron; FI, feed intake, GE, gross

76 energy; Ht, hematocrit; Hb, hemoglobin; IDCE, Ileal digestible energy coefficient IDE, ileal

77 digestible energy; SMB, soybean meal.

78

79  **Introduction**

80  Iron (Fe) is an essential mineral routinely supplemented in broiler feeds intending to prevent

81  deficiencies that can impair growth and other metabolic processes such as oxygen transport,

82  deoxyribonucleic acid synthesis, and electron transport (Cook et al., 1992; Lieu et al., 2001;

83  Wijayanti et al., 2004; Shinde et al., 2011; Zoroddu et al., 2019). Anemia is the most common

84  outcome of the dietary deficiency of Fe, which can be diagnosed through the assessment of

85  hematocrit, hemoglobin, and ferritin in the blood (Worwood, 1990; Miller, 2013).

86  Iron is widely present in nature, including ingredients used in animal feeding (NRC,

87  1994). Animal by-products have Fe mostly bound into hemoglobin, myoglobin, ferritin,

88  cytochromes, as well as in lower proportions of Fe-containing enzymes (Dale et al., 2002;

89  Theil, 2004; Lozoff et al., 2006, Allen and Peerson, 2009). On the other hand, plant feedstuffs

90  are expected to have Fe widely found in phytate complexes (Maenz, 1999; Hurrell et al., 2003;

91  Cowieson et al., 2006); this varies with the composition of soil, climate, and crop systems

92  originating the plants (Yu et al., 2000; Gupta et al., 2008). Mineral compounds added to broiler

93  feeds, such as limestone and phosphates, have high contents of Fe when compared to all other

94  routinely used major feed ingredients (Park et al., 2004, Ma et al., 2016, Lu et al., 2022).

95  When it comes to its overall availability for poultry, Fe is impacted by its molecular

96  presentation in feed ingredients. For instance, Fe is highly available from animal by-products

97  since heme Fe has a preferential intestinal absorption pathway (Tako and Glahn, 2011). On the

98  other hand, Fe in limestone and phosphates are expected to be of low availability because it is

99  prevalently present in the form of low solubility oxides (Abbaspour et al., 2014; Sun et al.,

100  2022). Availability of Fe from plant feedstuffs is expected to be low because of its

101  complexation in phytates (Lopez et al., 2002; Hunt, 2003; Akter et al., 2017).

102  Absorption and transport of dietary Fe across the intestinal mucosa occurs from both

103  heme as well as inorganic forms (Conrad et al., 2000). However, associated receptors at the

104 enterocyte surface differ with heme transporter protein 1 (HCP1) being responsible for heme

105 Fe absorption (Conrad and Umbreit, 2002; Shayeghi et al., 2005) whereas the divalent metal

106 carrier 1 (DMT1) for inorganic Fe (Mackenzie and Garrick, 2005; Mckie, 2008).

107 Corn and SBM are the main feedstuffs utilized in poultry feeds, which have 0.19% and

108 0.34% estimated contents of phytic acid in corn and SBM, respectively (Rostagno et al., 2017).

109 The addition of supplemental phytase in broiler feeds is now economically mandatory because

110 it releases P from phytate at comparative lower costs when compared to other P sources

111 (Ravindran et al., 2000; Rutherfurd et al., 2012; Cowieson et al., 2013; Naves et al., 2016, Walk

112 and Rama Rao, 2020). Phytases are also expected to increase the availability of Fe, however,

113 the literature in this matter is limited (Akter et al., 2015).

114 Common recommendations for Fe supplementation in broiler feeds are highly variable,

115 denoting a lack of supporting research. For instance, recommendations from the NRC (1994),

116 Rostagno et al. (2017), Cobb (2018) and Aviagen (2022) are 80, 52.8, 40, and 20 mg/kg Fe,

117 respectively. Poultry excreta in general have high contents of Fe, which increases as diets with

118 excessive levels of Fe are consumed (Bao et al., 2007; Nollet et al., 2007; Nollet et al., 2008).

119 Contaminating impacts of excessive contents of trace minerals in animal feeds has led to the

120 establishment of upper limits for total Fe in feeds of 450 ppm in the European Union (EFSA,

121 2016).

122 The objective of the present study was to evaluate Fe requirements of broiler chickens

123 using Fe sulfate, a traditional source. Phytase was added in excess in the feeds, such that the

124 assessment of Fe released from phytate could be detected under its extensive degradation.

125 Evaluations were conducted in live performance as well as expanded to other metabolic

126 responses sensitive to dietary Fe.

127

128 **Material and Methods**

129    All procedures used in the present study were approved by the Ethics and Research

130    Committee of the Federal University of Rio Grande do Sul, Porto Alegre.

131

132    **Bird Husbandry and Dietary Treatments**

133    A total of 1,280 one-day-old male Cobb x Cobb 500 chicks were randomly placed into

134    80 wire cages ($0.9 \times 0.4$ m$^2$). Each cage was equipped with one trough feeder and one drinker,

135    which allowed *ad libitum* access to water and mash feeds. Temperature at placement was 32ºC,

136    which was adjusted weekly to maintain bird comfort throughout the study. Lighting was

137    provided 24 h continuously throughout the first week and then run in a 16 light and 8 dark

138    schedule. All cages were daily checked for sick and dead birds with dead bird body weight

139    being registered as observed.

140    Birds were given a common Fe-deficient diet (analyzed total $31.3 \pm 3.79$ mg/kg Fe)

141    produced with corn, polished white rice and isolated soy protein from 1 to 7 d. Starting at 8 d,

142    birds were fed a corn-SBM diet not supplemented with Fe ($53.3 \pm 1.41$ mg/kg analyzed) to 28

143    d (Table 1). Birds were randomly allocated into treatments using a 2 X 5 factorial arrangement

144    of feeds with or without phytase or having 5 graded increases of supplemental Fe. The trial

145    was replicated once, with 8 replicates per treatment each time; therefore, there were a total 10

146    treatments with 16 replications of 8 birds.

147    Dietary treatments were formulated with energy and nutrients to optimize live

148    performance as usual in commercial integrations. Each feeding treatment was manufactured

149    once, mixed in batches of 400 kg, stored at -20 ºC and provided as mash in both study

150    replications. The phytase utilized in the present study was a commercially available product

151    added at 100 g per ton (Ronozyme HiPhorius, 40,000 FYT/g, Novozymes A/S, Bagsvaerd,

152    Denmark), to deliver 4,000 FYT/kg ($4,452 \pm 487$ analyzed). Phytase was added in excess of its

153    commercial recommendation (1,000 FYT), such that the vast majority of phytate present in

154 corn and SBM was overwhelmingly degraded. Phytase was added into the feeds without

155 attributing value for P and Ca to avoid any confounding effects on overall performance.

156 Supplementation of Fe was from laboratory grade Fe sulfate heptahydrate ($FeSO_4 7H_2O$) at 0,

157 10, 20, 30 and 40 mg/kg (Sigma Aldrich, St. Louis, MO). Calcium carbonate and phosphoric

158 acid were also laboratory grade and had no significant Fe content. Analyzed phytase in the

159 feeds, from the lowest to the highest dietary Fe contents were $4{,}463 \pm 227$; $4{,}083 \pm 118$; $4{,}493$

160 $\pm 761$; $4{,}777 \pm 691$; $4{,}448 \pm 387$ FYT/kg. Analyzed Fe in the feeds with and without phytase

161 were $53.3 \pm 1.41$, $65.5 \pm 0.59$, $77.2 \pm 1.97$, $87.6 \pm 1.72$, $97.7 \pm 1.33$ mg/kg. All feeds were

162 added with 1% indigestible marker (Celite, Celite Corp., Lompoc, CA) and had an average

163 geometric diameter of $1.118 \, \mu m \pm 1.72$. Phytase and supplemental Fe were included in the

164 feeds in 1 kg mixes diluted with SBM. Phytase activity in feeds was analyzed as done by

165 Engelen et al. (1994) and expressed in phytase units (FYT, defined as the activity that releases

166 one $\mu$mol of inorganic phosphate from 5.0 mM sodium phytate/min at pH 5.5 and 37 ºC).

167 Analyses of Fe using the atomic absorption spectrophotometric method of Association of

168 Official Analytical Chemists (method 968.08; AOAC, 2016).

169

170 **Growth Performance, Total excreta, Ileal Contents**

171 Body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) corrected

172 for the weight of dead birds were evaluated at 8, 14, 21, and 28 d. Excreta were collected twice

173 daily on wax paper from 21 to 24 d being immediately mixed and pooled by cage and stored at

174 -20 ºC until analysis. Ileal contents were collected from all birds at 28 d after euthanasia by

175 electrical stunning using 45 V for 3 s from a section of intestine between Meckel's diverticulum

176 to approximately 2 cm cranial to the ileo-cecal junction. Contents were flushed with distilled

177 water into plastic containers, pooled by cage, immediately frozen in liquid nitrogen, and stored

178 in a freezer at -20 ºC until lyophilized (Christ Alpha 2-4 LD Freeze Dryer, Newtown, UK).

179    Diet and freeze-dried samples of ileal digesta were ground to pass a 0.5-mm screen in a grinder

180    (Tecnal, TE-631/2, São Paulo, Brazil).

181

182    **Analysis and calculations**

183         Dry matter (DM) analysis of samples was performed after oven drying the samples at

184    105 °C for 16 h (method 934.01; AOAC International, 2006). Ileal digesta, excreta, and feed

185    samples were analyzed for gross energy (GE) using a calorimeter calibrated with benzoic acid

186    as a standard (IKA Werke, Parr Instruments, Staufen, Germany). Calculations of ileal

187    digestible energy (IDE) and apparent metabolizable energy (AME) were done afterwards.

188    Crude protein (N x 6.25) was determined by the combustion method (method 968.06; AOAC

189    International, 2006). Phytate in feed samples was determined by the method described by Latta

190    and Eskin (1980) with Inductively Coupled Plasma - Atomic Emission Spectrometry (ICP-

191    AES). Acid insoluble ash, ileum samples and excreta were determined as described by

192    Vogtmann et al. (1975) and Choct and Annison (1992). Apparent ileal digestibility, total tract

193    retention, IDE and AME were calculated using the following equations (Kong and Adeola,

194    2014): digestibility (%) = $[1 - (M_i/M_o) \times (E_o/E_i)] \times 100$, IDE and AME (kcal/kg) = $GE_i - [GE$

195    $\times (M_i/M_o)]$, where $M_i$ represents the concentration of acid insoluble ash in the diet in grams per

196    kilogram of DM; $M_o$ represents the concentration of acid insoluble ash in the excreta and ileal

197    digesta in grams per kilogram of DM output; $E_i$ represents the concentration of DM, PB, GE,

198    Fe in the diet in milligrams per kilogram of DM; and $E_o$ represents the concentration of DM,

199    PB, GE, Fe in the excreta and ileal digesta in milligrams per kilogram of DM. Retention of Fe

200    was determined (Zhang et al., 2018) as follows: Fe retention (mg/bird) = [feed intake (g/bird)

201    x feed Fe content (mg/kg)] – [fecal output (g/bird) x fecal Fe content (mg/kg)]. The feed and

202    fecal Fe contents were based on their analyzed values on dry matter.

203

204 **Blood and Liver collection**

205    Blood sampling was drawn from 20 birds at 7 days for the assessment of hematological

206 parameters. Blood samples were taken through heart punctures collection from 2 broilers

207 randomly selected from each treatment on d 14, 21 and 28. Blood obtained was partially

208 transferred to 0.5mL test tubes containing EDTA for hematocrit (Ht) and hemoglobin (Hb)

209 analyzes. Determination of Ht was done using micro capillaries containing blood centrifuged

210 for 5 min at 15,650 to 18,510 x g. Concentration of Hb was determined using the

211 cyanmethemoglobin method as described by Crosby et al. (1954). Serum from centrifuged

212 blood (3 mL) was used for analysis of ferritin, which was done using an enzyme-linked

213 immunosorbent assay (ELISA) kit (Quimica Basica Ltda, Minas Gerais, Brazil) as described

214 by Andrews et al. (1994). Dilutions from 0 to 80% were previously prepared using human and

215 broiler chicken sera to check if linearity of responses existed (Table 2). Linearity was

216 determined by taking an average of 20 serum samples treated with dissociation reagent and

217 diluted as described in Table 2 with assay buffer and mixing them in the proportions indicated.

218 The measured concentrations were compared to expected values based on the ratios used.

219 Human sera were obtained from the Clinical Pathology Laboratory from Pontifical Catholic

220 University of Rio Grande do Sul from Brazil (PUCRS). Samples were anonymous and

221 previously used to evaluate ferritin content.

222    Livers were collected from five birds per cage after euthanasia by neck dislocation. All

223 collected samples were weighed and stored in plastic bags by cage at -20°C until analysis.

224 Livers were later submitted to ethyl ether extraction following previous acid hydrolysis with

225 hydrochloric acid (method 920.39, AOAC International, 1995). Samples were further ashed

226 and Fe content was determined as done with the feeds.

227

228 **Thigh collection and color evaluation**

229       Thigh muscles were collected immediately after euthanasia, had feathers manually

230 removed and were then subjected to a color assessment using the CIE (Commission

231 Internacionale de L'Eclairage) color values using the CIELAB trichromatic system as

232 luminosity (L*), redness (a*) and yellowing (b*) using a chromometer (HunterLab Labscan;

233 HunterLab, Virginia, USA). Evaluation of thigh color was done at three random locations on

234 its surface.

235

236 **Statistical Analysis**

237       Data were tested for homoscedasticity and normality of the variance prior to statistical

238 analyses (Shapiro and Wilk, 1965). Data were analyzed using the GLM procedure of SAS

239 Institute (SAS, 2011) with significance accepted as $P \leq 0.05$. Mean separation was done using

240 Tukey multiple-range test when the model effect was significant (Tukey, 1991).

241       Estimations of maximum responses to total dietary Fe were done using linear (L) and

242 quadratic polynomial (QP) regression models. The L model ($Y = \beta_1 + \beta_2 \times X$) has Y as the

243 dependent variable, X as the dietary level of Fe, $\beta_1$ as the intercept, and $\beta_2$ as the linear

244 coefficient. The QP model ($Y = \beta_1 + \beta_2 \times Fe + \beta_3 \times (Fe)^2$) has Y as the dependent variable as

245 a function of dietary level of Fe; $\beta_1$ as the intercept; $\beta_2$ as the linear coefficient and $\beta_3$ as the

246 quadratic coefficient. The maximum response for Fe was defined as $Fe = -\beta_2 \div (2 \times \beta_3)$.

247

248 **Results**

249       Analyzed Fe in the experimental feeds were close to the expected from feed formulation

250 (Table 1); therefore, feeds were considered acceptable for the experimental assessment

251 originally planned. Analyses of variance and regressions were conducted with the Fe analyzed

252 data.

253      No interactions between dietary Fe and phytase were found for any evaluated response

254    throughout the study ($P > 0.05$). The factorial analyses showed no effects of dietary Fe in live

255    performance (Table 3), ileal digestibility, total tract retention of DM and AME (Table 4), Ht

256    and Hb at d 28 (Table 5), or thigh muscle color (Table 6) ($P > 0.05$). However, the increased

257    supplementation of Fe led to higher retention, intake, and excretion of the mineral (Table 4),

258    as well as higher Ht at 14 and 21 d, Hb at 14 and 21 d, serum ferritin at 14, 21 and 28 d (Table

259    5), and Fe contents in the liver (Table 6) ($P < 0.05$). Regression analyses showed that increased

260    dietary Fe correlated linearly with increased Fe excretion and retention as well as liver Fe

261    content, Ht and Hb at d 14 and Ht at d 21. Regression equations were as follow: Fe excreted =

262    - 0.47612 + 0.4823x, $R^2 = 0.9117$, $P<0.001$; Fe retained = 0.4078 + 0.0696x, $R^2 = 0.7915$,

263    $P<0.001$; Ht at 14 = 23.3716 + 0.0635x, $R^2 = 0.2112$, $P<0.001$; Hb at d 14 = 7.3992 + 0.0238x,

264    $R^2 = 0.1204$, $P<0.001$; Ht at d 21 = 27.8750 + 0.0341x, $R^2 = 0.2228$, $P<0.001$. Increasing Fe

265    led to quadratic increases on Hb at d 21, serum ferritin at d 14, 21 and 28. Dietary Fe that

266    maximized Hb at 21 was 83.3 mg/kg whereas serum ferritin was maximum at 104.0, 91.9 and

267    88.3 mg/kg at d 14, 21 and 28, respectively. These responses were quadratically correlated to

268    the increasing dietary Fe as follow: Hb at 21 = 2.531354039 + 0.182135074x – 0.001086281$x^2$,

269    $R^2 = 0.4191$, $P<0.001$; serum ferritin at d 14 = 61.12394689 + 1.59480140x - 0.00818646$x^2$,

270    $R^2 = 0.7830$, $P<0.001$; serum ferritin at 21 = 41.70580102 + 2.52267152x – 0.01372836$x^2$, $R^2$

271    = 0.8107, $P<0.001$; serum ferritin at d 28 = 32.76252977 + 2.98391206x – 0.01690114$x^2$, $R^2$

272    = 0,3704, $P \leq 0.003$.

273      The test performed to validate the detection of chicken ferritin using a human ferritin

274    assay showed a linear relationship with chicken serum ferritin averaging 71.5% of human

275    values. Tests of linearity used in the experiment were based on the validation model described

276    by Bienboire-Frosini et al. (2017).

277

278    **Discussion**

279        Feeds in the present study were formulated with corn and SBM to have nutrients and

280    energy comparable to those used commercially as usual in broiler integrations, except for Fe.

281    Limestone and phosphates were replaced by laboratory grade calcium carbonate and

282    phosphoric acid having only 9.2 and 2.8 ppm Fe, respectively. Mineral premixes had no Fe;

283    therefore, any relevant change of Fe contents in feeds were obtained from corn and SBM or

284    from the Fe sulfate in supplemented feeds.

285        Results from the present research demonstrated that Fe from corn and SBM allowed to

286    maximize broiler growth from 7 to 28 d and, therefore, supplementation as generally suggested

287    is not necessary when these parameters are the only ones considered. Presently, trace mineral

288    supplementation ranges from as low as 20 mg/kg (Aviagen, 2022) to as high as 80 mg/kg (NRC,

289    1994). Other recommendations are intermediary: 52.8 mg/kg (Rostagno et al., 2017) and 40

290    mg/kg (Cobb, 2018). As demonstrated in the present study, all those recommendations seem

291    excessive since they do not result in growth improvements when birds are fed corn-SBM diets.

292    The contribution of Fe in non-supplemented broiler feeds derive from plant sources, which are

293    mostly corn and SBM, but also from limestone and phosphates. Phytate is expected to complex

294    with Fe in plant sources (Maenz, 1999; Bohn et al., 2008), which may be rendered available

295    for absorption when phytase is added to the feeds. On the other hand, total Fe present in

296    limestone is variable, ranging from 100 to 185 ppm Fe (Yang et al., 2011; Bai et al., 2021).

297    Availability of Fe from limestone and phosphates for poultry is unknown.

298        In the present study, Ht, Hb and serum ferritin were determined after birds were fed a

299    low Fe common feed at 7 d. Values found in the present study were $25.8 \pm 1.2\%$, $8.3 \pm 0.6$

300    g/dL and $113 \pm 6.4$ ng/mL, which are in a close range to those for one-day-old chicks

301    determined by Morita et al. (2009). It has also been previously reported that Ht and Hb

302    increased when birds were fed Fe supplemented feeds after a period of Fe deficiency (Taschetto

303    et al., 2017; Ebbing et al., 2019). Deaton et al. (1969) have observed that Ht and Hb are low in

304    the first week of age, increasing from the second week onwards with broilers. In the present

305    study, dietary Fe fed from 7 d to 21 d affected Ht and Hb; however, ferritin was affected

306    throughout the entire study. These are traditional parameters used to assess Fe status in different

307    species (Abbaspour et al., 2014, Ma et al., 2014; Manor et al., 2017). However, Ht and Hb did

308    not differ from each other at 28 d in the present study, which may have been due to the

309    accumulation of body Fe. Lin et al. (2020) found no difference for Ht between treatments when

310    supplementing 50 to 150 mg/kg Fe in poultry feeds. Similarly, the supplementation of Fe to 50

311    mg/kg did not show changes in Hb at 21 days (Aoyagi and Baker, 1995). Others have reported

312    that Ht and Hb did not differ, tending to stabilize when broilers were given increased dietary

313    Fe after 21 d (Liao et al., 2017; Abdel-Rahman et al., 2022).

314         Ferritin is a main intracellular Fe reservoir protein that maintains Fe in an accessible and

315    readily mobilized form (Mackenzie et al., 2008; Orino and Watanabe, 2008). Ferritin is

316    composed of an inner Fe subunit and an outer apoferritin subunit, which protects and stabilizes

317    the Fe core, preventing excessive Fe release and its toxic effects (Harrison and Arosio, 1996;

318    Torti and Torti, 2002). Serum ferritin is the main blood parameter when serum Fe status is

319    clinically evaluated (Wish, 2006; Knovich et al., 2009).

320         In the present study, serum ferritin was analyzed using a human serum ferritin assay after

321    the observation of parallel responses of dilutions of serum from both species. Genes of chicken

322    ferritin exhibit approximately 85% nucleotide identity in coding regions, which yield proteins

323    with a similarity of 93% of amino acid sequence to human ferritin (Stevens et al., 1987). Bai

324    et al. (2021) used a human serum assay to detect chicken serum ferritin with birds fed diets

325    having 139 and 609 mg/kg Fe and obtained 110.4 and 326.2 ng/mL of ferritin, respectively.

326    These authors concluded that the increased values signaled an adequate sensitivity of the

327    ferritin to dietary Fe. Analyzes of ferritin in poultry were also conducted in the liver or in the

328 ferritin to total protein ratio and all resulted in an increase in ferritin when birds were given

329 increased dietary Fe (Abdel-Rahman et al., 2022, Hu et al., 2022). Therefore, it can be

330 concluded that results found in this study are in line with research done in other species, such

331 as rats and humans after being submitted to increases in dietary Fe (Patterson et al., 2001; Yun

332 et al., 2011; Wang et al., 2014; Kaluza and Madej, 2015; Xiao et al., 2016).

333      The increase in Fe excretion, as well as in Fe body retention, occurred in parallel with

334 dietary Fe content in the present study. This have been previously reported by other authors

335 (Bao et al., 2007, Nollet et al., 2007, Faria et al., 2020). The frequent use of chicken excreta as

336 soil amendment certainly contributes to a constant increase in Fe in areas where this practice

337 is a routine. It is obviously expected that the linear excretion of Fe accompanied by its dietary

338 content is an indicator of excess when adequate growth and serum status is taken in

339 consideration. In parallel, liver Fe content on day 28 was increased similarly as Fe retained.

340 Previous studies have reported increased Fe in liver along with its increases in feeds and it can

341 build up in the liver, just like other minerals. (Vahl and Van`T Klooster, 1987; Cao et al., 1996;

342 Ma et al., 2016; Akter et al., 2017; Han et al., 2022). Excess dietary Fe, on the other hand, does

343 not seem to increase the risk of toxicity (Spears, 1999; Bai et al., 2021).

344      Addition of exogenous phytase in poultry feeds is commercially mandatory nowadays

345 since they increase P availability from plant feedstuffs, reducing feed costs with a

346 corresponding reduction in the use of phosphates (Bougouin et al., 2014; Walters et al., 2019).

347 Secondary benefits, such as the increased availability of Ca, protein, amino acids and other

348 minerals have also been reported when birds are fed phytase (Woyengo and Nyachoti, 2010;

349 Cowieson et al., 2017; Sommerfeld et al., 2018; Ajuwon, et al., 2020; Song et al., 2021).

350      In the present study broilers fed phytase showed higher BWG and lower FCR when

351 compared to those that were not fed the enzyme. Phytase improvements occurred regardless of

352 Fe content in the feeds. The contents of non-phytate P (nPP) and total Ca in the feeds in the

353  present study were as usual in commercial feeds and similar to commercially used

354  recommendations (0.45% nPP and 1.00 % Ca from the NRC, 1994; 0.43% nPP and 0.91% Ca

355  from Rostagno et al., 2017; 0.42% nPP and 0.84% Ca from Cobb, 2018; 0.42% nPP and 75%

356  Ca from Aviagen, 2022). Since all essential trace minerals, other than Fe, were supplemented

357  in the experimental feeds, benefits of phytase on further availability of Fe could be assessed.

358  Several reports have shown improvements in live performance in broilers resulting from

359  phytase added in feeds at higher levels than those needed to maximize P availability (Pirgozliev

360  et al., 2008; Cowieson et al., 2014; Muszy and Tomaszewska, 2017; Broch et al., 2018; Gautier

361  et al., 2018). Improvements in ileal digestibility responses (DM, IDEC, IDE and CP) as well

362  as in total tract retention (DM and AME) observed in the present study have also been reported

363  by many authors (Gehring et al., 2013; Wu et al., 2015; Farhadi et al., 2017; Lee et al., 2017;

364  Pieniazek et al., 2017; Truong et al., 2017; Leyva-Jimenez et al., 2019; Woyengo and Wilson,

365  2019; Bassi et al, 2021).

366       The increased retention and ileal digestibility of Fe obtained when birds were fed phytase

367  compared to those that were not feed the enzyme are in agreement with *in vitro* data from Akter

368  et al. (2015) as well as with results of studies conducted with rats, humans and pigs (Pallauf et

369  al., 1999; Hurrell et al., 2003, She et al., 2018).

370

371  **Conclusions**

372       Phytase supplementation promotes improvements in broiler growth, which were not

373  related to increases in availability of P and Ca. The effects of phytase on Fe digestibility were

374  evident from the present results. Increases in Fe supplementation to levels that exceed the total

375  supply of this mineral in commercial feeds did not lead to benefits in live performance;

376  however, blood parameters were positively affected. Excess Fe in the diet leads to an increase

377  in its content in the liver, as well as in excreta, which eventually leads to an increased disposal

378     of this mineral in the environment.

379

380     **Acknowledgments**

381     

383

384     **Conflict of interest**

385     All authors declare that they have no conflict of interest.

386

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737 **Table 1.** Ingredient and nutrient composition of with or without phytase and with graded
738 increases of supplemental Fe.

| Item | Fe-deficient | Basal Feed |
|---|---|---|
| | Pre-starter (1 to 7 d) | Starter (8 to 28 d) |
| Ingredient, %[1] | | |
| Rice, polished and broken | 51.35 | - |
| Corn 7.8 | 12.26 | 50.58 |
| Soybean meal 46 | 13.00 | 38.61 |
| Soy protein isolate 89 | 15.16 | - |
| Soybean oil | 0.50 | 4.51 |
| Calcium carbonate | 2.18 | 2.55 |
| Phosphoric acid | 1.18 | 1.42 |
| Common salt | 0.11 | 0.47 |
| DL-Methionine, 99% | 0.36 | 0.32 |
| L-Lysine HCl, 76% | 0.13 | 0.17 |
| L-Threonine, 98.5% | 0.10 | 0.08 |
| Choline chloride | 0.16 | 0.06 |
| Vitamin and mineral mix[2] | 0.23 | 0.23 |
| Kaolim (diluent) | 3.28 | - |
| Celite (indigestible marker) | - | 1.00 |
| Total | 100.00 | 100.00 |
| Energy and nutrients, % or as noted[3] | | |
| $AME_n$, kcal/kg | 2,950 | 3,000 |
| Crude Protein | 22.9 (22.9 ± 0.52) | 21.9 (21.2 ± 0.62) |
| Ca | 1.10 (1.07 ± 0.02) | 1.05 (1.02 ± 0.03) |
| Available P | 0.50 | 0.48 |
| Total P | 0.69 (0.68 ± 0.02) | 0.67 (0.67 ± 0.03) |
| Phytate | - | 0.23 (0.22 ± 0.01) |
| Na | 0.22 | 0.20 |
| Choline, mg/kg | 1,600 | 1,600 |
| Dig. Lys | 1.28 | 1.22 |
| Dig. TSAA | 0.99 | 0.91 |
| Dig. Thr | 0.84 | 0.79 |
| Dig. Trp | 0.24 | 0.24 |
| Dig. Arg | 1.47 | 1.37 |
| Dig. Val | 0.96 | 0.93 |
| Dig. Ile | 0.84 | 0.86 |
| Fe, mg/kg[4] | 28.68 (31.30 ± 3.79) | 56.6 (53.33 ± 1.41) |
| Phytase, FYT/kg[5] | - | 4,000 (4,452 ± 487) |

739 [1] Calcium carbonate and phosphoric acid were laboratory grade and had only trace amounts of Fe (9.2 and 2.8
740 ppm, respectively).
741 [2] Composition per kilogram of feed: vitamin A, 12,000 IU; vitamin $D_3$, 3,000 IU; 60C, 50 mg; vitamin E, 100 IU;
742 vitamin $K_3$, 6 mg; vitamin B12, 35 µg; thiamin, 3 mg; riboflavin, 15 mg; vitamin B6, 6 mg; niacin, 40 mg;
743 pantothenic acid, 25 mg; folic acid, 4 mg; biotin, 0.3 mg, Zn (zinc), 100 ppm; Mn (manganese), 100 ppm; Cu
744 (copper), 15 ppm, Se (selenium), 0.45 mg and I (iodine), 2 mg.
745 [3] Analyzed values are within parentheses.
746 [4] Formulated Fe were 56.6, 66.6, 76.6, 86.6, 96.6 mg/kg; analyzed Fe in the feeds with and without phytase were
747 53.33 ± 1.41, 65.45 ± 0.59, 77.19 ± 1.97, 87.57 ± 1.72, 97.65 ± 1.33 mg/kg.
748 [5] Ronozyme HiPhorius 40,000 FYT/g, Novozymes A/S, Bagsvaerd, Denmark.

749 **Table 2**. Relationship between serum ferritin in chickens and humans after dilution at the same proportions of the respective sera[1].

| Dilution, %[2] | Ferritin, ng/mL | | | Chicken to human ferritin ratio | % Recovery (chicken)[3] |
| | Human | Observed chicken | Expected chicken | | Observed to Expected |
|---|---|---|---|---|---|
| 0 | 240.5 ± 31.6 | 173.9 ± 20.1 | 173.9 | 0.72 | 100.0 |
| 20 | 192.1 ± 25.3 | 135.6 ± 15.5 | 139.2 | 0.71 | 97.4 |
| 40 | 144.0 ± 18.9 | 103.4 ± 14.3 | 104.4 | 0.72 | 99.0 |
| 60 | 95.9 ± 12.6 | 67.5 ± 9.5 | 69.6 | 0.70 | 97.0 |
| 80 | 48.0 ± 6.3 | 34.8 ± 4.7 | 34.8 | 0.72 | 100.0 |

750 [1]Human sera ferritin associated with diluted human sera: $Y= 240.2900000 – 2.4051000x$, $R^2 = 0.0952$; $P = 0.0018$; broiler chicken sera ferritin associated with diluted broiler
751 sera: $Y= 172.300000 – 1.7317500x$, $R^2 = 0.9252$; $P \leq 0.001$.
752 [2]Ferritin dilutions from human and chicken sera were performed with assay buffer enzyme-linked immunosorbent assay (ELISA) kit (Quimica Basica Ltda., Minas Gerais,
753 Brazil).
754 [3]Linearity of observed and expected sera ferritin values in broiler chickens ($Y= 1.0037 + 0.9581$, $R^2 = 0.99$), determined with 20 dissociation reagent-treated samples diluted
755 with assay buffer and mixed as recommended.

756 **Table 3**. Growth performance of broilers as affected by feeds with or without phytase and with graded increases of supplemental Fe[1].

| Item | 8 to 14 d | | | 15 to 21 d | | | 22 to 28 d | | | 8 to 28 d | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BWG, g | FCR | FI, g | BWG, g | FCR | FI, g | BWG, g | FCR | FI, g | BWG, g | FCR | FI, g |
| Phytase, FYT/Kg[2] | | | | | | | | | | | | |
| 0 | 260[b] | 1.160[a] | 303 | 473 | 1.343[a] | 636 | 559[b] | 1.442[a] | 805 | 1,293[b] | 1.347[a] | 1,742 |
| 4,000 | 270[a] | 1.122[b] | 302 | 483 | 1.315[b] | 633 | 578[a] | 1.388[b] | 801 | 1,330[a] | 1.307[b] | 1,737 |
| Fe, mg/kg[3] | | | | | | | | | | | | |
| 56.6 | 266 | 1.136 | 303 | 477 | 1.313 | 627 | 572 | 1.412 | 812 | 1,315 | 1.324 | 1,741 |
| 66.6 | 266 | 1.139 | 303 | 481 | 1.312 | 630 | 565 | 1.415 | 799 | 1,312 | 1.318 | 1,729 |
| 76.6 | 264 | 1.150 | 304 | 476 | 1.324 | 629 | 568 | 1.442 | 818 | 1,310 | 1.338 | 1,751 |
| 86.6 | 265 | 1.143 | 303 | 476 | 1.358 | 645 | 568 | 1.390 | 789 | 1,309 | 1.328 | 1,737 |
| 96.6 | 264 | 1.136 | 299 | 480 | 1.338 | 641 | 567 | 1.408 | 797 | 1,311 | 1.326 | 1,738 |
| SEM | 1.222 | 0.004 | 1.570 | 2.579 | 0.006 | 3.654 | 2.988 | 0.006 | 4.509 | 4.121 | 0.004 | 6.137 |
| *Probability <* | | | | | | | | | | | | |
| Phytase | <0.0001 | <0.0001 | 0.6797 | 0.0464 | 0.0260 | 0.7692 | 0.0012 | <0.0001 | 0.7147 | <0.0001 | <0.0001 | 0.6740 |
| Fe | 0.9466 | 0.8293 | 0.9075 | 0.9563 | 0.2881 | 0.4307 | 0.9671 | 0.1024 | 0.2628 | 0.9821 | 0.4562 | 0.8596 |
| Phytase X Fe | 0.7673 | 0.3092 | 0.3600 | 0.6503 | 0.7270 | 0.2791 | 0.7555 | 0.7096 | 0.4897 | 0.7850 | 0.4869 | 0.4617 |

757 [a>b] Means with different letters in the same column indicate significant differences ($P \leq 0.05$).

758 [1]BWG = body weight gain; FCR = feed conversion ratio corrected for the weight of dead birds; FI = feed intake.

759 [2]Ronozyme HiPhorius 40,000 FYT/g, Novozymes A/S, Bagsvaerd, Denmark; average analyzed phytase in the Fe supplemented feeds was $4,452 \pm 487$ FYT/kg.

760 [3]Analyzed Fe in the feeds with and without phytase were $53.33 \pm 1.41$, $65.45 \pm 0.59$, $77.19 \pm 1.97$, $87.57 \pm 1.72$, $97.65 \pm 1.33$ mg/kg.

761 **Table 4**. Apparent ileal digestibility and total tract retention responses of broilers as affected by increased dietary Fe with or without phytase, on
762 dry matter (DM) basis[1].

| Item | Apparent ileal digestibility | | | | | Total tract retention | | | | Intake | Excretion |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | DM, % | IDEC, % | IDE, kcal/kg | CP, % | Fe, % | DM, % | AME, kcal/kg | Fe, % | Fe, mg/bird | Fe, mg/bird | |
| Phytase, FYT/Kg[2] | | | | | | | | | | | |
| 0 | 67.2[b] | 71.8[b] | 3,235[b] | 77.6[b] | 10.6[b] | 68.5[b] | 3,326[b] | 13.1[b] | 5.5[b] | 42.1 | 36.6 |
| 4000 | 69.8[a] | 73.5[a] | 3,311[a] | 80.1[a] | 11.8[a] | 71.2[a] | 3,393[a] | 14.2[a] | 6.0[a] | 42.0 | 36.0 |
| Fe, mg/kg[3] | | | | | | | | | | | |
| 56.6 | 68.7 | 73.1 | 3,290 | 79.2 | 11.6 | 69.6 | 3,356 | 14.1 | 4.1[e] | 29.1[e] | 25.0[e] |
| 66.6 | 68.7 | 72.7 | 3,267 | 78.4 | 11.2 | 70.4 | 3,362 | 13.8 | 4.9[d] | 35.6[d] | 30.7[d] |
| 76.6 | 68.4 | 72.3 | 3,264 | 78.4 | 11.4 | 69.4 | 3,339 | 13.5 | 5.9[c] | 43.9[c] | 38.0[c] |
| 86.6 | 68.8 | 72.6 | 3,270 | 79.0 | 11.1 | 70.0 | 3,368 | 13.4 | 6.4[b] | 48.1[b] | 41.7[b] |
| 96.6 | 67.9 | 72.6 | 3,271 | 79.2 | 10.8 | 69.7 | 3,372 | 13.5 | 7.2[a] | 53.3[a] | 46.1[a] |
| SEM | 0.300 | 0.266 | 11.888 | 0.233 | 0.145 | 0.254 | 7.229 | 0.125 | 0.138 | 1.017 | 0.891 |
| *Probability <* | | | | | | | | | | | |
| Phytase | <0.0001 | 0.0015 | 0.0019 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | 0.0002 | 0.8651 | 0.2927 |
| Fe | 0.8310 | 0.9148 | 0.9629 | 0.4427 | 0.4071 | 0.5724 | 0.5069 | 0.2093 | <0.0001 | <0.0001 | <0.0001 |
| Phytase X Fe | 0.3960 | 0.7714 | 0.8789 | 0.0852 | 0.8506 | 0.3429 | 0.5062 | 0.5305 | 0.4160 | 0.1957 | 0.3270 |

763 [a>b] Means with different letters in the same column indicate significant differences (P < 0.05).
764 [1]DM = Dry matter; IDCE = Ileal digestible energy coefficient; IDE = Ileal digestible energy; CP = Crude protein.
765 [2]Ronozyme HiPhorius 40,000 FYT/g, Novozymes A/S, Bagsvaerd, Denmark; analyzed phytase in the Fe supplemented feeds were (from the lowest to the highest Fe content
766 feeds) 4,452 ± 487 FYT/kg.
767 [3]Analyzed Fe in the feeds with and without phytase were 53.33 ± 1.41, 65.45 ± 0.59, 77.19 ± 1.97, 87.57 ± 1.72, 97.65 ± 1.33 mg/kg.
768 [4]Retention, intake, excretion of Fe (mg/kg), respectively: $Y = 0.4078 + 0.0696x$, $R^2 = 0.7915$, P<0.001; $Y = -0.0683 + 0.5519x$, $R^2 = 0.9155$, P<0.001; $Y = -0.47612 + 0.4823x$,
769 $R^2 = 0.9117$, P<0.001; X=dietary Fe content.

770 **Table 5**. Blood parameters of broilers as affected by increased dietary Fe with or without phytase[1].

| Item | Ht, % | | | Hb, g/dL | | | Serum ferritin, ng/mL | | |
|---|---|---|---|---|---|---|---|---|---|
| | d 14 | d 21 | d 28 | d 14 | d 21 | d 28 | d 14 | d 21 | d 28 |
| Phytase, FYT/Kg[2] | | | | | | | | | |
| 0 | 28.2 | 30.6 | 31.6 | 9.3 | 9.8 | 9.9 | 133 | 151 | 156 |
| 4000 | 28.2 | 30.4 | 31.7 | 9.1 | 9.9 | 10.1 | 133 | 151 | 160 |
| Fe, mg/kg[3] | | | | | | | | | |
| 56.6 | 26.9$^b$ | 29.4$^b$ | 31.5 | 8.5$^b$ | 9.1$^b$ | 10.1 | 124$^d$ | 137$^c$ | 142$^b$ |
| 66.6 | 26.9$^b$ | 30.5$^a$ | 31.1 | 9.1$^{ab}$ | 10.0$^a$ | 10.1 | 129$^c$ | 148$^b$ | 161$^a$ |
| 76.6 | 28.6$^{ab}$ | 30.4$^a$ | 32.4 | 9.4$^{ab}$ | 10.0$^a$ | 10.2 | 135$^b$ | 156$^a$ | 158$^a$ |
| 86.6 | 29.3$^a$ | 30.9$^a$ | 31.8 | 9.5$^a$ | 10.1$^a$ | 10.0 | 139$^a$ | 156$^a$ | 165$^a$ |
| 96.6 | 29.2$^a$ | 31.1$^a$ | 31.5 | 9.5$^a$ | 10.1$^a$ | 9.9 | 139$^a$ | 158$^a$ | 164$^a$ |
| SEM | 0.243 | 0.126 | 0.2705 | 0.121 | 0.063 | 10.1 | 0.704 | 0.952 | 1.413 |
| *Probability* < | | | | | | | | | |
| Phytase | 0.9536 | 0.3733 | 0.9200 | 0.5395 | 0.7435 | 0.2314 | 0.3932 | 0.9766 | 0.0675 |
| Fe | 0.0002 | <0.0001 | 0.5815 | 0.0160 | <0.0001 | 0.4046 | <0.0001 | <0.0001 | <0.0001 |
| Phytase X Fe | 0.1253 | 0.5886 | 0.2039 | 0.1684 | 0.9689 | 0.6000 | 0.9173 | 0.9732 | 0.7689 |

771 $^{a>b}$ Means with different letters in the same column indicate significant differences ($P < 0.05$).

772 [1] birds (20 samples) showed at 7 day the following values for hematocrit (Ht), hemoglobin (Hb) and ferritin, $25.8 \pm 1.2\%$, $8.3 \pm 0.6$ g/dL and $113 \pm 6.4$ ng/mL, respectively.

773 [2] Ronozyme HiPhorius 40,000 FYT/g, Novozymes A/S, Bagsvaerd, Denmark; analyzed phytase in the Fe supplemented feeds were (from the lowest to the highest Fe content
774 feeds) $4,452 \pm 487$ FYT/kg.

775 [3] Analyzed Fe in the feeds with and without phytase were $53.33 \pm 1.41$, $65.45 \pm 0.59$, $77.19 \pm 1.97$, $87.57 \pm 1.72$, $97.65 \pm 1.33$ mg/kg.

776 [4] Ht at d 14 and 21, Hb at d 14 and 21, and ferritin at d 14, 21 and 28, respectively: $Y = 23.3716 + 0.0635x$, $R^2 = 0.2112$, $P<0.001$; $Y = 27.8750 + 0.0341x$, $R^2 = 0.2228$, $P<0.001$;
777 $Y = 7.3992 + 0.0238x$, $R^2 = 0.1204$, $P<0.001$; $Y = 2.531354039 + 0.182135074x - 0.001086281x^2$, $R^2 = 0.4191$, $P<0.001$; $Y = 61.12394689 + 1.59480140x - 0.00818646x^2$,
778 $R^2 = 0.7830$, $P<0.001$; $Y = 41.70580102 + 2.52267152x - 0.01372836x^2$, $R^2 = 0.8107$, $P<0.001$; $Y = 32.76252977 + 2.98391206x - 0.01690114x^2$, $R2 = 0,3704$, $P\leq0.003$.
779 X=dietary Fe content.

**Table 6**. Fresh liver Fe concentration and thigh muscle color of broilers as affected by increased dietary Fe with or without phytase at 28 d.

| Item | Fresh Liver, mg/kg[4] | L*[1] | a* | b* |
|---|---|---|---|---|
| Phytase, FYT/Kg[2] | | | | |
| 0 | 125.4 | 60.2 | 16.3 | 6.7 |
| 4000 | 125.3 | 60.5 | 16.0 | 6.2 |
| Fe, mg/kg[3] | | | | |
| 56.6 | 111.5$^d$ | 59.9 | 16.1 | 6.3 |
| 66.6 | 120.1$^c$ | 60.2 | 16.5 | 6.5 |
| 76.6 | 126.8$^b$ | 60.5 | 16.5 | 6.4 |
| 86.6 | 132.1$^a$ | 60.6 | 16.1 | 6.7 |
| 96.6 | 136.3$^a$ | 60.6 | 15.8 | 6.4 |
| SEM | 1.089 | 0.188 | 0.108 | 0.181 |
| *Probability <* | | | | |
| Phytase | 0.9382 | 0.3998 | 0.1551 | 0.0596 |
| Fe | <0.0001 | 0.7310 | 0.1917 | 0.9180 |
| Phytase X Fe | 0.9227 | 0.2131 | 0.5811 | 0.2321b |

$^{a>b}$ Means with different letters in the same column indicate significant differences (P ≤ 0.05). [1]Lightness (L*) range from 100 (white) to 0 (black), whereas positive a* values are measures of redness and negative a* values are measures of greenness; positive b* values are measures of yellowness and negative b* values are measures of blueness.

[2]Ronozyme HiPhorius 40,000 FYT/g, Novozymes A/S, Bagsvaerd, Denmark; analyzed phytase in the Fe supplemented feeds were (from the lowest to the highest Fe content feeds) 4,452 ± 487 FYT/kg.

[3]Analyzed Fe in the feeds with and without phytase were 53.33 ± 1.41, 65.45 ± 0.59, 77.19 ± 1.97, 87.57 ± 1.72, 97.65 ± 1.33 mg/kg.

[4]Fe in Fresh liver at 28 d: Y = 104.6332 + 0.2719x, $R^2$ = 0.1938, P < 0.001.

**CAPÍTULO III**[1]

1  Dietary contribution of iron from limestone and dicalcium phosphate to broiler chickens

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5

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19

20 ABSTRACT

21 Limestone and phosphates are very rich in iron (Fe); however, its contribution from these

22 sources have not been thoroughly investigated in chickens. The present research was conducted

23 to evaluate growth performance and blood parameters of broilers when using limestone and

24 dicalcium phosphate as sources of Fe. A total of 576 one-day-old male Cobb x Cobb 500 were

25 allocated into 72 battery cages, with 6 treatments and 12 replicates of 8 chicks at placement.

26 Chicks were fed diets formulated with corn, soybean meal (SBM), laboratory grade calcium

27 carbonate and phosphoric acid (having traces of Fe). All chicks were fed a common pre-starter

28 without Fe supplementation (analyzed total $58.2 \pm 2.4$ mg/kg Fe) from placement to 7 d.

29 Allocation of birds to dietary treatments was completely randomized on d 8. Treatments had

30 increasing Fe derived from commercial limestone and dicalcium phosphate (analyzed Fe 7,218

31 and 4,783 mg/kg, respectively) progressively replacing calcium carbonate and phosphoric acid

32 to provide graded increases in total Fe (analyzed Fe in the feeds were $57.6 \pm 2.1$, $92.0 \pm 2.3$,

33 $124.1 \pm 2.7$, $159.3 \pm 3.1$, $187.2 \pm 3.2$, $223.7 \pm 3.6$ mg/kg, respectively). There were no effects

34 for dietary Fe on growth performance, hematocrit, and hemoglobin at the end of the study on

35 day 28 ($P > 0.05$). Increasing dietary Fe from commercial limestone and dicalcium phosphate

36 led to a linear reduction in the percent ileal digestibility of Fe. However, linear increments in

37 Fe retention, serum ferritin and liver Fe occurred when compared to feeds without Fe derived

38 from limestone and phosphate dicalcium. It is concluded that Fe from limestone and dicalcium

39 phosphate can be partially utilized by broiler chickens. It was estimated that the Fe retained

40 from limestone and dicalcium phosphate is of 1.9%. Broilers fed corn-soy feeds (58.2 mg/kg

41 Fe) do not require supplemental Fe.

42

43 **Key words:** broiler, iron, performance.

**INTRODUCTION**

44

45      Iron (Fe) is an essential mineral that is routinely supplemented in broiler feeds intending

46 to prevent dietary deficiencies that can affect commercial performance. The essentiality of Fe

47 for animals is mainly related to the synthesis of hemoglobin (Zoroddu et al., 2019); therefore,

48 anemia is the most notable signal of Fe deficiency in humans and animals (Worwood, 1990).

49 Minor dietary quantities of Fe are needed because of its participation in the electron transport

50 chain, deoxyribonucleic acid repair and synthesis, found in cytochromes, Fe-containing

51 enzymes and ferritin (Zhang, 2014; Puig et al., 2017).

52      Absorption of Fe is directly related to the form it is present in feeds with heme Fe being

53 preferentially absorbed. For instance, in humans, heme Fe is absorbed at a rate of 25.0%

54 (Roughead and Hunt, 2000) compared to non-heme Fe at 7.0% (Roughead et al., 2002).

55 Absorption of Fe occurs mainly in the duodenum and proximal jejunum (Zhang, 2010);

56 however, the efficiency of this process is dependent on the oxidative state of Fe at the brush

57 border (Sharp and Srai, 2007). At physiological pH, Fe is oxidized, and therefore in the ferric

58 ($Fe^{3+}$) state; however, absorption of non-heme Fe occurs only as in ferrous state ($Fe^{2+}$) (Han et

59 al., 1995) in a process mediated by the divalent metal cation transporter 1 (DMT1) (Conrad

60 and Umbreit, 2002). Some reduction of $Fe^{3+}$ to $Fe^{2+}$ occurs by duodenal cytochrome B (Dcytb)

61 present at the luminal side of duodenal enterocytes (Conrad et al., 2000), which then allows for

62 the further absorption of $Fe^{2+}$ by the DMT1 system. Feeds provided to poultry are frequently

63 voided of animal proteins and, therefore, without heme Fe, and therefore with a reduced rate

64 of Fe absorption.

65      The wide presence of Fe in nature contrasts with its variability in terms of availability for

66 animals. Heme Fe is the predominant form of Fe in animal protein feedstuffs, as part of

67 hemoglobin and myoglobin (Wijayanti et al., 2004). On the other hand, Fe in plant feedstuffs

68 is highly complexed with phytate (Cowieson et al., 2006), and, therefore, of reduced

69  availability in diets without phytase (Feijó et al., 2023). High contents of Fe can be found in

70  feedstuffs originated from rocks, such as limestone and phosphates (NRC, 1994; Lima et al.,

71  1995, Rostagno et al., 2017). These are believed to bear Fe mostly in the $Fe^{3+}$ state, and

72  therefore of a potentially lower availability for poultry when compared to heme Fe and $Fe^{2+}$

73  (Park et al., 2004). Supplementation of Fe, such as in Fe sulfate, is usually recommended in

74  commercial broiler feeds (Rostagno et al., 2017; Cobb, 2018; Aviagen, 2022).

75      Feijó et al. (2023), have recently shown that broilers fed corn-soy feeds with phytase did

76  not require Fe supplementation in order to optimize bird live performance. Concerns with

77  excessive usage of trace minerals in animal feeds has led to regulations that limit total Fe in

78  feeds. For instance, an upper limit of 450 mg/kg has been established for by the European

79  Union (EFSA, 2016). Since Fe is widely spread in nature, this value may be difficult to manage

80  due to the high contents of Fe in limestone and phosphates, which are routinely included in

81  animal feeds to provide Ca and P. The objective of the present research was to investigate Fe

82  availability from a commercial type of feed for broilers without animal protein having the bulk

83  of dietary Fe from limestone and dicalcium phosphate. The responses evaluated in this

84  investigation included growth performance, but also variables that are highly sensitive

85  indicators of Fe organic status.

86                          **MATERIAL AND METHODS**

87      All procedures used in the present study were approved by the Ethics and Research

88  Committee of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil.

89

90  ***Bird Husbandry and Dietary Treatments***

91      A total of 576 one-day-old male Cobb x Cobb 500 (body weight = 46.2 ± 0.6 g) chicks

92  were randomly placed into 72 wire cages ($0.9 \times 0.4$ m$^2$). Each cage was equipped with one

93  trough feeder and one drinker, which allowed *ad libitum* access to water and mash feeds.

94  Temperature at placement was 32ºC, which was adjusted weekly to maintain bird comfort

95  throughout the study. Lighting was provided 24 h continuously throughout the first week and

96  then run in a 16 light : 8 dark schedule. All cages were daily checked, and the body weight of

97  dead birds being registered as observed.

98      Feeds utilized in this study were formulated with corn and soybean meal (SBM), which

99  were previously analyzed by NIRS (proximate and amino acids), as well as limestone,

100  dicalcium phosphate, and laboratory grade Ca carbonate and phosphoric acid (Table 1).

101  Calcium, P, and Fe in corn, SBM, limestone, dicalcium phosphate, calcium carbonate and

102  phosphoric acid were analyzed by Inductive Coupled Plasma Atomic Emission Spectroscopy

103  (ICP-Spectro Flamme, Spectro Analytical Instruments, Kleve, Germany) (Anderson, 1999).

104      Chicks were fed a common pre-starter feed expected to be Fe deficient ($58.2 \pm 2.4$ mg/kg

105  Fe) from placement to 7 d. Starting at 8 d, birds were randomly allocated into treatments having

106  6 graded increases of formulated Fe (55.5, 88.5, 121.5, 154.5, 187.5, 220.5 mg/kg Fe) obtained

107  by proportionally exchanging laboratory grade Ca carbonate and phosphoric acid, without any

108  significant Fe contents by commercially available limestone and dicalcium phosphate.

109  Treatments were replicated 12 times using 8 chicks per cage on day 8. Feed formulation had

110  energy and nutrients targeting to optimize live performance as usual in commercial integrations

111  (2,950 kcal/kg AME and 23% CP in the pre-starter and 3,000 kcal/kg AME and 22% CP in the

112  starter). All nutrients were balanced throughout the feeds to meet the usual recommendations,

113  except for Fe (Table 1). Each feeding treatment was manufactured in one 400 kg batch and

114  then stored at -20 ºC until use.

115      Analyzed Fe contents in feeds were $57.6 \pm 2.1$, $92.0 \pm 2.3$, $124.1 \pm 2.7$, $159.3 \pm 3.1$, 187.2

116  $\pm 3.2$, $223.7 \pm 3.6$ mg/kg, respectively. All feeds were added with 1% indigestible marker

117  (Celite, Celite Corp., Lompoc, CA) and had an average geometric diameter of 1.169 μm $\pm$ 1.58.

118  The oxidation state of Fe in limestone and dicalcium phosphate was analyzed with a

119  conventional Mossbauer spectrometer working in the transmission geometry and using a drive

120  with a triangular reference signal at constant acceleration. The magnetic properties of the

121  samples were studied using a PPMS-Physical Property Measurement System, Model 6000

122  (Quantum Design, San Diego, CA), equipped with a superconducting magnet as described by

123  Beltrán et al. (2015). Drinking water had no significant Fe content.

124

125  *Growth Performance, Total excreta, Ileal Contents*

126  Body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) corrected

127  for the weight of dead birds were evaluated at 8, 14, 21, and 28 d. Excreta were collected twice

128  daily on wax paper from 24 to 26 d being immediately mixed and pooled by cage and stored at

129  -20 ºC until analysis. Ileal contents were collected from all birds at 28 d after euthanasia by

130  electrical stunning using 45 V for 3 s from a section of intestine between Meckel's diverticulum

131  to approximately 2 cm cranial to the ileo-cecal junction. Contents were flushed with distilled

132  water into plastic containers, pooled by cage, immediately frozen in liquid nitrogen, and stored

133  in a freezer at -20ºC until lyophilized (Christ Alpha 2-4 LD Freeze Dryer, Newtown, UK). Diet

134  and freeze-dried samples of ileal digesta were ground to pass a 0.5-mm screen in a grinder

135  (Tecnal, TE-631/2, São Paulo, Brazil).

136

137  *Analyses and Calculations*

138  Ileal digesta, excreta, as well as feed samples were analyzed for dry matter (DM) at 105

139  °C for 16 h (method 934.01; AOAC International, 2006) and for Fe. Acid insoluble ash contents

140  in diets and ileum samples were determined using the method described by Vogtmann et al.

141  (1975), and Choct and Annison (1992). Apparent ileal digestibility of Fe was calculated using

142  the equations from Kong and Adeola (2014). Total Fe retention was determined as described

143  by Feijo et al. (2023) by the difference of the total intake and the total excretion of Fe from

144 days 24 to 26 of age. In parallel, the retention of Fe originated only from limestone and

145 dicalcium phosphate was estimated by using the differences between intake and excretion of

146 after correcting Fe contents of the treatment without limestone and dicalcium phosphate to

147 zero.

148

149 *Blood and Liver Collection*

150      Blood sampling was obtained from 20 birds on day 7 after euthanasia by neck dislocation

151 for an initial assessment of hematological parameters. Samples were later collected by heart

152 puncturing from one bird randomly selected from each replication on day 28. Blood obtained

153 was partially transferred to 5 mL test tubes containing EDTA for hematocrit (Ht) and

154 hemoglobin (Hb) analyzes. Determination of Ht was done using micro capillaries containing

155 blood centrifuged for 5 min at 15,650 to 18,510 x g. Concentration of Hb was determined using

156 the cyanmethemoglobin method as described by Crosby et al. (1954). Serum from centrifuged

157 blood (3 mL) was used for analysis of ferritin, which was done using an enzyme-linked

158 immunosorbent assay (ELISA) kit (Quimica Basica Ltda, Minas Gerais, Brazil) as described

159 by Andrews et al. (1994) and previously done by Feijo et al. (2023).

160      Livers were collected from five birds per cage after euthanasia by neck dislocation on

161 day 28. All collected samples were weighed and stored in plastic bags by cage at -20°C until

162 analysis. Livers were later submitted to ethyl ether extraction following previous acid

163 hydrolysis with hydrochloric acid (method 920.39, AOAC International, 1995). Samples were

164 further ashed and Fe content determined as done with the feeds.

165

166 *Statistical Analysis*

167      Data were tested for homoscedasticity and normality of the variance prior to statistical

168 analyses (Shapiro and Wilk, 1965). Data were analyzed using the GLM procedure of SAS

169 Institute (SAS, 2009) with significance accepted as P ≤ 0.05 using analyzed Fe. Mean

170 separation was done using Tukey multiple-range test when the model effect was significant

171 (Tukey, 1991). All responses to total dietary Fe were tested using linear (L) and quadratic

172 polynomial (QP) regression models. Regressions were done using the total dietary Fe, but also

173 the Fe originated only from the increments of limestone and dicalcium phosphate such that

174 differences in Fe utilization by birds could be assessed.

175

176                                         **RESULTS**

177         The formulated depletion feed provided from placement to 7 d and the feeds provided in

178 the experimental period from 8 to 28 d are presented in Table 1. Analyzed Fe in laboratory

179 grade Ca carbonate and phosphoric acid as well as commercially available limestone and

180 dicalcium phosphate were 9.2, 2.8, 7,218 and 4,783 mg/kg, respectively. Analyzed Fe in

181 experimental feeds were close to the expected from feed formulations (Fe formulated were

182 55.5, 88.5, 121.5, 154.5, 187.5, 220.5 mg/kg Fe whereas analyzed Fe were 57.6 ± 2.1, 92.0 ±

183 2.3, 124.1 ± 2.7, 159.3 ± 3.1, 187.2 ± 3.2, 223.7 ± 3.6 mg/kg). Therefore, feeds were considered

184 acceptable for the experimental assessment originally planned. Analyses of variance and

185 regressions were conducted with the Fe analyzed data. The oxidation state of Fe in the

186 commercial limestone and dicalcium phosphate utilized in the present study indicated that,

187 from the total Fe present, limestone had 62% $Fe^{2+}$ and 38% $Fe^{3+}$ whereas dicalcium phosphate

188 had 100% $Fe^{3+}$. Blood analyses performed on day 7 produced the following values for Ht, Hb

189 and ferritin: 26.2 ± 1.6%, 8.8 ± 0.5 g/dL and 121 ± 5.2 ng/mL, respectively.

190         There were no effects of dietary Fe on live performance (Table 2) (P > 0.05). Increased

191 dietary Fe from the gradual inclusion of commercial limestone and dicalcium phosphate led to

192 a reduction in the percent ileal digestible and retained Fe (Figure 1). Adjustments for percent

193 ileal digestible Fe was linear (Y = 13.0788 − 0.0461x, $R^2$ = 0,8140, P < 0.001). Linear

194 adjustments were also found for the total intake, excretion, and retention of Fe per bird ($Y = -$

195 $4.8589 + 0.5474x$, $R^2 = 0.9717$, P<0.001; $Y = - 0.8489 + 0.5573x$, $R^2 = 0.9701$, $P < 0.001$; Y

196 $= 4.0101 + 0.0099x$, $R^2 = 0.2142$, $P < 0.001$, respectively). These responses are presented on

197 Table 3. On the other hand, it was found linear adjustment for Fe retained from d 26 to 28

198 relative to that Fe from only from limestone and dicalcium phosphate ($Y = 0.2408 + 0.0099x$,

199 $R^2 = 0.2240$, $P < 0.001$). Linear adjustment also provided the best statistical adjustment for

200 actual retention rate when the Fe retained relative to that consumed was only from limestone

201 and dicalcium phosphate ($Y = 0.1974 + 0.0190x$, $R^2 = 0.2625$, $P < 0.001$) (Figure 2).

202       Hematocrit and Hb at 28 days were not affected by dietary Fe; however, serum ferritin

203 and liver Fe contents increased as dietary Fe had increments ($Y = 142.8394 + 0.0702x$, $R^2 =$

204 $0.3699$, $P < 0.001$; $Y = 110.5263 + 0.0752x$, $R^2 = 0.6390$, $P < 0.001$, respectively) (Table 4).

205 Linear regressions were obtained with ferritin and liver Fe content as a function of the Fe sole

206 originated from limestone and dicalcium phosphate ($Y= 1.3810 + 0.0702x$, $R^2= 46.19$, P <

207 $0.001$; $Y = 0.2735 + 0.752x$ $R^2 = 70.22$, $P < 0.001$) (Figure 3).

208

209                     **DISCUSSION**

210       Feeds used in this experiment were formulated with corn and SBM and contained

211 nutrients and energy as usual in broiler integrations. The mineral premixes had all essential

212 trace minerals except for Fe. Therefore, any relevant changes in Fe content in the experimental

213 feeds originated from the gradual replacement of Ca carbonate and phosphoric acid by

214 commercial limestone and dicalcium phosphate.

215       Live performance results obtained in the present study showed that diets with the lowest

216 Fe content (57.6 mg/kg feed) had no differences from those having increases in Fe to the

217 highest content of 223.7 mg/kg. This can be compared to recent results from Feijo et al. (2023)

218 and indicates that recommendations for Fe supplementation, as suggested by Rostagno et al.

219    (2017) of 52.8 mg/kg, from Cobb (2018) of 40 mg/kg, and Aviagen (2022) of 20 mg/kg, are

220    not needed.

221        Limestone and phosphates are sedimentary rocks and are expected to contain Fe in

222    similar oxidation states (Lerman et al., 1967). Analyses performed in the limestone and

223    dicalcium phosphate in present study, however, showed that the original Fe oxidation states in

224    limestone were 62% as $Fe^{2+}$ and 38% as $Fe^{3+}$, whereas Fe in dicalcium phosphate were 100%

225    $Fe^{3+}$. Limestone utilized in animal feeds is obtained by mining and is minimally processed prior

226    to marketing (Despotou et al., 2016), whereas dicalcium phosphate is produced through

227    reacting mixtures of phosphoric acid and limestone (Lima et al., 1995). In the present study,

228    limestone and dicalcium phosphate were evaluated altogether as a source of Fe because of their

229    similar chemical origin as well as because, in practical terms, they are the usual sources used

230    to supplement Ca and P in broiler diets.

231        Non-heme Fe is absorbed by the DMT1 system which carries only $Fe^{2+}$ (Andrews et

232    al., 2002). On the other hand, the actual oxidative state of Fe is dependent on the availability

233    of oxygen in its surrounding environment. Therefore, the existing pH after feed intake is

234    determinant in affecting the oxidation state when Fe is at the intestinal brush border. After feed

235    entrance into the gastrointestinal tract, it is quickly presented into environments having very

236    low pH in the gizzard-pro ventriculus, remaining in contact with hydrochloric acid from 30 to

237    60 minutes (Van der Klis et al., 1990; Dänicke et al., 1999). Because the digesta exiting the pro

238    ventriculus is highly acidic, a soluble $Fe^{2+}$ is expected to be dominant. However, Fe absorption

239    occurs at the upper portion of small intestine (Sharp and Srai, 2007) where an immediate

240    buffering in the duodenal lumen brings the pH close to neutrality (Singh et al., 2014). This

241    quick transition to duodenal neutrality would maintain the $Fe^{3+}$ state (McKie et al., 2001). The

242    existing duodenal cytochrome B (Dcytb) system at the brush-border of duodenal enterocytes

243    can reduce $Fe^{3+}$, however, at lower rates when compared to $Fe^{2+}$ (Frazer and Anderson, 2001).

244    It is widely known that the overall absorption of Fe is low throughout the domestic

245    animal species (Bao et al., 2007; Nollet et al., 2008; Faria et al., 2020). Absorption is the sole

246    mechanism in healthy animals by which Fe stores can be regulated since there is no

247    physiological means for its excretion. A reduction in the percentage of ileal digestible Fe in the

248    present study was observed as limestone and dicalcium phosphate were gradually included in

249    the diets. The utilization of Fe from feedstuffs may be better understood when retention, instead

250    of digestibility, is considered since the stores of body Fe control the rate at which Fe is taken

251    from the feed. Even the more favorable forms of utilization, as in heme or $Fe^{2+}$, have reduced

252    rates of absorption in humans when the system is saturated (Ludwiczek et al., 2004). The body

253    balance of Fe is done through the communication among the sites of absorption, utilization and

254    storage, and this communication is mediated by hepcidin (Pigeon et al., 2001). In the present

255    study, and increased net retention of Fe per bird was observed as dietary Fe increased.

256    However, the rate of retention was very low either when related to the total Fe consumed (1,0%,

257    Figure 1) as when it was related to the increase in Fe originated exclusively from limestone

258    and dicalcium phosphate (1.9%, Figure 2). To the authors knowledge, the utilization of Fe from

259    limestone and phosphates have never been demonstrated or, as well, have had a proposed

260    utilization by poultry that would reduce the need for supplemental Fe in premixes.

261    In the present study, there were no differences for Ht and Hb at 28 days. Feijó et al.

262    (2023) observed that broilers fed diets supplemented with Fe sulfate had Ht and Hb increasing,

263    regardless of the dietary content until d 21, then stabilizing afterwards to 28 d. Absorbed Fe is

264    minimally lost in healthy birds, and, therefore, Fe accumulated to day 21 may have reached the

265    maximum capacity to produce red blood cells. Several reports found no difference for Ht and

266    Hb between diets with different Fe contents exceeding 50 mg/kg Fe, which, therefore, seems

267    to be an indication of an adequate dietary level for poultry (Liao et al., 2017; Lin et al., 2020;

268    Abdel-Rahman et al., 2022).

269        Ferritin is a non-toxic Fe storage complex that allows Fe to be available when needed by

270        the organism and it is also the main parameter for evaluating Fe status in humans (Orino and

271        Watanabe, 2008; Miller, 2013). In the present research, serum ferritin increased as limestone

272        and dicalcium phosphate was added in the diets. In the present research, 223.7 mg/kg of dietary

273        Fe led to 158 ng/ml ferritin, which was linearly translated into an increase in ferritin of 7.0

274        ng/ml for every 100 mg/kg Fe originated from limestone and dicalcium phosphate (Figure 3).

275        Some authors have highlighted that increased values tend to signal adequate sensitivity of

276        ferritin to Fe in broiler diets (Bai et al, 2021; Abdel-Rahman et al., 2022, Hu et al., 2022).

277        A reference minimal value for ferritin that has been stablished as a reference for adequate

278        dietetic Fe in humans is at or above 30 ng/ml (Guyatt et al., 1992; Munoz et al., 2009), whereas

279        excess ferritin denotes excessive absorption, as usually due in hemochromatosis (Fleming and

280        Sly, 2002). No reference exists for a minimal ferritin blood content in chickens; however, from

281        the present study it can be concluded that 146 ng/mL, as similarly obtained in the present study

282        and as indicated by Feijo et al. (2023), is an adequate value to optimize broiler growth.

283        According to the present results, the sensitivity of serum ferritin to dietary Fe is corroborated

284        by investigations conducted with pigs, rats, and humans (Smith et al., 1984, Yun et al., 2011,

285        Gondolf et al., 2013). In the present research, there were differences among treatments in the

286        total contents of Fe in livers. Increased hepatic ferritin expression suggests that this is the major

287        storage protein of Fe (Basclain et al., 1998) and, therefore, the parallel increases of ferritin and

288        liver Fe are expected. It seems that an upper limit for Fe storage in the liver is out of range in

289        commercial type diets (Ma et al., 2016; Akter et al., 2017; Han et al., 2022).

290        Commercial type broiler diets formulated without animal protein need to include sources

291        of Ca and P to obtain an adequate balance for these minerals. This is mostly done by adding

292        limestone and dicalcium phosphate in feeds. Both mineral sources have high contents of Fe

293        which, however, contrasts with their low digestibility and retention. In practical term, Fe from

294  these sources seem not to be needed since Fe present in corn and SBM (57.6 mg/kg) allows

295  maximum live performance. Increases in serum ferritin and liver Fe contents are indicators of

296  Fe status but also indicators of absorbed Fe that are in excess for use in animal metabolism. In

297  the present study, rates of increase in ferritin and liver Fe of 7% and 7.5%, respectively, were

298  found for those indicators.

299

300  **ACKNOWLEDGMENTS**

303

304  **DISCLOSURES**

305  All authors declare that they have no conflict of interest or personal relationships that could

306  have appeared to influence the work reported in this article.

307

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486 **Table 1.** Ingredient and nutrient composition with graded increases of Fe.

| Item | Fe deficient diet | Starter (mg/kg) | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 to 7 d | 55.5 | 88.5 | 121.5 | 154.5 | 187.5 | 221.5 |
| Ingredient, %[1] | | | | | | | |
| Corn, 7.8 CP | 50.08 | 50.79 | 51.09 | 51.40 | 51.70 | 51.98 | 52.27 |
| Soybean meal, 46 CP | 41.03 | 38.56 | 38.51 | 38.46 | 38.40 | 38.35 | 38.30 |
| Soybean oil | 3.49 | 4.44 | 4.33 | 4.23 | 4.13 | 4.03 | 3.93 |
| Calcium carbonate | 2.53 | 2.42 | 1.93 | 1.45 | 0.96 | 0.48 | - |
| Phosphoric acid | 1.52 | 1.45 | 1.16 | 0.87 | 0.57 | 0.29 | - |
| Limestone | - | - | 0.21 | 0.42 | 0.63 | 0.84 | 1.07 |
| Dicalcium phosphate | - | - | 0.42 | 0.85 | 1.27 | 1.67 | 2.09 |
| Common salt | 0.47 | 0.47 | 0.47 | 0.47 | 0.47 | 0.47 | 0.47 |
| DL-Methionine, 99% | 0.34 | 0.32 | 0.32 | 0.32 | 0.32 | 0.32 | 0.32 |
| L-Lysine HCl, 76% | 0.17 | 0.17 | 0.17 | 0.17 | 0.17 | 0.17 | 0.18 |
| L-Threonine, 98.5% | 0.09 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 |
| Choline chloride | 0.04 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 |
| Vitamin and mineral mix[2] | 0.23 | 0.23 | 0.23 | 0.23 | 0.23 | 0.23 | 0.23 |
| Celite (indigestible marker) | - | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Total | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |
| Energy and nutrients, % or as noted[3] | | | | | | | |
| $AME_n$, kcal/kg | 2,950 | 3,000 | 3,000 | 3,000 | 3,000 | 3,000 | 3,000 |
| Crude Protein | 22.9 | 21.9 | 21.9 | 21.9 | 21.9 | 21.9 | 21.9 |
| Ca | 1.05 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Available P | 0.50 | 0.48 | 0.48 | 0.48 | 0.48 | 0.48 | 0.48 |
| Total P | 0.80 | 0.77 | 0.77 | 0.77 | 0.77 | 0.77 | 0.77 |
| Na | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 |
| Choline, mg/kg | 1,600 | 1,600 | 1,600 | 1,600 | 1,600 | 1,600 | 1,600 |
| Dig. Lys | 1.28 | 1.22 | 1.22 | 1.22 | 1.22 | 1.22 | 1.22 |
| Dig. TSAA | 0.96 | 0.92 | 0.92 | 0.92 | 0.92 | 0.92 | 0.92 |
| Dig. Thr | 0.83 | 0.79 | 0.79 | 0.79 | 0.79 | 0.79 | 0.79 |
| Dig. Val | 0.97 | 0.93 | 0.93 | 0.93 | 0.93 | 0.93 | 0.93 |
| Fe, mg/kg[4] | 57.6 (58.2 ± 2.4) | 55.5 | 88.5 | 121.5 | 154.5 | 187.5 | 220.5 |

487 [1]Calcium carbonate, acid phosphoric, limestone and dicalcium phosphate had 9.2, 2.8, 7,218 and 4,783 mg/kg Fe,
488 respectively).
489 [2]Composition per kilogram of feed: vitamin A, 12,000 IU; vitamin $D_3$, 3,000 IU; vitamin C, 50 mg; vitamin E,
490 100 IU; vitamin $K_3$, 6 mg; vitamin B12, 35 µg; thiamin, 3 mg; riboflavin, 15 mg; vitamin B6, 6 mg; niacin, 40
491 mg; pantothenic acid, 25 mg; folic acid, 4 mg; biotin, 0.3 mg; Zn, 100 mg; Mn, 100 mg; Cu, 15 mg; Se, 0.45 mg
492 and I, 2 mg.
493 [3]Analyzed values are within parentheses.
494 [4]Analyzed Fe in the starter feeds were 57.6 ± 2.1, 92.0 ± 2.3, 124.1 ± 2.7, 159.3 ± 3.1, 187.2 ± 3.2, 223.7 ± 3.6
495 mg/kg.

496 **Table 2**. Growth performance of broilers as affected by feeds graded increases of Fe[1].

| Fe, mg/kg[2] | 8 to 14 d | | | 15 to 21 d | | | 22 to 28 d | | | 8 to 28 d | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BWG, g | FCR | FI, g | BWG, g | FCR | FI, g | BWG, g | FCR | FI, g | BWG, g | FCR | FI, g |
| 55.5 | 320 | 1.188 | 381 | 575 | 1.385 | 795 | 644 | 1.422 | 914 | 1,540 | 1.358 | 2,090 |
| 88.5 | 329 | 1.189 | 391 | 553 | 1.395 | 771 | 652 | 1.439 | 938 | 1,535 | 1.368 | 2,100 |
| 121.5 | 316 | 1.190 | 376 | 555 | 1.392 | 772 | 650 | 1.421 | 921 | 1,521 | 1.360 | 2,069 |
| 154.5 | 323 | 1.182 | 381 | 556 | 1.376 | 764 | 650 | 1.440 | 937 | 1,528 | 1.362 | 2,082 |
| 187.5 | 320 | 1.183 | 377 | 568 | 1.376 | 782 | 642 | 1.417 | 910 | 1,530 | 1.352 | 2,069 |
| 220.5 | 322 | 1.180 | 380 | 575 | 1.390 | 799 | 647 | 1.441 | 928 | 1,544 | 1.363 | 2,108 |
| SEM | 3.457 | 0.005 | 3.946 | 4.503 | 0.006 | 5.568 | 5.941 | 0.007 | 7.982 | 7.098 | 0.004 | 10.106 |
| *Probability <* | 0.9413 | 0.9952 | 0.9147 | 0.5223 | 0.8977 | 0.3692 | 0.9971 | 0.8949 | 0.8916 | 0.9582 | 0.8854 | 0.8487 |

497 [a>b] Means with different letters in the same column indicate significant differences ($P \leq 0.05$).

498 [1]BWG = body weight gain; FCR = feed conversion ratio corrected for the weight of dead birds; FI = feed intake.

499 [2]Analyzed Fe in the feeds were $57.6 \pm 2.1$, $92.0 \pm 2.3$, $124.1 \pm 2.7$, $159.3 \pm 3.1$, $187.2 \pm 3.2$, $223.7 \pm 3.6$ mg/kg.

**Table 3**. Apparent ileal digestibility of Fe and retention responses of broilers as affected by increased dietary Fe, on dry matter (DM) basis.

| Fe, mg/kg[1] | Ileal digestibility Fe, % | Intake | Excretion | Fe retained mg/bird |
|---|---|---|---|---|
| | | Fe, mg/bird | | |
| 55.5 | 11.32$^a$ | 31.88$^f$ | 27.54$^f$ | 4.34$^b$ |
| 88.5 | 8.29$^b$ | 51.41$^e$ | 46.38$^e$ | 5.03$^{ab}$ |
| 121.5 | 6.74$^c$ | 67.89$^d$ | 62.39$^d$ | 5.50$^{ab}$ |
| 154.5 | 5.43$^{cd}$ | 84.90$^c$ | 79.34$^c$ | 5.56$^{ab}$ |
| 187.5 | 4.53$^{de}$ | 103.64$^b$ | 97.67$^b$ | 5.97$^a$ |
| 220.5 | 3.25$^e$ | 125.54$^a$ | 119.48$^a$ | 6.06$^a$ |
| SEM | 0.341 | 3.774 | 3.704 | 0.143 |
| *Probability<* | <0.0001 | <0.0001 | <0.0001 | 0.0030 |

[a>b] Means with different letters in the same column indicate significant differences (P < 0.05).

[1] Analyzed Fe in the feeds were $57.6 \pm 2.1$, $92.0 \pm 2.3$, $124.1 \pm 2.7$, $159.3 \pm 3.1$, $187.2 \pm 3.2$, $223.7 \pm 3.6$ mg/kg.

[2] Ileal digestible Fe (%), intake, excretion, and retention of Fe (mg/bird), respectively: $Y = 13.0788 - 0.0461x$, $R^2 = 0,8140$, $P < 0.001$; $Y = -4.8589 + 0.5474x$, $R^2 = 0.9717$, $P<0.001$; $Y = -0.8489 + 0.5573x$, $R^2 = 0.9701$, $P < 0.001$; $Y = 4.0101 + 0.0099x$, $R^2 = 0.2142$, $P < 0.001$, $X$ = dietary Fe content.

507 **Table 4**. Blood parameters and fresh liver Fe concentration of broilers as affected by increased
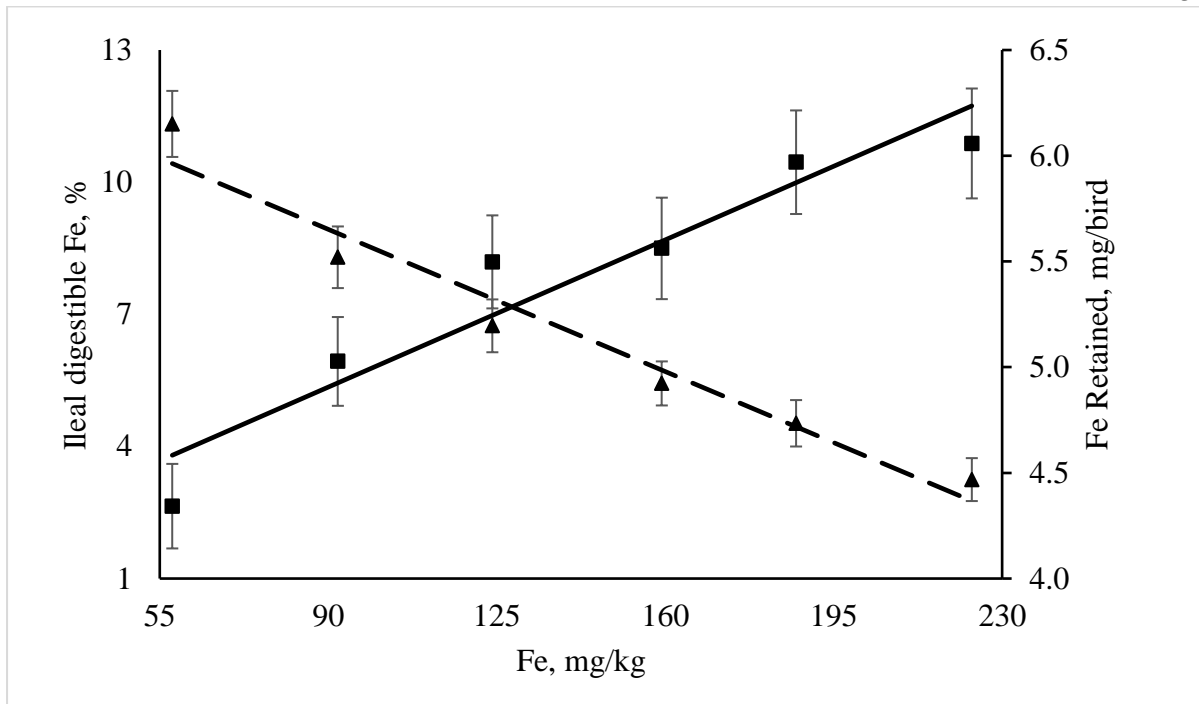508 dietary Fe[1].

| Fe, mg/kg[2] | Ht, % | Hb, g/dL | Ferritin, ng/mL | Fresh liver, mg/kg |
|---|---|---|---|---|
| 55.5 | 31.2 | 10.8 | 146[c] | 114.6[d] |
| 88.5 | 31.2 | 10.7 | 151[bc] | 117.0[cd] |
| 121.5 | 31.5 | 10.8 | 152[b] | 120.3[bc] |
| 154.5 | 31.3 | 10.9 | 153[ab] | 123.1[ab] |
| 187.5 | 31.4 | 10.7 | 155[ab] | 125.1[a] |
| 220.5 | 31.3 | 10.7 | 158[a] | 126.5[a] |
| SEM | 0.180 | 0.071 | 0.769 | 0.627 |
| *Probability <* | 0.9981 | 0.9386 | <0.0001 | <0.0001 |

509 [a>b] Means with different letters in the same column indicate significant differences ($P < 0.05$).
510 [1]Birds (n=20) had hematocrit (Ht), hemoglobin (Hb) and ferritin values as follow: $26.2 \pm 1.6\%$, $8.8 \pm 0.5$ g/dL
511 and $121 \pm 5.2$ ng/mL, respectively.
512 [2]Analyzed Fe in the feeds were $57.6 \pm 2.1$, $92.0 \pm 2.3$, $124.1 \pm 2.7$, $159.3 \pm 3.1$, $187.2 \pm 3.2$, $223.7 \pm 3.6$ mg/kg.
513 [3]Ferritin and Fe content in the liver at 28 d, respectively: $Y = 142.8394 + 0.0702x$, $R^2 = 0.3699$, $P < 0.001$; $Y = $
514 $110.5263 + 0.0752x$, $R2 = 0.6390$, $P < 0.001$, $X$ = dietary Fe content.

Fig. 1. Apparent ileal digestibility of Fe (triangle, long dash line) and Fe retained (solid squares, solid line) associated with total dietary Fe: $Y = 13.0788 - 0.0461x$, $R^2 = 0,8140$, $P < 0.001$ and $Y = 4.0101 + 0.0099x$, $R^2 = 0.2142$, $P < 0.001$, respectively.
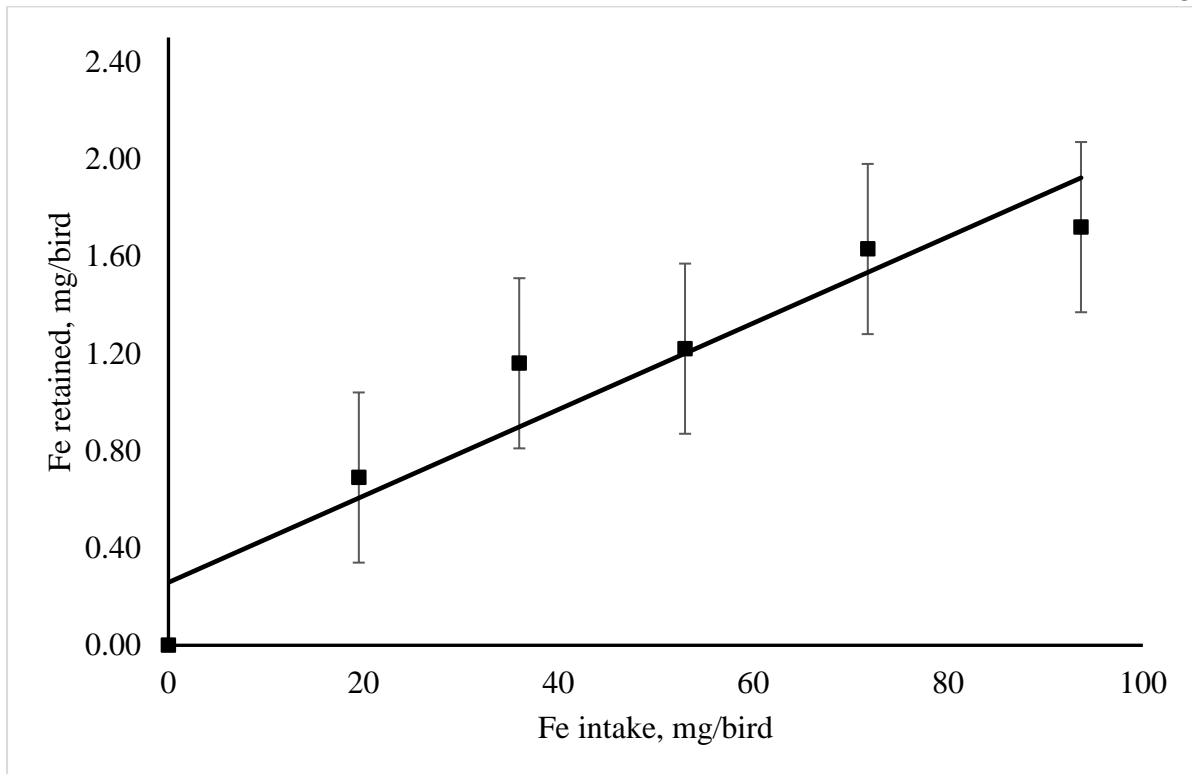
519
520  Fig. 2. Fe retained associated only with the intake of Fe from limestone and dicalcium phosphate (solid squares,
521  solid line): Y = 0.1974 + 0.0190x, $R^2$ = 0.2625, P < 0.001.

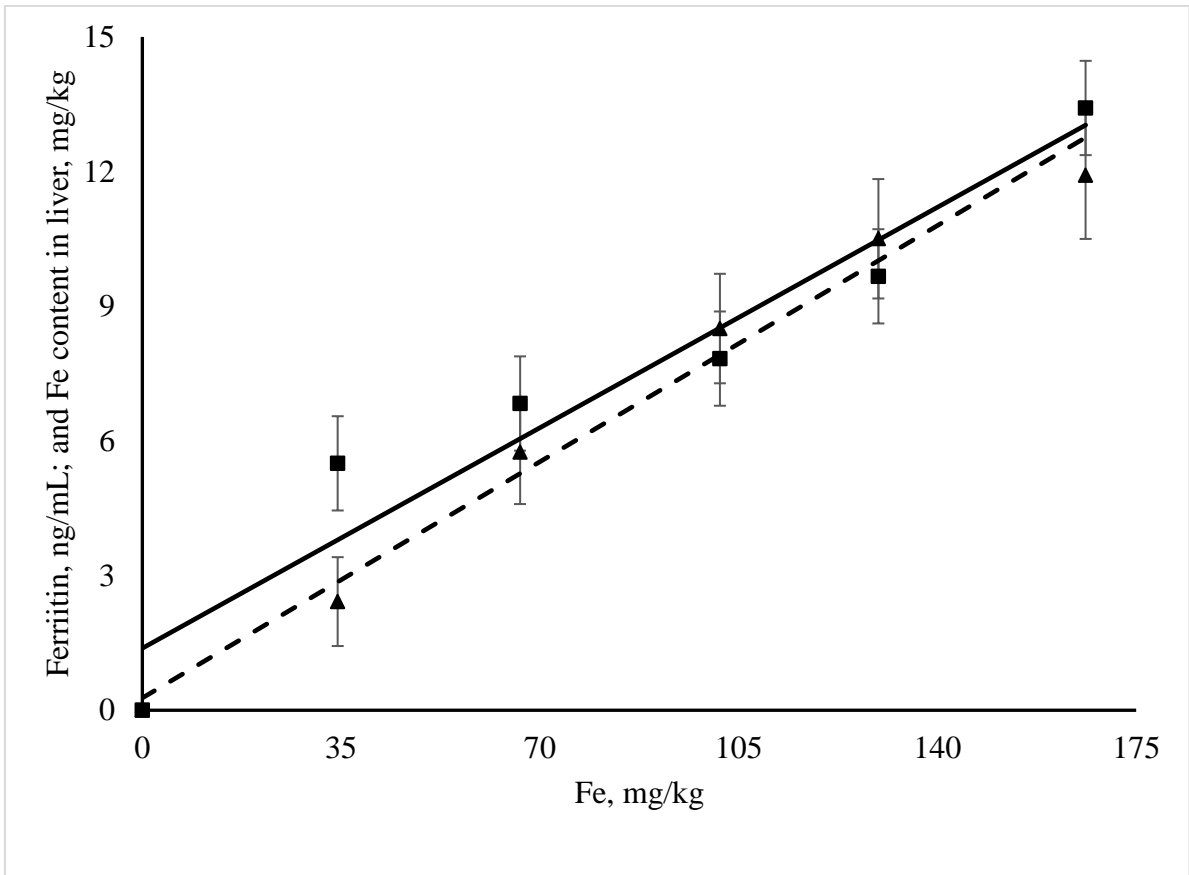Fig. 3 Ferritin (solid squares, solid line) and Fe content in the liver (triangle, long dash line) associated with Fe only from limestone and dicalcium phosphate: $Y = 1.3810 + 0.0702x$, $R^2 = 46.19$, $P < 0.001$; and $Y = 0.2735 + 0.0752x$ $R^2 = 70.22$, $P < 0.001$, respectively.

**CAPÍTULO IV**

# CONSIDERAÇÕES FINAIS

O estudo demonstrou que suplementação de fitase promove melhorias no crescimento dos frangos de corte, que não foram relacionadas com aumentos na disponibilidade de P e Ca. Na presente pesquisa ficou evidente que a fitase promove melhorias na digestibilidade de Fe.

A isenção do Fe do milho e da soja leva a uma correlação linear com o Fe à medida que calcário e fosfato bicálcico são adicionados à dieta. Esses ingredientes, possuem grandes quantidades de Fe, no entanto esse micromineral se encontra de uma forma menos disponível, o que contrasta com sua baixa taxa de retenção, em torno de 1,90%.

Aumentos na suplementação de Fe de origem do sulfato ferroso heptahidrato, assim como do calcário e fosfato bicálcico para níveis que excedam a oferta total deste mineral em dietas comerciais não levaram a benefícios no desempenho vivo; no entanto, os parâmetros sanguíneos e conteúdo de Fe no fígado foram afetados positivamente a medida que o houve aumento no Fe retido.

Frangos alimentados com rações base de milho e farelo de soja não necessitam de suplementação de Fe em pré-misturas. O excesso de Fe nas dietas acarreta em aumento de Fe nas excretas, o que pode levar ao aumento da disposição deste mineral no meio ambiente.

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**APÊNDICES**

**Apêndice 1.** Instruções para publicação na revista Journal Animal Science.

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The layout of the Journal is compatible with the OUP LaTeX template. More information can be found here.

### Title Page

Required items on the page are:

1. Running title: short, succinct title no more than 45 keystrokes (characters plus spaces) in length with first and proper nouns capitalized
2. A title with the first word and proper nouns capitalized. Species of subject is encouraged. The title should be unique. The Journal does NOT support multipart series.
3. Full names (given name, middle initial, family name) of all authors
4. Institutions of the authors with location denoted with a symbol (*, †, ‡, §, #,||, and ¶) behind the author last name
5. Department, city, state, country, and postal code (Please note: the country must be listed for each affiliation)
6. Acknowledgements of consortia, grants, experiment station, or journal series number are given as a numerical footnote to the title

### Lay Summary

Authors are required to submit a Lay Summary as part of the article, in addition to the main text abstract. The Lay Summary should clearly summarize the focus and findings of the article for non-expert readers, and will be published as part of the article online and in PDF. The Lay Summary follows the Title Page in the main manuscript file and is limited to 200 words. Authors should avoid technical and discipline-specific abbreviations and language whenever possible to engage a larger non-scientific audience. Abbreviations are defined at first use.

### Teaser Text

Authors are required to provide 1-2 sentence(s) clearly describing the impact of the research described in the manuscript. Teaser text is used in the Journal table of contents to entice readers. It follows the Lay Summary in the main document file.

## Abstract

A single paragraph of no more than 2,500 keystrokes (characters plus spaces) that summarizes the results in an understandable form using statistical evidence (*P*-values).  Abbreviations are defined at first use in the ABSTRACT and again in the body of the manuscript.

## Key words

List up to 6 words in alphabetical order and separated by a comma.  Capitalize only proper nouns.  Do NOT use abbreviations. Place at the end of the ABSTRACT.

## List of Abbreviations

A comprehensive list of all abbreviations used in the manuscript and their definition can be found in the Excel spreadsheet. An example format is MRF, myogenic regulatory factor. The List should not contain standard JAS Abbreviations, diets or treatment descriptions. Abbreviations must be defined at first use in the manuscript text but not in tables and figures unless unique.
Download an MS Excel spreadsheet of *JAS* standard abbreviations.
Plural abbreviations do not contain a final "s" because the context of an abbreviation implies whether it is singular or plural. Use of the standard 3-letter abbreviations for amino acids (e.g., Ala) is acceptable in *JAS*. Use of the internationally recognized chemical symbols for chemical elements (e.g., P and S) is acceptable in *JAS*. Except for N (not italicized), which is the recognized abbreviation for nitrogen and newton (unit of force), chemical symbols for elements are reserved for elements (e.g., C is for carbon and never for control).

## Introduction

A clear justification for conducting the research with a stated hypothesis and objective(s) is required.  The rationale for the experiments should place the work into the context of existing literature.  There is NO word limit on the section but brevity is encouraged.

## Materials and Methods

The American Society of Animal Science (ASAS) supports rigor, reproducibility and transparency in science and seeks to ensure that publications of the society reflect these values while also minimizing the burden on authors in preparation of scientific results for publication. There are many available resources describing principles and practices to enhance rigor, reproducibility, and transparency in science. Authors considering the Journal of Animal Science are encouraged to consult these resources  when during preparation of their submissions.
The manuscript must include a statement of institutional animal care and use committee (IACUC), or country-specific equivalent, approval of all animal procedures. The *IACUC statement should appear as the first item in MATERIALS AND METHODS* and should specify which publicly available animal care and use standards were followed.  A clear description of all biological, analytical and statistical procedures is required with each section denoted by a short descriptive title (i.e., Animals and

sampling, Western blot, Immunocytochemistry, Experimental design and analysis, etc). Materials used must include the product name and vendor at first mention. When a commercial product is used as part of an experiment, the manufacturer name and location must be given parenthetically and the generic name should be used subsequently. No ™, ®, or © symbols should be used. Sex, breed, age, species are included in the animal descriptions. Provide evidence of assay validation, or suitable published reference, as well as inter/intra-assay CV, as needed. Appropriate statistical methods should be used with experimental unit defined. Numbers of biological and experimental replicates should be stated. State the threshold for significance ($P <$ 0.05) and definition of tendency if used.

## Results

Experimental results are presented in tables and figures. The results should contain sufficient detail to allow the reader to interpret the data. Quantitative measures of significance ($P$-values) should be presented. Authors may use either absolute $P$-values or a defined significance level as long as usage is consistent.

## Discussion

The section contains the interpretation of the results. It should be clear and concise, address the biological mechanisms and their significance, and integrate the results into existing literature. The Discussion may offer an interpretation that is consistent with the data. Do NOT include any reference to tables and figures or include $P$-values in the Discussion. Authors have the option to create a single RESULTS AND DISCUSSION section.

## Disclosure of potential conflicts of interest

### Authors
The Journal requires all authors to disclose any potential conflict of interest at the point of submission. It is the responsibility of the corresponding author to ensure that conflicts of interest of all authors are declared to the Journal.
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The corresponding author must submit a completed and signed International Committee of Medical Journal Editors (ICMJE) disclosure of potential conflicts of interest (COI) form for each author at initial submission. A form must be submitted even if there are no interests to disclose, in which case the disclosure form and manuscript should state "none declared." In addition, the manuscript must include a concise and accurate summary of any conflicts of interest declared in the ICMJE forms.

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**Editors and editorial board members**
At initial submission, the corresponding author must declare if the Editor-in-Chief, an Editor, or an Editorial Board Member of the Journal is an author of or contributor to the manuscript. Another Editor without a conflict of interest will oversee the peer review and decision-making process. If accepted, a statement will be published in the paper describing how the manuscript was handled.

**Literature Cited**
Papers in the section must be published or 'in press'. All references must include the DOI, if available. Authors are encouraged to use the most recent reference style for the Journal of Animal Science in the reference management software of their choice. The format for references are

**Journal articles**
Perez, V. G., A. M. Waguespark, T. D. Bidner, L. L. Southern, T. M. Fakler, T. L. Ward, M. Steidinger, and J. E. Pettigrew. 2011. Additivity of effects from dietary copper and zinc on growth performance and fecal microbiotia of pigs after weaning. J. Anim. Sci. 89:414–425. doi:10.2527/jas.2010-2839.

**Abstracts**
Centon, J. R., G. E. Erickson, T. J. Klopfenstein, K. J. Vander Pol, and M. A. Greenquist. 2007. Effects of roughage source and level in finishing diets containing wet distillers grains on feedlot performance. J. Anim. Sci. 85(Suppl. 2):76. (Abstr.) doi:10.2527/jas.2006-354.

**Books and chapters in books**
AOAC. 1990. Official methods of analysis. 15th ed. Assoc. Off. Anal. Chem., Arlington, VA.
NRC. 2000. Nutrient requirements of beef cattle. 7th rev. ed. Natl. Acad. Press, Washington, DC.
Robinson, P. H., E. K. Okine, and J. J. Kennelly. 1992. Measurement of protein digestion in ruminants. In: S. Nissen, editor, Modern methods in protein nutrition and metabolism. Academic Press, San Diego, CA. p. 121–127.

**Conference proceedings**
Bailey, E. A., J. R. Jaeger, J. W. Waggoner, G. W. Preedy, L. A. Pacheco, and K. C. Olson. 2012. Effect of weaning method on welfare and performance of beef calves during receiving. Proc. West. Sec. Amer. Soc. Anim. Sci. 63:25-29.

**Tables and Figures**

Tables and figures should be placed at the end of the manuscript, following the references. Tables and figures should be numbered consecutively, in the order in which they are cited in the manuscript. Tables and figures must be prepared so they can be understood without referring to information in the body of the manuscript.
**Tables**

1. Tables can be created in in Word using the Table function.
2. All tables should be editable in the manuscript file.
3. Each table should be placed on a separate page. Tables should fit on a single 8.5 X 11-inch page in either landscape or portrait view.
4. Every column should include a heading.
5. Align column values to the decimal point whenever possible. Columns containing a mix of values, symbols and words may be aligned to the center of the heading. Columns using ± should be aligned to the symbol.
6. Units (e.g., kg) are separated from descriptor by a comma.
7. Numerals are used to reference footnotes. Each footnote should begin on a new line immediately below the table.
8. Lowercase, superscript letters are used to indicate significant differences among means within a row or column and to reference footnotes explaining how to interpret the letters.
9. The order of footnotes below the table is numbers first followed by letters and special symbols.
10. If reporting significance, the column heading is P-value.

## Figures

1. Figures should be submitted as JPEG, TIFF, or EPS files only.
2. Figures must be high-resolution with a minimum resolution of 300 dpi and a maximum resolution of 600 dpi.
3. All figures must have a title and legend. The legend should be a brief description that allows the reader to interpret the results.
4. Axes descriptors are separated from units (i.e., kg, mm, mL) by a comma. Do NOT place units within parentheses.
5. Use Times New Roman font no smaller than 8 point following figure reduction.

## Supplemental Material

Authors may include supplemental tables, figures, or other forms of supplemental material (e.g., detailed data sets, Excel files, videos). Supplemental materials should be included in a separate file.

Supplemental materials must undergo peer review and, thus, should be in a format that is easily accessible (i.e., does not require dedicated software or software that is not generally available) to most reviewers and readers.

## Additional Usage Notes

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Authors of papers that contain original quantitative trait loci (QTL) or DNA marker association results for livestock are strongly encouraged to make their data available in an electronic form to one of the publicly available livestock QTL databases after the manuscript appears on the JAS Advance Articles website (https://academic.oup.com/jas/advance-articles).
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The use of names of commercial products should be minimized. When a commercial product is used as part of an experiment, the manufacturer name and location (city and state if in the US; city, administrative region or district [e.g., province], and country if outside the US) or a website address must be given parenthetically at first mention in text, tables, and figures. The generic name should be used subsequently. No ™, ®, or © symbols should be used.

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The mission of the American Society of Animal Science (ASAS) is to "foster the discovery, sharing, and application of scientific knowledge concerning the responsible use of animals to enhance human life and wellbeing" (see ASAS's History and Mission).

The *Journal of Animal Science*, which is published by ASAS, accepts manuscripts presenting information for publication with this mission in mind.

The Editor-in-Chief, Managing Editor, and Section Editors establish the editorial policies of *JAS*, subject to review by the publications committee and ASAS Board of Directors. The views expressed in articles published in *JAS* represent the opinions of the author(s) and do not necessarily reflect the official policy of the institution with which an author is affiliated, the ASAS, or the *JAS* Editor-in-Chief. Authors are responsible for ensuring the accuracy of collection, analysis, and interpretation of data in manuscripts and ultimately for guaranteeing the veracity of the contents of articles published in *JAS*.

## General Usage

- For general style and form, authors should follow that recommended in Scientific Style and Format: The CSE Manual for Authors, Editors, and Publishers. 7th ed. Council of Science Editors, Reston, VA.
- For American English spelling and usage, consult Merriam-Webster Online.
- For SI units, the National Institute of Standards and Technology provides a comprehensive guide.
- Abbreviations are not used to begin sentences. Words must be spelled out.
- "Sex" should be used, rather than "gender." Gender is more appropriate for describing a role in society than for describing biological sex.
- The hierarchy for brackets and parentheses is [ ( ) ]. For example, $[(2 + 3) \times (12 \div 2)] \times 2 = 60$.
- Meat shear force should be expressed in kilograms (kg), although newtons (N) may also be acceptable.
- Report time using the 24-h system (e.g., 1410 h rather than 2:10 p.m.).
- Use italics to designate genus and species.
- Names of muscles are not italicized.
- Specify the basis (i.e., as-fed or dry matter) for dietary ingredient and chemical composition data listed in text or in tables. Similarly, specify the basis for tissue composition data (e.g., wet or dry basis).
- Calculations of efficiency should be expressed as output divided by input (i.e., gain:feed, not feed:gain).
- A diet is a feedstuff or a mixture of feedstuffs; a ration is the daily allotment of the diet.
- The word "Table" is capitalized and never abbreviated.

- Except to begin a sentence, the word "Figure" should be abbreviated to "Fig."
- Except to begin a sentence, experiment and equation should be abbreviated to Exp. And Eq., respectively, when preceding a numeral (e.g., Exp. 1).
- Avoid jargon unfamiliar to scientists from other disciplines. Do not use the term "head" to refer to an animal or group of animals. Instead, use animal, sow, ewe, steer, heifer, cattle, etc.
- Avoid bi- as a prefix because of its ambiguity; biweekly means twice per week and once every 2 weeks.
- Breed and variety names should be capitalized (e.g., Landrace and Hereford).
- Trademarked or registered names should be capitalized, but no ™ or ® symbols should be used.

## Contact Information

For information on the scientific content of the journal, contact the Editor-in-Chief, Dr. Elisabeth Huff Lonergan, American Society of Animal Science, P.O. Box 7410, Champaign, Illinois 61826-7410; e-mail: elonerga@iastate.edu.
For questions about submitting a manuscript and ScholarOne Manuscripts, contact Mkaj Wang; e-mail: jas.editorialoffice@jjeditorial.com.
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## Care and Use of Animals

All authors submitting to *JAS* must complete the Care and Use of Animals statement during submission certifying that any research that involves animals has followed established standards for the humane care and use of animals and must specify which standards were used. Only investigations that have followed high standards for the humane care and use of animals in research will be reported in *JAS*. Also, the manuscript must include a statement of institutional animal care and use committee (IACUC), or country-specific equivalent, approval of all animal procedures. The IACUC statement should appear as the first item in MATERIALS AND METHODS and should specify which publically available animal care and use standards were followed (e.g., ADSA-ASASPSA Guide for Care and Use of Agricultural Animals in Research and Teaching; Primary Industries Ministerial Council, Model code of practice for the welfare of animals: the sheep). The manuscript should describe anesthetics, analgesics, tranquilizers, and care taken to minimize pain and discomfort during preoperative, operative, and postoperative procedures. If research requires discomfort to the animals or stressful conditions, justification for these conditions must be evident in papers published in *JAS*.

## Protection of Human Subjects

In the United States, federally funded or regulated research involving human subjects must comply with Code of Federal Regulations (CFR), Title 45 Public Welfare, Part 46 Protection of Human Subjects. However, CFR 45 Part 46.101(b) exempts some research from these regulations. For all exempted research and other details, see this page. Exempted research includes that in which the only involvement of human subjects is for "taste and food quality evaluation and consumer acceptance if 1) wholesome foods without additives are consumed or 2) a food is consumed that contains a food ingredient at or below the level and for a use found to be safe, or agricultural chemical or environmental contaminant at or below the level found to be

safe, by the Food and Drug Administration or approved by the Environmental Protection Agency or the Food Safety and Inspection Service of the U.S. Department of Agriculture." If human subjects were used in exempted research and the research was in compliance with CFR 45 Part 46, or equivalent regulations where the research was conducted, authors must state in MATERIALS AND METHODS or acknowledgements that they were in full compliance. If human subjects were used in research that was not exempted in CFR 45 Part 46, or equivalent regulations where the research was conducted, authors must certify that the research received prior approval from an appropriate Institutional Review Board.

## Types of Articles

### Research Articles
Results of research contained in manuscripts submitted to *JAS* must not have been published in or submitted to another peer reviewed scientific journal prior to receiving a decision from *JAS*. Previous presentation at a scientific meeting or the use of data in field-day reports or similar documents, including press publications or postings to personal or departmental websites, does not preclude the publication of such data in *JAS*.

Articles simultaneously posted to websites and submitted to *JAS* should carry a disclaimer on the website that this version of the paper has not undergone *JAS* peer review and is not to be considered the final published form of the article. If the article has been published in *JAS*, the author should include the complete *JAS* citation.

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Special Topics. This Section includes Biographical or Historical Sketches and Contemporary Issues in the animal sciences. Contemporary Issues include topics such as environmental concerns, legislative proposals, systems analysis, and various "newsworthy" scientific issues. Even though Contemporary Issues manuscripts do not have to include original data, authors' assertions should be substantiated with references to established information from credible published sources. Special Topics papers will be subject to peer review in a manner similar to other *JAS* submissions. Because of the nature of these manuscripts, their format may vary from that of standard scientific articles, although the ABSTRACT must be consistent with keystroke (characters and spaces) limitations defined earlier in this document. Teaching articles should be submitted to Translational Animal Science.

Short Communications. JAS will consider publication of short communications that are hypothesis-driven and report novel results. Submitted papers should follow JAS guidelines for headings and format, but are restricted to 2 figures or tables or a combination of 1 figure/1 table. The words "Short Communication:" should begin the title. The final published paper will be published Open Access using the current pricing structure.

Technical Notes. A technical note is used to report a new method, technique, or procedure of interest to *JAS* readers. When possible, a technical note should include a comparison of results from the new method with those from previous methods, using appropriate statistical tests. The advantages and disadvantages of the new procedure

should be discussed. When typeset for publication, a technical note shall not exceed 10 pages (approximately 18 Microsoft Word document pages), including tables and figures. "Technical note:" shall be the first portion of the title of such manuscripts. The review process for a technical note will be the same as that for other manuscripts. Information that is more extensive or detailed than necessary for a Technical note may be presented in an e-supplement (see E-Supplements).

Letters to the Editor. A letter judged suitable for publication will be printed in a "Letters to the Editor" section of *JAS*. The purpose of this section is to provide a forum for scientific exchange relating to articles published in *JAS*. To be acceptable for publication, a letter must adhere to the following guidelines. 1) Only a letter that addresses matters of science and relates to information published in *JAS* will be considered. In general, a letter should not exceed 5,000 keystrokes and should contain no more than 5 citations. 2) A letter should provide supporting evidence based on published data for the points made or must develop logical scientific hypotheses. A letter based on conjecture or unsubstantiated claims will not normally be published. No new data may be presented in a letter. 3) The Editor-in-Chief will evaluate each letter and determine whether a letter is appropriate for publication. If a letter is considered appropriate, the author(s) of original *JAS* article(s) will be invited to write a letter of response. Normally both letters will be published together. 4) All letters will be subject to acceptance and editing by the Editor-in-Chief and editing by a technical editor.

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The journal publishes invited review articles only.

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## Review of Manuscripts

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Advance Articles and Author Proofs

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JAS Ethics Policy
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Quality Guidelines for JAS Figures
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Where ethically feasible, *JAS* strongly encourages authors to make all data and software code on which the conclusions of the paper rely available to readers. We suggest that data be presented in the main manuscript or additional supporting files, or deposited in a public repository whenever possible. Information on general repositories for all data types, and a list of recommended repositories by subject area, is available here.

**Data and Software Citation**

*JAS* supports the Force 11 Data Citation Principles and requires that all publicly available datasets be fully referenced in the reference list with an accession number or unique identifier such as a digital object identifier (DOI). Data citations should include the minimum information recommended by DataCite:

    [dataset]* Authors, Year, Title, Publisher (repository or archive name), Identifier

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If there is an article describing the software, it is recommended to cite both the software and the article.

**Apêndice 2.** Instruções para publicação na revista Poultry Science.

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First self-published in 1921, Poultry Science is an internationally renowned monthly journal, known as the authoritative source for a broad range of poultry information and high-caliber research. The journal plays a pivotal role in the dissemination of preeminent poultry-related knowledge across all disciplines. Poultry Science is an Open Access journal with no subscription charges, meaning authors who publish here can make their research immediately, permanently, and freely accessible worldwide while retaining copyright to their work.

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## SUBMISSION

All manuscripts are submitted and reviewed via the journal's Editorial Manager manuscripts submission site at https://www.editorialmanager.com/psj. New authors should create an account prior to submitting a manuscript for consideration.

The submission system guides you stepwise through the process of entering your article details and uploading your files. The system converts your article files to a single PDF file used in the peer-review process. Editable files (e.g., Word) are required to typeset your article for final publication. All correspondence, including notification of the Editor's decision and requests for revision, is sent by e-mail.

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You can use this list to carry out a final check of your submission before you send it to the journal for review. Please check the relevant section in this Guide for Authors for more details.

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One author has been designated as the corresponding author with contact details (please note that co-corresponding authors are not allowed):

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All necessary files have been uploaded:

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Include keywords
All tables (including titles, description, footnotes)
Figure legends
Ensure all figure and table citations in the text match the files provided
Figure files (figures must be uploaded as separate files, not embedded in the manuscript)

Supplemental files (where applicable)

Conflict of Interest statement

Further considerations

Manuscript has been 'spell checked' and 'grammar checked'
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1 IU = 0.67 mg of d-α-tocopherol

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*Article:*

Bagley, L. G., and V. L. Christensen. 1991. Hatchability and physiology of turkey embryos incubated at sea level with increased eggshell permeability. Poult. Sci. 70:1412-1418.

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## TABLES

Tables must be created using the MS Word table feature and inserted in the manuscript after the references section. When possible, tables should be organized to fit across the page without running broadside. Be aware of the dimensions of the printed page when planning tables (use of more than 15 columns will create layout problems). Place the table number and title on the same line above the table. The table title does not require a period. Do not use vertical lines and use few horizontal lines. Use of bold and italic typefaces in the table should be done sparingly; you must define such use in a footnote. Each table must be on a separate page. To facilitate placement of all tables into the manuscript file (just after the references) authors should use "section breaks" rather than "page breaks" at the end of the manuscript (before the tables) and between tables.

Units of measure for each variable must be indicated. Papers with several tables must use consistent format. All columns must have appropriate headings. Abbreviations not found on the inside front cover of the journal must be defined in each table and must match those used in the text. Footnotes to tables should be marked by superscript numbers. Each footnote should begin a new line. Superscript letters shall be used for the separation of means in the body of the table and explanatory footnotes must be provided [i.e., "Means within a row lacking a common superscript differ ($P < 0.05$)."]; other significant P-values may be specified. Comparison of means within rows and columns should be indicated by different series of superscripts (e.g., a,b,… in rows; x-z … in columns) The first alphabetical letter in the series (e.g., a or A) shall be used to indicate the largest mean. Lowercase super- scripts indicate $P \leq 0.05$. Uppercase letters indicate $P \leq 0.01$ or less.

Probability values may be indicated as follows: *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and †$P \leq 0.10$. Consult a recent issue of Poultry Science for examples of tables.

Generally, results should be presented to the significant figure of the instrument used to collect the data. For example, results should not be presented to 5 digits when the instrument used only reads to 2 digits.

## MISCELLANEOUS USAGE NOTES

### Abbreviations
• Abbreviations shall not be used in the title, key words, or to begin sentences, except when they are widely known throughout science (e.g., DNA, RNA) or are terms better known by abbreviation (e.g., IgG, CD). A helpful criterion for use of abbreviation is whether it has been accepted into thesauri and indexes widely used for searching major bibliographic databases in the scientific field. Abbreviations may be used in heads within the paper, if they have been first defined within the text. The inside back cover of every issue of the journal lists abbreviations that can be used without

definition. The list is subject to revision at any time, so authors should always consult the most recent issue of the journal for relevant information. Abbreviations are allowed when they help the flow of the manuscript; however, excessive use of abbreviations can confuse the reader. The suitability of abbreviations will be evaluated by the reviewers and editors during the review process and by the technical editor during editing. As a rule, author-derived abbreviations should be in all capital letters. Terms used less than three times must be spelled out in full rather than abbreviated. All terms are to be spelled out in full with the abbreviation following in bold type in parentheses the first time they are mentioned in the main body of the text. Abbreviations shall be used consistently thereafter, rather than the full term.

• The abstract, text, each table, and each figure must be understood independently of each other. Therefore, abbreviations shall be defined within each of these units of the manuscript.

• Plural abbreviations do not require "s." Chemical symbols and three-letter abbreviations for amino acids do not need definition. Units of measure, except those in the standard Poultry Science abbreviation list, should be abbreviated as listed in the CRC Handbook for Chemistry and Physics (CRC Press, 2000 Corporate Blvd., Boca Raton, FL, 33431) and do not need to be defined.

• The following abbreviations may be used without definition in Poultry Science:


A adenine
ADG average daily gain
ADFI average daily feed
AME apparent metabolizable energy
AMEn nitrogen-corrected apparent metabolizable energy
ANOVA analysis of variance
B cell bursal-derived, bursal-equivalent derived cell bp base pairs
BSA bovine serum albumin
BW body weight
C cytosine
cDNA complementary DNA
cfu colony-forming units
CI confidence interval
CP crude protein
cpm counts per minute
CV coefficient of variation
d day
df degrees of freedom
DM dry matter
DNA deoxyribonucleic acid
EDTA ethylenediaminetetraacetate
ELISA enzyme-linked immunosorbent antibody assay
EST expressed sequence tag
g gram
g gravity
G guanine
GAT glutamic acid-alanine-tyrosine

GLM general linear model
h hour
HEPES N-2-hydroxyethyl piperazine-N'-ethane-sulfonic acid
HPLC high-performance (high-pressure) liquid chromatography
i.m. intramuscular
i.p. intraperitoneal
i.v. intravenous
ICU international chick units
Ig immunoglobulin
IL interleukin
IU international units
kb kilobase pairs
kDa kilodalton
L liter*
L:D hours light:hours darkness in a photoperiod (e.g., 23L:1D)
m meter
µ micro M molar
MAS marker-assisted selection
ME metabolizable energy
MEn nitrogen-corrected metabolizable energy
MHC major histocompatibility complex
mRNA messenger ribonucleic acid
min minute
mo month
MS mean square
n number of observations
N normal
NAD nicotinamide adenine dinucleotide
NADH reduced nicotinamide adenine dinucleotide
NRC National Research Council
NS not significant
PAGE polyacrylamide gel electrophoresis
PBS phosphate-buffered saline
PCR polymerase chain reaction
pfu plaque-forming units
ppm parts per million
QTL quantitative trait loci
r correlation coefficient
r2 coefficient of determination, simple
R2 coefficient of determination, multiple
RH relative humidity
RIA radioimmunoassay
RNA ribonucleic acid
rpm revolutions per minute
s second
s.c. subcutaneous
SD standard deviation
SDS sodium dodecyl sulphate
SE standard error
SEM standard error of the mean

SRBC sheep red blood cells
SNP single nucleotide polymorphism
T thymine
TBA thiobarbituric acid
T cell thymic-derived cell
TME true metabolizable energy
TMEn nitrogen-corrected true metabolizable energy
Tris tris(hydroxymethyl)aminomethane
TSAA total sulfur amino acids
U uridine
USDA United States Department of Agriculture
UV ultraviolet
vol/vol volume to volume
vs. versus
wt/vol weight to volume
wt/wt weight to weight
wk week
yr year
*Also capitalized with any combination, e.g., mL.

## International words and phrases

Non-English words in common usage (defined in recent editions of standard dictionaries) will not appear in italics (e.g., in vitro, in vivo, in situ, a priori). However, genus and species of plants, animals, or bacteria and viruses should be italicized. Authors must indicate accent marks and other diacriticals on international names and institutions. German nouns shall begin with capital letters.

## Capitalization

Breed and variety names are to be capitalized (e.g., Single Comb White Leghorn).

## Number style

Numbers less than 1 shall be written with preceding zeros (e.g., 0.75). All numbers shall be written as digits. Measures must be in the metric system; however, US equivalents may be given in parentheses. Poultry Science requires that measures of energy be given in calories rather than joules, but the equivalent in joules may be shown in parentheses or in a footnote to tables. Units of measure not preceded by numbers must be written out rather than abbreviated (e.g., lysine content was measured in milligrams per kilogram of diet) unless used parenthetically. Measures of variation must be defined in the Abstract and in the body of the paper at first use. Units of measure for feed conversion or feed efficiency shall be provided (i.e., g:g).

## Nucleotide sequences

Nucleotide sequence data must relate to poultry or poultry pathogens and must complement biological data published in the same or a companion paper. If sequences are excessively long, it is suggested that the most relevant sections of the data be

published in Poultry Science and the remaining sequences be submitted to one of the sequence databases. Acceptance for publication is contingent on the submission of sequence data to one of the databases. The following statement should appear as a footnote to the title on the title page of the manuscript. "The nucleotide sequence data reported in this paper have been submitted to Embank Submission (Mail Stop K710, Los Alamos National Laboratories, Los Alamos, NM 87545) nucleotide sequence database and have been assigned the accession number XNNNNN." Publication of the description of molecular clones is assumed by the editors to place them in the public sector. Therefore, they shall be made available to other scientists for research purposes.

Nucleotide sequences must be submitted as camera- ready figures no larger than 21.6 x 27.9 cm in standard (portrait) orientation. Abbreviations should follow Poultry Science guidelines.

**Gene and protein nomenclature**

Authors are required to use only approved gene and protein names and symbols. For poultry, full gene names should not be italicized. Gene symbols should be in uppercase letters and should be in italics. A protein symbol should be in the same format as its gene except the protein symbol should not be in italics.

General usage

• Note that "and/or" is not permitted; choose the more appropriate meaning or use "x or y or both."

• Use the slant line only when it means "per" with numbered units of measure or "divided by" in equations. Use only one slant line in a given expression (e.g., g/d per chick). The slant line may not be used to indicate ratios or mixtures.

• Use "to" instead of a hyphen to indicate a range. Insert spaces around all signs (except slant lines) of operation (=, -, +, x, >, or <, etc.) when these signs occur between two items.

• Items in a series should be separated by commas (e.g., a, b, and c).

• Restrict the use of "while" and "since" to meanings related to time.

• Appropriate substitutes include "and," "but," or "whereas" for "while" and "because" or "although" for "since."

• Leading (initial) zeros should be used with numbers less than 1 (e.g., 0.01).

• Commas should be used in numbers greater than 999.

•Registered (®) and trademark (©) symbols should not be used, unless as part of an article title in the References section. Trademarked product names should be capitalized.

**FIGURES/ILLUSTRATIONS**

**General points**

• Submit each illustration as a separate file.

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• Provide captions to illustrations separately.

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**SUPPLEMENTARY DATA**

Supplementary material such as applications, images and sound clips, can be published with your article to enhance it. Submitted supplementary items are published exactly as they are received (Excel or PowerPoint files will appear as such online). Please submit your material together with the article and supply a concise, descriptive caption for each supplementary file. If you wish to make changes to supplementary material during any stage of the process, please make sure to provide an updated file. Do not annotate any corrections on a previous version. Please switch off the 'Track Changes' option in Microsoft Office files as these will appear in the published version. Ensure that the supplementary material is referred to in the main manuscript at an appropriate point in the text. Supplementary material will be available online only and will not be copyedited, so ensure that it is clearly and succinctly presented, and that the style conforms to the rest of the paper. Also ensure that the presentation will work on any Internet browser. It is not recommended for the files to be more than 2 MB each, although exceptions can be made at the editorial office's discretion.

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### Reporting sex- and gender-based analyses

### Reporting guidance

For research involving or pertaining to humans, animals or eukaryotic cells, investigators should integrate sex and gender-based analyses (SGBA) into their research design according to funder/sponsor requirements and best practices within a field. Authors should address the sex and/or gender dimensions of their research in their article. In cases where they cannot, they should discuss this as a limitation to their research's generalizability. Importantly, authors should explicitly state what definitions of sex and/or gender they are applying to enhance the precision, rigor and reproducibility of their research and to avoid ambiguity or conflation of terms and the constructs to which they refer (see Definitions section below). Authors can refer to the Sex and Gender Equity in Research (SAGER) guidelines and the SAGER guidelines checklist. These offer systematic approaches to the use and editorial review of sex and gender information in study design, data analysis, outcome reporting and research interpretation - however, please note there is no single, universally agreed-upon set of guidelines for defining sex and gender.

### Definitions

Sex generally refers to a set of biological attributes that are associated with physical and physiological features (e.g., chromosomal genotype, hormonal levels, internal and external anatomy). A binary sex categorization (male/female) is usually designated at birth ("sex assigned at birth""), most often based solely on the visible external anatomy of a newborn. Gender generally refers to socially constructed roles, behaviors, and identities of women, men and gender-diverse people that occur in a historical and cultural context and may vary across societies and over time. Gender influences how people view themselves and each other, how they behave and interact and how power is distributed in society. Sex and gender are often incorrectly portrayed as binary (female/male or woman/man) and unchanging whereas these constructs actually exist along a spectrum and include additional sex categorizations and gender identities such as people who are intersex/have differences of sex development (DSD) or identify as non-binary. Moreover, the terms ""sex"" and ""gender"" can be ambiguous—thus it is important for authors to define the manner in which they are used. In addition to this definition guidance and the SAGER guidelines, the resources on this page offer further insight around sex and gender in research studies.

**VITA**

Julmar da Costa Feijó, filho de Jorge de Souza Feijó e Carmen Neide Araújo da Costa, nasceu em Nhamundá, Amazonas, no dia 13 de novembro de 1994. Cursou o ensino fundamental na Escola Municipal Pe. Zezinho Finlândia e o ensino médio na Escola Estadual Profª Enery Barbosa dos Santos em Nhamundá, AM. Em 2013, ingressou no curso de Zootecnia da Universidade Federal do Amazonas, Manaus, AM, obtendo o grau de Zootecnista em 2017. Iniciou, em março de 2018, o mestrado em Ciência Animal, na área de concentração em Nutrição e Produção de Não Ruminantes, na Universidade Federal do Amazonas, Manaus, AM. Obteve o título de mestre em Ciência Animal em novembro de 2019. Em abril de 2020, ingressou no curso de Doutorado em Zootecnia, área de concentração em Nutrição e Metabolismo Animal pelo Programa de Pós-Graduação em Zootecnia na Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, desenvolvendo o trabalho de tese sobre a exigência e digestibilidade de ferro para frangos de corte. Submeteu-se à banca de defesa de Tese em dezembro de 2023 pela Universidade Federal do Rio Grande do Sul em Porto Alegre, RS.