UNIVERSIDADE ESTADUAL DO OESTE DO PARANÁ
CAMPUS DE MARECHAL CÂNDIDO RONDON
PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOTECNIA

JOMARA BROCH

FITASE EM DIETAS PARA FRANGOS DE CORTE

MARECHAL CÂNDIDO RONDON
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Tese apresentada à Universidade Estadual do Oeste do Paraná como parte das exigências do Programa de Pós-Graduação em Zootecnia, área de concentração em Nutrição e Produção Animal, para a obtenção do título de "Doutor".

Orientador: Prof. Dr. Ricardo Vianna Nunes
Co-Orientadora: Profª. Drª. Cinthia Eyng
Co-Orientador: Prof. Dr. Gene Michael Pesti

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JOMARA BROCH

Fitase em dietas para frangos de corte

Tese apresentada ao Programa de Pós-Graduação em Zootecnia em cumprimento parcial aos requisitos para obtenção do título de "Doutora em Zootecnia", Área de Concentração "Produção e Nutrição Animal", Linha de Pesquisa "Produção e Nutrição de Não-Ruminantes", APROVADA pela seguinte Banca Examinadora:

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ÁREA DE CONCENTRAÇÃO: PRODUÇÃO E NUTRIÇÃO ANIMAL
LINHA DE PESQUISA: PRODUÇÃO E NUTRIÇÃO DE NÃO-RUMINANTES

STATEMENT OF REMOTE PARTICIPATION IN DEFENSE COMMITTEE OF DOCTORAL THESIS / UNIOESTE – MAL. CÂNDIDO RONDON

At 01:30 PM of 29/03/2019 (Day/Month/Year), I attended synchronously with other members of the examination committee who sign the physical Ata of this public act, the Defense Examination of the Doctoral thesis of the candidate **JOMARA BROCH** titled "Fitase em dietas para frangos de corte", enrolled in the Graduate Program in Zootecnia of Universidade Estadual do Oeste do Paraná – Unioeste (Western Paraná State University), *Campus* of Marechal Cândido Rondon, State of Paraná, Brazil.

Considering the thesis evaluation, the questions and comments of all the committee members and the student replies, I state with this document, for recording purposes, my decision that the candidate can be considered: APPROVED.

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DEDICATÓRIA

Aos meus pais, Delmir e Silvani Broch,
dedico este trabalho e todas as conquistas...

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FITASE EM DIETAS PARA FRANGOS DE CORTE

RESUMO

O objetivo deste estudo foi avaliar os efeitos de fitases em diferentes dietas para frangos de corte. No primeiro experimento, cinco tratamentos foram distribuídos em um delineamento inteiramente casualizado, com oito repetições. Os tratamentos consistiram de uma dieta controle positivo (CP), controle negativo (CN); e CN+1000, 2000 ou 3000 FYT kg^{-1} fitase. De 1 a 21 dias de idade, o ganho de peso (GP), consumo de ração (CR) e conversão alimentar (CA) elevaram-se devido ao aumento dos níveis de fitase ($P<0,05$) e de 1 a 42 dias os melhores resultados para GP, CR e CA foram obtidos utilizando 2051, 1992 e 2101 FTY kg^{-1}, respectivamente. Aos 42 dias de idade, os maiores valores do índice de Seedor (IS) e matéria seca (MS) foram obtidos com 1553 e 1765 FTY kg^{-1}, respectivamente. Aos 21 dias de idade, o conteúdo de cálcio (Ca) no sangue diminuiu com o aumento da fitase. O fósforo (P) no sangue apresentou comportamento quadrático, com o máximo registrado com 1680 FYT kg^{-1} de fitase. O conteúdo de Ca na tíbia elevou-se devido ao aumento da fitase aos 21 dias de idade ($P<0,05$). Os coeficientes de digestibilidade ileal aparente da matéria seca, matéria mineral, proteína bruta e energia bruta apresentaram respostas quadráticas, com os maiores coeficientes obtidos com a inclusão de 1164, 1592, 1085 e 1342 FYT kg^{-1}, respectivamente. Uma alta dose de 2973 FYT kg^{-1} apresentou o melhor GP entre 1 e 21 dias de idade. Dos 22 aos 42 dias, 2051 FYT kg^{-1} e 2101 FYT kg^{-1} apresentaram os melhores GP e CA, respectivamente. O segundo e terceiro experimentos foram divididos em duas fases (1-21 e 22-42 d). Quinze tratamentos foram distribuídos em um esquema fatorial 3x5, combinando dietas de alto (AF), médio (MF) e baixo (BF) fitato com dietas CP, CN (com redução de 0,15% de Ca e P) e CN+0, 500, 1000 ou 1500 FTU kg^{-1} de fitase. De 1 a 21 dias de idade, o CR atingiu o ponto máximo com 1051 FTU kg^{-1} de fitase nas dietas BF. A cinza na tíbia das aves que receberam BF apresentou uma resposta máxima com 1101 FTU kg^{-1}. O Ca sanguíneo apresentou comportamento linear em aves recebendo dietas AF e quadrático nas que receberam BF. O P sanguíneo apresentou resposta quadrática em aves alimentadas com dietas AF, MF e BF. O teor de Ca nas tíbias dos frangos recebendo dieta BF apresentou resposta linear crescente com o aumento dos níveis de fitase ($P<0,05$). Em geral, o conteúdo de P nas tíbias das aves alimentadas com dietas contendo AF foi maior do que nas dietas de BF ou MF ($P<0,05$). A suplementação de fitase melhora o desempenho e as características ósseas das aves. O uso de 1101 FTU kg^{-1} é recomendado para melhores características ósseas

em dietas LP. Aos 42 dias aves recebendo tratamento CN apresentaram menor (P<0,05) GP, FQ e MS comparado às aves do tratamento CP, pelo teste de Dunnett. O Ca e P sanguíneo das aves do grupo AF recebendo CN e CN+500 FTU kg^{-1} apresentaram maior concentração (P<0,05) que BF. O teor de P na tíbia de aves alimentadas com dietas contendo BF apresentou comportamento quadrático (P<0,05) e o nível que forneceu a resposta máxima foi 470 FTU kg^{-1}. A suplementação de fitase apresentou resposta positiva em dietas com redução de Ca e P. A fitase melhora o desempenho das aves com base na análise de regressão, com 952 FTU kg^{-1}, sem afetar negativamente os demais parâmetros avaliados.

Palavras-chave: avicultura, enzima exógena, fósforo fítico, ingredientes.

PHYTASE IN DIETS FOR BROILERS

ABSTRACT

The aim of this study was to evaluate the effects of phytases in broiler's diets. In the first experiment, five treatments were distributed in a completely randomized design, with eight replications. The treatments consisted of a positive control diet (PC), a negative control diet (NC); and the NC diet + 0, 1000, 2000 or 3000 FYT kg^{-1} phytase. From 1 to 21 days of age, weight gain (WG), feed intake (FI) and feed conversion ratio (FCR) raised due to the increasing levels of phytase ($P<0.05$), and from 1 to 42 days the best results for WG, FI and FCR were obtained using 2051, 1992 and 2101 FTY kg^{-1}, respectively. At 42 days of age, the highest Seedor Index (SI) and dry matter (DM) values were obtained with 1553 and 1765 FTY kg^{-1}, respectively. At 21 days of age, blood calcium (Ca) content decreased with increasing phytase. Blood phosphorus (P) exhibited quadratic behavior, with the maximum recorded at 1680 FYT kg^{-1} phytase. Tibia Ca raised due to the increasing phytase at 21 days of age ($P<0.05$). The apparent ileal digestibility coefficients of dry matter, mineral matter, crude protein and crude energy showed quadratic responses, with the highest coefficients obtained for the inclusion of 1164, 1592, 1085 and 1342 FYT kg^{-1} phytase, respectively. A high dose of 2973 FYT kg^{-1} had the best WG from 1 to 21 days of age. From 22 to 42 days, 2051 FYT kg^{-1} and 2101 FYT kg^{-1} showed the best WG and FCR, respectively. The second and third experiments were divided into two phases (1-21 and 22-42 d). Fifteen treatments were distributed in a 3x5 factorial arrangement, with high (HP), medium (MP) and low (LP) phytate and PC, NC (reduction of 0.15% of Ca and P) and NC diet plus 0, 500, 1000 or 1500 FTU kg^{-1} of phytase. From 1 to 21 days of age, FI peaked with supplementation of 1051 FTU kg^{-1} phytase to the LP diets. BA of broilers receiving LP showed a maximum response at 1101 FTU kg^{-1}. Ca blood had a linear behavior for broilers fed with HP and quadratic for those into LP treatments. Blood P showed quadratic responses for broilers fed HP, MP and LP diets. Ca tibia content of broilers receiving LP diets had a linear response, increasing phytase levels increased Ca content ($P<0.05$). In general, bone P of birds fed with diets containing HP was higher than those into LP or MP diets ($P<0.05$). Phytase supplementation improves the performance and bones of birds. The use of 1101 FTU kg^{-1} is advised for better bone characteristics in LP diet. At 42 broilers WG, BS and DM were lower compared to the PC, by Dunnett's Test. Serum Ca and P of birds of HP group receiving the NC and NC + 500 FTU kg^{-1} had a higher concentration ($P<0.05$) than LP. Bone P of birds fed with diets containing

LP had a quadratic behavior ($P<0.05$) and the levels that provided the maximum response were 470 FTU kg^{-1}. Phytase supplementation had a positive response in diets with reduced Ca and P. Phytase improves broilers performance based on regression analysis, with 952 FTU kg^{-1} without having a negative impact on the other parameters evaluated.

Keywords: poultry, exogenous enzymes, phytic acid, feedstuffs.

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1. INTRODUÇÃO

A avicultura é uma atividade de ciclo rápido e índices zootécnicos muito bons, os quais representam um papel de grande destaque econômico e social para o Brasil. Os frangos de corte atualmente apresentam elevada taxa de crescimento, altos índices produtivos para produção de carne e uma elevada eficiência no aproveitamento dos nutrientes das dietas. Contudo, essa excelência produtiva possui um alto custo de produção e o principal fator responsável por tornar a atividade onerosa é a nutrição.

A determinação das exigências nutricionais é de fundamental importância na produção de frangos de corte para otimizar cada vez mais o desenvolvimento e desempenho desses animais (Adedokun e Adeola, 2013), bem como para se desenvolver alternativas que possibilitem a redução dos custos, com dietas balanceadas.

Entre os nutrientes essenciais para uma ótima nutrição animal, tem se os minerais e na categoria dos macrominerais o fósforo (P) é um dos principais elementos e o mais oneroso (2 a 3% do custo total) a ser incluído na dieta. Este elemento é fundamental no metabolismo e desenvolvimento das aves, exerce papel fisiológico importante no organismo e possui relação direta com a saúde e o desenvolvimento das aves e dos ossos. Além disso, está relacionado a sérios problemas ambientais quando depositado de maneira imprópria na natureza, sendo considerado um dos principais poluentes da água e do solo (Munir e Maqsood, 2013).

As dietas para aves são compostas principalmente por produtos de origem vegetal, nos quais a maior parte do P se encontra na forma indisponível, denominada fitato; em torno de 2,5 a 4,0 g kg^{-1} (Ravindran, 1995). O fitato possui baixa solubilidade no intestino delgado, sendo mal absorvido pelas aves e sua carga negativa o confere a capacidade de formar quelatos, produzindo sais insolúveis com minerais, que reduzem a digestibilidade dos nutrientes da dieta (Wilkinson et al., 2014).

Visto que a maior parte do P contido nos alimentos utilizados nas dietas para aves se encontra na forma indisponível, fontes inorgânicas são utilizadas para fornecer as exigências deste mineral. No entanto, estas possuem um alto custo e são de fontes finitas (recursos naturais não renováveis). Para aumentar a disponibilidade deste P fítico, a fitase é adicionada às rações e possibilita a hidrólise em um nível eficaz (Bedford e Schulze, 1998).

Fatores como a relação Ca:P da dieta, a proporção em relação a outros minerais, a concentração de aminoácidos e a vitamina D podem influenciar a absorção do P (Adedokun e Adeola, 2013). Além disso, o tipo de dieta, a fonte e a quantidade de ácido fítico, aliados ao

tipo da fitase nos ingredientes utilizados, também podem interferir na utilização e aproveitamento do P pelas aves.

A molécula de fitato e os nutrientes ligados a ela não podem ser absorvidos no trato digestivo sem degradação enzimática realizada pelas fitases (Gupta et al., 2015). As fitases são as enzimas exógenas mais utilizadas em dietas comerciais para animais não ruminantes e caracterizam-se por reduzir os efeitos antinutricionais do fitato. Elas são capazes de disponibilizar o P que ocorre naturalmente na forma de fitato e, assim, reduzir a quantidade de P inorgânico suplementado na dieta e também melhorar a disponibilidade de outros minerais, de aminoácidos e energia. Além disso, contribuem para reduzir o impacto negativo da excreção de P inorgânico no ambiente (Munir e Maqsood, 2013).

Diante disso, o efeito de fitases foi avaliado em diferentes dietas para frangos de corte, sobre desempenho, parâmetros sanguíneos, características ósseas, rendimento de carcaça e cortes.

2. Revisão

2.1. Cálcio e fósforo na nutrição de frangos de corte

O Ca e o P são os elementos minerais mais abundantes do organismo e os principais cátions da dieta. Cerca de 98% do Ca encontra-se como fosfato de cálcio ($Ca_3(PO_4)_2$) no esqueleto, os outros 2% estão distribuídos nos fluidos extracelular e celular, exercendo papel essencial no metabolismo, coagulação do sangue, ativação enzimática e função neuromuscular (Pond et al., 2005). Aproximadamente 80% do P ocorrem como constituintes dos ossos e 20% como componentes de compostos orgânicos, exercendo papel no metabolismo (ATP, creatinina, enzimas), em ácidos nucleicos (DNA, RNA) e em fosfolípidos de membrana (France et al., 2010).

Os ossos servem como armazéns de minerais que são mobilizados quando a absorção é inadequada, para satisfazer as necessidades do corpo. A formação e mineralização do tecido ósseo ocorrem no período fetal, com a competição dos osteoblastos (células responsáveis pela formação óssea), tanto para a síntese da matriz proteica quanto para sua subsequente mineralização. O processo de renovação do osso ao longo da vida para manter suas propriedades biomecânicas é dado pela ação dos osteoclastos (células responsáveis pela reabsorção óssea), que digerem o tecido ósseo produzindo uma saída da fase mineral para a

corrente sanguínea. Posteriormente, ocorre a formação de um novo tecido pela ação dos osteoblastos, que necessita a entrada de Ca e P para a mineralização (Gómez Alonso et al., 2004).

O Ca e o P estão intimamente relacionados, e a deficiência ou excesso de qualquer um interferirá na utilização e metabolismo do outro. A regulação da homeostase destes minerais é mantida através dos sistemas esquelético e endócrino, os rins e o intestino delgado. O sistema hormonal é constituído por calcitonina, hormônio da paratireoide (PTH) e vitamina D (1,25 dihidroxicolecalciferol) ou calcitriol.

A modulação da absorção, depósito e excreção para manter os níveis séricos de Ca e P constantes dependem de fatores dietéticos: fonte dos nutrientes (que afeta a digestibilidade ou disponibilidade), concentração de Ca, P e vitamina D_3; fatores fisiológicos: status do hormônio da paratireoide, status reprodutivo e pH do sangue, e fatores ligados ao animal: linhagem e idade (Adedokun e Adeola, 2013). Por isso, de acordo com estes autores, é importante compreender a interação entre os diversos fatores, principalmente Ca, P e vitamina D_3 e adaptar estas relações de acordo com as diferentes linhagens e idade das aves, ingredientes da ração e até mesmo aos níveis de inclusão de fitase.

O Ca é absorvido principalmente no duodeno e jejuno, por difusão simples, paracelular e não-saturável, e a capacidade de absorção é estimulada diretamente pela vitamina D e dependente da quantidade ingerida e da biodisponibilidade dietética deste mineral (Pond et al., 2005). O controle da homeostase do Ca é realizado através das interações entre PTH, calcitonina e vitamina D ativa ($1,25(OH)_2D_3$) em receptores específicos, havendo um equilíbrio entre a absorção intestinal e as perdas por excreção renal. Quando a dieta é deficiente ou há um aumento nas exigências de Ca, ocorre uma redução na absorção e na concentração plasmática desse mineral. Isso estimula a secreção de PTH, que leva à ativação da 1-α-hidroxilase no rim, promovendo a formação da vitamina D ativa ($1,25(OH)_2D_3$), ou calcitriol), e liberação do Ca e PO_4 dos ossos aumentando a reabsorção óssea, resultando no aumento da absorção e metabolismo do Ca no intestino delgado e reabsorção do Ca no rim. Em contrapartida, o excesso de Ca circulante desencadeia reações contrárias ao PTH; há um estímulo para secreção de calcitonina, que leva à reabsorção do Ca tubular, favorecendo o depósito de Ca nos ossos e aumento da excreção renal, reestabelecendo a homeostase desse elemento no organismo (Gómez Alonso et al., 2004).

A absorção do P ocorre através do cruzamento da membrana da borda em escova intestinal por transporte ativo, e também é estimulado pela vitamina D ($1,25(OH)_2D_3$). O esquema geral é semelhante ao do Ca, mas com o PO_4 a regulação principal é entre as

entradas e as perdas renais, sendo necessária uma concentração adequada de PO4 sérico para produzir uma mineralização. O PTH é o principal regulador da excreção renal de fosfatos, inibindo a reabsorção tubular. Altos níveis de fosfato no sangue estimulam a secreção de PTH (que promovem sua eliminação renal) e inibem a 1-α-hidroxilase renal, que diminui a síntese de vitamina D ($1,25(OH)_2D_3$) e, portanto, sua absorção intestinal e reabsorção renal. Em contrapartida, quando há deficiência de P na dieta, ou as exigências de P são elevadas, a concentração de P plasmático reduz. A baixa concentração plasmática de P leva à formação da vitamina D ativa ($1,25(OH)_2D_3$) no rim que, por sua vez, leva ao aumento da absorção de P no intestino delgado e reabsorção de P no rim. Ao mesmo tempo, a mobilização óssea é induzida a manter uma concentração normal de P plasmático. Devido aos mecanismos de regulação hormonal, a calcemia e a fosfatemia tendem a mover-se na direção oposta, mantendo um produto constante, exceto quando há déficit no sistema de vitamina D ou destruição óssea em massa (Gómez Alonso et al., 2004).

2.2. Ácido Fítico

O ácido fítico (mio-inositol 1,2,3,4,5,6 - hexaquis (dihidrogênio) fostato) (IUPAC- IUB, 1977), é um ácido livre essencial durante a germinação das sementes e responsável por suprir as necessidades de biossíntese dos tecidos em crescimento das plantas. Os sais do ácido fítico, descritos como fitatos, correspondem a uma mistura de minerais, como potássio, magnésio e cálcio, presentes como quelato e armazenados na forma de fósforo (P) em cereais, legumes e óleos (Pallauf e Rimbach, 2009).

O teor de ácido fítico e a disponibilidade do P para os animais é altamente variável (Tabela I). Esta variabilidade pode depender das condições de crescimento da planta, do tamanho das partículas e dos processos tecnológicos utilizados no beneficiamento dos cereais (Tahir et al., 2012), além dos métodos utilizados para sua determinação. Sementes oleaginosas, grãos integrais e leguminosas representam as fontes mais concentradas, já as raízes, tubérculos e outros vegetais geralmente apresentam quantidades mais baixas; na maioria dos grãos o fitato é isolado na camada de aleurona, o que o torna mais concentrado no farelo, já nas leguminosas, é encontrado na camada de cotilédone (Nissar et al., 2017).

Tabela 1. Fósforo total, fítico e disponível nos ingredientes.

Ingredientes	P total (%)	P fítico (%)	P disponível (%)
Arroz, farelo	1.71	1.37	0.35
Aveia, grão	0.38	0.16	0.22
Canola, farelo	1.14	0.75	0.39
Carne e ossos, farinha (48%)	5.79	-	5.21
Cevada, grão	0.35	0.20	0.15
Mandioca, integral raspa	0.08	0.06	0.02
Milho, grão (7,86%)	0.24	0.18	0.06
Milho, glúten (60%)	0.52	0.47	0.05
Penas e vísceras, farinha	1.15	-	1.15
Soja, farelo (45%)	0.55	0.36	0.19
Trigo, farelo	0.94	0.45	0.49
Trigo, grão	0.32	0.22	0.10

Fonte: Rostagno et al. (2017)

O fitato é carregado negativamente nas diversas condições de pH (ácido, neutro e básico). Isso lhe confere a capacidade de se precipitar com as moléculas carregadas positivamente da dieta, secreções endógenas do trato gastrointestinal e a proteína dietética, formando complexos resistentes à hidrólise. Desta forma, a digestibilidade dos nutrientes da digesta é reduzida, acarretando na sua utilização incompleta pelos animais (Woyengo e Nyachoti, 2013). A capacidade de ligação dos grupos de fosfato a cátions é afetada pela sua distribuição no anel de mio-inositol; os complexos são mais solúveis com a redução e mais fracos com a remoção dos grupos de fosfato (Nissar et al., 2017).

As dietas típicas de frangos de corte contêm em torno de 2,5 a 4,0 g de fitato kg^{-1}. Para que o P seja utilizado, o fitato deve ser hidrolisado para que os íons de fosfato inorgânico sejam liberados, e isto dependerá da capacidade enzimática das aves. A degradação do fitato no trato digestivo das aves pode ser atribuída à uma ou mais fitases e elas são possíveis através de três fontes: fitases da secreção digestiva intestinal; atividade de fitase proveniente de bactérias residentes ou atividade da fitase endógena presente em alguns ingredientes (Ravindran, 1995).

2.3. Mecanismos de atuação das fitases

As fitases hidrolisam o fitato em uma molécula de inositol e seis moléculas inorgânicas de fosfato; assim, se o fitato é hidrolisado em seguida os seus efeitos antinutricionais são reduzidos, podendo ser utilizado pelas aves (Ravindran, 1995). A suplementação de fitase em dietas de aves é uma prática comum, utilizada em larga escala e importante, devido à atividade inadequada da fitase endógena do trato digestivo das aves. A fitase melhora a utilização do P fítico e reduz a excreção de P no ambiente, isto atrai um grande interesse científico e comercial (Munir e Maqsood, 2013).

As enzimas exógenas são aditivos que não têm função nutricional direta, mas ajudam no processo digestivo melhorando a digestibilidade dos nutrientes da dieta. Desde o final da década de 80, elas desempenham papel importante no aumento da eficiência na produção de carne e ovos, através da sua capacidade de alterar o perfil nutricional dos ingredientes das rações (Bedford e Partridge, 2001).

Na nutrição animal, as enzimas exógenas são responsáveis por degradar fatores antinutricionais presentes em muitos ingredientes da ração, aumentar a disponibilidade de alguns nutrientes, complementar as enzimas produzidas por animais jovens que, devido à imaturidade do sistema digestivo tem produção insuficiente, reduzir a grande variabilidade nos valores nutritivos dos alimentos, melhorando assim a precisão nas formulações de rações (Munir e Maqsood, 2013). No entanto, a resposta das enzimas está associada a três componentes: enzima, substrato e a ave. Este conjunto deve ser considerado para garantir e melhorar os benefícios da utilização das enzimas (Ravindran, 2013).

É importante que as enzimas utilizadas sejam específicas ao substrato disponível, para que possam agir com eficiência. Por exemplo, o ambiente considerado ideal deve ser aquoso, pois a umidade é essencial para a mobilidade e solubilidade da enzima e do substrato; altas temperaturas podem resultar em desnaturação e redução da atividade enzimática e a relação substrato *vs* enzima deve ser adequada, sendo que quanto mais substrato, melhor e maior a área de atuação para a enzima (Ravindran, 2013).

A relação substrato *vs* enzima está relacionada com a eficácia da enzima. A partir daí, é importante considerar que a presença de substratos nos ingredientes é bastante variável e dependente da localização desse substrato na matriz do ingrediente, da presença de outros possíveis fatores antinutricionais e da diferença na acessibilidade ou solubilidade da enzima (Olukosi, 2013).

A hidrólise do fitato em ortofosfato e fosfatos de inositol é conseguida enzimaticamente com fitase. Este método reduz o conteúdo de ácido fítico nos grãos, sem reduzir o seu conteúdo mineral (Gupta et al., 2015). A fitase (mio-inositol 1,2,3,4,5,6 - hexaquis fosfato fosfohidrolases) é a única enzima conhecida que pode iniciar a desfosforilação gradual do fosfato no carbono 1, 3 ou 6 no anel inositol do fitato, gerando uma série de ésteres fosfatos mio-inositol inferiores (IP 6 ⇒IP 5 ⇒IP 4 ⇒IP 3 ⇒IP 2 ⇒IP 1). Através dessa sucessão de reações de desfosforilação, são produzidos seis radicais de P inorgânico e inositol (Selle e Ravindran, 2007), além da liberação de cálcio, ferro, zinco e outros metais.

As fitases podem ser divididas em três grupos: com base no mecanismo catalítico, têm-se as fitases ácidos histidina, de cisteína ou ácido roxo; com base no pH, dividem-se em fitases ácidas e alcalinas; e também com base no carbono no anel de mio-inositol de fitato em que a desfosforilação é iniciada, em 3-fitases (EC 3.1.3.8), 6-fitases (CE 3.1.3.26) e 5-fitases (EC. 3.1.3.72) (Greiner e Konietzny, 2006).

A atividade da fitase foi detectada em muitas espécies de plantas como trigo, centeio, cevada, ervilha, feijão, soja, milho, arroz, alface, espinafre, grama, pólen de lírio, etc, mas pelo fato do processo de produção a partir de plantas ser oneroso e demorado, a produção de fitase de origem microbiana é a mais desenvolvida (Gupta et al., 2015).

A atividade de fitase é expressa em FTU, que corresponde à quantidade de fitase que libera 1 mol de fosfato inorgânico por minuto a partir de 0,0051 mol L^{-1} fitato de sódio em pH de 5,5 e à uma temperatura de 37°C (AOAC, 2000). Contudo, em termos práticos, a especificação padrão de mensuração estabelecida para atividade de fitase é diferente das condições reais *in vivo* dos animais e, além disso, muitas características associadas à composição da dieta e características dos animais podem influenciar a atividade da enzima *in vivo.*

A atuação da fitase está relacionada às características ligadas aos animais (espécie, idade, condições fisiológicas), aos fatores dietéticos (concentração e fonte de fitato, e minerais), e à origem e nível da fitase adicionada à dieta (Dersjant-Li et al., 2015). O nível dietético de fósforo (P) também pode influenciar na resposta da fitase, por isso, níveis muito altos ou baixos devem ser evitados; altos níveis de Ca ou alta relação Ca:P pode reduzir a resposta da fitase; e a vitamina D exerce influência indireta na atividade da fitase através do aumento da absorção de Ca, limitando a formação de fitatos de Ca insolúveis, resistentes à hidrólise da enzima (Kornegay, 2001).

Propriedades como estabilidade, resistência à protease, inativação pelo HCl no estômago e a origem da enzima são essenciais para a ação eficiente das fitases na alimentação

dos animais (Oluski, 2013). Outro aspecto importante é o local da atividade de diferentes tipos de fitases no trato digestório do animal; pesquisas sugerem que a parte superior do trato digestivo é o principal local.

O nível do pH no estômago das aves está entre 2,5 a 3,5; ou seja, valores muito abaixo de 5,5, valor da mensuração padrão da atividade da fitase, portanto a atividade "real" in *vivo* é muito variável. Como o ácido fítico (e fitato) dissocia-se e é solúvel em pH ácido (por exemplo, estômago), a formação dos minerais e complexos ocorre principalmente em pH mais elevado, como do intestino. Assim, os ácidos fíticos se complexam com cálcio, proteínas e aminoácidos, além de interagirem com enzimas endógenas, resultando na redução da digestibilidade dos nutrientes (Dersjant-Li et al., 2015).

Deste modo, uma hidrólise prévia do fitato pela fitase na parte superior do trato digestivo é essencial para uma melhora na digestibilidade dos nutrientes; isto resultará em uma molécula de inositol e seis moléculas inorgânicas de fosfato (mais aminoácidos, minerais entre outros nutrientes). Em casos de uma hidrólise incompleta, normalmente pode restar IP4 e IP3, que são muito resistentes ao ataque das fitases. Assim, o sucesso de altas doses de fitase depende da sua especificidade ao substrato e também da destruição destes ésteres de fosfatos remanescentes e da geração do inositol através do esforço conjunto da fitase exógena e das fosfatases da mucosa.

2.4. Suplementação de fitase em dietas para frangos de corte: efeitos extra fosfóricos

A fitase tem sido utilizada para reduzir o custo da dieta através da possibilidade de redução de fontes de fosfato inorgânico, energia, calcário e aminoácidos sintéticos. Esses efeitos estão ligados a uma matriz de liberação de nutrientes para uma determinada dose da enzima e o valor criado dependerá dos preços dos vários nutrientes deslocados (Cowieson et al., 2015). Antigamente, utilizava-se uma dose fixa de 500 FTU kg^{-1} em ração de frangos, por exemplo, mas com os avanços das pesquisas, e devido a fatores econômicos, é grande o interesse do uso de doses mais elevadas.

Um pré-requisito para a formulação de rações é a equivalência do P da fitase, no entanto, este valor ainda não está bem definido. Os valores para equivalência da fitase são conflitantes e os critérios de resposta utilizados para avaliar estes valores possuem um efeito importante sobre os resultados. Segundo Selle e Ravindran (2007) o valor geral determinado para a equivalência de P da fitase (840 FTU kg^{-1} = 1.0 g kg P) não é exatamente o valor real

sugerido na prática. Isto porque os resultados são afetados pelo teor e a fonte de P, nível de Ca, tipo de dieta, espécie e idade do animal (animais jovens tendem a responder melhor às enzimas do que animais mais velhos) (Anselme, 2006); além do tipo e a quantidade de cereais, os fatores antinutricionais e as enzimas utilizadas (Munir e Maqsood, 2013).

A magnitude da resposta da fitase pode ser mais significativa com o aumento dos níveis de inclusão nas dietas, provavelmente devido à maior degradação do fitato, pois quando este é hidrolisado os seus efeitos antinutricionais são eliminados (Kornegay, 2001). Além disso, a degradação do fitato se correlaciona positivamente com grandes aumentos na retenção de P, concentração de cinzas na tíbia, ganho de peso, consumo de ração, eficiência alimentar, retenção de nitrogênio, energia metabolizável aparente e retenção de Ca; resultados que são mais pronunciados com altos níveis de inclusão (Selle e Ravindran, 2007).

Alguns resultados sugerem que o aumento dos níveis de P dietético pode impedir as respostas ao aumento dos níveis de inclusão de fitase, existem duas explicações para isso: o produto final da hidrólise do fitato, o P inorgânico, inibe a atividade catalítica da fitase (Lei e Stahl, 2000); e o aumento da liberação de P, devido à ação da fitase, pode provocar um desequilíbrio entre o Ca e P no trato gastrointestinal do animal. Outra explicação é que altos níveis de fitase podem alterar o balanço eletrolítico da dieta, pois o fitato e a fitase influenciam a secreção de sódio no lúmen intestinal (Ravindran et al., 2013).

Outro aspecto muito importante é o modelo utilizado para interpretação dos resultados. Como todas as enzimas, as fitases exibem uma cinética de Michaelis-Menten com retornos marginais decrescentes (Shirley e Edwards, 2003). As respostas são melhores descritas ou modeladas por métodos capazes de ajustar transições suaves de porções ascendentes a platôs. A relação entre dose e resposta da fitase foi instituída como log-linear, ou seja, é preciso um aumento logarítmico da dose para manter um incremento linear de resposta (Kornegay, 2001). Com o nível de fitase expresso na base Log, as respostas tendem a ser menores por unidade de fitase dietética, com maiores respostas observadas em doses Log mais altas de fitase. Essa transformação permite um espaçamento de pontos dos dados mais semelhante e remove platôs da resposta da enzima. Assim, Log torna-se o modelo mais apropriado para interpretação dos dados (Shirley e Edwards, 2003).

Altas doses de fitase podem ser benéficas, no entanto, é necessário adequar os níveis de nutrientes e os demais fatores dietéticos, para que as vantagens sejam perceptíveis (Selle e Ravindran, 2007). Também é preciso considerar que a atuação da fitase está relacionada às características ligadas ao animal (espécie, idade, condições fisiológicas), aos fatores dietéticos (concentração e fonte de fitato, concentração de minerais) e à origem e nível da enzima

adicionada à dieta (Dersjant-Li et al., 2015). Também, é muito importante escolher o modelo apropriado, pois os dados se ajustam melhor a um modelo específico, então eles podem fornecer estimativas diferentes dos níveis de uso que maximizam os lucros (Bedford et al., 2016).

Em estudo realizado por Boney e Moritz (2017) com frangos de corte, os autores constataram melhora na conversão alimentar e aumento da disponibilidade de P, além de influências benéficas na saúde intestinal, possivelmente devido à uma redução da irritação do intestino. Os autores especulam que a eficácia da fitase pode ser afetada dependendo da composição dos ingredientes utilizados e da presença de fatores antinutricionais.

Ao avaliar se a eficácia da fitase poderia ser afetada por uma fonte de proteína da dieta, Kaczmarek et al. (2016) observaram que a fitase melhorou o ganho de peso corporal, a taxa de conversão alimentar e a deposição de Ca e P nas tíbias, independentemente da fonte proteica. A melhora no conteúdo de cinzas, Ca e P na tíbia indica um aumento na mineralização óssea, referente ao aumento na disponibilidade de minerais liberados pela fitase a partir do complexo mineral do fitato. A desfosforilação do ácido fítico pela fitase provavelmente levou a uma melhor mineralização óssea via maior digestibilidade ileal do Ca e P.

Ainda segundo os autores, é possível que ocorra variações na degradação do fitato em diferentes ingredientes, isto depende da localização dos fitatos, o que pode torná-los mais resistentes ao ataque direto da fitase. A eficácia da fitase sobre a digestibilidade dos aminoácidos também parece depender do ingrediente utilizado na dieta, estando ligada ao tipo e concentração da proteína; ressaltando que proteínas formam complexos insolúveis com ácido fítico em pH baixo, já reportado em diversas literaturas.

Os resultados encontrados por Cowieson et al. (2015) em experimentos realizados com frangos de corte recebendo altas doses de fitase apontaram melhora no desempenho, aumento na retenção de Ca e P, resistência da tíbia, teor de cinzas e concentrações de inositol no plasma. Os resultados sugeriram que o efeito benéfico de altas doses de fitase pode ser conferido através de mecanismos similares ao da insulina e que os efeitos da fitase são eficazes na melhoria do desempenho das aves alimentadas com dietas com níveis adequados ou não de Ca e P.

Ao avaliarem os benefícios da suplementação de fitase em dietas para frangos de corte, Milica et al. (2012) constataram que a adição de fitase proporcionou uma redução na mortalidade e melhora no desempenho das aves, além da redução dos efeitos negativos das dietas com níveis reduzidos de P total e disponível. Os resultados das análises histológica,

física e química das tíbias das aves indicaram que as mudanças dependem da deficiência de P e da adição de fitase, mas, de modo geral, a fitase foi mais eficiente em dietas com um nível reduzido de fosfato dicálcico.

Os efeitos da fitase sobre as propriedades histológicas, mecânicas e químicas da tíbia também foram avaliadas por Qian et al. (1996). No experimento, foi observado que a deficiência do P influenciou o grau de conversão da cartilagem em osso e a ordem do desenvolvimento histológico da tíbia provocando uma mineralização defeituosa ou desorganizada da matriz extracelular da zona de cartilagem hipertrófica. Já as melhoras das características histológicas da tíbia foram devido à suplementação de fitase e ao P inorgânico; as tíbias foram mais longas e largas e houve uma melhora na força de ruptura, ou seja, ocorreu uma melhor mineralização óssea. Além dos benefícios sobre as características ósseas, a fitase melhorou o ganho de peso corporal e o consumo de ração. Os resultados sugerem que a fitase melhora a qualidade da dieta por meio da liberação de outros minerais e nutrientes, além de aumentar a disponibilidade de P e promover o crescimento e desenvolvimento dos ossos, assim, a quantidade de P inorgânico adicionado pode ser reduzida.

As informações sobre os efeitos da fitase em dietas com redução nutricional sobre rendimentos e características de qualidade de carcaça ainda são limitadas. Os resultados do trabalho realizado por Driver et al. (2006) indicaram que dietas com deficiência de Ca e P, durante as fases inicial e final, afetam a integridade dos diferentes ossos das aves de diferentes maneiras durante o abate e o processamento. A resistência de ruptura da tíbia e fêmur (ossos longos) parece ser influenciada pelo conteúdo de Ca e P de dietas iniciais, pois é nesta fase que o desenvolvimento ósseo é mais ativo; enquanto que a incidência de clavículas (osso curto) com ruptura foi influenciada apenas pelo tipo de dieta durante a fase final, pois é mais sensível às flutuações nos níveis de Ca e P a curto prazo. Assim, conclui-se que a qualidade da carcaça depende dos níveis de Ca e P e também da idade da ave.

Além de todos os benefícios supracitados, a fitase é apontada como responsável no aumento da digestibilidade do P e redução da excreção fecal de P e isto é muito importante, pois os resíduos fosfatos dos animais representam um grande problema ambiental, pois são contaminantes de reservatórios de água, através do escoamento superficial ou lixiviação (Selle e Ravindran, 2007, Munir e Maqsood, 2013).

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3. HIGH LEVELS OF DIETARY PHYTASE IMPROVES BROILER PERFORMANCE

Abstract

The objective of this study was to evaluate the effects of dietary phytase on broilers from 1 to 42 days of age. Five treatments were distributed in a completely randomized design, with eight replicates of 23 birds per experimental unit (per averages). The treatments consisted of a positive control diet (PC), a negative control diet (NC); and the NC diet + 1000, 2000 and 3000 FYT kg^{-1} phytase. The effects of dietary treatments on performance, bone quality and blood minerals were determined. From 1 to 21 days of age, WG, FI and FCR increased with increasing levels of phytase ($P<0.05$), and from 1 to 42 days, body weight peaked with 2000 FTY kg^{-1} ($P<0.05$). The best results for WG, FI and FCR were obtained using 2051, 1992 and 2101 FTY kg^{-1}, respectively. At 42 days of age, the Seedor Index (SI) and bone-dry matter (DM) were maximized at lower levels of phytase than for WG or FCR. The highest SI and DM values were obtained with 1553 and 1765 FTY kg^{-1}, respectively. At 21 days of age, blood Ca content decreased with increasing phytase levels. Blood P exhibited quadratic behavior, with the maximum recorded at 1680 FYT kg^{-1} phytase. Tibia Ca increased with increasing phytase at 21 days of age ($P<0.05$). Blood P at 42 days of age was lower than at 21 days but did not vary between treatments. The apparent ileal digestibility coefficients of dry matter, mineral matter, crude protein and crude energy showed quadratic responses, with the highest coefficients obtained for the inclusion of 1164, 1592, 1085 and 1342 FYT kg^{-1} phytase, respectively. It is concluded that phytase improves broiler performance based on regression analyses. A high dose of 2973 FYT kg^{-1} had the best WG from 1 to 21 days of age. From 21 to 42 days, 2051 FYT kg^{-1} and 2101 FYT kg^{-1} showed the best weight gain and fed conversion ratio, respectively. These recommendations do not negatively affect the other parameters evaluated.

Keywords: Bone parameters; enzyme; phosphorus; poultry production.

3.1. Introduction

Characterized by the rapid cycling and efficient conversion of plant to animal protein, poultry production is one of the most advanced agribusiness production chains in the world. Modern broilers high growth rates, high production rates of meat, and efficient use of nutrients in diets. However, evaluation of the nutritional requirements of the birds must be constantly re-evaluated in order to optimize the maximum performance of these animals (Adedokun and Adeola, 2013).

Minerals have a high degree of nutritional importance and are considered essential elements in the metabolism and development of animals. Phosphorus is one of the main minerals present in diets and is essential for the development of birds, playing a major physiological role in the body, with its dietary deficiency related to constant bone problems. Phosphorus is also considered one of the main pollutants of soil and water when applied excessively to the environment (Lukić et al., 2009).

Several factors can affect the use of phosphorus by animals, including calcium and phosphorus levels in the diet, vitamin D and its active forms, its relationship with other minerals such as sodium, chlorine and potassium, the type of diet used, and the amount of phytic acid present in the diet (Adedokun and Adeola, 2013).

Phytic acid, also called phytate or phytin, is an essential component of seeds and is responsible for meeting the biosynthesis needs of growing plant tissues. This compound complexes to positively charged molecules such as dietary proteins, amino acids and proteolytic enzymes, reducing the digestibility of amino acids. During the digestion of lipids, the calcium-phytate complex can react with fatty acids to form insoluble soaps in the intestinal lumen. Phytate can also bind to starch, inhibiting the action of amylase and consequently reducing the digestibility of carbohydrates (Kornegay, 2001; Woyengo and Nyachoti, 2013).

Broiler diets are based on feed ingredients from plant sources, seeds or seed products, with 60 to 80% of their phosphorus content in the form of phytate and thus unavailable to broilers. Typical broiler diets contain from 2.5 to 4.0 g kg^{-1} of phytate (Ravindran, 1995). As broilers cannot hydrolyse phytate since they do not synthesise specific digestive phytases, the use of exogenous sources of phosphorus, such as minerals or feeds of animal origin, is necessary to avoid P deficiency in the poultry metabolism.

Exogenous enzymes have been used to provide more nutrients from feed, allowing the nutritionist greater flexibility in choosing the types of ingredient to be used in feed

formulation. In addition, enzymes have an important role in reducing the negative environmental impact of animal production through reducing waste excretion.

Phytases are the enzymes responsible for hydrolyzing one phytate molecule to inositol and six inorganic phosphate molecules (Yao et al., 2012). When phytate is hydrolyzed, its inhibitory effects are eliminated (Kornegay, 2001), with the magnitude of the phytase response more significant with increasing inclusion levels in diets, likely due to higher phytate degradation. Phytate degradation is known to correlate with large increases in P retention, tibia ash concentration, weight gain, feed intake, feed efficiency, nitrogen retention, apparent metabolizable energy and Ca retention, all of which are more pronounced with a high level of dietary phytase inclusion (Selle and Ravindran, 2007). However, like all enzymes, phytases exhibit Michaelis-Menten kinetics with diminishing marginal returns (Shirley and Edwards, 2003); responses are best described or modelled by methods capable of fitting smooth transitions from ascending to plateau portions.

The objective of this study was to evaluate the effects of dietary phytase on broilers from 1 to 42 days of age.

3.2. Material and methods

This study was conducted according to the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiment. It was carried out in the Poultry Sector of the Experimental Station of the State University of the West of Paraná - UNIOESTE, *Campus* Marechal Cândido Rondon – PR, Brazil. A total of 920 male one-day-old Cobb 500 broiler chicks, were used at this experiment. The animals received feed and water *at libitum*, with a continuous 24h lighting program.

The broilers chicks were distributed in a completely randomized design with five treatments and eight replicates per treatment in 40 pens (experimental unit – EU, with 1.76 m^2 each, with a stocking density of 13.07 birds per m^2). Each pen contained a tubular feeder, nipple drinkers, a heating source (250-watt infrared lamps) and a concrete floor coated with pine shavings. The treatments consisted of; 1) a positive control diet (PC) which aimed to provide the nutritional requirements of the animals; 2) negative control diet (NC) with nutritional reduction of 0.12% of calcium and 0.14% of phosphorus; 3 - 5) NC diet with addition of 1000, 2000 and 3000 FYT kg^{-1} phytase (RONOZYME ® HiPhos GT, DSM Nutritional Products, Kaiseraugst, Switzerland) is a microbial 6-phytase expressed through the use of synthetic genes in Apergillus oryzae with phytase activity of 10000 phytase units (FYT) per g. One phytase unit is defined as the amount of enzyme that releases 1 µmol of

inorganic phosphate under standard conditions (0.25 M acetate buffer pH 5.5, 37ºC and 5 mmol sodium phytate).

The experimental diets were mash, isoprotein and isocaloric. They were formulated based on corn, soybean meal and 3.00% of wheat bran according to Brazilian Tables for Poultry and Swine (Rostagno et al., 2011). Birds were fed pre- starter (1 to 7 days), starter (8 to 21 days), grower (22 to 35 days) and finisher (36 to 42 days) diets (Table 1). Celite® was used as indigestible marker at finisher phase.

Feed intake and body weights were recorded at 21 and 42 days of age, to evaluate the performance of the birds. Feed intake and feed conversion ratio were determined and corrected using the weight of dead birds, according to Sakomura and Rostagno (2007).

Performance graphs were performed to compare the phytase response curve in Log 10 (Phytase + 100) and FYT. For this, a correction factor was calculated for each variable (WG, FI, FCR):

X (Mean of the 21-day performance variable of each treatment) / Y (Mean of the 42-day performance variable of each treatment) = Correction factor

Correction factor * The mean of the variable of each treatment at 42 days of age

This makes the values of 42 days equivalent to the values of 21, allowing a better graphic visualization.

For the evaluation of bone development, two birds at 21 and 42 days of age with mean group weights (±5%) were weighed and sacrificed using cervical dislocation according to resolution number 1000/2012 of the CFMV. The legs were separated and deboned to obtain tibiae.

After deboning, the left tibiae were weighed to the nearest ± 0.0001 g and its length was determined using a digital caliper (accuracy of 0.01 mm). The bone density was calculated by dividing the bone weight (mg) by its length (mm), thus obtaining the Seedor Index (SI) (SEEDOR et al., 1991). After its determination, tibiae were stored individually at -20ºC for further analysis.

Determination of bone breaking strength was performed after bone thawing at room temperature. The tibiae were individually supported on the epiphyses regions. A force load of 200 kgf at the speed of 5 mm s^{-1} was applied in the central region of each bone using a probe TA-TPB and a Texturometer (CT3 Texture Analyzer, Brookfield).

After the bone strength was measured, the tibiae were weighed on an analytical balance (± 0.0001 g) and then analyzed for dry matter analysis (Silva and Queiroz, 2002) after which the samples were weighed, ashed overnight at 600 C, and weighed again (*Adapted* Hall

et al., 2003). The percentage of tibiae ash was calculated as the proportion of the dry, pre-ashed tibiae multiplied by 100.

To determine the amount of calcium and phosphorus in the bones, the ashes were placed in a sand bath (250ºC) in a solution of HCl (6 M) to solubilize the minerals. Calcium was measured using an atomic absorption apparatus (GBC-932AA) and phosphorus using a spectrophotometer (UV/VIS GBC-916).

At 21 and 42 days of age, two birds per pen, were randomly chosen, fasted for 6 h and blood samples were collected via brachial puncture. Blood was rested for coagulation and centrifuged (Centrifuge Baby I 206 BL) at 3000 rpm for 10 min to obtain serum, which was stored at -20 °C. To perform the analyzes the serum was thawed at room temperature, centrifuged at 3000 rpm for 5 min and calcium and phosphorus analyzes were performed using a high performance automatic spectrophotometer (Flexor EL 200 Biochemical Analyzer) with automatic calibration (Elical) and commercial kits (Elitech).

To evaluate the incidence of tibial dyschondroplasia, the left leg tibia of 42 day old birds were decalcified with 50% formic acid and 20% sodium citrate (Fernandes et al., 2007). After decalcification, the bone was embeded in paraffin (Beçak and Paulete, 1976). The sections were made with microtomes at 5 μm thickness and stained with Hematoxylin-Eosin, for observation of the epiphyseal disk area and measurements of the areas to characterize the incidence of tibial dyschondroplasia.

For analysis of tibial epiphyseal cartilage slides, three distinct regions characterized by the morphological appearance were considered: resting zone, proliferative cartilage zone and hypertrophic cartilage zone. The images were measured with the aid of a computerized image analyzer PROPLUS IMAGE 4.1.

At 42 days of age, four birds were selected per pen to evaluate carcass yield and parts: by wing, whole leg, bone in breast, breast, boneless breast meat and abdominal fat (fat removed from around the cloaca and gizzard).

At 42 days of age, four birds were selected to determine the ileal digestibility of nutrients. The ileum contents were collected, weighed and freeze-dried (Liotop, L 101) for 48 h after being weighed again and then ground in a ball mill (Tecnal). Dry matter, mineral matter, crude protein and insoluble acid ash were determined in the feed samples and digesta by the methods of Silva e Queiroz (2001). Gross energy was determined by bomb calorimetry (Calorimeter C2000, IKA). Insoluble acid ash was used as an inert marker (Sakomura and Rostagno, 2016). Dry matter, crude protein digestibility coefficients and the digestible energy values were calculated according Sakomura and Rostagno (2016).

Data were analyzed by SAS softwer package (Statistical Analysis System, 2011). Polynomial regression between levels of inclusion of the enzyme was performed excluding the positive control treatment. In addition, the Dunnett`s test was performed at 5% probability to compare each experimental mean (NC; NC + 1000; 2000 and 3000 FYT kg^{-1}) with the control mean (PC). Dunnett's test controls the experiment wise error rate and is more powerful than tests designed to compare each mean with each other mean.

3.3. Results

From 1- 21 days of age, weight gain (WG), feed intake (FI) and feed conversion ratio (FCR) showed an improvement with increasing levels of phytase ($P<0.05$). WG and FI values were significantly different ($P<0.05$) compared to the positive control (PC) treatment according to Dunnett´s test. Broilers that received available P and Ca deficient diets (negative control - NC), without phytase supplementation, exhibited the lowest WG and reductions in FI. Broilers receiving 3000 FYT kg^{-1} achieved the best WG compared to the positive control (PC) treatment (Table 2).

Performance was increased by phytase addition in a quadratic manner ($P<0.05$) from 1 to 42 days of age. The best results for WG, FI and FCR_C were obtained using 2051, 1992 and 2101 FTY kg^{-1}, respectively. All birds in the NC treatment exhibited significantly different performance ($P<0.05$) with respect to those in the PC treatment according to Dunnett`s test. Broilers in the NC treatment had lower WG, FI and worse FCR_C; however, broilers receiving 2000 FYT kg^{-1} achieved better FCR_C than those in PC.

Economic simulations were generated to evaluate the different models. Four logarithmic equations were generated (according to performance data of 42 days of age) for FI ($y = 7.8885 \ln(x) + 425.7$) and WG ($y = 26.209 \ln(x) + 2416.4$), and polynomial for FI ($y = -2E\text{-}05x^2 + 0.0751x + 4247.3$) and WG ($y = -9E\text{-}05x^2 + 0.2262x + 2417.5$) to perform economic analysis simulations.

No significant differences were observed between treatments in terms of the Seedor Index (SI), breaking strength (BS), dry matter (DM) and mineral matter percentage (MM) in the tibiae for birds of 21 days of age ($P>0.05$). From 1 - 42 days of age SI and DM values were significantly different ($P<0.05$) compared to the positive control (PC) treatment according to Dunnett´s test. Broilers that received NC treatment exhibited the lowest SI and DM. Due to phytase supplementation the highest SI was obtained with the addition of 1553 FTY kg^{-1} (Table 3).

At 21 days of age, whereas blood Ca decreased in a linear manner, P increased and then decreased, with the maximum achieved using 1680 FYT kg^{-1} phytase. According to Dunnett's test, a significant difference ($P<0.05$) in both Ca and P was recorded with the inclusion of 2000 or 3000 FYT kg^{-1}, which had lower values in relation to PC. Broilers in the NC treatment had lower blood P than those in PC. A significant difference ($P<0.05$) was also observed in tibiae Ca content at 21 days of age due to phytase addition. Broilers in the NC treatment had lower tibiae Ca levels compared to those in the PC treatment. There was no difference ($P>0.05$) in the tibiae P content of birds aged 21 days.

The concentration of P in the blood at 42 days of age exhibited a quadratic behavior similar to that recorded at 21 days. However, a higher concentration of P was obtained with the use of 2033 FYT kg^{-1} phytase, while Ca levels were significantly different ($P<0.05$) from the control in treatments involving the addition of 1000, 2000 and 3000 FYT kg^{-1} phytase according to Dunnett´s test. For P, only birds in the NC treatment differed ($P<0.05$) from those in PC (Table 4). No significant differences were observed between treatments in the tibiae Ca and P levels for birds of 42 days of age ($P>0.05$).

No differences in tibiae growth plate, hypertrophic cartilage zone and total tibial epiphysis of broilers (Table 5) were observed between treatments at 42 days of age ($P>0.05$).

Carcass yield and cuts were consistent (i.e. not significantly different) among treatments (Table 6).

The apparent ileal digestibility coefficients of dry matter (AIDCDM), mineral matter (AIDCMM) and crude energy (AIDCCE) increased and then decreased with increasing phytase, with maximum recorded after the inclusion of 1164, 1592 and 1085 FYT kg^{-1} phytase, respectively (Table 7).

Significant differences ($P<0.05$) in AIDCMM were recorded according to Dunnett's test. Birds in the NC+1000, 2000 and 3000 FYT kg^{-1} treatments exhibited higher apparent ileal digestibility coefficients in relation to those in the PC treatment for MM. Birds receiving NC+3000 FYT kg^{-1} had lower AIDCE in relation to those in the PC treatment.

3.4. Discussion

Diets with reduced nutritional levels had negative effects on broiler performance at 21 and 42 days of age. Nutritional reduction (Ca and available P) in the negative control diet were also responsible for decreasing performance at the same ages of broiler's life (Walk et al., 2013).

Supplementation of 3000 FYT kg^{-1} in the starter phase (1-21 days of age) produced significant improvements in broiler WG. Considering the total study period (1-42 days of age), supplementation of 2000 FYT kg^{-1} phytase was sufficient for the broilers to achieve the best nutrient utilization, converting 1.63 kg of feed into 1 kg of meat, representing approximately 3.68% more meat when compared to the PC ration. These improvements in performance may be associated with phytate hydrolysis provided by phytase supplementation when compared to broilers receiving the NC diet (Walk et al., 2013).

Diets formulated with corn, soybean meal and wheat bran contain sufficient levels of phytate (0.21%, 0.36% and 0.45%, respectively; Rostagno et al., 2017) to negatively interfere with a bird's performance. Thus, the use of high levels of phytase, in diets containing an adequate amount of substrate could improve broiler performance via the attenuation of the anti-nutritional effects of phytate (Walk et al., 2013).

From the performance data obtained here (Table 2), it can be inferred that the inclusion of phytase had a positive effect on diets, with an increase in available nutrients, especially in previously deficient levels of Ca and available P. Exogenous phytase increases the digestibility of many dietary nutrients, mainly P, that are attached to phytate, which can then be released and absorbed in the small intestine (Adeola; Cowieson, 2011). In addition to increasing animal performance, dietary phytase supplementation also allows for a reduction in the use of inorganic P, which has a high cost in feed formulation, thereby increasing the use of phytate as a source of available P (Pieniazek et al., 2016) whilst reducing environmental pollution through decreased faecal phytate P.

Figure 1 shows the WG, FI, FCR values obtained in the present study plotted against the log10 transformation [log10([Phytase]+100)] of dietary phytase levels (Graphs A, C, E) and against linear increases in phytase level (Graphs B, D, F). According to Kornegay (2001), the relationship between phytase dose and response has been established as log-linear, that is, a logarithmic increase in dose is required to maintain a linear increase in response. Here, the linear plots reveal how the responses appear quadratic (R2 a little greater for second-order lines). In contrast, in the log-linear plots the R2 values are practically identical for first- and second-order lines, demonstrating that the [log10([Phytase]+100)] linear depictions are better.

Data analysis conducted using phytase level expressed on a log-scale-basis (log10 [phytase + 100]) indicates that a higher level of phytase may enhance the degradation and use of phytate phosphorus in broiler diets. Responses tend to be lower per unit of dietary phytase, with larger responses more commonly observed at higher logarithmic doses of dietary

phytase. This transformation allows for a more equal spacing of data points and removes many of the plateaus from the phytase response. Thus, Log is the most appropriate model for data interpretation (Shirley and Edwards, 2003). The enzyme has logarithmic effect and thus the use of log in this case would be the best answer because the quadratic model may be a statistical, but not necessarily biological, adjustment of the data.

All the performance variables showed a linear response at 21 days of age, indicating that the higher the phytase level, the better the performance of the bird. This result is in contrast to that observed at 42 days, which had a quadratic effect confirming the presence of a maximum value that can be considered the recommended dose to obtain maximal technical performance. In this case, based on the two equations plotted on the graph for 21 and 42 days, the point of intersection between the two lines thus likely represents the best recommended phytase dose from an economic perspective.

Using the presented equations in this study for the simulation of the economic analyzes and considering the price of ton of feed (US$ 260), the kilogram of chicken (US$ 0.75) and phytase (US$ 1.1) per ton of feed, then the inclusion for maximum return will be 3000 FYT (US$0.835) according to log model and 1200 FYT (US$ 0.794) quadratic model. However, simulating the phytase cost of US$ 3 per ton of feed to obtain a maximum financial return, it will require the inclusion of 1400 FYT (US$ 0.818) and 1100 FYT (US$ 0.784) using log and quadradic model, respectively. From 1-42 of bird's age, the inclusion of 2051 and 2101 FYT showed the best performance for WG and FCR, respectively. According to these economic simulations to obtain maximum financial return, it is very important to choose appropriate model due to how the data will better fit in specific model, then they will provide different estimates of levels of use that maximize profits. At this way, the phytase inclusion will depend on the market price and the model used to adjust the responses (Bedford et al., 2016).

The Seedor Index (SI) is directly related to bone density, that is, as SI increases the greater the bone density and thus also bone strength, resistance and weight. In the present study the results obtained for BW and SI correlate with the higher BS and better performance of the birds that received PC diets, without phytase supplementation.

Oliveira et al. (2014) also observed that older birds exhibit an increase in the resistance or breaking strength of the tibiae, reinforcing the existence of a positive correlation between these variables (age and BS). Such results are of great importance regarding the search for improvements in bone problems faced by modern broiler chickens, which may

occur due to genetic improvement, and for a reduction in losses in both the field and slaughterhouse.

Birds aged 21 days had a higher average deposition of tibiae (47.57%) compared to the bones of birds aged 42 days (41.07%). These results are in agreement with those found by Oliveira et al. (2014), who observed the marked deposition of bone mineral matrix in birds up to 21 days of age, followed by a decline until slaughter.

However, despite the reduction in tibiae mineral content at 42 days of age, bone breaking strength was not affected ($P>0.05$), likely because this characteristic is related to both the inorganic and organic parts of the bone (Oliveira et al., 2014). In the present study, the tibiae of birds aged 21 days contained 1.03% more organic matter than the tibiae of birds slaughtered at 42 days of age.

In general, birds in all treatments showed similar tibiae mineralization. However, birds at 21 days had lower ($P<0.05$) Ca content in the tibiae when receiving NC diet. Han et al. (2016) reported similarly low levels of tibiae Ca and poor tibiae mineralization in animals under Ca-deficient diets, which also resulted in low levels of bone resistance to breaking, length, weight and ash. However, in the present study the lower Ca content at 21 days did not affect tibia weight, breaking strength or ash content.

According to Shirley and Edwards (2003), birds fed NC diets deficient in total phosphorus had elevated plasma Ca and very low plasma P. However, birds fed diet with phytase supplementation restored the homeostatic balance between these minerals, increasing P levels and slightly decreasing Ca levels in plasma. This pattern was observed in the present study, with birds fed the NC diet presenting a higher level of Ca and lower P in relation to those in treatments with phytase, with the exception of Ca at 42 days.

Although bone histological analysis revealed no statistically significant differences between treatments, the results do indicate that phytase is able to combat the development of tibial dyschondroplasia (TD). Indeed, the area of the tibia hypertrophic cartilage zone (A2), which is considered the main affected region in TD according to Oviedo-Rondón et al. (2001) and Murakami et al. (2003), was greater in the present study in birds in the NC treatment. However, phytase supplementation did not result in significant reductions in TD incidence. According to Punna and Roland (2001), the dietary supplementation of phytase can effective reduce the TD due to the improvements of phytate P and Ca digestion and its utilization by broilers chickens.

In general, it can be inferred that phytase supplementation was not sufficient to affect the selected carcass characteristics and cuts because of the balanced NC diet used;

similarly, in a study by Singh et al (2003), which they did not observe differences in carcass yield of broilers receiving diets supplemented with phytase.

In poultry farming, high performance is associated with adequate bone mineralization, which is fundamental to supporting the great muscular development advocated by the recent genetic evolution in production. Chickens with a developmental disability can suffer bone fractures during harvesting, transportation and slaughter, potentially leading to considerable losses through carcasses discarded at the slaughterhouse (Cardoso Júnior et al., 2010). Thus, adequate bone deposition has a direct effect on production and meat yield.

Although phytase at 1280 FYT kg^{-1} can be considered the optimum level for nutrient digestibility, in order to obtain the best performance, it is necessary to use a higher dose of around 2050 FYT kg^{-1}. This level guarantees the action of the enzyme and the release of previously unavailable dietary nutrients, with subsequent absorption and utilization by the birds.

3.5. Conclusion

Phytase improves broiler performance based on regression analysis. At 21 days the high dose of 2973 FYT kg^{-1} improved weight gain. Considering the total period of 42 days, 2051 FYT kg^{-1} and 2101 FYT kg^{-1} had better weight gain and feed conversion ratio, respectively. It may be suggested that dietary phytase was able to hydrolyze the phytate, releasing nutrients and improving broilers performance. These recommendations do not negatively affect the other parameters evaluated. However, the inclusion level of phytase may depend on the market price and the model used to adjust the performance responses of the broilers.

Conflict of interest statement

The authors declare there are not any conflicts of interest.

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501 Table 1. Composition and nutrient specifications of the experimental diets used for broilers

Ingredient (g kg^{-1})	Pre-starter		Starter		Grower		Finisher	
	PC	NC	PC	NC	PC	NC	PC	NC
Corn	525.1	537.9	586.2	599.0	612.9	625.7	626.5	639.4
Soybean meal (45%)	369.4	367.1	315.9	313.5	293.5	291.2	265.3	263.0
Wheat bran	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
Soybean oil	28.2	23.8	24.6	20.2	27.2	22.8	35.0	30.6
Monobical. phosphate	17.7	10.6	14.6	7.4	12.2	5.0	9.9	2.8
Limestone	12.0	12.6	12.4	13.0	10.9	11.5	10.9	11.6
Salt	5.1	5.1	4.8	4.8	4.6	4.6	4.4	4.4
DL-Methionine (98%)	3.65	3.64	3.43	3.42	2.02	2.01	2.20	2.19
Byo-Lys (51.7%)	3.06	3.11	3.40	3.44	2.78	2.83	2.39	2.44
L-Threonine (99%)	1.29	1.30	0.83	0.83	0.47	0.47	0.37	0.37
L-Valine (99%)	0.89	0.90	0.52	0.53	0.15	0.15	0.16	0.16
L-Isoleucine (99%)	0.24	0.25	0.02	0.03	0.00	0.00	0.00	0.00
Vitamin[a]	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Mineral[b]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Choline chloride (60%)	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60
Salinomycin 12%	0.55	0.55	0.55	0.55	0.55	0.55	0.000	0.00
BHT	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Avilamycin 10%	0.05	0.05	0.05	0.05	0.05	0.05	0.00	0.00
Inert (sand)	0.00	0.30	0.00	0.30	0.00	0.30	0.00	0.30
Celite®	0.00	0.00	0.00	0.00	0.00	0.00	10.00	10.00
Nutrient specification (g kg^{-1})								
Met. En. (MJ kg^{-1})	12,35	12,35	12,56	12,56	12,77	12,77	12,97	12,97
Crude protein	220.0	220.0	200.0	200.0	190.0	190.0	178.0	178.0
Calcium	9.2	8.0	8.6	7.4	7.5	6.3	7.0	5.8
Total phosphorus	7.0	5.7	6.2	4.9	5.7	4.4	5.2	3.9
Av. phosphorus	4.7	3.3	4.0	2.6	3.5	2.1	3.0	1.6
Sodium	2.2	2.2	2.1	2.1	2.0	2.0	2.0	2.0
Dig. Lysine	13.0	13.0	12.0	12.0	11.0	11.0	10.0	10.0
Dig. met+cys	9.4	9.4	8.8	8.8	7.2	7.2	7.1	7.1
Dig. Threonine	8.5	8.5	7.4	7.4	6.8	6.8	6.3	6.3

	Dig. Tryptophane	2.5	2.5	2.2	2.2	2.1	2.1	1.9	1.9
	Dig. Valine	10.0	10.0	8.8	8.8	8.1	8.1	7.6	7.6
	Dig. Isoleucine	0.87	0.87	0.76	0.76	0.72	0.72	0.67	0.67

[a] Vitamin premix for birds. Levels per kilogram product: Vit. A (min) 2.7 g. Vit. D3 (min) 0.75g. Vit. E (min) 0.06 g. Vit. K3 (min) 2.5 g. Vit. B1 (min) 1.5 mg. Vit. B2 (min) 6 g. Vit. B6 (min) 3 g. Vit. B12 (min) 0.0012 µg. Pantothenic acid (min) 12 g. Niacin (min) 25g. Folic acid (min) 800 mg. Biotin (min) 60 mg. Selenium (min) 0.25 g.

[b] Roligomix - Mineral premix for birds. Levels per kilogram product: Copper (min) 20g. Iron (min) 100g. Manganese (min) 160g. Cobalt (min) 2 g. Iodine (min) 2 g. Zinc (min) 100g.

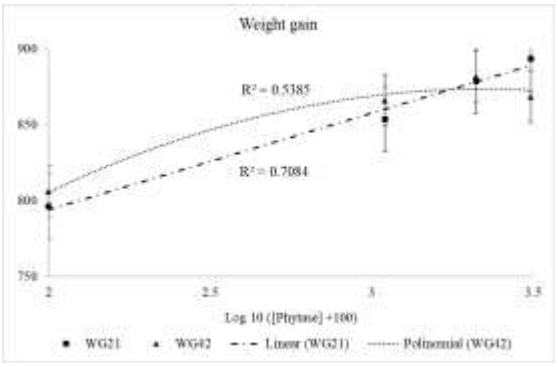
Table 2. Broiler performance from 1 to 21 and 1 to 42 days of age supplemented with phytase or inorganic phosphorus

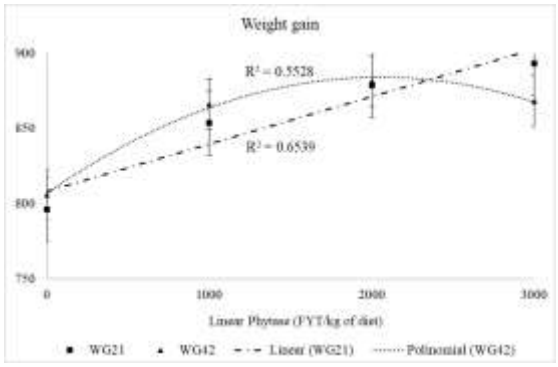
Treatments	21 days old			42 days old		
	WG (g)	FI (g)	FCR (g/g)	WG (g)	FI (g)	FCR_C (g/g)
PC#	855a	1230a	1.439	2569a	4416a	1.690a
NC*	796b	1176b	1.477	2415b	4220b	1.757b
NC + 1000 FYT kg^{-1}*	854a	1231a	1.442	2596a	4405a	1.662a
NC + 2000 FYT kg^{-1}*	879a	1240a	1.411	2642a	4431a	1.630b
NC + 3000 FYT kg^{-1}*	894b	1260a	1.410	2602a	4394a	1.651a
Average	856	1227	1.439	2565	4373	1.678
CV (%)	2.83	2.90	2.61	3.53	2.98	2.78
SEM	14.38	15.56	0.02	41.30	51.81	0.02
P (Dunnett)	<0.001	<0.001	0.007	<0.001	0.016	<0.001
P (Regression)	<0.001	0.001	0.007	<0.001	0.005	<0.001
	<0.001(L)	<0.001(L)	<0.001(L)	<0.001(Q)	0.010(Q)	0.015(Q)

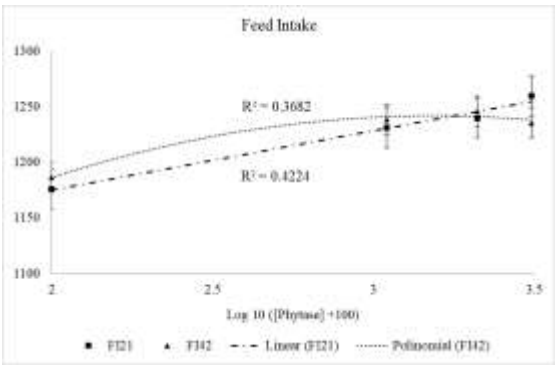
Polynomial Regression Equations	R^2	FYT for 1st derivation	Estimated response
WG_{21}= 856.581+0.0316551*FYT	0.65	-	-
FI_{21}= 1187.58+0.026021*FYT	0.38	-	-
FCR_{21}=1.466959+0.0000230009*FYT	0.55	-	-
WG_{42}= 2417.70+0.226123*FYT-0.0000551180*FYT^2	0.55	2051	2650
FI_{42}= 4224.78+0.221749*FYT-0.0000556494*FYT^2	0.37	1992	4446
$FCR_{C\ 42}$=1.75649-0.000122340*FYT+0.0000000291141*FYT^2	0.31	2101	1.627

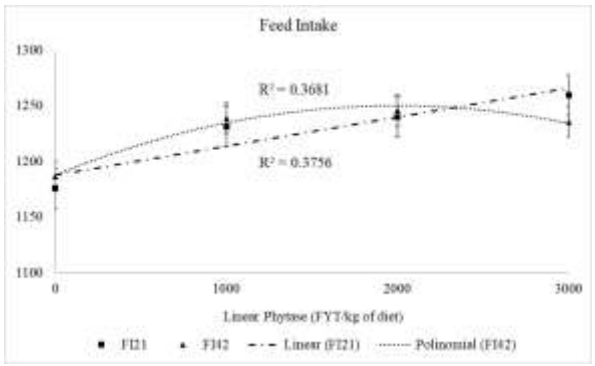
PC: positive control; NC: negative control; BW= body weight; WG= weight gain; FI= feed intake; FCR: feed conversion ratio= FCR_C = FCR corrected; Q= quadratic; L=linear; CV= coefficient of variation; * Regression analysis; # Control for the Dunnett`s Test; Means followed by a or b in the same column differ at the 5% level of significance Dunnett's Test.

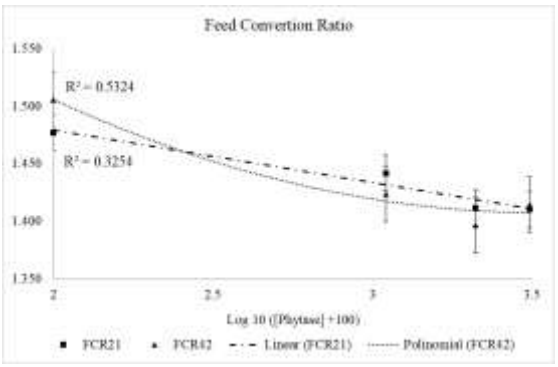
Figure 1. Weight gain, feed intake and feed conversion ratio in base Log (Graphics A, C, E) and in base FYT (Graphics B, D, F).











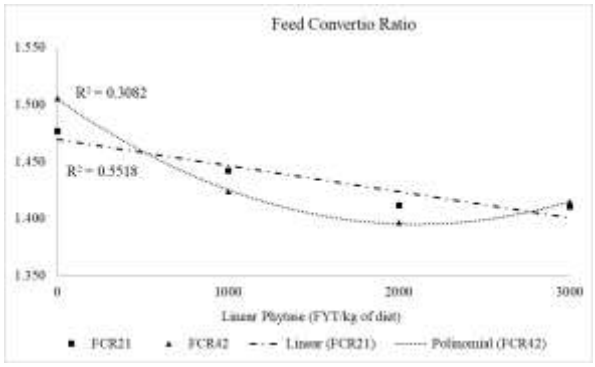


Table 3. Bone quality of the tibiae of broiler chickens at 21 and 42 days old supplemented with phytase or inorganic phosphorus

Treatments	21 days old				42 days old			
	Seedor Index	Breaking Strength (Kgf mm^{-1})	Dry matter (g kg^{-1})	Bone ash (g kg^{-1})	Seedor Index	Breaking Strength (Kgf mm^{-1})	Dry matter (g kg^{-1})	Bone ash (g kg^{-1})
PC#	70.45	15.51	435.04	480.06	142.44a	28.46	466.63a	405.64
NC*	67.22	15.02	444.64	476.31	133.80b	23.90	426.57b	384.43
NC + 1000 FYT kg^{-1}*	70.55	15.33	428.31	469.25	145.16a	28.30	470.75a	436.19
NC + 2000 FYT kg^{-1}*	69.88	15.93	424.33	474.41	149.24a	26.70	456.19a	410.73
NC + 3000 FYT kg^{-1}*	66.40	15.52	428.04	478.50	134.71a	27.84	452.48a	416.37
Average	68.90	15.26	432.07	475.71	141.07	27.04	454.52	410.67
CV (%)	9.68	18.90	7.74	5.05	6.55	16.66	3.79	5.69
SEM	1.82	0.68	8.35	5.93	3.76	1.63	11.04	13.47
P (Dunnett)	0.209	0.966	0.458	0.745	0.007	0.253	0.020	0.081
P (Regression)	0.121	0.940	0.416	0.684	0.008	0.071	0.040	0.058
	-	-	-	-	0.001(Q)	-	-	-

Polynomial Regression Equations	R^2	FYT for 1st derivation	Estimated response
SI_{42}=133.229 +0.0200987*ENZ-0.00000647177*ENZ^2	0.33	1553	149
MSO_{42} = 429.9954 + 0.0423427*ENZ – 0.0000119979*ENZ2	0.27	1765	467

PC: positive control; NC: negative control; Q= quadratic; CV= coefficient of variation; * Regression analysis; # Control for the Dunnett`s Test; Means followed by a or b in the same column differ at the 5% level of significance Dunnett's Test.

Table 4. Mineral content in bones and blood of broiler chickens at 21 and 42 days of age supplemented with phytase or inorganic phosphorus

Treatments	21 days old				42 days old			
	Ca Bone ($g\ kg^{-1}$)	P Bone ($g\ kg^{-1}$)	Ca Blood ($mg\ dl^{-1}$)	P Blood ($mg\ dl^{-1}$)	Ca Bone ($g\ kg^{-1}$)	P Bone ($g\ kg^{-1}$)	Ca Blood ($mg\ dl^{-1}$)	P Blood ($mg\ dl^{-1}$)
PC[#]	214.9a	111.0	9.27a	5.82a	178.5	75.2	5.38a	3.68[a]
NC*	210.6b	107.8	9.02a	3.67b	183.4	78.1	5.69a	2.11b
NC + 1000 FYT kg^{-1}*	211.0a	111.1	9.31a	5.80a	186.5	75.9	6.22b	3.82[a]
NC + 2000 FYT kg^{-1}*	214.0a	117.4	6.54b	4.66b	181.4	75.8	6.01b	3.84[a]
NC + 3000 FYT kg^{-1}*	190.2b	126.0	5.68b	4.68b	182.9	77.6	5.93b	3.73[a]
Average	208.1	114.6	7.96	4.93	182.5	76.5	5.85	3.44
CV (%)	8.45	8.76	5.79	8.4	4.32	2.12	11.40	12.29
SEM	10.61	6.23	0.40	0.23	0.37	0.10	0.18	0.20
P (Dunnett)	0.006	0.213	<0.001	<0.001	0.700	0.294	0.008	<0.001
P (Regression)	0.231	0.649	<0.001	<0.001	0.811	0.383	0.181	<0.001
	-	-	0.014(L)	<0.001(Q)	-	-	-	<0.001(Q)

Polynomial Regression Equations	R^2	FYT for 1st derivation	Estimated response
Ca_{21}= 9.43763-0.00153474*ENZ	0.78	-	-
P_{21}= 3.89139+0.00177776*ENZ-0.000000529125*ENZ^2	0.45	1680	5.38
P_{42}= 2.18632+0.00186011*ENZ-0.000000457531*ENZ^2	0.73	2033	4.08

PC: positive control; NC: negative control; Q= quadratic; CV= coefficient of variation; * Regression analysis; [#] Control for the Dunnett`s Test; Means followed by a or b in the same column differ at the 5% level of significance Dunnett's Test.

Table 5. Growth plate (A1), hypertrophic cartilage zone (A2) and total tibial epiphysis (A3) of broilers at 42 days of age supplemented with phytase or inorganic phosphorus

Treatments	42 days old		
	A1	A2	A3
PC[#]	23.00	24.87	57.33
NC*	20.66	27.54	52.84
NC + 1000 FYT kg^{-1}*	21.04	27.11	57.98
NC + 2000 FYT kg^{-1}*	20.37	26.21	57.62
NC + 3000 FYT kg^{-1}*	20.19	25.01	57.21
Average	20.77	26.15	56.60
CV (%)	17.55	18.08	11.29
SEM	1.61	1.99	2.75
P (Dunnett)	0.505	0.859	0.700
P (Regression)	0.794	0.873	0.594

PC: positive control; NC: negative control; CV= coefficient of variation; Q=quadratic; * Regression analysis; [#] Control for the Dunnett`s Test.

Table 6. Carcass yield and cuts (g kg^{-1}) of 42 days old broilers supplemented with phytase or inorganic phosphorus

Treatments	Carcass	Wing	Whole leg	Bone in breast	Breast	Boneless breast meat	Abdominal fat
PC#	742.9	102.3a	298.4	381.5	895.2	341.6	21.6
NC*	735.8	105.3b	294.4	385.9	884.8	341.5	19.5
NC + 1000 FYT kg^{-1}*	741.8	102.1a	299.6	376.2	895.3	337.0	20.6
NC + 2000 FYT kg^{-1}*	743.7	102.2a	298.4	386.1	891.5	344.3	19.1
NC + 3000 FYT kg^{-1}*	742.0	102.1a	295.9	386.5	899.3	347.7	19.5
Average	741.2	102.8	297.4	383.2	893.2	342.4	20.0
CV (%)	2.32	4.78	7.26	5.34	2.44	6.38	22.71
SEM	3.05	0.89	3.79	3.65	3.90	3.87	0.81
P (Dunnett)	0.375	0.041	0.874	0.207	0.096	0.385	0.189
P (Regression)	0.320	0.023	0.813	0.092	0.081	0.217	0.612

Polynomial Regression Equations	R^2	FYT for 1st derivation	Estimated response
Wing= 104.372+0.000950905*FYT	0.37	-	-

PC: positive control; NC: negative control; CV= coefficient of variation; Q=quadratic; L=linear; * Regression analysis; # Control for the Dunnett`s Test; Means followed by a or b in the same column differ at the 5% level of significance Dunnett's Test.

Table 7. Ileal digestibility (g kg^{-1}) of broilers at 42 days of age supplemented with phytase or inorganic phosphorus

Treatments	42 days old			
	AIDCDM	AIDCMM	AIDCCP	AIDCCE
PC[#]	622	349a	707	642a
NC*	618	354a	699	638a
NC + 1000 FYT kg^{-1}*	638	444b	717	658a
NC + 2000 FYT kg^{-1}*	621	421b	710	638a
NC + 3000 FYT kg^{-1}*	602	382b	692	615b
Average	620	390	705	638
CV (%)	3.57	8.86	3.70	3.08
SEM	8.44	17.66	9.27	8.29
P (Dunnett)	0.053	<0.001	0.358	0.002
P (Regression)	0.024	<0.001	0.216	0.001
	0.018(Q)	<0.001(Q)	-	0.003(Q)

Polynomial Regression Equations	R^2	FYT	Response
AIDCDM= 619.781+0.022229*ENZ-0.0000095532ENZ^2	0.25	1164	630
AIDCMM= 358.977+0.102851*ENZ-0.0000322999ENZ^2	0.49	1592	440
AIDCCE= 639.806+0.0237379*ENZ-0.0000109437ENZ^2	0.40	1085	650

PC: positive control; NC: negative control; AIDCDM= apparent ileal digestibility coefficient of dry matter; AIDCMM= apparent ileal digestibility coefficient of mineral matter; AIDCCP= apparent ileal digestibility coefficient of crude protein; AIDCCE= apparent ileal digestibility coefficient of crude energy; CV= coefficient of variation; Q=quadratic; * Regression analysis; [#] Control for the Dunnett`s Test; Means followed by a or b in the same column differ at the 5% level of significance Dunnett's Test.

(Artigo nas normas da Revista British Poultry Science)

4. PHYTASE AND PHYTATE INTERACTIONS IN BROILERS CHICKENS AT 21 DAYS OF AGE

Abstract. This study was conducted to evaluate the effects of different levels of phytase in diets with different amounts of phytate on live performance and bone characteristics of broiler chickens at 21 days.

2. A total of 2,625 male, 1-d-old Cobb 500 broilers were allocated to fifteen dietary treatments. Treatments consisted of a 3x5 factorial arrangement, with high (HP), medium (MP) and low (LP) phytate (2.45, 2.34, 2.23 g kg^{-1} of phytate P, respectively) and a positive control (PC); negative control (NC) with a reduction of 0.15% of calcium (Ca) and 0.15% of phosphorus (P) and NC diet plus 0, 500, 1000 or 1500 FTU kg^{-1} of phytase.

3. FI peaked with supplementation of 1051 FTU kg^{-1} phytase to the LP diets. With 1000 FTU kg^{-1} there was no differentiation between FI by broilers from HP, MP or LP diets. Bone ash (BA) of broilers receiving LP showed a maximum response at 1101 FTU kg^{-1}. Birds receiving the NC diet had a larger hypertrophic cartilage zone A2 ($P<0.05$) than those receiving the PC diet. Serum Ca and P of birds receiving the NC treatment and LP diet were lower than broilers fed the MP and HP diets. Broilers in the NC+500 FYT kg^{-1} treatments had lower tibia P levels compared to those in the PC treatment; also, broilers receiving HP diets had a higher tibia Ca content than those receiving LP diets ($P<0.05$). In general bone P of birds fed diets containing HP was higher than those into a LP or MP diets ($P<0.05$).

4. Phytase supplementation improved the performance and bones of birds. The use of 1101 FTU kg^{-1} resulted in better bone characteristics when fed with the lowest phytate level, this level does not negatively affect the other parameters evaluated.

Keywords: Bone mineralization; Feedstuffs; Growth; Poultry; Phosphorus.

4.1. Introduction

Broiler diets are mainly composed of vegetable feedstuffs. These ingredients are usually composed of high P amounts in phytate form (60 to 80%). The phytate form is found in plants, is largely unavailable to be used for broilers. Phytate is negatively charged under many pH conditions (acidic, neutral, and basic), due to its, phytate has the ability to form a complex with positively charged molecules in the diet, especially divalent cations. Forming complexes with other nutrients can reduce the digestibility of those nutrients in the digesta, which makes them unavailable for use by animals (Woyengo and Nyachoti, 2013).

Phytase (myo-inositol (1,2,3,4,5,6) hexaquisphosphate phosphohydrolases) represents a subgroup of phosphatases that are able of initiating the phytate dephosphorylation (myo-inositol (1,2,3,4,5,6) hexaquisphosphate). In theory, the enzymatic hydrolysis of a phytate generates a series of lower myo-inositol phosphate esters, through a succession of dephosphorylation reactions, to produce inositol and six radicals of inorganic P (Selle and Ravindran, 2007).

The ability of phytase to degrade phytate can be affected by factors such as the amount and source of P, dietary calcium (Ca) levels, animal species and age (Anselme, 2006), the presence of antinutritional factors, the type and amount of cereals used (Munir and Maqsood, 2013), concentrations and phytate sources of diets, and the level and type of phytase used (Ravindran et al., 2008).

Another important factor is the location of the phytate in the seeds. In small grains, phytate is found mainly in the bran (aleurone, forehead and pericarp layer). In maize it is mainly in the germ, in legumes it is accumulated in the cotyledon and in soybean it is distributed throughout the seed. However, in many other seeds, phytate localization has yet to be determined or has no specific location (Kornegay, 2001).

In order to maximize the feed utilization by poultry, as well reducing the feed costs, the use of animal by-products in the diets has become a common practice in some countries; since the growth in livestock and demand for animal proteins, has led to large volumes of these by-products (Carvalho et al., 2012). Additionally, is considered a rational and economic way to feed livestock animals.

Among the animal feedstuffs used in broiler diets, meat and bone meal and poultry by-products have been proven to be good protein sources, Ca and P. According to Rostagno et al. (2017) meat and bone meal has 8.55 to 14.1% of total Ca; and 4.59 to 7.54 of total P, with 4.13 to 6.79% of that being available P. The poultry by-products contain 4.06 to 4.34% of total Ca; and 2.37 to 2.54% of P that is available. These animal by-products are relatively inexpensive ingredients that allow nutritionists to reduce or replace the amount of inorganic P in diets.

The objective of the current study was to evaluate the effects of different levels of phytase levels on diets formulated based on vegetable feedstuffs, vegetable plus animal, and animal origin (high, medium and low phytate, respectively) on live performance and bone characteristics of broiler chickens at initial phase.

4.2. Material and methods

The experiment was conducted at the Poultry Sector of the Experimental Station of the State University of the Western of Paraná – UNIOESTE. Experimental birds

were handled with care to avoid unnecessary discomfort, and all experimental procedures were approved by the University ethical review committee.

Management of birds

A total of 2,625 male, 1-d-old Cobb 500 broilers were housed in a controlled environment in 105-floor pens, seven replicate pens per treatment of 25 birds per pen and 7 replicate pens per treatment. Each pen was 1.96 m^2 with a concrete floor covered with pine shavings as bedding and equipped with a semiautomatic feeder and nipple drinkers. Throughout the experimental period the room temperature was maintained within the zone of thermal comfort, lighting was provided for 24 h per day. Feed was provided ad libitum, and birds had free access to water during the entire experimental period.

Dietary treatments

Chicks were randomized by weight and distributed into a 3x5 factorial design, consisting of 15 treatments. Three diets were formulated to contain high (HP), medium (MP) and low (LP) phytate (2.45, 2.34, 2.23 g kg^{-1} of phytate P, respectively) (Table 1). Fifteen experimental diets were formulated with different phytate contents combined with a positive control (PC) diet which aimed to provide the calcium (Ca) and phosphorus (P) requirements of the birds; negative control (NC) with a reduction of 0.15% of the Ca and 0.15% of the P and NC diet plus 0, 500, 1000 or 1500 FTU kg^{-1} of phytase (Potenzya F is a fungical 3-phytase expressed through the use of synthetic genes in Apergillus oryzae, no stable term, with phytase activity of 5000 phytase units (FYT) per g). One phytase unit is defined as the amount of enzyme that releases 1 µmol of inorganic phosphate under standard

conditions (0.25 M acetate buffer pH 5.5, 37ºC and 5 mmol sodium phytate). The experimental diets were formulated according to the feed composition and nutritional requirements for a starter phase (1-21 days), proposed by Rostagno et al. (2017). Phytase activity in the diets was determined using the ISO 30024 protocol (International Organization for Standardization (2009). The analyses were performed on all dietary treatments; a pool of starter and grower feed samples were sent to a commercial laboratory CBO (Valinhos, SP, Brazil). The analysis of the added enzyme to the experimental feed showed that concentrations of phytase were 0, 573, 1227, 1850 FTU kg^{-1} in the experimental feed.

Performance, blood and bone analyses

Weight gain (WG), feed intake (FI) and feed conversion ratio (FCR) were determined at 21 d of age. Mean individual bird weight and feed intake was calculated and corrected using the weight of dead birds, which was recorded daily, according to Sakomura and Rostagno (2016).

On d 21, 2 birds per pen were randomly chosen, fasted for 6 h and blood samples were collected via brachial puncture. Blood was coagulated and centrifuged at 1008 g rpm for 10 min to obtain serum, which was stored at -20°C. To perform the analyzes the serum was thawed at room temperature, centrifuged at 1008 g for 5 min and then Ca, P, and alkaline phosphatase (ALP) analyses were performed using a high-performance automatic spectrophotometer (Flexor EL 200, Elitech, Paris, France) with specific kits, calibrated with standards (Elical, Elitech).

For the evaluation of bone development, 2 birds with mean group weights (±5%) were euthanized by electronarcosis followed by exsanguination, according to Normative Resolution Nº. 37 of February 15, 2018 of CONCEA. Legs were

127 separated and deboned to obtain tibia. After deboning, the left tibia was weighed to
128 the nearest ± 0.0001 g and their lengths were determined using a digital caliper
129 (accuracy of 0.01 mm). The Seedor Index (SI) (Seedor et al., 1991) was calculated
130 by dividing the bone weight (mg) by its length (mm). After this determination, tibia
131 was individually stored at -20ºC for further analysis.
132 Determination of bone breaking strength (BS) was performed after bone thawing at
133 room temperature. The tibia was individually supported on the epiphysis. A force
134 load of 200 kgf at the speed of 5 mm s^{-1} was applied in the central region of each
135 bone using a probe TA-TPB and a Texturometer (CT3 Texture Analyzer,
136 Brookfield).
137 Broken tibia was used for tibia ash determination. The tibia was weighed on an
138 analytical balance (± 0.0001 g) and dry matter (DM) analyzed (AOAC, 1995 - Index
139 nº 920.39), after which the samples were weighed, ashed overnight at 600 ºC, and
140 weighed again (*after* Hall et al., 2003). The percentage of bone ash (BA) was
141 calculated as the proportion of the dry, pre-ashed tibia multiplied by 100.
142 To determine the amount of Ca and P concentration in the bones, the ashes were
143 placed in a sand bath (250ºC) with HCl (6 M) solution to solubilize the minerals. Ca
144 was measured using an atomic absorption apparatus (GBC-932AA) and P using a
145 spectrophotometer (UV/VIS GBC-916).
146 To evaluate the incidence of tibial dyschondroplasia (TD), the left leg tibia was
147 decalcified with 50% formic acid and 20% sodium citrate (Fernandes et al., 2007).
148 After decalcification, the bone was embedded in paraffin (Beçak and Paulete, 1976).
149 The sections were made with microtomes at 5 μm thickness and stained with
150 Hematoxylin-Eosin, for observation of the epiphyseal disk area and measurements of
151 the areas to characterize the incidence of TD. For analysis of tibial epiphyseal

cartilage slides, two distinct regions characterized by the morphological appearance were considered: growth plate (A1) and hypertrophic cartilage zone (A2). The images were measured with the aid of a computerized image analyzer PROPLUS IMAGE 4.1.

The left tibias were used to determine radiographic bone mineral densitometry (BMD), which was performed at the Dentistry Clinic of the Universitary Hospital of Cascavel. The tibia was used to determine the optical densitometry in radiographic images compared to an aluminum scale with 10 degrees for 1 mm (penetrometer). The bones were radiographed with a dental X-ray machine (Orthopantomograph OP 300) at 85 kVp, 6.3 mA and 10 s of exposure time. The digital images were analyzed using Adobe Photoshop CS6. Five areas of each penetrometer degree (1–5 mm) were analyzed, and an equation was used from the values obtained. In addition, six areas of each bone were evaluated, and the obtained value was applied in the equation to determine BMD expressed as millimeters of aluminum (mmAl). Higher values indicated greater radiopacity and greater bone density.

Statistical analysis

Statistical analysis was performed using SAS - Version 9.1. An analysis of variance and subsequent polynomial regression between the inclusion levels of the enzyme was performed excluding the positive control (PC) treatment. In addition, the Dunnett`s Test was performed at the 5% probability level to compare the PC treatment with the other treatments. Tukey`s Test was performed to compare the means of each phytate content.

4.3. Results

No significant interaction (P>0.05) was found on weight gain (WG) and feed conversion ratio (FCR) (Table 2). The feed intake (FI) was higher and FCR was worst (P<0.05) in broilers fed diets with high phytate (HP) compared with those fed with low phytate (LP). WG and FI values were significantly different (P<0.05) compared to the positive control (PC) treatment according to Dunnett`s Test. Broilers that received diets negative control (NC), without phytase supplementation, exhibit the lowest WG and reductions in FI. Broilers receiving 1000 and 1500 FTU kg^{-1} achieved the best WG and FCR compared to the PC treatment. Regression equations for FI and WG had the best fit with quadratic adjustment and the levels that provided the maximum responses were estimated at 233 and 1180 FTU kg^{-1}, respectively. FCR showed a linear improvement with increasing levels of phytase (P=0.0 1).

The interaction between phytate and phytase significantly influenced the FI. Feed consumption was increased (11%) (P=0.003) in birds fed on HP diets with nutritional reduction of Ca and P without phytase, when compared to birds on the LP diets. Phytase supplemented to the NC diet at 500 FTU increased (P=0.031) (4.1%) the FI of birds fed HP, when compared to birds on the LP diets. With 1000 FTU kg^{-1} of inclusion there was no differentiation between FIs. A quadratic effect was observed only between phytase supplementation and LP diet and the level of phytase that provided the maximum FI responses was estimated at 1051 FTU kg^{-1}.

No effects of phytate and phytase levels (Table 4) were observed (P>0.05) for Seedor Index (SI), growth plate (A1) and bone mineral density (BMD). Broilers fed NC diets without phytase supplementation, exhibited the lowest breaking strength (BS), dry matter (DM) and bone ash (BA) content, and a higher value for hypertrophic cartilage zone (A2), by Dunnett`s Test, regardless of the level of phytate in the diet.

Most measurements had best fit with quadratic adjustments and the equations derived showed the greatest values for supplementation at 1140 (BS), 1008 (DM), 1304 (BA) FTU kg^{-1}. For A2, 1308 FTU kg^{-1} may provide a tendency of tibial dyschondroplasia. However, the phytate and phytase interaction significantly influenced the DM and BA (Table 5). Tibia DM of broilers fed diets with HP and receiving the NC without enzyme was 7.52% and 8.34% higher than broilers fed diets with MP and LP, respectively; BA was higher in birds fed NC+1000 FTU kg^{-1} with LP than broilers fed diets with MP. A quadratic effect was observed in DM content in broilers receiving diets with MP (P=0.005) and LP (P=0.001) and the greater level of phytase was 1074 and 1049 FTU kg^{-1}, respectively. An increasing linear effect (P<0.007) was observed in content BA in broilers receiving HP diets, on the other hand, BA percentage of broilers receiving LP was obtained with the addition of 1101 FTU kg^{-1}. A significant interaction between phytase supplementation and phytate content was detected in serum Ca, P and alkaline phosphatase (ALP) (Table 6) and bone Ca and P (Table 7). Serum Ca and P of birds receiving LP was lower (P<0.05) compared to MP and HP, only for the NC treatment. Broilers receiving HP and supplementation of 1000 FTU kg^{-1} of phytase had a higher concentration of serum P when compared to the LP. Blood Ca linearly increased (P=0.043) in broilers fed with HP and quadratic effect was observed (P=0.013) when broilers were fed with LP diets; and the level that was determined as providing the maximum response value 1029 FTU kg^{-1} phytase. A quadratic response of blood P was observed for broilers fed different phytase levels for all phytate concentration (HP, MP and LP) in the diets, and the levels that were determined as providing the maximum response value was 1067, 995 and 992 FTU kg^{-1} phytase, respectively. For ALP, only broilers fed with MP had a quadratic behaviour (P=0.018) and the levels that was determined as providing the

maximum response value was 934 FTU kg^{-1} phytase. Bone P content of broilers fed LP diet and receiving NC was lower (P=0.007) than MP. Broilers fed diets with HP and receiving 1000 FTU kg^{-1} had higher (P<0.05) P tibia content. Tibia Ca content in broilers fed LP diets had a linear adjustment, increasing phytase levels there was an increasing Ca content (P=0.048).

4.4. Discussion

Diets with reduced nutritional level had negative effects on broilers performance. The reductions in WG and FI and worse in FCR were due to the reduction of 0.15% of Ca and 0.15% of P; levels much below the recommendation for broilers diets at 21 d. It was clear that regardless of the phytase level, broilers fed diets with HP had a worse FCR.

Phytase supplementation was responsible for attenuating this negative effect of reducing Ca and P while keeping a similar performance to the birds fed with PC treatment and also promoting an improvement in FCR. This improvement on broilers performance is due to the increased P availability, other minerals and nutrients and the possibility of better diet quality from a higher nutrient digestibility, a result of the phytase action (Amerah et al., 2014; Qian et al., 1996).

Birds that received NC and HP had higher FI than broilers fed LP treatment. However, broilers receiving 500 FTU kg^{-1} achieved a FI similar between HP and MP, showing the effect of phytase on making similar diets with different phytate contents. The FI response peaked at 1050 FTU kg^{-1} in broilers fed LP and MP diets may be associated with the diet composition, which had a lower fiber content in relation a HP diet. High dietary fiber results in higher viscosity of the digesta, reducing intake and, consequently, nutrient digestibility and bird performance (Broch

et al., 2017). However, this phytase-induced improvement in FI was not reflected in the WG and FCR of the same group of birds.

The positive effect of enzyme also happens with some bone characteristics such BS, DM, BA and A2. Bone mineralization increased due to the availability of minerals released from the phytate mineral complex diets (Gautier et al., 2017), meeting the requirements of skeletal development; these data are close with previous studies (Broch et al., 2018; Boney e Moritz, 2017; Cowieson et al., 2015; Milica et al., 2012; Qian et al., 1996). The abnormal bone development is a sign of a P deficiency and no phytase supplement, and this can affect the degree of conversion of cartilage to bone in the tibia and the histological development of tibia (Qian et al., 1996). Broilers fed with NC diet had defective or disorganized mineralization of the extracellular matrix of cartilage compared to those fed with PC, confirmed by A2 results. However, with supplementation of phytase this effect reverts due to the improvements of phytate P and Ca digestion, and its utilization by broilers chickens (Broch et al., 2018).

The higher DM concentrations were observed in broilers fed NC and HP diets, which match with FI results. The maximum achieved DM bone concentration in broilers into MP and LP groups was very similar ~1062 FTU kg^{-1}. BA of broilers fed with LP diets with the maximum achieved using 1101 FTU kg^{-1}; and agrees with a higher BA deposition observed in broilers supplemented 1000 FTU kg^{-1} into LP treatment. On the contrary, broilers into HP with increased phytase inclusion had an increase in BA concentration; this may be due to the greater substrate content in vegetable origin diets. Morgan et al. (2016) found that 47% of phytate in wheat bran was susceptible to the effects of phytase, that is, it could be removed if there was sufficient phytase; this suggests that our vegetable origin diets (HP) with 3% wheat bran could be improved with the use of high doses of phytase.

279 The concentration of serum P responded in relation to dietary Ca and non-phytic P
280 levels; birds receiving P deficient diets had a high concentration of Ca and lower
281 concentration of P in plasma. Phytase supplementation causes an increase in P levels
282 and decrease of Ca levels in plasma, restoring the homeostatic balance between these
283 minerals (Shirley and Edwards, 2003). The enzyme ALP is an indicator of increased
284 bone formation activity; high concentrations of ALP are associated with increased
285 formation of bone tissue. However, the reduction of this enzyme associated with
286 diets supplemented with phytase may reflect the reduction of ALP because of the
287 increase of P availability.
288 High P contents in blood are supposed to be related to a dynamic bone growth; when
289 bone growth decreases, P is transferred to a lesser extend into the bones and thus the
290 serum contents are higher. However, the P content may show broad variations which
291 may be due to a difference in FI and due a different digestibilities of feedstuffs and
292 thus, despite equal P concentrations in the diet, the availability of this mineral can
293 vary which will be noticeable in blood concentrations (Goetting-Fuchs et al., 2012).
294 The calculated phytate P concentrations in diets used in the present study were
295 around 2.45 (HP), 2.34 (MP) and 2.23 (LP) g kg^{-1} diets; therefore, it could be
296 expected that phytase responses would be more pronounced in HP diets, due to the
297 higher amount of available substrate. However, the phytase effect was more
298 pronounced into LP diet, affecting the most of variables in a quadratic manner
299 confirming the presence of a maximum value that can be considered the
300 recommended dose to obtain maximal technical performance. However, at some
301 point the enzymes become saturated and the reaction rate levels off, not happening
302 additional effect.

High phytase levels appear to be more effective in diets with LP content. At LP concentrations more phytase is required to maintain the product supply as the available substrate is depleted, while at HP concentrations even a low level of phytase is saturated with phytate, and thus most of the degradation of the substrate originates from high molecular weight (and more antinutritional) (Cowieson et al., 2016).

4.5. Conclusion

In conclusion, our study findings revealed that phytase supplementation improves broiler`s performance and bones quality. The use of 1101 FTU kg^{-1} is recommended for better bone characteristics in LP diets, this recommended level should negatively affect the other parameters evaluated.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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461 **Table 1.** *Ingredient composition and nutrient specification of starter (1-21 d) diets.*

Ingredients (g kg^{-1})	HP		MP		LP	
	PC	NC	PC	NC	PC	NC
Maize	539.0	553.5	577.3	590.9	610.0	624.5
Soybean meal (45%)	335.9	333.4	282.2	280.2	287.1	284.6
Gluten feed meal	20.0	20.0	20.0	20.0	20.0	20.0
Wheat bran	30.0	30.0	30.0	30.0	-	-
Soybean oil	31.1	26.2	17.7	13.1	10.1	05.2
Meat & bone meal	-	-	20.0	20.0	20.0	20.0
Poultry by-product meal	-	-	18.5	18.5	18.5	18.5
Monocalcium phosphate	15.44	7.77	7.71	0.04	8.1	0.39
Limestone	11.70	11.85	8.01	8.17	7.9	8.03
Salt	3.31	3.30	2.82	2.81	2.81	2.81
Byo-Lys (51.7%)	4.50	4.57	5.63	5.66	5.53	5.60
DL-Methionine (98%)	2.96	2.94	3.10	3.08	3.07	3.06
L-Threonine (99%)	0.82	0.82	1.16	1.15	1.10	1.11
L-Valine (98.5%)	0.38	0.38	0.67	0.65	0.63	0.64
Mineral[b]	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin[a]	1.50	1.50	1.50	1.50	1.50	1.50
Na bicarbonate	1.50	1.50	1.50	1.50	1.50	1.50
Choline chloride	0.60	0.60	0.60	0.60	0.60	0.60
Salinomycin (12%)	0.55	0.55	0.55	0.55	0.55	0.55
BHT	0.20	0.20	0.20	0.20	0.20	0.20
L-Isoleucine (99%)	-	-	0.36	0.38	0.29	0.31
Avilamycin (10%)	0.05	0.05	0.05	0.05	0.05	0.05
Inert (sand)	-	0.40	-	0.40	-	0.40
Nutrient specification (g kg^{-1})						
Met. En (MJ kg^{-1})	12,56	12,56	12,56	12,56	12,56	12,56
Crude protein	213.9	213.9	213.9	213.9	213.9	213.9
Calcium	8.56	7.06	8.56	7.06	8.56	7.06

Total P	6.49	5.08	6.53	5.13	6.45	5.01
Av. P	4.20	2.70	4.17	2.67	4.17	2.67
Phytate P	2.44	2.46	2.33	2.35	2.22	2.24
Sodium	1.90	1.90	1.90	1.90	1.90	1.90
Dig. lysine	12.26	12.26	12.26	12.26	12.26	12.26
Dig. met+cys	8.84	8.84	8.84	8.84	8.84	8.84
Dig. threonine	7.97	7.97	7.97	7.97	7.97	7.97
Dig. valine	9.44	9.44	9.44	9.44	9.44	9.44
Dig. isoleucine	8.33	8.32	8.22	8.22	8.22	8.22

462 HP: high phytate; MP: medium phytate; LP: low phytate; PC: positive control; NC: negative control.
463 [a]Vitamin premix for birds. Levels per kilogram product: Vit. A (min) 2.7g, Vit. D3 (min) 0.75g, Vit. E
464 (min) 0.06g, Vit. K3 (min) 2.5g, Vit. B1 (min) 1.5mg, Vit. B2 (min) 6g, Vit. B6 (min) 3g, Vit. B12
465 (min) 0.0012µg, Pantothenic acid (min) 12g, Niacin (min) 25g, Folic acid (min) 800mg, Biotin (min)
466 60mg, Selenium (min) 0.25g. [b] Mineral premix for birds. Levels per kilogram product: Copper (min)
467 20g, Iron (min) 100g, Manganese (min) 160g, Cobalt (min) 2g, Iodine (min) 2g, Zinc (min) 100g.
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484 **Table 2**. *Effect of phytase and phytate on broiler performance at 21 d of age.*

Treatments	FI (g)	WG (g)	FCR (g g^{-1})
HP	1220[a]	860	1422[a]
MP	1210[ab]	870	1385[ab]
LP	1200[b]	860	1390[b]
PC	1202	860	1414
NC+0*	1107*	820*	1426
NC+500 FTU kg^{-1}*	1202	870	1402
NC+1000 FTU kg^{-1}*	1203	890*	1381*
NC+1500 FTU kg^{-1}*	1203	890*	1370*
SEM	0.005	0.005	0.004
P Phytate	0.034	0.577	<0.0001
P Enzyme	<0.0001	<0.0001	<0.0001
P Interation	0.046	0.071	0.188
P Regression	0.016(Q)	0.004(Q)	<0.0001(L)

485 HP: high phytate; MP: medium phytate; LP: low phytate; PC: positive control; NC: negative control;
486 FI: feed intake; WG: weigh gain; FCR: feed conversion ratio; Q: quadratic; L: linear; *Regression
487 analysis; Means followed by * in the same column differ at the 5% level of significance by Dunnett's
488 Test; Means followed by different letter in the same line differ at the 5% level of significance by
489 Tukey`s Test.
490 FI: $1168.657143+0.550619*FTU-0.001183*FTU^2$; R^2: 0.25; FTU for maximum response: 233;
491 Maximum response: 1233.
492 WG: $819.1457072+0.1227582*FTU-0.0000520*FTU^2$; R^2: 0.34; FTU for maximum response: 1180;
493 Maximum response: 892.
494 FCR: $1426.370859+ 0.000056866*FTU$; R^2:0.25.
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504 **Table 3**. I*nteractions between phytase and phytate on broiler feed intake at 21 d of*
505 *age.*

Treatments	Feed Intake (g)			
	HP	MP	LP	P Tukey
NC*	1208[a]	1155[b]	1139[b]	0.003
NC+500 FTU kg^{-1}*	1245[a]	1208[ab]	1194[b]	0.031
NC+1000 FTU kg^{-1}*	1226	1213	1244	0.396
NC+1500 FTU kg^{-1}*	1228	1246	1211	0.461
P Regression	0.496	0.503	0.011(Q)	

506 HP: high phytate; MP: medium phytate; LP: low phytate; PC: positive control; NC: negative control;
507 Q: quadratic; *Regression analysis; Means followed by different letter in the same line differ at the 5%
508 level of significance by Tukey`s Test.
509 FI_{LP}: 1134.900000+0.184943*FTU-0.000088*FTU^2; R^2: 0.46; FTU for maximum response: 1051;
510 Maximum response: 1232.

511 **Table 4**. *Effect of phytase and phytate on broiler bone characteristics at 21 d of age.*

Treatments	SI	BS (kgf $mm^{-1)}$	DM (g kg^{-1})	BA (g kg^{-1})	A1 (mm^2)	A2 (mm^2)	BMD (mmAl)
HP	70.43	13.41	412.3	466.1	13.13	37.60	2.91
MP	73.46	14.31	414.4	469.9	13.07	36.46	2.92
LP	71.49	13.09	410.2	467.5	13.16	36.89	3.02
PC	70.61	13.88	425.4	479.2	12.98	35.53	3.03
NC+0*	70.18	11.32*	389.4*	437.2*	12.55	41.83*	2.92
NC+500 FTU kg^{-1}*	73.18	13.87	416.9	469.0	13.45	36.95	2.97
NC+1000 FTU kg^{-1}*	73.60	14.47	415.4	473.2	13.22	36.35	2.93
NC+1500 FTU kg^{-1}*	71.40	14.46	414.3*	480.7	13.39	35.16	2.90
SEM	0.62	0.28	0.19	0.22	0.18	0.39	0.04
P Phytate	0.117	0.101	0.469	0.547	0.979	0.452	0.516
P Enzyme	0.272	0.0005	<0.0001	<0.0001	0.565	<0.001	0.889
P Interation	0.223	0.157	0.0001	0.002	0.982	0.997	0.870
P Regression	0.141	0.029(Q)	0.0001(Q)	0.001(Q)	0.328	0.005(Q)	0.722

512 HP: high phytate; MP: medium phytate; LP: low phytate; PC: positive control; NC: negative control; SI: Seedor index; BS: breaking strength; DM: dry matter; BA: bone ash;
513 A1: growth plate; A2: hypertrophic cartilage zone; BMD: bone mineral density; Q: quadratic; *Regression analysis; Means followed by * in the same column differ at the 5%
514 level of significance by Dunnett's Test.
515 BS: 11.38670238+0.00583626*FTU-0.00000256*FTU^2; R^2: 0.20; FTU for maximum response: 1140; Maximum response: 15.
516 DM: 390.8542857+0.0576276*FTU-0.000286*FTU^2; R^2: 0.32; FTU for maximum response: 1008; Maximum response: 420.
517 BA: 438.7583333+0.0633929*FTU-0.0000243*FTU^2; R^2: 0.51; FTU for maximum response: 1304; Maximum response: 480.
518 A2: 41.54051329-0.00960195*FTU+0.00000367*FTU^2; R^2: 0.42; FTU for maximum response: 1308; Maximum response: 35.26.
519
520

Table 5. *Interaction between phytase and phytate on broiler tibia dry matter (DM) and bone ash (BA) at 21 d of age.*

Treatments	DM (g kg^{-1})				BA (g kg^{-1})			
	HP	MP[1]	LP[2]	P Tukey	HP[3]	MP	LP[4]	P Tukey
NC*	411.1^{a}	380.2^{b}	376.8^{b}	<0.0001	441.0	446.7	424.0	0.060
NC+500 FTU kg^{-1}*	413.4	420.0	417.4	0.661	462.7	473.3	471.0	0.113
NC+1000 FTU kg^{-1}*	410.5	416.2	419.5	0.539	474.3^{ab}	459.1^{b}	486.1^{a}	0.021
NC+1500 FTU kg^{-1}*	404.8	420.2	418.0	0.120	477.3	484.0	480.6	0.689
P Regression	0.406	0.005(Q)	0.001(Q)		0.007(L)	0.902	<0.0001(Q)	

HP: high phytate; MP: medium phytate; LP: low phytate; PC: positive control; NC: negative control; Q: quadratic; L: linear; *Regression analysis; Means followed by different letter in the same line differ at the 5% level of significance by Tukey`s Test.

[1]DM_{MP}: 382.8071429+0.0768857*FTU-0.0000358*FTU^2; R^2: 0.55; FTU for maximum response: 1074; Maximum response: 424.

[2]DM_{LP}: 378.5214286+0.0883000*FTU-0.0000421*FTU^2; R^2: 0.61; FTU for maximum response: 1049; Maximum response: 425.

[3]BA_{HP}: 441.0814286+5.5192202*FTU; R^2: 0.50.

[4]BA_{LP}: 424.5314286+0.1158629*FTU-0.0000526*FTU^2; R^2: 0.83; FTU for maximum response: 1101; Maximum response: 488.

538 **Table 6**. I*nteraction between phytase and phytate on broiler calcium (Ca), phosphorus (P) and alkaline phosphatase (ALP) blood at 21 d of age.*

Treatments	Blood Ca (mg dl^{-1})				Blood P (mg dl^{-1})				Blood ALP (U l^{-1})			
	HP[1]	MP	LP[2]	P Tukey	HP[3]	MP[4]	LP[5]	P Tukey	HP	MP[6]	LP	P Tukey
NC*	9.63[a]	9.62[a]	8.86[b]	0.002	5.53[a]	4.86[a]	3.83[b]	0.003	898.07	1046.79	937.64	0.436
NC+500 FTU kg^{-1}*	9.99	10.00	9.70	0.384	6.26	6.22	6.24	0.987	939.43	713.50	829.29	0.080
NC+1000 FTU kg^{-1}*	10.09	9.94	9.78	0.329	6.37[a]	6.46[ab]	5.89[b]	0.016	791.36	752.21	726.07	0.868
NC+1500 FTU kg^{-1}*	10.08	9.70	9.70	0.181	6.32	6.14	5.89	0.139	816.00	799.86	605.77	0.089
P Regression	0.043(L)	0.148	0.013(Q)		0.013(Q)	0.0005(Q)	0.0001(Q)		0.575	0.018(Q)	0.933	

539 HP: high phytate; MP: medium phytate; LP: low phytate; PC: positive control; NC: negative control; Q: quadratic; L: linear; *Regression analysis; Means followed by
540 different letter in the same line differ at the 5% level of significance by Tukey`s Test.
541 [1]Ca_{HP}: 9.638750000+0.000861429X; R^2= 0.25.
542 [2]Ca_{LP}: 8.892760714+0.001904307*FTU-0.000000925*FTU^2; R^2: 0.42; FTU for maximum response: 1029; Maximum response: 9.87.
543 [3]P_{HP}: 5.549571429+0.001668429*FTU-0.0000007815*FTU^2; R^2: 0.46; FTU for maximum response: 1067; Maximum response: 6.44.
544 [4]P_{MP}: 4.890330579+0.003316901*FTU-0.0000016675*FTU^2; R^2: 0.58; FTU for maximum response: 995; Maximum response: 6.54.
545 [5]P_{LP}: 4.017768595+0.004710384*FTU-0.000002375*FTU^2; R^2: 0.64; FTU for maximum response: 992; Maximum response: 6.35.
546 [6]ALP_{MP}: 1028.632143+0.7118071*FTU-0.000381*FTU^2; R^2: 0.30; FTU for maximum response: 934; Maximum response: 1361.

547 **Table 7**. I*nteraction between phytase and phytate on broiler calcium (Ca) and phosphorus*
548 *(P) bone at 21 d of age.*

Treatments	Bone Ca (g kg^{-1})				Bone P (g kg^{-1})			
	HP	MP	LP[1]	P Tukey	HP	MP[2]	LP	P Tukey
NC*	18.28	19.03	16.30	0.071	10.20^{ab}	11.10^{a}	8.83^{b}	0.007
NC+500 FTU kg^{-1}*	18.92	19.80	19.96	0.703	9.97	9.92	9.37	0.484
NC+1000 FTU kg^{-1}*	20.35	18.37	18.19	0.092	11.04^{a}	9.45^{bc}	9.42^{c}	0.006
NC+1500 FTU kg^{-1}*	19.88	19.26	20.52	0.272	11.14^{a}	9.58^{b}	10.66^{ab}	0.035
P Regression	0.575	0.938	0.048(L)		0.109	0.012(L)	0.363	

549 HP: high phytate; MP: medium phytate; LP: low phytate; PC: positive control; NC: negative control; L:
550 linear; *Regression analysis; Means followed by different letter in the same line differ at the 5% level of
551 significance by Tukey`s Test.
552 [1]Ca_{LP}: 16.77700000 + 0.00416233*FTU; R^2: 0.43.
553 [2]P_{MP}: 11.09831683-0.00298137*FTU; R^2: 0.42.

(Artigo nas normas da Revista Animal Feed Science and Technology)

5. INFLUENCE OF PHYTATE AND PHYTASE ON PERFORMANCE, BONE AND BLOOD PARAMETERS OF BROILERS AT 42 DAYS OF AGE

Abstract

The objective of this study was to evaluate the effect of diets containing different levels of phytate and phytase on broilers at 42 d of age. Broilers were distributed in a 3x5 factorial design, with seven replicates per treatment. The treatments consisted of a combination of diets containing high (HP), medium (MP) and low (LP) phytate and a positive control diet (PC), negative control diet (NC), and NC + 0, 500, 1000 or 1500 FTU kg^{-1} of phytase. Broilers that received the NC diet exhibited the lowest weight gain WG ($P<0.05$) while broilers supplemented with 1000 FTU kg^{-1} had 2.84% higher WG ($P<0.05$) compared to the PC. Broilers that received NC treatment had the lowest breaking strength (BS) (11.85% lower) and dry matter (DM) (4.92% lower) compared to the PC. Serum Ca and P of birds of HP group receiving the NC and NC+500 FTU kg^{-1} had a higher concentration ($P<0.05$) than LP. Serum P of birds fed diets containing MP and LP had a quadratic behavior ($P<0.05$) and the levels that provided the maximum responses were 1090 and 1110 FTU kg^{-1}, respectively. Broilers in the NC and NC+500 and 1000 FYT kg^{-1} had lower tibia Ca levels compared to those in the PC treatment; also, broilers receiving HP diets had a higher ($P<0.05$) tibia Ca content than those receiving MP. Bone P of birds fed diets containing LP had a quadratic behavior ($P<0.05$) and the levels that provided the maximum response was 470 FTU kg^{-1}. Phytase supplementation had a positive response in diets with reduced Ca and P. Phytase improves broiler performance based on regression analysis, with 952 FTU kg^{-1}.

Keywords: feedstuffs, nutrition, poultry production, phosphorus.

5.1. Introduction

Phytic acid is the main storage form of phosphorus (P) in cereal grains, legumes and protein. Phosphorus can be found in plant material as a mixed salt known as phytate, which represents 50-85% of the total P content in plant seeds (Pallauf and Rimbach, 1997; Cowieson et al., 2016).

Phytate has a low solubility in the small intestines, therefore it is poorly absorbed by broilers and since it carries a negative charge, it is a potent mineral chelator that forms

587 insoluble salts with minerals. In addition, phytate can also reduce the digestibility of protein
588 and energy (Wilkinson et al., 2014). Poultry diets are mainly composed of corn and soybean
589 meal in which most of P is in the phytate form. According to Ravindran (1995) broiler diets
590 contain about 2.5 to 4.0 g kg^{-1} of phytate.

591 Phytases are capable of initiating phytate dephosphorylation by generating a series
592 of lower myo-inositol phosphate esters through a succession of dephosphorylation reactions
593 to produce inositol and six inorganic P radicals (Selle and Ravindran, 2007). However, the
594 effectiveness of the enzyme is influenced by the characteristics of the animals (species, age,
595 physiological conditions), dietary factors such as phytate concentration and source,
596 concentration of minerals as well as the origin and level of phytase added to the diet
597 (Dersjant-Li et al., 2015).

598 Phytate utilization may vary between diets and its effects depend on the
599 ingredients used in the diets, mineral concentrations, protein content, and phytate solubility.
600 Gastrointestinal pH has an influence on phytate susceptibility because the addition of H^+
601 ions into the phosphate groups of phytate makes it susceptible to the phytase effects (Maenz
602 et al., 1999). The efficiency of phytate P use can also be affected by genetics. Modern
603 broilers show rapid growth, consume more feed and have a higher passage rate than older
604 broilers breeds, which may interfere with the use of phytate P and may contribute to the
605 inability of commercial chickens to use phytate P (Zhang et al., 2003).

606 This study was designed to evaluate the effect of phytase supplementation in diets
607 composed of high, medium and low phytate content on performance, bone characteristic,
608 blood parameters and processing yield of broilers from 42 days.

609

610 **5.2. Material and methods**

611 The experiment was conducted at the Experimental Station of the West Paraná
612 State University – Unioeste, *Campus* Marechal Cândido Rondon – PR, Brazil.
613 Experimental birds were handled with care to avoid unnecessary discomfort and all
614 experimental procedures were approved by the University ethical review committee.

615 Male Cobb 500 broilers chicks (n= 2,625) were obtained from a commercial
616 hatchery on the day of hatch. Chicks were randomized by weight and distributed into a 3x5
617 factorial design, consisting of 15 treatments with each treatment containing 7 replicates of
618 25 birds per experimental unit (EU). Treatments consisted of diets having high (HP),
619 medium (MP) and low phytate (LP) concentrations, formulated having high (HP), medium

(MP) and low (LP) phytate concentration based on vegetable ingredients, vegetable plus animal ingredients and animal ingredients, respectively. The treatments were composed by a positive control (PC) diet which aimed to provide the nutritional requirements of the animals; negative control (NC) with nutritional reduction of 0.15% calcium (Ca) and reduction of 0.15% phosphorus (P), and NC diet plus 0, 500, 1000 or 1500 FTU kg^{-1} of phytase. Phytase was added at the rate of 100 mg kg^{-1} diets to provide 500 phytase units (FTU) per kg of diet, 200 mg kg^{-1} diets to provide 1000 FTU kg^{-1} of diet, and 300 mg kg^{-1} diets to provide 1500 FTU kg^{-1} of diet. One FTU is defined as the amount of enzyme necessary to release one µmole of inorganic phosphate per minute from 5.0 mM sodium phytate at pH 5.5 and 37°C.

All diets were fed in mash form and birds were given *ad libitum* access to feed and water. The experimental diets were formulated according to the feed composition and nutritional requirements for a starter phase from 1 to 21 days and grower phase from 22 to 42 days (Table 1 and 2), proposed by Rostagno et al. (2017).

Phytase activity in the diets was determined using the ISO 30024 (International Organization for Standardization, 2009). The analyses were performed on all dietary treatments; a pool of starter and grower feed samples were sent to a commercial laboratory CBO (Valinhos, SP, Brazil) to determine the phytase activity (Table 3). Weight gain (WG), feed intake (FI) and feed conversion ratio (FCR) were recorded at 42 days of age. Mean individual bird weight and feed intake was calculated, taking into consideration mortalities, according to Sakomura and Rostagno (2016).

At 42 days of age two birds per pen were randomly selected, fasted for 6 h and blood samples were collected via brachial puncture. Blood was coagulated and centrifuged at 1008 g rpm for 10 min to obtain serum, which was stored at -20 °C. To perform the analyzes, serum was thawed at room temperature, centrifuged at 1008 g for 5 min and then Ca, P, and alkaline phosphatase (ALP) analyses were performed using a high-performance automatic spectrophotometer (Flexor EL 200, Elitech, Paris, France) with specific kits, calibrated with standards (Elical, Elitech).

Evaluation of bone development was conducted at 42 days of age. Two birds with mean group weights (±5%) were euthanized by eletronarcose followed by exsanguination, according to Normative Resolution Nº. 37 of February 15, 2018 of CONCEA. Legs were separated and deboned to obtain tibia. After deboning, the left tibia was weighed to the nearest ± 0.0001 g and their lengths were determined using a digital

caliper (accuracy of 0.01 mm). The Seedor Index (SI) (Seedor et al., 1991) was calculated by dividing the bone weight (mg) by its length (mm). After SI determination, tibia was stored individually at -20ºC for further analysis. Determination of bone breaking strength (BS) was performed after bone thawing at room temperature. Tibia was individually supported on the epiphyses regions. A force load of 200 kgf at the speed of 5 mm s^{-1} was applied in the central region of each bone using a probe TA-TPB and a Texturometer (CT3 Texture Analyzer, Brookfield). After BS was measured, tibia was weighed on an analytical balance (± 0.0001 g) and dry matter analyzed (AOAC, 1995). Samples were weighed, ashed overnight at 600°C and weighed again (*Adapted* Hall et al., 2003). The percentage of tibia ash was calculated as the proportion of the dry, pre-ashed tibia multiplied by 100. To determine the amount of Ca and P in the bones, the ashes were placed in a sand bath (250ºC) in a solution of HCl (6 M) to solubilize the minerals. Calcium was measured using an atomic absorption apparatus (GBC-932AA) and phosphorus using a spectrophotometer (UV/VIS GBC-916).

To evaluate the incidence of tibial dyschondroplasia, the left tibia of (n=105) of 42-day old birds were decalcified with 50% formic acid and 20% sodium citrate (Fernandes et al., 2007). After decalcification, the bone was embedded in paraffin (Beçak and Paulete, 1976). The sections were made with microtomes at 5 μm thickness and stained with Hematoxylin-Eosin, for observation of the epiphyseal disk area and measurements of the areas were used to characterize the incidence of tibial dyschondroplasia. For analysis of tibial epiphyseal cartilage slides, two distinct regions characterized by the morphological appearance were considered: growth plate (A1) and hypertrophic cartilage zone (A2). The images were measured with the aid of a computerized image analyzer PROPLUS IMAGE 4.1.

The left tibias (n=105) were used to determine radiographic bone mineral densitometry (BMD), which was performed at the Dentistry Clinic of the Hospital Universitário de Cascavel. The tibiotarsus was utilized to determine the optical densitometry in radiographic images compared to an aluminum scale with 10 degrees for 1 mm (penetrometer). The bones were radiographed with a dental X-ray machine (Orthopantomograph OP 300) at 85 kVp, 6.3 mA and 10 s of exposure time. The digital images were analyzed using Adobe Photoshop CS6. Five areas of each penetrometer degree (1–5 mm) were analyzed, and the equation was calculated from the values obtained. In addition, six areas of each bone were performed, and the obtained values were applied

in the equation to determine bone mineral density expressed as millimeters of aluminum (mm Al). Higher values indicated greater radiopacity and greater bone density. At the end of the experiment, four birds per pen were selected to evaluate carcass yield and cuts which included: wings, legs, breast, breast fillet, and abdominal fat (removed from around the cloaca and gizzard).

All data were analyzed using SAS - Version 9.1. An analysis of variance and subsequent polynomial regression between levels of inclusion of the enzyme was performed excluding the PC treatment. In addition, the Dunnett`s Test was performed at the 5% probability level to compare the PC treatment with the other treatments. Tukey`s Test was performed to compare the means of each phytate content.

5.3. Results

The analyzed phytase activity was higher than calculated values (Table 3). There were no interactions (P>0.05) between phytase supplementation and dietary phytate content on broilers performance (Table 4). No effects (P>0.05) were observed for enzyme supplementation and phytate content on feed intake (FI) and feed conversion ratio (FCR). Only broiler`s weight gain (WG) showed a quadratic response (P<0.05) with the level of phytase inclusion with the maximum response being calculated at 952 FTU kg^{-1}. Weight gain was significantly different (P<0.05) when phytase was added compared to the positive control (PC) treatment by Dunnett´s test. Broilers that received experimental diets low in available P and Ca (negative control - NC) and without phytase supplementation, exhibited the lowest WG (3.19% lower than PC). Broilers receiving 1000 FYT kg^{-1} achieved 2.84% higher WG compared to the PC treatment.

There were no interactions (P>0.05) between phytase supplementation and levels of dietary phytate on bone characteristics (Table 5). Seedor index (SI), bone ash (BA), growth plate (A1) and bone mineral densitometry (BMD) were not influenced (P>0.05) by enzyme supplementation neither by phytate content. Breaking strength (BS) had a quadratic effect (P=0.008), and the highest BS was obtained using 1023 FTU kg^{-1}. Broilers that received NC treatment, without phytase supplementation, had 11.85% and 4.92% lower BS and DM compared to the PC by Dunnett's Test. Hypertrophic cartilage zone (A2) was higher in broiler receiving LP diets when compared to birds on the MP diets (P <0.05).

No significant (P>0.05) interaction was found on alkaline phosphatase (ALP). There was an interaction (P<0.05) between phytase supplementation and phytate content on blood Ca and P (Tables 6 and 7). Serum Ca and P concentration of broilers fed diets with HP and receiving the NC and NC+500 FTU kg^{-1} was higher (P<0.05) than broilers fed diets with LP. In addition, broilers fed the NC+1500 FTU kg^{-1} and HP diet had a lower concentration (P<0.05) of serum Ca compared to birds fed diets with MP and LP. Serum P of birds fed diets containing MP and LP had a quadratic effect (P<0.05) and the levels that provided the maximum responses were 1090 and 1110 FTU kg^{-1}, respectively.

A significant difference (P<0.05) was also observed in tibia Ca content due to phytase addition. Broilers fed NC and NC+500 and 1000 FYT kg^{-1} treatments had lower tibia Ca levels compared to that fed PC treatment. In addition, broilers receiving HP diets had a higher tibia Ca content than those receiving MP (P<0.05). For tibia P content, there was an interaction (P<0.05) between phytase supplementation and phytate content. Bone P of birds fed diets containing LP had a quadratic behavior (P<0.05) and the level that provided the maximum response was 470 FTU kg^{-1}. Broilers receiving MP diets had a higher P content (P<0.05) than broilers fed LP diets.

Phytase level and phytate content did not influence (P>0.05) carcass yield and cuts of broilers (Table 7). Only abdominal fat of birds that received the NC treatment was lower (P<0.05) compared to PC treatment.

5.4. Discussion

Phytase supplementation increased phytate hydrolysis regardless of phytate level, as indicated by the lack of phytate × phytase interaction. Higher doses of phytase than standard levels exerted an additive effect, which was also manifested in higher WG in broilers fed diets with 1000 FTU kg^{-1} phytase compared to the PC treatment.

The phytate content in the diets did not influence broiler performance likely because the phytate concentration among diets was not large enough to show statistical differences. In a study conducted by Morgan et al. (2016), there was an improvement in WG and FCR of broilers fed diets with highly susceptible phytate, this means susceptible to phytase degradation, compared to those fed diets with low susceptible phytate; suggesting the occurrence of higher phytate hydrolysis in broilers fed the highly susceptible diet. According to the authors, the fraction of susceptible phytate indicates the "active" fraction of phytate that interferes in the digestion process and the higher level of

hydrolysis of this fraction may be correlated with better performance. In phytate-rich diets, a greater phytate hydrolysis occurs along the gastrointestinal tract regardless of the presence or absence of phytase. Other factors such as the ingredients being used, mineral and protein concentrations, phytate solubility, and gastrointestinal pH can also influence phytate susceptibility (Morgan et al., 2016).

An improvement of bone parameters is directly related to an increase in bone mineralization. Diets supplemented with phytase likely increased availability of P, Ca and other minerals released from the phytate mineral complex (Singh et al., 2003; Gautier et al., 2017). Effects of phytase could be observed under increasing hydrolysis of phytate antinutritional effects on divalent cations, making the bone characteristics of phytase supplemented broilers similar to those receiving the PC treatment. This positive action of the enzyme on bone characteristics was similar to the response observed on broiler`s WG. Better performance could be associated with an adequate bone mineralization, which is essential to sustain the muscular development. However, bone growth and mineralization are less pronounced during the finisher phase of the broilers and may explain the lack of statistical difference among some of the variables measured. Our data indicate an adequate bone development without disorders with a stabilized development of muscles, ligaments, and tendons, parameters which dependent on the bone state and stabilizes as the birds grow (Amoroso et al., 2013).

According to the interaction observed in blood samples, there are evidence that broilers fed diets with HP without phytase (NC) appear to be trying to digest and absorb Ca and P. This behavior was evident with the increase of Ca and P in the blood. As birds mature, there is a reduction in bone development, an increase in muscle development, and accumulation of fat, so that even if physiologically the bird does not require the same levels of Ca and P as in the initial phase, its absorption occurs, however this is not mobilized to the tissues which results in increase of circulating Ca and P. This effect is reversed when phytase was added at 500, 1000, and 1500 FTU kg^{-1} and there is stabilization in high, medium and low phytate of diet. In this case, phytase at higher doses is more effective in diets with HP.

The enzyme alkaline phosphatase (ALP) indicates the degree of bone remodeling. In the growth phase of the animal, higher concentrations of ALP indicate an increase in the formation of the bone tissue. However, mature animals, such as 42 days-old broilers have already undergone the process of bone formation and thus have lower levels

784 of ALP, which may explain why in the present study, the plasma concentration of ALP
785 was not influenced by the treatments.

786 Birds in treatments NC and NC+500 and NC+1000 FTU kg^{-1} had a lower Ca
787 deposition of tibia compared to the PC bones of bird, but with the inclusion of 1500 FTU
788 kg^{-1} the values were similar to PC, then the efficacy of phytase was observed. However,
789 the lower Ca content did not affect tibia BS and BA. According to interaction observed the
790 phytase was effective on P deposition, which made the NC diets similar to PC diets.

791 Phytase enzymes cause the liberation of inorganic P and Ca from the phytate
792 molecule, which results in higher P or Ca utilization resulting in an improvement in bone
793 mineralization (Perney et al., 1993). Bone characteristics such SI, BS, DM, BA, BMD, it
794 supposed to increase as more P is deposited into bone.

795 Phytate level and phytase supplementation did not influence carcass and cuts
796 weights. Shibata et al. (2012) evaluated diets with two levels of phytic acid (0.06 and
797 0.12%) and reported no differences on BW and parts (wing, leg, and breast) weights. In
798 addition, Singh et al. (2003) and Broch et al. (2018) reported that phytase supplementation
799 did not influence carcass yield and cuts. However, abdominal fat weight decreased
800 significantly in broilers receiving NC treatment. This difference may be associated with the
801 lower availability of nutrients.

802 Phytate and nutritional reduction of minerals can affect animal performance and
803 bone characteristics. However exogenous enzymes allows for greater flexibility during
804 feed formulation by increasing the available of nutrients from feed and reducing anti-
805 nutritional factors such as phytate (Broch et al., 2018). There are limited reported studies in
806 the literature investigating the influence of phytase in broiler diets with feed ingredients of
807 animal origin as the majority of previous studies were based on vegetable diets.

808 Feed formulas with high level of feed ingredients from animal origin have
809 potentially higher levels of Ca and P and lower level of phytate. Therefore, phytase
810 responses tend to be less pronounced in diets containing LP, due to their lower substrate
811 content. It's important to consider that P availability and solubility can vary among
812 phosphate sources, as well as interactions among minerals on P precipitation in the poultry
813 digesta (Hamdi et al., 2017).

814 Thus, it is desirable to investigate the impact of phytase on the performance of
815 broilers offered diets containing different phytate concentrations (Lio et al., 2016). Also, it
816 should be considered that phytases differ in their ability to hydrolyze phytate and this

difference is dependent on the concentration and source of phytate in the diet and the phytase features, which may be related to the kinetics of individual phytases, energy, amino acid density, animal genetic and age (Dos Santos et al., 2014; Cowieson et al., 2016).

5.5. Conclusion

The data from the current study showed that the phytase supplementation had a positive response in diets with reduced Ca and P. Phytase improved broiler performance based on regression analysis, with 952 FTU kg^{-1} being the optimum inclusion level without having a negative impact on the other parameters evaluated. The overall effect of phytase could have been more pronounced if greater difference in phytate concentration would have been used. It should also be considered that the requirements of development of tibias of birds reduce with advancing age.

Conflict of interest statement

The authors declare there are not any conflicts of interest.

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928 phytate phosphorus bioavailability: Heritability and genetic correlations with
929 growth and feed utilization traits in a randombred chicken population. Poult. Sci.,
930 82, 1075-1079.

931 Table 1. Composition and nutrient specifications of the experimental diets used during the
932 starter phase (1-21 days) for broilers

Ingredients (g kg^{-1})	HP		MP		LP	
	PC	NC	PC	NC	PC	NC
Corn	539.0	553.5	577.3	590.9	610.0	624.5
Soybean meal (45%)	335.9	333.4	282.2	280.2	287.1	284.6
Gluten feed meal	20.0	20.0	20.0	20.0	20.0	20.0
Wheat bran	30.0	30.0	30.0	30.0	-	-
Soybean oil	31.1	26.2	17.7	13.1	10.1	05.2
Meat & bone meal	-	-	20.0	20.0	20.0	20.0
Poult. bypro. meal	-	-	18.5	18.5	18.5	18.5
Monob. phosphate	15.44	7.77	7.71	0.04	8.1	0.39
Limestone	11.70	11.85	8.01	8.17	7.9	8.03
Byo-Lys (51.7%)	4.50	4.57	5.63	5.66	5.53	5.60
Salt	3.31	3.30	2.82	2.81	2.81	2.81
DL-Methionine (98%)	2.96	2.94	3.10	3.08	3.07	3.06
Vitamin[a]	1.50	1.50	1.50	1.50	1.50	1.50
Na bicarbonate	1.50	1.50	1.50	1.50	1.50	1.50
L-Threonine (99%)	0.82	0.82	1.16	1.15	1.10	1.11
Choline chloride	0.60	0.60	0.60	0.60	0.60	0.60
Avilamycin	0.55	0.55	0.55	0.55	0.55	0.55
L-Valine (99%)	0.38	0.38	0.67	0.65	0.63	0.64
Mineral[b]	0.50	0.50	0.50	0.50	0.50	0.50
BHT	0.20	0.20	0.20	0.20	0.20	0.20
L-Isoleucine (99%)	-	-	0.36	0.38	0.29	0.31
Avilamycin 10%	0.05	0.05	0.05	0.05	0.05	0.05
Inert (sand)	-	0.40	-	0.40	-	0.40
Nutrient specification (g kg^{-1})						
Met. En (MJ kg^{-1})	12,56	12,56	12,56	12,56	12,56	12,56
Crude protein	213.9	213.9	213.9	213.9	213.9	213.9
Calcium	8.56	7.06	8.56	7.06	8.56	7.06
Total P	6.49	5.08	6.53	5.13	6.45	5.01
Av. P	4.20	2.70	4.17	2.67	4.17	2.67

Phytate P	2.44	2.46	2.33	2.35	2.22	2.24
Sodium	1.90	1.90	1.90	1.90	1.90	1.90
Dig. lysine	12.26	12.26	12.26	12.26	12.26	12.26
Dig. met+cys	8.84	8.84	8.84	8.84	8.84	8.84
Dig. threonine	7.97	7.97	7.97	7.97	7.97	7.97
Dig. valine	9.44	9.44	9.44	9.44	9.44	9.44
Dig. isoleucine	8.33	8.32	8.22	8.22	8.22	8.22

PC: positive control; NC: negative control; HP: high phytate; MP: medium phytate; LP: low phytate.

[a] Vitamin premix for birds. Levels per kilogram product: Vit. A (min) 2.7g, Vit. D3 (min) 0.75g, Vit. E (min) 0.06g, Vit. K3 (min) 2.5g, Vit. B1 (min) 1.5mg, Vit. B2 (min) 6g, Vit. B6 (min) 3g, Vit. B12 (min) 0.0012µg, Pantothenic acid (min) 12g, Niacin (min) 25g, Folic acid (min) 800mg, Biotin (min) 60mg, Selenium (min) 0.25g. [b] ROLIGOMIX - Mineral premix for birds. Levels per kilogram product: Copper (min) 20g, Iron (min) 100g, Manganese (min) 160g, Cobalt (min) 2g, Iodine (min) 2g, Zinc (min) 100g.

960 Table 2. Composition and nutrient specifications of the experimental diets used during the
961 grower phase (22-42 days) for broilers

Ingredients (g kg^{-1})	HP		MP		LP	
	PC	NC	PC	NC	PC	NC
Corn	599.0	613.4	629.3	644.0	662.0	676.5
Soybean meal (45%)	261.1	258.6	211.9	209.2	229.6	227.0
Gluten feed meal	35.0	35.0	28.0	28.0	18.0	18.0
Wheat bran	35.0	35.0	35.0	35.0	-	-
Soybean oil	31.9	26.9	23.5	18.5	17.2	12.2
Feather meal	-	-	10.0	10.0	10.0	10.0
Poult. bypro. meal	-	-	28.0	28.0	30.0	30.0
Monob. phosphate	11.99	4.32	7.67	-	7.67	-
Limestone	10.67	10.82	9.55	9.71	9.29	9.45
Byo-Lys (51.7%)	4.83	4.90	5.81	5.90	5.22	5.29
Salt	3.52	3.51	3.21	3.21	3.19	3.18
DL-Methionine (98%)	2.28	2.27	2.42	2.40	2.48	2.47
Vitamin[a]	1.20	1.20	1.20	1.20	1.20	1.20
Na bicarbonate	1.00	1.00	1.00	1.00	1.00	1.00
L-Threonine (99%)	0.54	0.54	0.78	0.78	0.70	0.70
Choline chloride	0.55	0.55	0.55	0.55	0.55	0.55
Avilamycin	0.55	0.55	0.55	0.55	0.55	0.55
L-Valine (99%)	0.18	0.18	0.40	0.41	0.36	0.36
Mineral[b]	0.50	0.50	0.50	0.50	0.50	0.50
BHT	0.20	0.20	0.20	0.20	0.20	0.20
L-Isoleucine (99%)	-	-	0.30	0.32	0.23	0.24
Avilamycin 10%	0.05	0.05	0.05	0.05	0.05	0.05
L-Thriptofane	-	-	0.13	0.14	0.10	0.11
Inert (sand)	-	0.40	-	0.40	-	0.40
Nutrient specification (g kg^{-1})						
Met. En. (MJ kg^{-1})	12,98	12,98	12,98	12,98	12,98	12,98
Crude protein	194.0	194.0	194.0	194.0	194.0	194.0
Calcium	7.32	5.82	7.32	5.82	7.32	5.82
Total P	5.69	4.29	5.60	4.19	5.46	4.05

Av. P	3.42	1.92	3.42	1.92	3.42	1.92
Phytate P	2.39	2.41	2.25	2.27	2.11	2.13
Sodium	1.85	1.85	1.85	1.85	1.85	1.85
Dig. lysine	10.78	10.78	10.78	10.78	10.78	10.78
Dig. met+cys	7.87	7.87	7.87	7.87	7.87	7.87
Dig. threonine	7.01	7.01	7.01	7.01	7.01	7.01
Dig. tryptophane	1.98	1.98	1.94	1.94	1.94	1.94
Dig. valine	8.41	8.41	8.41	8.41	8.41	8.41
Dig. isoleucine	7.42	7.40	7.33	7.33	7.33	7.33

PC: positive control; NC: negative control; HP: high phytate; MP: medium phytate; LP: low phytate.

[a] Vitamin premix for birds. Levels per kilogram product: Vit. A (min) 2.7g, Vit. D3 (min) 0.75g, Vit. E (min) 0.06g, Vit. K3 (min) 2.5g, Vit. B1 (min) 1.5mg, Vit. B2 (min) 6g, Vit. B6 (min) 3g, Vit. B12 (min) 0.0012µg, Pantothenic acid (min) 12g, Niacin (min) 25g, Folic acid (min) 800mg, Biotin (min) 60mg, Selenium (min) 0.25g. [b]ROLIGOMIX - Mineral premix for birds. Levels per kilogram product: Copper (min) 20g, Iron (min) 100g, Manganese (min) 160g, Cobalt (min) 2g, Iodine (min) 2g, Zinc (min) 100g.

Table 3. Analyzed phytase activity in experimental feed

Expected activity	Measured in mash		
	HP	MP	LP
0 FTU kg^{-1}	0	0	0
500 FTU kg^{-1}	520	590	610
1000 FTU kg^{-1}	1210	1280	1230
1500 FTU kg^{-1}	1650	2000	1840

HP: high phytate; MP: medium phytate; LP: low phytate.

979 Table 4. Effect of dietary phytate and phytase on broiler performance at 42 d of age

Treatments	FI (g)	WG (g)	FCR (g g^{-1})
HP	4400	2850	1.545
MP	4320	2800	1.547
LP	4270	2820	1.518
PC	4300	2820	1.527
NC+0*	4260	2730*	1.563
NC+500 FTU kg^{-1}*	4390	2860	1.553
NC+1000 FTU kg^{-1}*	4340	2900*	1.498
NC+1500 FTU kg^{-1}*	4340	2840	1.530
Mean	4330	2830	1.530
CV (%)	4.76	3.98	4.65
SEM	0.020	0.011	0.007
P Phytate	0.066	0.430	0.399
P Enzyme	0.226	<0.001	0.087
P Interation	0.234	0.553	0.138
P Regression	0.259	<0.001(Q)	0.534

980 HP: high phytate; MP: medium phytate; LP: low phytate; PC: positive control; NC: negative control; FI: feed
981 intake; WG: weight gain; FCR: feed conversion ratio; Q: quadratic; CV: coefficient of variation; *Regression
982 analysis; Means followed by * in the same column differ at the 5% level of significance by Dunnett's Test.
983 WG: $2702.317564+0.422784*FTU-0.000222*FTU^2$; R^2: 0.37; FTU for maximum response: 952; Maximum
984 response: 2904.

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993 Table 5. Effect of dietary phytate and phytase on bone characteristics of broiler at 42 d of age

Treatments	SI	BS (kgf mm^{-1})	DM (g kg^{-1})	BA (g kg^{-1})	A1 (mm^2)	A2 (mm^2)	BMD (mmAl)
HP	143.54	30.09	479.2	418.2	24.25	57.77^{ab}	3.79
MP	144.65	30.98	479.1	404.9	23.74	54.10^{b}	3.82
LP	145.46	31.35	487.2	412.4	23.72	60.42^{a}	3.88
PC	144.60	32.06	489.5	415.6	23.90	56.23	3.85
NC+0*	145.53	28.26*	465.4*	407.5	23.23	57.28	3.87
NC+500 FTU kg^{-1}*	145.02	30.64	479.9	410.5	22.89	61.69	3.90
NC+1000 FTU kg^{-1}*	146.36	33.06	492.8	410.7	23.94	56.19	3.76
NC+1500 FTU kg^{-1}*	141.21	31.27	487.2	418.1	25.72	55.53	3.76
Mean	144.56	31.06	483.3	412.6	23.93	57.40	3.83
CV (%)	7.27	15.77	5.04	6.04	14.85	19.17	8.19
SEM	1.04	1.04	0.24	0.25	0.36	1.08	0.03
P Phytate	0.786	0.556	0.597	0.136	0.704	0.048	0.522
P Enzyme	0.417	0.009	0.003	0.560	0.080	0.346	0.567
P Interation	0.905	0.849	0.112	0.362	0.051	0.164	0.730
P Regression	0.304	0.031(Q)	0.462	0.395	0.138	0.280	0.443

994 HP: high phytate; MP: medium phytate; LP: low phytate; PC: positive control; NC: negative control; SI: Seedor Index; BS: breaking strength; DM: dry matter; BA: bone
995 ash; A1: growth plate; A2: hypertrophic cartilage zone; BMD: bone mineral density; Q: quadratic; CV: coefficient of variation; *Regression analysis; Means followed by *
996 in the same column differ at the 5% level of significance by Dunnett's Test; Means followed by different letter in the same line differ at the 5% level of significance by
997 Tukey`s Test.
998 BS: $28.04408333+0.00855555*FTU-0.00000418*FTU^2$; R^2: 0.13; FTU for maximum response: 1023.4; Maximum response: 32.42.

999 Table 6. Effect of dietary phytate and phytase on blood and bone parameters of broiler at
1000 42 d of age

Treatments	Ca Blood (mg dl^{-1})	P Blood (mg dl^{-1})	ALP Blood (U l^{-1})	Ca Bone (g kg^{-1})	P Bone (g kg^{-1})
HP	9.32[a]	5.96[a]	186.88	19.25[a]	9.87
MP	9.18[ab]	5.34[b]	190.11	17.61[b]	10.23
LP	8.92[b]	5.36[b]	205.38	18.48[ab]	9.72
PC	9.54	5.95	206.17	19.40	10.19
NC+0*	9.16	4.99*	207.33	17.24*	9.33*
NC+500 FTU kg^{-1}*	9.39	5.80	203.93	18.09*	9.68
NC+1000 FTU kg^{-1}*	8.98	5.66	180.93	18.01*	9.79
NC+1500 FTU kg^{-1}*	9.03	5.63	187.07	19.44	10.79
Mean	9.22	5.64	196.78	18.43	9.95
CV (%)	9.09	13.05	30.35	10.14	10.61
SEM	0.08	0.07	5.91	0.19	0.11
P Phytate	0.047	<0.001	0.393	<0.001	0.050
P Enzyme	0.128	<0.001	0.468	<0.001	<0.001
P Interation	<0.001	<0.001	0.160	0.376	0.031
P Regression	0.534	0.003(Q)	0.322	0.438	0.153

1001 HP: high phytate; MP: medium phytate; LP: low phytate; PC: positive control; NC: negative control; Ca:
1002 Calcium; P: Phosphorus; ALP: Alkaline phosphatase; Q= quadratic; CV= coefficient of variation; *
1003 Regression analysis; Means followed by * in the same column differ at the 5% level of significance by
1004 Dunnett's Test. Means followed by a different letter in the same column differ at the 5% level of significance
1005 by Tukey`s Test
1006 P_{blood}: 4.723000000+0.002319095*FTU-0.000001174*FTU^2; R^2: 0.20; FTU for maximum response: 987.7;
1007 Maximum response: 5.9.
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Table 7. Interaction between phytate and phytase on blood and bone gof broilers at 42 days of age

Treatments	Ca Blood (mg dl^{-1})				P Blood (mg dl^{-1})				P Bone (g kg^{-1})			
	HP	MP	LP	P Tukey	HP	MP[1]	LP[2]	P Tukey	HP	MP	LP[3]	P Tukey
NC+0*	10.14[a]	8.67[b]	8.67[b]	<0.001	6.26[a]	4.37[b]	3.70[b]	<0.001	9.02	9.56	9.42	0.469
NC+500*	10.27[a]	9.25[ab]	8.64[b]	0.003	6.61[a]	5.47[b]	5.32[b]	<0.001	9.96[ab]	10.35[a]	8.58[c]	0.003
NC+1000*	8.48	9.34	9.12	0.061	5.46	5.72	5.46	0.245	9.17	10.46	9.72	0.121
NC+1500*	8.39[b]	9.45[a]	9.25[a]	0.001	5.57	5.65	5.68	0.883	10.73	10.77	10.89	0.940
P Regression	0.128	0.098	0.140		0.611	0.001(Q)	<0.001(Q)		0.07	0.06	0.008 (Q)	

HP: high phytate; MP: medium phytate; LP: low phytate; NC: negative control; Q= quadratic; * Regression analysis; Means followed by different letter in the same line differ at the 5% level of significance by Tukey`s Test

[1] P_{MP}: 4.194321429+0.003021500*FTU-0.000001386*FTU^2; R^2: 0.50; FTU for maximum response:1090; Maximum response: 5.84.

[2] P_{LP}: 3.443285714+0.004506571*FTU-0.000002029*FTU^2; R^2: 0.90; FTU for maximum response:1110.5; Maximum response: 5.95.

[3] P_{LP}: 9.302970297- 0.001876040*FTU+ 0.000001997*FTU^2; R^2: 0.54; FTU for maximum response:469.7; Maximum response: 8.86.

Table 8. Effect of dietary phytate and phytase on carcass yield and cuts (g) of broiler at 42 d of age

Treatments	Carcass	Wing	Whole leg	Breast	Breast fillet	Abdominal fat
HP	706.5	103.4	292.7	405.9	354.3	19.3
MP	708.0	103.6	288.6	411.8	358.4	18.7
LP	705.8	10.53	288.4	403.2	350.2	19.4
PC	708.4	102.4	290.1	398.3	344.5	21.1
NC+0*	705.8	106.4	285.6	410.3	357.2	17.3*
NC+500 FTU kg^{-1}*	704.6	103.5	291.1	408.3	353.3	19.7
NC+1000 FTU kg^{-1}*	707.5	104.5	293.8	399.4	351.6	19.6
NC+1500 FTU kg^{-1}*	709.4	101.9	292.7	409.7	355.0	20.5
Mean	707.1	103.8	290.7	405.2	352.3	1.97
CV (%)	1.48	5.81	4.36	3.97	5.69	18.63
SEM	0.104	0.06	0.13	0.16	0.20	0.04
P Phytate	0.783	0.409	0.248	0.094	0.313	0.562
P Enzyme	0.559	0.100	0.197	0.062	0.817	0.012
P Interation	0.960	0.702	0.556	0.428	0.776	0.051
P Regression	0.361	0.087	0.078	0.142	0.632	0.055

HP: high phytate; MP: medium phytate; LP: low phytate; PC: positive control; NC: negative control; Q= quadratic; CV= coefficient of variation; * Regression analysis; Means followed by * in the same column differ at the 5% level of significance by Dunnett's Test.