UNIVERSIDADE ESTADUAL PAULISTA – UNESP

CÂMPUS DE JABOTICABAL

AVALIAÇÃO DE FITASES MICROBIANAS EM DIETAS DE FRANGOS DE CORTE

Gabriel Villela Dessimoni Pinto

Zootecnista

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Gabriel Villela Dessimoni Pinto
Orientadora: Profa. Dra. Nilva Kazue Sakomura

Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Doutor em Zootecnia

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CAMPUS DE JABOTICABAL

FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS DE JABOTICABAL

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TÍTULO: AVALIAÇÃO DE FITASES MICROBIANAS EM DIETAS DE FRANGOS DE CORTE

AUTOR: GABRIEL VILLELA DESSIMONI PINTO

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DADOS CURRICULARES DO AUTOR

GABRIEL VILLELA DESSIMONI - Nasceu em 02 de janeiro de 1986 em Lavras – Minas Gerais no Brasil, onde viveu por 17 anos cercado por animais de fazenda acompanhado pelo seu avô. É filho de Ricardo Gomide Dessimoni Pinto e Nísia Andrade Villela Dessimoni Pinto. Ingressou em fevereiro de 2004 no Curso de Zootecnia pela Universidade Federal dos Vales do Jequitinhonha e Mucuri - UFVJM, em Diamantina – MG, sendo aluno de iniciação científica, trabalhando com ovinocultura; estagiou na EMBRAPA Milho e Sorgo, atuando na "Integração Lavoura-Pecuária" na cidade de Sete Lagoas – MG; atuou dando consultorias pela Empresa Jr. e obteve o título de Zootecnista em dezembro de 2008. Após este período ingressou na pós graduação e obteve o título de mestre, sendo bolsista CNPq pela mesma instituição, trabalhando com aditivos dentro da avicultura. Em janeiro de 2012, mudou-se para Jaboticabal –SP para cursar o doutorado dando continuidade à linha de pesquisa estabelecida, sendo também financiado pelo CNPq. Após o término do doutorado, Gabriel pensa em seguir uma carreira de oportunidades.

“*Feliz o homem que encontrou a sabedoria, o homem que alcançou o entendimento! Ganha-la vale mais do que a prata, e seu lucro mais do que o ouro. É mais valiosa do que as pérolas; nada que desejas a iguala. Em sua direita: longos anos; em sua esquerda: riqueza e honra! Os seus caminhos são deliciosos, e os seus trilhos são prosperidade. É uma árvore de vida para os que a colhem, e felizes são os que a retêm!*”

(Provérbios 3:13-14)

DEDICATÓRIA

Dedico primeiramente a Deus pela oportunidade e até mesmo pelos obstáculos colocados em meu caminho para que eu pudesse vence-los e me tornar mais forte a cada dia.

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A minha esposa Aline pela força, apoio, companheirismo e compreensão.

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SUMÁRIO

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CERTIFICADO

Certificamos que o Protocolo nº 013022/14 do trabalho de pesquisa intitulado **"Estudo da eficácia dos níveis de fitases na performance, mineralização dos ossos e parâmetros da cama em frangos de corte alimentados com dietas à base de milho e soja"**, sob a responsabilidade da Prof.ª Dr.ª Nilva Kazue Sakomura está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), em reunião ordinária de 03 de setembro de 2014.

Jaboticabal, 03 de setembro de 2014.

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C E R T I F I C A D O

Certificamos que o Protocolo nº 018760/14 do trabalho de pesquisa intitulado **"Desempenho e digestibilidade de dietas de frangos de corte contendo fitase e carboidrases",** sob a responsabilidade da Profª Drª Nilva Kazue Sakamura está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), em reunião ordinária de 09 de outubro de 2014.

Jaboticabal, 09 de outubro de 2014.

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AVALIAÇÃO DE FITASES MICROBIANAS EM DIETAS DE FRANGOS DE CORTE

RESUMO – O objetivo do estudo foi avaliar fitases originárias de diferentes microrganismos sobre o desempenho, características ósseas, excreção e digestibilidade dos nutrientes, bem como a biodisponibilidade do fósforo e avaliação econômica. O método da curva padrão foi utilizada para obter a biodisponibilidade de P, em resposta das variáveis de desempenho e mineralização óssea. O estudo compreendeu a realização de quatro experimentos. No primeiro, foram avaliadas fitases de origem *Buttiauxella sp* (A; expressa em *Trichoderma reesei*) e *E. coli* (B; expressa em *S*. *pombe*). Um total de 2.160 frangos de corte da linhagem Cobb 500, com 1 dia de idade, foram distribuídos em um delineamento experimental inteiramente casualizado, contendo nove tratamentos e oito repetições, cada repetição contendo 30 aves. Os tratamentos foram: T1 - Controle Positivo (PC) - dieta com total atendimento nutricional às exigências das aves; T2 – Controle Negativo 1 (CN1) - redução de 0,14% de P e 0,13% de Ca, em relação ao CP; T3 – Controle negativo 2 (CN2) - redução de 0,17% de P e 0,16% de Ca, em relação ao CP; T4 – CN2 + fitase A (250 FTU / kg); T5 – CN2 + fitase A (500 FTU / kg); T6 - CN2 + fitase A (1000 FTU / kg); T7 - CN2 + fitase B (250 FTU / kg); T8 - CN2 + fitase B (500 FTU / kg); T9 - CN2 + B fitase (1000 FTU / kg). Um segundo experimento foi realizado para avaliar a resposta da inclusão de uma fitase bacteriana (*E. Coli*) em dietas com total atendimento às exigências e redução nutricional. Um total de 896 frangos de corte machos Cobb (500) foram divididos em 32 unidades experimentais, cada unidade contendo 28 aves. O delineamento experimental foi o inteiramente casualizado, com quatro tratamentos e oito repetições. Os tratamentos foram: Controle Positivo (PC) - dieta com total atendimento às exigências nutricionais das aves; Controle Negativo (NC) – dieta com redução de 100kcal/kg de energia metabolizável, 0,14% de Pdisp e 0,11% de Ca, em relação ao CP; NC + Fitase (500FTU / kg); PC + Fitase (500FTU / kg). O terceiro experimento foi realizado para avaliar a inclusão de fitase *E. coli* (expressa em *Pichia pastoris*). Um total de 1890 frangos de corte machos, com 1 dia de idade, da linhagem Cobb 500 foram distribuídos em seis tratamentos e sete repetições contendo 45 aves cada, em um delineamento experimental inteiramente casualizado. Os tratamentos foram: controle positivo (PC) - dieta com total atendimento nutricional às exigências das aves; Controle Negativo (NC1) - redução de 0,06% do Pdisp, em relação ao CP; Controle Negativo 2 (NC2) - redução de 0,12% de Pdisp, em relação ao CP; NC2 + Fitase (FTU 120); NC2 + Fitase (FTU 180); NC2 + Fitase (FTU 240). No quarto experimento, foram comparadas 4 fitases, sendo 2 fúngicas (F1 e F2) e 2 bacterianas (B1 e B2), adicionadas a dietas a base de milho e farelo de soja. Um total de 2205 pintos machos Cobb 500 de 1 dia foram distribuídos em sete tratamentos e sete repetições contendo 45 aves cada, distribuídos em um delineamento experimental inteiramente casualizado. Os tratamentos foram: Controle Positivo (CP) - dieta com total atendimento às exigências nutricionais das aves; Controle Negativo (CN1) - redução de 0,06% de Pdisp, em relação ao CP; Controle Negativo 2 (CN2) - redução de 0,12% de Pdisp, em relação ao CP; CN2 + fitase fúngica 1 (F1); CN2 + fúngica fitase 2 (F2); CN2 + fitase bacteriana 1 (B1) e CN2 + fitase bacteriana 2 (B2). Todas as fitases foram adicionadas na concentração de 500 FTU/kg. Foram realizadas analises multivariadas exploratória nos dados. No primeiro estudo verificamos que a fitase derivada de *Buttiauxella sp* têm uma maior eficiência

para ganho de peso e consumo de ração em concentração de 500 FTU/kg, em relação a fitase *E. coli*, com 1000 FTU/kg. Ambas as fitases reduziram teores de fósforo total e fósforo solúvel na cama. A biodisponibilidade calculada com base no teor de cinzas da tíbia e ganho de peso, indicou que a fitase *E. coli* é mais eficaz em depositar minerais nos ossos, enquanto que a fitase *Buttiauxella sp* é a mais eficaz para o deposição de peso. O conteúdo mineral dos ossos (P, Zn e cinzas) e força de deflexão das tíbias foram melhoradas pela adição de ambas as fitase. As fitases utilizadas a uma concentração de 1000 FTU/kg resultaram em melhor relação custo/benefício, considerando o preço fosfato bicálcico de U$ 0,50/kg. No segundo estudo, a adição de fitase *E. coli* aumentou 7,0 e 5,3% o consumo de ração, 8,9 e 8,8% o ganho de peso e melhorou 2 e 5 pontos da conversão alimentar nas fases iniciais e de crescimento, respectivamente em relação a dieta CN. A inclusão "on top" de fitase (adição no CP), melhorou 2 pontos na conversão alimentar, em relação a dieta CP. A inclusão de fitase em dieta com redução nutricional melhora a digestibilidade da matéria seca (MS), proteína bruta (PB), fósforo (P), energia digestível aparente na matéria natural (ED na MN) e na matéria seca, em relação a dieta sem fitase, bem como os teores de cinzas, cálcio e fósforo nas tíbias. A inclusão de fitase na dieta com total atendimento nutricional ("*on top*") melhora a digestibilidade de PB, ED na NM, cálcio (Ca) e P, em relação a mesma dieta, sem a enzima. A deposição de cinzas nas tíbias das aves alimentadas com adição "*on top*" de fitase é superior às que não receberam fitase. Considerando o custo da fonte de fósforo inorgânico, foi obtido uma economia de U$ 3,72 / tonelada de ração, com a inclusão da fitase. No terceiro estudo, a fitase *E. Coli* (expressa em *Pichia pastoris*), demonstrou ser eficiente, melhorando o ganho de peso, consumo de ração, conversão alimentar, viabilidade e as características ósseas das aves alimentadas com redução de P na dieta. No entanto, os níveis mais próximos ao teor máximo estudado (240 OTU) apresentaram os melhores resultados. A substituição de fosfato bicálcico por fitase foi economicamente viável quando o custo por OTU/g não excedeu U$0,000014, U$0,000012 e U$0,000010, para as concentrações de 120, 180 e 240 OTU, respectivamente. No último experimento, com base na análise multivariada: análise de agrupamento pelo método hierárquico, análise de componentes principais e análise discriminante confirmaram que a dieta controle positivo e controle negativo tem propriedades muito específicas e contrastantes. As fitases fúngicas (F1 e F2) apresentam um mesmo padrão de respostas, bem como fitases bacterianas (B1 e B2). Ambas as fitases foram superiores, quando comparado ao controle negativo, no entanto, eles não foram capazes de igualar às respostas da dieta controle positivo. As fitases bacterianas foram mais eficientes em recuperar as respostas de desempenho, sendo mais semelhantes à dieta controle positivo. Todas as fitases foram efetivas, conforme suas avaliações, entretanto, a forma de utiliza-las dependerá do objetivo de cada produção.

Palavras-chave: fitato, desempenho, nutrientes, custo, multivariada, tíbia

EVALUATION OF MICROBIAL PHYTASES IN BROILER DIETS

ABSTRACT - The objective of this study was to evaluate phytases from different microorganisms on performance, bone characteristics, excretion and digestibility of nutrients, as well as phosphorus bioavailability and costs. The standard curve method was used to obtain the phosphorus bioavailability in response of performance and bone mineralization. The study was divided in four trials. In the first study were evaluated phytases from *Buttiauxella* sp (A; expressed in *Trichoderma reesei*) and *E. coli* (B; expressed in *S. pombe*) origins. A total of 2,160 male day-old Cobb 500 chicks were distributed in a completely randomized design to nine treatments and eight replicates containing 30 birds each. The treatments were: T1 - Positive Control (PC) – meeting all requirements of the birds; T2 - Negative Control 1 (NC1) – reduction of 0.14% P and 0.13% Ca; T3 - Negative Control 2 (NC2) - reduction of 0.17% P and 0.16% Ca; T4 - NC2 + phytase A (250 FTU/kg); T5 - NC2 + phytase A (500 FTU/kg); T6 - NC2 + phytase A (1000 FTU/kg); T7 - NC2 + phytase B (250 FTU/kg); T8 - NC2 + phytase B (500 FTU/kg); T9 - NC2 + phytase B (1000 FTU/kg). Regressions were performed on performance data, bone mineralization and excretion of nutrients to determine the optimal level inclusion of each phytase. A second trial was conducted to evaluate the response of the inclusion of a new bacterial phytase (*E. Coli*) supplemented on diets meeting all requirements of the birds, or with nutritions reduction. A total of 896 male day-old Cobb 500 broilers were divided into 32 experimental units, each unit consisting of 28 birds. The design was completely randomized with four treatments and eight replications. The treatments were: Positive Control (PC) - diet meeting the nutritional requirements of the birds; Negative Control (NC) – reduction of 100kcal ME/kg; 0.14% $_{av}$P and 0.11% Ca; NC + Phy (500FTU/kg); PC + Phy (500FTU/kg). The third trial was conducted to evaluate the inclusion of *E. coli* phytase (expressed in *Pichia pastoris*). A total 1890 male day-old Cobb 500 chicks were assigned to six treatments and seven replicates with 45 birds each, distributed in a completely randomized design. The treatments were: Positive Control (PC) - diet meeting the nutritional requirements of the birds; Negative Control (NC1) - reduction of 0.06% $_{av}$P; Negative Control 2 (NC2) - reduction of 0.12% $_{av}$P; NC2 + Phytase (120 FTU); NC2 + Phytase (180 FTU); NC2 + Phytase (240 FTU). At the fourth trial four phytases were compared, being two fungal (F1 and F2) and two bacterial (B1 and B2), supplemented in a corn and soybean meal based diets. A total of 2,205 male day-old Cobb 500 chicks were assigned to seven treatments and seven replicates containing 45 birds and were distributed in a completely randomized design. The treatments were: Positive Control (PC) - diet meeting the nutritional requirements of the birds; Negative Control 1 (NC1) - reduction of 0.06% $_{av}$P; Negative Control 2 (NC2) - reduction of 0.12% $_{av}$P; NC2 + Fungal Phytase 1 (F1); NC2 + Fungal Phytase 2 (F2); NC2 + Bacterial Phytase 1 (B1); and NC2 + Bacterial Phytase 2 (B2). All phytases were included in the concentration of 500 FTU/kg. Exploratory multivariate analyzes of the data were performed. In the first study it was found that phytase derived from *Buttiauxella* sp have a higher efficiency for body weight gain and feed intake in concentrations around 500 FTU/kg, while *E. Coli* phytase acts better around 1000 FTU/kg. Both phytases decreased the content of total phosphorus and soluble phosphorus in litter. The bioavailability calculated based on ash content and body

weight gain, indicated that *E. coli* phytase is more efficient in depositing minerals in the bones, while *Buttiauxella* sp phytase is more efficient for body weight gain. Mineral contents (P, Zn and Ash) and deflection force of tibias were improved by adding both phytases to the diets. The phytases in concentration of 1000 FTU/kg resulted in better cost/benefit ratio, considering the dicalcium phosphate price of $0.50. In the trial 2, the *E. coli* phytase addition increased ($p<0.05$) of 7.0 and 5.3% in FI, 8.9 and 8.8% in BWG and improved 2 and 5 points in the FCR in initial and growth phases, respectively, when compared to NC. The inclusion "on top" of phytase (PC + PHY) provided an improvement of 2 points in FCR in comparison to PC diet. Phytase supplementation in diets with reduced nutritional diets improves dry matter (DM), crude protein (CP), apparent digestible energy (ADE) in DM, ADE in natural matter (NM), and phosphorus (P) digestibility when compared to NC diet. Phytase supplementation on top (in PC diet) improved digestibility of CP, ADE in NM, Ca and P, when compared to the same diet with no phytase supplementation. Ash deposition on tibia of broilers fed diets with phytase supplementation on top is higher than those who does not received phytase. Considering the costs of the sources of inorganic phosphorus, it was achieved a saving of U$ 3.72/ton of produced feed with phytase supplementation. In the third study, the *E. coli* phytase (expressed in *Pichia pastoris*) showed to be efficient, improving body weight gain, feed intake, feed conversion, viability and bone characteristics of birds fed diets P-deficient. However, the levels closest to the maximum content studied (240 OTU) showed the best results. The substitution of dicalcium phosphate for phytase economically viable when the cost per FTU/g did not exceed U$0.000014, U$0.000012 and U$0.000010, to concentrations of 120, 180 and 240 OTU, respectively. In the last trial the multivariate analysis: cluster analysis by hierarchical method, principal component analysis and discriminant analysis confirmed that the positive and negative control has very specific and contrasting properties. Fungal phytases (F1 and F2) showed the same pattern, as well as bacterial phytases (B1 and B2). Both phytases showed a superior process when compared to the negative control, therefore they were not able to equalize to the positive control diet. Bacterial phytases were more efficient to recover the performance of broilers, being more similar to positive control diets. All phytases were effective, according to their evaluations. However, its supplementation method will depend on the purpose of each production.

Keywords: phytate, performance, nutrients, cost, multivariate, tibia

ABREVIAÇÕES

A Phytase - Phytase extracted from bacteria of the genus *Buttiauxella*;

Ash (21) – Tibia Ash at 21 Days;

Ash (35) – Tibia Ash at 35 Days;

B Phytase - Phytase extracted from bacteria of the genus *E. Coli*;

B1 – Bacterial phytase (produced by *Escherichia coli*);

B2 – Bacterial phytase (produced by *Escherichia coli*);

BWG - Body Weight Gain;

BWG (21) - Body Weight Gain at 21 Days;

BWG (35) - Body Weight Gain at 35 Days;

Ca – Calcium;

$_t$Ca – Total calcium;

DDTW (21) - Defatted Dry Tibia Weight at 21days;

DDTW (35) - Defatted Dry Tibia Weight at 35days;

DTW - Defatted Dry Weight;

F1 – Fungal phytase (produced by *Peniophora lyci*);

F2 – Fungal phytase (produced by *Aspergillus niger*);

FCR - Feed Conversion Reeder

FI – Feed Intake;

FI (21) - Feed Intake at 21 Days;

FI (35) - Feed Intake at 35 Days;

FI (21) - Feed Efficience at 21 Days;

FI (35) - Feed Efficience at 35 Days;

FTU - One Unit of Phytase Activity;

G1 - Group one;

G2 - Group two;

ISP - intake of supplementary phosphorus;

L - Linear Model;

Mg - Magnesium;

$_{t}$**N** - Total Nitrogen;

Na - Sodium;

NC1 - Negative Control 1;

NC2 - Negative Control 2;

P -Phosphorus;

$_{av}$**P -** Available phosphorus;

$_{t}$**P** - Total Phosphorus;

PB –Phosphorus Bioavailability;

PB (21) – Phosphorus Bioavailability at 21 Days;

PB (35) – Phosphorus Bioavailability at 35 Days;

PC - Positive Control;

PCOM - Principal Components;

PR - Phosphorus Released;

Q - Quadratic Model;

QP - Quadratic Plateau Model;

VIA - Viability;

WSP - Water Soluble Phosphorus;

Zn - Zinc;

CAPÍTULO 1 – CONSIDERAÇÕES GERAIS

1.1. INTRODUÇÃO

A principal fonte de armazenamento de fósforo (P) em grãos e cereais é na forma de fitato que, nos vegetais, tem a função de reserva e previne o estresse oxidativo, impedindo a morte do embrião. O fitato, apesar de ser uma molécula rica em P, apenas um terço deste nutriente é disponível para não ruminantes (FERKET et al., 1993).

O termo fitato é comumente descrito pelos nutricionistas como a molécula de ácido fítico complexada com nutrientes. Quando presente no trato digestório, tem um efeito antinutricional de reduzir a biodisponibilidade dos nutrientes e se complexar com enzimas gástricas digestivas. Entretanto, a molécula de ácido fítico possui algumas características de acordo com o local e estado onde se encontram.

De uma forma mais simples, poderíamos dizer que a molécula presente nos ingredientes de origem vegetal, que constituem as rações, é denominada de fitina. Esta, por sua vez, constitui a forma de armazenamento de fósforo para as plantas. Tem como característica se solubilizar em pH baixo, presente no início do trato gastrointestinal dos não ruminantes, passando a forma livre, o ácido fítico. O fato do ácido fítico ser solúvel em pH baixo, cria condições para a sua hidrólise, através da fitase que deve atuar de maneira consistente nestas condições. À medida que o bolo alimentar prossegue pelo trato gastrointestinal, ocorre o aumento do pH, principalmente a partir do duodeno, pela liberação do suco pancreático. Então, o ácido fítico forma cristais complexando com os nutrientes e se precipita (SELLE et al., 2006).

A fitase cliva a cadeia de myo-inositol hexafosfato do ácido fítico, liberando inositol e fosfato inorgânico no estômago. Esta hidrólise evita que a molécula se ligue a moléculas carregadas positivamente, formando quelatos em pH mais básico (duodeno), melhorando a disponibilidade de Ca, Fe, Mg, Zn e Cu, proteína e energia (NAHM, 2007), bem como a atividade das enzimas endógenas.

A fitase vem sendo comercializada desde 1991, sendo a enzima mais estudada e mais utilizada na nutrição de monogástricos (SELLE; RAVINDRAN, 2007). É um mercado que movimenta cerca de US$ 1 bilhão. Segundo, Cowieson et al. (2008),

este mercado tem um crescimento de 10 a 15% ao ano. Com o alto custo das fontes inorgânicas de fósforo e estudos que comprovam a eficácia da enzima e sua viabilidade econômica, a aplicação de fitase tem sido uma ferramenta aplicada em quase a totalidade das formulações de dietas para aves.

Com o advindo da engenharia genética, diversas fitases tem sido produzidas, havendo uma crescente demanda para suas avaliações. Existe uma busca para fitase com pH de atuação mais amplo e melhor termoestabilidade (BESLIN, 2014). Desta forma, o presente estudo objetivou avaliar diferentes fitases sobre o desempenho, características ósseas, excreção e digestibilidade dos nutrientes, bem como a biodisponibilidade do fósforo. E ainda, uma abordagem utilizando análises multivariadas exploratórias para comparação entre fitases fúngicas e bacterianas.

1.2. REVISÃO DE LITERATURA

A partir de 1857, Louis Pasteur notou haver uma relação entre fermentação e atividade biológica das leveduras. A partir deste momento, as enzimas vem sendo amplamente estudadas (LEHNINGER, 1994).

As enzimas de modo geral, são compostos proteicos capazes de atuar em substratos específicos, aumentando a velocidade de uma reação química. Enzimas destinadas a nutrição animal sofrem influência de algumas condições encontradas no trato digestório como as condições de temperatura, pH, coenzimas e inibidores. As mesmas devem ser capazes de resistir às condições desfavoráveis que possam ocorrer durante o processo de preparação da ração ou que existam naturalmente no trato gastrointestinal (SOTO SALANOVA et al.,1996; MCCLEARY, 2001). Para uma atuação eficiente, é necessário ter quantidade satisfatória de substrato na dieta, dosagem correta da enzima e estabilidade nas condições encontradas no trato digestivo como: pH baixo, ação de enzimas proteolíticas, como a pepsina e também durante a fabricação da ração como a temperatura durante o processo de peletização (CAMPESTRINI; SILVA; APPELT, 2005).

1.2.1. Fitase e seus benefícios

A fitase foi descoberta em 1907 por Suzuki, porém, somente na década de 90, seu emprego na produção animal tornou-se economicamente viável. Produzida a partir *Aspergillus niger*, essa foi a primeira fitase a surgir no mercado (SELLE; RAVINDRAN, 2008).

Sua atividade é expressa como sendo a quantidade de enzima que hidrolisa 1 micromol de fósforo inorgânico por minuto, proveniente de 1,5 micromol de fitato de sódio em pH 5,5 à temperatura de 37ºC (ENGELEN et al., 1994).

A fitase deve ser capaz de hidrolisar a molécula de fitato durante a digestão, antes de chegar ao duodeno, local onde o fitato se quela com cátions e se precipita, tornando sua hidrólise impraticável. Desta forma, segundo Igbasan (2010), para uma fitase atuar de maneira eficaz, ela deve apresentar estabilidade em pH, resistência ao processo de produção da ração, resistência à ação proteolítica e estabilidade no trato digestivo.

Diversos estudos apontam que a fitase é capaz de melhorar a digestibilidade do fósforo, aminoácidos, proteína e energia (RUTHERFORD et al., 2003; SANTOS et al., 2008), assim como de outros minerais como Ca, Na e Cu (CHUNG et al., 2013).

Um estudo realizado por Fukayma et al. (2008), aplicando-se fitase em concentrações de 500 a 1000 FTU em dietas com redução de energia metabolizável, cálcio e fósforo, observaram que a adição de 750 FTU proporcionou o máximo desempenho e os melhores resultados de mineralização e resistência ósseas de frangos de corte aos 20 dias de idade.

Rutherford et al. (2012), trabalhando com níveis de suplementação de 1000 e 2000 FTU de fitase, verificaram melhoras de até 16% na digestibilidade ileal aparente total, um aumento de até 35% no teor mineral das tíbias, além de melhora na digestibilidade aparente ileal dos aminoácidos treonina, tirosina e histidina em 14, 9 e 7%, respectivamente, após a inclusão de fitase.

Laurentiz et al. (2009), verificaram que a adição de 500 FTU/kg de ração de fitase, além de melhorar o desempenho de frangos de corte em dietas Ca e P deficientes, reduziram excreção de fósforo, contribuindo para uma menor contaminação do meio ambiente através da cama dos frangos. Augspurger e Baker

(2004) estudando a suplementação de uma fitase *E. coli* (500 FTU) em dietas a base de milho e farelo de soja deficiente em Ca (4,8 g/kg) com fitase de E. coli (500 FTU/kg) concluíram que a fitase liberou em média 0,90 g de Ca/kg de ração com base nas cinzas da tíbia.

Khodambashi et al. 2013, avaliando a inclusão de 500 FTU de fitase em dietas com redução de Ca e P, também observaram um maior teor de cinzas nas tíbias, além de melhor relação altura das vilosidades e profundidade de cripta no duodeno, jejuno e íleo de frangos de corte, em comparação com frangos alimentados com a dieta contendo total atendimento nutricional.

Diversos estudos também indicaram que a suplementação de fitase melhora o desempenho de frangos de corte, proporcionando melhores resultados no ganho de peso, consumo de ração, eficiência alimentar (DENBOW et al, 1995; MITHCELL; EDWARDS, 1996; SINGH; KHATTA, 2003; KARIM, 2006; SELLE; RAVINDRAN, 2007; COWIESON; COWIESON, 2011; LIU et al., 2014).

1.2.2. Tipos de fitases

As fitases disponíveis no mercado são denominadas fúngicas ou bacterianas, de acordo com o microrganismo que são produzidas. Podem também ser classificadas de acordo com a Comissão de Nomenclatura da União Internacional de Bioquímica e Biologia Molecular (NCIUBMB) e pela Nomenclatura Bioquímica (JCBN), de acordo com o local da primeira hidrólise na molécula do fitato como: mio-inositol hexaquifosfato 3-fosfohidrolase, denominada 3-fitase e mio-inositol hexaquifosfato 6-fosfohidrolase, denominada 6-fitase ou fitato 6-fosfatase. A 3-fitase hidrolisa o fosfato na posição D-3 (L1) do anel do mio-inositol presente na molécula do fitato, enquanto a 6-fitase hidrolisa o fosfato na posição L-6 (D4) (RAGON et al., 2008).

Existem também fontes de fitases de origem animal, parcialmente purificada do intestino de ratos, aves, novilhos e homens (BITAR e REINHOLD, 1972) e vegetais, que representam avanços científicos importantes. Porém, sua aplicação prática ainda é limitada. Em compensação, fontes microbianas apresentam características que

permitem um alto rendimento e produção em maior escala, tornando-se amplamente utilizadas na indústria (KIM; MULLANEY; PORRES, 2006).

Segundo Nascimento (2011), poucas espécies selvagens de bactérias são utilizadas para produção de fitase, principalmente devido ao baixo rendimento e por apresentarem uma faixa ótima de pH não adequada ao sistema digestivo dos monogástricos. Por isso, a busca concentra-se mais nas bactérias termo-ácidas-tolerantes ou fungos.

A indústria da fitase abrange diversos produtos que apresentam características particulares, resultados de engenharia genética específicas de cada empresa. Sendo as maiores diferenças provenientes das variações de microrganismos, bem como dos processos de produção e extração. Por consequência, as fitases atuam de forma distintas, influenciadas pelo pH do estômago, ação de enzimas digestivas, tempo de atuação e estabilidade dentro do sistema digestivo.

O processo de produção de fitases mais comumente utilizados, são realizados por dois métodos: fermentação líquida submersa e fermentação em estado sólido. A fermentação líquida submersa nada mais é que o cultivo de microrganismos em um meio líquido rico em substrato que utilizam os nutrientes e excretam as enzimas para o meio de cultura. A fermentação em estado sólido é o crescimento do microrganismo em substratos sólidos na ausência de líquido na forma livre (PANDEY et al., 2003). A fermentação no estado sólido é uma técnica mais econômica devido a utilização de resíduos agroindustriais como substrato, maior facilidade em separar a fitase, maior produtividade e menor utilização de água. Geralmente são utilizados como substratos os farelos, cascas e bagaços (PANDEY et al., 2003).

Para aplicação da técnica de fermentação em estado sólido, deve-se escolher adequadamente o microrganismo, pois os fungos são capazes de crescer em ambiente com escassa água e alta concentração de sólidos (GUTIÉRREZ – ROJAS, et al. 1995). Segundo Salmon, 2011, a produção de fitases fúngicas se mostra mais vantajosa pela facilidade de extração da enzima do ponto de vista industrial, já que os fungos sintetizam enzimas extracelulares que são lançadas em um substrato externo e, portanto, elimina-se a etapa de rompimento celular de enzimas intracelulares.

A produção comercial de fitase é facilmente atingida com culturas microbianas. Mas, para que se processe a enzima em escala comercial, é necessário que o

microrganismo a produza extracelularmente. Além disso, as fitases também podem ser produzidas industrialmente por fermentação submersa utilizando a tecnologia do DNA recombinante com micro-organismos geneticamente modificados (SALMON, 2011). Desta maneira, muitas empresas utilizam desta tecnologia para expressar os genes, principalmente de bactérias, em organismos com parede celular, como os fungos.

Outro fator importante que deve-se levar em consideração é a estabilidade da fitase. Heinzl (1996) demonstrou que a fitase atua otimamente em pH 5 a 6,5 (mantendo-se estável na faixa de pH 2 a 7), bem como a 60ºC com amplo limite de temperatura.

O que se pode observar, através das pesquisas, é que as fitases de origem bacteriana tem uma maior estabilidade no sistema digestivo das aves, sofrendo menor degradação por parte das enzimas digestivas gástricas, além de uma faixa mais ampla de atuação no trato digestivo (WYSS et al., 1999; AUGSPURGER et al., 2003; PILLAI et al., 2006).

Augspurger et al. (2003) observaram uma maior liberação do P por fitase produzida por *E. coli* quando expressa em levedura em comparação com a fitases fúngicas para pintos jovens e suínos. De acordo com o autor, a maior liberação de P por *E. coli* foi proveniente do pH ideal de atuação desta enzima ser mais baixo que as fúngicas.

Segundo Pillai et al. (2006), fitases *E. coli* parecem ser mais resistentes à degradação da pepsina. Rodriguez et al. (1999) relataram um aumento de 30% na atividade de fitase *E. coli* depois de serem incubados em pepsina a um pH de 2, enquanto que outras fitases fúngicas tiveram uma diminuição 58 a 77% na sua atividade sob as mesmas condições experimentais.

1.2.3. Fósforo e suas implicações

O elemento fósforo é um não-metal originários das rochas, pertencente ao grupo XA da tabela periódica, com massa atômica de 31u. É o único macro nutriente inexistente na atmosfera, em sua forma natural.

O fósforo das rochas, que se mineraliza no solo, é incorporado nas plantas pelas raízes passando a participar da cadeia trófica dos consumidores. Através dos excrementos ou pela morte dos animais, o mesmo é devolvido ao solo. No entanto, parte deste fosforo é transportado para o lençol freático por lixiviação, incorpora-se na cadeia trófica de rios e lagos, até chegar ao mar. Uma parte se perde nos solos profundos, não sendo mais aproveitado, a menos que haja o aparecimento de algas que o reincorpore na cadeia trófica.

O fósforo é um nutriente associado ao combustível biológico de alta energia dos seres vivos, tais como ATP, ADP e fosfocreatina, bem como às moléculas de glucose-6-fosfato e glicerol fosfato. Ele também é constituinte dos ácidos nucleicos e dos fosfolípidos, que têm um papel vital na construção de membrana plasmática das células. É um componente das coenzimas NAD e FAD, participando no metabolismo de carboidratos, lipídios e proteínas.

É um dos principais constituintes dos componentes estruturais dos tecidos esqueléticos. Atua também como principal tampão intracelular no corpo e está envolvido no equilíbrio ácido-base do plasma (CROMWELL, 1996). O fósforo também participa da homeostase do cálcio no organismo. É um regulador da excreção renal e também auxilia na utilização de vitaminas. Tanto o excesso quanto a deficiência interferem na absorção de cálcio no metabolismo (MCDOWELL, 2003).

É um mineral indispensável para a agricultura e nutrição animal. Todavia, há uma preocupação por parte dos ambientalistas quanto a sua utilização inconsciente em grande escala. Segundo Lana (2009), as reservas conhecidas e de exploração a baixo custo podem se esgotar em um prazo de quarenta a cem anos. Desta forma, a utilização consciente deste nutriente se torna cada vez mais necessária, devido ao ritmo acelerado de seu consumo.

Na Europa e América do Norte, a adição de P ao solo tem sido superior à sua remoção, tornando o balanço positivo e, consequentemente os níveis de P no solo se

tornam acima dos requeridos pelas culturas. Este fato leva também a elevadas e contínuas perdas de P por escoamento superficial e drenagem. Segundo Lelis et al. (2009) o fósforo e nitrogênio presentes no solo em forma de ácido fítico é disponibilizado pela microbiota do solo, e por lixiviação, contaminam os mananciais de água, beneficiando o crescimento de algas aquáticas. Ao serem decompostas, as algas aumentam a demanda por oxigênio na água, podendo levar a morte de animais aquáticos.

1.2.4. Fator antinutricional do fitato

O fitato ($C_6H_{18}O_{24}P_6$) é uma molécula cíclica simples, carreando seis fosfatos, com teor de fósforo de 28,16%. Apresenta algumas características específicas de acordo com o ambiente em que se encontram. Em pH ácido o fitato poderá estar levemente carregado negativamente ou em pH neutro/alcalino, fortemente carregado negativamente, conforme demonstrado nas figuras 1 e 2, respectivamente.

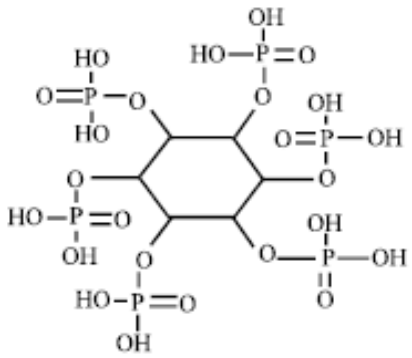

Figura 1. Estrutura do ácido fítico em pH ácido, elucidando o encontrado no proventrículo e moela de aves (Adaptado de COULIBALY et al., 2011)

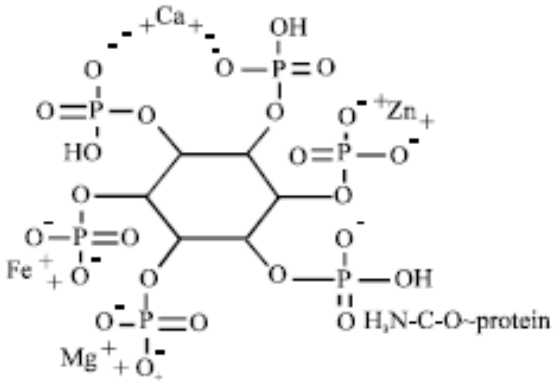

Figura 2. Etrutura do ácido fítico em pH básico, elucidando o encontrado a partir do duodeno em aves (Adaptado de COULIBALY et al., 2011)

Conforme demostrado nas figuras, o pH influencia a afinidade do fitato por cátions no trato gastrintestinal. Este fato está correlacionado com a constante de dissociação da molécula (SANTOS, 2012). A fitase deve ser capaz de hidrolisar a molécula de fitato durante o processo digestivo caracterizado pela fase ácida do trato das aves (proventrículo e moela) e suínos (estômago). Nestas condições o ácido fítico se encontra solúvel porém associado às proteínas que tem seus pontos isoelétricos na faixa de 4,5. Quando o pH no trato digestivo fica abaixo do ponto isoelétrico da proteína, ela fica carregada positivamente, permitindo que o ácido fítico (ânion) se ligue a ela, alterando-a e tornando-a insolúvel (REDDY et al., 1989). Esta interação fitato-proteína, acarreta em menor digestibilidade, pois reduz a atuação das proteases digestivas, resultando no aumento de secreção de pepsina, acarretando em perdas endógenas (VAINTRAUB; BULMAGA, 1991, KONIETZNY; GREINER, 2002, KIES et al., 2006, COWIESON; SELLE; RAVINDRAN, 2008).

O tempo de permanência, da digesta no trato digestório de frangos de corte é de aproximadamente 4h e 30 min. Sendo aproximadamente 50 min no papo, 90 min no proventrículo e moela, 5 a 8 minutos no duodeno, 20 a 30 min no jejuno, 50 a 70 min no íleo e 25 min no reto (GAUTHIER, 2002). Como a fitase atua apenas em pH ácido, onde o substrato fitato esta solúvel e passível de hidrólise, a mesma tem um tempo para atuação de aproximadamente 2h e 20min.

O fitato tem uma maior afinidade por íons de cobre e zinco em pH básico (SELLE & RAVINDRAN, 2007). No entanto, em dietas com concentrações altas de cálcio há formação de complexos Ca-fitato formando sais que se precipitam e reduzem a disponibilidade dos minerais, em pH básico (LEI & PORRES, 2003).

Segundo Selle, Cowieson e Ravindran (2009), é improvável que o Ca esteja ligado com o fitato nos ingredientes de origem vegetal, uma vez que eles possuem baixa concentração desse mineral. Entretanto, como as aves tem uma exigência maior de Ca, fontes externas deste mineral fazem parte da formulação das dietas, passando a existir formação significativa de complexos no trato gastrointestinal. Nestas condições, estima-se que aproximadamente um terço do Ca dietético forme complexos com o fitato durante a passagem da ração pelo intestino (NAVES, 2014).

Podemos observar a partir da figura 3 como a molécula de ácido fítico aumenta a afinidade por cátions a medida que o pH se torna mais básico.

Figura 3 – Solubilidade do fitato em pH de 2,0 a 7,0 na presença de Ca em concentração similar ao encontrado no trato gastrintestinal dos animais (Fonte: Universidade de Maryland, dados não publicados; citado por Gomes, 2013)

Cowieson e Acamovic (2004) verificaram que existia um incremento na produção de mucina e na excreção de sódio e outros minerais quando frangos de corte recebiam dietas com fitato. A maior secreção de muco é uma resposta à agressão do sal de fitato à parede intestinal e também ao aumento principalmente de pepsina gástrica. Segundo os mesmos, com a diminuição da digestão das proteínas ligadas à molécula de fitato, o organismo secreta mais HCl para ativação da pepsina que foi liberada para compensar as perdas.

De acordo com Cowieson e Cowieson (2011), o fitato diminui a solubilidade da proteína através da interação eletrostática, fazendo com que os nutrientes apresentem menos água ao seu redor, reduzindo sua solubilidade e consequentemente a digestibilidade.

Todos estes fatores inerentes ao fitato, reduzem a eficiência na produção animal e aumentam a carga de poluentes através das excretas das aves pela diminuição da digestibilidade dos nutrientes. Portanto, torna-se necessário a utilização de forma racional do fósforo, através da aplicação de tecnologias que visem a otimização da produção, minimizando impactos ambientais e a um custo acessível. Neste contexto, o uso da fitase tem se mostrado uma ferramenta importante para os nutricionistas modernos, que visam uma produção sustentável.

2. REFERÊNCIAS

AUGSPURGER, N. I.; BAKER, D. H. Phytase improves dietary calcium utilization in chicks, and oyster shell, carbonate, citrate, and citrate malate forms of calcium are equally bioavailable. **Nutrition Research.** v. 24, p. 293-301, 2004.

AUGSPURGER, N. I.; WEBEL D. M.; LEI X. G.; BAKER, D D. H. Efficacy of an E. coli phytase expressed in yeast for releasing phytate-bound phosphorus in young chicks and pigs. **J. Anim. Sci.** v. 81, p. 474–483, 2003.

BESLIN, J. J. Phytase - A Key to Unlock Phytate Complex. **Int. J. Pure App. Biosci.** v.2 n.6, p.304-313, 2014.

BITAR, K.; REINHOLD, J.G. Phytase and alkaline phospatase activities in intestinal mucosae of rat, chicken, calf and man. **Biochimica Biophysica Acta**. v.268, p.442-52, 1972.

CAMPESTRINI E.; SILVA V. T. M.; APPELT M. D. Utilização de enzimas na alimentação animal. **Revista Eletrônica Nutritime**, v. 6, n. 2, p.254-267, 2005.

CHUNG, T. K.; RUTHERFURD S. M.; THOMAS D. V.; MOUGHAN, P. J. Effect of two microbial phytases on mineral availability and retention and bone mineral density in low-phosphorus diets for broilers. **British Poultry Science**, v.54, p.362-373, 2013.

COULIBALY, A.; B. KOUAKOU AND J. CHEN. Phytic acid in cereal grains: Structure, healthy or harmful ways to reduce phytic acid in cereal grains and their effects on nutritional quality. **Am. J. Plant Nutr. Fertiliz. Technol**, v.1, p. 1-22, 2011.

Cowieson, A.J.; Acamovic, T.; Bedford, M.R. The effect of phytase and phytic acid on the endogenous loss from broiler chickens. **British Poultry Science**, v. 45, p. 101 – 108, 2004.

COWIESON, A.J.; COWIESON, N.P. Phytate and the thermodynamics of water. **Australian Poultry Symposium**, p. 11, 2011.

COWIESON, A.J.; SELLE, P.H. E RAVINDRAN, V. Uso de Fitase e suas implicações na digestão e absorção de nutrientes. **Anais**... Conferência APINCO, 2008.

CROMWELL, G.L. Metabolism and role of phosphorus, calcium, and vitamin D3 in swine nutrition. **Phytase in Animal Nutrition and Waste Management**. p. 101- 110, 1996.

DENBOW, D. M.; RAVINDRAN, V.; KORNEGAY, E. T.; SELF, B. B. ; HULET, R. M. Improving phosphorus availability in soyabean meal for broilers by supplemental phytase. **Poult. Sci.**, v.74, p.1831-1842, 1995.

Engelen, A. J.; Van Der Heeft F. C.; Randsdorp, P. H.; Smit E. L. Simple and rapid determination of phytase activity. **Journal of AOAC International**, v.77, p.760-764, 1994.

FERKET, P. R. Pratical use of feed enzymes for turkeys and broilers. **Journal Applied of Poultry Research**, v.2, p.75-81, 1993

FUKAYAMA, E. H.; SAKOMURA, N. K.; DOURADO, L. R. B.; NEME, R.; FERNANDES, J. B. K.; MARCATO, S. M. Efeito da suplementação de fitase sobre o desempenho e a digestibilidade dos nutrientes em frangos de corte. **Revista Brasileira de Zootecncia**, v.37, n.4, p.629-635, 2008.

GAUTHIER, R. Intestinal health, the key to productivity: the case of organic acids. Em: CONVENCION ANECA-WPDC. **Anais… Puerto Vallarta.** WPDC, p. 1-14, 2002.

GOMES, G. A.* 2013.** Fitase e suas implicações na digestão e absorção de nutrientes**.** ***[S.l.]: Engormix* Web Site*, *2013*. http://pt.engormix.com**.** Acess*o em 09 f*ev. 2015.

GUTIÉRREZ-ROJAS, M; AURIA, R.; BENET,J.C.; REVAH, S. A mathematical model for solid state fermentation of mycelial fungi on inert support. **The Chemical Engineering Journal**, v. 60, p. 189-198, 1995.

HEINZL, W. Technical specifications of natuphos. **BASF Technical Syniposium**. World Congress Center, Atlanta, Georgia. January 23, p. 39-70, 1996.

IGBASAN, F. A.; MÄNNER, K; MIKSCH, G.; BORRISS R.; FAROUK, A.; SIMONE, O. Comparative studies on the in vitro properties of phytases from various microbial origins, **Archiv für Tierernaehrung**, v. 53, p. 353-373, 2000.

KARIM, A. Responses of broiler chicks to non-phytate phosphorus levels and phytase supplementation. International Journal of Poultry Science, v.5, p.251-254, 2006.

KHODAMBASH N.; EMAMI I.**;** ZAFARI NAEINI S.**;** RUIZ-FERIA C. A**.** Growth performance, digestibility, immune response and intestinal morphology of male broilers fed phosphorus deficient diets supplemented with microbial phytase and organic acids**.** **Livestock Science**, v. 157, n. 2, p. 506 – 513, 2013.

KIES, A.K.; DE JONGE, L.H.; KEMME, P.A.; JONGBLOED, A.W.. Interaction between protein, phytate, and microbial phytase. In vitro studies. **J. Agric. Food Chem**. v.54, p.1753–1758, 2006.

KIM, T.; MULLANEY, E. J.; PORRES, J. M. Shifting the ph profile of *Aspergillus niger* phy A phytase to match the stomach ph enhances its effectiveness as an animal feed additive. **Applied and Environmental Microbiology**. v.72, n.6, p. 4397-4403, 2006.

KONIETZNY, U.; AND GREINER, R. Molecular and catalytic properties of phytate-degrading enzymes. **Int. J. Food Sci. Technol**. v. 37, p. 791–812, 2002.

LANA, R. P. Uso racional de recursos naturais não-renováveis: aspectos biológicos, econômicos e ambientais. **Revista Brasileira de Zootecnia**, v. 38, p. 330-340, 2009.

LAURENTIZ, A. C.; JUNQUEIRA, O. M.; FILARDI, R. S.; DUARTE, K. F.; ASSUENA, V.; SGAVIOLI, S. Desempenho, composição da cama, das tíbias, do fígado e das excretas de frangos de corte alimentados com rações contendo fitase e baixos níveis de fósforo. **Revista Brasileira de Zootecnia**, v.38, n.10, p.1938-1947, 2009.

LEHNINGER, A. L. 1994. **Principios de Bioquímica.** Sarvie, São Paulo, Brasil.

LEI, X. G.; PORRES, J. M. Phytase enzymology, applications, and biotechnology. **Biotechnology Letters**, v.25, p.1787-1794, 2003.

LELIS, G.R.; ALBINO, L.F.T.; TAVERNARI, F.C. E ROSTAGNO, H.S. Suplementação dietética de fitase em dietas para frangos de corte. **Revista Eletrônica Nutritime**, v.6, n.2, p.875- 889, 2009.

LIU, S. Y.; CADOGAN D. J.; PÉRON A.; TRUONG H. H.; SELLE P. H. Effects of phytase supplementation on growth performance, nutrient utilization and digestive dynamics of starch and protein in broiler chickens offered maize, sorghum and wheat based diets. **Anim. Feed Sci. Tech**. v. 197, p.164–175, 2014.

MCCLEARY B.V. Analysis of feed enzymes. In: BEDFORD, M.R. & PARTRIDGE, G.G. **Anais… Enzymes in farm animal nutrition.** Oxford: CAB PUBLISHING, 2001.

MCDOWELL, L. **Minerals in animal and human nutrition**. Amsterdan: Elsevier Science, 2003. p. 235-270.

MITCHELL, R. D.; EDWARDS, H. M. Effect of phytase and 1,25 dihydroxycholecalciferol on phytate utilization and the quantitative requirement for calcium and phosphorus in young broiler chickens. **Poult. Sci.**, v. 75, p. 95-110, 1996.

NAHM, K.H. Efficient phosphurus utilization in poultry feeding to lessen the environmental impact of excreta. **World´s Poultry Science Journal**, v.63, p.625-645, 2007.

NASCIMENTO, J. C. S. *Produção, caracterização bioquímica e purificação de fitase produzida por Aspergillus niger var. phoenicis URM 4924****. 2011. 99 f. Dissertação (Mestrado em Biociência Animal) - Universidade Federal de Pernambuco, Recife, 2011****.*

NAVES, L. P. Efeito antinutricional do ácido fítico sobre o cálcio e uso de fitase em dietas de frangos de corte. **Revista Eletrônica Nutritime**. v.11, n. 03, p. 3399-3409, 2014.

PANDEY, A. Solid state fermentation. **Biochemical Engineering Journal**, v.13, n.2/3, p.81-84, 2003.

PILLAI, P. B.; O'CONNOR-DENNIE, T.; OWENS C. M.; EMMERT, J. L. Efficacy of an *Escherichia coli* Phytase in Broilers Fed Adequate or Reduced Phosphorus Diets and Its Effect on Carcass Characteristics. **Poultry. Science**, v. 85, n. 10, p. 1737-1745, 2006.

RAGON, M.; AUMELAS, A.; CHEMARDIN, P.; GALVEZ, S.; MOULIN G.; BOZE H. Complete hydrolysis of myo-inositol hexakisphosphate by a novel phytase *from Debaryomyces castellii* CBS 2923. **Applied Microbiology and Biotechnology**, v.78, p.47-53, 2008.

REDDY N. R., PIERSON M. D., SATHE S. K. AND SALUNKHE D. K. Phytates in cereals and legumes. **CRC Press, Inc**., Boca Raton, Florida, p. 152, 1989.

RODRIGUEZ, E.; PORRES, J. M.; HAN, Y.; LEI, X. G. Different sensitivity of recombinant *Aspergillus niger* phytase (rPhyA) and *Escherichia coli* pH 2.5 acid phosphatase (r-AppA) to trypsin and pepsin in vitro. **Arch. Biochem. Biophys**. v. 365, p. 262–267, 1999.

RUTHERFURD, S. M.; CHUNG, T. K.; MOREL, P. C. H.; MOUGHAN, P. J. Effect of Microbial Phytase on Ileal Digestibility of Phytate Phosphorus, Total Phosphorus, and Amino Acids in a Low-Phosphorus Diet for Broilers. **Poultry Science**, v. 83, p. 61–68, 2003.

RUTHERFURD, S. M.; CHUNG, T. K.; THOMAS, D. V.; ZOU, M. L.; MOUGHAN, P.J. Effect of a novel phytase on growth performance, apparent metabolizable energy, and the availability of minerals and amino acids in a low-phosphorus corn-soybean meal diet for broilers. **Poult Sci**. v.91, n. 5, p. 1118-1127, 2012.

SALMON, D.N.X. **Desenvolvimento de um bioprocesso para a produção, caracterização e recuperação da fitase de Schizophyllum commune obtida por fermentação em estado sólido**. 2011. 107p. Dissertação (Mestrado em Processos Biotecnológicos) - Universidade Federal do Paraná, Curitiba, 2011.

SANTOS, F. R.; HRUBY, M., PIERSON, E. E. M.; REMUS, J. C., SAKOMURA, N. K. Effect of Phytase Supplementation in Diets on Nutrient Digestibility and Performance in Broiler Chicks. **J. Appl. Poult. Res.** v. 17, p. 191–201, 2008.

SANTOS, T.T. Phytate: anti-nutrient for poultry and swine. **Feedstuffs**, v. 84p. 1-3., 2012.

SELLE, P. H.; COWIESON, A. J.; RAVINDRAN, V. Consequences of calcium interactions with phytate and phytase for poultry and pigs. **Livestock Science**, v. 124, p.126-141, 2009.

SELLE, P. H.; RAVINDRAN, V. BRYDEN, W. L.; SCOTT, T. A. Influence of dietary phytate and exogenous phytase on amino acid digestibility in Poultry: **Resumos. Journal of Poultry Science**, v. 43, p.89, 2006

SELLE, P. H.; RAVINDRAN, V. Phytate-degrading enzymes in pig nutrition. **Livestock Science**, v.133, p. 9-122, 2008.

SELLE, P.H.; RAVINDRAN, V. Microbial phytase in poultry nutrition. **Animal Feed Science and Technology**, v. 135, p. 1-41, 2007.

SINGH, P. K.; KHATTA, V. K. Effect of phytase supplementation on the performance of broiler chickens fed wheat based diets. **Indian J. Anim. Nutr.**, v.20, p.57-62, 2003.

SOTO-SALANOVA; M. F.; GARCIA, O.; GRAHAM, H. PACK, M. Uso de enzimas em dietas de milho e soja para frangos de corte. Em: CONFERÊNCIA APINCO DE CIÊNCIA E TECNOLOGIA AVÍCOLA, 1996, Campinas. **Anais...** Campinas: FACTA, p.71-76. 1996.

VAINTRAUB, I. A. AND V. P. BULMAGA. Effect of phytate on the in vitro activity of digestive proteinases. **J. Agric. Food Chem**. v.39, p. 859-861, 1991.

WYSS, M.; BRUGGER, R.; KRONENBERGER, A.; RÉMY, R.; FIMBEL, R.; OESTERHELT, G.; LEHMANN, M.; VAN LOON, A. P. G. M. Biochemical characterization of fungal phytases (myo-inositol hexakisphosphate phosphohydrolases): catalytic properties. **Applied and Environmental Microbiology** v. 65, n. 2, p. 367-373, 1999.

CAPÍTULO 2 - COMPARISON OF AN *Escherichia coli* AND A *Buttiauxella* PHYTASES ON PHOSPHORUS BIOAVAILABILITY, LITTER COMPOSITION, BONE CHARACTERISTICS AND FEED COSTS

ABSTRACT - An experiment was conducted to evaluate the effects of two microbial phytases on performance, bioavailability of phosphorus, bone mineralization, nutrient excretion and economic evaluation in diets for broiler chickens. A total 2,160 Cobb 500 male day old chicks were assigned to nine treatments and eight replicates containing 30 birds each were distributed in a completely randomized design. The treatments were: T1 - Positive Control (PC); T2 - Negative Control 1 (NC1) – reduction of 0.14% P and 0.13% Ca; T3 - Negative Control 2 (NC2) - reduction of 0.17% P and 0.16% Ca; T4 - NC2 + phytase A (250 FTU/kg); T5 - NC2 + phytase A (500 FTU/kg); T6 - NC2 + phytase A (1000 FTU/kg); T7 - NC2 + phytase B (250 FTU/kg); T8 - NC2 + phytase B (500 FTU/kg); T9 - NC2 + phytase B (1000 FTU/kg). Standard curve method was utilized to obtained the bioavailability of P in response of performance variables and bone mineralization. Regressions were performed on performance data, bone mineralization and excretion of nutrients to determine the optimal level inclusion of each phytase. The phosphorus content in bone proved to be the best parameter to evaluate bioavailability. Phytase A have a higher efficiency for body weight gain and feed intake in concentrations around 500 FTU/kg, while phytase B, around 1000 FTU/kg. There was reductions in levels of total phosphorus and soluble phosphorus with the addition of phytase in the diet. Through the bioavailability calculated by ash content and tibia weight gain, was also observed that phytase B is more efficient in depositing ash in the bones and in reducing nutrient excretion in litter, while the phytase A is most effective for body weight gain. Mineral contents as P, Zn and Ash and deflection force of tibias were improved by adding phytase to the diets. Both phytases in concentrations of 250 FTU/kg generated greater return, estimated using the dicalcium phosphate price of $0.50.

Keywords: enzyme, broiler, phytase, bone, litter

1. INTRODUCTION

The phosphate reserves in the world that can be explored at low cost will soon become increasingly scarce (Lana, 2009). Therefore, there is an increasing trend in the cost of phosphorus sources. In this sense, the use of exogenous phytase in plant origin diets is an alternative to reduce the use of inorganic phosphate in feed formulation and to reduce the feed cost. In Brazil, due to high costs of phosphorus sources and their economic viability, phytase is the most common exogenous enzyme used in poultry feed. Today the total enzyme market is estimated to be about 3-5 billion dollars per year worldwide (Adeola and Cowieson, 2011).

Corn and soybean meal are commonly used in the diet of broilers and have around 66% their total phosphorus content in the form of phytate, making the phosphorus unavailable to birds (Ravindran et al., 1995). Inclusion of phytase in the diet can improve the availability of phosphorus and reduce P excretion by birds, and therefore reduces the contamination of the environment (Musapuor et al., 2006).

The poultry manure contaminates the ground and water because of its high level of phosphorus and nitrogen through a process called translocation (Conffey, 1992). The phosphorus that reaches the water sources, stimulates the growth of algae by a process called eutrophication. The decomposition of the algae affects the water quality, reducing its oxygen content and increasing the competition of plants and aquatic animals for oxygen, causing their death (Cromwell et al., 1995).

The importance of phytase on economic and environmental aspects is increasing in recent years, and the industries are searching for another way of producing phytase, such as different microorganisms (Kaur et al., 2009). So there is always the need to collect information on new phytases.

Therefore, studying the proper dosage, considering performance, bone parameters, nutrient excretion and cost analysis are extremely important for the evaluation of phytase. In this way, the main objective of this study was to evaluate diets with Ca and P reduction supplemented with different phytases on performance, bone composition, litter analysis and phosphorus bioavailability. As well as to compare both phytases, to determine their optimal utilization levels and cost/benefit analysis.

2. MATERIALS AND METHODS

2.1. Birds and housing

The trial was conducted at the Laboratory of Poultry Sciences, Department of Animal Science, Faculty of Agriculture and Veterinary Sciences - UNESP, Jaboticabal, Brazil.

All experimental procedures were approved by the Ethics Committee on Animal Use (CEUA - 013022/14).

A total 2,169 Cobb 500 male day old chicks were housed in an experimental facility, with tunnel type system to control the environment. The control of temperature, humidity and air exchange were performed automatically by exhaust fans and climate control system, according to the age of the birds (COBB, 2006). During the starter phase incandescent lamps were used as heating source according to the needs of poultry. New shaving litter was used in all experimental plots. Nipple drinkers were used during all rearing phases. Infant tubular feeders were used until 14 days of age, then replaced to adult tubular feeders.

Water and feed were offered ad libitum during all the trial period. The lighting program was set at 24 hours of light.

The birds were vaccinated against coccidiosis on the 1st day of life (via ocular), against Gumboro disease at six days old (low strain), New Castle disease at 11 days old and strengthening Gumboro at 16 days of age (strong strain), administered via water.

2.2. Experimental design

The birds were weighed (53 ± 1g) and distributed in a completely randomized design with nine treatments and eight replicates of 30 chicks each. Each treatment had similar body weight means.

The experimental treatments were: **T1 -** Positive Control (PC); **T2 -** Negative Control 1 (NC1) – reduction of 0.14% $_{av}$P and 0.13% $_{t}$Ca; **T3 -** Negative Control 2 (NC2)

- reduction of 0.17% $_{av}$P and 0.16% $_{t}$Ca; **T4 -** NC2 + phytase A (250 FTU/kg); **T5 -** NC2 + phytase A (500 FTU/kg); **T6 -** NC2 + phytase A (1000 FTU/kg); **T7 -** NC2 + phytase B (250 FTU/kg); **T8 -** NC2 + phytase B (500 FTU/kg); **T9 -** NC2 + phytase B (1000 FTU/kg).

2.3. Experimental diets

The positive control diet was formulated to meet the nutritional requirements of broilers (NRC, 1994), except for energy and amino acids, which were reduced based on the phytase matrix for all diets (reduction 62 kcal/kg ME and aminoácids: lysine, methionine, methionine + cysteine, threonine, tryptophane and valine, based on 500 U/kg contribution from phytase B) The negative control diets had also reduced levels of Ca and $_{av}$P. The Table 1 describes the control diets and their nutritional compositions for starter (1-14 days), grower (15-28 days) and finisher (29-42 days) phases. The phytases A and B, both at concentrations of 10,000 FTU/g were bacterial phytases of *Buttiauxella sp*. (expressed in *Trichoderma reesei*) and *E. coli* (expressed in *S. pombe*), respectively (DuPont - Danisco Animal Nutrition - Brabrand, Denmark). The diets of the other treatments were based on the same formulation, with phytase added as replacement of inert filler, according to the respective inclusion level.

Table 1. Composition of experimental diets and calculated nutritional values

Ingredients	Starter (1-14 days)			Grower (15-28 days)			Finisher (29-42 Days)		
	PC	NC1	NC2	PC	NC1	NC2	PC	NC1	NC2
Corn	60.30	62.09	62.29	66.75	67.89	68.00	70.03	71.29	71.45
Corn gluten meal 60%	3.00	3.58	3.60	4.79	4.83	4.96	3.99	3.96	3.95
Soybean meal 45% CP	31.10	29.96	29.90	23.19	23.05	22.97	20.79	20.59	20.58
Soy Oil	0.86	0.22	0.15	1.22	0.81	0.73	1.55	1.13	1.08
L-Lysine HCl 99%	0.41	0.44	0.44	0.39	0.39	0.40	0.31	0.32	0.32
DL-Methionine 98%	0.29	0.28	0.28	0.22	0.21	0.21	0.16	0.16	0.16
L-Threonine 90%	0.15	0.15	0.15	0.08	0.08	0.08	0.03	0.03	0.03
L-Valine 99%	-	-	-	0.02	0.02	0.01	-	-	-
Inert[1]	-	-	0.05	-	-	0.05	-	-	0.05
Salt	0.48	0.48	0.48	0.46	0.46	0.46	0.41	0.41	0.41
Dolomitic limestone	1.12	1.28	1.31	1.10	1.26	1.28	1.05	1.20	1.23
Dicalcium phosphate 18,5%	2.12	1.36	1.19	1.61	0.85	0.69	1.52	0.76	0.59
Choline chloride 60%	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07
Premix Vit.[2]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Premix Min.[3]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
TOTAL (%)	100	100	100	100	100	100	100	100	100
ME (kcal/kg)(%)	2961	2961	2961	3093	3093	3093	3141	3141	3141
Crude protein (%)	22.39	22.39	22.40	20.29	20.35	20.40	18.84	18.84	18.87
Crude fiber (%)	2.71	2.72	2.73	2.45	2.46	2.46	2.37	2.38	2.38
Calcium (%)	1.05	0.92	0.89	0.90	0.77	0.74	0.85	0.72	0.69
Total phosphorus (%)	0.72	0.51	0.45	0.66	0.47	0.42	0.62	0.43	0.38
Available phosphorus (%)	0.50	0.36	0.33	0.40	0.26	0.23	0.38	0.24	0.21
Phytic phosphorus	0.23	0.23	0.23	0.23	0.23	0.23	0.22	0.22	0.22
Sodium (%)	0.21	0.21	0.21	0.20	0.20	0.20	0.18	0.18	0.18
Lysine (%)	1.30	1.30	1.30	1.11	1.11	1.11	0.98	0.98	0.98
Met+Cys (dig) (%)	0.91	0.91	0.91	0.81	0.81	0.81	0.72	0.72	0.72
Methionine (dig) (%)	0.60	0.60	0.59	0.52	0.52	0.51	0.44	0.44	0.44
Threonine (dig) (%)	0.85	0.85	0.85	0.71	0.71	0.71	0.62	0.62	0.62
Tryptophane (dig) (%)	0.22	0.22	0.22	0.19	0.19	0.19	0.17	0.17	0.17
Valine (dig) (%)	0.91	0.91	0.91	0.85	0.85	0.85	0.77	0.77	0.77

[1] The phytases were included in diets at 500g/ton;

[2] Folic Acid 1000mg, Pantothenic Acid 15000mg; Antioxidant 0.5g; Niacin 40000mg, Selenium 300mg, Biotin 60mg, Vit B11800mg, Vit B12 12000mg; Vit B2 6000mg, Vit B6 2800mg, Vit D_3 2,000,000 IU; Vit E 15000mg, Vit K3 1800mg;

[3] Mn 150000mg; Zn 100000mg; 100000mg Fe, Cu 16000mg, I 1500mg.

2.4. Performance

At 42 days old, birds and feed leftovers were weighed. The performance parameters evaluated were: feed intake (**FI**), body weight gain (**BWG**) and feed conversion rate (**FCR**). Mortality was recorded daily and was used to adjust the total number of chicks to determine feed intake and feed conversion ratio (Sakomura e Rostagno et al., 2007).

2.5. Analysis of tibias

At 28 days of age, three birds from each replicate were sampled based on the average weight (± 5%), slaughtered by electro-narcosis followed by exsanguination and had their tibias removed, placed in labeled plastic bags according to the experimental pen and frozen. Once thawed, the tibias were stripped, identified and dried in a ventilated oven at 100ºC for 24 hours. The excess of fat of the tibias were removed by immersion in ethyl ether, until the total disappearance of fat. They were dried in a ventilated oven again at 100°C for 24 hours. After that, the tibias were individually weighed on a precision scale and then were ground in a ball mill ahead of determination of dry matter, ether extract, ash, calcium, phosphorus and zinc (Silva and Queiroz, 2009).

The weight of the defatted tibia was determined as the percentage of dry tibia after ether extraction. The ash percentage was determined based on the weight of ash from the dry defatted tibia. Calcium (**Ca**), Phosphorus (**P**) and Zinc (**Zn**) were determined from defatted dry tibia by nitro perchloric digestion (Silva and Queiroz, 2009).

To assess bone strength at 42 days of age, one bird from each replicate was sampled based on the average weight (± 5%), slaughtered by electronarcosis followed by exsanguination and had their tibias removed, placed in labeled plastic bags and frozen. Once thawed, the tibias were stripped and subjected to the strength test. For this was used the Instron Corporation IX Automated Materials Testing System 1.09. A force was applied in the epiphysis in the central region of the bone with constant

velocity (10 mm/sec), with 80mm of distance between the support. The result was obtained considering the time to break the bone.

2.6. Analysis of nutrients in the litter

At 42 days, samples of litter were collected at three points of each experimental pen. These points were standardized, far from feeders and drinker areas. The three sub-samples for each experimental unit were mixed and thoroughly homogenized.

All samples were processed on the day of collection in a knife mill with a sieve of one millimeter. Total and soluble phosphorus were determined using spectrophotometric molybdate-vanadate, after nitro-perchloric digestion, as described by Leytem et al. (2007). Nitrogen was determined according to the methodology of Silva and Queiroz (2009) and corrected for dry matter.

2.7. Phosphorus Bioavailability

Phosphorus bioavailability was calculated using standard curve concept. To establish the standard curve, the intake of supplementary phosphorus (ISP) of treatments PC, NC1 and NC2 were calculated, according to formula:

$$\mathrm{ISP}(\mathrm{g}) = \frac{(\text{feed intake}(\mathrm{g}) \times \text{suplementary dietary P}(\%))}{100}$$

Supplementary dietary P was considered null in the most deficient treatment (NC2); 0.03 for NC1; and 0.17% for PC; being these values the difference of $_{av}$P between NC2 and the other treatments.

The ISP was associated to the responses of performance (BWG) and bone characteristics (ash, P and deflection force (**DF**)) variables to construct the standard curve.

The results of performance and bone characteristics variables of the treatments with phytase addition were confronted with their respective standard curve, in order to obtain the total phosphorus released (**PR**) in grams. The formula below demonstrates the calculation to obtain the nutritional matrix (bioavailability) of $_{av}$P of the phytase enzyme, being considered the feed intake of the bird and the phosphorus released.

$$BP(\%) = \frac{PR(g)}{FI(g)} \times 100$$

2.8. Cost of diets analysis

The cost analysis of the diets was performed to determine the economy of including phytases at different levels. For these calculations data of phosphorus bioavailability of BWG and the dicalcium phosphate cost were used.

The calculation of the amount of inorganic dicalcium phosphate which could be replaced by phytases inclusions (250, 500 and 1000 FTU) in the diet, based on the bioavailability found for these phytases inclusions.

The price of dicalcium phosphate was recorded on first quarter of 2015, with a value of US$ 0.50/kg (MFRural, 2015). The prices of phytase A and B were considered as US$17.00 and US$15.00, respectively.

2.9. Statistical analysis

Data were analyzed by GLM method (general linear model) of SAS 9.0 (SAS Institute, 2002). The errors in the statistical models were normally and independently distributed with constant variance. Three orthogonal contrasts were constructed in order to verify the effectiveness of phytases for the evaluated variables in this study:

C1 = T1 vs (T4, T5, T6, T7, T8, T9), to verify, in a general way, whether the diets reduced of Ca and P supplemented with phytase are equivalent or not to diets meeting the nutritional requirements of the birds.

C2 = T3 vs (T4, T5, T6, T7, T8, T9), to verify how much of the evaluated variables responses is due to phytase supplementation.

C3 = (T4, T5, T6) vs (T7, T8, T9), to verify if there are diferences between both phytases inclusion in diets with reduced levels of Ca and P.

Regression analyses were fitted on performance, litter quality parameters, and bone minerals data to determine utilization level of phytases, using T3 as the zero level of phytase inclusion, T4 and T7, T5 and T8, T6 and T9, as levels 250, 500 and 1000 FTU/kg for phytases A and B, respectively. Linear (L), quadratic (Q) and quadratic-plateau (QP) models were used to analyse the different enzymes levels on the variables responses.

The QP model was adjusted according to Robbins et al. (2006), $Y_i = L + U\left(R - Phy_i\right)^2$, i = 1,2...n_1, n_1+1,...,n; in which (R - Phy_i)^2 = 0 for i ≥ n_1 + 1, n_1 is the number of observations up to the breaking point, n is the number of observation pairs, estimated by the equation. The Y_i is the response of the estimated variable to the phytase level in the diet; Phyi is the level of phytase in the diet; L is asymptotic response of the function; U is slope at the breaking point; and R is the phytase level estimated by the breaking point.

3.0 RESULTS

3.1 Phytase to maximize the performance of broilers

Different concentrations of phytase affect the feed intake and body weight gain of broilers (Table 2).

Table 2. Feed intake (FI), body weight gain (BWG) and feed conversion (FCR), of broilers submitted to phytase at different concentrations at 42 days and contrast analysis

Treatments	n	FI (g)	BWG (g)	FCR (g:g)
T1 - PC	8	4586.6	2660.7	1.720
T2 - NC 1	8	4455.5	2579.1	1.728
T3 - NC 2	8	4315.5	2511.9	1.718
T4 - NC 2 + 250 A	8	4529.3	2649.2	1.710
T5 - NC 2 + 500 A	8	4539.5	2666.5	1.703
T6 - NC 2 + 1000 A	8	4639.6	2722.2	1.705
T7 - NC 2 + 250 B	8	4443.1	2605.8	1.706
T8 - NC 2 + 500 B	8	4489.7	2630.1	1.707
T9 - NC 2 + 1000 B	8	4569.0	2679.6	1.705
SEM[1]		131.6	82.4	0.021
p-value		0.0040	0.0023	0.2478
		P-VALUE CONTRAST		
CONTRASTS		FI (g)	BWG (g)	FCR
C1: T1 vs (T4, T5, T6, T7, T8, T9)		0.1061	0.3657	0.1296
C2: T3 vs (T4, T5, T6, T7, T8, T9)		0.0004	0.0002	0.1219
C3: (T4, T5, T6) vs (T7, T8, T9)		0.1060	0.1257	0.9711

[1] SEM = Standard error of the means for treatments effects;
n = number of observations.

The **C2** contrast, which evaluates phytase action, showed significant effect ($p \leq 0.01$) to feed intake and body weight gain. The **C1** and **C3**, which compare PC and the NC2 with phytase, and the comparison between both phytase groups, were not significant for any of the performance variables evaluated ($p > 0.05$).

To obtain the concentration of phytase that leads to the maximum response of broilers, the L, Q and QP models were adjusted to describe the gradual inclusion of enzymes in the diet (Table 7). No model was adjusted to feed conversion since there was no response in function of the phytases concentrations studied.

The equations obtained to BWG and FI of phytases A and B were adjusted to QP, and are shown in Table 7. For Phytase A, the higher BWG (2,692g) occurred at a concentration of 489 FTU/kg and the maximum FI (4,586g) at 460 FTU/kg.

For phytase B, QP effect on BWG and FI, promoted higher BWG (2,678g) at 949 FTU/kg and the maximum FI (4,567g) at 1032 FTU/kg.

3.2. Effects of phytases on composition and quality of bone

In the evaluation of a phytase, it is important to consider the minerals that were available to animal. In this case, bones are an excellent criteria because the highest concentrations of Ca, P and Zn.

Table 3 presents the data of mineral composition and deflection force of tibias, and statistical results.

Table 3. Results of composition: calcium (Ca) phosphorus (P), zinc (Zn) and ash (Ash) of 28 days and deflection force (DF) of 42 days of broilers tibias and contrast analysis

Treatments	n	Ca (mg/g)	P (mg/g)	Zn (mg/g)	Ash (%)	DF (N)
T1 - PC	8	203.1	286.4	188.9	38.1	264.5
T2 - NC 1	8	194.3	255.3	165.8	35.1	227.4
T3 - NC 2	8	181.4	229.0	156.9	34.1	189.8
T4 - NC 2 + 250 A	8	200.1	261.3	176.2	35.4	237.1
T5 - NC 2 + 500 A	8	195.0	263.0	180.8	36.4	269.6
T6 - NC 2 + 1000 A	8	183.7	263.3	187.6	37.3	249.8
T7 - NC 2 + 250 B	8	172.3	222.0	177.0	35.0	245.0
T8 - NC 2 + 500 B	8	182.0	262.3	173.6	37.1	233.5
T9 - NC 2 + 1000 B	8	199.3	265.0	188.7	38.3	242.7
SEM[1]		11.8	13.0	7.1	1.77	42.52
p-value		<0.0001	<0.0001	<0.0001	<0.0001	0.0252
P-VALUE CONTRAST						
CONTRASTS		Ca	P	Zn	Ash	DF
C1: T1 vs (T4, T5, T6, T7, T8, T9)		0.0035	<0.0001	<0.0001	0.0093	0.1063
C2: T3 vs (T4, T5, T6, T7, T8, T9)		0.1074	<0.0001	<0.0001	0.0004	0.0009
C3: (T4, T5, T6) vs (T7, T8, T9)		0.0166	0.0014	0.3940	0.4348	0.3405

[1] SEM = Standard error of the means for treatments effects;
n = number of observations.

Trough C2 ($p \leq 0.05$), both phytases increased P, Zn and Ash deposition, and showed higher DF in comparison to NC. However, the levels of Ca, P, Zn and Ash were lower than those observed to PC ($p \leq 0.05$), as demonstrated by C1.

When comparing different enzymes groups (C3), it is possible to verify that enzyme A showed 4.5 and 5.1% higher deposition of Ca and P, respectively, than phytase B. C3 was not significant ($p > 0.05$) to Zn, Ash and DF.

The response of the variables Ca, P, Zn and Ash as a function of different concentrations of phytase were analyzed by Q, QP and L models, as shown in Table

7. According to the fitted models, the maximum levels of Ca (198.5 mg/g), P (271.3 mg/g), Zn (186.8 mg/g) and ash (37.4 %) were obtained with concentrations of 492, 694, 770 and 1073 FTU/kg, respectively, from phytase A. Models fitted for phytase B showed that the maximum levels of P (275.4 mg/g), Zn (188.9 mg/g) and ash (39.6%) would be obtained with 1367, 1225, 1750 FTU/kg of phytase B, respectively. The derivation of the equation obtained for Ca showed a minimum value of 175.6 mg/g of bone at the concentration of 183 FTU/kg of phytase B.

3.3. Litter composition

It is well known that the phytase used in cereal-based diets for broilers promotes the breaking of the bonds of phytate chain, releasing minerals, carbohydrates and amino acids, making them available to the animals. Thus, there is an increase in the bioavailability of these nutrients, which affects the excreta profile of birds. Data of litter composition at 42 days are expressed on Table 4.

Table 4. Total nitrogen ($_t$N), water soluble phosphorus (WSP) and total phosphorus (tP) in litter of broilers fed different levels of phytase and contrast analysis results

Treatments	n	$_t$N (% DM)	WSP (mg/kg)	tP (g/kg)
T1 - PC	8	3.51	1257.6	10.85
T2 - NC	8	3.74	936.2	9.52
T3 - NC 2	8	3.37	865.5	9.01
T4 - NC 2 + 250 A	8	3.35	1030.5	9.06
T5 - NC 2 + 500 A	8	3.82	1134.2	7.96
T6 - NC 2 + 1000 A	8	3.62	1136.0	8.04
T7 - NC 2 + 250 B	8	3.65	862.0	7.62
T8 - NC 2 + 500 B	8	3.19	1008.5	7.97
T9 - NC 2 + 1000 B	8	3.37	1057.0	7.94
SEM [1]		0.27	149.7	0.62
p-value		0,0004	<0,0001	<0,0001
P-VALUE CONTRAST				
CONTRASTS		$_t$N	WSP	tP
C1: T1 vs (T4, T5, T6, T7, T8, T9)		0.7643	0.0001	<0.0001
C2: T3 vs (T4, T5, T6, T7, T8, T9)		0.2921	0.0038	0.0003
C3: (T4, T5, T6) vs (T7, T8, T9)		0.0235	0.0060	0.0073

[1] SEM = Standard error of the means for treatments effects;
n = number of observations.

From C3 it is possible to observe that $_t$N in litter was higher to the birds that received diets supplemented with phytase A. However, none model was able to describe the responses with gradative phytase inclusion. C1 and C2 analysis showed that the inclusion of phytases reduce WSP and tP excretion, reducing, their concentrations in litter. With C3 analysis, it is observed that phytase B presents lower $_t$N and tP excretion than phytase A. In the other hand, phytase A presents lower WSP excretion, when compared to phytase B.

To evaluate the effect of different phytase on nutrients excretion, litter composition data were analyzed by Q, QP and L models (Table 7). Results showed that the maximum WSP excretion (1,140 mg/kg of litter) was at 645 FTU/kg of phytase A. For Pt, the same enzyme in the concentration of 953 FTU/kg, resulted in a lower excretion of Pt in litter (7.99 g/kg of litter).

Increasing phytase B caused a linear increase of WSP in litter. Our results indicated that Pt had a quadratic effect, and its first derivative, set equal to zero, determined the concentration of the enzyme (666FTU/kg) which provided the smaller amount of Pt in litter (7.53 g g/kg of litter). Based on the results obtained here, the addition of phytase to broilers diets reduced the concentration of total phosphorus in litter, while there was an increasing trend in the concentrations of WSP with increasing phytase concentration in the diets.

The values of WSP and Pt in the diets supplemented with phytases were smaller when compared to PC diet without reducing Ca and P (without phytase). Nitrogen content in litter did not show any significant response.

3.4. Bioavailability of phosphorus

To obtain the standard curve, the parameters Ash and P, evaluated at 28 days, and BWG and DF, evaluated at 42 days, were used.

Linear response was obtained, considering phosphorus in the tibia (P), with the three increasing levels of ISP (PC, NC1 and NC2). The regression obtained was: $P = 236.8(\pm 3.9) + 15.07(\pm 2.03)x$, with R^2 = 0.71, ($p < 0.0001$), where x is the quantity in

grams or percentage of phosphorus, which was released by the enzyme for phosphorus in the tibia (P).

Considering the percentage of tibia ash, a linear regression was obtained with three increasing levels of ISP (PC, NC1 and NC2), ($p < 0.05$). $Ash = (34.23(\pm 0.42) + 1.15(\pm 0.22)x$, with $R^2 = 0.55$, ($p < 0.0001$), where x is the quantity in grams or percentage of phosphorus, which was released by the enzyme to percentage tibia ash (ash).

Phosphorus bioavailability obtained from P and ash variables are shown in Table 5.

Table 5. Intake of supplemental phosphorus (ISP), phosphorus release (PR) and phosphorus bioavailability (PB) calculated by phosphorus (P) and ash (Ash) of tibia at 35 days

Treatments	ISP (g)	$PR^{(P)}$ (g)	$PB^{(P)}$ (%)	$PR^{(Ash)}$ (g)	$PB^{(Ash)}$ (%)
PC	3.414	-	-	-	-
NC 1	0.597	-	-	-	-
NC 2	0	-	-	-	-
NC 2 + 250 A	-	1.625	0.083	0.974	0.058
NC 2 + 500 A	-	1.814	0.091	1.918	0.098
NC 2 + 1000 A	-	2.081	0.101	2.694	0.149
NC 2 + 250 B	-	0	0	0.676	0.031
NC 2 + 500 B	-	1.546	0.072	2.438	0.090
NC 2 + 1000 B	-	2.007	0.099	3.526	0.177
SEM[1]		0.866	0.044	1.618	0.085

[1] SEM = Standard error of the means for treatments effects (n = 8).

Linear response was obtained, considering body weight gain (BWG) with the three increasing levels of ISP (PC, NC1 and NC2). The regression obtained was: $BWG = 2560.9(\pm 34.8) + 13.6(\pm 6.0)x$, with $R^2 = 0.22$, ($p < 0.0043$), where x is the quantity in grams or percentage of phosphorus, which was released by the enzyme for BWG.

Considering the deflection force (DF), a linear regression was adjusted with three increasing levels of ISP (PC, NC1 and NC2), ($p < 0.05$). $DF = 201.8(\pm 10.1) + 8.4(\pm 2.2)x$, with $R^2 = 0.37$, ($p < 0.0001$), where x is the quantity in grams or percentage of phosphorus, which was released by the enzyme to percentage DF.

The data of phosphorus bioavailability obtained from BWG and DF are presented in Table 6.

Table 6. Intake of supplemental phosphorus (ISP), phosphorus release (PR) and phosphorus bioavailable (PB) calculated by body weight gain (BWG) and deflection force (DF) of tibias

Treatments	ISP $^{(BWG)}$ (g)	PR $^{(BWG)}$ (g)	PB $^{(BWG)}$ (%)	PR $^{(DF)}$ (g)	PB $^{(DF)}$ (%)
PC	7.797	-	-	-	-
NC 1	1.337	-	-	-	-
NC 2	0	-	-	-	-
NC 2 + 250 A	-	6.502	0.140	4.227	0.113
NC 2 + 500 A	-	7.782	0.170	8.101	0.174
NC 2 + 1000 A	-	11.886	0.250	5.739	0.167
NC 2 + 250 B	-	3.305	0.072	5.163	0.103
NC 2 + 500 B	-	5.097	0.073	3.847	0.080
NC 2 + 1000 B	-	8.747	0.188	4.887	0.123
SEM[1]		6.361	0.132	5.420	0.116

[1] SEM = Standard error of the means for treatments effects (n = 8).

The variable that best fit the standard curve was the amount of P in bones (R^2 = 0.72), while BWG and Ash variables showed lower R^2, being 0.22 and 0.57, respectively.

Table 7. Equations adjusted in function of enzymes concentration to describe performance, litter and bones of broilers

PHY	VAR	MODEL	Equation	Max Min	R^2 Ad	p - value
	FI	QP	$FI = 4585.7(\pm38.5) - 0.0013(\pm0.0011) \times [460.1(\pm202.2) - PHY_I]^2$	460	0.40	<0.01
	BWG	QP	$BWG = 2692.2(\pm24.4) - 0.0008(\pm0.0006) \times [488.9(\pm201.6) - PHY_I]^2$	489	0.42	<0.01
	WSP	QP	$WSP = 1140.0(\pm45.6) - 0.0007(\pm0.0006) \times [644.5(\pm283.6) - PHY_I]^2$	645	0.41	<0.01
PHY	tP	QP	$tP = 7.99(\pm0.22) + 0.0000017(\pm0.000001) \times [952.5(\pm418.9) - PHY_I]^2$	953	0.35	<0.01
A	Ca	Q	$Ca = 181.49(\pm3.97) + 0.069(\pm0.02)PHY_i - 0.00007(\pm0.000002){PHY_I}^2$	492	0.34	<0.01
	P	Q	$P = 227.94(\pm5.58) + 0.125(\pm0.028)PHY_i - 0.00009(\pm0.000003){PHY_I}^2$	694	0.52	<0.01
	Zn	QP	$Zn = 186.8(\pm2.1) - 0.00005(\pm0.00002) \times [770.2(\pm132.4) - PHY_I]^2$	770	0.78	<0.01
	Ash	QP	$Ash = 37.36(\pm0.85) - 0.000003(\pm0.000003) \times [1073.2(\pm614.6) - PHY_I]^2$	1073	0.31	<0.01
	FI	QP	$FI = 4567.5(\pm48.3) - 0.0002(\pm0.0002) \times [1031.5(\pm485.5) - PHY_I]^2$	1032	0.41	<0.01
	BWG	QP	$BWG = 2677.7(\pm29.2) - 0.0002(\pm0.0002) \times [949.0(\pm413.4) - PHY_I]^2$	949	0.41	<0.01
	WSP	L	$WSP = 865.5(\pm39.5) + 0.203(\pm0.076)PHY_i$	-	0.26	<0.01
PHY	tP	Q	$tP = 8.86(\pm0.21) - 0.001(\pm0.01)PHY_i + 0.0000003(\pm0.0000009){PHY_I}^2$	666	0.37	<0.01
B	Ca	Q	$Ca = 176.65(\pm5.14) - 0.011(\pm0.026)PHY_i + 0.00003(\pm0.00002){PHY_I}^2$	183	0.36	<0.01
	P	Q	$P = 219.36(\pm6.44) + 0.082(\pm0.033)PHY_i - 0.00003(\pm0.00003){PHY_I}^2$	1367	0.53	<0.01
	Zn	Q	$Zn = 158.9(\pm3.3) + 0.049(\pm0.017)PHY_i - 0.00002(\pm0.00001){PHY_I}^2$	1225	0.62	<0.01
	Ash	Q	$Ash = 33.51(\pm0.67) + 0.007(\pm0.003)PHY_i - 0.000002(\pm0.000003){PHY_I}^2$	1750	0.52	<0.01

(FI) Feed Intake; (BWG) Body Weight Gain; (FCR) Feed Conversion Ratio; (Ca) Amount of Calcium present in the tibia of broilers at 28 days; (P) Amount of phosphorus present in the tibia of broilers at 28 days; (Zn) Zinc quantity present in the tibia of broilers at 28 days; (Ash) Ash content present in the tibia of broilers at 28 days; (N) Concentration of Water Soluble Phosphorus present in litter; (WSP) Concentration of Water Soluble Phosphorus present in litter; (tP) concentrations of total phosphorus present in litter; (Phy_i) Concentration of phytase.

3.5. Cost of diet analysis

Economic viability is an important aspect that should be considered when including phytase in poultry diets. To know the level of phytase added that ensures the best economic income is an important tool for the success of the company.

Since BWG is the most important performance parameter, it was chosen to calculate the economic viability of the phytases, being calculated its cost limit.

Figure 1 shows the savings when using phytase in different concentrations, in partial replacement of dicalcium phosphate. The savings were calculated considering phosphorus releasing through phytase, using BWG as parameter.

The tested phytases were able to replace part of dietary dicalcium phosphate and generate an optimal financial return at 1000 FTU, with US$ 5.06/ton feed and US$ 3.58/ton feed of economy to phytases A and B, respectively.

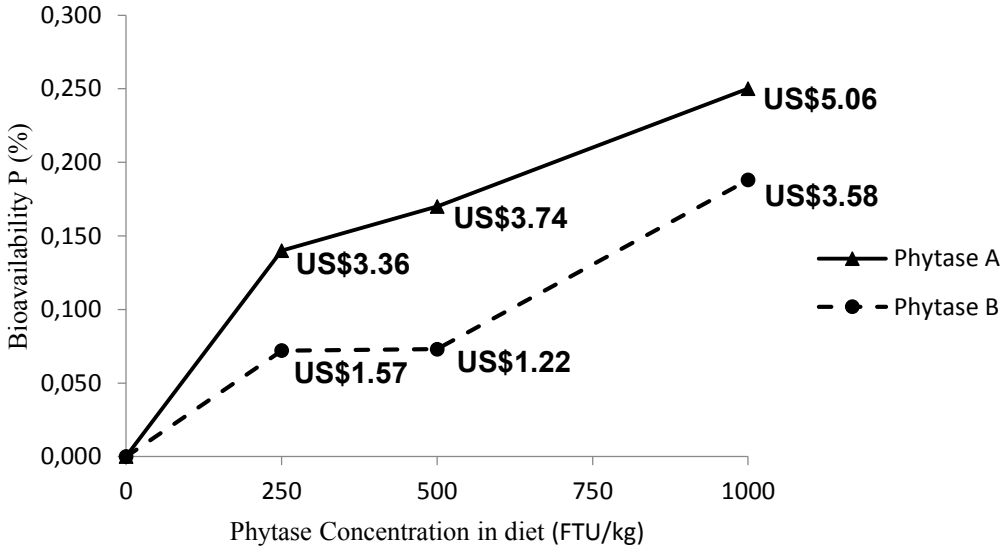

Figure 1. Savings per ton of feed using phytase in different concentrations in partial replacement of dicalcium phosphate.

The tested phytases were able to replace part of dietary dicalcium phosphate and generate an optimal financial return at 1000 FTU, with US$ 5.06/ton feed and US$ 3.58/ton feed of economy to phytases A and B, respectively.

4. DISCUSSION

In order to evaluate phytase and its optimal concentration, the effects of two different bacterial phytases on performance, bone mineralization, nutrient excretion, bioavailability of phosphorus and economic evaluation of broilers were studied in this experiment.

The inclusions of phytase on NC2 diet were able to increase feed intake, and, consequently body weight gain of the birds. Phytase utilization completely recovered the lost performance caused by Ca and P reduction, being equivalent to PC diet. However, the phytase supplementation did not affect FCR. Previous studies (DENBOW et al., 1998; AHMAD et al., 1999) showed that the enzyme activity does not alter the feed conversion and/or efficiency, and greater weight gain is explained by the result of higher feed intake when phytase is added to the diet, as ocurred in this study. According to Cowieson et al. (2011), the phytate present inthe feed per se can be an appetite suppressant while phytase increases the intake of diets deficient in P until the requirement is met, but further increases in phytase continue to stimulate consumption. This may explain the quadratic and linear-plateau effects of increasing levels of phytases A and B on feed intake.

The optimal level of phytase A in the diet to achieve maximum BWG (2,692 g) in broilers was 489 FTU/kg, which is close to the recommended amount by the most companies and researchers (Huff et al., 1998; Gomide et al., 2007; Schlegel and Nys, 2009). For phytase B, the QP effect on performance variables estimated as 1000FTU/kg for the highest body weight gain and feed intake. Denbow et al. (1998), working with additions to 1200 FTU/kg of fungal phytase, observed a linear increase in growth rate, feed intake and body weight. In this study, the L model was also significant, with R^2 very similar to the proposed QP model, however it was considered the model described by being more consistent, due the phytate content in foods is depleted, reducing the activity of enzyme by lack of substrate.

Although there was no statistical difference between the two phytases (C3) to BWG, FI and FCR, when evaluated by regression, phytase A presented lower optimum level than phytase B to FI and BWG, reaching the maximum response to these variables before enzyme B.

Results showed that the level of phytase in the diet affects bone mineralization. Both phytases increased P, Zn and Ash deposition, however, these responses did not reach the bone mineralization observed in the birds receiving PC diet. The same was observed to deflection force. Some studies verified that the phytase supplementation in phosphorus deficient diets are not completely able to mineralize the bone of the broilers until 21 days (Catalá-Gregory et al., 2006; Viveiros et al., 2006).

None phytases were able to improve Ca deposition, unlike the study carried out by Sebastian et al. (1996), however, phytase A showed higher deposition of this mineral than phytase B.

Besides the enzyme A showed higher P and Ca deposition, it reached the threshold more rapidly, with the maximum levels of (694, 770, and 1073FTU/kg) vs (1367, 1225 and 1750FTU/kg) of phytase B to P, Zn and Ash respectively, giving conditions to affirm that it was more efficient. In Brenes et al. (2003) study, phytase inclusion increased tibia ash (up to 4%), Ca (up to 2%), P (up to 1%) and Zn (up to 4%) content. In the present study, Ca showed the minimum value (175.6 g/kg bone) with 183FTU/kg of Phytase B. It was expected that the addition of phytase would increase the content of this mineral in the tibia. One possible explanation is that the inclusion of 250 FTU/kg of this phytase did not improved Ca deposition on tibias, and thus, the equation had a negative quadratic coefficient.

Based on regression equation, the level of 952FTU/kg of phytase A resulted in lower tP (7.99 g) in litter. Considering that the fitted model was the QP, the use of higher phytase level will not result in highest reduction in tP excretion. For phytase B, according to Q model, was estimated the concentration of 666 FTU/kg to the lower amount of tP (7.53 g) in litter. Comparing with the positive control treatment, a reduction of around 26% of tP content in the litter was observed with 500FTU/kg of Phytase A. This reduction was expected, since the diets using phytase contained less amount of inorganic phosphate versus the positive control diets. As in this work, several studies have also demonstrated the reduction of tP in litter with the use of phytase (Denbow et al., 1998; Smith et al., 2004; Shelton et al., 2004; Mcgrath et al., 2005; Angel et al., 2006; Powell et al., 2008).

The results for WSP levels showed opposite behavior as tP, since it increased with increasing concentration of both phytases in the diet. Maximum excretion of WSP

(1140mg/kg litter) was achieved with phytase A (645 FTU/kg). The increasing levels of phytase B promoted a linear increase in litter WSP. Despite the increasing trend of WSP in litter with increasing concentrations of phytase, these values were 862, 1008 and 1057 mg/kg of litter to 250, 500 and 1000 FTU/kg, respectively, which were numerically lower than the control diet which was not supplemented with phytase (1257.6 mg/kg of litter). According to Angel et al. (2005), WSP contents can be changed by microorganisms present in digesta that will transform part of the insoluble P to soluble. It seems that using 500FTU/kg of phytase A led to a reduction of approximately 10% WSP and 27% Pt compared to the positive control, without any negative effect on performance of broilers. This reduction is highly beneficial, since the WSP is highly leached by rains and becomes readily available to algae, and it also affects the water quality due to accelerated eutrophication (Sonzogni et al., 1982). Some studies have shown that the addition of phytase to poultry diets reduced the concentration of litter WSP about 35.6% (Applegate et al., 2003) and 29% (Mcgrath et al., 2005).

Controversy exists about the effects of using phytase on the excretion of WSP and WSP in relationship with Pt (Moore et al., 1998; Sims et al., 1999; Delaune et al., 2004; Saylor et al., 2001). Phytase releases phosphate groups from the phytate molecule, making them available P to be used by the animal (Rapp et al., 2001). If dietary concentrations of inorganic phosphorus are not adequately reduced when phytase is added, the excretion of available P increases. Therefore, there is a potential for increases in WSP excreted, as well as an increase in the proportion of P which is WSP (Angel et al., 2005).

Based on our results, it seems that the concentration of phytase did not affect the nitrogen content in litter. Phytase is an enzyme which breaks down phytate and thus improves the P, Ca, Na, Cu, energy and amino acid availability and digestibility in broilers (Chung et al., 2013). One possible explanation is that the nitrogen of the litter was volatilized before the analysis was performed.

The bioavailability of phosphorus results for each phytase, demonstrated that phytase A had a numerically higher value for available P than phytase B, when calculated by BWG. However, phytase B showed higher bioavailability when evaluated

with tibia Ash. This fact shows the higher efficiency of phytase A to body weight gain, while phytase B is more efficient to minerals on tibia deposition.

The bioavailability of P showed a linear effect by BWG variable. Augspurger et al. (2003) also obtained linear effects on the performance of broilers, while Pereira et al. (2012) found a quadratic effect for these variables. According to Pereira et al. (2012), the linear response is the one that best describes the standard curve, since each increase in x-axis is an increase in the y-axis, and thus the estimates of phosphorus released by phytase are more reliable.

Phosphorus in bones showed the best fit to elaborate the standard curve (R^2=0.72). The ash content of the bones did not show the best measure to calculate the bioavailability (R^2=0.57) unlike to that observed in other studies (Garcia et al., 2006; Pereira et al., 2012).

The variable P in bones was the best fit model for the standard curve. The bioavailability of P found here for the two phytases studied corroborates to other previous studies and demonstrates the positive effect of exogenous phytase in poultry feed (Snow et al., 2004; Manangi and Coon, 2008; Powell et al., 2011).

With the better nutrients utilization, especially of dietary phosphorus, there is a reduction of $_t$P and WSP excretion when phytases are included in the diet. The concentration of 500 FTU/kg of phytase A reduced the concentration of $_t$P (26%) and WSP (10%) in litter when compared to PC treatment. This effect may help to prevent contamination of the environment. The performance and bone strength also showed higher values in this range of concentration. Therefore, considering the environment and performance, 500 FTU/kg of phytase A would be recommended, without considering the costs.

Phytase B presented lower $_t$N, $_t$P and WSP excretion, lower efficiency for body weight gain, but presented higher bone ash, demonstrated by bioavailability calculated using ash data, when compared to phytase A. It was possible to determine the optimal level that leads to the best performance as 1000 FTU/kg. The concentration that reduced $_t$P content in litter was 666 FTU/kg, while for bone mineralization and bone strengh best responses was observed to 1000 FTU/kg, thus being this the recommendation level of phytase B.

Considering the cost of dicalcium phosphate replacement to phytase, the recommendation would be 1000 FTU to both enzymes. Together with cost, others aspect should be considered, as performance and nutrient excretion. Considering that the body weight gain of the birds that received 1000FTU/kg of phytase A was 8.37% higher than the NC and 2.31% higher than the PC, with no changes on FCR, this may reflect on benefits of phytase. The best benefit of lower nutrient excretion were also observed in inclusion of 1000 FTU.

Results from our study showed that both phytases have proven to be efficient in releasing phytate P, however, phytase derived from Buttiauxella spp. proved to be more efficient than that from E. coli considering BWG, however, E. coli phytase showed higher efficiency to reduce nutrients excretion.

Since phytase generates ambient and bone characteristics beneficial effects in all concentrations, it should be considered the cost of phytase and phosphate as the major factor of its inclusion.

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5. REFERENCES

Adeola, O., and A. J. Cowieson. 2011. Board-Invited Review: Opportunities and challenges in using exogenous enzymes to improve nonruminant animal production. J. Anim. Sci. 89:3189-3218.

Ahmad, T., S. Rasool, M. Sarwar, A. Haq, Z. Hasan. 1999. Effect of microbial phytase produced from a fungus *Aspergillus niger* on bioavailability of phosphorus and calcium in broiler chickens. Anim. Feed. Sci. Tech. 83:103-114.

Angel, R., W. W. Saylor, A. D. Mitchell, W. Powers, and T. J. Applegate. 2006. Effect of dietary phosphorus, phytase, and 25-hydroxycholecalciferolon broiler chicken bone mineralization, litter phosphorus, and processing yields. Poult. Sci. 85:1200–1211.

Applegate, T. J., B. C. Joern,, D. L. Nussbaum-Wagler, and R. Angel. 2003. Water soluble phosphorus in fresh litter is de- pendent upon phosphorus concentration fed but not on fungal phytase supplementation. Poult. Sci. 82:1024–1029.

Augspurger, N.R., D. M. Webel, X. G. Lei, and D. H. Baker. 2003. Efficacy of an *E. coli* phytase expressed in yeast for releasing phytate bound phosphorus in young chicks and pigs. J. Anim. Sci. 81:474-483.

Brenes, A., A. Viveros, I. Arija, C. Centeno, M. Pizarro, C. Bravo. 2003. The effect of citric acid and microbial phytase on mineral utilization in broiler chicks. Anim. Feed. Sci. Tech. 110:201–219.

Catalá-Gregori, P., V. García, F. Hernández, J. Madrid, and J. J. Cerón. 2006. Response of Broilers to Feeding Low-Calcium and Phosphorus Diets Plus Phytase Under Different Environmental Conditions: Body Weight and Tibiotarsus Mineralization. Poult. Sci. 85:1923-1931.

Chung, T.K., S. M. Rutherfurd, D. V. Thomas, P. J. Moughan. 2013. Effect of two microbial phytases on mineral availability and retention and bone mineral density in low-phosphorus diets for broilers. Brit. Poultry Sci. 54:362-373.

COBB. Broiler Management Guide. COBB 500. S.l.: s.n., 2008, 65p.

Conffey, M. T. 2992. An industry perspective on environmental ans waste management issues: Challenge for the feed industry. In: GEORGIA NUTRITION CONFERENCE, Athens. Proceedings…Athens: University of Georgia, p. 144-148.

Cowieson A.J., P. Wilcock, and M. R. Bedford. 2011. Super dosing effects of phytase in poultry and other monogastrics. World Poultry Sci. J. 67:225-236.

Cromwell, G. L., R. D. Coffey, H. J. Monegue, J. H. Randolph. 1997. Efficacy of low activity, microbial Phytase in improving the bioavailability of phosphorus in corn soybean meal diets pigs. J. Anim Sci. 75:3174-3186.

Delaune, P. B., P. A. Moore Jr., D. K. Carman, A. N. Sharpley, B. E. Haggard, and T. C. Daniel. 2004. Development of a phosphorus index for pastures fertilized with poultry litter factors affecting phosphorus runoff. J. Environ. Qual. 33:2183–2191.

Denbow, M. D., E. A. Grabau, G. H. Lacy, E. T. Kornegay, D. R. Russell, and P. F. Umbeck. 1998. Soybeans Transformed with a Fungal Phytase Gene Improve Phosphorus Availability for Broilers. Poult. Sci. 77:878-881.

Garcia, A. R., A. B. Batal, and N. M. Dale. 206. Biological Availability of Phosphorus Sources in Prestarter and Starter Diets for Broiler Chicks. J. Appl. Poult. Res. 15:518–524.

Gomide, E. M., P. B. Rodrigues, R. T. F. Freitas, E. T. Fialho. 2007. Planos nutricionais com a utilização de aminoácidos e fitase para frangos de corte mantendo o conceito de proteína ideal nas dietas. R. Bras. Zootec. 36:1769-1774.

Huff, W. E., P. A. Moore, P. W. Waldroup Jr, A. L. Waldroup, J. M. Balog, G. R. Huff, N. C. Rath, T. C. Daniel, V. Raboy. 1998. Effect of Dietary Phytase and High Available Phosphorus Corn on Broiler Chicken Performance. Poult. Sci. 77:1899–1904.

Kaur, P., G. Kunze, T. Satyanarayana. 2009. Yeast Phytases: Present Scenario and Future Perspectives. Crit. Rev. Biotechnol. 27:93-109.

Lana, R. P. 2009. Uso racional de recursos naturais não-renováveis: aspectos biológicos, econômicos e ambientais. R. Bras. Zootec. 38:330-340.

Leytem, A.B., P. W. Plumstead, R. O. Maguire, P. Kwanyuen, J. Brake. 2007. What aspect of dietary modification in broilers controls litter water-soluble phosphorus: dietary phosphorus, phytase, or calcium? J. Environ. Qual. 36:453–463.

Manangi, M. K., and C. N. Coon. 2008. Phytate phosphorus hydrolysis in broilers in response to dietary phytase, calcium, and phosphorus concentrations. Poult. Sci. 87:1577–1586.

Mcgrath, J.M., J. T. Sims, R. O. Maguire, W. W. Saylor, R. Angel, and B. L. Turner. 2005. Broiler diet modification and litter storage: Impacts on phosphorus in litters, soils, and runoff. J. Environ. Qual. 34:1896–1909.

MFRural. (2015). Price of animal food. MFRural. Assessed Jan. 2015. http://www.mfrural.com.br.

Moore, P. A., M. L. Self-Davis, T. C. Daniel, W. E. Huff, D. R. Edwards, D. J. Nichols, W. F. Jaynes, G. R. Huff, J. M. Balog, N. C. Rath, P. W. Waldroup, And V. Raboy. 1998. Use of high available phosphorus corn and phytase enzyme additions to broiler diets to lower phosphorus levels in poultry litter. Natl. Poult. Waste Manage. Symp. J. P. Blake and P. H. Patterson, ed. Auburn Univ. Printing Service, Auburn, AL. 346–352

Musapuor A., M. Afsharmanesh, H. Moradi Shahrbabak. 2006. Use of Microbial Phytase for Decrease of Pollutant Due to Environmental Poultry Excreta Phosphorus. Int. J. Agric. Biol. 08:35–37.

NRC. 1994. National Research Council, Nutrient requirements of poultry, Washington: National Academy Press, 9th revised ed.

Pereira, R., J. F. M. Menten, G. G. Romano G. G, C. S. L. Silva, K. C. Zavarize, N. A. Barbosa, 2012. Eficiência de uma fitase bacteriana na liberação de fósforo fítico em dietas de frangos de corte. Arq. Bras. Med. Vet. Zoo. 64:137-144.

Powell, S., S., Johnston, L. Gaston, and L. L. Southern. 2008. The Effect of Dietary Phosphorus Level and Phytase Supplementation on Growth Performance, Bone-Breaking Strength, and Litter Phosphorus Concentration in Broilers. Poult. Sci. 87: 949-957.

Powell, S., T. D. Bidner, and L. L. Soutjer. 2011. Phytase supplementation improved growth performance and bone characteristics in broilers fed varying levels of dietary calcium. Poult. Sci. 90:604–608.

Rapp, C., H. J. Lantzsch, and W. Drochner. 2001. Hydrolysis of phytic acid by intrinsic plant and supplemented microbial phytase (*Aspergillus niger*) in the stomach and small intestine of mini pigs fitted with re-entrant cannulas. 2. Phytase activity. J. Anim. Physiol. Anim. Nutr. 85:414–419.

Ravindran, V., W. L. Bryden, and E. T. Kornegay. 1995. Phytates: Occurrence, bioavailability and implications in poultry nutrition. Poult. Avian Biol. Rev. 6:125–143.

Robbins, K. R., A. M. Sexton, and L. L. Southern. 2006. Estimation of nutrient requirements using broken-line regression analysis. J. Anim. Sci. 84:155–E165.

Rostagno, H. S., L. F. T. Albino, J. L. Donzele, P. C. Gomes, R. F. Oliveira, D. C. Lopes, A. S. Ferreira, L. S. T. Barreto, R. F. Euclides. 2011. Tabelas brasileiras para aves e suínos: composição de alimentos e exigências nutricionais. 3.ed. Viçosa: Universidade Federal de Viçosa. 252p.

Sakomura, N. K., H. S. Rostagno. 2007. Métodos de pesquisa em nutrição de monogástricos. Funep, Jaboticabal, São Paulo/Brasil.

SAS Institute. 2002. SAS user's guide: statistics, version 9.0. Cary: SAS Institute.

Saylor, W. W., J. T. Sims, G. W. Malone, and M. F. Lavahun. 2001. Use of phytase and high-available phosphorus corn in broiler diets: Impact on litter phosphorus levels and solubility. Proc. Maryland Nutr. Conf., Univ. Maryland, College Park. 43–57.

Sebastian, S., S. P. Touchburn, E. R. Chavez, and P. C. Lague. 1996. The Effects of Supplemental Microbial Phytase on the Performance and Utilization of Dietary

Calcium, Phosphorus, Copper, and Zinc in Broiler Chickens Fed Corn-Soybean Diets. Poult. Sci. 75:729-736.

Schlegel, P., Y. Nys, and C. Jondreville. 2009. Zinc availability and digestive zinc solubility in piglets and broilers fed diets varying in their phytate contents, phytase activity and supplemented zinc source. Animal. 14:200-209.

Shelton, J. L., L. L. Southern, L. A. Gaston, and A. Foster. 2004. Evaluation of the nutrient matrix values for phytase in broilers. J. Appl. Poult. Res. 13:213–221.

Silva, D.J., A. C. Queiroz. 2009. Análise de alimentos: métodos químicos e biológicos 3. ed. Viçosa, MG: UFV, 235 p.

Sims, J. T., M. F. Lavahun, G. W. Malone, W. W. Saylor, and V. Raboy. 1999. Abstract: Effects of microbial phytase, high available phosphorus corn, and dietary phosphorus level on broiler production. 2. Effects on broiler litter composition. Poult. Sci. 78:102.

Smith, D.R., P.A. Moore, D.M. Miles, B.E. Haggard, And T.C. Daniel. 2004. Decreasing phosphorus runoff losses from land-applied poultry litter with dietary modifications and alum addition. J. Environ. Qual. 33:2210–2216.

Snow, J. L., D. H. Baker, and C. M. Parsons. 2004. Phytase, citric acid and 1α-hydroxycholecalciferol improve phytate phosphorus utilization in chicks fed a corn-soybean meal diet. Poult. Sci. 83:1187–1192.

Sonzogni, W. C. 1982. Bioavailability of phosphorus inputs to lakes. J. Environ. Qual. 11:555-563.

Viveros, A., A. Brenes, I. Arija, and C. Centeno. 2002. Effects of microbial phytase supplementation on mineral utilization and serum enzyme activities in broiler chicks fed different levels of phosphorus. Poult. Sci. 81:1172 -1183.

CAPÍTULO 3 - PHYTASE AFFECTS THE PERFORMANCE, NUTRIENTS DIGESTIBILITY, MINERALS OF TIBIA AND DIET COST

ABSTRACT - An experiment was conducted to evaluate the response of the inclusion of phytase (Phy) on the performance, digestibility of nutrients, minerals of tibia, bioavailability of phosphorus and diet cost. A total of 896 male broilers (Cobb 500) were divided into 32 experimental units, each unit consisting of 28 birds. The design was a completely randomized design with four treatments and eight replications. The treatments were: Positive Control (**PC**) - diet meeting the nutritional requirements of the birds; Negative Control (**NC**) - energy, Ca and $_{av}$P reduction; -100kcal/kg; -0,14% $_{av}$P and -0,11% Ca); NC + Phy (500FTU/kg); PC + Phy (500FTU/kg). The phytase addition increased ($p<0.05$) of 7.0 and 5.3% in FI, 8.9 and 8.8% in BWG and improved 2 and 5 points in the FCR in initial and growth phases, respectively compared to NC diet without supplementation. The NC + Phy also presented a FI similar to PC in initial and growth phases. The inclusion "on top" of phytase (Phy + PC) improved 2 points ($p <0.05$) in feed conversion at the initial phase, compared to PC diet. There was an increase in digestibility by 0.6%, 1.7%, 123 kcal/g, 112.2 kcal/g and 18.3% for dry matter (**DM**), crude protein (**CP**), apparent digestible energy (**ADE**) in DM, ADE in natural matter (**NM**), and phosphorus (**P**), respectively, in NC+Phy compared to NC diet. The phytase supplemented on top increased digestibility of CP (0.3%), ADE in NM (70 cal/g), Calcium (**Ca**) (7.8%) and P (12.5%), compared to PC diet. Ash, P and Ca were higher in the NC + Phy compared those of the NC diet without enzyme. The PC + PHY showed higher ash, but the deposition of Ca and P were similar in relation to the PC. Considering the costs of dicalcium phosphate (U$0.50/kg) and phytase (U$6.00/kg), it was possible to save US$ 3.72/ton of produced feed.

Key words: phytase, bioavailability, phosphorus, economic, digestibility

1. INTRODUCTION

The high cost of inorganic phosphate has influenced the direction of research and the industry to obtain other sources of phosphorus or improve the availability of phosphorus to animals on diets. The enzyme phytase has been highlighted in nutrition of non-ruminants as a tool to reduce the inclusion of inorganic phosphorus in the diet and also decrease nutrient excretion through the excrement of animals (Waldroup et al., 2000; Granã et al., 2013; Ghada et al., 2013). With the growth and narrowing of an increasingly demanding market about the sustainable issue, there is a pressure on poultry production in the world resulting in an increase in the importance of phytase. Phytase market moves billions of dollars per year (Adeola and Cowieson, 2011), generating many jobs directly or indirectly.

Corn and soybean meal have a large amount of phosphorus (P) contained in the inositol hexaphosphate complex molecule, which is a molecule formed ionizable phosphate (Lehninger, 1994) with capacity to retain cations, carbohydrates, amino acids and some digestive enzymes (Sohail et al., 1999; Ravindran et al., 1999; Sebastian et al., 1998). Because birds do not produce endogenous phytase to break this structure and release the P and other nutrients contained in the molecule, the association between phytase and cereal generates a great potential in releasing these nutrients.

Although phytase has been studied, new enzymes have been produced and different responses has been obtained. In this sense, the present study aimed to evaluate the inclusion of a phytase produced by *E. coli* in broiler diets based on corn and soybean meal, with or without nutrient reductions on performance, nutrient digestibility, phosphorus bioavailability, bone minerals and its economic viability.

2. MATERIALS AND METHODS

2.1. Birds and Housing

The trial was conducted at the Laboratory of Poultry Sciences of Department of Animal Science, Faculty of Agriculture and Veterinary Sciences - UNESP, Jaboticabal, Brazil.

All experimental procedures were approved by the Ethics Committee on Animal Use (CEUA - 018760/14).

The birds were housed in an experimental facility, with tunnel type system to control the environment. The control of temperature, humidity and air exchange were performed automatically by exhaust fans and climate control system, according to the age of the birds (Cobb Guidelines, 2006). During the starter phase, incandescent lamps were used as heating source according to the needs of poultry. Shaving litter was used in all experimental plots, as well as nipple drinkers and tubular feeders. Infant tubular feeders were used until 14 days of age, then substituted by adult tubular feeders.

Water and feed were offered ad libtum during the whole trial period. The lighting program was set at 24 hours of light.

All birds were vaccinated with the challenge of the region: at the hatchery against Marek and Gumboro diseases, at 12 days of age the birds were vaccinated against New Castle, via water.

2.2. Experimental design

A total of 896 Cobb 500 strain male day-old broiler chicks were weighed (46 ± 1g each) and distributed in each treatment with similar body weight means. The birds were distributed in a completely randomized design with four treatments and eight replicates, allocated to 32 pens (1.0 x 3.0 m) of 28 chicks each.

The experimental treatments were: Positive Control (PC) - diet meeting the nutritional requirements of the birds; Negative Control (NC) - reduction of 100kcal/kg ME; 0,14% $_{av}$P and 0,11% $_{t}$Ca; NC + Phytase (500FTU/kg); PC + Phytase (500FTU/kg).

2.3. Experimental diets

The Positive Control diets were formulated to meet the nutritional requirements of broilers based on the recommendations of the Brazilian Tables for Poultry and Swine (Rostagno et al., 2011). The Negative Control diets had reductions of energy, Ca and $_{av}$P. Table 1 describes the control diets and their nutritional compositions for starter (1-14 days), grower (15-28 days) and finisher (29-42 days) phases. The phytase were produced by bacteria of the genus *E. Coli* (Phytafeed GR 5000G), produced by Pintaluba Group (Reus, Spain).

Table 1. Composition of experimental and calculated nutritional values Positive Control (PC) and Negative Control (NC) diets

Ingredients	1 to 14 days		15 to 21 days		22 to 42 days	
	PC (%)	NC (%)	PC (%)	NC (%)	PC (%)	NC (%)
Corn	52.215	55.831	57.861	61.477	62.329	65.963
Soybean Meal 45%	41.293	40.587	34.489	33.783	30.957	30.230
Soy Oil	2.865	0.500	2.911	0.546	3.460	1.089
Limestone	0.807	1.018	0.848	1.059	0.770	0.981
Dicalcium Phosphate 18,5	1.707	0.942	1.563	0.798	1.223	0.458
Salt	0.431	0.429	0.426	0.425	0.399	0.397
DL-Methionine 99%	0.289	0.285	0.308	0.304	0.260	0.256
L-Lysine 98%	0.155	0.169	0.293	0.307	0.377	0.399
L-Threonine 98%	0.038	0.040	0.100	0.102	0.064	0.066
Min Vit Premix [1]	0.200	0.200	0.200	0.200	0.160	0.160
Indicator[2]	-	-	1.000	1.000	-	-
TOTAL	100	100	100	100	100	100
Nutritional levels (%)						
ME (Kcal/kg)	3000	2900	3050	2950	3170	3070
CP	23.63	23.63	21.20	21.20	20.00	20.00
Ca	0.881	0.771	0.841	0.731	0.717	0.607
$_{av}$P	0.436	0.296	0.401	0.261	0.335	0.195
Phytic phosphorus	0.240	0.244	0.227	0.232	0.224	0.228
Na	0.215	0.215	0.210	0.210	0.198	0.198
K	0.916	0.914	0.808	0.806	0.757	0.754
Met+Cys (dig)	0.915	0.915	0.876	0.876	0.804	0.804
Met (dig)	0.611	0.609	0.598	0.595	0.536	0.534
Lys (dig)	1.271	1.271	1.217	1.217	1.101	1.101
Thr (dig)	0.826	0.826	0.791	0.791	0.715	0.715
Trp (dig)	0.271	0.269	0.234	0.231	0.215	0.213

[1] Folic acid 437.5mg; Pantotenic Acid 6250mg; Biotin 44mg. Cloridrohiquinolina Hidroxio de Tolueno Butilado 100mg; Niacin 18750mg; Selenium 187.5mg; Vit A 4687500UI. Vit B1 1250mg; Vit B12 7500MGC; Vit B2 3125mg; Vit B6 1750mg; Vit D3 1187500UI; Vit E 17500UI; Vit K3 940mg; Mn 44g; Zn 40.65g; Fe 31.25g; Cu 6250mg; I 625mg

[2] Indicator a source of silica - Celite

Diets with phytase inclusion were included 100g/ton (500FTU/kg)

2.4. Performance parameters

At 21 and 42 days old, birds and feed leftovers were weighed. Performance parameters evaluated were: feed intake (**FI**), body weight gain (**BWG**), feed conversion rate (**FCR**) and viability (**VIA**). Mortality was recorded daily and was used to adjust the total number of chicks to determine feed intake and feed conversion ratio.

2.5. Digestibility of Nutrients

The digestibility of nutrients was determined by the ileal digestibility using as indicator a source of silica, Celite, added to all experimental diets, at 14 days of age at 1%.

At 21 days of age 10 birds from each experimental unit were randomly chosen and slaughtered by electronarcosis followed by exsanguination. Immediately after slaughter, the ileum was exposed by abdominal incision, and a segment of 20 cm, which ends at 4 cm from the ileo cecal junction was removed and its contents collected in a plastic container identified by treatment and repetition. The broilers were stimulated to consume feed four hours before slaughter to collect ileum intestinal contents.

After collection, the digesta were frozen for later analysis. Thawing was accomplished at room temperature, and the samples were homogenized, weighed again, frozen and submitted to lyophilization at a temperature of -80°C for 72 hours. After that, the samples were ground in micromill A11 basic (IKA) and, along with samples of the experimental diets were sent to the laboratory for analysis.

Were determined dry matter, gross energy, nitrogen, phosphorus, calcium and acid insoluble ash (**AIA**) of the samples of digesta and diets. Nitrogen was analyzed by Kjeldahl method, dry matter (final drying), calcium and phosphorus were determined by nitro perchloric digestion (Silva and Queiroz, 2009). For the determination of gross energy, the samples were pelleted and subjected to combustion in calorimetric bomb. For the calculations of digestibility, AIA, the indigestible fraction present in diets and digesta was determined.

Were determined the digestibility of dry matter (**DM**), crude protein (**CP**), digestible energy (**ADE**), calcium (**Ca**) and phosphorus (**P**), calculated based on the analysis of diets and ileal digesta, according to Sakomura and Rostagno (2007).

2.6. Analysis of tibias

At 21 days of age, two birds from each experimental unit were sampled and had their tibias removed, placed in labeled plastic bags according to the experimental pen and frozen. Once thawed, the tibias were stripped, identified and dried in a ventilated oven at 100ºC for 24 hours. The excess of fat was removed by immersion of the tibias in ethyl ether, until the total disappearance of fat. They were dried in ventilated oven again at 100°C for 24 hours. After that the tibias were individually weighed on a precision scale and then were ground in ball mill to determine ash, Ca and P (Silva and Queiroz, 2009).

2.7. Phosphorus Bioavailability

Phosphorus bioavailability was calculated using standard curve concept. To establish the standard curve, the intake of supplementary phosphorus (ISP) of treatments PC and NC were calculated, according to formula:

$$\mathrm{ISP}(\mathrm{g}) = \frac{(\text{feed intake}(\mathrm{g}) \times \text{suplementary dietary P}(\%))}{100}$$

Supplementary dietary P was considered null in the most deficient treatment (NC) and 0.14% for PC; being these values the difference of $_{av}$P between NC and the other treatments.

The ISP was associated to the responses of performance (BWG) and bone (ash and phosphorus) variables to construct the standard curve.

The results of performance and bone characteristics variables of the treatments with phytase addition were confronted with their respective standard curve, in order to obtain the total phosphorus released (**PR**) in grams. The formula below demonstrates the calculation to obtain the nutritional matrix (bioavailability) of $_{av}P$ of the phytase enzyme, being considered the feed intake of the bird and the phosphorus released.

$$BP(\%) = \frac{PR(g)}{FI(g)} \times 100$$

2. 8. Cost analysis

The cost analysis of the diets was performed to determine the economy of including phytases at different levels. For these calculations it were used data of phosphorus bioavailability of BWG and the dicalcium phosphate cost.

The calculation of the amount of inorganic dicalcium phosphate which could be replaced by phytase inclusion (100g/ton; 500 FTU) in the diet, based on the bioavailability found for this phytase inclusion, the linear equations were obtained, taking into account the cost of the enzyme and the phosphate, as well as their inclusion to obtain a better economic profit.

The cost of dicalcium phosphate and enzyme (Phytafeed GR 5000G) were recorded on January 2015, with a value of US$ 0.50/kg, U$$6.00/kg, respectively.

2.9. Statistical analysis

Data were analyzed by general linear models procedures of SAS (SAS Version 9.00). The errors in the statistical models were normally and independently distributed with constant variance. The viability variable were Adjusted by ASEN(SQUARE ROOT (%VIABILITY/100). The means were tested by Student Newman Keuls - SNK multiple-range test with 5% of significance.

3. RESULTS

3.1. Performance

To verify the efficiency of phytase used in diets with nutritional reduction (NC) on the performance of broilers, it must prove that nutritional reduction affect bird performance. Subsequently, it should be evaluated the phytase included in this diet in order to verify the response in relation of birds to the deficient diet and /or diet that meets the requirement of the bird.

The effect of phytase supplementation in diets with and without nutrient reduction on feed intake, body weight gain, feed conversion and viability of broilers during initial (1-21) and grower (22-42 days) phases were showed in Table 2.

Table 2. Effect of phytase supplementation on broilers performance

Treatment	Feed Intake (g)	Body Weight Gain (g)	Feed Conversion	Viability (%)[1]
	Initial (1 to 21 days)			
CP	1224 **A**	959 **A**	1.276 **B**	99.78
NC	1119 **B**	841 **C**	1.332 **D**	99.55
NC + PHY	1197 **A**	916 **B**	1.307 **C**	99.78
PC +PHY	1222 **A**	974 **A**	1.255 A	99.78
SEM [2]	20	19	0.017	0.05
p-value	<0.0001	<0.0001	<0.0001	0.8871
	Grower (1 to 42 days)			
CP	4954 **A**	3156 **A**	1.570 **A**	99.31
NC	4675 **B**	2845 **B**	1.650 **C**	99.26
NC + PHY	4942 **A**	3083 **A**	1.603 **B**	97.92
PC +PHY	4912 **A**	3158 **A**	1.555 **A**	100.00
SEM [2]	104	68	0.011	0.09
p-value	0.0001	<0.0001	<0.0001	0.2288

[1] Viability - Adjusted by ASEN(SQUARE ROOT (%mortality/100)).
[2] SEM = Standard error of the means for treatments effects (n = 8).
Means with no common letter in row differ significantly (P≤0.05) by SNK test.

There was significant effects ($p<0.05$) between the PC and NC diets for all performance variables, except for viability, in both phases. The reduction of energy, Ca and P studied, decreased feed intake at 8.6 and 5.6%, weight gain in 12.3 and 9.9% and increased feed conversion in 5 and 8 points in the initial and total phase, respectively, in comparison to positive control.

The inclusion of phytase in diet with nutritional reduction increased (p<0.05) FI, BWG and decreased FCR in relation to NC diet in all phases. Phytase addition increased of 7.0 and 5.7% in FI, 8.9 and 8.4% in BWG and improved 2 and 5 points in the FCR in initial and total phases, respectively, compared to NC diet without enzyme supplementation. The NC + Phy also presented a FI similar to PC in initial and total phase, and the BWG also did not differ in total phase.

The inclusion "on top" of phytase (PC + PHY) significantly affected (p <0.05) feed conversion at the initial phase. There was an improvement in feed conversion of 2 points compared to PC diet.

3.2. Digestibility

Evaluation of digestibility is important to assess the efficiency of phytase on the availability of nutrients. Table 3 shows the effect of phytase supplementation in diets containing adequate levels of nutrients and diets with reduction of ME, Ca and P on the ileal digestibility, dry matter, crude protein, apparent digestible energy in natural and dry matter, calcium and phosphorus.

Table 3. Effect of phytase supplementation on broilers diets on nutrients digestibility at 21 days.

Treatments	DM (%)	CP (%)	ADE (cal/g DM)	ADE (cal/g NM)	Ca (%)	P (%)
CP	95.55 ^{AB}	82.50 ^{B}	3376.5 ^{A}	3023.7 ^{B}	54.82 ^{C}	60.97 ^{B}
NC	95.32 ^{B}	82.59 ^{B}	3263.8 ^{B}	2900.5 ^{C}	64.33 ^{A}	57.75 ^{C}
NC + PHY	95.90 ^{A}	83.99 ^{A}	3387.6 ^{A}	3011.7 ^{B}	64.76 ^{A}	68.34 ^{A}
PC +PHY	95.80 ^{A}	84.60 ^{A}	3450.9 ^{A}	3093.9 ^{A}	59.11 ^{B}	68.58 ^{A}
SEM [1]	0.54	1.14	59.25	52.85	3.14	2.16
Probability	0.014	0.0003	<0.0001	<0.0001	<0.0001	<0.0001

[1] SEM = Standard error of the means for treatments effects (n = 8)
Means with no common letter in row differ significantly (P≤0.05) by SNK test.

It was observed significant effects of phytase addiction on all variables evaluated (p<0.05). The ADE and P of NC were lower compared to PC, however, the digestibility of Ca was higher. There was a reduction of 113 and 123 cal/g in the

digestibility of energy in DM and NM, 5.3% in the coefficient of P digestibility and an increase of 17.3% in the coefficient of Ca digestibility in the NC compared to PC.

The inclusion of phytase in the NC significantly increased all variables except the digestibility of Ca. There was an increase in digestibility by 0.6%, 1.7%, 123 kcal/g, 112.2 kcal/g and 18.3% for DM, CP, ADE in DM, ADE in NM and P, respectively, compared to NC diet. The NC + PHY also increased the digestibility of CP, Ca and P compared to PC diet.

The phytase supplemented on top increased digestibility of CP (0.3%), ADE in NM (70 cal/g), Ca (7.8%) and P (12.5%), compared to PC diet.

3.3. Minerals Deposition in Tibias

Evaluation efficacy of phytase on mineral deposition in the tibia is important because the bone tissue has the largest amount of these nutrients, and can be used to determine the efficiency of utilization for deposition.

Table 4 shows the results of Ash, Phosphorus and Calcium in the tibias of broilers at 21 days of age, according to dietary treatments.

Table 4. Effect of enzymes supplementation on broilers tibias at 21 days.

Treatment	Ash (%)	Phosphorus (mg/g of bone)	Calcium (mg/g of bone)
CP	38.35 **B**	75.93 **A**	137.25 **A**
NC	35.21 **C**	67.64 **B**	129.83 **B**
NC + PHY	38.71 **B**	74.20 **A**	138.10 **A**
PC +PHY	40.72 **A**	77.16 **A**	142.68 **A**
SEM [1]	1.44	2.89	6.16
p-value	<0.0001	<0.0001	0.0059

[1] SEM = Standard error of the means for treatments effects (n = 8).
Means with no common letter in row differ significantly (P≤0.05) by SNK test.

Dietary treatments affected all variables evaluated (p<0,05). The reduction of ME, Ca and P on the NC diets was sufficient to ensure a low deposition of ash, Ca and P in the tibia of the birds.

Ash, P and Ca were higher in the NC + Phy when compared to those of the NC diet without enzyme, but they were similar to those of PC.

The PC + PHY showed higher ash, but the deposition of Ca and P were similar in relation to the PC.

3.4. Bioavailability of Phosphorus

The phosphorus bioavailability by adding phytase in broiler diets it is important in diet formulation because of economic and environmental benefits.

To calculate phosphorus bioavailability, the performance data (BWG and FCR) and tibia minerals (Ash and P) were used.

Linear response was obtained, considering BWG, with two increasing levels of supplemental P intake (PC = 1.71g and NC = 0). The regression obtained was: $BWG = 840.58(\pm\ 7.84) + 5.99\ (0.93)x$, where x is the quantity in grams of phosphorus, which was released by the enzyme for BWG.

Considering FCR, the linear regression adjusted with two increasing levels of supplemental P intake (PC = 1.71g and NC = 0), was: $FCR = 1.33(\pm\ 0.01) - 0.03(0.001)x$, where x is the quantity in grams of phosphorus, which was released by the enzyme for FCR.

For bone ash, a linear regression was fitted with two increasing levels of supplemental P intake (PC = 1.71g and NC = 0). $Ash = 35.59(\pm\ 0.65) + 1.63(0.50)x$, where x is the quantity in grams of phosphorus, which was released by the enzyme for ash in tibias to 21 days.

Linear response was also obtained considering the P content in tibia with two increasing levels of supplemental P intake (PC = 1.71g and NC = 0). $P = 68.69(\pm\ 1.35) + 3.98(1.03)x$, where x is the amount in grams of phosphorus released by the enzyme for phosphorus in tibias to 21 days.

Phosphorus bioavailability data obtained from BWG, FCR, ash and P are presented in Table 5.

Table 5. Means and standard error of total phosphorus released by phytase (PR), phosphorus bioavailability (PB), coefficient of variation (CV) and coefficient of determination (R^2) of broilers submitted to phytase at 500FTU concentration

Variables	PR (g)	PB (%)	CV (%)	R^2	Probability
BWG (g)	1.078 ± 0.261	0.090 ± 0.020	1.93	0.93	< 0.0001
FCR (g/g)	0.769 ± 0.387	0.070 ± 0.031	1.42	0.70	0.0007
Ash (%)	1.913 ± 1.080	0.160 ± 0.090	3.91	0.52	0.0082
P (mg/g)	1.384 ± 0.755	0.116 ± 0.063	4.14	0.60	0.0031

A linear response obtained with the two increasing levels of supplemental P intake in diets was used to get the standard line and to calculate bioavailability of P (in grams and percentage of phosphorus intake) as shown in Table 5.

The linear regression based on BWG and P had higher coefficients of determination (R^2=0.93 and 0.60) and were used to calculate the P bioavailability.

3.5. Cost analysis

One of the advantages use of phytase in the broiler chickens diet is the economic aspect. Thus, an economic evaluation was performed in order to quantify how much is possible to save by reducing dicalcium phosphate from the diet and supplementing phytase, based on the results of bioavailability of phosphorus.

The variables BWG and tibia P showed consistent values (with higher R^2) for performance and bone parameters, respectively.

The equation that describes the savings obtained by adding phytase in the diet, as a function of the cost of dicalcium phosphate using P bioavailability and BWG variable is: $Y = (4.865 \times A) - (I \times B)$, where Y is the economic response in US$/ton; A is the cost per kilogram of dicalcium phosphate; I is the inclusion of the enzyme in kilogram per ton and B is the cost of kilograms of Phytase. Considering the costs of the sources of inorganic phosphorus and phytase and the inclusion (A= U$ 0.50; B= U$ 6.00 and I = 0.1 kg/ton (500FTU)), it was possible to save US$ 1.83 per ton of produced feed.

Taking into account the bioavailability calculated for variable P in the tibia, we have the following equation: $Y = (6.270 \times A) - (I \times B)$. Considering the costs of the

sources of inorganic phosphorus, phytase and the inclusion (A= U$ 0.50; B= U$ 6.00 and I = 0.1 kg/ton (500FTU)), it was possible to save US$ 3.72 per ton of feed produced.

4. DISCUSSION

Nutritional reduction (ME, Ca and P) in the NC diet was significant for reducing bird performance. Thus, it was possible verify the responses to the use of phytase on broiler performance. Birds fed deficient diet, decreased feed intake and had lower weight gain. The energy reduction is usually compensated by an increased intake (Leeson et al., 1996). However, the reduction of phosphorus acts conversely, reducing the feed intake of birds (Viveiros et al., 2002). When the birds fed P-deficient diets, there is a decrease in serum of T3, T4 and GH hormones (Parmer et al., 1987) and, according to the theory of Emmans (1981), the bird eats because of its growth. Therefore, low levels of GH (growth hormone) in P-deficient diets may have a direct relationship to the development of the bird, since a bird that presents lower levels of this hormone will have its reduced weight gain, explaining therefore the lower feed intake.

A nutritional reduction in the NC diet also increased feed conversion compared to PC diets. In this case, it is clear that the animal, in addition to the reduced growth, became less efficient to use diet. With the addition of the enzyme, there was a significant improvement in feed conversion, recovering some of the lost performance. Therefore, the phytase was effective in releasing nutrients and improving performance. Sebastian et al. (1996); Ahmad et al. (2000), and Rutherfurd et al. (2012) showed a positive effect for weight gain and feed consumption with the inclusion of phytase in the diets with phosphorus reduction (0.3%, 0.35 and 0.35% avP, respectively), however the inclusion of phytase had no effect on feed conversion. Ahmad et al. (2000) attributed the better performance of broilers subjected to inclusion of phytase in diets due to increased feed intake. In the current study, the improvement of performance of birds fed phytase inclusion in diets can be explained by the higher feed intake and also for better digestibility.

The digestibility of P was 18.34% higher with the addition of phytase increasing bioavailability of P, as demonstrated in Table 5. Thus, the enzyme was effective in the breakdown of phosphorus-phytic acid complex, releasing minerals and making available for absorption, increasing feed intake (Santos et al., 2008). The increase in feed intake of NC + Phy treatment resulted in greater weight gain of the birds, an improvement of 8.84% for BWG compared to NC, from 1 to 42 days. Other studies have also demonstrated a greater weight gain due to increased feed intake with the addition of phytase to the diet (Cowieson et al., 2011; LIU et al., 2014.).

The phytase supplemented "on top" was able to act on the phytate molecule releasing the nutrients and improved digestibility of crude protein, Ca and P compared to the PC. Thus, the PC + Phy showed better FCR without affecting BWG compared to PC. According to Walk et al. (2014), improvements in feed conversion ratio may be attributed to breakdown of phytate and inositol utilization with phytase.

Other studies have also found that the inclusion of phytase in broilers diets improve ileal digestibility of phosphorus, amino acids, protein and energy (Rutherfurd et al., 2003; Santos et al., 2008). Studies of O'Connor-Dennie and Emmert (2012), corroborate with our results, they also observed an improvement in feed conversion when adding phytase to P-deficient diets. However, Ahmad et al. (2000) showed a greater weight gain but did not observe effect on feed conversion of birds with "on top" supplemental phytase.

According to Watson et al. (2006), phytase is more effective in the P-deficient diets rather than P-sufficient diets. In this study, it was also confirmed this statement. In diets with nutrient reductions of ME, Ca and P the phytase promoted grater benefits compared to the addition in diet formulated according to requirement. However, the inclusion of phytase "on top" also proved to be efficient.

It is important to consider that regardless of how the phytase is being used, the improvement of digestibility and bioavailability of nutrients leads to less excretion to the environment, reducing soil and water contamination.

The addition of phytase in the PC diet improved the digestibility of Ca, however, digestibility of was higher when phytase was supplemented in NC diet. As the calculation digestibility takes into account what has been consumed on which was not absorbed, by reducing the nutrient, its efficiency of utilization may have been higher.

Another hypothesis, according Kort et al. (2009), the formation of insoluble complexes between Ca and phosphate can be reduced by decreasing the concentration of Ca, improving the digestibility of both minerals. Ravindran et al. (2008) observed an improvement in the digestibility of Ca and P in diets supplemented with phytase. In the current study was also observed that phytase improved the digestibility of these minerals when supplemented "on top". With the inclusion in NC, we observed improvements in the digestibility of P, but not for Ca. Sebastian et al. (1996) and Powell et al. (2011) also reported no increase in the digestibility of Ca. It is known that one of the factors that influence the bioavailability of minerals is the interactions that occur between them (Bremner and Beattie, 1995; Cozzolino, 2007). Some authors have reported an increase in the apparent ileal digestibility of P by reducing the Ca:P ratio in the broilers diets or just reducing the concentrations of Ca (Plumstead et al., 2008; Walk et al., 2012; Adeola and Walk, 2013). The significant increase in P digestibility with the use of phytase can be explained by the breakdown of the structure of phytate, releasing molecules of phosphorus. Although there are other ions associated with the phytate molecules, such as Ca, Mg, Fe and Cu, the proportion is inferior to the phosphorus molecules, which in turn decreases the Ca:P ratio. Manangi and Coon (2008) observed that broilers have a better ability to utilize non-phytic P using a low Ca:P ratio, due to an increase of endogenous phytase (Applegate et al., 2003) or by reduction in the activity of dicalcium phosphate precipitation in the small intestine (Walk et al., 2012b).

Evidence of increased availability and digestibility of minerals in this study were demonstrated by ash, calcium and phosphorus content in the tibias of broilers. According to Sakomura and Rostagno (2007), bone deposition should be the main item of study on the efficacy of phytase, since the highest concentration of minerals are present in the bones of the animal. If the tibia is mineralized, this indicates that the mineral was available and used by the animal. The mineral content, Ca and P in the tibia are in agreement with animal performance and diet digestibility observed in our study.

The lowest levels of minerals found in the tibia of the broilers fed NC diets can be explained by the deficiency of phosphorus, which reduces the rate of bone matrix formation and delay the onset of mineralization (Baylink et al., 1971), and also can

increase the levels of bone reabsorption (Bruin et al., 1975). Parmer, et al. (1987) evaluated low levels of P in broiler diets on bone parameters and found that phosphorus deficient diet significantly reduced the amount of bone ash, as well as the length and width of the bone.

The inclusion of phytase in the NC improved the concentration of ash, calcium and phosphorus in bones, being similar to those of PC diet. Ahmad et al. (2000) also found similar values of ash, Ca and P in the tibias of broilers in treatments with reduced Ca and P, using phytase compared with treatments without nutrient reduction. Rutherfurd et al. (2012) observed an increase of tibia ash content of broiler with P reduced diets and using phytase. O'Connor-Dennie and Emmert (2012), using phytase (500FTU) derived from *E. coli* found an increase in tibia ash determined and P release of at least 0.083%.

With "on top" inclusion, Ahmad et al. (2000) observed an increase in the content of all minerals compared to the control diet, which was not observed in the current study, where there were higher levels of ash only in relation to the PC. However, according to Jiang et al. (2013), the mineral content in the tibia is closely related to the concentration of P available to the animal. Thus, the phytase released the phosphorus present in phytate molecule.

In addition, phytase also improved P bioavailability. Based on the performance and bone variables evaluated, BWG and tibia P best represented the bioavailability. These were used to calculate the economic viability. Although the bioavailability of P calculated from bone ash is the most widely used, the variable P in bone showed a better fit. Despite the ashes have not been used for calculations of economic viability in this study, this parameter is correlated with the amount of phosphorus in the bones. Pillai et al. (2006) found an R^2 of 0.72 and a bioavailability of 0.16% for phosphorus calculated based on tibia ash of broilers using phytase derived from *E. coli*, in our study we found the same results.

Based on bioavailability of 0.090% for BWG and 0.116% for tibia P, it was determined the minimum cost of dicalcium phosphate (kg) to save using phytase. If the price of dicalcium phosphate was more than US$0.12 and US$0.10/kg, according to BWG and tibia P, respectively, becomes economic viable the phytase supplementation.

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5. REFERENCES

Adeola, O., and A. J. Cowieson. 2011. Board-Invited Review: Opportunities and challenges in using exogenous enzymes to improve non-ruminant animal production. J Anim. Sci. 89:3189-3218.

Adeola, O., and C. L. Walk. 2013. Linking ileal digestible phosphorus and bone mineralization in broiler chickens fed diets supplemented with phytase and highly soluble calcium. Poult. Sci. 92:2109–2117.

Ahmad, T., S. Rasool, M. Sarwar, A. Haq, Z. Hasan. 2000. Effect of microbial phytase produced from a fungus *Aspergillus niger* on bioavailability of phosphorus and calcium in broiler chickens. Anim. Feed Sci. Tech. 83:103±114.

Applegate, T. J., R. Angel, H. L. Classen. 2003. Effect of dietary calcium, 25-hydroxycholecalciferol, or bird strain on small intestinal phytase activity in broiler chickens. Poult. Sci. 82:1140–1148.

Baylink, D., J. Wergedal, M. Stauffer. 1971. Formation, mineralization and resorption of bone in hypophosphatemic rats. J. Clin. Invest. 50:2519- 2530.

Bremner, I., and J. H. Beattie. 1995. Copper and zinc metabolism in health and disease: speciation and interactions. Proc. Nutr. Soc. 54:489-99.

Bruin, W. J., D. J. Baylink, J. E. Wergedal. 1975. Acute inhibition of mineralization and stimulation of bone resorption mediated by hypophosphatemia. Endocrinology 96:394-399.

COBB. Broiler Management Guide. COBB 500. S.l.: s.n., 2008, 65p.

Cowieson, A. J., P. Wilcock, M. R. Bedford. 2011. Super-dosing effects of phytase in poultry and other monogastrics. World Poultry Sci. J. 67:225-236.

Cozzolino, S. M. F. 2007. Biodisponibilidade de nutrientes. SP: Manole, 67-175.

De Kort, E., M. Minor, T. Snoeren, T. Van Hooijdonk, E. Van Der Linden. 2009. Calcium-binding capacity of organic and inorganic ortho and polyphosphates. Dairy Sci. Technol. 89:283–299.

Emmans, G. C. 1981. Animal Growth and feed intake. A collection of papers. SAS, Edinburgh p1981-1990.

Ghada, A. A., N. D. Enas, S. K. Seham, M. A. K. Madeha, M. H. Emtenan, H. Zeinab, M. H. Eman, M. I. Helal. 2013. A novel phytase enzyme for poultry feed. World Appl. Sci. J. 26:194-199.

Graña, A., F. C. Tavernari, G. R. Lelis, L. F. T. Albino, H. S. Rostagno, P. C. Gomes. 2013. Evaluation of nutrient excretion and retention in broilers submitted to different nutritional strategies. Braz. J. Poult. Sci. 15:161-168.

Jiang, X., F. Luo, M. Qu, V. Bontempo, S. Wu, H. Zhang, H. Yue, G. Qi. 2013. Effect of non-phytate phosphorus levels and phytase sources on the growth performance, serum biochemical and tibial parameters of broiler chickens. Ital. J. Anim. Sci. 12:375-380.

Leeson, S., L. Caston, J. D. Summers. 1996. Broiler response to diet energy. Poult. Sci. 75:529-535.

Lehninger, A. L. 1994. Principios de Bioquímica. Sarvie, São Paulo, Brasil.

Liu, S. Y., D. J. Cadogan, A. Péron, H. H. Truong, P. H. Selle. 2014. Effects of phytase supplementation on growth performance, nutrient utilization and digestive dynamics of starch and protein in broiler chickens offered maize, sorghum and wheat based diets. Anim. Feed Sci. Tech. 197:164–175.

Manangi, M. K., and C. N. Coon. 2008. Phytate phosphorus hydrolysis in broilers in response to dietary phytase, calcium, and phosphorus concentrations. Poult. Sci. 87:1577–1586.

O'Connor-Dennie, T., and J. Emmert. 2012. The effect of phytase source, wheat middlings, and citric acid on phosphorus utilization, growth performance, and carcass yield in broilers. Jpn. J. Trop. Agr. 89:240.

Parmer, T. G., L. B. Carew, F. A. Alster. 1987. Thyroid Function, Growth Hormone, and Organ Growth in Broilers Deficient in Phosphorus. Poult. Sci. 66:1995- 2004.

Pillai, P. B., T. O'connor-Dennie, C. M. Owens, J. L. Emmert. 2006. Efficacy of an *Escherichia coli* phytase in broilers fed adequate or reduced phosphorus diets and its effect on carcass characteristics. Poult. Sci. 85:1737–1745.

Plumstead P. W., A. B. Leytem, R. O. Maguire, J. W. Spears, P. Kwanyuen, J. Brake. 2008. Interaction of calcium and phytate in broiler diets: Effects on apparent pre cecal digestibility and retention of phosphorus. Poult. Sci. 87:449–458.

Powell, S., T. D. Bidner, L. L. Southern. 2011. Phytase supplementation improved growth performance and bone characteristics in broilers fed varying levels of dietary calcium. Poult. Sci. 90:604–608.

Ravindran, V., S. Cabahug, G. Ravindran, W. Bryden. 1999. Influence of microbial phytase on apparent ileal amino acid digestibility of feeds tuffs for broilers. Poult. Sci. 78:699 -706.

Ravindran, V., A. J. Cowieson, P. H. Selle. 2008. Influence of dietary electrolyte balance and microbial phytase on growth performance, nutrient utilization, and excreta quality of broiler chickens. Poult. Sci. 87:677–688.

Rostagno, H. S., L. F. T. Albino, J. L. Donzele, P. C. Gomes, R. F. M. Oliveira, D. C. Lopes, A. S. Ferreira, S. L. T. Barreto, R. F. Euclides. 2011. Tabelas brasileiras para aves e suínos: composição de alimentos e exigências nutricionais. 3a ed. Universidade Federal de Viçosa, Viçosa, Minas Gerais - Brazil.

Rutherfurd, S. M., T. K. Chung, P. C. H. Morel, P. J. Moughan. 2003. Effect of microbial phytase on ileal digestibility of phytate phosphorus, total phosphorus, and amino acids in a low-phosphorus diet for broilers. Poult. Sci. 83:61–68

Rutherfurd, S. M., T. K. Chung, D. V. Thomas, M. L. Zou, P. J. Moughan. 2012. Effect of a novel phytase on growth performance, apparent metabolizable energy, and the availability of minerals and amino acids in a low-phosphorus corn-soybean meal diet for broilers. Poult. Sci. 91:1118–1127.

Sakomura, N. K., and H. S. Rostagno. 2007. Métodos de pesquisa em nutrição de monogástricos. Funep, Jaboticabal, São Paulo - Brasil.

Santos, F. R, M. Hruby, E. E. M. Pierson, J. C. Remus, N. K. Sakomura. 2008. Effect of phytase supplementation in diets on nutrient digestibility and performance in broiler chicks. J. Appl. Poult. Res. 17:191–201.

SAS. SAS user's guide: statistics, version 9.0. Cary: SAS Institute, 2002.

Sebastian, S., S. P. Touchburn, E. R. Chávez. 1998. Implications of phytic acid and supplemental microbial phytase in poultry nutrition: a review. World's Poult. Sci. 54:27-47.

Sebastian, S., S. P. Touchburn, E. R. Chavez, P. C. Lague. 1996. Efficacy of supplemental microbial phytase at different dietary calcium levels on growth performance and mineral utilization of broiler chicks. Poult. Sci. 75:1516–1523.

Silva, D. J., and A. C. Queiroz. 2009. de. Análise de alimentos: métodos químicos e biológicos 3. ed. Viçosa, MG: UFV, 235 p.

Sohail, S. S, and D. A. Roland. 1999. Influence of supplemental phytase on performance of broilers from four to six weeks of age. Poult. Sci. 78:550–558.

Viveiros, A., A. Brenes, I. Arija, C. Centeno. 2002. Effects of microbial phytase supplementation on mineral utilization and serum enzyme activities in broiler chicks fed different levels of phosphorus. Poult. Sci. 81:1172-1183.

Waldroup, P. W., J. H. Kersey, E. A. Saleh, C. A. Fritts, F. Yan, H. L. Stilborn, R. C. Jr Crum, V. Raboy. 2000. Nonphytate phosphorus requirement and phosphorus

excretion of broiler chicks fed diets composed of normal or high available phosphate corn with and without microbial phytase. Poult. Sci. 79:1451-1459.

Walk, C. L., E. K. Addo-Chidie, M. R. Bedford, O. Adeola. 2012a. Evaluation of a highly soluble calcium source and phytase in the diets of broiler chickens. Poult. Sci. 91:2255–2263.

Walk, C. L., M. R.Bedford, A. P. Mcelroy. 2012b. In vitro evaluation of limestone, dicalcium phosphate, and phytase on calcium and phosphorus solubility of corn and soybean meal. Poult. Sci. 91:674–682.

Walk, C. L., T. T. Santos, M. R. Bedford. 2014. Influence of superdoses of a novel microbial phytase on growth performance, tibia ash, and gizzard phytate and inositol in young broilers. Poult. Sci. 93:1172-1177.

Watson, B. C., J. O. Matthews, L. L. Southern, J. L. Shelton. 2006. The effects of phytase on growth performance and intestinal transit time of broilers fed nutritionally adequate diets and diets deficient in calcium and phosphorus. Poult. Sci. 85:493–497.

CAPÍTULO 4 - STUDY OF SUPPLEMENTAL PHYTASE DERIVED OF *Escherichia coli* IN DIFFERENT CONCENTRATIONS ON PERFORMANCE, BONE MINERALIZATION AND COST OF BRILERS DIETSOF BROILERS

ABSTRACT - The trial was conducted to evaluate the inclusion of *E. coli* phytase (expressed in *Pichia pastoris* genus) in small concentrations on performance, weight and ash of bones, as well as to determine the bioavailability of P and cost/benefit of its use in diets based on corn and soybean meal and deficient in phosphorus. A total 1,890 Cobb male day old chicks were assigned to six treatments and seven replicates with 45 birds each, distributed in a completely randomized design. The treatments were: Positive Control (PC) - diet meeting the nutritional requirements of the birds; Negative Control (NC1) - reduction of 0.06% $_{av}$P; Negative Control 2 (NC2) - reduction of 0.12% $_{av}$P; NC2 + Phytase (120 OTU); NC2 + Phytase (180 OTU); NC2 + Phytase (240 OTU), being 1 OTU equivalent to approximately 2 FTU. The standard curve methodology was applied to performance and bone variables to calculate the bioavailability of P. The results obtained with the inclusion of different concentrations of phytase were compared with the standard curve to calculate the bioavailability of phosphorus. Regressions were performed with performance, bones and bioavailability of phosphorus to determine the optimal level of each enzyme. With different phytase inclusions, it was possible to verify a gradual increase on body weight gain, feed intake, feed conversion, viability and even the bone characteristics of birds fed diets containing reduction of P. The closest levels to the highest studied (240 OTU or 500 FTU) showed the best results. The replacement of dicalcium phosphate by phytase supplementation is economically viable when the cost per OTU does not exceed US$ 1.4×10^{-5}, US$ 1.2×10^{-5} and US$ 1.0×10^{-5} for the concentrations of 120, 180 and 240 OTU, respectively.

Keywords: additives, enzyme, broiler, phytate, bone, litter

1. INTRODUCTION

With the shortage of natural sources of inorganic phosphorus and consequently the increase in the price of dicalcium phosphate, the use of phytase in broiler diets becomes a relevant measure for providing the phosphorus of plants to animals. Phytase acts on the phosphate group links the phytic acid molecule, releasing phosphorus and other minerals such as calcium, copper, iron and zinc, as well as energy and amino acids (Silva et al., 2006).

Diets based on corn and soybean meal present small amount of phytate in comparison to other ingredients such as canola and rice bran, for example (Eeckhout and De Paepe, 1994). Thus, it is neither necessary nor economically feasible to include high amounts of phytase to these diets. In addition to greater economic viability, smaller phytase concentrations have some advantages, such as improved retention of non-mineral nutrients as amino acids and energy, whereas high doses have a greater effect on phosphorus retention, but not on these nutrients (Cowieson et al., 2006; Karadas et al. 2010; Bedford et al., 2011; Walk et al. 2012).

Recent studies have found high inclusions of phytase on broilers diets for maximum response (Cowinson et al., 2006), however the production of concentrated enzyme is difficult to obtain (Nascimento, 2011) and the inclusion of large amounts becomes economically unviable. In this sense, it is necessary to evaluate the behaviour of gradual phytase inclusion levels up to 240 OTU (approximately 500 FTU), with the purpose to achieve greater economic gains in production, especially in diets based on corn and soybean meal.

This study aimed to evaluate gradual inclusion levels of *E. coli* phytase (*Pichia pastoris* genus) on performance, weight and bone ash, as well as to determine the bioavailability of P and cost/benefit of its usage in diets P deficient based on corn and soybean meal.

2. MATERIAL AND METHODS

2.1. Birds and Housing

The trial was conducted at the Laboratory of Poultry Sciences of Department of Animal Science, Faculty of Agriculture and Veterinary Sciences - UNESP, Jaboticabal, Brazil.

The birds were housed in an experimental facility, with tunnel type system to control the environment. The control of temperature, humidity and air exchange were performed automatically by exhaust fans and climate control system, according to the age of the birds (Cobb Guidelines, 2006). During the initial phase, incandescent lamps were used as heating source according to the needs of poultry. Shaving litter was used in all experimental pens, as well as nipple drinkers and tubular feeders. Infant tubular feeders were used until 14 days of age, then substituted by adult tubular feeders.

Water and feed were offered ad libtum during the whole trial period. The lighting program was set at 24 hours of light.

All birds were vaccinated with the challenge of the region: at the hatchery against Marek and Gumboro diseases, at 12 days of age the birds were vaccinated against New Castle, administered via water.

2.2. Experimental design

A total of 1,890 day-old male broiler chicks (Cobb 500) were weighed (± 45g each) and distributed in each treatment with similar body weight means. The birds were distributed in a completely randomized design with six treatments and seven replicates, allocated to 42 pens (1.0 x 3.0 m) of 45 chicks each.

The experimental treatments were: Positive Control (**PC**) - diet meeting the nutritional requirements of the birds; Negative Control (**NC1**) - reduction of 0.06% $_{av}$P; Negative Control 2 (**NC2**) - reduction of 0.12% $_{av}$P; NC2 + Phytase (120 FTU); NC2 + Phytase (180 FTU); NC2 + Phytase (240 FTU).

2.3. Experimental diets

Diets were formulated to meet the nutritional requirements of broilers based on the recommendations of the Brazilian Tables for Poultry and Swine (Rostagno et al., 2005), except to $_{av}$P. The Negative Control diets had reduction of $_{av}$P in comparison to PC. Table 1 describes the control diets and their nutritional compositions for initial (1-21 days) and grower (22-35 days) phases.

The phytase included was produced by *E. coli* (expressed in *Pichia pastoris* genus), commercial name OptiPhos 4.000 PF, produced by Ammco Pharma. OptiPhos phytase concentration is calculated by Phytex method (USA), and is expressed in OTU. In a simpler way, it is possible to consider that each OTU (Phytex method) is equivalent to 2 FTU (AOAC/ISO method).

Table1. Positive Control (PC), Negative Control 1 (NC1) and Negative Control 2 (NC2) experimental diets for the phases from 1 to 21 and 22 to 35 days of age of the birds.

Ingredients (%)	1 - 21 days			22 - 35 days		
	PC	NC1	NC2	PC	NC1	NC2
Corn	55.253	55.505	55.757	61.458	61.710	61.962
Soybean meal (45%)	37.142	37.096	37.051	30.904	30.859	30.813
Soy oil	3.669	3.583	3.498	4.064	3.979	3.893
Limestone	1.379	1.333	1.559	1.132	1.358	1.585
Dicalcium phosphate	1.106	1.030	0.682	1.131	0.783	0.434
Salt	0.508	0.508	0.507	0.475	0.475	0.475
DL - Methionine	0.287	0.287	0.287	0.226	0.226	0.226
L - Lysine HCl	0.243	0.244	0.245	0.215	0.216	0.217
L - Threonine	0.074	0.074	0.074	0.055	0.055	0.055
Vitamin premix[1]	0.100	0.100	0.100	0.090	0.090	0.090
Mineral premix[2]	0.100	0.100	0.100	0.100	0.100	0.100
Coccidiostat	0.050	0.050	0.050	0.050	0.050	0.050
Choline Chloride 60%	0.070	0.070	0.070	0.070	0.070	0.070
Inert [3]	0.020	0.020	0.020	0.030	0.030	0.030
TOTAL	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
Nutritional levels						
ME (Kcal/kg)	3050	3050	3050	3150	3150	3150
Crude protein	22.5	22.5	22.5	20.00	20.00	20.00
Lysine	1.253	1.253	1.253	1.085	1.085	1.085
Metionine+Cystine	0.879	0.879	0.879	0.770	0.770	0.770
Digestible metionine	0.588	0.588	0.588	0.501	0.501	0.501
Digestible treonine	0.805	0.805	0.805	0.705	0.705	0.705
Crude Fibre	2.965	2.967	2.969	2.735	2.737	2.739
Calcium	0.900	0.900	0.900	0.830	0.830	0.830
Available phosphorus	0.345	0.285	0.225	0.295	0.235	0.175
Total phosphorus	0.601	0.541	0.481	0.534	0.474	0.415
Phytic phosphorus	0.231	0.232	0.232	0.222	0.222	0.222
Potassium	0.834	0.834	0.834	0.737	0.737	0.737
Chlorine	0.349	0.349	0.349	0.329	0.329	0.329
Sodium	0.220	0.220	0.220	0.207	0.207	0.207

[1]7.000.000.000 U.I. vit A; 2.200.000.000 U.I. vit D3; 11.000.000 mg vit E; 1.600.000 mg vit K3; 2.000.000 mg vit B1; 5.000.000 mg vit B2; 3.000.000 mg vit B6; 12.000.000 mcg vit B12; 35.000.000 mg nicotinic acid; 13.000.000 mg pantothenic acid; 800.000 mg folic acid; 100.000.000 mg Antioxidant; 1.000.000 mg. Vehicle Q.S.P. Addition 1.0 kg/t.

[2]8.000.000 mg Cu; 50.000.000 mg Fe; 70.000.000 mg Mn; 50.000.00 mg Zn; 1.200.000 mg I; 200.000 mg Se; 1.000.000 g Vehicle Q.S.P. Addition 1.0 kg/t.

[3] Phytase replaced by inert ingrediente on NC2 treatments to obtain the diets with 120, 180 and 240 OTU/Kg, being each OTU (Phytex method) is equivalent to 2 FTU (AOAC/ISO method).

2.4. Performance

At 21 and 35 days old, birds and feed leftovers were weighed. Performance parameters evaluated were: body weight (**BW**), feed intake (**FI**), body weight gain (**BWG**), feed conversion rate (**FCR**) and viability (**VIA**). Mortality was recorded daily and was used to adjust the total number of chicks to determine feed intake and feed conversion ratio.

2.5. Analysis of tibias

At 35 days of age, five birds of each experimental unit were slaughtered and had their tibiae removed, placed in identified plastic bags according to the treatment and replication and frozen. Once thawed, the tibias were stripped and dried in ventilated oven at 100°C for 24 hours. Fat excess was removed by immersing the tibias in ethyl ether, up to the total disappearance of fat. They were dried again in a ventilated oven at 100°C for 24 hours. After the tibias were individually weighed on precision scale, it was determined the defatted dry weight (**DTW**). Then, they were milled in a ball mill, a sample of about 2g was weighed and forwarded to muffle furnace with temperature of 600°C for approximately 4h, until the sample became white, in order to determine ashes (Silva and Queiroz, 2009).

2.6. Phosphorus Bioavailability

Phosphorus bioavailability was calculated using standard curve concept. To establish the standard curve, the intake of supplementary phosphorus (ISP) of treatments PC, NC1 and NC2 were calculated, according to formula:

$$\mathrm{ISP}(\mathrm{g}) = \frac{(\text{feed intake}(\mathrm{g}) \times \text{suplementary dietary P}(\%))}{100}$$

Supplementary dietary P was considered null in the most deficient treatment (NC2); 0.06 for NC1; and 0.12% for PC; being these values the difference of $_{av}$P between NC2 and the other treatments.

The ISP was associated to the responses of performance (BWG) at 21 and 35 and bone characteristics (ash (**ASH**) and dried tibia weight (**DTW**)) variables to construct the standard curve.

The results of performance and bone characteristics variables of the treatments with phytase addition were confronted with their respective standard curve, in order to obtain the total phosphorus released (**PR**) in grams. The formula below demonstrates the calculation to obtain the nutritional matrix (bioavailability) of $_{av}$P of the phytase enzyme, being considered the feed intake of the bird and the phosphorus released.

$$BP(\%) = \frac{PR(g)}{FI(g)} \times 100$$

2.7. Cost analysis

The cost analysis of the diets was performed to determine the economy of including phytases at different levels. For these calculations it were used data of phosphorus bioavailability of BWG and the dicalcium phosphate cost.

The calculation of the amount of inorganic dicalcium phosphate which could be replaced by phytase inclusions in the diet, based on the bioavailability found for each evaluated phytase inclusion. Thus, it was established the maximum cost (limit) for each phytase FTU of each enzyme that does not exceed the cost of inorganic phosphate defined at the evaluation period.

After determining a threshold cost by OTU (or FTU) for each phytase at different inclusions was generated a figure (graph) containing both phytases, being Y axis correspondent to the found phosphorus bioavailability and the X axis to the concentrations of phytases in the diet. For each inclusion of phytase, there is an

associated bioavailability and consequently a limit cost of its utilization, expressed in dollars.

The price of dicalcium phosphate was recorded on January 2015, with a value of US$ 0.50/kg (MFRural, 2015).

2.8. Statistical analysis

Data were analyzed by general linear models procedures of SAS (SAS Version 9.00). Regression analyses were applied on performance, bone and bioavailable of phosphorus data to determine the best utilization level of phytases, using NC as the zero level of phytase inclusion, and as levels 120, 180 and 240 OTU for phytase, respectively. Linear (L), quadratic (Q) and quadratic-plateau (QP) models were used to analyse the different enzymes levels on the variables responses.

The QP model was adjusted according to Robbins et al. (2006), $Y_i = L + U\left(R - Phy_i\right)^2$, i = 1,2...n_1, n_1+1,...,n; in which $(R - Phy_i)^2 = 0$ for $i \geq n_1 + 1$, n_1 is the number of observations up to the breaking point, n is the number of observation pairs, estimated by the equation. The Y_i is the response of the estimated variable to the phytase level in the diet; Phyi is the level of phytase in the diet; L is asymptotic response of the function; U is slope at the breaking point; and R is the phytase level estimated by the breaking point.

3. RESULTS

3.1. Performance

Increasing levels of phytase were evaluated on feed intake, weight gain, feed conversion and viability of broilers (Table 2). To obtain the concentration of phytase that provides the best performance, the Linear (L), quadratic (Q) or quadratic with plateau (QP) models were tested and the one that best fit to the variables was used on the regression analysis.

Considering the initial stage of 1 to 21 days (Table 2), none of the models was adjusted to feed conversion, thus there was no response in function of the studied phytase levels. Feed intake and body weight gain were adjusted by QP ($p \leq 0.05$) model, with plateau at 279 and 231 OTU, respectively. At these concentrations, there was an increase of 22 and 23% in FI and BWG, respectively, compared to NC, which has no phytase supplementation. Viability was adjusted by a linear model ($p \leq 0.05$) up to 21 days, as the concentration of phytase increased in the diet, there was reduction in mortality.

The performance up to 35 days of age of the birds (Table 2), BWG and FI have also been adjusted by a QP model, stabilizing at the levels of 272 and 263 OTU, respectively, being 30 and 35% more efficient for these two evaluated variables in relation to NC diet, without phytase.

Feed conversion and viability at 35 days were adjusted to quadratic model with maximum values (1.60 and 96%) achieved at 300 and 400 OTU, respectively.

Table 2. Feed intake (FI), body weight gain (BWG), feed conversion ratio (FCR) and viability (VIA) of broilers in the period from 1-21 and 1-35 days fed phosphorus deficient diet with increasing levels of phytase and regression analysis

		1 - 21 days		
Variables	**NC2**	**NC2 + (120 OTU)**	**NC2 + (180 OTU)**	**NC2 + (240 OTU)**
FI (g)	685 ± 27	786 ± 29	817 ± 40	832 ± 26
BWG (g)	504 ± 21	595 ± 27	611 ± 26	621 ± 20
FCR	1.36 ± 0.03	1.32 ± 0.02	1.33 ± 0.02	1.34 ± 0.03
VIA[b]	96.51 ± 3.11	97.46 ± 2.00	99.37 ± 1.08	99.37 ± 1.08
		Regression [a]		
FI (g)	$\mathrm{FI} = 835.2\,(\pm 19.8) - 0.0019\,(\pm 0.0009) \times \left[279.3\,(\pm 77.6) - Phy\right]^2$ *R^2= 0.80; p(<0.0001)*			
BWG (g)	$BWG = 619.8\,(\pm 8.3) - 0.0022\,(\pm 0.0008) \times \left[231.1\,(\pm 44.1) - Phy\right]^2$ *R^2= 0.82; p(<0.0001)*			
FCR	- *ns**			
VIA	$VIA = 96.40\,(\pm 0.68) + 0.013\,(\pm 0.004)\,Phy$ *R^2= 0.27; p=0.0042*			
		1 - 35 days		
Variables	**NC2**	**NC2 + (120 OTU)**	**NC2 + (180 OTU)**	**NC2 + (240 OTU)**
FI (g)	1867 ± 139	2235 ± 77	2378 ± 60	2388 ± 77
BWG (g)	1191 ± 67	1.485 ± 39	1570 ± 44	1594 ± 58
FCR	1.57 ± 0.05	1.51 ± 0.03	1.51 ± 0.03	1.50 ± 0.02
VIA[b]	73.65 ± 9.72	81.59 ± 5.25	83.22 ± 3.64	84.44 ± 4.00
		Regression [a]		
FI (g)	$\mathrm{FI} = 2423.5\,(\pm 50.9) - 0.0076\,(\pm 0.0026) \times \left[271.5\,(\pm 53.2) - Phy\right]^2$ *R^2= 0.86; p(<0.0001)*			
BWG (g)	$BWG = 1610.8\,(\pm 26.5) - 0.0061\,(\pm 0.0015) \times \left[263.3\,(\pm 37.2) - Phy\right]^2$ *R^2= 0.91; p(<0.0001)*			
FCR	$FCR = 1.56\,(\pm 0.02) - 0.0006\,(\pm 0.0003)\,Phy + 0.000001(\pm 0.000001)Phy^2$ *R^2= 0.28; p=0.0302*			
VIA	$VIA = 83.0\,(\pm 1.8) - 0.00025\,(\pm 0.0003) \times \left[194.4\,(\pm 129.4) - Phy\right]^2$ *R^2= 0.32; p=0.0082*			

[a] Regression analysis, considering the phytase inclusion levels (0, 120, 180 and 240 OTU/kg), being each OTU (Phytex method) equivalent to 2 FTU (AOAC/ISO method).
[b] Viability = 100 – mortality (%). Adjusted by ASEN (SQUARE ROOT (Viability / 100)).
ns* no significant (p>0.05).

3.2. Tibias

The weight of the defatted dry tibia and its ash concentration were evaluated as indicative of minerals release by phytase (Table 3).

Table 3. Ash content and dry defatted tibia weight (DTW) of broilers at 35 days fed phosphorus deficient diets with increasing levels of phytase and regression analysis

Variables	NC2	NC2 + (120 OTU)	NC2 + (180 OTU)	NC2 + (240 OTU)
DTW (g)	3.858 ± 0.122	4.758 ± 0.177	4.772 ± 0.228	4.895 ± 0.123
ASH (%)	32.67 ± 1.45	35.02 ± 1.47	37.61 ± 1.49	37.30 ± 2.33
Regression [a]				
DTW (g)	$DTW = 4.83\,(\pm 0.05) - 0.00004(\pm 0.00001) \times \left[166.2\,(\pm 32.9) - Phy\right]^2$ *R^2= 0.87; p(<0.0001)*			
ASH (%)	$\mathrm{ASH} = 38.48\,(\pm 3.67) - 0.00004(\pm 0.00005) \times \left[388.7\,(\pm 374.5) - Phy\right]^2$ *R^2= 0.56; p(<0.0001)*			

[a] Regression analysis of DTW and ASH, considering the phytase inclusion levels (0, 120, 180 and 240 OTU/kg), being each OTU (Phytex method) equivalent to 2 FTU (AOAC/ISO method)

Quadratic-plateau model were adjusted for both evaluated variables. To DTW, the plateau was stabilized in 266 OTU with response of 4.83g, and this value was 25% higher than NC. The maximum percentage of ash was determined at 388 OTU, with a response of 38.5% of tibia ash, 18% higher than the NC. Control diet showed values of 5.23g and 39.13% for DTW and ASH, respectively.

Phosphorus bioavailability

The bioavailability of phosphorus was calculated using the standard curve method, considering BWG of birds up to 21 and 35 days of age, were adjusted by QP model (Table 4 and 5).

The maximum bioavailability found using DTW as parameter was obtained using 145 OTU of enzyme, with 0.054% P released, and 382 OTU to ash in the tibia, with 0.071% P provided by phytase.

Table 4. Body weight gain (BWG) of broilers fed diets with decreasing levels of available phosphorus to determine the standard curve and bioavailability of phosphorus at 21 days for the treatments with increasing levels of phytase and regression analysis

Variable	PC	NC1	NC2
BWG	656.5 ± 32.9	653.4 ± 11.1	504.2 ± 21.3
Standard Curve [a]	$BWG = 527.195(\pm 14.374) + 143.124(\pm 21.154)x$ R^2=0.71; p(<0.0001)		
	Phosphorus Bioavailability		
Variables	**NC2 +(120 OTU)**	**NC2 + (180 OTU)**	**NC2 + (240 OTU)**
BWG (g)	0.477 ± 0.190	0.584 ± 0.180	0.657 ± 0.138
BWG (%)	0.060 ± 0.022	0.071 ± 0.019	0.079 ± 0.015
	Regression [b]		
BWG (g)	$Bioav(g) = 0.655(\pm 0.069) - 0.000009(\pm 0.000004) \times [258.2(\pm 63.9) - Phy]^2$ R^2=0.78; p(<0.0001)		
BWG (%)	$Bioav(\%) = 0.078(\pm 0.006) - 0.000001(\pm 0.0000005) \times [240.4(\pm 46.3) - Phy]^2$ R^2=0.80; p(<0.0001)		

[a] Standard curve to determine phosphorus bioavailability calculated from supplemental phosphorus intake of treatments PC, NC1 and NC2, when x is the quantity in grams or percentage of phosphorus, which was released by the enzyme for BWG

[b] Regression analysis of bioavailable phosphorus, considering the phytase inclusion levels (0, 120, 180 and 240 OTU/kg), being each OTU (Phytex method) equivalent to 2 FTU (AOAC/ISO method).

Table 5. Performance of broilers fed diets with decreasing levels of available phosphorus to determine the standard curve and bioavailability of phosphorus at 35 days for the treatments with increasing levels of phytase and regression analysis

Variable	PC	NC1	NC2
BWG	1704.9 ± 45.3	1627.1 ± 32.2	1191.1 ± 67.2
Standard Curve [a]	$BWG = 1292.00(\pm 36.50) + 170.17(\pm 18.60)\,x$ R^2=0.82; p(<0.0001)		
	Phosphorus Bioavailability		
Variables	**NC2 +(120 OTU)**	**NC2 + (180 OTU)**	**NC2 + (240 OTU)**
BWG (g)	1.401 ± 0.230	1.903 ± 0.258	2.039 ± 0.340
BWG (%)	0.062 ± 0.009	0.080 ± 0.010	0.085 ± 0.011
	Regression [b]		
BWG (g)	$Bioav(g) = 2.10\,(\pm 0.24) - 0.00002(\pm 0.000007) \times \left[302.6\,(\pm 65.7) - Phy\right]^2$ R^2=0.90; p(<0.0001)		
BWG (%)	$Bioav(\%) = 0.085(\pm 0.003) - 0.000001(\pm 0.0000003) \times \left[245.7\,(\pm 24.1) - Phy\right]^2$ R^2=0.95; p(<0.0001)		

[a] Standard curve to determine phosphorus bioavailability calculated from supplemental phosphorus intake of treatments PC, NC1 and NC2, when x is the quantity in grams or percentage of phosphorus, which was released by the enzyme for BWG

[b] Regression analysis of bioavailable phosphorus, considering the phytase inclusion levels (0, 120, 180 and 240 OTU/kg), being each OTU (Phytex method) equivalent to 2 FTU (AOAC/ISO method).

The plateau of phytase utilization at 21 days was set at 240 OTU with a bioavailability of 0.079% P. Considering up to 35 days, the plateau was reached at 270 OTU with a bioavailability of 0.085% P.

Regarding the bone variables at 35 days of age of the birds, the standard curve for DTW showed better R^2 when compared to ASH (Table 6).

Table 6. Ash content and dry deffated tibia weight of broilers at 35 days fed diets with decreasing levels of available phosphorus to determine the standard curve and bioavailability of phosphorus at 35 days for the treatments with increasing levels of phytase and regression analysis

Variable	PC	NC1	NC2
DTW (g)	5.233 ± 0.125	4.982 ± 0.308	3.858 ± 0.122
Ash (%)	39.13 ± 2.12	36.46 ± 4.05	32.67 ± 1.50
Standard Curve [a]	$DBW = 4.014(\pm 0.103) + 0.683(\pm 0.080)x$		
	R^2 = 0.79; p(<0.0001)		
Standard Curve [a]	$Ash = 32.891\ (\pm 0.933)\ +\ 3.223\ (\pm 0.727)\ x$		
	R^2 = 0.51; p(<0.0001)		
Phosphorus Bioavailability			
Variables	**NC2 +(120 OTU)**	**NC2 + (180 OTU)**	**NC2 + (240 OTU)**
DTW (g)	1.089 ± 0.260	1.109 ± 0.333	1.289 ± 0.180
DTW (%)	0.049 ± 0.011	0.047 ± 0.014	0.054 ± 0.007
Ash (g)	0.661 ± 0.457	1.465 ± 0.462	1.603 ± 0.397
Ash (%)	0.029 ± 0.020	0.061 ± 0.019	0.067 ± 0.016
Regression [b]			
DTW (g)	$Bioav(g) = 1.199\ (\pm 0.063) - 0.00004\ (\pm 0.00002) \times \left[172.1\ (\pm 36.0) - Phy\right]^2$		
	R^2 = 0.84; p(<0.0001)		
DTW (%)	$Bioav(\%) = 0.0503\ (\pm 0.002) - 0.000002\ (\pm 0.000001) \times \left[145.3\ (\pm 45.1) - Phy\right]^2$		
	R^2 = 0.84; p(<0.0001)		
Ash (g)	$Bioav(g) = 1.880\ (\pm 1.540) - 0.000009\ (\pm 0.00002) \times \left[445.3\ (\pm 513.7) - Phy\right]^2$		
	R^2 = 0.59; p(<0.0001)		
Ash (%)	$Bioav(\%) = 0.0713\ (\pm 0.0404) - 0.0000005\ (\pm 0.0000006) \times \left[381.8\ (\pm 333.4) - Phy\right]^2$		
	R^2 = 0.59; p(<0.0001)		

[a] Standard curve to determine phosphorus bioavailability calculated from supplemental phosphorus intake of treatments PC, NC1 and NC2, when x is the quantity in grams or percentage of phosphorus, which was released by the enzyme for DTW or ash;

[b] Regression analysis of bioavailable phosphorus, considering the phytase inclusion levels (0, 120, 180 and 240 OTU/kg), being each OTU (Phytex method) equivalent to 2 FTU (AOAC/ISO method).

3.4. Economic analysis

Economic viability is an important aspect that should be considered when including phytase in poultry diets. To know the level of phytase added that ensures the greatest economic return to the producer is an important tool for the success of the company.

Since BWG is the most important performance parameter, it was chosen to calculate the economic viability of the phytase, being calculated its cost limit. The cost limit is the price of each u/g of phytase that would be equivalent to the cost of dicalcium phosphate.

The Figure 1 shows cost limit for each phytase FTU, calculated from the P bioavailability obtained for body weight gain, considering the price of dicalcium phosphate as US$ 0.50.

The phytase had higher cost benefit when used at the level of 120 OTU/kg and each OTU can cost up to US$ 0.000014. But this scenario changes with the price of dicalcium phosphate.

If the phytase has a concentration of 4000 u/g, multiplying 5000 by US$ 0.000010 (each OTU cost of phytase), it is possible to say this phytase may cost up to US$ 0.04/g or US$ 40.00/kg, by using a concentration of 240 OTU/kg in diet.

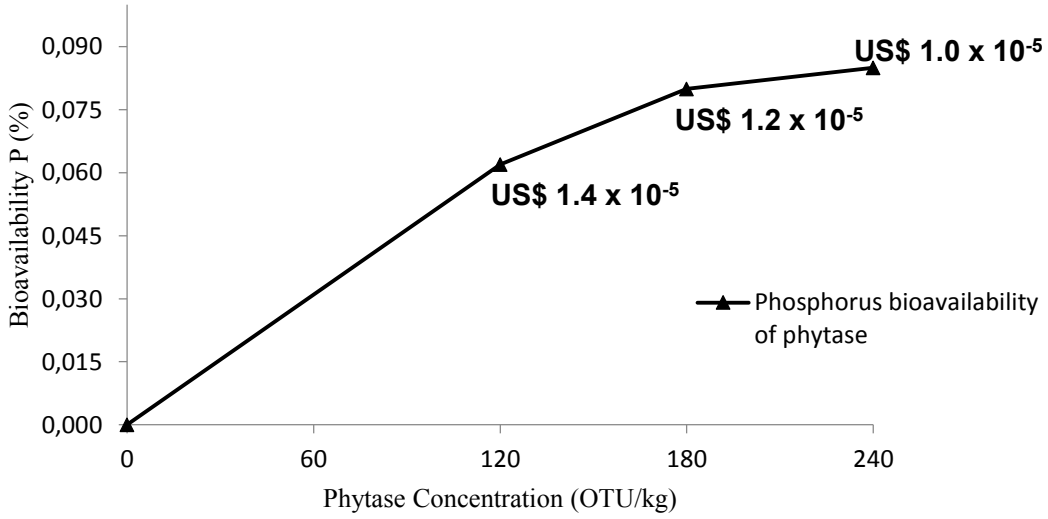

Figure 1. Limit cost per OTU of phytase to replace the inorganic phosphate.

4. DISCUSSION

For an enzyme acts efficiently, it is necessary to have the specific substrate in the diet and correct level, as well as its ability to overcome barriers found in the stomach, such as low pH and proteolytic enzymes, such as pepsin (Campestrini et al., 2005). Thus, there are two different implications for the performance variables that have been adjusted by Q and QP models, as follows, the FI and BWG up to 21 days and FI, BWG, FCR and VIA at 35 days. At first, the concentrations of enzyme were used in this study, the plateau response, or maximum point, may have been a consequence of the reduction in catalytic activity, influenced by enzyme concentration, and still having substrate (phytic acid) in the digest, which could be cleaved by phytase. The phytic acid is soluble at acidic pH, and the beginning of the gastrointestinal tract is the optimal condition for the action of phytase. However, the period that the digest remains under this condition is a short time. Most phytases are not able to degrade the six phosphate groups from the inositol molecule in time and with restrictions in the intestine of poultry (Greiner et al., 2000). In this case, it means that the inclusion of higher levels of phytase to the values established by the plateau could provide a higher response than those predicted by the models. Studies with inclusions of up to 24,000 FTU phytase in phosphorus deficient diets have shown performance responses with high phytase concentrations (Shirley and Edwards Jr, 2003; Coewinson et al., 2006; Bedford et al., 2012).

The other implication would be that the maximum set point or plateau predicted by the models would be a result of the reduction or disappearance of phytic acid by the action of phytase, with no further response to higher phytase additions.

It is known that as the digesta moves into the small intestine, there is an increase in pH through the duodenal secretions and phytic acid form crystals by complexing itself with nutrients and precipitates (Selle et al, 2007). This precipitation leads to losses as the reduction in feed intake and, consequently, decrease in body weight gain (Santos et al., 2008; Cowieson et al., 2011), as well as the reduction on nutrients availability, resulting in larger environmental excretions (Waldroup et al., 2000). Therefore, the increase in feed intake, body weight gain and feed conversion of the birds, seen in this

study, showed that the phytase was effective to degrade phytic acid, reducing the anti-nutritional effect of phytate.

Some authors attribute the greater weight gain to the increased feed intake with phytase supplementation (Santos et al., 2008; Cowieson et al., 2011; Liu et al., 2014). This study demonstrated increases of up to 30% (272 OTU) and 35% (263 OTU) on FI and BWG at 35 days of age, respectively, in relation to NC without phytase. Ravindran et al. (2008), evaluating the phytase supplementation in diets P deficient, have also observed a greater response on weight gain, feed intake and feed conversion ratio.

The quadratic model for FCR at 35 days, in this study, generated the optimal level of phytase usage at 300 OTU, with response of 1.50 versus 1.56 observed with NC diet. Chung et al. (2013) have also observed a better feed conversion rate for birds fed phytase, however it were not observed improvements in weight gain and feed intake. Several studies corroborate the results of the current study, and indicate that phytase supplementation provides improvements on weight gain, feed intake and feed conversion ratio (Denbow et al., 1995; Mitchell, Edwards, 1996; Singh et al., 2003; Karim, 2006; Selle et al., 2007).

Another aspect that is important to highlight is the viability of birds. As the reduction of P in deficient diets were severe, the mortality of birds reached 26.35% at 32 days for the birds fed diets with P reductions without phytase supplementation. However, as the enzyme was included in the diets, the viability improved. Powell et al. (2011), working with P reductions observed mortality of less than 6% in all treatments with phytase inclusions and 13.89% for the treatment with no enzyme.

Phytase also plays an important role in bone development, through the release of Ca and P, as well as other bivalent minerals that compose this tissue (Augspurger and Baker, 2004; Onyango et al., 2005). This greater availability of minerals and effectiveness of phytase activity can be evidenced when the dry defatted tibia weight and the ash content of broilers at 35 days are analysed through QP model, with an improvement of 25 and 18%, respectively. Other studies also demonstrate an increase in bone ash content of broilers with phytase in P deficient diets (Selle et al., 2009; Kozłowski et al., 2010; Rutherfurd et al., 2012).

The minerals deposition in bone is a good parameter for phytase evaluation, since this tissue has the highest concentrations of minerals in the body. Besides the

bone integrity is a characteristic which is correlated with the ash content and weight of the tibia, this is a problem faced by the industry in poultry production.

The bioavailability of P can be measured by digestibility trials or the standard curve methodology, the latter being easy to perform and the most used (Augspurger & Ugalde, 2009). Therefore this methodology was used for the calculation of the P released by the studied levels of phytase in the diets.

The bioavailability of P calculated from the body weight gain at 21 and 35 days, and from the weight and ashes of defatted tibia, expressed by QP models, explain the higher feed intake, body weight gain, weight and ash content as increased the phytase inclusion. Pillai et al. (2006), working with the same enzyme determined the bioavailability of 0.09% P released with 250 FTU/kg (125 OTU), through the ashes. Maybe the methodology used to characterize the enzyme was not considered (Phytex). In this study however, it was observed a lower value of phosphorus release (0.067%) with 240 OTU (500 FTU), considering ash of tibias, and 0.79% considering the body weight gain. Results presented by Augspurguer et al. (2003) agree with our findings, that the enzyme supplementation up to 1000 FTU/kg was not able to release quantities exceeding 0.067% P, considering bone ash of broilers. Biehl et al. (1995) found 0.116% P bioavailability, using 1200 FTU/kg of phytase (Natuphos).

As demonstrated in Figure 1, the phytase supplementation is economically viable when the cost per OTU/g does not exceed U$0.000014, U$0.000012 and U$0.000010 for the concentrations of 120, 180 and 240 OTU/kg, equivalent to approximately 240, 360 and 500 FTU, respectively. For a phytase at a concentration of 4000 U/g, for example, are needed 60g/ton addition to formulate a diet of 240 OTU/kg. Considering an intermediate price per kilo of phytase (U$10.00), the cost for each gram of enzyme would be U$0.01, being the cost for each FTU of U$0.0000025 below the set limit for the economically feasible use. In this example, would be spent U$0.60 with phytase compared to U$2.30 inorganic phosphate, thus a saving of U$ 1.70 per ton of feed.

Phytase used in small concentrations demonstrated to be efficient, improving weight gain, feed intake, feed conversion, viability and even the bone characteristics of birds fed P deficient diets in any tested concentration. However, the ideal inclusion

level which presented the best response, is close to the higher level studied, 240 OTU (500 FTU).

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5. REFERENCES

Augspurger, N. R., and D. H. Baker. 2004. Phytase improves dietary calcium utilization in chicks, and oyster shell, carbonate, citrate, and citrate-malate forms of calcium are equally bioavailable. Nutr. Res. 24:293–301.

Augspurger, N., and E. Ugalde. 2009. Comparative phytase utilization in pigs and broiler chickens. In: Congresso sobre manejo e nutrição de aves e suínos. Campinas. Anais... Campinas: CBNA. p.17-128.

Augspurger, N. I., D. M. Webel, X. G. Lei, and D. H. Baker. 2003. Efficacy of an E. coli phytase expressed in yeast for releasing phytate-bound phosphorus in young chicks and pigs. J. Anim. Sci. 81:474–483.

Bedford, M.R., Wyatt, C., Knox, A., Santos, T.T. 2011. Turkey performance between 0 and 84 days when diets supplemented with high doses of phytase, International Poultry Symposium, Atlanta.

Bedford, M.R. 2012. Alternate uses of phytases – Superdosing. Asian Poultry Magazine, p. 8-11.

Biehl, R. R., D. H. Baker, and H. F. DeLuca. 1995. 1α-hydroxylated cholecalciferol compounds act additively with microbial phytase to improve phosphorus, zinc and manganese utilization in chicks fed soy-based diets. J. Nutr. 125:2407.–2416.

Campestrini, E., V. T. M. Silva, M. D. Appelt. 2005. Utilização de enzimas na alimentação animal. Rev. Eletrônica Nutritime. 2:254-267.

Chung, T. K., S. M. Rutherfurd, D. V. Thomas, P. J. Moughan. 2013. Effect of two microbial phytases on mineral availability and retention and bone mineral density in low-phosphorus diets for broilers, British Poultry Science. 54:362-373.

COBB. Broiler Management Guide. COBB 500. S.l.: s.n., 2008, 65p.

Cowieson A. J., P. Wilcock, M. R. Bedford. 2011. Super-dosing effects of phytase in poultry and other monogastrics. 2011. World's Poultry Science Journal. 67:225-236.

Cowieson, A. J., T. Acamovic, M. R. Bedford. 2006. Supplementation of Corn–Soy-Based Diets with an Eschericia coli-Derived Phytase: Effects on Broiler Chick Performance and the Digestibility of Amino Acids and Metabolizability of Minerals and Energy. Poultry Science.85:1389-1397.

Denbow, D. M., V. Ravindran, E. T. Kornegay, Z. Yi, R. M. Hulet. 1995. Improving phosphorus availability in soybean meal for broilers by supplemental phytase. Poutry Science, Champaign. 74:1831-1842.

Eeckhout, W., M. De Paepe. 1994. Total phosphorus, phytate phosphorus and phytase activity in plant feedstuffs. Anim. Feed Sci. Technol. 47:19-29.

Greiner, R., N.-G. Carlsson, M. L. Alminger. 2000. Stereo-specificity of myo-inositol hexakisphosphate dephosphorylation by a phytate-degrading enzyme of *Escherichia coli*. J. Biotechnol. 84:53–62.

Karadas, F., V. Pirgozliev, A. C. Pappas, T. Acamovic, M. R. Bedford. 2010. Effects of different dietary phytase activities on the concentration of antioxidants in the liver of growing broilers. Journal of Animal Physiology and Animal Nutrition. 94:519-526.

Karim, A. 2006. Responses of broiler chicks to non-phytate phosphorus levels and phytase supplementation. International Journal of Poultry Science. 5:251-254.

Kozłowski, K., J. Jankowski, H. Jeroch. 2010. Efficacy of different levels of *Escherichia coli* phytase in broiler diets with a reduced P content. Polish Journal of Veterinary Sciences. 13:431-436.

Liu, S. Y., D. J. Cadogan, A. Péron, H. H. Truong, P. H Selle. 2014. Effects of phytase supplementation on growth performance, nutrient utilization and digestive dynamics of starch and protein in broiler chickens offered maize-, sorghum- and wheat-based diets. Animal Feed Science and Technology. 197:164–175.

Mitchell, R. D., H. M. Edwards Jr. 1996. Effects of phytase and 1,25-dihydroxycholecalciferol on phytate utilization and the quantitative requirement for calcium and phosphorus in young broiler chickens. Poultry Science, Champaign. 75:95-110.

MFRural. (2015). Price of animal food. MFRural. Assessed Jan. 2015. http://www.mfrural.com.br.

Onyango, E. M., M. R. Bedford, O. Adeola. 2005. Efficacy of an evolved *Escherichia coli* phytase in diets of broiler chicks. Poult. Sci. 84:248–255.

Pillai, P. B., T. O'Connor-Dennie, C. M. Owens, J. L. Emmert. 2006. Efficacy of an *Escherichia coli* Phytase in Broilers Fed Adequate or Reduced Phosphorus Diets and Its Effect on Carcass Characteristics. Poultry Science. 85:1737-1745.

Powell, S., T. D. Bidner, L. L. Southern. 2011. Phytase supplementation improved growth performance and bone characteristics in broilers fed varying levels of dietary calcium. Poultry Science 90:604-608.

Ravindran, V., A. J. Cowieson, P. H. Selle. 2008. Influence of dietary electrolyte balance and microbial phytase on growth performance, nutrient utilization, and excreta quality of broiler chickens. Poultry Science. 87: 677–688.

Robbins, K. R., A. M. Sexton, L. L. Southern. 2006. Estimation of nutrient requirements using broken-line regression analysis. J. Anim. Sci. 84:155–165.

Rostagno, H. S., L. F. T. Albino, J. L. Donzele, P. C. Gomes, R. F. M. Oliveira, D. C. Lopes, A. S. Ferreira, S. L. T. Barreto, R. F. Euclides. 2011. Tabelas brasileiras para aves e suínos: composição de alimentos e exigências nutricionais. Universidade Federal de Viçosa. 3a ed. 252p.

Rutherfurd, S. M., T. K. Chung, D. V. Thomas, M. L. Zou, P. J. Moughan. 2012. Effect of a novel phytase on growth performance, AME and the availability of minerals and amino acids in a low-phosphorus corn-soyabean meal diet for broilers. Poultry Science. 91:1118–1127.

Sakomura, N. K., and H. S. Rostagno. 2007. Métodos de pesquisa em nutrição de monogástricos. Funep, Jaboticabal, São Paulo/Brasil.

SAS Institute. 2002. SAS user's guide: statistics, version 9.0. Cary: SAS Institute.

Santos, F. R., M. Hruby, E. E. M. Pierson, J. C. Remus, N. K. Sakomura. 2008. Effect of Phytase Supplementation in Diets on Nutrient Digestibility and Performance in Broiler Chicks. J. Appl. Poult. Res. 17:191–201.

Selle, P. H., V. Ravindran, G. G. Partridge. 2009. Beneficial effects of xylanase and/or phytase inclusions on ileal aminoacid digestibility, energy utilisation, mineral retention and growth performance in wheat-based broiler diets. Animal Feed Science and Technology. 53: 303–313.

Selle, P. H., and V. Ravindran. 2007. Microbial phytase in poultry nutrition. Animal Feed Science and Technology. 135:1-41.

Silva, D. J., and A. C. Queiroz. 2009. Análise de alimentos: métodos químicos e biológicos 3. ed. Viçosa, MG: UFV, 235 p.

Shirley, R. B., and H. M. Edwards Jr. 2003. Graded levels of phytase past industry standards improves broiler performance. Poultry Science. 82:671-680.

Silva, Y. L., P. B. Rodrigues, R. T. F. Freitas, A. G. Bertechini, E. T. Fialho, E. J. Fassani, C. R. Pereira. 2006. Redução de proteína e fósforo em rações com fitase para frangos de corte, no período de 1 a 21 dias de idade. Desempenho e teores de minerais na cama. Revista Brasileira de Zootecnia. 35:833-841.

Singh, P. K., and V. K. Khatta. 2003. Effect of phytase supplementation on the performance of broiler chickens fed wheat based diets. Indian Journal of Animal Nutrition. 20:57-62.

Waldroup, P. W., J. H. Kersey, E. A. Saleh, C. A. Fritts, F. Yan, H. L. Stilborn, R. C. Crum Jr., V. Raboy. 2000. Non phytate phosphorus requirement and phosphorus excretion of broiler chicks fed diets composed of normal or high available phosphate corn with and without microbial phytase. Poultry Science. 79:1451-1459.

Walk, C. L., M. R. Bedford, A. P. McElroy. 2012. Influence of limestone and phytase on broiler performance, gastrointestinal pH, and apparent ileal nutrient digestibility. Poultry Science. 91:1371–1378.

CAPÍTULO 5 – EXPLORATORY MULTIVARIATE ANALYSIS TO EVALUATION THE EFFECT OF INCLUSION OF DIFFERENT ORIGINS OF PHYTASES IN BROILERS DIETS

ABSTRACT - The trial was conducted to evaluate differences between phytases, from fungal and bacterial origins, used in corn and soybean meal based diets on performance, bone mineralization and bioavailability of P. A total of 2,205 Cobb 500 male day-old chicks were assigned to seven treatments and seven replicates. Each experimental unit contained 45 birds and were distributed in a completely randomized design. The treatments were: Positive Control (PC) - diet meeting the nutritional requirements of the birds; Negative Control 1 (NC1) - reduction of 0.06% $_{av}$P; Negative Control 2 (NC2) - reduction of 0.12% $_{av}$P; NC2 + Fungal Phytase 1 (F1); NC2 + Fungal Phytase 2 (F2); NC2 + Bacterial Phytase 1 (B1); and NC2 + Bacterial Phytase 2 (B2). Due to the nature of dependence contained in the original set of variables, the effects of the treatments were subjected to multivariate exploratory methods. The cluster analysis by hierarchical method, principal component analysis, linear discriminant analysis and variance analysis performed with the scores of the first factor were in agreement and confirmed that the treatment that meets all the requirement of the bird (PC) has very specific and contrasting properties of the diet with phosphorus reduction (NC). Fungal phytases can be considered as having the same pattern, as well as bacterial phytases. Both phytases showed a superior process when compared to the negative control, however they were not able to equalize to the positive control diet, and bacterial phytases were more similar to positive control than fungal phytases.

Keywords: multivariate, phytase, performance, bone.

1. INTRODUCTION

Phytate is an anion present in cereals, and has the characteristic to chelate with minerals such as Ca, Mg, Zn or Fe, and crystallize forming salts in alkaline pH. Besides being a molecule rich in phosphorus unavailable for non-ruminants, due to the lack of significant amounts of endogenous digestive enzymes capable of degrading this molecule, phytate also has the property of chelating with proteins, amino acids and endogenous enzymes. This specific quality of phytate at alkaline pH disadvantage digestibility and absorption of nutrients (Ravindran et al., 1999). In this sense, the phytase supplementation becomes important to reduce these anti-nutritional effects.

Usually, industrial phytase for animal nutrition are classified by their origin (fungal or bacterial) and also by where they do the first hydrolysis in the phytate molecule: 3-phytase - do the first hydrolysis in carbon 3 of the myo-inositol-hexakisphosphate molecule or 6-phytase - do the first hydrolysis at carbon 6.

For a phytase act expressively is necessary that it is active at low pH, when the phytate is soluble, it should not be denatured by the feed manufacturing process, should not be impacted by digestive enzymes and must act efficiently in a short space of time (Zyla et al., 2004). From the time when the digesta reaches the intestine and the pancreatic secretion increase the pH, the formation of crystals occurs and therefore the phytase is active, the substrate (phytate) is no longer available (Selle, 2007).

There are some studies suggesting that the new generation of bacterial phytase (*E. coli*), are more efficient in phosphorus releasing in comparison to fungal phytases (Augspurger et al., 2003; Pillai et al., 2006). However, it is known that there are a structure of correlation between the performance variables, bone quality and bioavailability of phosphorus. Thus, a multivariate analysis is important to provide a higher level of information and exploratory results which correspond to their techniques and methods, whose analysis corresponds to all the variables in the theoretical interpretation of the obtained dataset (Costello and Osborne, 2005).

In this sense, the present study aimed to evaluate the differences between fungal and bacterial phytase, taking into account the performance, weight and bone ash and the bioavailability of P, using techniques of exploratory multivariate analysis. In addition to determine whether the use of cluster analysis techniques for hierarchical

method, principal component analysis and discriminant analysis are consistent and can be used for phytases evaluation.

2. MATERIAL AND METHODS

2.1. Birds and Housing

The trial was conducted at the Laboratory of Poultry Sciences of Department of Animal Science, Faculty of Agriculture and Veterinary Sciences - UNESP, Jaboticabal, Brazil.

The birds were housed in an experimental facility, with tunnel type system to control the environment. The control of temperature, humidity and air exchange were performed automatically by exhaust fans and climate control system, according to the age of the birds (Cobb Guidelines, 2006). During the starter phase, incandescent lamps were used as heating source according to the needs of poultry. Shaving litter was used in all experimental plots, as well as nipple drinkers and tubular feeders. Infant tubular feeders were used until 14 days of age, then substituted by adult tubular feeders.

Water and feed were offered ad libtum during the whole trial period. The lighting program was set at 24 hours of light.

All birds were vaccinated with the challenge of the region: at the hatchery against Marek and Gumboro diseases, at 12 days of age the birds were vaccinated against New Castle, administered via water.

2.2. Experimental design

A total of 2205 day-old male broiler chicks (Cobb) were weighed (±45g each) and distributed in each treatment with similar body weight means. The birds were distributed in a completely randomized design with seven treatments and seven replicates, allocated to 49 pens (1.0 x 3.0 m) of 45 chicks each.

The experimental treatments were: Positive Control (**PC**) - diet meeting the nutritional requirements of the birds; Negative Control 1 (**NC1**) - reduction of 0.06% $_{av}$P; Negative Control 2 (**NC2**) - reduction of 0.12% $_{av}$P; NC2 + Fungal Phytase 1 (**F1**); NC2+ Fungal Phytase 2 (**F2**); NC2 + Bacterial Phytase 1 (**B1**); and NC2 + Bacterial Phytase 2 (**B2**).

2.3. Experimental diets

The diets were formulated to meet the nutritional requirements of broilers based on the recommendations of the Brazilian Tables for Poultry and Swine (Rostagno et al., 2005), except to P. The Negative Control diets had reduction of $_{av}$P in comparison to PC. Table 1 describes the control diets and their nutritional compositions for starter (1-21 days) and grower (22-35 days) phases. Fungal and bacterial origin phytases were included in the NC2 treatments. The fungal phytases were produced by *Peniophora lyci* (F1) and *Aspergillus niger* (F2) and bacterial phytases were produced by *E. coli* (B1 and B2), from different companies.

Table1. Positive Control (PC), Negative Control 1 (NC1) and Negative Control 2 (NC2) experimental diets for the phases from 1 to 21 and 22 to 35 days of age of the birds

Ingredients (%)	1 - 21 days			22 - 35 days		
	PC	NC1	NC2	PC	NC1	NC2
Corn	55.253	55.505	55.757	61.458	61.710	61.962
Soybean meal (45%)	37.142	37.096	37.051	30.904	30.859	30.813
Soy oil	3.669	3.583	3.498	4.064	3.979	3.893
Limestone	1.379	1.333	1.559	1.132	1.358	1.585
Dicalcium phosphate	1.106	1.030	0.682	1.131	0.783	0.434
Salt	0.508	0.508	0.507	0.475	0.475	0.475
DL - Methionine	0.287	0.287	0.287	0.226	0.226	0.226
L - Lysine HCl	0.243	0.244	0.245	0.215	0.216	0.217
L - Threonine	0.074	0.074	0.074	0.055	0.055	0.055
Vitamin premix[1]	0.100	0.100	0.100	0.090	0.090	0.090
Mineral premix[2]	0.100	0.100	0.100	0.100	0.100	0.100
Coccidiostat	0.050	0.050	0.050	0.050	0.050	0.050
Choline Chloride 60%	0.070	0.070	0.070	0.070	0.070	0.070
Inert [3]	0.020	0.020	0.020	0.030	0.030	0.030
TOTAL	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
Nutritional levels						
ME (Kcal/kg)	3050	3050	3050	3150	3150	3150
Crude protein	22.50	22.50	22.50	20.00	20.00	20.00
Lysine	1.253	1.253	1.253	1.085	1.085	1.085
Metionine+Cystine	0.879	0.879	0.879	0.770	0.770	0.770
Digestible metionine	0.588	0.588	0.588	0.501	0.501	0.501
Digestible treonine	0.805	0.805	0.805	0.705	0.705	0.705
Crude Fibre	2.965	2.967	2.969	2.735	2.737	2.739
Calcium	0.900	0.900	0.900	0.830	0.830	0.830
Available phosphorus	0.345	0.285	0.225	0.295	0.235	0.175
Total phosphorus	0.601	0.541	0.481	0.534	0.474	0.415
Phytic phosphorus	0.231	0.232	0.232	0.222	0.222	0.222
Potassium	0.834	0.834	0.834	0.737	0.737	0.737
Chlorine	0.349	0.349	0.349	0.329	0.329	0.329
Sodium	0.220	0.220	0.220	0.207	0.207	0.207

[1]7.000.000.000 U.I. vit A; 2.200.000.000 U.I. vit D3; 11.000.000 mg vit E; 1.600.000 mg vit K3; 2.000.000 mg vit B1; 5.000.000 mg vit B2; 3.000.000 mg vit B6; 12.000.000 mcg vit B12; 35.000.000 mg nicotinic acid; 13.000.000 mg pantothenic acid; 800.000 mg folic acid; 100.000.000 mg Antioxidant; 1.000.000 mg. Vehicle Q.S.P. Addition 1.0 kg/t.

[2]8.000.000 mg Cu; 50.000.000 mg Fe; 70.000.000 mg Mn; 50.000.00 mg Zn; 1.200.000 mg I; 200.000 mg Se; 1.000.000 g Vehicle Q.S.P. Addition 1.0 kg/t.

[3] Phytase replaced by Inert ingredient on NC2 treatments to obtain the diets with 500 FTU/Kg.

2.4. Performance characteristics evaluated

At 21 and 35 days old, birds and feed leftovers were weighed. The performance parameters evaluated were: feed intake at 21 days (**FI(21)**), feed intake at 35 days (**FI(35)**), body weight gain at 21 days (**BWG(21)**), body weight gain at 35 days (**BWG(35)**), feed efficiency at 21 days (**FE(21)**) and feed efficiency at 35 days (**FE(35)**). Mortality was recorded daily and was used to adjust the total number of chicks to determine feed intake and feed efficiency.

2.5. Analysis of tibias

At 35 days of age, five birds of each experimental unit were slaughtered and had their tibiae removed, placed in identified plastic bags according to the treatment and replication, and frozen. Once thawed, the tibias were stripped and dried in ventilated oven at 100°C for 24 hours. Fat excess was removed by immersing the tibias in ethyl ether, up to the total disappearance of fat. They were dried again in a ventilated oven at 100°C for 24 hours. After the tibias were individually weighed on precision scale, it was determined the defatted dry tibia weight at 21 (**DDTW (21)**) and 35 (**DDTW (35)**) days. Then, they were milled in a ball mill, a sample of about 2g was weighed and forwarded to muffle furnace with temperature of 600°C for approximately 4h, until the sample became white, in order to determine ashes at 21 (**Ash (21)**) and 35 (**Ash (35)**) days (Silva and Queiroz, 2009).

2.6. Phosphorus Bioavailability

Phosphorus bioavailability was calculated using standard curve concept. To establish the standard curve, the intake of supplementary phosphorus (ISP) of treatments PC, NC1 and NC2 were calculated, according to formula:

$$ISP = \frac{\left(\text{feed intake}\left(g\right) \times \text{suplementary dietary P}\left(\%\right)\right)}{100}$$

Supplementary dietary P was considered null in the most deficient treatment (NC2); 0.06 for NC1; and 0.12% for PC; being these values the difference of $_{av}P$ between NC2 and the other treatments.

The ISP was associated to the responses of performance (BWG) at 21 and 35 and bone characteristics (ash (**ASH**) and dried tibia weight (**DTW**)) variables to construct the standard curve.

The results of performance and bone characteristics variables of the treatments with phytase addition were confronted with their respective standard curve, in order to obtain the total phosphorus released (**PR**) in grams. The formula below demonstrates the calculation to obtain the nutritional matrix (bioavailability) of $_{av}P$ of the phytase enzyme, being considered the feed intake of the bird and the phosphorus released.

$$BP\left(\%\right) = \frac{PR\left(g\right)}{FI\left(g\right)} \times 100$$

2.7. Statistical analysis

Multivariate approaches were used to analyse the effect of PC, NC, F1, F2, B1 and B2 treatments: cluster analysis by hierarchical method, principal component analysis and linear discriminant analysis and variance analysis performed with the scores of the first factor.

Cluster analysis by hierarchical method is used to know the structure of groups contained in a database when there is no a priori information on the number of groups. It was processed using the Euclidean distance between treatments samples and the connection method groups the hard method.

The principal component analysis is applied to resize the set of original variables into new latent variables, preserving the relevant information. These new variables are linear combinations of the original variables and the eigenvectors are built with the eigenvalues of the correlation matrix of the original variables. The eigenvectors here are called principal components (**PC**), and distributed themselves along the directions of maximum variance. According to the Kaiser criterion, eigenvalues above unity will be accepted, because they preserve the relevant information. The coefficients of the linear functions defining the factors were used to interpret their meaning, using the sign and relative size of the coefficients. The correlation between each variable (xi), and a principal component (PC_h) is calculated by:

$$r_{xy}(PC_h) = \frac{a_{jh}\sqrt{\lambda_h}}{S_j}$$

Where: S_j is the standard deviation of the variable j; a_{jh} is the j variable coefficient in the h-th principal component and λ_h is the h-th eigenvalue of the covariance matrix. The effect of the treatments PC, NC, F1, F2, B1 and B2 were tested with analysis of variance (ANOVA) and the differences between the average of treatments were tested with the Tukey multicomparison test of means.

Fisher's linear discriminant analysis is a linear combination of the original variables, which is characterized by determining the variables that best discriminate between two or more structurally different and mutually exclusive populations. Discrimination is achieved by the weights of each variable which maximizes the variance between groups on the variance in the groups (Hair et al., 2005). A diferênça entre os grupos foi realizada pelo teste multivarado Wilk´s Lambda.

All multivariates analyzes were performed in Statistica software version 7.0 (Statsoft, Inc. 2004). For the cluster and principal components analysis the original variables were standardized (zero mean and unit variance).

3. RESULTS AND DISCUSSION

Multivariate analysis is an exploratory technique that was used for comparison between fungal and bacterial origin of phytases, as well as their individual evaluations.

Only one eigenvalue was greater than unity, that the origins of the treatments can be grouped and represented basically by a single variable. Thus, the first principal component characterized by the variables shown in Table 2 was enough to preserve the original information relevant and accounted for 77.24% of the variance contained in the original set of variables. According to the variables contained therein, it can be used as a performance index.

The principal component one (PC1) are described in Table 3. All the loads have the same sign characterizing direct correlation between them, that is, when one value increases (or decreases), the other value also increases (or decreases). The variables feed efficiency at 21 and 35 days had no significant loads.

The analysis of variance processed with the scores of the first factor demonstrated to exist differences between the applied treatments ($p<0.01$), and after applying Tukey multiple comparison test it was possible to observe that F1 and F2 treatments do not differ, and the same is true for B1 and B2 treatments. Os tratamentos NC, F1 e F2 diferem dos tratamentos B1 e B2. The PC and NC treatments differ between them and from the others, and showed contrasting results, with the PC treatment of better performance. Figure 1 shows the resulting dendrogram of cluster analysis by hierarchical method.

Table 2. Means and stander deviation of the variables used in this study

Treatments	Ash (21) %	DDTW (21) G	Ash (35) %	DDTW (35) g	FI (21) g	BWG (21) g	FI (35) g	BWG (35) g	PB (21) %	PB (35) %
PC	34.1 ± 0.5	2.1 ± 0.1	39.1 ± 2.1	5.2 ± 0.1	862 ± 77	644 ± 56	1666 ± 47	1048 ± 49	0.120 ± 0.00	0.120 ± 0.00
NC	26.7 ± 2.7	1.7 ± 0.1	32.7 ± 1.5	3.9 ± 0.1	713 ± 94	523 ± 66	1133 ± 153	677 ± 54	0.000 ± 0.00	0.000 ± 0.00
F1	29.6 ± 1.9	1.9 ± 0.1	36.3 ± 2.3	4.5 ± 0.2	775 ± 17	587 ± 23	1390 ± 98	885 ± 56	0.032 ± 0.01	0.061 ± 0.01
F2	30.4 ± 1.4	1.9 ± 0.1	35.8 ± 1.5	4.7 ± 0.1	791 ± 21	596 ± 15	1493 ± 30	926 ± 22	0.039 ± 0.01	0.071 ± 0.01
B1	33.1 ± 0.6	2.2 ± 0.1	38.3 ± 1.1	4.9 ± 0.3	854 ± 13	639 ± 09	1630 ± 42	1029 ± 31	0.053 ± 0.01	0.010 ± 0.01
B2	32.7 ± 2.1	2.0 ± 0.1	37.0 ± 1.3	4.9 ± 0.2	836 ± 32	636 ± 28	1591 ± 27	1003 ± 37	0.052 ± 0.01	0.095 ± 0.01

Number of observations n = 7

Ash (21) – ash at 21 days; DDTW (21) – dried deffated tíbia weight at 21 days; Ash (35) – ash at 35 days; DDTW (35) – dried deffated tíbia weight at 35 days; FI (21) – feed intake at 21 days; BWG (21) – body weight gain at 21 days; FI (35) – feed intake at 35 days; BWG (35) – body weight gain at 35 days; PB(21) – phosphorus bioavailability at 21 days; PB(35) – phosphorus bioavailability at 35 days.

Table 3. Correlation between each original variable and the first principal component (PCOM)

Variables	**PCOM**
Ash (21)	-0.8718
DDTW (21)	-0.8048
Ash (35)	-0.7889
DDTW (35)	-0.9242
FI (21)	-0.8283
BWG (21)	-0.8446
FI (35)	-0.9455
BWG (35)	-0.9311
PB (21)	-0.8586
PB (35)	-0.9706

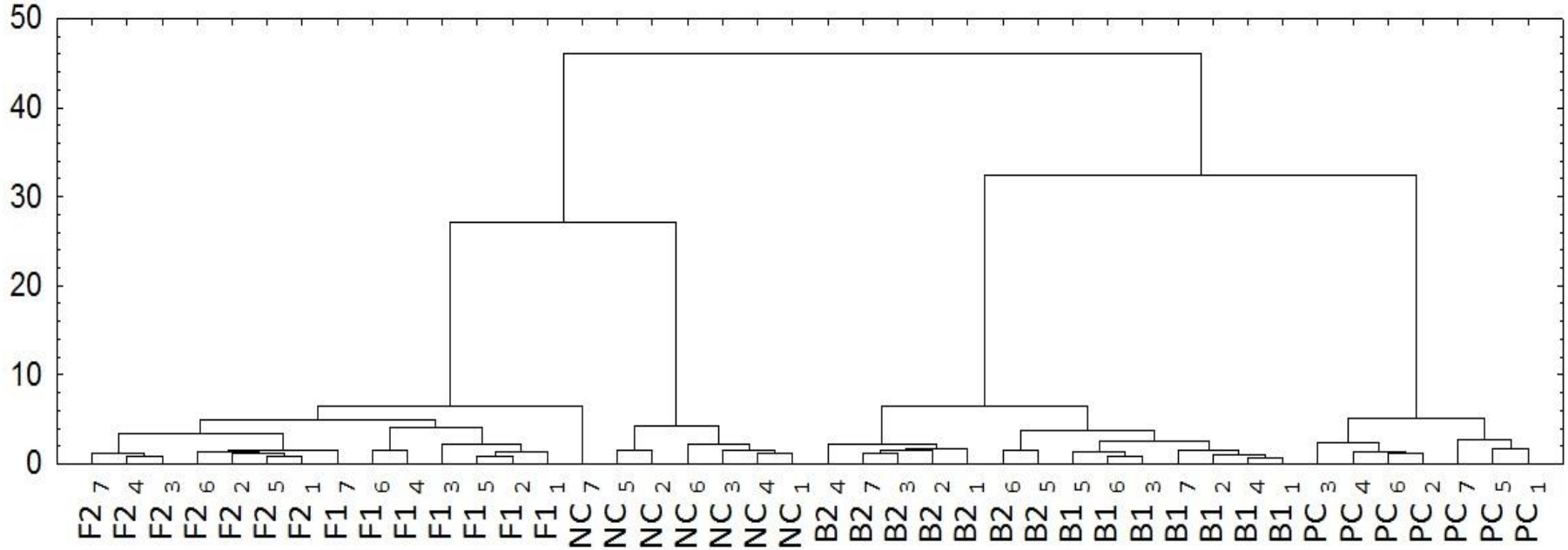

Figure 1. Dendrogram showing the structure of groups between positive control (PC), negative control (NC), NC + fungal phytase 1 (F1); NC + fungal phytase 2 (F2); NC + bacterial phytase 1 (B1) and NC + bacterial phytase 2 (B2) treatments.

The dendrogram is a specific type of diagram, or iconic representation, that organizes certain factors and variables (Figure 1). Based on statistical analysis of performance data, bones and bone bioavailability, it is employed a quantitative method that leads to clusters and its hierarchical ordering. The structure of groups shows an organization of the treatments in agreement with the results obtained from the ANOVA. It is seen that there is a drastic separation of NC, F1 and F2 treatments in a group and CP, B1 and B2 in the other group, as well as two other distinct groups, namely the PC and NC treatments. These results are also consistent with the results obtained with Fisher linear discriminant analysis, which distribution of obtained classification between the treatments is shown in Figure 2.

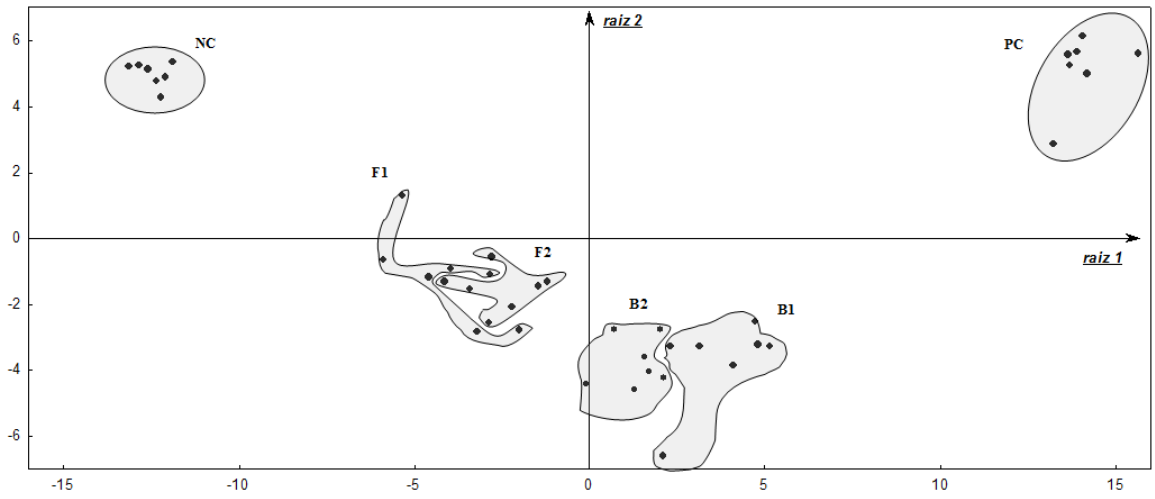

Figure 2. Discrimination of positive control (PC), negative control (NC), NC + fungal phytase 1 (F1); NC + fungal phytase 2 (F2); NC + bacterial phytase 1 (B1) and NC + bacterial phytase 2 (B2) treatments, obtained by linear discriminant analysis of Fischer.

The difference between treatments in the discriminant analysis was proven by Wilk's Lambda test ($p<0.001$). F1 and F2 treatments and B1 and B2 treatments can be considered as having the same response. Although there is no significant difference between the bacterial phytases, the linear discriminant analysis of Fisher and the cluster analysis by hierarchical method showed that phytases B1

and B2, although produced by the same organism (*E. Coli*), have different responses, but not enough to be different by analysis of variance. Table 2 demonstrates the data used to build the centroids. It is possible to observe, in despite of no significance between B1 and B2, that the main difference between these phytases is associated with the weight of the tibia at 21 days (Figure 3), and B1 phytase showed weight 11.3% higher, in relation to B2.

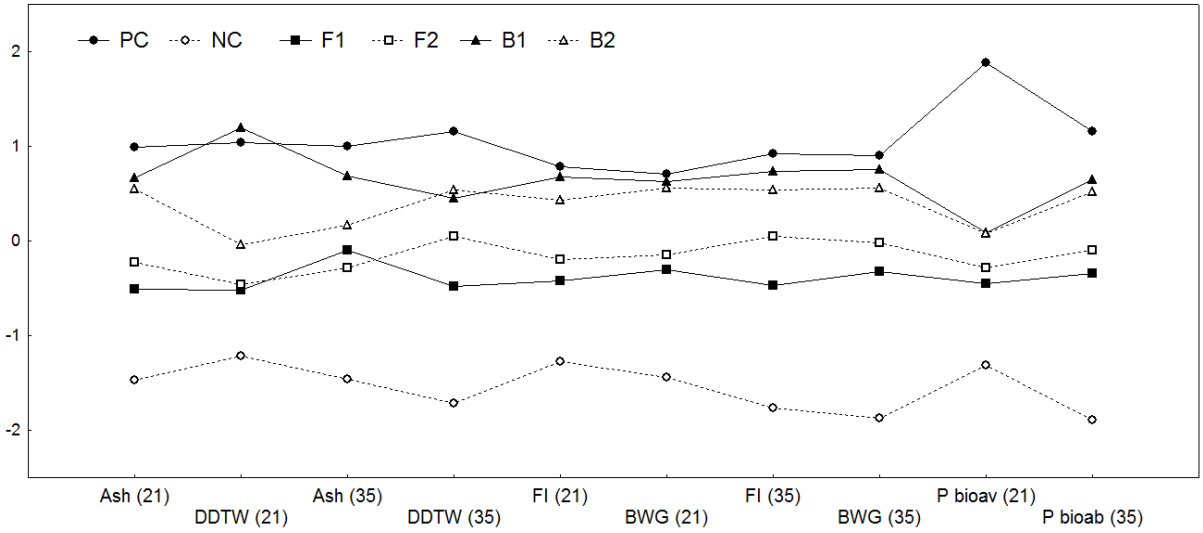

Figure 3. Distribution of centroid of positive control (PC), negative control (NC), NC + fungal phytase 1 (F1); NC + fungal phytase 2 (F2); NC + bacterial phytase 1 (B1) and NC + bacterial phytase 2 (B2) treatments built with the means of the variables Ash (21), DDTW (21), Ash (35), DDTW (35), FI (21), BWG (21), FI (35), BWG (35), PB (21) and PB (35).

Considering that the PC is composed of a diet that meets the requirement of the bird, it is possible to conclude through the distance of the groups and the centroids of each treatment, that the bacterial phytases have a higher efficiency over the fungal ones, being closer to the PC diet. Studies have shown that bacterial phytases derived from *E. coli* have less action from the low pH of the stomach (Wyss et al., 1999; Augspurger et al., 2003) and from the gastric digestive enzymes such as pepsin (Rodriguez et al., 1999a). Rodriguez et al. (1999b) determined the optimum pH for the activity of the enzyme derived from *E. coli* (pH 2.5 to 3.5) and for a fungal phytase (pH 4.0 to 4.5). It is evident that the range of action of the bacterial enzyme is different from the fungal. The

bacterial operates more concisely in lower pH, as founded in the gizzard and proventriculus of the bird (pH ± 2.5 to 3.6), resulting in greater efficiency. The fungal phytases usually hydrolyze the myo-inositol molecule at the carbon 3, while the bacterial begin the hydrolysis at the carbon 6 (Konietzny and Greiner, 2002; Pereira et al., 2012). There is no evidence that the site of the first hydrolysis influence the effectiveness of the enzymes, however, this fact could have a relationship to the separation of the groups G1, related to fungal phytases, and G2, related to the bacterial.

Adeola et al. (2006) also observed that *E. coli* phytases have a higher bioavailability of phosphorus in relation to the fungal. Onyango et al. (2005) studied the activity of different bacterial and fungal phytases, and found that the bacterial phytase has a better performance throughout the digestive system of birds in relation to fungal phytase.

According to McGinnis (2007), a commercial bacterial phytase had a higher ability to break down the phytate in the duodenum of growing pigs, compared to phytases of fungal origin. The improved efficiency of the bacterial enzyme can also be explained by Prata et al. (2007), who found that a phytase originated from *E. coli* genetically modified showed a higher hydrolysis of the substrate velocity, especially when hydrolyzing the esters IP5 and IP6, in relation to other fungal phytases.

4. CONCLUSION

The multivariate techniques were consistent and confirmed that the treatment that meets all the requirement of the bird (PC) and the diet with phosphorus reduction (NC) have very specific and contrasting properties.

Fitases de mesma origem apresentam a mesma resposta quando adicionadas em dietas com reduções de fósforo. However, bacterial phytases showed better response in comparison to the fungal ones.

The techniques described can be used for evaluation and comparison of phytases.

The effect of treatments can be validated due to concordance between the four applied approaches.

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5. REFERENCES

Adeola, O., O. A. Olukosi, J. A. Jendza, R. N. Dilger, M. R. Bedford. 2006. Response of growing pigs to *Peniphora lycii* e *Escherichia coli* derived phytase or varying ratios of calcium to total phosphorus. Journal of Animal Science. 82: 637-644.

Augspurger, N. I., D. M. Webel, X. G. Lei, D. H. Baker. 2003. Efficacy of an *E. coli* phytase expressed in yeast for releasing phytate-bound phosphorus in young chicks and pigs. J. Anim. Sci. 81:474–483.

COBB. Broiler Management Guide. COBB 500. S.l.: s.n., 2008, 65p.

Costello, A. B, J. W. Osborne. 2005. Best practices in exploratory factor analysis: Four recommendations for getting the most from your analysis. Practical Assessment Research & Evaluation. 10:13-24.

Hair J. F., R. E. Anderson, R. L. Tatham, et al. 2005. Análise Multivariada de Dados. 5. ed. Porto Alegre, Bookman. 593p.

Konietzny, U., R. Greiner. 2002. Molecular and catalytic properties of phytase degrading enzymes (phytases). Journal Food Science Technology. 37:791-812.

McGinnis, L. M., M. R. Widmer, C. L. Wright, T. M. Parr, H. H. Stein. Site of activity within the digestive tract of growing pigs of an evolved *E. coli* derived and a fingal derived commercial phytase. 2007. Journal of Animal Science. 85:87.

Onyango, E. M., M. R. Bedford, O. Adeola. 2005. Phytase activity along the digestive tract ofthe broiler chick: A comparative study of an *Escherichia coli* derived and *Peniophora lycii* phytase. Canadian Journal of Animal Science. 85:61-68.

Pereira, R., J. F. M. Menten, G. G. Romano, C. L. S. Silva, K. C. Zavarize, N. A. A. Barbosa. 2012. Eficiência de uma fitase bacteriana na liberação de fósforo fítico em dietas de frangos de corte. Arq. Bras. Med. Vet. Zootec. 64:137-144.

Pillai, P. B., T. O'Connor-Dennie, C. M. Owens, J. L. Emmert. 2006. Efficacy of an *Escherichia coli* Phytase in Broilers Fed Adequate or Reduced Phosphorus Diets and Its Effect on Carcass Characteristics. Poultry Science. 85:1737-1745.

Prata, R., C. Batie, S. Betts, S. S. Basu. 2007. XX Congreso Latinoamericano de Avicultura, Porto Alegre, Brazil, p.13-15.

Ravindran V., S. Cabahug, G. Ravindran, L. Bryden. 1999. Influence of microbial phytase on apparent ileal amino acid digestibility of feedstuffs for broilers. Poultry Sci. 78:699-706.

Rodriguez, E., J. M. Porres, Y. Han, X. G. Lei. 1999a. Different sensitivity of recombinant *Aspergillus niger* phytase (r- PhyA) and *Escherichia coli* pH 2.5 acid phosphatase (r-AppA) to trypsin and pepsin in vitro. Arch. Biochem. Biophys. 365:262–267.

Rodriguez, E., Y. Han, X. G. Lei. 1999b. Cloning, sequencing, and expression of an *Escherichia coli* acid phosphatase/phytase gene (appA2) isolated from pig colon. Biochem. Biophys. Res. Commun. 257:117–123.

Rostagno, H. S., L. F. T. Albino, J. L. Donzele, P. C. Gomes, R. F. M. Oliveira, D. C. Lopes, A. S. Ferreira, S. L. T. Barreto, R. F. Euclides. 2011. Tabelas brasileiras para aves e suínos: composição de alimentos e exigências nutricionais. Universidade Federal de Viçosa. 3a ed. 252p.

Sakomura, N. K., H. S. Rostagno. 2007. Métodos de pesquisa em nutrição de monogástricos. Funep, Jaboticabal, São Paulo/Brasil.

Selle, P. H., V. Ravindran. 2007. Microbial phytase in poultry nutrition. Animal Feed Science and Technology, 135:1-41.

Silva, D. J., A. C. Queiroz. 2009. Análise de alimentos: métodos químicos e biológicos 3. ed. Viçosa, MG: UFV, 235 p.

Statsoft, Inc. 2004. Statistica (data analysis software system), version 7.

Wyss M, Brugger R, A. Kronenberger, R. Rémy, R. Fimbel, G. Oesterhelt, M. Lehmann, A. P. G. M. Van Loon. 1999. Biochemical characterization of fungal phytases (myo-inositol hexakisphosphate phosphohydrolases): catalytic properties. Applied and Environmental Microbiology. 65:367-373.

Zyla, K., J. Koreleski, S. Swiatkiewicz, A. Wikiera, M. Kujawski, J. Piironen, D. R. Ledoux. 2000. Effects of phosphorolytic and cell wall-degrading enzymes on the performance of growing broilers fed wheat-based diets containing different calcium levels. Poult. Sci. 79:66–76.

CAPÍTULO 6 – CONSIDERAÇÕES FINAIS

Os resultados obtidos nestes estudos, poderão auxiliar na tomada de decisão quanto a utilização de diferentes fitases em dietas para frangos de corte à base de milho e farelo de soja.

No capítulo 2, a fitase derivada de *Buttiauxella* apresentou sua máxima eficiência em concentrações próximas a 500 FTU, enquanto a fitase derivada de *E. coli* mostrou ser mais eficiente em torno de 1000 FTU. Nestas condições, as fitases são capazes de biodisponibilizar o fósforo fítico, podendo substituir boa parte do fosfato bicálcico na dieta, sem afetar o desempenho e ossos das aves, reduzindo a excreção de fósforo para o ambiente.

Sabendo-se como as fitases atuam sobre o ganho de peso, consumo de ração, conversão alimentar, excreção de fósforo (total e solúvel), peso, cinzas e resistência óssea, a enzima deve se tornar uma ferramenta a ser utilizada em de acordo com as condições inerentes a cada produção. Logo, a escolha da fitase e sua concentração, tem respostas específicas para cada variável avaliada. Desta forma sua escolha dependerá do objetivo de cada produção, relacionados a fatores ambientais, custos de produção, desempenho e qualidade óssea.

A inclusão de fitase é utilizada, quase em sua totalidade, em dietas contendo reduções nutricionais, com objetivo de suprir suas carências. No Capítulo 3, foi verificado que a utilização "*on top*" de fitase derivada de *E. coli* afeta beneficamente a a conversão alimentar e cinzas ósseas de frangos de corte. Nestas condições, pode representar um maior retorno financeiro devido a melhora na eficiência na utilização da dieta e melhora na qualidade óssea, reduzindo as perdas na produção e no abate de frangos de corte.

O emprego de fitase *E. coli* nas concentrações de 120, 180 e 240 FTU, também trouxeram benefícios em dietas deficientes em fósforo (Capítulo 4). A máxima eficiência da fitase foi estabelecida em valores próximos ao máximo avaliado no estudo (240 FTU). No entanto, com o aumento dos níveis de fitase à dieta, sua eficiência passa a ficar cada vez mais reduzida, talvez pela degradação de

praticamente todo o substrato da dieta ou pelo alcance do máximo potencial de produção do animal. Este fato pode ser confirmado verificando a inclinação das retas referentes aos gráficos dos capítulos 2 (p. 59) e 4 (p. 111), que apresentaram a biodisponibilidade do fósforo em função da concentração de fitase. Ambas as fitases apresentam uma maior inclinação nos níveis mais baixos estudados.

Por fim, uma forma de analisar e comparar fitases pode ser descrita no capítulo 5, utilizando análises multivariadas exploratórias. Com ela, foi possível verificar que as fitases bacterianas (*E. coli*) são mais eficientes para o desempenho das aves e mineralização óssea, além de apresentarem uma maior liberação do fósforo em relação as fitases fúngicas. Este fato, implica em maiores estudos no campo da engenharia genética em busca da fitase ideal e no direcionamento para produção de fitases de origem bacteriana.