EFFECT OF DURATION OF THE TRIAL ON THE EFFICACY OF A NOVEL 3-PHYTASE IN BROILERS AND LAYING HENS

Ph.D. Thesis

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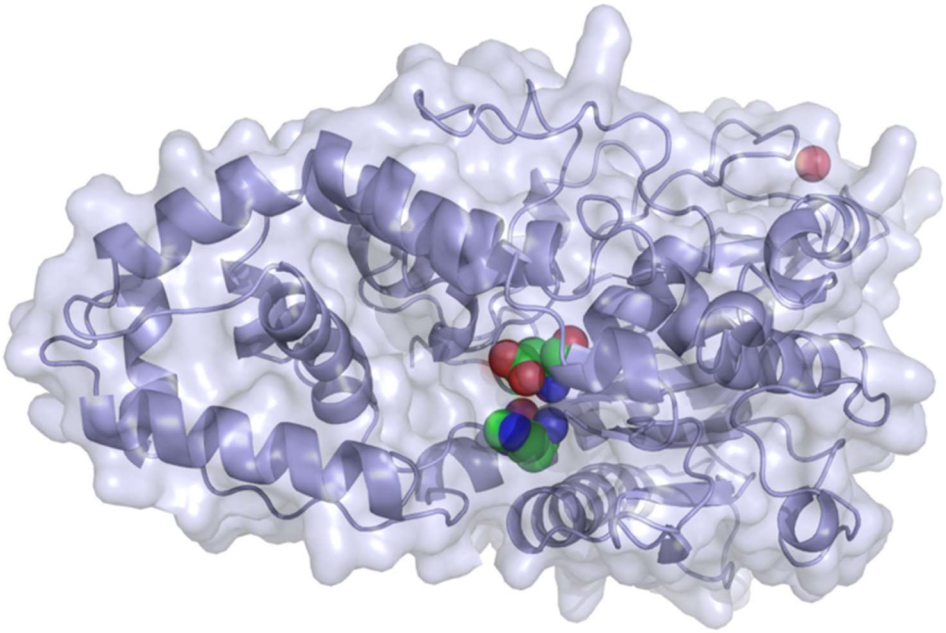

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Cover image

3d ribbon/surface model of 3-phytase from *Aspergillus niger*. The catalytic centre (His-59 and Asp-339) is emphasized. From PDB 1IHP. Ref.: D. Kostrewa *et al.* 1997. Crystal structure of phytase from Aspergillus ficuum at 2.5 A resolution. Nat. Struct. Biol., 4, 185-190. http://doi.org/10.1038/nsb0397-185 Creative Commons CC0 License.

Abstract

This thesis tackles the effect of duration of the trial on the efficacy of a novel 3-phytase in broilers and laying hens. Calcium and phosphorus are critical in poultry nutrition because of their involvement in different physiologic and metabolic activities, particularly bone and egg mineralization. Exogenous microbial phytase is commonly used in poultry diets to ameliorate the deleterious effect of phytate on bird performance and the environment, as well as to reduce feeding costs. There are many evidences of the effectiveness of dietary addition of phytase in improving phosphorus (P) digestibility in poultry, but there are still inconsistencies among studies on the effects of phytase on the different response criteria studied. These inconsistencies can be due to many factors being one of them the duration of the trials. In this study, two objectives were pursued: 1) To investigate the effects of reducing the mineral (Ca and P) content and adding different doses of a new 3-bacterial phytase in laying hens diets in a short and long-term experiment. 2) To investigate the effects of reducing the mineral (Ca and P) content and adding different doses of a new 3-bacterial phytase in broiler diets in a short and long-term experiment. To this aim, two experiments were designed:

Experiment 1) A total of 192 laying hens were used to evaluate the effect of dietary mineral content and phytase dose on nutrient utilization, egg production and quality and bone mineralization of young laying hens. Four dietary treatments were studied: PC, positive control with no added phytase, 4.07% Ca and 0.61% P; NC, negative control with no added phytase, 2.97% Ca and 0.37% P; and P500 and P1000, where NC diet was supplemented with phytase at 500 and 1000 FTU/kg, respectively. Hens' performance and egg traits were registered from 22 to 31 weeks of age. Coefficients of total tract apparent digestibility (CTTAD) of nutrients were determined at 25 and 31 weeks of age. Apparent ileal digestibility (AID) and blood content of Ca and P, as well as bone traits, were

determined at 31 weeks of age. The results of this study showed that Ca and P retention was higher in birds on PC diet at 25 weeks, but not at 31 weeks of age compared to those on NC diet ($p < 0.05$). Birds fed P1000 feed had the highest CTTAD values for dry and organic matter at both ages ($p < 0.001$). Coefficient of total tract apparent digestibility of Ca was significantly higher in P1000 diet than in NC diet at 31 weeks of age ($p < 0.001$). Birds fed with P500 diet at 25 weeks of age and P1000 at 31 weeks of age showed higher CTTAD and retention of P, but lower excretion of P than those fed NC diet ($p < 0.05$). Phytase inclusion linearly increased AID of dry matter and P ($p < 0.001$). Hens fed P500 feed had the greatest body weight at the end of the trial ($p < 0.05$) and P1000 birds had the best feed conversion ratio ($p < 0.05$). Fowl fed a PC diet produced eggs with higher shell thickness and yolk color than those fed on NC diet ($p < 0.05$). Phytase inclusion linearly increased the yolk color ($p < 0.05$). Tibia of laying hens fed with PC had significantly higher ash content than those on NC diet ($p < 0.05$), and birds fed with P1000 presented intermediate values.

Experiment 2) In this experiment two trials differing in duration (short and long-term trials), were conducted to evaluate the effects of providing deficient (NC) or correct (PC) Ca and P levels and different doses of a new phytase (250, 500 and 1000 FTU/kg feed) in broiler feeds on growth performance, nutrient digestibility and retention and tibia mineralization. A total of 80 and 490 male chicks (Ross) of 21 days and 1 day of age were used in the short (17 days) and long (42 days) term trials, respectively. Birds were weighed and feed intake registered periodically during the experimental period. In both trials, 80 animals were selected at 28 days of age for a nutrient balance trial in metabolic cages and, at the end of the digestibility trial (38 days of age), one chick per cage (8 animals per treatment) was euthanized to determine tibia mineralization. The results of this study showed that in the long-term trial, chicks fed NC diets showed a lower ($p <0.05$) performance compared to chicks fed PC, 500 and 1000 FTU/kg feed during

the starting period, and a significantly higher digestibility for P than those fed the PC diet. Regarding the effects of phytase, feeding 250 to 500 FTU/kg diets increased most of the nutrients' digestibility in the short-term trial while no effects on nutrients other than P were detected in the long-term trial. Tibia mineralization increased linearly with phytase addition ($p < 0.05$) only in the long-term trial.

In conclusion, from the results of two experiments it can be concluded that it would be advisable to increase the dose of phytase in the feed of laying hens to obtain long-term benefits. In addition, the age of the animals is key in determining the effects of mineral levels and phytase addition in broiler feeds and being more noticeable in young broilers. In terms of mineral and nutrients digestibility, the duration of the trial is key due to a possible adaptation phenomenon of birds to low P supplies by increasing its digestibility and retention in long compared to short-term trials both in broilers and laying hens.

Resumen

Esta tesis aborda el efecto de la duración de la prueba sobre la eficacia de una nueva 3-fitasa en pollos de engorde y gallinas ponedoras. El calcio y el fósforo son críticos en la nutrición de las aves debido a su participación en diferentes actividades fisiológicas y metabólicas, particularmente en la mineralización ósea. La fitasa microbiana exógena se usa comúnmente en las dietas de las aves para mejorar el efecto nocivo del fitato en el rendimiento de las aves y el medio ambiente, así como para reducir los costos de alimentación. Hay muchas evidencias de la efectividad de la adición dietética de fitasa para mejorar la digestibilidad del fósforo (P) en aves, pero todavía hay inconsistencias entre los estudios sobre los efectos de la fitasa en las diferentes condiciones de estudio. Estas inconsistencias pueden deberse a muchos factores, siendo uno de ellos la duración de los ensayos. En este estudio se persiguieron dos objetivos: 1) Cómo la inclusión dietética de fitasa, a dosis normal y sobredosis, podría afectar la utilización de nutrientes y el rendimiento en gallinas ponedoras jóvenes. 2) Investigar los efectos de reducir el contenido de minerales (Ca y P) y agregar diferentes dosis de una nueva 3-fitasa bacteriana en dietas de pollos de engorde en un experimento a corto y largo plazo. Así, se diseñaron dos experimentos:

Experimento 1) Se utilizó un total de 192 gallinas ponedoras para evaluar el efecto del contenido de minerales en la dieta y la dosis de fitasa sobre la utilización de nutrientes, la producción y calidad de los huevos y la mineralización ósea de gallinas ponedoras jóvenes. Se estudiaron cuatro tratamientos dietéticos: PC, control positivo sin fitasa añadida, 4,07% Ca y 0,61% P; NC, control negativo sin fitasa añadida, 2,97% Ca y 0,37% P; y P500 y P1000, donde la dieta NC se complementó con fitasa a 500 y 1000 FTU/kg, respectivamente. El desempeño de las gallinas y las características del huevo se controlaron desde las 22 a las 31 semanas de edad. Los coeficientes de digestibilidad aparente del tracto total (CTTAD) de los nutrientes se

determinaron a las 25 y 31 semanas de edad. La digestibilidad ileal aparente (DIA) y el contenido sanguíneo de Ca y P, así como las características óseas, se determinaron a las 31 semanas de edad. Los resultados de este estudio mostraron que la retención de Ca y P fue mayor en las aves con dieta PC a las 25 semanas, pero no a las 31 semanas de edad en comparación con las aves con dieta NC ($p < 0,05$). Las aves P1000 tuvieron los valores CTTAD más altos para materia seca y orgánica en ambas edades ($p < 0.001$). La CTTAD de Ca fue significativamente mayor en la dieta P1000 que en la dieta NC a las 31 semanas de edad ($p < 0,001$). Las aves alimentadas con dieta P500 a las 25 semanas de edad y P1000 a las 31 semanas de edad mostraron mayor CTTAD y retención de P, pero menor excreción de P que las alimentadas con dieta NC ($p < 0,05$). La inclusión de fitasa incrementó linealmente la AID de materia seca y P ($p < 0.001$). Las gallinas P500 alimentadas tenían el mayor peso corporal al final del ensayo ($p < 0,05$) y las aves P1000 tenían el mejor índice de conversión alimenticia ($p < 0,05$). Las aves alimentadas con una dieta PC produjeron huevos con mayor grosor de la cáscara y color de la yema que las alimentadas con la dieta NC ($p < 0,05$). La inclusión de fitasa aumentó linealmente el color de la yema ($p < 0,05$). La tibia de las gallinas ponedoras alimentadas con PC tuvo un contenido de cenizas significativamente mayor que las de la dieta NC ($p < 0,05$), y las aves alimentadas con P1000 presentaron valores intermedios.

Experimento 2) En este experimento se realizaron dos ensayos de diferente duración (ensayos a corto y largo plazo) para evaluar los efectos de proporcionar niveles deficientes (NC) o correctos (PC) de Ca y P y diferentes dosis de una nueva fitasa (250, 500 y 1000 FTU/kg de alimento) en alimentos para pollos de engorde sobre el rendimiento del crecimiento, la digestibilidad y retención de nutrientes y la mineralización de la tibia. Se utilizaron un total de 80 y 490 pollitos machos (Ross) de 21 días y 1 día de edad en los ensayos a corto (17 días) y largo (42 días), respectivamente. Las aves se pesaron y se registró el consumo de

alimento periódicamente durante el período experimental. En ambos ensayos, se seleccionaron 80 animales a los 28 días de edad para un ensayo de equilibrio de nutrientes en jaulas metabólicas y, al final del ensayo de digestibilidad (38 días de edad), se sacrificó un pollito por jaula (8 animales por tratamiento) para determinar la mineralización de la tibia. Los resultados de este estudio mostraron que en la prueba a largo plazo, los pollitos alimentados con dietas NC mostraron un rendimiento más bajo ($p < 0.05$) en comparación con los pollitos alimentados con PC, 500 y 1000 FTU/kg de alimento durante el período de inicio y. una digestibilidad significativamente mayor para P que aquellos alimentados con la dieta PC. Con respecto a los efectos de la fitasa, la alimentación con dietas de 250 a 500 FTU/kg aumentó la digestibilidad de la mayoría de los nutrientes en el ensayo a corto plazo, mientras que no se detectaron efectos sobre los nutrientes distintos del P en el ensayo a largo plazo. La mineralización de la tibia aumentó linealmente con la adición de fitasa ($p < 0,05$) solo en el ensayo a largo plazo.

Como conclusión de los resultados de dos experimentos, se puede concluir que sería recomendable aumentar la dosis de fitasa en el pienso de las gallinas ponedoras para obtener beneficios a largo plazo y la edad de los animales es clave para determinar los efectos del mineral. los niveles y la adición de fitasa en los alimentos para pollos de engorde y siendo más notorios en los pollos jóvenes. En términos de digestibilidad de minerales y nutrientes, la duración de la prueba es clave debido a un posible fenómeno de adaptación de las aves a suministros bajos de P, al aumentar su digestibilidad y retención en ensayos a largo plazo en comparación con ensayos a corto plazo tanto en pollos como en gallinas ponedoras.

Resum

Aquesta tesi aborda l'efecte de la durada de l'assaig sobre l'eficàcia d'una nova 3-fitasa en polls d'engreix i gallines ponedores. El calci i el fòsfor són crítics en la nutrició de les aus de corral per la seva implicació en diferents activitats fisiològiques i metabòliques, especialment en la mineralització òssia. La fitasa microbiana exògena s'utilitza habitualment en les dietes d'aus de corral per millorar l'efecte nociu del fitat sobre el rendiment dels ocells i el medi ambient, així com per reduir els costos d'alimentació. Hi ha moltes evidències de l'eficàcia de l'addició dietètica de fitasa en la millora de la digestibilitat del fòsfor (P) a les aus de corral, però encara hi ha inconsistències entre els estudis sobre els efectes de la fitasa en els diferents criteris de resposta estudiats. Aquestes inconsistències poden ser degudes a molts factors, un d'ells la durada dels assaigs. En aquest estudi es van perseguir dos objectius: 1) Com la inclusió dietètica de fitasa, a dosi normal i sobredosi, podria afectar la utilització dels nutrients i el rendiment en gallines ponedores joves. 2) Investigar els efectes de reduir el contingut de minerals (Ca i P) i afegir diferents dosis d'una nova 3-fitasa bacteriana a les dietes de pollastre en un experiment a curt i llarg termini. Així doncs, es van dissenyar dos experiments:

Experiment 1) Es van utilitzar un total de 192 gallines ponedores per avaluar l'efecte del contingut de minerals de la dieta i la dosi de fitasa sobre la utilització de nutrients, la producció i la qualitat d'ous i la mineralització òssia de les gallines ponedores joves. Es van estudiar quatre tractaments dietètics: PC, control positiu sense fitasa afegida, 4,07% Ca i 0,61% P; NC, control negatiu sense fitasa afegida, 2,97% Ca i 0,37% P; i P500 i P1000, on la dieta NC es va complementar amb fitasa a 500 i 1000 FTU/kg, respectivament. El rendiment de les gallines i els trets dels ous es van controlar de les 22 a les 31 setmanes d'edat. Els coeficients de digestibilitat aparent total del tracte (CTTAD) dels nutrients es van determinar a les 25 i 31 setmanes d'edat. La digestibilitat ileal aparent (AID) i el contingut

sanguini de Ca i P, així com els trets ossis, es van determinar a les 31 setmanes d'edat. Els resultats d'aquest estudi van demostrar que la retenció de Ca i P era més alta en els ocells amb dieta PC a les 25 setmanes, però no a les 31 setmanes d'edat en comparació amb els que feien dieta NC ($p < 0,05$). Els ocells P1000 tenien els valors CTTAD més alts de matèria seca i orgànica a les dues edats ($p <0,001$). El CTTAD de Ca va ser significativament més alt a la dieta P1000 que a la dieta NC a les 31 setmanes d'edat ($p <0, 001$). Els ocells alimentats amb dieta P500 a les 25 setmanes d'edat i P1000 a les 31 setmanes d'edat van mostrar un CTTAD i una retenció de P més alts, però una excreció menor de P que els alimentats amb dieta NC ($p <0, 05$). La inclusió de fitases va augmentar linealment l'AID de matèria seca i P ($p <0, 001$). Les gallines P500 alimentades tenien el pes corporal més gran al final de l'assaig ($p < 0, 05$) i les aus P1000 van tenir la millor relació de conversió d'alimentació ($p < 0, 05$). Les aus alimentades amb una dieta de PC van produir ous amb un gruix de closca i un color de rovell més alts que els alimentats amb dieta NC ($p <0, 05$). La inclusió de fitases va augmentar linealment el color del rovell ($p <0, 05$). La tíbia de les gallines ponedores alimentades amb PC tenien un contingut de cendra significativament més alt que les que feien dieta NC ($p <0, 05$), i els ocells alimentats amb P1000 presentaven valors intermedis.

Experiment 2) En aquest experiment es van realitzar dos assaigs de durada diferent (assaigs a curt i llarg termini), per avaluar els efectes d'aportar nivells deficients (NC) o correctes (PC) de Ca i P i diferents dosis d'una nova fitasa (250, 500 i 1.000 FTU/kg d'alimentació) en els broilers sobre el rendiment de creixement, la digestibilitat i retenció de nutrients i la mineralització de la tíbia. Es van utilitzar un total de 80 i 490 pollets mascles (Ross) de 21 dies i 1 dia d'edat en els assaigs a curt (17 dies) i llarg (42 dies), respectivament. Els ocells es van pesar i es va registrar periòdicament la ingesta d'aliments durant el període experimental. En ambdós assaigs, es van seleccionar 80 animals als 28 dies d'edat per a un assaig d'equilibri de nutrients en gàbies metabòliques i, al final de

l'assaig de digestibilitat (38 dies d'edat), es va sacrificar un pollet per gàbia (8 animals per tractament). determinar la mineralització de la tíbia. Els resultats d'aquest estudi van mostrar que en l'assaig a llarg termini, els pollets alimentats amb dietes NC van mostrar un rendiment inferior (p <0, 05) en comparació amb els pollets alimentats amb PC, 500 i 1000 FTU/kg d'alimentació durant el període inicial, i. una digestibilitat significativament més alta per a P que els alimentats amb la dieta de PC. Pel que fa als efectes de la fitasa, l'alimentació de dietes de 250 a 500 FTU/kg va augmentar la major part de la digestibilitat dels nutrients en l'assaig a curt termini, mentre que no es van detectar efectes sobre nutrients diferents de P en l'assaig a llarg termini. La mineralització de la tíbia va augmentar linealment amb l'addició de fitasa (p <0, 05) només en l'assaig a llarg termini.

Com a conclusió dels resultats de dos experiments, es pot concloure que seria aconsellable augmentar la dosi de fitasa en l'alimentació de les gallines ponedores per obtenir beneficis a llarg termini i l'edat dels animals és clau per determinar els efectes dels minerals. nivells i addició de fitasa en els pinsos de pollastre i sent més notable en pollastres joves. Pel que fa a minerals i nutrients cavar estibilitat, la durada de l'assaig és clau a causa d'un possible fenomen d'adaptació dels ocells a baixes aportacions de P augmentant la seva digestibilitat i retenció en assaigs llargs en comparació amb a curt termini tant en gallines ponedores com en pollastres.

Abbreviations

ADFI: average daily feed intake

ADG: average daily gain

AID: apparent ileal digestibility

AME: apparent metabolizable energy

aP: available phosphorus

AppA-So: The appA-So gene, encoding a phytase from Serratia odorifera

BW: body weight

Ca: calcium

Co: cobalt

CP: crude protein

CTTAD: coefficients of total tract apparent digestibility

Cu: copper

DFI: daily feed intake

DM: Dry matter

EE: Ether extract

FCR: feed conversion ratio

Fe: iron

GE: gross energy

GLM: general lineal model

K: potassium

ME: metabolizable energy

Mg: magnesium

Mn: manganese

N: nitrogen

NC: negative control with no added phytase

Ni: nickel

nPP: non-phytate P

NSP: non-starch polysaccharides

OM: organic matter

P: phosphorus

PC: positive control with no added phytase

SEM: standard error of the means

Zn: zinc

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Introduction

Chapter I

INTRODUCTION

Feed has a great economic impact on the production of broilers because it represents 70 to 80 percent of the total production cost (Gunasekar, 2007). Proper nutrition is based on the principle that the animal receives the right amount of nutrients, such as protein, carbohydrates, fats, vitamins, and minerals, to participate in all biochemical processes in the body. Among the minerals required by poultry, phosphorus (P) and calcium (Ca) are the most important not only for optimal growth, but also for the mineralization and that participate in the processes of metabolism and absorption of nutrients. Phosphorus is one of the essential minerals and nutrients for birds and its deficiency leads to negative effects such as skeletal deformities, disorders of metabolic processes and ultimately poor use of nutrients and reduced performance (Scott et al., 1982). In addition, P is the most expensive mineral in the final cost of feed (Gomes et al., 2004). Cereals and plant proteins make up the bulk of bird feed. Unfortunately, much of the P in plant sources is not available to birds, because the availability of P from plant foods is affected by an anti-nutritional factor called phytate. Phosphorus is primarily in the form of phytate, that is stored in plant seeds, and form a variety of insoluble salts with most minerals including P, Ca, magnesium (Mg), zinc (Zn) and copper (Cu). In addition, phytate can also difficult the access to other nutrients including proteins, starch, and lipids.

In feeds, P is added in the form of phosphate. Global crude phosphate reserves are the most important sources for maintaining the P cycle (Rodehutscord, 2008). However, mineral P reserves are limited in the environment. For this reason, P supplements are an important factor in significantly increasing the cost of feed in bird feed. In fact, in the diet formulation, P is the third most expensive nutrient after energy and protein. Also, the high P content of slurry and manure suppose a challenge, with the accumulation of P in soils and the threat to surface water quality that may result from P losses to waterways due to runoff or leaching. In addition, over-enrichment of nutrients and excretion of undigested phosphate

through faeces can cause other adverse environmental effects, such as surface water atrophy and thus pose a significant threat to aquatic life. Hence, P has a major environmental impact if not managed properly.

Therefore, improving the digestibility of P from dietary plant sources can be effective in reducing the final cost of feed, the levels of mineral supplementation and the release of undigested nutrients into the environment.

Monogastric animals have almost no endogenous phytase or very little secretion, which is an enzyme that can liberate P, Ca, protein, and other phytate-binding nutrients and increase P digestibility. For this reason, microbial external phytase is commonly used in diets to reduce the harmful effects of phytate on P digestibility, the environment and feed costs (Correll, 1999; Tejedor et al., 2001). The use of phytase in poultry diets may release cations and other nutrients also limited by phytate-P complexes, improving growth performance and metabolism in broilers. There are many commercial phytases currently on the market, but not all of them work in the same way, and the activity or efficiency of phytase supplementation depends on several factors, such as dose, ratio of Ca and P in the diet, diet composition, genotype, and the age of birds (Adeola and Cowieson, 2011).

Effective nutrient management is the key to sustainable production and can reduce the release of P into the environment. This is especially important in highly efficient poultry (with rapid growth or high laying index) that require a high-quality feed, with supplements that enable animals to use effectively the nutrients in their feed. The use of exogenous phytases is key to provide P to animals minimizing the use of mineral phosphates. However, there are still unknowns to be resolved that may lead to a poor interpretation of the results, or inconsistencies among studies with phytases. One of these issues is the effect of the duration of the exposure to experimental diets on phytase effects at different levels (digestibility, performance, health and mineral retention). The present

Doctoral Thesis addresses this specific effect. In the following lines, the use of exogenous enzymes in poultry feeding with special emphasis on phytase effects is reviewed.

1. USE OF EXOGENOUS ENZYMES IN POULTRY NUTRITION

As mentioned above, feed is the largest cost in the poultry industry system, accounting for about 70% of the total production costs. Birds naturally produce enzymes as natural catalysts to digest nutrients, but it is worth noting that the nutrient digestion efficiency in the poultry diet is not 100%. The rate of nutrient digestion depends on the feed or raw material chemical composition, being between 1 to 40 percent of them not digested, appearing in the faeces. Additionally, there are some components (or antinutritional factors) present in the raw materials most frequently used in poultry feeds (cereals, oilseed meals…), that reduces, relevantly, the digestion of certain nutrients. Some of these components are xyloses, glucans, pectins, or phytates, which birds cannot digest without the help of exogenous enzymes in the feed. On the other hand, birds do not have the enzymes needed to break down completely other nutrients such as cellulose, and consequently raw materials rich in fibre are generally avoided in poultry feeding.

1.1. Objectives of exogenous enzymes in poultry

In the last decades, the inclusion of exogenous enzymes has been promoted to improve growth performance and feed efficiency in poultry feeding. Although improving the nutritive value of feedstuffs is the principal rationale for using enzyme technology (Bedford and Partridge, 2001), there are several other reasons for boosting the use of enzymes in animal feed (Marsman et al., 1997; Ravindran et al., 1999; Hong et al., 2002; Malathi and Devegowda, 2001). Some of them are described below.

a) Complement endogenous gastrointestinal activity. Some nutrients, such as starch and protein, are not completely digested in the small intestine of poultry, especially in young animals with immature digestive tracts. A young broiler is a highly efficient animal in terms of feed conversion to body weight gain but, due to its immature digestive system, almost 25% of the amount of energy and 50% of nitrogen (N) is excreted through faeces (Ravindran, 1995). Therefore, the addition of exogenous enzymes can help them to improve feed efficiency at this age.

b) Removing anti-nutritional factors. Most of the raw materials used today in the animal feed industry could have different anti-nutritional agents, such as non-starch polysaccharides (NSP) or phytate, which may adversely reduce digestion of nutrients and affect poultry performance. For example, the presence of NSP fractions such as xylans or glucans in some grains, such as wheat and barley, increases the viscosity in the gastrointestinal tract, which greatly impairs nutrient uptake. This anti-nutritional effect can be reduced by the addition of industrial xylanases or β-glucanases, which break down the polymers of hemicellulose and xylan and β-glucan, respectively. There are many studies that have shown the effects of adding exogenous enzymes on NSP hydrolysis, reducing viscosity, improving the use of other nutrients and poultry performance, and decreasing the excretion of nutrients, especially N, P, Zn and Cu (Esmaeilipour, 2011; Abd El-Hack et al., 2017a,b; Berwanger et al., 2017). In addition, the use of microbial enzymes addressed to the lysis of phytate, which is often added to commercial pig and poultry diets to improve the availability of P and other nutrients, is very widespread. Exogenous phytase also seems to improve the availability of other nutrients, e.g the hydrolysis of phytate throughout the gastrointestinal tract (Leske and Coon, 1999) or at the ileal level (Rutherfurd et al., 2002) has been shown to improve the digestibility of amino acids at the ileal level (Ravindran et al., 1999a). It has been shown that adding microbial phytases to diets containing

phytate can double and triple the P released from phytate (Van der Klis et al., 1997; Um et al., 2000; Camden et al., 2001; Shirley and Edwards, 2003). The role of enzymes in improving the use of feed nutrients has been studied extensively, with the enzyme phytase having the largest share (approximately 60%; Adeolaand Cowieson, 2011).

c) Improving animal welfare and gut health. The use of exogenous enzymes in poultry is also positively related with gut health. In one hand, it has been widely reported that the use of enzymes degrading soluble NSP, including xylans, glucans or pectins, will reduce faecal moisture and the presence of leg injuries due to bed humidity, increasing general animal welfare (Amerah, 2015; Alagawany et al., 2015; Aftab and Bedford, 2018). On the other hand, derived from the increased digestibility, enzyme can change the amount and shape of the substrates present in the digestive tract and, consequently, the microbiota pattern of the gut (Danicke et al., 1999; Yuan et al., 2017). Additionally, with the use of enzymes, lower levels of nutrients are available to the harmful bacteria present in the digestive tract end points, which ultimately improves the health of the digestive system (Bedford, 2001; Persia et al., 2002; Sun et al., 2015; Hosseini and Afshar, 2017).

d) Reducing the environmental impact of excreta. The poultry industry faces the challenge of decreasing environmental impact of livestock production. Reducing the amount of nutrients (such as P or N) excretion through faeces due to increased digestibility of feed will help to reduce environmental pollution. For example, one of the benefits of using glucanases in grain-fed birds is reducing the amount of waste excreted into the environment and reducing the problems caused by watery stools such as dirty eggs (Choct, 2001). In previous studies, it has been shown that manipulating the protein and P levels of poultry diet by adding amino acid and phytase enzymes can reduce the excretion of P, N, and Cu and, on the other hand, minimize the environmental pollution caused by

these elements when released into the environment (Schoulten et al., 2003; Campestrini et al., 2005; Silva et al., 2008). More than half of the P and N consumed is excreted by birds.

However, several other reasons have been accepted for the widespread use of enzymes in poultry feed. These reasons include:

1) Increasing the range of raw materials, as well as more alternatives in the formulation of diets, with the approach of reducing or eliminating the restrictions related to the use of raw materials with low digestibility.
2) Reduce fluctuations in nutritional value between the same raw materials. The use of enzymes increases the nutritional value of low-quality raw materials and reduces differences in the nutritional value between high quality and low-quality raw materials. In fact, enzymes increase the accuracy of dietary formulations.
3) Decrease faeces moisture and consequently reduce litter moisture in birds consuming diets with high levels of NSP. Low litter quality leads to plantar ulcers, chest burns and reduced carcass quality.
4) Improve the morphological characteristics of intestinal tissue and thus increase the digestibility and absorption of feed.
5) Increase herd uniformity by improving poor animal performance.

1.2. Properties of enzymes

To better understand the limitations of using enzymes, it is necessary to know their properties. Enzymes are produced by all living organisms as natural catalysts. Food is not digested without the presence of enzymes. Enzymes are proteins and are composed of amino acid chains with peptide bonds (Ferket, 1993). The catalytic properties of enzymes are related to the three-dimensional shape and position of amino acids within their molecule. In fact, creating

conditions that significantly alter the structure of the enzyme often leads to a decrease in its activity. As a result, enzymes are overly sensitive to the environment in which they operate and perform best only at the right temperature and humidity, as well as the right concentration of substrate and the desired pH (Acamovic et al., 1996; Rastogi et al., 2007; Mulisa, 2016).

The main factors affecting enzyme activity are (adapted from Mulisa, 2016):

A) Moisture content: Enzymes need a hydrated liquid environment to perform their catalytic activities. Moisture is required for the movement and solubility of both enzymes and substrates.

B) Temperature: In general, the activity of enzymes is normal up to 40ºC and decreases significantly with further increase in temperature due to changes in the structure and protein nature of the enzyme.

C) pH: Most enzymes are denatured (altered and destroyed protein structure) in environments with high and low pH. In general, the best pH range for enzyme activity is around 4 – 6, depending on the enzyme. If the enzymes are resistant and stable in the process of acidification which takes place during the passage of the gastrointestinal tract, these substances will act for a longer period. Resistance to pH changes means the ability of an enzyme to resume its activity after a temporary change in pH. This feature is especially important for enzymes that need to work in the gut without breaking down.

D) Amount and concentration of enzyme: In theory, the reaction ratio between the enzyme and the substrate is related to the concentration of enzyme. The rate of this reaction increases as the amount of enzyme increases, because there are more active sites available for the interaction with the targeted substrate and these reactions continue if the relevant enzyme and substrate are present. However, in practice, due to the limitations of the animal digestive system, this relationship is not so and in fact it is nonlinear.

E) Origin: The origin of the enzyme production (fungi, bacteria, or yeast) is a major factor in how enzymatic reactions are performed. In fact, enzymes produced with different microbial sources have different efficiencies in the gastrointestinal tract of animals.

F) Substrates or raw materials: In different raw materials, substrates, nutrients, and anti-nutrients are present in a complex combination, affecting enzyme access to nutrients. The physical and chemical structure of most substrates is still not well understood. For example, the chemical structure of NSP is very different in different raw materials. Therefore, a better understanding of substrates will help to increase the efficiency of using enzymes.

From an enzymatic point of view, raw materials in feed can be divided into three groups (Acamovic et al., 1996):

1) Raw materials for which the digestive system of birds themselves produce enzymes suitable for their digestion (starch, proteins, and lipids).

2) Raw materials for which birds do not produce the enzymes needed to digest them (cellulose).

3) Raw materials for which birds do not produce the enzymes needed to digest them (or produce them at very low levels) and have anti-nutritional effects (β-glucans, pentosans and phytates).

1.3. Types of exogenous enzymes

The use of exogenous enzymes, especially carbohydrases and phytases, is almost ubiquitous in poultry and pig feed, because, as mentioned above, the inclusion of plants in feed supposes the inclusion of some low-digestibility compounds, as well as some anti-nutritional agents such as NSP and phytic acid (Slominski et al., 2006). Some of the most widely used enzymes in the past few years include cellulase (β-glucanases), xylanases and related enzymes, phytases, proteases,

lipases, and galactosidase enzymes. Non-starch polysaccharides in cereals (such as barley, wheat, rye, and triticale) are undesirable antinutritional carbohydrates because they reduce the digestion and absorption of all nutrients in the diet, especially fat and protein. Today, gluconases, xylanases, and β-glucanases are used in almost all wheat and barley-based diets worldwide. Recently, there has been considerable interest in the use of phytase as a feed additive, as it not only increases phosphate availability in plants, but also reduces environmental pollution. In recent years, the use of phytases has increased compared to glucanases as the main enzyme used in poultry diets (Khatak et al., 2006). Several other enzymatic products are currently being evaluated in the feed industry, including proteases to improve protein digestion, lipases to improve lipid digestion, β -galactosidases to neutralize certain antioxidants in non-cereal foods, and even amylase to aid starch digestion in the early stages of animals. The most important are described below:

Xylanases: Xylanases randomly break down soluble and insoluble NSPs in the fibre part of plant cell walls, and reduce digestion viscosity, improve digestion, nutrient release, and food permeability (Adeola and Bedford, 2004; Collins et al., 2005). Xylanase is generally added to broiler diets to improve energy expenditure and growth performance. Adding wheat-based diets with phytase and xylanase compounds increases nutrient and energy intake as well as growth performance in broilers (Selle et al., 2009).

Galactosidases: They are mainly used for diets that are formulated with a high percentage of soybean, because soybean contains more than 40% of carbohydrates that are not digested, and this causes viscous substances in the intestine. In soybean and canola meals arabinans, arabinogalactans, galactans, galactomannans, mannans, and pectic polysaccharides predominate (Graham and Aman, 1991). This is probably part of the anti-nutritional effects of soybean

meal. Anti-nutritional activity of these cell wall NSPs had impaired impacts on growth rate and feed efficiency (Kalantar et al., 2015).

Amylases: Their action on starch by hydrolysis of 1,4 glycosidic linkages, increases its digestibility and complements the endogenous amylase function of the bird and releases energy for metabolism. Stefanello et al., (2017) found that supplementing amylase with diets containing different varieties of maize improved bird performance in energy use. On the other hand, by increasing the digestibility of starch, it reduces the amount of glucose as the potential substrate for non-beneficial bacteria becomes the second part of the gastrointestinal tract (Weurding et al. 2001).

Pectinases: They degrade pectin α-1,4- linked anhydrogalacturonic acid and, as a result, breaks down indigestible pectin and reduce digestive viscosity. It was shown that the addition of pectinase to chickpea-based diets increased feed intake and eventually increased growth rate (Igbasan et al., 1997).

Proteases: They hydrolase storage and structural proteins, increasing their digestibility and disrupting the interaction of proteins with starch and fibre in the diet. They reduce the effect of some dietary anti-nutritional agents, such as trypsin and lectin inhibitors in soybean meal and other plants, thereby increasing the digestibility of nutrients. Various studies have reported that supplementing the diet of broilers with protease enzyme improves the digestibility of amino acids in ingredients or complete diets (Adebiyi and Olukosi, 2015; Cowieson and Roos, 2016).

B-glucanases: They degrade β-glucan by cleaving β-1,3(4) glycosidic linkages and breaks down fibre. Supplementation of barley-based poultry diets with exogenous β-1,3-1,4-glucanases improves feed utilization efficiency, growth and improves the use of low-cost feed materials (Bedford, 2000).

Lipases: They increase the fat digestibility by its hydrolysis and thereby improving its digestibility. Al-Marzooqi et al. (1999) showed that increasing the level of lipase supplementation (0 to 11,250,000 U/kg feed) in diets containing 4% fat of mixed animal and plant fats increased the digestibility of ME and EE in broilers.

Beta-mannanases: β-Mannan is an anti-nutritive fibre found in soybean meal and other vegetable sources. β-Mannanases breaks down the β-Mannan and as a result β-mannanase releases mannan-oligosaccharides of different lengths. β-mannanase hydrolyzes glycosidic bonds between mannan structures and thus improves the nutritional value of the feed. β-mannase appears to reduce the viscosity of the ileum and thus create an environment that enhances nutrient digestion and gastrointestinal health (Liu et al. 2015; Ahirwar et al. 2016; Tewoldebrhan et al. 2017). β-mannase supplementation has been reported to increase feed conversion ratio, mean daily increase and carcass weight in poultry (Wu et al. 2005; Li et al. 2010; Cho and Kim, 2013).

Phytases: Most P in plant-based animal feed is found in phytate storage form. On the other hand, P is a major dietary requirement for feeding poultry to ensure bone growth, eggshell quality, and growth, among others. Phosphorus in phytate is not available to poultry because it lacks the phytase enzyme, and phytase breaks down this phytate molecule. Therefore, the inclusion of phytase enzyme in poultry feed is required to release P bound to phytate. Several studies have examined the effect of phytase on P digestion in birds. For example, in a study by adding 1000 FTU/kg of phytase to corn soybean meal diets of the poultry increased ileal P digestibility from 54.7 to 66.2% (Camden et al., 2001).

Multienzyme complexes: The use of multi-enzymes complexes containing several enzymes is another recent topic in poultry feeding. All living organisms, including microorganisms, plants, and animals, produce enzymes. Since

enzymes are very specific for their catalytic reactions, when an enzyme cocktail containing several activities is used in a broiler diet, it is more likely to have greater effect than when they are used separately. Additionally, we must bear in mind that in the formulation of commercial feed, the source of cereals and fibrous by-products can change frequently depending on the price and availability. Considering the variability of these in the different types of NSPs that they contain, the supplementation of a multi-enzymatic complex would a priori produce a greater coverage in the improvement of the nutritional value of the different formulas.

For example, complete *in vitro* dephosphorylation of phytate has been shown to occur only if a combination of enzymes is used (Zyla et al., 1996). Another study showed that the use of an enzymatic cocktail consisting of mannase, β -glucanase and xylanase improved feed conversion ratio (FCR) in broilers and increased NSP degradation and the major NSP monomers including arabinose, xylose and mannose in the ileum and cecum (Hanseo Ko et al., 2021). Also, Attia et al. (2008) showed that the use of a multi enzyme can reduce the cost of poultry rations and Woyengo et al. (2019) reported increases in P and CP digestibility through the addition of amylase to a basal diet containing 1000 FTU/kg phytase.

Although positive effects of including multienzyme complexes in poultry feeding are found, there are many inconsistencies on the results regarding the effects of using several enzymes on poultry performance among studies. These effects seem to depend on the effect of diet composition, age, type of chickens, composition, and type of enzymatic mixture (Attia et al., 2003; Choct M. 2006; Hussein et al., 2019).

2. PHYTATE AND PHYTASES

Phytate is an anti-nutrient found in most plant foods but is a useful source of nutrients because it is the most important form of P storage in plants. In general,

the three terms phytate, phytin, and phytic acid are used to describe the phytase substrate, but the most common term is phytate, which refers to a mixed salt of phytic acid (myo-inositol hexaphosphate, IP^6). In fact, phytic acid contains six phosphate groups crosslinked with a hexagonal hydroxyl myoinositol group (Rimbach, 1997; Figure 1). Around 60-80% of total P in grains and its by-products is in the form of phytate. Phosphorus is the third most valuable nutrient after energy and protein due to its high nutritional and environmental importance in poultry and swine nutrition (Letourneau-Montimy et al., 2011).

Due to its special chemical structure, IP^6 binds to positively charged molecules and nutrients and forms a very stable insoluble complex. In fact, this is an important anti-nutrient property of IP^6. This special property makes it compatible with other minerals such as Mg, K, Cu, Zn, Ni, Co, Mn, Fe and Ca, forming insoluble complexes, and even strongest bonds with Cu and Zn (Selle et al., 2009).

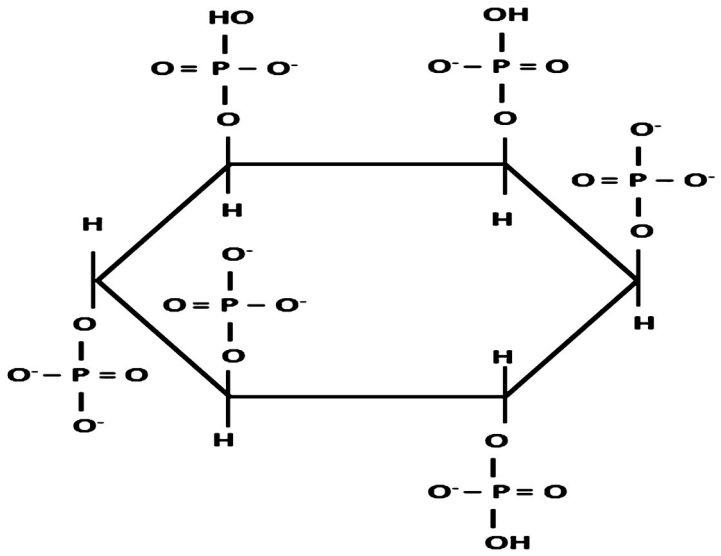

Figure 1: Structure of phytic acid at neutral pH, based on the Anderson model (Source: Erdman, 1979).

Due to the degradation of phytate, P and other restricted nutrients are released and made available to poultry for absorption. Various technologies such as heat treatment, mechanical processing, storage, soaking, germination, and fermentation are used to better degrade phytate (Oloffs et al., 2000; Humer et al.,

2013; Kraler et al., 2014). However, one of the most practical and effective methods for degrading phytate in poultry feed is the use of exogenous microbial phytase enzyme. Microbial external phytase is commonly used to reduce the harmful effects of phytate on yield, environment and reduce feed costs (Amerah et al., 2014).

Phytases (inositol hexaphosphate phosphohydrolase) are important enzymes that can stepwise hydrolyze phytate and release phytate-bound P (Haefner et al., 2005). The removal of the phosphate group starts with a fully phosphorylated phytic acid (IP^6), followed by penta- (IP^5), tetra- (IP^4), tri- (IP^3), di- (IP^2) and monoesters of inositol in descending order of preference (Yu S et al., 2012).

The lack of an international standard unit for measuring phytase activity has led to considerable confusion in the commercial feed industry and a comparison of the efficiency of different phytase sources. The defined unit of measurement of phytase activity depends on the assay conditions such as the concentration of substrate (sodium phytate) used, temperature and pH. Phytase activity is usually expressed as FTU (Phytase Units). One FTU is the activity of a phytase required to release 1 μmol of inorganic phosphate per minute from an excess of 15 M sodium phytate substrate at 37ºC and pH 5.5 (Greiner and Konietzny, 2006). This definition provides a useful measure of the amount of phytase activity and represents a simple measurement under well-defined assay conditions.

There are four possible sources for phytases (Greiner et al., 1997; Sandberg and Andlid, 2002; Greiner and Konietzny, 2006):

A. Intestinal phytase found in digestive secretions, usually at low concentrations (animal origin). Animal phytases are produced by intestinal mucosa and are largely found in the duodenum.

B. Phytase originating from microbiota in the digestive tract.

C. Endogenous phytase from plant feedstuffs. The detected phytase activity in most plant feedstuffs is considerably low and due to a narrow pH spectrum of activity, more susceptible to proteolytic digestion and thermal destruction during feed processing.

D. Phytase produced by exogenous microorganisms. Microbial sources are more widely used for commercial phytase production. Previous research has shown that phytases of microbial origin are auspicious for use in the commercial biotechnology of enzymes due to their catalytic properties and ease of enzyme production (Haefner et al., 2005) and most scientific work has been done on microbial phytases.

Several distinct phytases produced by exogenous microorganisms are now commercially available. They are classified according to **1)** their origin (fungal or bacteria), **2)** the carbon in the myo-inositol ring of phytate at which dephosphorylation is initiated (3 or 6-phytase) and the presence of any protection against high temperatures (coated or non-coated).

As mentioned above, phytases fall into two categories depending on the site where the hydrolysis of the phytate molecule is initiated: 3-Phytase (EC 3.1.3.8; myo inositol hexakis phosphate-3-phosphohydrolase) preferentially hydrolyzes the ester bond at the third position of myo inositol hexakis phosphate to produce d-myo-Ins-1, 2, 4, 5, 6-pentakisphosphate and orthophosphate, whereas 6-phytase (EC 3.1.3.26, myo inositol hexakis phosphate-6phosphohydrolase) hydrolyzes the ester bond at the 6th position of myo inositol hexakisphosphate to produce d-myo-Ins-1, 2, 3, 4, 5-pentakisphosphate and orthophosphate. It has been reported that 3-phytases are primarily of microbial origin and 6-phytases are mainly isolated from plant sources (Cosgrove and Irving, 1980), but there are exceptions, e.g., soya phytases are 3-phytases and *E. coli* phytases are 6-phytases (Sandberg and Andlid, 2002). It has been observed that new generation bacterial phytases have specific properties for IP^6 and IP^5 and have a higher resistance to

proteolytic digestion than phytase fungi (Adeola and Cowieson, 2011). In literature studies, large variations in the equivalent inorganic P value of phytases have been reported, and this may be related to different factors such as animal species, method of analysis, feed composition (level of phytate and Ca in the diet), adaptation time and type of phytase used.

2.1. Factors affecting phytase activity

Many factors can have an influence on *in vivo* phytase activity, which can be divided into enzyme, dietary and animal-related factors.

2.1.1. Enzyme related factors affecting phytase activity

Numerous research has been done in the last decade to develop phytases in animal nutrition. However, more research is needed to make optimal use of them.

An ideal phytase for use in animal feed must have several quality criteria:

A) Effective and maximum release of phytate phosphate in the gastrointestinal tract

B) Stability in the feed preparation process

C) Stability during feed storage

D) Low cost

The effective release of phytate phosphate in the gastrointestinal tract depends on factors such as temperature, pH, humidity, and phytase resistance to endogenous enzymes such as protease.

2.1.1.1. Optimal temperature, pH, and humidity range

Phytases, like other enzymes, are sensitive to temperature because their structure is made up of proteins and can therefore be denatured at high temperatures. The optimum temperature range for the activity of most phytases is between 44 to 60

°C, but in the meantime, some of them show optimal activity at higher or lower temperatures. For example, the temperature required for the maximum activity of *A. fumigatus* and *β. amyloliquefaciens* phytases is higher (70°C). Some studies have proven that phytses from *A. fumigatus* maintained at 90°C for 20 minutes only decrease their optimal activity a 10% (Kim et al., 1998; Choi et al., 2001). Other phytases require temperature for optimal activity of 25 °C or lower such as Enterobacter aerogenes phytase (Vohra and Satyanaryana, 2003; Lei et al., 2007). The phytase origin has an impact of the optimal temperature for maximum activity. For example, the optimal activity of fungal phytases is between 50-60 °C, but yeast phytases require higher temperatures (60-75 °C; Lei et al., 2007). Also, plant phytases usually had lower optimum temperatures than microbial phytases, being grain phytases the ones that require the lowest temperatures for optimum activity (Konietzny and Greiner, 2002).

As previously explained, due to the protein structure of phytases, phytases with a higher optimal temperature are preferred by the feed industry since in the pelletizing process, temperature can reach 80 to 85°C for a few seconds, and at this temperature some phytases will be denatured. It is also possible to add phytases to feeds after the pelleting process, in the form of liquid phytases sprayed after cooling the pellets. This practice can provide an opportunity for enzymes sensible to pelleting temperatures.

One of the important factors that affect the solubility of phytate is pH, and phytate is more soluble at lower pH values, which is why the complexes formed between phytic acid and minerals are soluble in the acidic conditions of the gastrointestinal tract. (Schlemmer et al., 2009).

Phytases have different pH optimal ranges. The optimal activity of most microbial phytases is around pH 5.0, but there are exceptions, such as Bacillus phytases, which shows the best optimal activity at pH 8.0 (Vijayaraghan et al., 2013). However, the source of phytases also influences the optimal pH. Various

studies have reported that the optimal activity of most plant phytases is around pH 5.0, while the optimal pH for microbial phytase activity is 2 to 6 (Yu et al., 2012).

As shown in Figure 2, according to various studies (Kumar et al., 2003; Morales et al., 2011), *E. coli* phytases are active at a lower pH range than fungal phytases. Also, the phytase activity curve at different pH values can be different for *E. coli*, due to the technology of production and expression of bacteria.

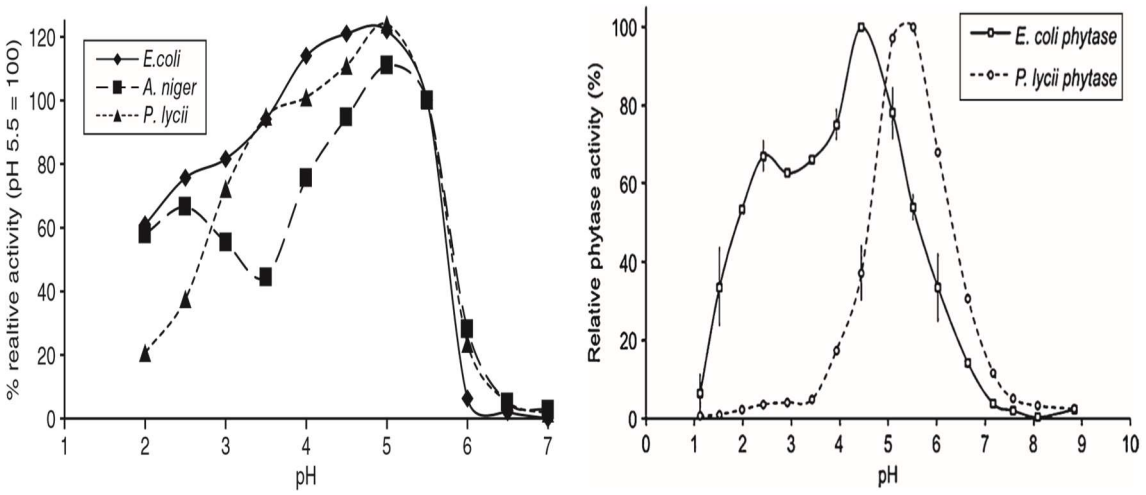

Figure 2: Relative activity of different commercial phytases. Left-hand figure compares three commercial phytases (*A. niger*, *E. coli* and *P. lycii*) when using the activity at pH 5.5 as 100% (Source: Kumar et al., 2003). Right-hand figure compares two commercial phytases (*E. coli* and *P. lycii*); the maximum phytase activity recorded was considered as 100% (Source: Morales et al., 2011).

Enzymes have a specific optimum pH. When they are placed in an environment that has a different pH, their activity decreases, but they often maintain their stability. If the optimal pH is provided their activity is reversed, as shown by Kumar et al. (2003).

As mentioned earlier, phytase activity is generally measured at 37 °C and pH 5.5, however, these are not actually real conditions, as gastric pH is significantly lower than pH 5.5. Therefore, different commercial phytases are significantly

different due to the need for a pH for optimal activity *in vivo*. It has been reported that one of the most effective ways to prevent the reduction of the anti-nutritional effects of phytate is the complete hydrolysis of phytate in the upper gastrointestinal tract as quickly as possible (Yu et al., 2012). Therefore, maintaining the effect of enzymes added to the diet in monogastric animals at the acidic pH of the stomach is of particular importance. If enzymes are resistant to acidification of substances that pass through the gastrointestinal tract, these enzymes can maintain their optimal activity for a longer period and by resisting pH changes during passage through the gastrointestinal tract. The enzyme can resume its activity after a temporary change in pH, and this is a very important feature for enzymes that must function in the intestine without proteolytic decomposition. Therefore, one of the signs of the effectiveness of a phytase in the stomach and upper part of the small intestine is to have an optimal pH.

Another important factor that has a great impact on the activity of phytase enzyme is humidity because enzymes need a liquid or aqueous environment to perform their catalytic activities and moisture is one of the basic needs for the movement and solubility of enzymes and substrates. In addition to temperature and pH, phytase activity is directly dependent on the amount of moisture (Carlson and Poulsen, 2003; Haraldsson et al., 2004). Most phytases are found in cereal grains in the aleurone and scutellum, thus in the layers of dry cereals, where phytases are inactivated due to lack of moisture (Oatway et al., 2001).

2.1.1.2. Phytase resistance to endogenous protease

Because of their protein structure, phytases may be affected by the activity of endogenous proteases in the gastrointestinal tract of animals and may be hydrolyzed. In a study conducted by Kumar et al. (2011), the response of phytases derived from *P. lycii*, *A. niger* and *E. coli* to endogenous protease activity was evaluated. Phytase derived from E. *coli* was more resistant to protease than the other two. This may partly explain the difference in bio sensitivity between

commercial phytases in monogastric animals (Morales et al., 2011). In another study, two commercial phytases (*E. coli and P. lycii*) were incubated with pepsin or crude trout extract of rainbow trout. After 1 hour of incubation, *P. lycii* phytase activity was rapidly reduced, while *E. coli* phytase maintained its activity for 4 hours (Figure 3). They reported that *E. coli* phytase has a high resistance to protease. Therefore, there is a great variety in the resistance of different commercial phytases to proteases in the stomach.

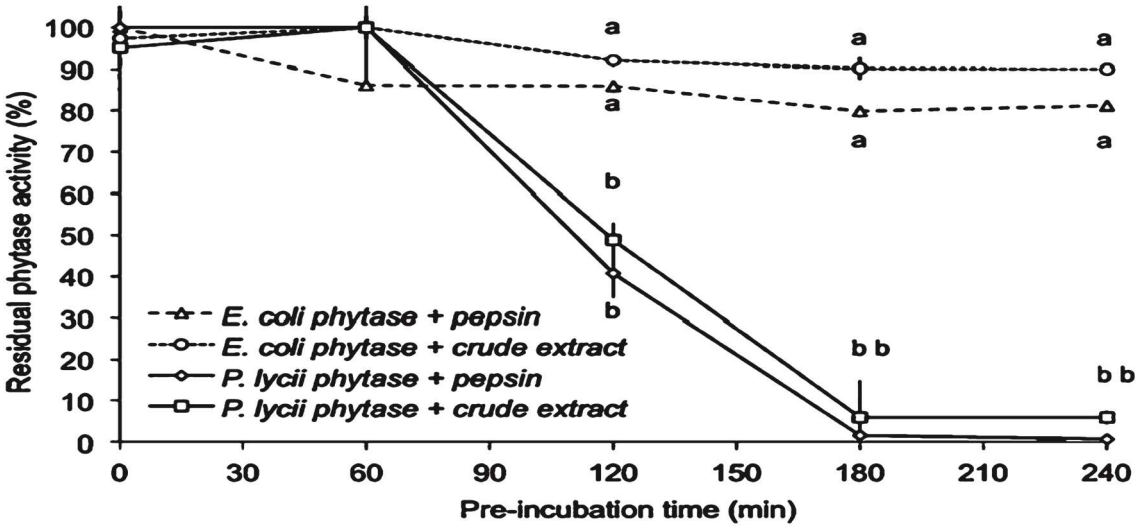

Figure 3: Residual phytase activity of *E. coli* and *P.lycii* phytase after incubation with pepsin or a gastric crude extract from trout stomach for up to 4h (Morales et al., 2011).

2.1.1.3. Phytase activity in the presence of ions

Some ions can reduce or inhibit phytase activity by forming insoluble complexes with the enzyme phytase. In this regard, Zn and Cu often stop phytase activity (Konietzny et al., 1995; Konieczny and Greiner 2002). Also, in the study of Konietzny and Greiner (2002) it was reported that fluoride is a known inhibitory agent for acid phosphatase and has inhibitory effects on the activity of acid phytases and is often active in the presence of ions with a concentration of 5 mM normally. The phytase enzyme from *A. fumigatus* has been reported to be an exception to this rule (Wyss et al., 1999).

2.1.1.4. Dosing and super-dosing phytase

Two of the most important factors affecting the optimal activity of phytase enzyme are the level of dietary phytate and the phytase dose. The release of P bound to phytate by exogenous phytase is directly affected by the dose of phytase enzyme. Supplementing a diet with doses greater that the commercial (500 FTU/kg), such as 1500 FTU/kg or above, could improve mineral utilization and performance (Kies et al., 2006; Walk et al., 2014; Manobhavan et al., 2016). The use of extraordinary doses of phytase in the diet of broilers has been reported to improve the digestibility of amino acids, minerals, energy and thus animal performance (Cowieson et al. 2011, 2013). Bone length and proximal epiphyseal width are higher in broilers fed phosphorus-containing diets containing 1000 and 2500 FTU phytase than in broilers fed diets supplemented with 500 FTU phytase, and these varied quality characteristics Bone has been reported by various studies using an extraordinary dose of phytase (Rutherfurd et al. 2012; Chung et al. 2013; Manobhavan et al. 2016). In a study by Kim et al. (2017), they studied the effect of extra dose of phytase on egg production performance and quality in laying hens, and the results of this study showed that the use of an extraordinary dose of 20,000 FTU/kg phytase It had a positive effect on egg production in laying hens' diets but did not have a beneficial effect on egg quality. On the other hand, Farhadi et al. (2017) reported that high doses of phytase enzyme in the diet of broilers can cause excessive dryness of the substrate and as a result cause dermatitis of the lower leg.

Thus, it is generally accepted that using high doses of phytase may improve the use of phosphate and other nutrients in the diet, thereby reducing costs and the negative impact of livestock on the environment.

However, for a more economical and efficient use of phytase, it is important to better understand the activity of phytase *in vivo*. Phytase that works over a wide range of temperatures and pH and is active up to the stomach and upper intestine

(along with several other properties, such as resistance to endogenous enzymes) will be an ideal phytase for animal feeding. In addition, the dose used for phytase should be calculated by considering the composition of the diet and its uses.

2.1.2. Animal related factors affecting phytase activity

Animal related factors, including species and age of animals, can have a relevant effect on the efficacy of added phytase in the gastrointestinal tract.

2.1.2.1. Species

One of the animal-related factors that can affect the optimal performance of the enzyme phytase is the animal species. Because monogastric animals such as pigs, birds, and fish (as well as humans) lack the phytase enzyme in their digestive system or their phytase activity is very low, they are unable to utilize the P in the phytate structure. Therefore, in order to supply P, it is necessary to add P in mineral and absorbable form to their food, which in turn increases feed costs and environmental pollution (Lei and Stahl, 2001; Lei and Porres, 2003; Naves et al., 2012; Dersjant-Li et al., 2015). The physiology of digestion and absorption of nutrients in pigs and birds is quite different from ruminants and other monogastric animals.

In pigs and birds, the phytase-producing microbiota is located in their large intestine, which is assumed to release phosphate in this part of the gastrointestinal tract. As phosphate is not able to be absorbed in this place, it is excreted and out of reach of the animal (Nitsan et al., 1991; Nir et al., 1993). Marounek et al. (2010) demonstrated with a diet based on wheat, corn and SBM without microbial phytase supplementation that the highest specific phytase activity (per gram of digestion) in layers was observed in cecum and that, in general, the phytase activity was moderate in the small intestine and low in the crop and stomach.

The addition of exogenous phytase changes the site where phytase has its main activity from the large intestine (endogenous phytase) to the stomach and upper small intestine (exogenous microbial phytase) in most monogastric species (pigs, poultry, and fish) (Kemme et al., 1998; Marounek et al., 2010). In the case of pigs, a study of phytase activity in the gastrointestinal tract of young pigs using a diet containing *A. niger* phytase showed that the highest percentage of digestion occurs in the upper gastrointestinal tract (Kornegay, 1996). Another study in young piglets using a diet containing *E. coli* phytase found that phytase had the highest activity in the stomach and upper jejunum, and no activity was observed in the lower jejunum or ileum (Pagano et al., 2007). In this study, pigs fed phytase supplementation showed dose-dependent phytase activity in the upper gastrointestinal tract and reduced P and Ca concentrations in the large intestine. Also in laying hens, Marounek et al. (2010) demonstrated that a high total phytase activity was found in the caeca, an intermediate level in the small intestinal content and mucosa, and a low level in the crop and stomach, but the highest (approximately 45.9%) phytase activity per gram of digestion was in the caeca.

Thus, the addition of microbial phytase in the diet is necessary to increase the hydrolysis of phytate in the upper gastrointestinal tract and improve P utilization in pigs and poultry.

On the other hand, birds and pigs show differences in their digestive physiology that can affect phytase effects such as the speed at which food passes through the gastrointestinal tract. In birds, feed passes through the gastrointestinal tract faster than pigs, so in birds it takes about 2-4 hours, but in pigs, it takes more time and takes about 12-24 hours (Johansen et al. 1993). Another important difference between different monogastric species is viscosity. Intestinal viscosity is higher in birds, and these differences can also lead to different responses to the addition of exogenous enzymes (Bedford et al., 1992a).

Limited information is available on the activity of phytases for cold-water fish, but one study reported that species of fish with stomachs had also the highest phytase activity in the upper gastrointestinal tract (Yan et al., 2002).

2.1.2.2. Age

The effects of phytase on P digestibility have been extensively studied in the last two decades, but most studies on broilers have been around 21 days old and less attention has been paid to the effect of age on optimal phytase performance. The growing period is one of the most critical stages in poultry breeding, during which the nutrients necessary for the optimal growth of all body organs and other metabolic functions must be accurately provided. During the first week of life, the gut forms a small portion of the body mass, and the next two weeks are critical for the body to reach its maximum growth (Obst and Diamonds, 1992). Throughout this time, the bird's ability to extract nutrients may be limited, resulting in restrictions on growth. Additionally, the digestion of young birds is not yet fully developed, and the passage of feed occurs much faster than in older birds. Therefore, the effect of phytases in young animals are expected to be greater than in older birds, especially in the first weeks of breeding, since they have lower enzymatic secretions than adult birds (Marounek et al., 2010; Li et al., 2015; Babatunde et al., 2019a, b).

In the case of laying hens, they are usually reared until the age of 76-72 weeks and egg production begins around the age of 19 weeks. At the age of 28-26 weeks, egg production increases sharply and reaches the peak of production and then decreases. It has been reported that the availability and persistence of P and Ca decreases in older hens (Al-Batshan et al., 1994). From these results it can be concluded that there is probably a direct relationship between reduced P availability and egg production in old age.

In other study by Cambra-Lopez et al., (2020), the effect of pig age on the efficacy of a new microbial 3-phytase, in barley-wheat-based diets in weaned, growing and finishing pigs was examined. The results showed that phytase has the potential to increase the digestibility of Ca and especially P in growing pigs. Digestible energy (DE) also increased with the addition of phytase in weaned pigs (+0.69 MJ/kg of dry matter (DM); $P < 0.001$), and the inclusion of 3-phytase in the diet increased the average daily weight from 46 to 94 days (+0.07 kg/d; $P < 0.05$). The authors reported that age affects phytase activity and thus the ability to digest P and Ca, being this greater in weaned and growing pigs.

2.1.3. Dietary related factors affecting phytase activity

One of the factors that significantly affects phytase activity is the concentration of minerals in the diet, especially Ca and P. As an example, the formation of calcium phosphate deposits due to the high concentration of Ca in the diet can reduce P uptake through the intestine in poultry. It has been reported that calcium levels higher than 4-6 g/kg reduce the hydrolysis of phytate by phytase and leads to poorer performance and bone mineralization in poultry (Sebastian et al., 1996b). Also, in high levels of Ca in feeds can promote that phytate binds to cations such as Fe^{3+} and Ca^{2+} in the small intestine, reducing the solubility of phytate and thus reducing its availability by phytase (Grases et al., 2003). It has been reported that in layin hens without the addition of phytase and by increasing dietary Ca from 30 to 40 g/kg, phosphorus phytate degradation was reduced from about 33% to 9%, but with the addition of 500 FTU kg^{-1} phytase, this reduction was reduced from about 76% to 65% (Van der Klis et al., 1997).

Therefore, the Ca content in the diet can have a great impact on P phytate utilization and optimal phytase activity. It has also been reported that due to the high solubility of Ca in fine limestone, the particle size of limestone can affect phytase activity (Manangi and Coon, 2007).

As a result of these interactions, other studies have reported that reducing the ratio of Ca:P in the diet increases phytase efficiency and improves P digestion and retention and performance in pigs fed low P phytase supplemented diets (Qian et al., 1996; Liu et al., 1998).

Total dietary P levels can also affect the degradation of ileal P phytate. Libert et al. (1993) measured the digestibility of P ileal phytate with and without the use of exogenous phytase in broilers. In this study, it was observed that the disappearance of P phytate at the end of the small intestine in birds without and with the addition of 1000 FTU kg-1 *A. niger* phytase was 16.1% and 62.5%, respectively, at a dietary total P level of 4.8 g kg−1 and 23% and 65.4%, respectively, at a dietary total P level of 3.6 g kg−1.

2.2. A novel long-term phytase

As mentioned before, there are many commercial phytases available nowadays for animal feeding in the market. In a recent study, Salaet et al. (2021) has reported a novel phytase highly stable overtime with a low Michaelis-Menten constant (Km), showing significant advantages over other conventional phytases. The *AppA-So* protein retained more than 85% of its initial activity after incubation in different pH conditions (pH 2.5−6.5) at 37 °C for 3 h (Figure 4).

To obtain this phytase, the *AppA-So* gene encoding a phytase from Serratia odorifera was cloned and heterologously expressed in *Komagataella phaffii*. The open reading frame of *AppA-So* comprised 1281 bp that encoded a 426-amino acid protein, including a 27- amino acid signal peptide. The purified recombinant phytase showed optimal activity at 55 °C and pH 4.5, exhibiting enzymatic activity between pH 3.7 and 5.8, with a specific activity of 1123 U/mg at pH 4.5 and 37 °C (Salaet et al., 2021).

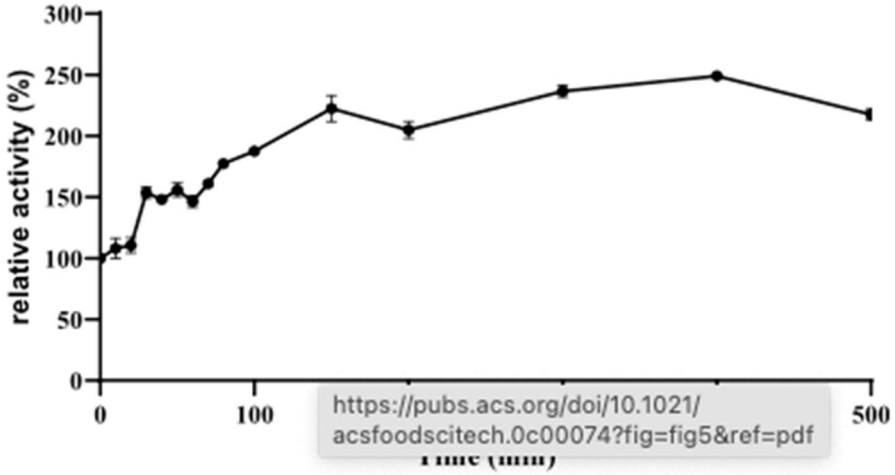

Figure 4. Maintenance of phytase activity over time. The specific activity per minute (measured following the ISO 30024 method) was determined at different time points after addition of the substrate (Salaet et al. 2021).

The comparison of this phytase with other well-known phytases suggests that the *S. odorifera* phytase has the lowest Km and highest stable activity over time, making it very suitable for use in the animal feed industry. With these conditions, this novel phytase could maintain a high speed even with low concentrations of the substrate.

When used in pigs, its inclusion has improved both P and Ca digestibility, with improvements in growth, feed conversion, and bone development observed until the pigs are 154 days in age (Cambra-López et al., 2020). On the other hand, a high dose of this phytase improved the average daily gain and the tibia weight in broilers (Hamdi et al., 2018).

However, there is no information about the effectiveness of this novel phytase both in short- and long-term periods in poultry, to determine its potential as a new commercial phytase for broiler and hen nutrition. The present Doctoral Thesis comprise the results obtained in two studies focused on determining the main effects of adding a novel phytase in short and long-term experiments conducted in broilers and laying hens.

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OBJECTIVES

Dietary mineral availability and phytase effectivity depend on many dietary, animal, and environmental factors. Most of these factors have been widely studied. However, the possible effect of the duration of the trials on mineral availability and phytase effectiveness has been not studied in deep in poultry. Our hypothesis is that, as animal digestive physiology usually shows adaptative mechanisms, duration of trial could affect the biological response of the exogenous phytase in the animals. This Doctoral Thesis explored this effect using a new 3-phytase, with the aim of highlighting the importance of the duration of the trials to evaluate correctly mineral nutrition in poultry.

To achieve this goal, two partial objectives were defined in this Doctoral Thesis:

- To determine the effect of mineral content and phytase dose on performance, nutrient utilization, and bone mineralisation in laying hens varying the duration of the trial and animal age.
- To determine the effect of mineral content and phytase dose on performance, nutrient utilization, and bone mineralisation of broilers varying the duration of the trial.

These two objectives have been addressed in two separate chapters:

Chapter 1: Effect of dietary mineral content and phytase dose on nutrient utilization, performance, egg traits and bone mineralization in laying hens from 22 to 31 weeks of age.

Chapter 2: The duration of the trial influences the effects of mineral deficiency and the effective phytase dose in broilers' diets.

Chapter I

Effect of Dietary Mineral Content and Phytase Dose on Nutrient Utilization, Performance, Egg Traits and Bone Mineralization in Laying Hens from 22 to 31 Weeks of Age

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Simple Summary

The aim of this work was to elucidate how the dietary inclusion of phytase, at a normal dose and overdosed, could affect the utilization of nutrients and performance in young laying hens. When a diet deficient in Ca and P was applied, the dietary inclusion of phytase at low doses (500 FTU/kg) led to an improvement in the digestive efficiency of P in the first weeks after introduction. However, when these deficient diets were maintained in the long term, laying hens improved their digestive utilization of both Ca and P, a higher dose of phytase (1000 FTU/kg) being required to achieve greater P availability. This overdosage also provided additional extraphosphoric advantages, slightly improving access to other nutrients and the feed conversion rate of the hens.

Abstract

A total of 192 laying hens were used to evaluate the effect of dietary mineral content and phytase dose on nutrient utilization, egg production and quality and bone mineralization of young laying hens. Four dietary treatments were studied: PC, positive control with no added phytase, 4.07% Ca and 0.61% P; NC, negative control with no added phytase, 2.97% Ca and 0.37% P; and P500 and P1000, where NC diet was supplemented with phytase at 500 and 1000 FTU/kg, respectively. Hens' performance and egg traits were controlled from 22 to 31 weeks of age. Coefficients of total tract apparent digestibility (CTTAD) of nutrients were determined at 25 and 31 weeks of age. Apparent ileal digestibility (AID) and blood content of Ca and P, as well as bone traits, were determined at 31 weeks of age. Ca and P retention was higher in birds on PC diet at 25 weeks, but not at 31 weeks of age compared to those on NC diet ($p < 0.05$). P1000 birds had the highest CTTAD values for dry and organic matter at both ages ($p < 0.001$). CTTAD of Ca was significantly higher in P1000 diet than in NC diet at 31 weeks of age ($p < 0.001$). Birds fed with P500 diet at 25 weeks of age and P1000 at 31

weeks of age showed higher CTTAD and retention of P, but lower excretion of P than those fed NC diet ($p < 0.05$). Phytase inclusion linearly increased AID of dry matter and P ($p < 0.001$). P500 hens fed had the greatest body weight at the end of the trial ($p < 0.05$) and P1000 birds had the best feed conversion ratio ($p < 0.05$). Fowl fed a PC diet produced eggs with higher shell thickness and yolk color than those fed on NC diet ($p < 0.05$). Phytase inclusion linearly increased the yolk color ($p < 0.05$). Tibia of laying hens fed with PC had significantly higher ash content than those on NC diet ($p < 0.05$), and birds fed with P1000 presented intermediate values. It can be concluded that it would be advisable to increase the dose of phytase in the feed of laying hens to obtain long-term benefits.

Keywords: laying hen; phytase; age; dose; digestibility; egg quality; mineralization

1. INTRODUCTION

Phytases have been widely studied by scientists in the field of nutrition, environmental protection and biotechnology. These enzymes can release phosphate from phytate, which is the main form of phosphorus (P) storage in grains frequently used in animal diets. On the other hand, monogastric animals such as birds, pigs and fish lack phytase enzyme in their digestive system, or its activity is very low. Therefore, it is necessary to add mineral P in diets in the form of inorganic phosphate to meet the animals' requirements. However, mineral phosphates are limited, expensive and non-renewable resources, and their use can cause environmental problems (Lei and Stahl, 2001). In addition, phytate is also known as an anti-nutritional agent, as phytate can form insoluble complexes, binding minerals and other nutrients (such as vitamins, proteins and amino acids), reducing their availability and absorption (Woyengo and Nyachoti, 2017). Production of high-quality eggs worldwide has a great impact on the economic dynamism of the egg industry. Laying hens have a metabolism that is highly

dependent on the availability of minerals, such as Ca and P, in order to maintain effective egg production without compromising their mineral health status. This is especially important in young laying hens, where a reduction in the dietary level of minerals is promoted to encourage their feed intake (Scott et al., 1971) at peak laying time. Ca and P requirements seem to be affected by the hens' age and production level, e.g., several studies have shown that the quality of eggshells decreases with age (Pelicia et al., 2009). Additionally, the dietary Ca to P ratio is also relevant at these ages in laying hens (Pelicia et al., 2009; Bougouin et al., 2014). Low rates reduce Ca absorption in the intestine, lead to decrease in shell quality, and could have severe long-term negative effects on Ca metabolism and bone reserves. However, high rates do not provide sufficient P and may cause a decrease of skeletal mineral content in laying birds. In this context, dietary inclusion of exogenous phytases could contribute to reducing mineral supplementation in young laying hens, promoting their feed intake, egg production and bone mineralization (Lim et al., 2003), although some studies have observed no positive effects (Hughes et al., 2009).

Dietary exogenous phytase supplementation is widely used to improve dietary P availability in broilers (Selle and Ravindran, 2007), as phytase hydrolyzes the phytate present in grains, releasing the phytate-P, with the associated reduction in P excretion. However, the number of studies on the use of exogenous phytases in laying hens is smaller, and some authors affirm that the benefits of supplementing layer diets with phytase are still under discussion (Liebert el al., 2005; Bougouin et al., 2014). Although some authors have indicated that phytase inclusion in the diet at 250–500 FTU units can improve dietary P absorption (Boling et al., 2000; Francesch et al., 2005), there is no consensus on its possible effect on improving dietary energy and protein utilization, and therefore, on laying hens' performance and bone mineralization (Selle and Ravindran, 2007). In fact, to improve the utilization of these nutrients, some authors mention that superdosing these exogenous phytases (1000 FTU or more) could eliminate

phytates from the diet, contributing to an improvement in the nutritional value of the diet (Cowieson et al., 2011). Another aspect not frequently considered is the possible appearance of compensatory effects when P-deficient diets are used, as some studies indicate that the length of trials could affect the efficacy of phytases in layers (Bougouin et al., 2014).

In this context, the present work is focused on elucidating how the inclusion level of phytases in P-deficient diets provided in short and long term could affect the utilization of P, but also the rest of the nutrients, and their possible effect on young laying hens' performance. Therefore, the aim of this study was to evaluate the effect of dietary inclusion level of a 3-phytase at 500 and 1000 FTU/kg on nutrient digestibility and egg production and quality, as well as on bone mineralization in young laying hens from 22 to 31 weeks of age.

2. MATERIALS AND METHODS

2.1. Animals and housing

A total of 288 laying hens (Lohmann Brown) at 16 weeks of age were initially used. Animals came from a commercial breeding farm. The study lasted for 46 days of rearing and 60 days of experimental period. Upon arrival, hens were randomly distributed into 72 cages (4 animals/cage) located in two environmentally controlled rooms (36 cages per room). Cages (60 × 50 × 120 cm) were equipped with a feeder trough, two nipple drinkers and all the environmental enrichment elements according to Directive 1999/74/CE. At week 22 of age (day 1 of the experimental period), 192 hens were weighed and randomly distributed in 4 different treatments, with 12 replicates/treatment (48 animals/treatment). Experimental feeds were provided from week 22 of age until week 31 of age. At week 28 of age, an indigestible marker (titanium dioxide, TiO_2) was added to the experimental diets at 4 g/kg. The marked feeds were provided until the end of the trial (week 31 of age). During the 106 days of study (rearing

and experimental period), room temperature was controlled and maintained around 20 °C.

2.2. Experimental diets

During the rearing period, all animals were fed a common commercial breeding feed based on corn, wheat and barley (weeks 16 and 17 of age) and laying feed based on wheat and corn (weeks 18 and 21 of age) until they reached a daily production of 1.07 eggs per hen. At 22 weeks of age, hens were assigned to one of the four dietary treatments: PC, positive control with no added phytase: Ca at 4.07% and P at 0.61%; NC, negative control with no added phytase: Ca at 2.97% and P at 0.37%; and two other diets in which NC diet was supplemented with ePhyt 1000® 3-phytase (Globalfeed) at 500 (P500) and 1000 (P1000) FTU/kg feed, respectively (see enzyme details at Cambra-López et al., 2020). All the diets were formulated following the recommendations given for laying hens by FEDNA 2019, except for Ca and P in the negative control, being isonutritive for the rest of the nutrients (Table 1). Feed and water were provided ad libitum throughout the experiment and diets were fed in mash form.

2.3. Laying performance and egg quality

Body weight (BW) was recorded per cage on arrival (16 weeks of age), at the start of the administration of experimental feeds (22 weeks of age) and at weeks 25 and 31 of age of the experimental period. Feed consumption was recorded at each weighing control to calculate average daily feed intake (DFI). Health status of the animals was checked daily and necropsies were performed from all dead animals. The number of eggs laid and their weight were daily monitored. Average laying index (egg/hen and day), egg weight (g) and egg mass (laying index x egg weight; g/day) were determined weekly. Average daily feed intake (DFI; g/day) and feed conversion ratio (FCR; g feed/g egg) were also calculated globally. Furthermore, the number of eggs with shell quality problems (soft

shelled eggs, shell-less eggs) was registered daily. On days 52, 53 and 59 of the experimental period, all the eggs laid in the last 24 h (approximately 150 eggs/treatment) were collected for egg quality measurements.

Table 1. Ingredients and chemical composition of the experimental diets (g/kg).

Ingredients and Chemical composition	Positive Control	Negative Control
Ingredients		
Corn grain	592.5	610
Soybean meal 44% CP	276	276
Soybean meal oil	25.4	25.4
DL-Methionine	1.6	1.6
Calcium carbonate	80.0	75.0
Dicalcium phosphate	16.0	3.5
Salt	3.45	3.45
Red coloring (canthaxanthin 10%)	0.05	0.05
Vit-min premix [1]	5	5
Chemical composition		
Dry matter	899	900
Ash	118	111
Crude protein	170	172
Ether extract	44.1	44.4
Gross energy (kcal/kg)	3652	3815
Apparent metabolizable energy (kcal/kg)	2819	2913
Metabolizable protein	90	87
Calcium	40.7	29.7
Total phosphorus	6.1	3.7
Phytate phosphorus	1.7	1.7
Non-phytate phosphorus [2]	4.4	2.0

[1] Provides per kilogram of feed: calcium: 200.61g, E5 manganese (manganese oxide): 13000 mg, E6 zinc (zinc oxide): 7400 mg, E4 copper (copper sulphate pentahydrate): 800 mg, E2 iodine (potassium iodide): 380 mg, E8 selenium (sodium selenite): 20 mg, E1 iron (carbonate ferrous): 3600 mg, E672 vitamin A: 1500000 UI, E671 vitamin D3: 300000 UI, vitamin K: 300 mg, vitamin B2: 600 mg, vitamin B12: 2000 mg, niacin: 3000 mg, calcium pantothenate: 1400 mg, pantothenic acid: 1288 mg, betaine: 10830 mg, choline chloride: 25500 mg, E320 butylhydroxyanisol (BHA): 4 mg, E321 butylhidroxytoluene (BHT): 44 mg, E324 ethoxyquin: 6.40 mg, dry matter: 956.54 g. [2]Calculated as the difference between total phosphorus and phytate phosphorus.

The sampled eggs were individually weighed and broken on a flat surface. Subsequently, the height of the inner thick albumen (Haugh units) was measured with an electronic albumen height gauge. The Haugh units were calculated $100 \times \log_{10} (H + 7.57 - 1.7W^{0.37})$, where H is the height of the albumen and W is the weight of the egg, according to Haugh (Haugh, 1937). The shells were broken in three parts and shell thickness was a mean value of measurements at these three locations taken by using a dial pipe gauge (3001 digital Baxlo, Instrumentos de

Medida y Precisión S.L., Barcelona, Spain). Additionally, yolk color was determined by the Roche yolk color fan (Hoffmann-La Roche Ltd., Basel, Switzerland; color scale from 15, dark orange, to 1, light pale).

2.4. Fecal and ileal digestibility

At weeks 25 and 31 of age, a nutrient retention balance was performed. Total excreta output and feed intake were measured quantitatively per cage (12 cages/treatment) for two days. During each of these 2-day collection periods, excreta were collected every 24 h, weighed and stored at 4 °C. At the end of the collection period, excreta were pooled per cage and homogenized. Representative samples were then taken and stored at −20 °C until analysis. Feed samples were dried at 105 °C for 24 h and then ground up. Excreta samples were dried at 80 °C for 48 h and then ground. Dry matter (DM), ash, crude protein (CP), gross energy (GE), Ca and P were determined in feeds and excreta samples. Ether extract (EE) and phytate-P and TiO_2 were also determined in feeds.

The coefficients of total tract apparent digestibility (CTTAD) for DM, organic matter (OM), GE, CP, Ca and P were calculated using the following equation:

$$CTTAD\ (\%) = \frac{[(Feed\ intake\ \times\ Nutrient_{feed}) - (Excreta\ output\ \times\ Nutrient_{excreta})]}{(Feed\ intake\ \times\ Nutrient_{feed})} \times 100$$

At the end of the trial (31 weeks of age), all birds were euthanized by stunning and exsanguination to obtain the ileal content. The ileum was removed by cutting the portion of the small intestine from Meckel's diverticulum to about 5 mm proximal to the ileocecal junction (Francesch et al., 2005; Adedokun et al., 2014). A 4 mL syringe full of room temperature distilled water was inserted at one end of the ileum and the digesta were carefully flushed out of the gut into a 10 cm diameter Petri dish (Rutherfurd et al., 2004; Gao et al., 2013) The digesta from all birds in a cage were pooled and stored at −80 °C until laboratory analysis. Ileal content was lyophilized and analyzed for DM, TiO_2, Ca and P.

The apparent ileal digestibility (AID) of Ca and total P was calculated by the relation:

$$AID\ (\%)\ =\ \left[1-\left(\frac{TiO_{2\ feed}\ \times Mineral_{digesta}}{TiO_{2\ digesta}\ \times Mineral_{feed}}\right)\right]\times 100$$

2.5. Bone mineralization and blood analysis

From the animals slaughtered at the end of the trial, one bird per cage (12 animals per treatment) was randomly selected to evaluate bone mineralization. The left tibia from this animal was obtained and frozen, after removing all the soft tissues, at −20 °C until analysis. Tibias were boiled to remove the remaining soft tissues, cleaned and dried at 110 °C for 12 h. Then, tibias were degreased in an ether solution for 48 h. Once cleaned and degreased, tibias were dried again at 110 °C for 12 h, weighed and then ash, Ca and P content was determined.

Blood samples from each animal were also collected at 31 weeks of age, into two 4 mL vacutainer tubes with serum clot activator, refrigerated and transported to the laboratory to determine Ca and P content.

2.6. Analytical methods

DM (934.01), ash (942.05), EE (920.39) with acid hydrolysis prior to ether extraction and CP (990.03) were analyzed according to AOAC methods (AOAC, 2019). GE was determined using an adiabatic bomb calorimeter (Gallenkamp Autobomb, Loughborough, UK). Mineral (Ca and P) content was analyzed by inductively coupled plasma atomic emission spectrometry (ICP-OES) (model Varian 720-ES, Varian Inc., Palo Alto, CA, USA), as described in Cambra-López et al. (AOAC, 2019). Phytate-P was analyzed by spectrophotometry according to the method described by Haug and Lantzch (Haug et al., 1983). TiO_2 concentration was analyzed in feeds and ileal content according to the methodology proposed by Short et al. (1996).

2.7. Statistical analyses

Data were analyzed using SAS System Software (version 9.1, SAS Institute Inc., Cary, NC, USA). The experimental unit was the cage for ADFI, FCR, body weight, egg production and nutrient balance traits; the egg for the egg quality traits; and the hen for the mineral content in tibia and blood.

Data on hen performance and egg production traits were analyzed in a repeated measures design taking into account the variation between animals and covariation within them. Covariance structures were objectively compared using the strictest criteria (Bayesian information criterion; Littell et al., 1998). The model included the treatment (PC, NC, P500 and P1000), the age (25 and 31 weeks of age) and their interactions as fixed effects. Random terms in the model included a permanent effect of each animal (p) and the error term (e), both assumed to have an average of zero, and variance σ_p^2 and σ_e^2. Data on CTTAD, AID, egg quality, bone and blood traits were analyzed according to the general lineal model (GLM) in a completely randomized design with a model accounting for the fixed effect of the treatment (PC, NC, P500 and P1000), the age (25 and 31 weeks of age) and their interactions. Additionally, polynomial orthogonal contrasts were applied to test linear (L) effects among treatments NC, P500 and P1000. Results were presented as least square means with their standard error of the means (SEM). Statistical significance level was set at 5% (0.05).

3. RESULTS

Table 2 shows the effect of dietary phytase inclusion on nutrient CTTAD, as well as on mineral retention and excretion for the laying hens' diets at 25 and 31 weeks of age. There was a clear effect of diet (mineral level and phytase) on all these parameters, as well as a significant diet x age interaction for CP, GE and P CTTAD; Ca and P retention; and P excretion. In general, the CTTAD of main nutrients was significantly higher at 31 than 25 weeks of age ($p < 0.001$). Among

diets, animals fed with the PC diet showed higher CTTAD of OM, CP and GE, as well as Ca retention and Ca and P excretion, but lower CTTAD of Ca and P, compared to those fed on NC diet ($p < 0.05$). Phosphorus retention was higher in animals on the PC diet at 25 weeks of age, but not at 31 weeks of age (diet x age; $p < 0.05$).

Table 2. Effect of mineral level (diet) and phytase inclusion on nutrient coefficient of total tract apparent digestibility (CTTAD, %), retention (ret; g/d animal) and excretion (exc; %) of diets in laying hens at 25 and 31 weeks of age.

Item					Ca			P		
	DM	OM	CP	GE	CTTAD	Ca Ret	Ca Exc	CTTAD	P Ret	P Exc
25 weeks of age										
PC	71.3 ^{b}	74.9 ^{a}	50.4 ^{b}	76.8 ^{a}	57.3 ^{b}	2.54 ^{a}	42.7 ^{a}	20.8 ^{b}	0.134 ^{a}	79.2 ^{a}
NC	71.8 ^{b}	73.4 ^{b}	46.0 ^{c}	75.9 ^{b}	63.6 ^{a}	2.05 ^{b}	36.4 ^{b}	24.2 ^{b}	0.089 ^{b}	75.8 ^{a}
P500	72.3 ^{ab}	74.7 ^{a}	51.9 ^{ab}	76.7 ^{ab}	63.5 ^{a}	2.44 ^{a}	36.5 ^{b}	30.1 ^{a}	0.128 ^{a}	69.9 ^{b}
P1000	72.8 ^{a}	75.1 ^{a}	53.0 ^{a}	76.7 ^{ab}	65.2 ^{a}	2.05 ^{b}	34.8 ^{b}	24.9 ^{b}	0.101 ^{b}	75.1 ^{a}
SEM	0.36	0.331	0.761	0.341	1.52	0.082	1.52	1.65	0.011	1.65
31 weeks of age										
PC	71.8 ^{b}	75.9 ^{a}	55.2 ^{a}	77.6 ^{a}	58.3 ^{c}	2.68 ^{a}	41.7 ^{a}	10.1 ^{c}	0.058 ^{c}	90.9 ^{a}
NC	72.7 ^{b}	74.6 ^{b}	55.0 ^{a}	76.8 ^{ab}	67.3 ^{b}	2.62 ^{ab}	32.7 ^{bc}	31.7 ^{b}	0.129 ^{b}	68.3 ^{b}
P500	72.2 ^{b}	74.6 ^{b}	54.1 ^{ab}	75.3 ^{c}	64.6 ^{b}	2.50 ^{ab}	35.4 ^{b}	29.9 ^{b}	0.120 ^{b}	70.1 ^{b}
P1000	73.9 ^{a}	76.2 ^{a}	52.2 ^{b}	76.5 ^{b}	70.6 ^{a}	2.47 ^{b}	29.4 ^{c}	38.9 ^{a}	0.179 ^{a}	61.1 ^{c}
SEM	0.445	0.348	0.994	0.389	1.66	0.086	1.66	2.15	0.011	2.19
p-value										
Diet	<0.001	<0.001	0.02	0.002	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Age	0.014	<0.001	<0.001	0.856	0.008	<0.001	0.008	0.052	0.208	0.077
Diet x Age	0.344	0.117	<0.001	0.002	0.366	0.006	0.366	<0.001	<0.001	<0.001

^{a,b,c,d} Means within a column and age not sharing superscripts differ at $p < 0.05$. Treatments: PC, positive control; NC, negative control; P500, negative control with phytase at 500 FTU/kg feed and P1000, negative control with phytase at 1000 FTU/kg feed. SEM: standard error of the mean. DM: dry matter; OM: organic matter; CP: crude protein; GE: gross energy; Ca ret: Ca retained; Ca exc: Ca excreted; P ret: P retained; P exc: P excreted.

Regarding the effect of phytase on nutrient digestibility, inclusion of the 3-phytase diet increased CTTAD of DM and OM at 25 and 31 weeks of age. Animals fed with P1000 had the highest CTTAD values for DM and OM at both ages (+1.0 and +1.7 percentage points compared to NC, respectively; $p < 0.001$). The effect of phytase inclusion on CTTAD of CP and GE was different depending on the age ($p < 0.01$). The CTTAD of CP in P1000 diet was higher at 25 weeks of age ($p = 0.02$) but lower at 31 weeks of age than in NC diet ($p < 0.002$). Regarding CTTAD of GE, it was significantly lower in P500 than in NC diet at 31 weeks of age ($p < 0.05$).

As for mineral utilization, the CTTAD of Ca was significantly higher in P1000 diet than in NC diet at 31 weeks of age ($p < 0.001$). Finally, dietary phytase inclusion improved CTTAD, retention and excretion of P at both ages, although at different levels of inclusion depending on the age (diet x age; $p < 0.05$). Animals fed with P500 diet at 25 weeks of age and P1000 at 31 weeks of age showed higher CTTAD ($p < 0.05$) and retention of P ($p < 0.05$), but lower excretion of P ($p < 0.05$) than those fed with NC diet.

Apparent ileal digestibility (AID) of Ca and P in 31-week-old layers, as well as their concentration in blood, is presented in Table 3. In general, values for AID of DM, Ca and P were lower than those of CTTAD presented in Table 2. Animals fed with PC diet showed lower Ca ileal digestibility and higher Ca and P concentration in blood than those on NC diet ($p < 0.05$). Dietary inclusion of the 3-phytase did not affect Ca ileal digestibility or blood concentration of Ca and P at 31 weeks of age. However, AID of both DM and P with P1000 diet was significantly higher than with NC diet ($p < 0.001$).

Table 3. Apparent ileal digestibility (AID) and blood concentration of calcium (Ca) and phosphorus (P) of laying hens at 31 weeks of age.

Traits	PC	NC	P500	P1000	SEM	*p*-Value
AID, %						
Dry matter[1,2]	68.24 [b]	68.82 [b]	66.58 [b]	76.50 [a]	1.37	<0.001
Ca	42.70 [c]	59.89 [ab]	53.14 [bc]	65.60 [a]	4.27	0.003
P[1]	19.89 [b]	22.91 [b]	29.67 [b]	52.96 [a]	3.52	<0.001
Blood concentration, mg/dL						
Ca	30.47 [a]	28.35 [b]	28.01 [b]	27.87 [b]	0.721	0.043
P	7.35 [a]	5.38 [b]	5.56 [b]	5.98 [b]	0.368	0.002

[a,b,c] Means with different superscripts differ ($p < 0.05$). Treatments: PC, positive control; NC, negative control; P500, negative control with 500 FTU/kg of phytase and P1000, negative control with 1000 FTU/kg of phytase. SEM: standard error of the mean. [1] Linear effect of the phytase inclusion. [2] Quadratic effect of the phytase inclusion ($p < 0.05$).

Table 4 presents the effect of dietary treatments on hens' performance and egg production traits from 22 to 31 weeks of age. Figure 1 shows that weekly egg mass evolution throughout the study was not affected at any time by the different

dietary treatments. Although hens' performance was not significantly affected by mineral level of the diet, animals fed with PC diet produced eggs with a higher shell thickness and yolk color than those on NC diet ($p < 0.05$). Regarding the effect of phytase inclusion, hens fed with P500 diets had the greatest body weight at the end of the trial ($p < 0.05$) and those on P1000 diets had the best FCR ($p < 0.05$). Hens fed with P1000 diets also had the lowest shell thickness values ($p < 0.05$). Dietary inclusion of phytase linearly increased the yolk color ($p < 0.05$), allowing us to achieve the values reached with the PC diet.

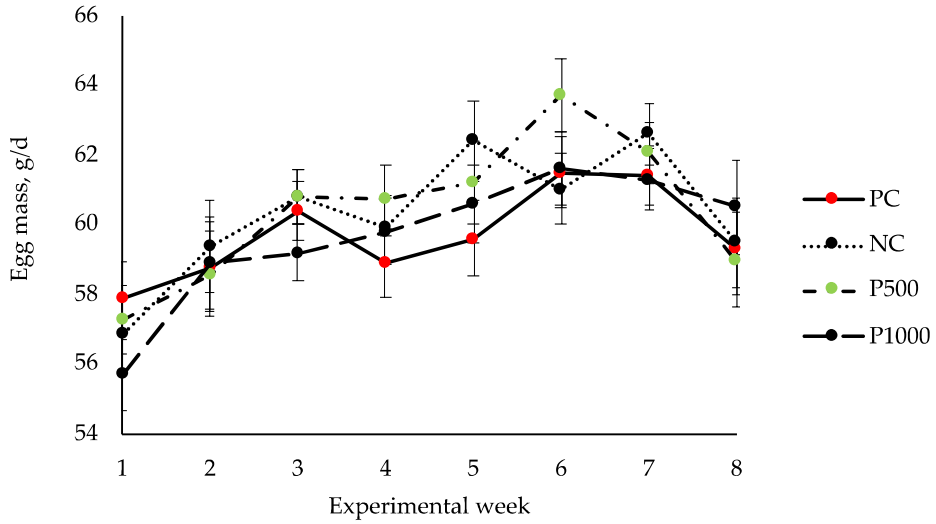

Figure 1. Egg mass evolution over the trial. Treatments: PC, positive control; NC, negative control; P500, NC with 500 FTU/kg of phytase and P1000, NC with 1000 FTU/kg of phytase. *p*-value of treatment = 0.614, *p*-value of week < 0.001, *p*-value of treatment × week = 0.724. Bars represent standard errors.

Finally, Table 5 shows the effect of diets on bone mineralization of young laying hens after 9 weeks of the treatment. Mineral level of the diet significantly affected main tibia mineralization traits. Tibia of laying hens fed with PC had significantly higher ash, Ca and P content than those with NC diet ($p < 0.05$). Dietary phytase inclusion did not significantly affect the main mineralization traits controlled.

However, tibia ash content of animals fed with P1000 had intermediate values, which were not significantly different from those of hens on PC diet.

Table 4. Effect of dietary phytase inclusion on performance and egg production traits of laying hens from 22 to 31 weeks of age.

Traits	PC	NC	P500	P1000	SEM	*p*-Value
Initial body weight, g	1749	1779	1753	1747	0.024	0.759
Final body weight, g	1872 ^{ab}	1851 ^{b}	1901 ^{a}	1840 ^{b}	0.017	0.074
ADFI, g/day	109.2	108	110.5	105.2	1.77	0.176
FCR, g feed/g egg	1.849 ^{a}	1.799 ^{ab}	1.830 ^{ab}	1.758 ^{b}	0.031	0.187
Average laying index	0.973	0.975	0.978	0.98	0.044	0.528
Average egg mass, g/day	59.28	60.56	59.66	59.84	0.696	0.594
Egg traits at 31 weeks of age:						
Shell thickness, mm ^{1,2}	0.382 ^{a}	0.371 ^{b}	0.375 ^{ab}	0.359 ^{c}	0.003	<0.001
Albumen height, mm	11.46	11.24	11.44	11.37	0.152	0.725
Haugh units	104.3	103.4	104.2	104.2	0.595	0.696
Yolk color ^{1,3}	13.81 ^{a}	13.57 ^{b}	13.80 ^{a}	13.95 ^{a}	0.059	<0.001

^{a,b,c} Least square means in a row not sharing superscripts differ at $p < 0.05$. Treatments: PC, positive control; NC, negative control; P500, negative control with 500 FTU/kg of phytase and P1000, negative control with 1000 FTU/kg of phytase. SEM: standard error of the mean; ADFI: average daily feed intake; FCR: feed conversion ratio. ^{1} Linear effect of the phytase inclusion ($p < 0.05$). ^{2} Quadratic effect of the phytase inclusion ($p < 0.05$). ^{3} Points in the Roche scale.

Table 5. Effect of dietary phytase inclusion on bone mineralization traits of laying hens at 31 weeks of age.

Traits	PC	NC	P500	P1000	SEM	*p*-Value
Tibia weight, g	6	5.96	5.81	5.81	0.197	0.845
Tibia weight, % BW	0.322 ^{a}	0.308 ^{ab}	0.301 ^{b}	0.299 ^{b}	0.007	0.113
Ash in tibia (% DM)	52.0 ^{a}	49.8 ^{b}	49.6 ^{b}	50.8 ^{ab}	0.463	0.001
Ca in tibia (% DM)	18.72 ^{a}	18.09 ^{b}	17.92 ^{b}	18.29 ^{ab}	0.235	0.064
P in tibia (% DM)	8.78 ^{a}	8.39 ^{b}	8.38 ^{b}	8.51 ^{b}	0.085	0.001

^{a,b} Least square means in a row not sharing superscripts differ at $p < 0.05$. Treatments: PC, positive control; NC, negative control; P500, negative control with 500 FTU/kg of phytase and P1000, negative control with 1000 FTU/kg of phytase. DM: dry matter; BW: body weight. SEM: standard error of the mean.

4. DISCUSSION

4.1. Nutrient utilization

Domestic animals excrete about 15 million tons of phosphorus into the environment every year (Cordell et al., 2009). Numerous studies have reported that mineral supplementation in commercial feeds increases phosphorus intake and its excretion rate, which could cause serious environmental problems, as phosphorus sources are limited and non-renewable resources (Mallin, 2000; Lei and Stahl, 2001; Lei and Porres, 2003; Lei et al., 2007). In our study, mineral

supplementation below commercial levels led to higher CTTAD and AID of Ca and higher CTTAD of P, regardless of the age of the young laying hens. In fact, higher mineral provision with PC diet (+25% Ca and +64% P), together with lower mineral digestibility compared to NC diet, led to higher Ca and P excretion. Other studies also showed higher Ca and P digestibility when diets were deficient in these minerals, indicating that this lower provision in minerals might increase their digestive efficiency. For instance, some studies have observed an increase in P digestibility in hens fed with P-deficient diets, both at fecal and ileal level (Sari et al., 2012), reducing P excreta (Meyer, and Parsons, 2011). In fact, Ren et al. (2020) reported that when dietary inorganic P was overdosed, it was mainly excreted by the laying hens. Regarding Ca, although some studies observed a decrease in Ca digestibility when dietary inorganic P was overdosed (Ren et al., 2020), others have observed the opposite behavior, decreasing Ca digestibility in Ca-deficient diets (Sari et al., 2012; Pongmanee et al., 2020).

The effect of a diet deficient in Ca and P on the use and retention of these minerals seems to change as we maintain this deficit over time. As expected, after only 3 weeks on the experimental diets, the hens with the deficient diet showed a lower daily retention of Ca and P, with a slight reduction in their excretion at 25 weeks. However, after 9 weeks on the deficient diet, these animals seemed to improve their digestive utilization of Ca and P, with no differences being observed in the daily retention of Ca, and this improvement was even greater for those on dietary P compared to those fed with PC, significantly reducing the excretion of both minerals. Recently, Bello and Korver (Bello, and Korver, 2019), in laying hens receiving nutritionally adequate and deficient diets in P from 30 to 70 weeks of age, also observed that hens fed the deficient diet increased both AID of P (from 40 to 53%) and the P retained (from 0.20 to 0.25 g/d) from 32 to 48 weeks of age. This is a relevant issue, as it could also affect the evaluation of phytase effectiveness in the long term.

Despite this improvement in daily mineral retention, the levels of Ca and P in the blood remained lower with the deficient diet. Previous works, where dietary Ca and P levels were similar to those evaluated in this research, showed contradictory results. Some studies observed that an increase in the dietary level of Ca and P did not lead to relevant modifications of these minerals in the blood (Boorman and Gunaratne, 2001; Imari et al., 2020; Ren et al., 2020). However, Sari et al. (2012) reported that a decrease in the P level of the diet led to a clear decrease in the blood P level of the hens (8.01 vs. 4.10 mg/dL), but without modifying the serum Ca level. In fact, Viveros et al. (2002) described a linear correlation between dietary non-phytate P and plasma P for different poultry species. Ren et al. (2020) associated these differences with the blood sample-collecting time used in the different trials.

Regarding the effects of dietary addition of phytase on mineral digestibility, retention and excretion, in the present work a clear interaction between the phytase level and age was found for P utilization. At 25 weeks of age, the P digestibility and retention were higher ($p < 0.05$) and P excretion was lower with the diet including phytase at 500 FTU/kg compared to the NC diet. However, at 31 weeks of age, the highest P digestibility (both fecal and ileal) and retention and the lowest P excretion were found in the group of animals fed the diet including 1000 FTU/kg. Therefore, the results of the present work could indicate that the recommended dose of the phytase for an effective use of P could be age-dependent, with a higher dose being required as the age of laying hens increases. The inclusion of phytase in laying hen diets can increase P digestibility and retention, as phytase hydrolyzes the phytate present in grains, releasing the phytate-P. However, the effective dose of phytase might change depending on the phytase, diet, other mineral levels and age, among other factors (Ahmadi, and Rodehutscord, 2012; Bougouin et al., 2014). Although there are not many studies evaluating the effect of age on the effectiveness of phytases in laying hens, most authors agree that their effectiveness decreases with age. Van der Klis et al. (1997)

found that Ca and P absorption at 36 weeks of age was significantly lower than at 24 weeks. More recently, a meta-analysis of the studies carried out with phytases in laying hens (Bougouin et al., 2014) described a negative correlation between age and the efficacy of phytase in terms of retention of P. These results could indicate that when diets with low levels of non-phytic P are used, the level of inclusion of phytase should increase with the age of the hens to ensure an adequate supply of P to these animals. However, we must also take into account in these studies the time from the introduction of mineral-deficient diets. As we have noted in the present work, laying hens can increase their Ca and P absorption efficiency when they are receiving a deficient diet for a long time, through an increase in the metabolism of renal and intestinal 1,25-hydroxycholecalciferol (Elaroussi et al., 1994). In this sense, Bello and Korver (Bello and Korver, 2019) observed how phytase supplementation in deficient diets at 30 weeks of age improved AID of P at 32 weeks of age, but this advantage disappeared thereafter (at 48 and 70 weeks of age). For this reason, we should avoid short-term trials to evaluate phytase effectiveness, as their commercial use will be long term in deficient diets, and we should probably recommend higher doses than those applied in short-term trials.

The highest values for dietary Ca utilization (both at fecal and ileal level) were obtained when P was also better used, at 31 weeks of age when phytase was overdosed at 1000 FTU/kg. Sometimes, the improvement observed in Ca digestibility with phytase inclusion is the consequence of a drop in digestibility in the control P-deficient diets, as there is an increase in the Ca/P ratio that can promote the formation of insoluble Ca phosphate and reduce Ca solubility in the digestive tract (Pongmanee et alj., 2020). In this work, there was no such fall of Ca digestibility in the NC diet compared to the PC diet, as the Ca/P ratio was barely modified. Therefore, we can assume that the improvement in the Ca utilization observed was mainly due to the fact that phytases are able to liberate not only P, but also Ca from Ca-phytate complexes (Selle and Ravindran, 2007).

Finally, when we overdosed the phytase at 1000 FTU/kg, an improvement in the use of DM and OM, even in that of CP at 25 weeks of age, was observed. In broilers, Dersjant-Li and Kwakernaak (Dersjant-Li and Kwakernaak, 2018) reported a linear increase in both ileal digestibility of total amino acids and apparent metabolizable energy (AME), its effect being different depending on the phytase used and independently of the available P. Selle et al. (2007), reviewing the main mechanisms proposed by the literature for the extraphosphoric effects, proposed that phytate could reduce the digestive utilization of dietary protein and energy by binding to amino acids, increasing mucin and then the loss of endogenous protein and compromising the Na+-dependent transport of starch, glucose and amino acids in the gut. In fact, Lei et al. (2011) observed that the CP and AME content of laying hens' diets could be slightly reduced thanks to the extraphosphoric consequences of phytase supplementation without penalties. However, these benefits could be slightly reduced in the long term, and this should be considered when formulating diets.

4.2. Laying hens' performance and egg quality

Differences in the Ca and P levels between PC and NC diets did not affect laying hens' performance during the 60-day experimental period, thus suggesting that laying hens can maintain optimal medium-term performance when fed a diet containing 2.0 g/kg non-phytate P (nPP), if feed intake is maintained within normal values. Previous reports indicated that diets containing 2.0–2.3 g/kg available P (aP) are enough to maintain hen performance when dietary Ca is within the range of 32.5–40.0 g/kg (Scott et al., 1999; Keshavarz, 2000; Hughes et al., 2008; Kozlowski and Jeroch, 2011). Boling et al. (2000) reported that P deficiency signs in older hens (70 weeks) occurred within only 3 weeks of consuming a diet with 1.0 g/kg aP, compared to 8 weeks in younger hens (20 weeks). The authors suggested that older hens may exhibit P deficiency symptoms sooner than younger hens. However, it seems that there are dietary

interactions between Ca and P in high egg-producing layers, as significant performance depression and high mortality rates are seen when low P content is combined with high Ca in the diet (Hartel, 1990). As the Ca/P ratio was not excessively modified in the present work, these young laying hens were not expected to show alterations in their reproductive performance when fed with a deficient diet from 22 to 31 weeks of age.

In terms of phytase addition, the dietary inclusion of the 3-phytase in the present study increased hens' final weight at 500 FTU/kg inclusion and improved FCR at 1000 FTU/kg. As mentioned above, extraphosphoric effects of phytase inclusion allow greater availability of other nutrients, especially when phytase is overdosed, which could slightly contribute to improving laying hens' performance. Similar results have previously been reported in other works (Viveros et al., 2002; Troesch et al., 2013), supporting the idea that the inclusion of phytases could allow a slight reduction in the level of other nutrients in the diet (Lei et al., 2011).

Literature results indicate that diets with 0.15–0.25% nPP and in the absence of phytase (Gordon and Roland, 1997; Van der Klis et al., 1997; Boling et al., 2000) and diets with 0.10–0.15% nPP supplemented with phytase (Gordon and Roland, 1997; Boling et al., 2000; Keshavarz, 2003; Keshavarz, 2003) are sufficient to maintain satisfactory egg production performance during the laying cycle. Hughes et al. (2009) showed no significant differences in egg production traits of laying hens fed with diets either containing 3.5 or 2.5 g/kg nPP, but those fed with 1.5 g/kg nPP had significantly reduced egg performance and higher incidence of soft-shelled and broken eggs compared to 3.5 g/kg nPP. In fact, the literature suggests that the addition of phytase to 1.0 and 2.0 g/kg nPP diets for hens could improve the hens' weight and feed efficiency (feed to egg mass ratio; Jalal and Scheideler, 2001; Scott et al., 2001), but in general it seems that extremely low levels of P are needed to affect these parameters (Punna et al., 1999; Ahmadi and Rodehutscord, 2012).

In our study, where diets with 2.0 and 4.4 g/kg nPP were compared, we did not observe significant differences in egg production, with or without phytase addition, but we observed both greater shell thickness and value for the yolk color with the diet including 4.4 g/kg nPP. Laying hens require Ca to form amorphous calcium carbonate and calcium phosphate during eggshell calcification (Murakami et al., 2007; Rodríguez-Navarro et al., 2015). However, most of the works reviewed in the literature show that the main determinant of the quality of the shell is the level of Ca and not so much the level of P. Bar et al. (Bar et al., 2002) already observed that an increase in the Ca level produced a clear improvement in the shell weight, while the modifications of the P level had no effect. In fact, most of the studies that evaluated the effect of the inorganic P level, at a constant Ca level, did not observe any significant effect on the eggshell characteristics (Van der Klis et al., 1997; Boorman and Gunaratne, 2001; Ren et al., 2020; Cheng et al., 2020). These results could explain why the PC diet allowed obtaining eggs with a greater shell thickness compared to NC diet, by providing a higher level of Ca, while the greater availability of P due to the inclusion of phytase did not lead to improvements in the shell quality. Regarding the yolk color, there seems to be an association between the dietary level of P and the intensity of yolk color. Several authors (Jang et al., 2008; Kozlowski and Jeroch, 2011; Englmaierova, 2012) have observed an increase in the intensity of the yolk color when they increased the level of inorganic P in the feed. In addition, several studies have reported a similar effect when phytase is added to feed (Kozlowski and Jeroch 2011; Brunelli and Pinheiro, 2012; Millán-Calleja et al., 2017; Dersjant-Li and Kwakernaak, 2018). Brunelli et al. (2012) associated this effect with the hydrolysis of phytic acid, as phytic acid has depigmenting properties (Gardoni et al., 2004).

4.3. Bone mineralization

Tibia quality has long been used to evaluate the phosphorus requirement of poultry species because it is a more sensitive indicator of phosphorus sufficiency than productive performance. In the present study, animals fed the NC diet showed lower ash, Ca and P retention in tibia compared with animals fed the PC diet, indicating that hens with the deficient diet started mobilizing bone mineral to support their eggshell formation. Similar results have also been observed in other short-term trials. Pongmanee et al. (2020) reported that laying hens fed with a Ca- and P-deficient diet from 25 to 37 weeks of age had significantly lower bone mineral density and content when compared with a diet meeting hens' requirement.

As regards phytase, previous studies showed that dietary phytase inclusion could increase bone Ca and P concentrations, breaking strength and ash content in laying hens fed Ca- and P-deficient diets after 17–22-week trials or in old laying hens (Lei and Stahl, 2001; Hughes et al., 2008). The percentage of P in tibia was not affected by phytase inclusion after 9 weeks of trial in this work, but a dose of 1000 FTU/kg in diets slightly increased Ca and ash content in tibia, reaching the levels found in the animals fed the PC diet. It seems that positive effects of phytase addition are more pronounced in older laying hens and long-term trials. Hughes et al. (2009) found that phytase addition to a deficient diet did not affect bone ash percentage at 42 weeks of age, but it was significantly improved at 61 weeks of age. In any case, there are already several studies indicating that, when enough phytase is introduced in the feed (2000 FTU/kg), the level of aP is not a limiting factor for the bone structure of laying hens in the long term (Cheng et al., 2020; Ren et al., 2020).

5. CONCLUSIONS

The results of this work allow us to conclude that when a diet deficient in Ca and P was applied, the dietary inclusion of phytase at low doses (500 FTU/kg)

afforded an improvement in the digestive efficiency of P during the first weeks after introduction. However, when this type of deficient diet is maintained in the long term, laying hens seem to improve their capacity for digestive utilization of both Ca and P, and it is necessary to include a higher dose of phytase (1000 FTU/kg) to achieve greater availability of dietary P. On the other hand, this overdosage allowed a series of additional extraphosphoric advantages, slightly improving access to other nutrients and the feed conversion rate of the hens, as well as favoring the recovery of some traits related to shell quality and bone mineralization that worsened with the deficient diet. Therefore, due to these compensation phenomena and the possible extraphosphoric effects, it would be advisable to increase the dose of phytase in the feed for laying hens in order to achieve long-term benefits.

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Chapter II

The Duration of the Trial Influences the Effects of Mineral Deficiency and the Effective Phytase Dose in Broilers' Diets

This Chapter has been submitted to Animals journal to be published with the collaboration of:

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Simple Summary

The aim of this work was to investigate the effects of reducing the mineral (Ca and P) content and adding different doses of a new 3-bacterial phytase in broiler diets in a short and long-term experiment. Mineral deficiency reduced growth performance only in young animals, increased Ca and P digestibility and reduced tibia mineralization, especially in the long-term trial. The effective dose of the new phytase varied with the response criteria and duration of the trial. Phytase increased feed efficiency at 500 FTU/kg, but only in young animals, and increased mineral digestibility and retention at a lower dose (250 FTU/kg) in the short compared with the long-term (500 FTU/kg) trial. Tibia mineralization increased with only 250 FTU/kg in the long-term trial. Therefore, the age of the animals and duration of the trial is key in determining the effects of mineral levels and phytase addition in broiler feeds and should be taken into account for future trials.

Abstract

Two trials differing in duration (short and long-term) were conducted to evaluate the effects of providing deficient (NC) or correct (PC) Ca and P levels, and different doses of a new phytase (250, 500 and 1000 FTU/kg feed) in broiler feeds on growth performance, nutrient digestibility and retention (28 days), and tibia mineralization. A total of 80 and 490 male chicks (Ross) of 21 days and 1 day of age were used in the short and long-term trials, respectively. In the long-term trial, chicks fed NC diets showed a lower ($p<0.05$) performance compared to chicks fed PC, 500 and 1000 FTU/kg feed during the starting period, and a significantly higher digestibility for P than those fed the PC diet. Regarding the effects of phytase, feeding 250 to 500 FTU/kg diets increased most of the nutrients' digestibility in the short-term, while only P digestibility in the long-term trial. Tibia mineralization increased linearly with phytase addition ($p<0.05$) only in the long-term trial. In conclusion, the effects of dietary mineral and

phytase levels on growth performance are more noticeable in young animals. In addition, the duration of the trial is key due to a possible adaptation phenomenon of birds to low P supplies.

Keywords: broilers; phytase; digestibility; mineralization; trial duration.

1. INTRODUCTION

Among the minerals required by poultry, phosphorus (P) and calcium (Ca) are the most important, not only because they are required for optimal growth rate, but also for bone mineralization. Phosphorus participates of metabolic processes and nutrients absorption, besides being one of the most expensive minerals in the feed´s final cost (Gomes et al., 2004). A deficiency in Ca and P, or inadequate level of Ca to P ratio affects bone growth and development. Specifically, reduced P in broiler diets have detrimental effects on growth performance and bone mineralization (Driver et al., 2005; Rao et al., 2006; Létour-neau-Montminy et al., 2010). Near two thirds of P in cereal grains and oilseeds are in the form of phytic acid (Adeola and Sands, 2003), which is considered the largest reserve of this element in plants. Because of lack of endogenous enzymes to hydrolyze phytate, P present in plants is biologically unavailable for monogastrics. Then, it is necessary to add inorganic P to poultry diets to meet P requirements, increasing the cost and environmental impact of these diets, or include phytases.

Phytases are enzymes extensively used to improve the availability of P. Many studies (Olukosi et al., 2007; Rutherfurd et al., 2012; Chen et al., 2013; Bougouin et al., 2014; Bata-bunde and Adeola, 2021; Wang et al., 2021) have shown that microbial phytases in P-deficient diets release the phytate bound P and improve the utilization of P and other nutrients in plant derived ingredients. This derives in increases in weight gain and bone ash percentages in broilers. The effective doses of phytase for broilers in the literature ranges from 250 to 12000 FTU/kg, being an inclusion level around 500 FTU/kg the most common (Rutherfurd et al.,

2012; Chen et al., 2013). This effective dose (minimum dose at which positive effects are observed) is generally fixed according to different response parameters such as growth performance, mineral digestibility, bone mineralization and also recently, insolitol liberation in the digesta and its concentration in plasma (Selle and Ravindran, 2007; Bougouin et al., 2014; Batabunde and Adeola, 2021; Bata-bunde et al., 2021; Kriseldi et al., 2021). However, the response of phytases in these parameters is variable among studies. For example, Batabunde et al. (2021) found a quadratic response to phytase in BW, feed intake, tibia ash, and a linear response in the apparent ileal digestibility (AID) of energy and nutrients. Other studies (Simons et al., 1990; Denbow et al., 1995) also observed that bird's performance reached a plateau when the phytase level was around 500 to 1,000 FTU/kg of diet. In contrast, Shirley and Edwards (2003) reported maximum performance and P retention in birds consuming up to 12,000 FTU/kg of diet.

Some of the factors that affect the animals' response to phytase are dietary Ca and phytate contents, experiment length, bird age, and phytase dose (Bougouin et al., 2014; Batabunde et al., 2019a; Batabunde et al., 2019b). In terms of experiment length, some experiments in laying hens demonstrate that prolonging phytase supplementation duration using P deficient diets had negative effects on P-retention. In this regard, Bougouin et al. (2014) stated that for each extra 10 d over the 92d mean duration of layer experiments, phytase-induced P retention decreased by 0.47 percentage units. Although this effect has been generally associated with age, previous studies suggested that compensatory effects can appear reducing the efficacy of phytases when Ca and P deficient diets are administrated for long periods of time to layers (Javadi et al., 2021). Studies in broilers also suggest that feeding low P diets during long feeding periods might reduce phytase efficacy in terms of P digestibility due to homeostatic adaptations in the digestive ability of the birds that increase P retention (Yan et al., 2005; Babatunde et al., 2019a). However, other response

criteria such as growth performance or tibia mineral retention are improved when low-P diets with phytases are fed during long periods.

In this context, the general objective of this study was to evaluate the consequences of reducing Ca and P content and adding different doses of a new 3-bacterial phytase in broiler P-deficient diets on growth performance, nutrient, and mineral utilization and re-tention, and to study the possible compensatory effects and optimum phytase doses comparing a short with a long-term experiment.

2. MATERIALS AND METHODS

2.1. Animals and housing

Two trials differing in duration were performed consecutively, a short-term and a long-term trial. In the short-term trial, 80 male chicks (Ross) of 21 days of age and 872 ± 27.4 (standard deviation; SD) g body weight (BW) were used. The study lasted 17 days in total. At 21 days of age, chicks were randomly distributed in 10 floor pens (1.3 x 1.3 m^2; 8 animals/pen) located in an environmentally controlled room and individually identified with a wing tag. Pens contained wood shavings to a depth of 10 cm and were provided with a single feed trough. A nipple watering line was provided for each 6 pens, with 3 nipples per pen. On day 7 of study (28 days of age), the 80 birds were housed in pairs (similar weight) in 40 metabolism cages (54 x 56 cm^2; 8 cages/treatment; 16 animals/treatment) for a period of 10 days.

In the long-term trial, 490 male chicks (Ross) of 1 day of age and 45.0 ± 0.93 (SD) g BW were used. The study lasted 42 days in total. At day 1 of age, chickens were randomly distributed in 35 floor pens (1.3 x 1.3 m^2; 14 animals/pen), located in two environmentally controlled rooms (20 and 15 pens in room 1 and 2, respectively). Pens contained wood shavings to a depth of 10 cm and were provided with a single feed trough. A nipple watering line was provided, with 3

nipples per pen. As in the short-term trial, 2 to 3 birds per pen (80 birds in total; 16 birds per treatment) were selected at 28 days of age according to its weight (average weight within each pen) and housed in pairs (similar weight) in 40 metabolic cages (54 x 56 cm^2; 8 cages/treatment; 16 animals/treatment) for a period of 10 days.

In both trials, cages were provided with a feed through with two spaces and a single nipple drinker. The last three days in the metabolic cages (35 to 38 days of age), the total amount of excreta produced per cage was daily collected for nutrient balance and retention study. On day 38 of the study, one chick randomly selected from each cage (8 animals/treatment), was slaughtered for bone analyses. In the long-term trial, animals that were not used for the nutrient balance and retention study were maintained in pens with 11-12 animals/pen until the end of the study (42 days of age) for performance calculations, health status and mortality records. Additionally, in the long-term trial, chicks randomly selected from cages for bone analyses were also blood sampled.

Throughout the study, room temperature was controlled, decreasing from 30°C on day 1 to 20°C on day 42 of rearing. Light program provided consisted in 0 h of darkness followed by a period of 24h light on d 1 and 2, and a progressive increase in the time of darkness until reaching 8 h of darkness on d 14 of the study.

2.2. Experimental diets

Experimental feeds were formulated based on corn, wheat, and soybean meal to fulfill the requirements of a grower feed in the short-term trial and starter and grower feeds in the long-term trial. Dietary treatments were provided from day 21 of age in the short-term trial and day 1 of age in the long-term trial. These consisted in five different feeds varying in the mineral level and the inclusion of phytase (FTU/kg of feed): PC, positive control without phytase and with total Ca (0.97% for starter and 0.95% on av. for grower feeds) and total P (0.65% for starter

and 0.63% on av. for grower feeds), levels recommended by FEDNA (2018) for growing broilers; NC, negative control without phytase and with low (below requirements) levels of Ca (0.73% for starter and 0.69% on av. for grower feeds) and total P (0.58% for starter and 0.51% on av. for grower feeds); and other three diets for which the NC diet was supplemented with ePhyt 1000® 3-phytase (Globalfeed) at 250 (P250), 500 (P500) and 1000 (P1000) FTU/kg feed, respectively (see enzyme details at Salaet et al., 2021). The NC based feeds were produced from a unique batch in both experiments. The ingredients and chemical composition of the experimental diets are presented in Table 1.

Feed and water were provided ad libitum during the experimental period and feed was provided in mash form. Phytase was added to feeds in a liquid form in the mixer.

2.3. Growth performance

In the short-term trial, chicks were weighed by group at 21 days of age and individually at 28 days of age (allocation in metabolic cages), 35 days of ages (beginning of the collection period) and 38 days of age (end of the collection period). In the long-term trial, chicks were weighed by group at d 1 of age and weekly over the trial. At 28 d of age (allocation in metabolic cages), birds were individually weighed and identified with a wing tag. In both trials, feed consumption was registered at each weighing control.

Health status of the animals was checked daily, and necropsies were performed on all dead animals. Body weight and feed intake were used to calculate the average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR).

2.4. Total tract digestibility

In both experiments, a total-tract digestibility trial was carried out to determine the coefficient of total-tract apparent digestibility (CTTAD) of Ca, P, and main nutrients, as well as Ca and P retention and excretion using metabolic cages.

Table 1. Ingredients and analysed chemical composition of positive control (PC) and negative control (NC) diets (% as-fed basis) in short- and long-term trials

	Short-term trail		**Long-term trail**			
	Grower feed		Starter feed		Grower feed	
	PC	NC	PC	NC	PC	NC
Ingredients, %						
Corn grain	25.6	25.7	19.1	19.3	25.6	25.9
Wheat grain	34.6	35.0	34.6	35	34.6	35.0
Soybean meal 44% CP	15	15.1	21.2	21.3	15	15.1
Extruded soybean meal	17.9	18	14.9	15	17.9	18
Soybean oil	2.81	2.81	5.6	5.6	2.81	2.81
L-lysine	0.41	0.41	0.5	0.5	0.41	0.41
DL-methionine	0.28	0.28	0.35	0.35	0.28	0.28
L-threonine	0.10	0.10	0.13	0.13	0.10	0.10
Calcium carbonate	0.88	0.78	0.73	0.63	0.74	0.64
Dicalcium phosphate	1.65	0.95	1.8	1.1	1.65	0.95
Salt	0.23	0.23	0.21	0.21	0.23	0.23
Sodium bicarbonate	0.22	0.22	0.3	0.3	0.22	0.22
Vitamin-mineral premix[1]	0.4	0.4	0.6	0.6	0.4	0.4
Chemical composition, %						
Dry matter	89.7	89.4	90.8	90.5	90.7	90.7
Ash	5.25	4.62	5.01	4.66	4.89	4.56
Crude protein	18.1	17.8	22.6	23.1	22.3	22.3
Ether extract	3.47	4.10	3.98	3.67	3.28	4.16
Gross energy (kcal/kg)	4119	4206	4150	4244	4020	4202
AME (kcal/kg)[2]	2863	2917	2884	2943	2794	2914
Calcium	0.91	0.68	0.97	0.73	0.98	0.70
Phosphorous	0.60	0.50	0.65	0.58	0.66	0.52
Phytate-phosphorous	0.21	0.18	0.24	0.23	0.23	0.24

Sixteen animals/treatment of 28 days of age were used. Animals were adapted during 7 days to metabolic cages. The last 3 days, feed intake and total excreta output were measured quantitatively per cage for the determination of dry matter (DM), ash, organic matter (OM), crude protein (CP), gross energy (GE), Ca and P. During the 3-day excreta collection period, excreta was collected every 24 h, weighed, and maintained at 4ºC. At the end of the collection period, excreta

were pooled by cage and homogenized. Representative samples were then obtained and stored at -20ºC until analyses. A representative sample of the different feeds was also taken before the start of the trial to analyze its composition.

2.5. Bone and blood sampling

In both experiments, at the end of the digestibility trial, one chick per cage (8 animals per treatment) were euthanized by stunning and exsanguination to obtain tibia bone. The left tibia was removed from each bird. After removing all the soft tissues, the tibia was frozen at -20ºC until analyses to determine tibia weight, as well as dry matter (DM), ash, Ca, and P content. Additionally, in the long-term trial, blood samples from each animal were collected into 4-mL vacutainer tubes with serum clot activator. Approximately 2 h after extraction, blood samples were refrigerated and transported to the laboratory. The tubes were centrifuged for 4 minutes at 4000 g and serum were collected to analyze Ca and P content.

2.6. Analytical methods

Feed samples were dried at 105ºC for 24 h and then grounded. Excreta samples were dried at 80ºC for 48 h and then grounded. DM (934.01), ash (942.05), ether extract (920.39) and CP (990.03) determinations were carried out according to AOAC (2000) procedures. Gross energy was determined using an adiabatic bomb calorimeter (Gallenkakmp, London, UK). Mineral (Ca and P) content in feeds and excreta was analyzed by inductively coupled plasma atomic emission spectrometry (ICP-OES) (model Varian 720-ES, Varian Inc., Palo Alto, CA, USA), as described in Cambra-López et al. (2020). Phytate-P in PC and NC feeds was analyzed by spectrophotometry according to the method described in Haugh and Lantzch (1993).

For the determination of minerals (ash, Ca, and P) in bones, tibias were boiled in order to remove the remaining soft tissues, cleaned and dried at 110º C for 12 h. After, tibias were degreased in an ether solution for 48 h. Once cleaned and degreased, tibias were dried again at 110ºC for 12 h, weighed and then introduced into a porcelain crucible and ashed at 550ºC for 12 h in a muffle furnace. Mineral (Ca and P) content in tibia bones was then analyzed as previously described, adding 0.05 g-ashed sample to the acid solution instead of 0.1 g as in feed and excreta.

2.7. Statistical analyses

The CTTAD of DM, organic matter (OM), GE, CP, Ca and P were calculated using the following equation:

$$CTTAD\ (\%) = \frac{[(Feed\ intake\ \times\ Nutrient_{feed}) - (Excreta\ output\ \times\ Nutrient_{excreta})]}{(Feed\ intake\ \times\ Nutrient_{feed})} \times 100$$

Where feed intake is the amount of feed consumed by cage in 3 days (g), $nutrient_{feed}$ is the concentration of nutrients in feed, excreta output is the amount of excreta produced by cage in 3 days (g) and $Nutrient_{excreta}$ is the concentration of nutrients in excreta.

Mineral retention was calculated as the amount of minerals ingested multiplied by their CTTAD. Ash and mineral content in tibias were expressed as the percentage or absolute amount of ash, Ca, and P per tibia, once degreased and dried.

Data was analyzed using SAS System software (Version 9.1, SAS Institute Inc., Cary, North Carolina, USA). The pen was the experimental unit for ADG, ADFI and FCR, and the cage for nutrient balance traits. For the mineral retention in tibia and blood parameters, bird was considered the experimental unit. The statistical model was performed using GLM procedure of SAS, and included the diet (PC, NC, P250, P500 and P1000) as the main effect and room (1 and 2) as a

block factor. Additionally, polynomial orthogonal contrasts were applied to test linear effects of phytase level. In growth performance analyses, the initial pen weight was used as a covariate. The percentage of dead animals among treatments was compared using a chi-square test (FREQ procedure). Statistical significance level was set at 5% ($P<0.05$).

3. RESULTS

3.1. Growth performance

In general, the health status of the animals was good in both trials. In the short-term trial one chick dead upon arrival and in the long-term a total of 28 animals died during the trial. In the long-term trial, beak abnormalities were detected in one chick (treatment NC) and three animals (treatments PC, 250 and 1,000) showed leg disorders at the end of the rearing period. The percentage of dead animals was not significantly different among treatments in the long-term trial (2.38, 9.52, 5.95, 9.52 and 5.95 for PC, NC, P250, P500 and P1000, respectively; $p = 0.442$). In the short-term trial, the diet given during the 17 days of the trial had no significant effect on any of the growth traits registered (Table 2).

However, significant differences between treatments were found in the long-term trial (Table 3). In this trial, chicks fed with PC diet showed a greater ADFI and ADG (+3.0 ± 0.7 g/d; $p = 0.024$) than those on NC diet during the starting period, but differences disappeared during the growing period and no differences were observed globally.

Regarding the phytase level, chicks fed with P500 and P1000 diets showed a lower ADFI (on av. -5.1 ± 0.8 g/d; $p < 0.001$) and better FCR than those on NC and P250 diets during the starting period. No differences were observed, again, among treatments with phytase and NC during the growing period.

Table 2. Short-term trial: Initial and final body weight (BW, g), daily weight gain (ADG, g/d), daily feed intake (ADFI, g/d) and feed conversion ratio (FCR, g feed/g weight) of broilers fed feeds including different levels of phytase from 21 to 38 days of age.

		Dietary treatment						
		PC	NC	P250	P500	P1000	SEM	P-value
BW	21 d	897	867	853	861	855	16	0.375
	38 d	2477	2486	2421	2419	2530	59	0.455
ADG	28 to 38 d	98.0	99.1	94.9	95.4	99.0	4.6	0.940
ADFI	28 to 38 d	163	162	155	153	161	7.0	0.766
FCR	28 to 38 d	1.67	1.64	1.64	1.62	1.63	0.03	0.793

Treatments: PC, positive control; NC, negative control; P250, negative control with phytase at 250 FTU/kg feed P500; negative control with phytase at 500 FTU/kg feed; and P1000, negative control with phytase at 1000 FTU/kg feed. Data represent mean values of 8 replicate cages of two chicks each per treatment. SEM: Standard error of the mean.

Table 3. Long-term trial: Average weight (BW, g), daily gain (ADG; g/d), feed intake (ADFI, g/d) and feed conversion ratio (FCR, g feed/g weight) of broilers fed feeds including different levels of phytase from 1 to 42 days of age.

		Dietary treatment						
		PC	NC	P250	P500	P1000	SEM	P-value
BW	1 d	45.2	44.8	44.6	45.6	45.4	0.327	0.161
	42 d	3297	3366	3324	3329	3328	41	0.808
Starter period (1 to 21 d):								
ADG		48.6^{b}	45.6^{a}	43.4^{a}	45.8^{a}	46.9^{ab}	0.734	<0.001
ADFI		65.3^{c}	62.1^{b}	61.3^{b}	56.3^{a}	57.0^{a}	0.794	<0.001
FCR		1.34^{b}	1.36^{b}	1.41^{c}	1.23^{a}	1.22^{a}	0.013	<0.001
Growing period (22 to 42 d):								
ADG		105.7	110.2	110.9	109.1	107.4	1.8	0.252
ADFI		168.8	174.3	172.2	166.9	172.1	2.86	0.388
FCR		1.60	1.58	1.55	1.53	1.60	0.024	0.158
Global period (1 to 42 d):								
ADG		75.2	75.5	74.6	75.2	74.8	0.99	0.965
ADFI		113.7^{b}	114.0^{b}	112.6^{ab}	107.8^{a}	110.3^{ab}	1.6	0.051
FCR		1.51^{b}	1.51^{b}	1.51^{b}	1.43^{a}	1.47^{ab}	0.02	0.013

^{a,b,c} Least square means in a row not sharing superscripts differ at P<0.05. Treatments: PC, positive control; NC, negative control; P250, negative control with phytase at 250 FTU/kg feed P500; negative control with phytase at 500 FTU/kg feed; and P1000, negative control with phytase at 1000 FTU/kg feed. Data represent mean values of 7 replicate pens per treatment. SEM: Standard error of the mean.

Animals on P500 diet had a lower ADFI (-6.2 ± 1.6 g/d; p = 0.031) and better FCR (-0.08 ± 0.01 g/d; p = 0.013) than those on NC diet during the global period.

3.2. Nutrient utilization

Table 4 shows the effect of the experimental diets on the CTTAD of main nutrients and on the excretion and retention of Ca and P. Diet had a great effect on nutrient utiliza-tion in the short-term trial. Chicks fed with PC diet showed a greater Ca and P excretion (+0.40 ± 0.06 and +0.21 ± 0.04 g/d animal, respectively; $p < 0.001$) but similar CTTAD and retention than those on NC diet. Regarding the phytase level, chicks fed with NC diet showed significantly lower CTTAD for DM, OM and Ca than those fed phytase added di-ets, lower CTTAD for CP and P than those on P250 and P500, and lower CTTAD for GE than those on P500 diet. Consequently, animals on NC diet showed a lower Ca retention than those fed with phytase added diets (on av. -0.53 ± 0.04 g/d animal; $p < 0.01$) and lower P retention than those on P500 and P1000 diets in the short-term trial (-0.09 ± 0.03; $P<0.05$). The CTTAD of DM, OM, Ca and Ca retention increased, linearly, with phytase addition. In the case of the long-term trial, chicks fed with PC diet showed also greater Ca and P excretion (+0.46 ± 0.05 and 0.25 ± 0.03 g/d animal, respectively; $p < 0.001$), but also a greater Ca retention (+0.12 ± 0.04 g/d animal; $p < 0.001$) and lower CTTAD for P than those on NC diet. Regarding phytase level, animals fed with the NC diet showed lower Ca retention (-0.18 ± 0.04 g/d animal; $p < 0.001$) that those on P500 and P1000 diets, and lower CTTAD for P and P retention (-0.07 ± 0.03 g/d animal; $p < 0.05$) than those on P500. The CTTAD of Ca increased linearly with phytase addition.

3.3. Bone mineralization and blood analyses

Finally, the effect of dietary treatment on bone mineralization and blood mineral concentration traits is presented in Table 5. In the short-term trial, neither the mineral level nor the inclusion of phytase in the diet significantly affected any of the mineralization parameters that we analyzed in tibias at 38 d of age, after 17 days of receiving the experi-mental diets.

Table 4. Effect of mineral and phytase inclusion levels on nutrient coefficient of total tract apparent digestibility (CTTAD, %), retention (g/d animal) and excretion (g/d animal) of broiler diets in both short- and long-term trials (35 to 38 days of age).

	Dietary treatment						
	PC	NC	P250	P500	P1000	SEM	P-value
Short-term trial:							
DM CTTAD[1]	67.4^{ab}	66.2^{a}	68.8^{b}	68.7^{b}	68.2^{b}	0.4	<0.001
OM CTTAD[1]	70.0^{ab}	68.4^{a}	70.3^{b}	70.9^{b}	70.1^{b}	0.4	0.006
CP CTTAD	58.9^{ab}	56.0^{a}	61.0^{b}	61.0^{b}	58.3^{ab}	0.9	0.001
GE CTTAD	70.2^{ab}	69.3^{a}	70.6^{ab}	71.5^{b}	70.9^{ab}	0.6	0.146
Ca CTTAD[1]	28.8^{a}	33.6^{a}	41.0^{b}	43.0^{b}	40.7^{b}	1.9	<0.001
Ca retention[1]	0.46^{ab}	0.40^{a}	0.52^{bc}	0.55^{c}	0.54^{bc}	0.03	0.004
Ca excretion	1.16^{b}	0.71^{a}	0.75^{a}	0.73^{a}	0.79^{a}	0.05	<0.001
P CTTAD	34.2^{a}	38.8^{a}	46.3^{b}	46.6^{b}	45.3^{ab}	2.0	<0.001
P retention	0.35^{a}	0.33^{a}	0.41^{ab}	0.42^{b}	0.42^{b}	0.02	0.019
P excretion	0.72^{b}	0.51^{a}	0.47^{a}	0.48^{a}	0.51^{a}	0.04	<0.001
Long-term trial:							
DM CTTAD	68.4	67.9	69.3	68.2	68.0	0.6	0.435
OM CTTAD	70.1	68.8	70.6	69.7	69.5	0.6	0.278
CP CTTAD	60.4	59.0	59.7	57.9	58.1	0.9	0.193
GE CTTAD	68.8	69.4	70.4	68.0	69.1	0.7	0.258
Ca CTTAD[1]	31.1^{a}	36.0^{ab}	34.6^{ab}	41.8^{b}	39.4^{b}	2.0	0.005
Ca retention	0.59^{b}	0.47^{a}	0.50^{a}	0.68^{c}	0.63^{bc}	0.03	<0.001
Ca excretion	1.31^{b}	0.85^{a}	0.94^{a}	0.96^{a}	0.96^{a}	0.05	<0.001
P CTTAD	36.8^{a}	42.1^{b}	40.2^{ab}	46.9^{c}	45.8^{bc}	1.4	<0.001
P retention	0.46^{ab}	0.43^{a}	0.40^{a}	0.50^{b}	0.48^{ab}	0.02	0.009
P excretion	0.81^{b}	0.56^{a}	0.59^{a}	0.57^{a}	0.60^{a}	0.03	<0.001

^{a,b,c} Least square means in a row not sharing superscripts differ at P<0.05. Treatments: PC, positive control; NC, negative control; P250, negative control with phytase at 250 FTU/kg feed P500; negative control with phytase at 500 FTU/kg feed; and P1000, negative control with phytase at 1000 FTU/kg feed. Data represent mean values of 8 replicate cages of two chicks each per treatment. [1] Linear effect of phytase level ($p < 0.05$). SEM: Standard error of the mean (n = 8).

However, when the animals received the experimental diets for 38 days (long-term trial), chicks fed with PC diet showed higher tibia weight and ash, Ca and P content in tibia than those fed NC diet ($p < 0.05$). Regarding phytase level, animals fed with the NC diet showed lower tibia weight and ash, Ca and P content in tibia than those fed phytase added diets (on av. -0.69 ± 0.15, -0.36 ± 0.10, -0.14 ± 0.05 and -0.08 ± 0.03 g, respectively; $p < 0.05$). These parameters showed a significant linear tendency to increase with phytase addition ($p < 0.05$). Dietary treatment did not significantly affect Ca and P content in the blood at 38 days of age in the long-term trial.

Table 5. Effect of mineral level and dietary phytase inclusion on bone mineralization traits and blood concentration of calcium (Ca) and phosphorus (P) of broilers in both short- (at 38 days of age after 17 days of treatment) and long-term trials (at 38 days of age after 38 days of treatment).

	Dietary treatment						
	PC	NC	P250	P500	P1000	SEM	P-value
Short-term trial:							
Tibia weight, g	5.27	4.72	4.74	4.57	4.91	0.26	0.348
Tibia weight, % BW	0.22	0.19	0.20	0.18	0.19	0.01	0.330
Ash in tibia, g	2.80	2.44	2.49	2.38	2.60	0.13	0.193
Ash in tibia, % DM	53.1	51.8	52.5	52.2	52.8	0.4	0.155
Ca in tibia, g	1.05	0.91	0.92	0.89	0.98	0.05	0.157
Ca in tibia, % DM	19.0	19.4	19.5	19.4	19.7	0.2	0.187
P in tibia, g	0.49	0.44	0.44	0.41	0.44	0.03	0.386
P in tibia, % DM	9.24	9.28	9.18	9.07	9.00	0.134	0.514
Long-term trial:							
Tibia weight, g[1]	4.82[b]	4.21[a]	4.85[b]	4.93[b]	4.91[b]	0.14	0.005
Tibia weight, % BW	0.18[ab]	0.17[a]	0.18[b]	0.19[b]	0.18[ab]	0.01	0.061
Ash in tibia, g[1]	2.60[b]	2.23[a]	2.61[b]	2.55[b]	2.62[b]	0.08	0.005
Ash in tibia, % DM	53.9	53.0	53.9	53.2	53.3	0.4	0.295
Ca in tibia, g[1]	0.99[b]	0.86[a]	1.00[b]	1.01[b]	1.00[b]	0.04	0.016
Ca in tibia, % DM	20.7	20.3	20.5	20.3	20.4	0.181	0.385
P in tibia, g[1]	0.47[b]	0.40[a]	0.47[b]	0.48[b]	0.48[b]	0.02	0.008
P in tibia, % DM	9.71	9.54	9.73	9.70	9.67	0.09	0.512
Minerals in blood:							
Ca (mg/dL)	11.9	12.1	11.8	12.5	11.7	0.4	0.510
P (mg/dL)	9.2	10.4	9.3	9.8	10.2	0.4	0.173

[a,b,c] Least square means in a row not sharing superscripts differ at P<0.05. Treatments: PC, positive control; NC, negative control; P250, negative control with phytase at 250 FTU/kg feed P500; negative control with phytase at 500 FTU/kg feed; and P1000, negative control with phytase at 1000 FTU/kg feed. Data represent mean values of 8 replicate animals per treatment. [1]Linear effect of phytase level ($p < 0.05$). SEM: Standard error of the mean (n = 8).

4. Discussion

Two trials were designed to explore the effect of the mineral level and phytase con centration in diets on growth performance, mineral and nutrient utilization and retention. The most important effects of mineral levels and phytase addition are discussed in the following lines.

4.1. Mineral levels

The effects of reducing Ca and P levels on performance were clear in the starter period for the long-term trial, but not in the growing period both in the long and the short-term trials. In the long-term trial, chicks fed the low Ca and P levels from day 1 to 21 of age showed lower ADG and ADFI compared with the animals fed the required Ca and P. These differences disappeared in the growing period, suggesting a possible age effect. However, the duration of the trial did not affect the animals' response to mineral-deficient diets in terms of performance. Some works in the literature also suggest that age has an influence on the utilization of nutrients by broilers, since the rate of nutrients utilization is greater in old compared with young animals (Li et al., 2015; Babatunde et al., 2019a, b). This and the fact that young animals are characterized by rapid growth of organs and tissues could explain that the consequences of mineral deficiency are more harmful in young compared with more mature animals. On the other hand, it must be taken into account that the sample size for growth performance parameters in the short-term trial is limited (n=16) and a greater number of animals would perhaps be required to extract more solid conclusions.

In terms of nutrients digestibility and retention, as expected, Ca and P excretion was greater in the PC compared with the NC treatment in both trials. However, Ca and P digestibilities were greater in the NC animals compared with the PC, although only significant in the case of P in the long-term trial. This could indicate that chicks fed mineral-deficient diets try to compensate their deficiency by increasing retention, and this effect seems to be more important as the duration of the deficiency is longer. In fact, Ca and P digestibility values were both higher at the same age in the long than in the short-term trial. In this regard, other studies in broilers also suggest that feeding low-P diets during long feeding periods might lead to homeostatic adaptations in the digestive ability of the birds that increase P retention (Yan et al., 2005; Babatunde et al., 2019a). Despite the increase in P digestibility with the NC diet in the long-term trial, this mechanism

was not enough to sustain the same level of tibia mineralization compared with animals fed PC diet. In the short-term trial, bone mineralization was not significantly affected by Ca and P deficiency. Many researchers reported that reduction in dietary P could be achieved without deleterious effects on bone mineralization if Ca is reduced concomitantly, and the reason might be that the Ca to P ratio is still within the range between 2 to 1 and 1 to 1, which is generally acceptable for poultry industry (Driver et al., 2005; Rao et al., 2006). In the present study, both Ca and P were concomitantly reduced, and the ratio was always greater than 1:1. This could be the reason for the lack of differences in tibia mineralization in the short-term trial. Therefore, the duration of the trial might increase the impact of feeding low mineral diets (independently of the final Ca to P ratio), with longer periods being more damaging for bone mineralization in broilers than shorter.

The Ca and P contents in serum and bone can well reflect the nutritional status of Ca and P in the body of broilers. Some studies reported a decrease in the Ca and P serum levels when mineral content in diets was lower (Karami et al., 2020; Javadi et al., 2021). However, in the present study, serum Ca and P concentrations determined in the long-term trial were numerically, although not significantly, greater in animals fed the NC. This agrees with the greater digestibility of these minerals, particularly P, in the NC compared with the PC of the long-term trial.

4.2. Phytase level

As for the mineral content of the diets, growth performance was affected by the phytase concentration in feeds only during the starter phase of the long-term trial. In this regard, the group of animals receiving 500 and 1000 FTU/kg feed of the new phytase showed lower ADFI and FCR compared with NC from 1 to 21 days of age. As birds control their feed intake based on the dietary energy level, the higher availability of some nutrients (including energy) in diets with added phytase could explain the reduced intake and improved conversion rate of

animals fed with added phytases. Also, an age effect on phytase efficacy according to which this effect is more evident in young animals due to the immaturity of the digestive enzymes and acids is reported by other studies in broilers (Babatunde et al., 2019a, b; Sens et al., 2021) and in pigs (Cambra-López et al., 2020).

On the other hand, the effect of phytase inclusion on nutrient digestion was particularly evident in the short-term trial compared with the long-term trial. In the case of mineral digestion, phytase addition improved Ca and P digestibility at the lowest phytase dosage (250 FTU/kg) in the short-term trial, compared with the NC treatment. In the long-term trial, this improvement was seen starting at 500 FTU/kg, and only for P. Additionally, the inclusion of 250 and 500 FTU/kg feed improved DM, OM, CP, and GE digestibility compared with the NC group in the short-term trial, but this improvement in other nutrients digestibility was not observed in the long-term trial. In this case, as the nutrient balance was carried out in animals of the same age in both trials, the difference can be attributed to the duration of the trial. As it has been mentioned for the mineral level, feeding low-P diets during long feeding periods might lead to physiological adaptations in the digestive ability of the birds that increase P retention (Yan et al., 2005; Li et al., 2015; Babatunde et al., 2019a; Javadi et al., 2021). Part of this adaptation is related to improved phytate P digestion (Yan et al., 2005). In fact, mineral digestibility of the NC group was greater in the long-term compared with the short-term trial, decreasing the room for improvement of the new phytase versus the NC group. This effect has important implications for the final effective doses determined for phytases. In the present study, the final effective doses for the ePhyt 1000® 3-phytase tested to increase P digestibility was 250 FTU/kg in the short-term trial and 500 FTU/kg in the long-term trial. The duration of the trial should therefore be considered when designing trials for evaluating the efficacy of a given phytase.

In terms of bone mineralization, as expected, the effect of phytase addition was more marked in the long-term trial. In this trial, animals fed NC with phytase showed increased tibia weight and ash, Ca, and P content (g) in tibia compared with NC at the lowest phytase doses (250 FTU/kg). In fact, animals fed with phytase were able to recover the levels of the PC for these parameters, contrary to animals from the short-term trial. Therefore, it seems that more time of phytase administration is needed to observe responses on tibia mineralization traits. Mineral content in serum was not different among treatments, although in the case of P it grew numerically with the inclusion of phytase. In this regard, other studies also observed increases in blood P concentration with phytase inclusion as a result of its effects on releasing P from the phytate (Wang et al., 2013; Babatunde et al., 2019a).

Thus, the evaluation of parameters such as growth performance, nutrient utilization and bone mineralization have proved valuable in determining mineral deficiency consequences and the efficiency of phytase in improving P bioavailability, as suggested by previous studies (Ravindran et al., 2000, 2001; Selle and Ravindran, 2007; Bougouin et al., 2014; Batabunde and Adeola, 2021). However, the effective doses of the new phytase in this study can vary from 250 to 500 FTU/kg depending on the design of the trial and the response criteria selected.

5. CONCLUSIONS

The results of this work permit us to conclude that the evaluation of parameters such as growth performance, nutrient utilization and bone mineralization have proved valuable in determining mineral deficiency consequences and the efficiency of phytase in improving P bioavailability. However, the age and duration of the trial can affect differently to these response criteria. Indeed, the effects of providing mineral (Ca and P) deficient and including phytase diets on growth performance depend on the age of the animals, being more noticeable in

young animals. In terms of mineral and nutrients digestibility, the duration of the trial is key due to the adaptation phenomena of birds to low P supplies by increasing its digestibility and retention. Bone mineralization recovery seems also to depend on the duration of the trial, being longer periods more effective for bone accumulation of minerals. The result from this study demonstrates the necessity of standardization of these factors in future studies using minerals and phytase.

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GENERAL DISCUSSION

The main objective of this Doctoral Thesis was to determine the effect of mineral content and phytase dose in diets of laying hens and broilers on performance, nutrient utilization, and bone mineralisation in the function of the duration of the trial. To achieve this goal, two studies have been separately conducted on hens and broilers. In the present general discussion, I will try to jointly discuss the results obtained in both studies to try to obtain effective conclusions on how the duration of the trials can affect the evaluation of mineral nutrition.

1. MINERAL CONTENT

Domestic animals excrete about 15 million tons of P into the environment every year (Cordell et al., 2009). Numerous studies have reported that mineral supplementation in commercial feeds increases P intake and its excretion rate, which could cause serious environmental problems, as P sources are limited and non-renewable resources (Mallin, 2000; Lei and Stahl, 2001; Lei and Porres, 2003; Lei et al., 2007). A possible solution could be the reduction of the dietary mineral levels, but it could affect nutrient utilization, poultry performance and bone mineralization.

Nutrient utilization

According to the results of the present Doctoral Thesis, in laying hens, mineral supplementation below commercial levels (-25% Ca and -64% P) led to higher digestibility and lower excretion of Ca and P. However, the response was different in function of the duration of the trial. The hens with the deficient diet showed lower daily retention of Ca and P in the short-term trial. However, these animals seemed to improve their digestive utilization of Ca and P when maintained in long term, disappearing the differences in Ca retention, and even increasing P retention compared to animals fed with the no-deficient diet. Something similar happens in broilers. Mineral supplementation below commercial levels (-25% Ca and -17% P) led to higher digestibility and lower

excretion of P and Ca. Furthermore, Ca and P digestibility values were both higher at the same age in the long than in the short-term trial. These results seem to indicate that when diets were deficient in minerals, digestive efficiency increased both in hens and broilers. This effect has also been previously reported by other studies in the literature (Meyer et al., 2011; Sari et al., 2012; Ren et al., 2020; Pongmanee et al., 2020). In addition, other studies also suggest that the use of these diets during long feeding periods might also lead to homeostatic adaptations in the digestive ability of the birds that increase P retention (Yan et al., 2005; Babatunde et al., 2019a).

Performance

According to the results obtained in our first study, in young laying hens, dietary Ca and P levels did not affect laying hens' performance (live weight, egg production…) during the 60-day experimental period, but the deficient mineral diet reduced the quality of the shell. It seems that laying hens can maintain optimal medium-term performance when fed a diet containing 2.0 g/kg non-phytate P (nPP) and Ca/P ratio is not excessively modified, when feed intake is maintained within normal values. However, egg shell quality could be affected when the Ca provided is not enough, as suggested by other studies in the literature (van der Klis et al., 1997; Boorman et al., 2001; Bar et al., 2002; Ren et al., 2020; Cheng et al., 2020). In the case of broilers, chicks fed the low Ca and P levels from day 1 to 21 of age showed lower growth performance compared with the animals fed the required Ca and P, but these differences disappeared in the growing period, suggesting a possible age effect (Li et al., 2015; Babatunde et al., 2019a,b). Therefore, all these results seem to indicate that the negative consequences of a mineral deficiency are more harmful in young compared with more mature animals.

Bone mineralization

Laying hens fed the mineral-deficient diet showed lower ash, Ca and P retention in tibia compared with animals fed a balanced diet in the long term, indicating that hens with the deficient diet started mobilizing bone minerals to support their eggshell formation, as described by Pongmanee et al. (2020). In a similar way, although broilers' bone mineralization was not affected by Ca and P deficiency in the short term, tibia mineralization was significantly reduced in the long term, according to other studies (Driver et al. 2005; Rao et al. 2006). Therefore, the duration of the trial might increase the impact of feeding low mineral diets (independently of the final Ca to P ratio), with longer periods being more damaging for bone mineralization in poultry than shorter.

2. PHYTASE DOSE

Another alternative to reduce the P level of the diets, the excretion of P, and the environmental impact associated to this excretion is the dietary inclusion of exogenous phytases. Their inclusion must improve P and other nutrients utilization, but it could affect nutrient utilization, poultry performance and bone mineralization in a different way in the function of their evaluation in the short and long term.

Nutrient utilization

The difference in nutrient utilization with the inclusion of phytase measured at 25 and 31 weeks of age in laying hens in our study was not enough to consider an age effect, but denoted relevant differences in respect with the duration of the phytase administration (3 or 9 weeks). In this regard, a clear interaction between the phytase level and the duration of the trial was found for P utilization. The P digestibility and retention were improved when the deficient diet included phytase at 500 FTU/kg in the short-term trial. However, a dose of 1000 FTU/kg was needed to improve P digestibility and retention in the long-term trial. Similar

results were also observed in broilers. Ous study showed that dietary phytase inclusion in a mineral-deficient diet improved main nutrients digestibility (DM, GE, CP, Ca and P) when included in the short term, but only Ca and P digestibility when fed in the long term. Additionally, the effective phytase dose increased with the duration of the trial, being 250 and 500 FTU/kg in the short and long-term trials, respectively. As mentioned above, poultry can increase their mineral absorption efficiency when they are receiving a deficient diet for a long time (Yan et al., 2005; Li et al., 2015; Babatunde et al., 2019a), through an increase in the metabolism of renal and intestinal 1,25-hydroxycholecalciferol (Elaroussi et al., 1994). Therefore, a greater amount of phytase seems to be required to improve P utilization when these deficient diets are used in the long term, and the duration of the trial should be considered when designing trials for evaluating the efficacy of a given phytase.

Performance

In laying hens, the dietary inclusion of phytase increased hens' final weight at 500 FTU/kg inclusion and improved FCR at 1000 FTU/kg, but it didn't affect egg production and quality traits. As the mineral-deficient diet did not reduce egg production, phytase inclusion neither affect hens' laying performance, that only seems affected by extremely low levels of P (Ahmadi et al. 2012; Punna et al. 1999). In addition, as Ca seems to be the main factor affecting shell quality, greater availability of P did not lead to shell quality improvements. In the case of broilers, chicks fed with a mineral-deficient diet including phytase from 500 FTU/kg also showed a better FCR during the first weeks of life. Both, the greater benefits observed of enzymes in young animals due to the immaturity of its digestive tract (specially in broilers) and the extraphosphoric effects of phytase inclusion might allow for a greater availability of other nutrients, especially when phytase is overdosed, which could slightly contribute to improving poultry growth performance (Viveros et al. 2002; Lei et al. 2011; Troesch et al. 2013). Also,

as birds control their feed intake based on the dietary energy level, the higher availability of energy could explain the improved FCR of animals fed with added phytases.

Bone mineralization

In this case, we have also observed agreement between the results obtained in chicks and hens. After 9 weeks of trial, with a phytase dose of 1000 FTU/kg in the diet, laying hens slightly increased Ca and ash content in the tibia, reaching the levels found in the animals fed the mineral-balanced diet. In the case of broilers, although no effect was observed in short term, chicks fed with a phytase dose of 250 FTU/kg in the diet in long term also were able to recover the levels of the mineral-based diet for tibia weight and ash, and Ca and P content in the tibia. It seems that positive effects of phytase addition on bone mineralization are more pronounced in older laying hens and long-term trials (Hughes et al. 2009), or after a long-time administration in broilers.

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GENERAL CONCLUSIONS

From the results of the present Doctoral Thesis, it can be concluded that:

1. When mineral-deficient poultry diets are provided, the digestive efficiency of animals increases to compensate for this limitation. In fact, the use of mineral-deficient diets for long periods could lead to digestive adaptations of the birds to increase P retention.
2. The negative consequences of the use of mineral-deficient diets in poultry performance are more harmful in young compared with more mature birds, being more damaging for bone mineralisation as the duration of low mineral diet administration is increased.
3. The inclusion of the new 3-phytase in laying hens and broilers improved P utilization. However, due to the digestive adaptations to the mineral-deficient diets with time, a greater amount of phytase seems to be needed to improve the P utilization as the duration of low mineral diet administration is increased, both, in laying hens (from 500 to 1000 FTU/kg feed) and broilers (from 250 to 500 FTU/kg feed).
4. In short-term trials, inclusion of the new 3-phytase also allowed for greater availability of other nutrients (extraphosphoric effects) in laying hens and broilers.
5. The positive effects of the new 3-phytase addition in the diet on growth performance and bone mineralization are more pronounced when they are provided in long term both in laying hens and broilers.

In conclusion, the use of mineral-deficient diets seems to cause digestive changes in the use of minerals, especially when they are used in the long term. However, the use of commercial phytases is usually also carried out during the whole production period. This must be taken into account when evaluating the potential of a phytase at a commercial level. From the results of the present Doctoral Thesis, the following practical recommendations are driven:

1. The trials addressed to evaluate the effects of phytase on nutrient utilization must be carried out in the short term, avoiding excessive low mineral levels in diets (better close to the levels at which they will be used commercially), to avoid digestive adaptation effects.

2. The trials addressed to evaluate the effects of phytase on growth performance and bone mineralization must be carried out in the long term, to give time for its effect to be relevant.

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EFFECT OF DURATION OF THE TRIAL ON THE EFFICACY OF A NOVEL 3-PHYTASE IN BROILERS AND LAYING HENS

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