Universitat Autònoma de Barcelona

DIETARY FACTORS INFLUENCING CALCIUM AND PHOSPHORUS UTILIZATION IN BROILER CHICKEN

Manel Hamdi

Universitat Autònoma de Barcelona

DIETARY FACTORS INFLUENCING CALCIUM AND PHOSPHORUS UTILIZATION IN BROILER CHICKEN

TESI DOCTORAL PRESENTADA PER:

Manel Hamdi

SOTA LA DIRECCIÓ DEL DOCTOR:

José Francisco Pérez Hernández

PER ACCEDIR AL GRAU DE DOCTOR DINS EL PROGRAMA DE DOCTORAT EN PRODUCCIÓ ANIMAL DEL DEPARTAMENT DE CIÈNCIA ANIMAL I DELS ALIMENTS

Bellaterra, 2016

FACULTAT DE VETERINÀRIA DE BARCELONA

José Francisco Pérez Hernández , professor titular del Departament de Ciència Animal i dels Aliments de la Facultat de Veterinària de la Universitat Autònoma de Barcelona,

Certifique:

Que la memòria titulada "**Dietary factors influencing calcium and phosphorus utilization in broiler chicken**", presentada per **Manel Hamdi** amb la finalitat d'optar al grau de Doctor en Veterinària amb menció internacional, ha estat realitzada sota la seva direcció i, considerant-la acabada, autoritzen la seva presentació perquè sigui jutjada per la comissió corresponent.

I perquè consti als efectes oportuns, signen la present a Bellaterra, 20 de setembre de 2016.

Dr. José Francisco Pérez Hernández

"Emporte dans ta mémoire, pour le reste de ton existence, les choses positives qui ont surgi au milieu des difficultés. Elles seront une preuve de tes capacités et te redonneront confiance devant tous les obstacles".

Paulo Coelho

A Nadia

A mes très chères Parents

Ce n'est pas par hasard, quand je suis arrivé jusqu'au bout.

C'est grâce à vous, seulement vous, qui m'a donné des racines et des ailes.

Agradecimientos/Acknowledges

Hace tres años, cuando acabé mi master, Francisco me hizo la propuesta de realizar una tesis bajo su supervisión. Sin pensarlmelo mucho, le dije que sí. Desde aquel día, empezó una nueva aventura en mi vida, la mejor, durante la cual he aprendido y disfrutado un montón.

Hoy es el momento de agradecer a todas aquellas personas que de una forma u otra me han ayudado a emprender esta aventura.

Primero, y como más importante, **Francisco**, te agradezco de una forma muy especial y sincera ofrecerme esta oportunidad y por todas las horas que dedicaste para el desarrollo de esta tesis. Gracias a tus ideas y tu alta disponibilidad a la hora de trabajar, hemos logrado buenos resultados. Tu paciencia y tu motivación han sido fundamentales para mi formación. He disfrutado muchísimo desarrollando este trabajo contigo.

David, muchas gracias por tu disponibilidad, humildad, ideas y consejos, siempre te molestaba con mis preguntas pero siempre tenías respuestas perfectamente adecuadas de un gran profesional.

Josep, **Ana, Roser, Susana** y **Lorena** gracias por aceptarme para formar parte de vuestro grupo, que para mí representa mi familia en España.

Rosa, creo que la mayoría de mi tiempo que he estado por aquí lo he pasado contigo en la granja o en el laboratorio, trabajando y ″peleando″. Miles de Gracias por tu ayuda y espero que tengas muy pronto todo lo que deseas en este mundo.

Olga, he aprendido mucho de ti durante mis largos días en el laboratorio. Te echamos mucho de menos en el grupo. A **Blas** y a **Carmen**, gracias por vuestra ayuda siempre que me hizo falta.

Agradezco a toda la gente maravillosa que conocí en Nutrición y que me ayudaron mucho, algunos de ellos ya les he visto acabar, como **Edgar, Gemma**, **Roger**, **Sergio**, **Ramon**, **Clara**, **Ester**…Y a otras que espero verles acabar muy pronto **Sergi**, **Emili**, **Paola**, **Yanan**, **Raquel**, **Laia**, **Marta**, **Agustina**, **Wellington**, **Lluís**, **Carmen**, **Anna**… que sin ellos nunca podría haber acabado, sobre todo aquellos días interminables de sacrificio en la granja.

A todo el equipo de la granja **Cristobal**, **Ramon**, **Sergi**, **Roger**, **Sonia**, **Pepe** y **Ramon** por haberme aguantado todas mis exigencias.

Un agradecimiento muy especial a mis mejores amigos **Inés** y **Ali** que siempre estaban presentes cuando les necesitaba.

Y para acabar, un agradecimiento muy especial a mis hermanos **Maher** y **Moez** y a toda mi familia que gracias al Skype y a pesar de la distancia han vivido conmigo el día a día de estos últimos años.

"La vie est un défi à relever, un bonheur à mériter, une aventure à tenter"

Mère Teresa

Resumen

El calcio y el fósforo son los dos principales macrominerales necesarios para alcanzar un crecimiento y mineralización ósea adecuados en aves. Las materias primas de piensos de origen vegetal contienen cantidades muy bajas de Ca y P. Éste último se presenta principalmente en forma de fitato poco digestible y en cantidad insuficiente para satisfacer las necesidades, por lo que se añaden habitualmente fuentes inorgánicas de Ca y P a las dietas de pollos broiler.

La hidrólisis del fósforo fítico mediante fitasa exógena ayuda a disminuir la incorporación de fuentes minerales, el impacto medioambiental de la producción de pollos de engorde y el coste de la dieta. Sin embargo, los minerales pueden unirse fácilmente al ácido fítico y formar complejos de fitato mineral, que pueden ser resistentes a la hidrólisis con fitasa, en función de los valores de pH.

El objetivo global de esta tesis fue investigar los factores que influyen en la utilización del Ca y el P en la dieta para pollos Broiler a diferentes edades.

Para alcanzar este objetivo, siete ensayos (capítulo 4 a 8) fueron diseñados.

El ensayo 1 se diseño para evaluar la interacción entre los niveles dietéticos de calcio y fósforo no fítico (NPP, con 1,150 FTU de fitasa) en los rendimientos productivos, mineralización oséa y en la retención de Ca y P del d 1 a 14 de vida. Los pollos fueron capaces de alcanzar su mayor ganancia de peso y una óptima mineralización ósea con el nivel 0.7% de Ca y 0.38% de NPP. El aumento del Ca en la dieta disminuyó el rendimiento y la mineralización de los huesos, especialmente con las dietas bajas en NPP. Mientras el aumento de Ca redujo su retención, el aumento de los niveles de NPP en la dieta aumentó de forma constante la retención de Ca, probablemente a través de un aumento en la deposición en el hueso.

El objetivo del **ensayo 2** fue mostrar el efecto de diferentes fuentes de Ca (carbonato cálcico, cloruro de calcio y un fosfato tricálcico encapsulado en grasa, TCP) a 4 niveles dietéticos de NPP (con 1,150 FTU de fitasa) sobre el rendimiento, la digestibilidad ileal del Ca y P y la mineralización ósea. Las fuentes de calcio también se evaluaron *in vitro* para medir la capacidad de unión al ácido (ABC) y la solubilidad del Ca a diferentes pH. El cloruro de Ca mostró la mayor solubilidad de Ca y la ABC más baja. El consumo de alimento y ganancia de peso al día 14 fueron mayores con el TCP y el carbonato cálcico que con el cloruro de Ca. La digestibilidad ileal del Ca fue mayor con el cloruro de Ca que con el carbonato cálcico o TCP.

En el capítulo 6, **el ensayo 3 y 4** tuvieron como objetivo explorar la influencia de diferentes fuentes de mono-, di- o tricalcio fosfato sobre el rendimiento de los animales, la mineralización ósea y la retención de minerales en pollos de engorde. En el ensayo 3, no se observaron interacciones entre la fuente de P y los niveles de NPP para las variables estudiadas; tampoco se observaron diferencias entre las fuentes de P. En **el ensayo 4**, los pollos de engorde alimentados con niveles más altos de MCP y TCP mostraron una mejora en FI, WG y G: F a diferentes edades en comparación con los niveles más bajos de MCP. El peso y las cenizas de la tibia, también fueron superiores para los animales alimentados con niveles altos de MCP y TCP que los alimentados con una dieta baja en MCP. No se observaron diferencias entre ambas fuentes.

En el capítulo 7, **los ensayos 5 y 6** fueron diseñados para evaluar la eficacia de una nueva fitasa, utilizada a diferentes dosis, y para comparar diferentes fitasas comerciales para pollos de carne. La inclusión de la nueva fitasa 1,000 FTU mejoró el crecimiento y la mineralización ósea en pollos de engorde hasta d 35 de edad, mostrando valores no diferentes a una dieta suplementada con P, un 0.2% más alta. No se observaron diferencias entre tipo de fitasas.

El ensayo 7, en el capítulo 8, fue diseñado para detectar la posible interacción entre la fuente de Cu ($CuSO_4$ y Cu_2O), y los diferentes niveles de inclusión sobre la utilización de Ca y P de pollos de engorde. Las dos fuentes de Cu también se evaluaron *in vitro* para medir la solubilidad del Cu y del fósforo fítico (PP), y la hidrólisis del PP mediante una fitasa a diferentes pH. Los resultados mostraron que el uso de 150 y 300 ppm Cu en forma de Cu_2O son adecuadas para garantizar un crecimiento adecuado de los pollos broiler, con una menor acumulación de Cu en los órganos en comparación con el $CuSO_4$.

Por todo ello, podemos considerar que, la utilización de altos niveles de Ca y P en la dieta de pollos o el uso de fuentes minerales altamente solubles, pueden producir respuestas negativas sobre los rendimientos productivos y la mineralización ósea.

Summary

Calcium and phosphorus are the two major macro-minerals required for proper growing performance and bone mineralization in poultry. Feed ingredients from plant sources contain very low amounts of Ca and P, mostly in phytate molecules. Thus, they are inadequate in meeting their requirements, and inorganic sources of Ca and P are usually added to poultry diets. The hydrolysis of phytate-P with exogenous phytase helps to decrease the incorporation of mineral sources, the environmental impact of broiler production, as well as the dietary cost. However, minerals can readily bind to phytic acid and form mineral phytate complexes that may be resistant to hydrolysis by phytase, depending on the pH values. The global aim of this thesis was to investigate dietary factors influencing Ca and P utilization by broiler chicks of different ages.

In order to achieve this objective, a series of seven trials (Chapters 4 to 8) were designed.

Trial 1 was designed to evaluate the interaction between dietary levels of Ca and non-phytate phosphorus (NPP, with 1,150 FTU of phytase) on broiler performance, bone ash and whole-body fractional retention of Ca and P from d 1 to 14 of life. Broilers achieved their greatest weight gain (WG) and bone mineralization with 0.7% Ca and 0.38% NPP. Increasing dietary Ca decreased performance and bone mineralization, especially of the low NPP diets. While increasing Ca reduced its fractional retention, the increase in the levels of dietary NPP steadily increased the fractional retention of Ca, likely through an increase in bone deposition.

The aim of **Trial 2** was to screen the influence of different Ca sources (limestone, Ca chloride, and a fat-encapsulated tri-calcium phosphate, TCP) at four dietary levels of NPP (with 1,150 FTU of phytase) on performance, ileal digestibility of Ca and P, and bone mineralization in broilers. Calcium sources were also evaluated *in vitro* to measure acid-binding capacity (ABC) and Ca solubility at different pHs. Ca chloride showed the highest solubility of Ca and the lowest ABC. The FI and WG on d14 were higher with TCP and limestone than with Ca chloride. Calcium ileal digestibility was higher with Ca chloride than with limestone or TCP.

In Chapter 6, **Trials 3 and 4** aimed to explore the influence of different sources of mono-, di- or tri-calcium phosphate on animal performance, bone mineralization and mineral retention in broilers. In Trial 3, no interactions were observed between the P source and the NPP levels for any variable; no differences were observed among P

sources. In Trial 4, broiler chickens fed with higher levels of MCP and TCP showed an improvement in FI, WG and G:F (gain feed ratio) at different stages, as compared to lower levels of MCP. Tibia weight and ash were also higher for animals fed with high MCP and TCP than for chicks of the low MCP diet. No differences were observed between either source.

In Chapter 7, **Trials 5 and 6** were designed to evaluate the efficacy of a new phytase and to compare different commercial phytases used at different levels in broiler diets. The inclusion of 1,000 FTU from the new phytase improved growth performance and bone mineralization in broilers up to d 35, reaching values no different of a diet supplemented with a 0.2% higher P content. No differences were observed among the phytase types.

Trial 7, in Chapter 8, was designed to screen the possible interaction between the Cu source ($CuSO_4$ and Cu_2O) supplemented at different levels on the utilization of Ca and P by broilers. The two Cu sources were also evaluated *in vitro* to measure Cu and phytic phosphorus (PP) solubility and PP hydrolysis by phytase at different pHs. The results showed that the use of 150 ppm Cu and 300 ppm Cu of Cu_2O is adequate to ensure broiler growth performance and a lower Cu organ accumulation in comparison to $CuSO_4$.

Taking the results all together, the use of high dietary levels of Ca and P or the use of highly soluble mineral sources can produce negative responses on bird performance and bone mineralization.

Index of contents

Index of tables

Index of figures

Abbreviations

ABC	Acid Binding Capacity
ADP	Adenosine Diphosphate
AMP	Adenosine Monoposphate
ATP	Adenosine Triphosphate
BWG	Body Weight Gain
Cu_2O	Dicopper Oxide
$CuSO_4$	Copper Sulfate
DCP	Di-calcium Phosphate
DM	Dry Matter
FCR	Feed Convertion Ratio
FI	Feed Intake
FTU	Phytase Units
G:F	Gain Feed Ratio
GIT	Gastro Intestinal Tract
$InsP_6$	Hexakis Dihydrogen Phosphate
MCP	Mono-calcium Phosphate
NPP	Non Phytic Phosphorus
PP	Phytic Phosphorus
SBM	Soy Bean Meal
TCP	Tri-calcium Phosphate
WG	Weight Gain

CHAPTER 1

General introduction

Calcium and phosphorus are the most abundant minerals in the body. They are essential for various biochemical pathways and skeletal integrity in poultry. The physiological roles of these two macro-minerals are intricately linked to each other (Selle *et al.*, 2009). Actual broilers selected for rapid growth require high levels of Ca and P for skeletal development and energy metabolism (Rama-Rao *et al.*, 2003). Supplementing broiler diet during this period with inadequate levels of Ca and P can lead to skeletal abnormality such as tibial dyschondroplasia, which is a common cause of deformity and lameness in broilers (Edwards, 2000). In poultry, bone breakage and associated infections contribute to low productivity and mortality (Rath *et al.*, 2000). Bone fragility is correlated with incidence of bone fragments in deboned meat products and with discoloration of meat adjacent to bone due to leaching of blood; the products may be less appealing to consumers (Rath *et al.*, 2000). An improper ratio of Ca and P could depress growth performance in broilers (Rama-Rao *et al.*, 2003; 2006) and affect bone mineralization. Driver *et al.* (2005b) confirmed that broken tibias and femurs during evisceration and carcass dissection were influenced by the low Ca and P content of diets fed during both the first 18 d and the last 19 to 35 d of age. In contrast, an excess of one or both minerals may reduce their availability due to the interaction between them, or may interfere with other micro-minerals (Cu, Zn, Mg …) and nutrients. For example, Ca can form soap precipitates with free saturated fatty acid, decreasing the energy digestibility of the diet and limiting animal growth (Edwards, 1960).

Calcium requirements for broilers have been measured following criteria to maximize performance and bone mineralization. As Ca is mainly stored in bones, Ca requirements for bone mineralization are usually higher than are those established to optimize body weight gain (Driver *et al.*, 2005b). However, the required values may depend on the age of the chicks and the composition of the diet, especially that of P and phytase incorporation.

Dietary Ca can be obtained from inorganic and organic sources, but there are little data on actual availability or digestibility, as opposed to relative Ca availability in feed ingredient sources. Additionally, Ca is considered a low-cost mineral with a low environment impact. This fact justifies that animal requirements are determined on a total Ca basis, and little information is known about the digestible Ca requirement for broilers and the availability of Ca sources. Other mineral factors, such as their buffering

capacity or their kosmotropic or chaostropic characteristics, may also be related to significant decreases in P solubility in the gizzard and may affect N and P digestibility (Tamim and Angel, 2003).

To cover broiler needs in P, inorganic sources as well as phytases are included in the diets. Vegetable ingredients are the major constituents of poultry diets. About two-thirds of the P of plant origin is presented as phytic acid (Cromwell, 1980). Phosphorus in phytic acid form is poorly available to monogastric animals (Sebastian *et al.*, 1996) because they do not possess significant amounts of endogenous phytase to hydrolyze phytic acid (Cooper and Gowing, 1983). Exogenous phytase is commonly added to broiler diets to improve the hydrolysis of phyate P, increase P digestibility, reduce P excretion in the environment and lower the cost of inorganic P addition (Shang *et al.*, 2015).

Since the first appearance of phytase in 1990, the poultry industry is now extensively using any of the different forms of phytase that have appeared on the market. From the results reviewed in the literature, it is clear that phytase is not able to release 100% of phytic P. Then, it has become necessary to assess the effectiveness and variability provided by the new sources of phytase or the dietary effects involved, and of course it is also necessary to define the dose-response effects of phytase in the diet.

Among the dietary effects involved on phytase efficacy, phytic acid has the capacity to bind or to chelate multivalent cations, including Ca, Zn, Fe and Cu (Tamim and Angel, 2003). The ability of the different metal ions to inhibit phytic phosphorus hydrolysis would be related to the stability of the complex as well as to the pH of the solution and the phytin-mineral molar ratio (Angel *et al.*, 2015). An excess of Ca in the poultry diet can cause antagonism with the absorption of other minerals through the formation of Ca-phytate complexes, which reduces the efficacy of phytase (Driver *et al.*, 2005a; Selle *et al.*, 2009). The use of high doses (therapeutic doses) of Cu in poultry diets has been shown to produce positive effects on bird health and immunity, and it is being used in some countries as growth promoters (Pesti and Bakalli, 1996). However, Cu is also able to chelate phytate and limit phytase hydrolysis. The solubility of this complex may depend on pH values (Selle and Ravindran, 2000), the complexes which precipitate at pH 6.5 are non-accessible for hydrolysis by phytase or absorption in the intestine. This effect may be different among different Cu sources.

The commercial development of phytase has provided the animal industry with a tool to decrease the need to supplement animal feeds with inorganic phosphorus, but the

use of a mineral source of P is still needed to meet animal requirements. Phosphorus supplementation of poultry feeds is routinely made by the addition of standard di-calcium phosphate. However, the characteristics of di-calcium phosphates obtained from different manufacturers will vary according to the origin of raw material (rock phosphate, phosphoric acid...) and also according to variations in industrial processing (Lima *et al.*, 1995). Economically, P is the third most expensive component in the poultry diet after energy and protein, so studies on P availability of the inorganic sources are of biological and economic importance (Lima *et al.*, 1997).

On this basis, it seems opportune to screen some the dietary factors mainly influencing Ca and P utilization by broiler chicks during the different phases of their short life. This thesis will also try to clarify the likely mechanisms behind these effects by studying the interaction factors observed in *in vitro* conditions.

CHAPTER 2

Literature Review

2.1. Importance of Calcium and Phosphorus for broiler chickens

2.1.1. Calcium

Calcium is, together with phosphorus, the main mineral retained in the body of broilers (Table 2.1). Calcium is the most abundant mineral in the body, and 99% is found in the skeleton. The skeleton not only provides a strong framework for supporting muscles and protecting delicate organs and tissues, including the bone marrow, but is also jointed to allow movement and is malleable to allow growth (Suttle, 2010).

The small proportion (1%) of body Ca that lies outside the skeleton is important to survival (Suttle, 2010). It can be found as free ion, bound to serum proteins and complexed to organic and inorganic acid. Indeed, it plays a role in blood coagulation, adhesion of molecules, neural transmission and signal transduction, muscle contraction, cellular motility, differentiation and proliferation, hormonal secretions and apoptosis (Brown, 2002). Calcium is necessary for muscular contraction that occurs as a result of neurotransmitter exocytosis (such as acetylcholine) at the neuromuscular synapse (Adeola *et al.*, 2005). Calcium is also involved in the release of hormones, such as insulin, by fusing secretory vesicles with lipid membranes and the expulsion of its contents in the target tissue.

In the poultry industry, Ca is mainly supplied with inorganic sources to reach Ca requirement levels in the diet, which has been described up to now on a total Ca basis. Calcium fortification of vegetable diets with limestone and calcium di-phosphate or mono-phosphate, together with a proper supplementation of vitamin D, reduces the risks of Ca deficiency in birds. However, there is still controversy in relation to the proper Ca levels and sources to provide to the animals as well as the digestive factors that may affect Ca absorption (Perry *et al.*, 1991).

2.1.2. Phosphorus

Phosphorus is the second most abundant mineral in the animal body, and about 80% is found in the bones (Table 2.1). Phosphorus is required for the formation of the organic bone matrix as well as the mineralization of that matrix. The remaining 20% of body P is widely distributed in the fluids and soft tissues of the body, where it serves a range of essential functions (Suttle, 2010).

Phosphorus is a component of nucleic acids, which are essential for cell growth and differentiation. As a phospholipid, it contributes to cell-membrane fluidity and integrity and to the myelination of nerves and, as a phosphate (PO_4^{3-}), it helps to maintain osmotic and acid-base balance (Suttle, 2010). Phosphorus also plays a vital role in a host of metabolic functions, including energy utilization and transfer *via* AMP, ADP and ATP (Adeola *et al.*, 2005). It is also involved in gluconeogenesis, fatty acid transport, amino acid and protein synthesis and activity of the sodium/potassium ion pump.

Through its involvement in many metabolic and structural processes, P is essential for animals to attain their optimum genetic potential in growth and feed efficiency as well as skeletal development. Because of the key role of P in growth and bone development and mineralization, the requirements of the animals for P are the highest during the time the animal is growing (Applegate and Angel, 2004). Therefore, growing broilers usually require P fortification of vegetable diets with di-calcium phosphate (DCP) or mono-calcium phosphate (MCP).

Table 2.1. Calcium and Phosphorus content in the chicken whole body and bones (g/kg).

	Ca	**P**
Whole body (g/kg)		
[1]Hatching	3.4	3.3
[1]7 weeks	6.8	5.1
Tibia content (g/kg DM)		
[2]Day 35, Male	168	80
[2]Day 35, Female	165	78

[1]Larbier and Leclerq, 1992 ; [2]Venäläinen *et al.*, 2006.

2.2. The symptoms of a Ca and P deficiency in poultry

As cited above, P and Ca are the main minerals in the whole body; they share a common storage in the bone structure and greatly affect each other during their absorption and metabolism (as we shall see later on). However, there are major differences between both minerals in relation to the consequences of a dietary deficit. In fact, Ca is considered a Type I nutrient (Table 2.2), while P is considered a Type II nutrient (nutrients are classified as either Type I or Type II based on the effect a deficiency has on the body).

Table 2.2. Examples of Type I and Type II nutrients.

Type I nutrients	Type II nutrients
All vitamins, Most trace elements, **Calcium**	Nitrogen, Sulfur, essential amino acids Potassium, Sodium, Magnesium, **Phosphorus**, Zinc, Water, Dietary sources of energy (including carbohydrate and fat)

(Emery, 2005)

Deficiency of Type I nutrients results in specific physical signs, such as anemia after Fe deficiency or scurvy after vitamin C deficiency (Emery, 2005). An animal responds to a deficiency of Type I nutrients by continuing its growth and consuming body stores, with an eventual reduction in bodily functions. Diagnosis is simple due to the symptoms, but also via measurement of the concentration of the nutrient itself in the whole body or storage tissues. Examples of other Type I nutrients, in addition to Ca, are Fe, Cu, Se and vitamins.

An animal responds to a deficiency of Type II nutrients by reducing growth and avidly conserving the nutrient to maintain the concentration of the nutrient in the tissues. The animal reduces excretion to conserve the nutrient, and a reduction of appetite usually accompanies this condition. Individuals with a Type II deficiency are stunted in growth and have no visual signs or differences from ″normal″ individuals. Other examples of Type II nutrients, in addition to P, are nitrogen, essential amino acids, K, Na or Zn.

Bone status is commonly used as an indicator of mineral adequacy in poultry diets. Well over 90% of Ca is found in the bones, where it combines with P to form calcium phosphate crystals or hydroxyapatite with the molecular formula $Ca_{10}(PO_4)_6(OH)_2$ (Scott *et al.*, 1982). Other elements including Na, Mg, Fe and Fl may also be incorporated into the hydroxyapatite crystal (Frandson and Spurgeon, 1992). With this description, it is easy to understand that a deficient Ca diet affects bone mineralization and strength (Reichmann and Connor, 1977), and is perhaps associated with increased risk of fractures (Blake and Fogelman, 2002).

The modern broiler chicken has been selected for rapid growth and increase in muscle mass, but it may also be associated with poor leg health and lameness due to reduced bone mineralization. Reducing Ca and P in the diet can also cause broken bones and bloody meat during processing of the carcass (Chen and Moran, 1995). In

particular, bone breakage during catching and transportation creates problems during processing (Gregory and Wilkins, 1992; Julian, 1998; Knowles and Wilkins, 1998).

Broken bones, especially fractured clavicle bones, may find their way into the meat, and must be removed at great expense. Hemorrhages in the meat are another major quality defect, which can lead to downgrading of the broiler carcass. This is very significant due to the increased current importance of selling cut-up chicken parts, in which the emphasis is no longer only on yield but also on characteristics such as bloody breast meat and broken bones (Gregory and Wilkins, 1990).

2.3. Phosphorus and Calcium requirements

The Ca and P requirements of domestic animals are usually discussed together because the requirement of each mineral depends on the concentration of the other in the diet. An excessive or deficient level of Ca or P in the diet often leads to a deficiency or excess of the other, which is due to the interactions between the two minerals concerning Ca and P availability and endogenous excretion (Al Masri, 1995). We shall try, in Chapters 4 and 7, to study the interaction between these two minerals more accurately.

2.3.1. Calcium

As referred stated above, requirements of Ca have been established based on its effect on performance, but mainly on bone mineralization. They are described on a total Ca basis (Table 2.3), and very few data on Ca availability or digestibility in feed ingredients and digestible Ca requirements for broilers are published. There are different causes that may justify this apparent lack of interest on improving the description on a digestible or available Ca basis. Calcium is considered, in contrast to P, to be a cheap nutrient, and its involvement in environmental contamination is low. However, recently, Angel *et al.* (2015) recommended moving towards a digestible Ca (dCa), because of the extensive use of phytase in poultry diets and the negative effects of Ca on phytase efficacy (Tamim and Angel, 2003).

It is generally assumed that Ca requirements levels include a wide safety margin to supply Ca requirements. Nevertheless, apart from the fact that inorganic Ca sources replace other ingredients in the diet, an excess in the levels of dietary Ca may interfere with the availability of other minerals, including P, Cu, Mn, Mg, and Zn (Maenz *et al.*, 1999).

Therefore, Ca requirements are affected by the criteria used to maximize or optimize and the age of the animals. According to Driver *et al.* (2005b), Ca requirements determined for 1-d-old to 16-d-old chicks (1.0% Ca; NRC, 1994) are adequate for maximum bone ash but excessive for all other measured variables. Both BWG and FCR were optimized at or below 0.625% dietary Ca, which may suggest that a lower total Ca concentration in general is desirable. Furthermore, significant sex differences were observed; males appeared to require more Ca than did females to maximize tibia ash but less Ca to optimize weight gain (0.49% ±0.11% vs. 0.62%±0.18% for males and females, respectively). Calcium requirements for FCR were very similar for both males and females (0.63% ±0.40% vs. 0.61% ±0.19%), respectively.

On the other hand, Ca must be soluble in the medium of the gastrointestinal digesta before absorption. Limestone, which is the main Ca source in the poultry industry, requires an acidic medium to reach a solubility of 80% (Walk *et al.*, 2012a). This implies that Ca can be mostly solubilized in the proventriculus and gizzard, but it becomes mostly insoluble in the small intestine. This may suggest that the current total Ca recommendation may be high, as they are associated with an intrinsically low available source. Recent reports suggest that the provision of an alternative, highly digestible Ca source at lower dietary concentrations may circumvent this problem (Bradbury *et al.*, 2012). Further studies are required to determine the Ca requirements of chicks based on the Ca source used in the diet.

2.3.2. Phosphorus

As shall be seen later, P in plants is present in different organic forms, such as phospholipids and proteins, but mostly as part of the phytic acid molecule. Phytic acid P has low availability for poultry, which implies that inorganic P must usually be added to the diet in order to meet P requirements in birds (Applegate and Angel, 2008). Phosphorus is also an expensive nutrient in the diet (approx. 150-300 $ per ton vs. 15-30 € per ton for Ca), and an environmental concern for the animal industry.

Table 2.3. Calcium and P Requirements for Broilers.

	FEDNA (2008)		NRC (1994)		INRA (1989)	
Age(d)	**1-15**	**16-37**	**1-21**	**22-42**	**1-15**	**16-35**
Calcium (%)	0.95-1.05	0.90-1	1	0.90	1.0-1.1	0.90-1.0
Phosphorus						
Total P (%)	0.65	0.60	-	-	0.67-0.70	0.66-0.69
Available P (%)	0.45	0.43	-	-	0.42-0.45	0.41-0.44
Non phytic P (%)	-		0.45	0.35	-	
Ca: NPP	-	-	2.2	2.57	-	
Ca: aP	2.11- 2.33	2.09-2.32	-		2.3- 2.4:1	2.19-2.27

Therefore, a greater effort has been made in order to improve and assure maximum P availability in the diet as well as to avoid an excess of P in the diet and excreta. The low digestibility of P in plant sources (Tamim and Angel, 2003; Tamim *et al.*, 2004) and the variable digestibility of P in inorganic sources (Coon and Leske, 1998) prompted the change in the use of P, from total P (tP, NRC, 1950) to inorganic P (iP, NRC, 1954), available P (aP, NRC, 1984), and non-phytic P (NPP, NRC, 1994).

Thus, the terms used to describe phosphorus requirements are:

- **Total P** (tP): This is generally referred to as phosphorus and encompasses any and all forms of phosphorus in the diet. It does not take into account differences on P availability (Angel *et al.*, 2002).
- **Digestible P** (dP): This refers to the P that is truly or apparently absorbed from the diet in the intestinal tract (i.e., feed P minus P within the distal ileum).
- **Retained P**: This refers to the P that stays in the body (i.e., feed P minus excreta P; Applegate and Angel, 2008).
- **Phytic P (PP):** In plants, IP6 (hexakis di-hydrogen phosphate) exists in its anionic form, phytate. In mature seeds, IP6 is found as a complex salt of Ca, Mg, and K, and in some cases it is bound to proteins and starches. Most of the P stored in seeds is present as phytin-P (PP) a form poorly available to poultry. This complexed or chelated molecule of $InsP_6$ is known as phytin (Angel *et al.*, 2002).
- **Non-phytic P (NPP):** Any P that is not bound to the phytin molecule, this NPP can be chemically determined by subtracting analyzed PP from analyzed P.

- **Available P** (aP): This refers to the P that is calculated to be absorbed from the diet by the animal (Angel *et al.*, 2002).

The difference between aP and NPP is that the term "aP" includes all absorbed forms of P, including inorganic P and a certain amount of organic P (including PP), whereas NPP excludes any PP available to the animal.

Many studies have been performed to determine the need for P and Ca in broilers at different life-stages. These studies are summarized in the following table (2.4).

Table 2.4. Different requirements of Ca and P for starter broilers depend on the criteria used.

Reference	Criteria	Age (wk)	Ca (%)	P (%)	NPP (%)	aP %	Ca:tP	Ca:NPP
Moran and Todd, 1994	Growth Bone ash	0-3	1.00	0.68	NS*	0.45	1.47:1	
Chen and Moran, 1995	Growth Bone ash	0-3	1.05	0.68	NS	NS	1.54:1	
Rama- Rao *et al.*, 1999	Growth	3-30 d	1	NS	0.44	NS		2.27:1
Angel *et al.*, 2000	Tibia/femur ash/strength	0-17 d	0.91		0.45-0.37	NS		2.21:1
[1]Driver *et al.*, 2005b	BWG Tibia ash	0-16d 0-16d	0.48-0.62 0.6-0.72	0.74 0.74	0.45 0.45		0.76:1 0.94:1	1.08:1 1.34:1
[2]Phillips *et al.*, 2012	BWG Tibia ash	1-10d 1-10d	1.16 1.17		0.51 0.69			2.25:1 1.70:1

1. Variable levels of Ca were evaluated with a fixed level of NPP (0.45).
2. Variable levels of Ca and P were factorially analyzed following surface analysis.
*NS: Not specified.

2.3.3. Ca:P ratio

Because of the complex interaction among Ca, P, vitamin D, and other calcitropic hormones, it is necessary to judiciously balance the amount of Ca and P added in the poultry diet (Lundy *et al.*, 1992; Rennie *et al.*, 1997, Rath *et al.*, 1999). The interactions of these two minerals are highly complex and are not easily interpreted. In the literature, Létourneau-Montminy *et al.* (2007, 2009) show the importance of the Ca: NPP ratios on growth performance and bone mineralization of broilers from 1 to 21 days. Similarly, Driver *et al.* (2005a) and Rama-Rao *et al.* (2006) studied the effects of changes in Ca and P or NPP intake in chickens from 1 to 16 days and from 1 to 42 days, respectively. Their work clearly demonstrates that the Ca:P ratio has a greater impact on quality and bone strength than does the intrinsic level of each mineral.

Historically, the ratio of Ca to P was defined for total Ca (tCa) and total P (tP) (NRC, 1950) in the diet. As the impact of phytate present in seeds and its impact on seed ingredient-based P availability began to be understood, a change was made to a tCa-to-available-P ratio (NRC, 1984). The tCa:aP ratios recommended were 2.22 to 2.28, depending on the age of broilers (from hatchling to 8 weeks of age). In 1994 (NRC, 1994), when the term ″NPP″ began being used to define P requirement, the tCa: NPP ratios recommended were 2.22 to 2.67, depending on growth stage. But according to Angel *et al.* (2015), because dCa in inorganic sources are widely different, it is better to use the ratio values between digestible nutrients. Furthermore, when phytase is introduced in the diet, we change the Ca source from a calcium phosphate (MCP or DCP) that usually has higher dCa (67%) to a limestone that usually has lower dCa (34.1%) (Angel, 2013). It is suggested that Ca may inhibit phytase activity (Applegate *et al.*, 2003), so it is important to know the dCa:dP ratio , when phytase is supplemented in the diet.

Al Masri (1995) describes a decrease in the availability of feed P from 0.66 to 0.30, as Ca: P ratios were changed from 1:1 to 2.5:1. The author states that increasing Ca concentration (i.e., from 0.66% to 1.58%) showed a greater effect on P absorption than on P retention, as the animals tended to reduce the endogenous P excretion in trying to conserve the nutrient (i.e., Type II nutrient).

In recent research, Anwar *et al.* (2016) described that increasing dietary Ca concentration from a 6.75 g/kg to 11.25 g/kg diet, when keeping the dietary P concentration constant (4.5 g/kg), decreased Ca digestibility and Ca retention.

High Ca or P levels in the intestine reduce the absorption of both (Al Masri, 1995). The solubility of mineral complexes decreased when Ca and P are supplemented at high levels. This level can increase ileal pH and reduces the absorption of both minerals (Shafey, 1993). We shall see this response in more detail in Chapter 5 and Chapter 8.

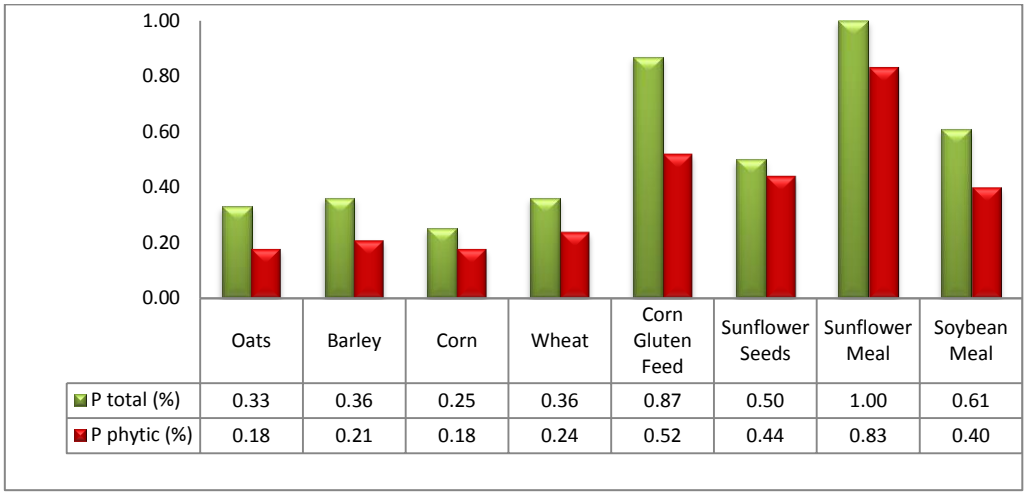
2.4. Phosphorus sources and availability

2.4.1. Vegetable sources of P

The P content in the raw materials used in animal feed presents a wide range of variation (Figure 2.5). In general, seeds (cereal grains, legumes and oilseeds) have a greater P content than do forages. The by-products of processing grains (wheat bran,

corn gluten or oilseed meal) are especially rich in P (Rebollar and Mateos, 1999). The level of P varies not only among sources but also within each source. Phosphorus content depends on soil type, cultivar, maturation state, culturing conditions, weather, etc., (Ravindran *et al.*, 1995; Rebollar and Mateos, 1999).

Most of the P in seeds is stored in the form of phytate, that is, the mixed salt of phytic acid (Myo-inositol 1, 2, 3, 4, 5, 6-hexakis di-hydrogen phosphate ($InsP_6$)), which is a phosphorolyted cyclic sugar alcohol. It contains 28.2% of bound P with molecular weight of 660 and represents, on average, 70% of the total P (tP) in feed ingredients commonly used in poultry diets (Figure 2.1) (Maenz, 2001; Kornegay, 2001; Catala-Gregori *et al.*, 2006).



	Oats	Barley	Corn	Wheat	Corn Gluten Feed	Sunflower Seeds	Sunflower Meal	Soybean Meal
P total (%)	0.33	0.36	0.25	0.36	0.87	0.50	1.00	0.61
P phytic (%)	0.18	0.21	0.18	0.24	0.52	0.44	0.83	0.40

Figure 2.1. Total and phytic P content (%) in different feedstuff (FEDNA, 2016).

Phytic acid location varies depending on the type of grain. In wheat and rye, as well as part of monocotyledons, phytate (between 80% and 90%) is located in the aleurone layers and in the pericarp, whereas corn and sorghum accumulate phytic P in the germ (Figure 2.2). In legumes, phytate is concentrated in cotyledons, and for oilseeds it is diffusely distributed throughout the seed associated with protein-rich globular bodies (Cosgrove, 1980; Sauveur, 1989).

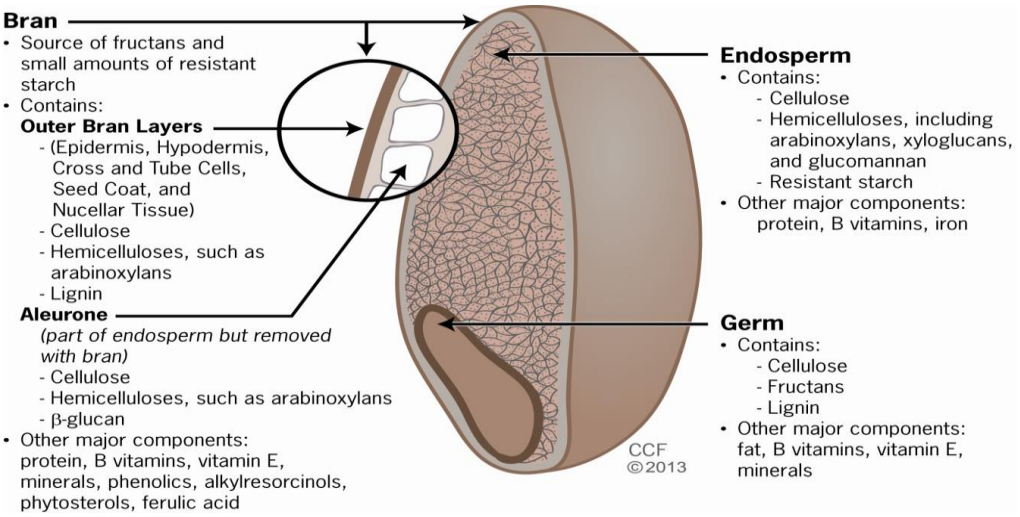


Figure 2.2. Schematic longitudinal section of a grain of wheat (Bernstein *et al.*, 2013).

Cereals contain between 0.2% and 0.3% phytic P; their by-products (except for maize and sorghum) are around 0.5% and 1.0%, and protein meals being between 0.3% and 0.9% (Pointillart, 1994). Maximal phytic acid levels are achieved at seed maturity immediately preceding desiccation (Raboy and Dickinson, 1987) and represent approximately 1% to 3% of the total weight in many cereals and oilseeds used in animal feeds (Cheryan, 1980; Angel *et al.*, 2002).

Seeds also accumulate mineral nutrients such as K, Mg, Ca, Fe, Zn, Cu, and Mn, which are used during germination in seedling growth (Iwai *et al.*, 2012). Phytic acid acts as a strong chelator of metal cations in mature seeds and binds them to form phytate, a salt of $InsP_6$ (Lott *et al.*, 2002; Raboy, 2009). Phytic acid is deposited in protein bodies (protein storage vacuoles) as a complex of chelated minerals and protein known as phytin (Prattley and Stanley, 1982).

Theoretically, the phytate molecule carries a maximum of twelve negative charges and could potentially chelate six Ca atoms in the digestive tract, but the affinity of phytate is greater for certain other divalent cations, including zinc and copper (Selle *et al.*, 2009) (Figure 2.3).

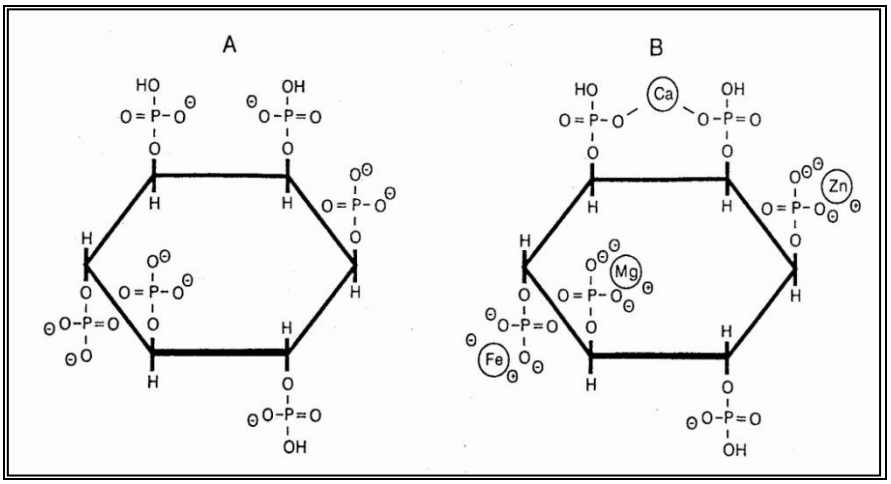


Figure 2.3. Structure of phytic acid (A) and phytic acid chelate with metal cations (B).

The order of stability of metal-phytate complexes was found to be: Cu > Zn> Co > Mn > Fe > Ca (Tamim and Angel, 2003). The maximum binding of divalent and trivalent cations occurs at pH 6, the normal pH of the duodenum, which is the major site of mineral absorption (Oberleas, 1973; Banks *et al.*, 2004a). The raw materials of animal origin, including the skeleton, are foods with high levels of P (FEDNA, 2011).

Thus, phytin is most commonly thought of as an anti-nutrient (Pallauf *et al.*, 1997; Angel *et al.*, 2002) because it reduces the availability of bound minerals for the animal.

From a practical standpoint, it is recognized that the availability of inorganic P and non-phytate organic P is similar and nearly 100% (range 80%-100%). On the contrary, the phytate P content is little available for, as the monogastric animals lack the precise enzyme (phytase), at least being sufficient to break and separate the P-inositol molecule (Kornegay, 1999). Hydrolysis of organic P in the gastrointestinal tract releases PO_4^{3-}, which is the only way that the animal can absorb and utilize P. The aP values described in the feedstuffs evaluation tables are values obtained without any exogenous phytase addition (Figure 2.4).

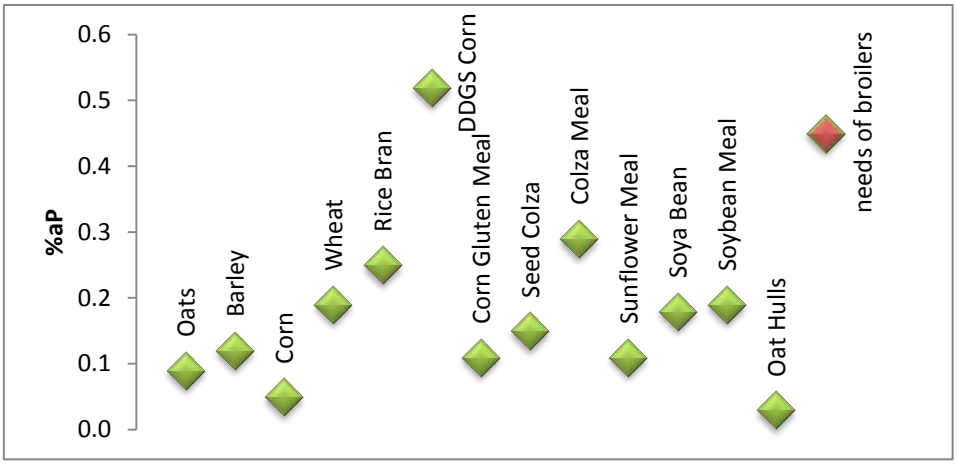


Figure 2.4. Comparison between available P content of some raw materials and broiler requirements (FEDNA, 2016**).**

2.4.1.1. Phytase

Phytase are phosphatases (myo-inisitol hexa-phosphate hydrolases) that dephosphorylate insoluble phytic acid in grains and oilseeds into orthophosphate and inositol phosphates Adeola and Cowieson, 2011) (Figure 2.5), making P fully available to monogastric animals (Gibson and Ullah, 1990). These enzymes are capable of hydrolyzing one or more phosphate groups from $InsP_6$, yielding P and a series of lower phosphoric esters (Harland and Morris, 1995; Angel *et al.*, 2002). Phytase hydrolyzes only phytate in solution, and with certain optimum conditions of pH and temperature that are variable according to the type of phytase (Wodzinski and Ullah, 1996).

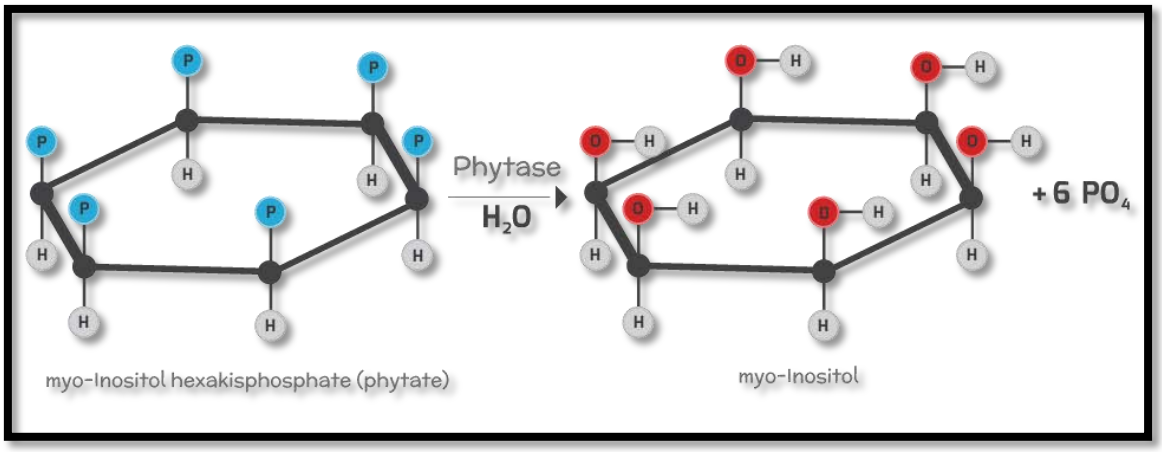


Figure 2.5. The mode of action of phytase.

2.4.1.1.1. Phytase activity

The activity of phytase is commonly expressed as FTU, which is defined as the amount of phytase required to liberate 1 mmol of inorganic phosphate per minute from 0.0051 mol/l sodium phytate at pH 5.5 and at a temperature of 37°C (Zyla *et al.*, 1995, AOAC). The pH level in the stomach of animals is far below pH 5.5 and, therefore, the ″real″ activity *in vivo* is different from the standard phytase activity measurement. In addition, many phytase characteristics, coupled with dietary and animal-related factors, can have an influence on phytase activity *in vivo* (Dersjant-Li *et al.*, 2015).

2.4.1.1.2. Type of Phytase

Generally, phytase has been categorized on two bases, depending on the site where the hydrolysis of the phytate molecule is initiated. The international Union of Biochemists (1979) acknowledges two classes of phytase with the following nomenclature: the 3-phytase (EC 3.1.3.8) initiates dephosphorization at the 3 position phosphate, while the 6-phytase (EC 3.1.3.26) commences at the 6-position; they produce different isomers of the lower inositol phosphates.

It has been stated that the 3-phytases are produced by microorganisms; the 6-phytases are mainly isolated from a plant source (Adeola and Cowieson, 2011). However, there are exceptions, as soybean phytases are 3-phytases and *Escherichia coli* phytases are 6-phytases (Sandberg and Andlid, 2002). The action of these 2 phytases differs in the location of phosphate removal, but also in how many phosphates they are able to remove from the myo-inositol hexaphophoric acid molecule and by their optimum activity at different pH levels. For example, the 3-phytase from *Aspergillus niger* (Natuphos) has 2 optimal pHs, namely 2.5 and 5.5, similar to the phytase from *Aspergillus ficuum,* which has optima pHs at 2.5 and 5.0 (Gibson and Ullah, 1990). The 6-phytase *Peniophora lycii* (Ronozyme) has two optima pHs, between 4.5 and 5.0.

Phytases are widely distributed in plants, animals, and microorganisms. In the digestive tract of animals, phytase can be present intrinsically in feed ingredients, produced by microflora present in the GIT, and from exogenous microbial phytases added to the diet (Hegeman and Grabau, 2001).

Endogenous intestinal phytase

The existence and role of intestinal phytase activity in poultry has been controversial (Abudabos, 2012). Its presence was first observed in chickens in 1938

(Krieger); later, Nelson (1976) concluded that intestinal phytase activity does not exist nor does it have a functional role in non-ruminants. In contrast, other results indicated the presence of phytase activity and a role for it in phosphorus utilization in chickens (Bitar and Reinhold, 1972; Maenz and Classen, 1998, Abudabos, 2012).

The origin of intestinal phytase is also disputed; some ascribe it to innate secretion (Bitar and Reinhold, 1972; Biehl and Baker, 1997) and others consider it as a product of gastrointestinal microflora (Wise and Gilburt, 1982; Kerr *et al.*, 2000). Maenz and Classen (1998), however, reported that intestinal brush-border-alkaline phosphatase (phytase) could contribute to degradation of phytate P; this phytase activity has been found in all segments of the small intestine. The specific and total activities of alkaline phosphatases in intestinal brush-border were highest in the duodenum and declined in the jejunum and ileum.

As intestinal enzymes are modified by dietary factors, it is possible that intestinal phytase may be affected by diet. In some studies, performed by McCuaig *et al.* (1972) and Applegate *et al.* (2003), significant differences were detected, but not in others (Maddaiah *et al.*, 1969; Biehl and Baker, 1997). Lowering the total P level of the starter diet resulted in a significant increase in phytase activity (Marounek *et al.*, 2010). A possible explanation for the increased utilization of phytate phosphorus with age is the suggestion that more endogenous phytase is present in the gastrointestinal tract of older animals.

Feedstufs phytase

It has been known, for more than 60 years, that some feedstuffs contain considerable phytase activity (Peers, 1953; Hill and Tyler, 1954b). Phytase activity varies greatly among species of plants. Among cereals, the highest phytase activities are found, in decreasing order, in rye, triticale, wheat and barley. High-protein feeds contain little activity, oats, maize and sorghum-negligible phytase activities (Eeckhout and de Paepe, 1994; Humer *et al.*, 2014). No correlation exists between the phytic P content in the grain and its phytase activity (Eeckout and De Paepe, 1994; Rebollar and Mateos, 1999). The majority of phytases in cereal grains are located in the aleurone layers and scutellum (Oatway *et al.*, 2001; Humer *et al.*, 2014). Diets including ingredients with high phytase activity, such as wheat bran and wheat, promote greater absorption of phytic P when diets are fed in mash form. Within wheat samples, phytase activity can

be highly variable (915 to 1581 FTU/kg; Eeckhout and De Paepe, 1994). Much of this variation can be explained through cultivar differences (Barrier-Guillot *et al.*, 1996; Applegate and Angel, 2004) and possibly through grain storage time and conditions.

The activity of this enzyme depends on moisture content, temperature and pH in dry cereals; phytases are inactive due to lack of moisture for activation (Humer *et al.*, 2014).

It is estimated that phytases contained in plants are at least 10% less efficient than those of fungal origin (Kornegay *et al.*, 1996). The reason might be the narrow range of pH at which plant phytases are active. Optimum pH for maximum activity is higher than is that found in the stomach of poultry (pH of 2.5-3.5), the principal point of action of phytases (Liebert *et al.*, 1993; Rebollar and Mateos, 1999). For example, 6-phytases from wheat have only one optimum pH, at 5.5 (Kies *et al.*, 2001).

Because vegetal phytases are active at a pH of 5 and are very sensitive to environmental conditions, pH too acidic or too alkaline may inactivate them irreversibly (Pointillart, 1994). Moreover, in certain regions of the gastrointestinal tract, where pH is 5-6, phytic acid can react with other minerals and precipitate, preventing the activity of phytase. In areas with lower pH (such as the proventriculus and gizzard in poultry), phytin is more soluble, but plant phytase is less active.

Optimal temperature ranges of plant phytases are from 45ºC to 60°C (Wodzinski and Ullah, 1996; Applegate and Angel, 2004). Plant phytases, nevertheless, may be partially or totally inactivated by over-heating or high steam-pelleting temperatures (Ravindran *et al.*, 1995). Blaabjerg *et al.* (2010) treated wheat with steam injection at approximately 70°C before pelleting at approximately 90°C, followed by cooling. The treatment led to a reduction in phytase activity by 74% (Humer *et al.*, 2014), whereas Jongbloed and Kemme (1990) reported that cold-pelleting did not exert negative effects on phytase activity. Producers that feed mash (diets that are not pelleted) diets may find some benefit from plant phytases, but they must consider the high inherent variability of vegetable phytase.

On the other hand, Phillippy (1999) also demonstrated that wheat phytase lost substantial activity when incubated with pepsin, a proteolytic digestive enzyme.

Phytase produced by industry

The first commercialized phytase was developed for use in The Netherlands in 1991 (Selle and Ravindran, 2007) to increase P availability from vegetable sources and,

as a consequence, reduce the inclusion of higher cost of organic phosphates supplementation and also to reduce P pollution from intensive agriculture (Onyango, 2005). Fungal phytase (*Aspergillus niger*) was the first generation of industrial phytase. Commercial phytases are typically produced using recombinant DNA technology, for example, a bacterial phytase gene being inserted into yeast for commercial production. This technology has greatly improved functional use of phytases by improving their thermostability, pH specificity, and resistance to breakdown by other digestive enzymes in the animal (Applegate and Angel, 2008). Table 2.5 presents some examples of currently commercial phytases.

Table 2.5. Some examples of currently commercially available 3- and 6-phytases and their characteristics.

Type	Protein origin	pH optima	Temperature Optima (ºC)	Commercial name
3	*A.niger*	2;5-5.5	65	Natuphos®
3	*A.niger*	6.0	-	Allzyme® SSF
3	*A.niger*	2.5	-	Finase® P/L
6	*E. coli*	4.5	55	Phyzyme® XP
6	*E. coli*	4.5	-	Quantum®
6	*E. coli*	-	-	Quantum Blue®
6	*E. coli*	3.4; 5.0	58	OptiPhos®
6	*Peniophora lycii*	4-5.5	50-55	Ronozyme®
6	*Buttiauxella spp.*	3.5-4.5	60	Axtra® PHY

(Dersjant-Li *et al.*, 2015)

2.4.1.1.3. Phytase efficacy

The addition of exogenous phytases in the diets of poultry has been shown to improve weight gain, mineral retention, energy utilization and amino acid digestibility (Ravindran *et al.*, 1999; Rutherfurd *et al.*, 2002; Augspurger *et al.*, 2003; Cowieson and Adeola, 2005). Phosphorus retention by broilers was improved from 50% to 60% by supplementing diets with a fungual phytase (Simons *et al.*, 1990; Kornegay *et al.*, 1996). Phytases also reduced the phytate-P excretion when they were supplemented to diets with little available P (Selle *et al.*, 2000). However, efficacy of phytase supplementation may depend on different factors (Ravindran *et al.*, 1995), such as:

1. The microbial source and form of the enzyme (coated, size of the particle, etc.),
2. Temperature, and optima pH of the enzyme,

3. The diet mineral concentration (Ca, Fe, Mg, Cu, and Zn), ingredients used or diet manufacturing methodology (pelleted, mash, or liquid),
4. Location of addition of phytase (post-pelleting or mixer),
5. Type and level of vitamin D metabolites,
6. The animal status (i.e., disease),
7. Resistance to endogenous protease.

The common recommended dose of phytase to be used is 500 FTU/kg in broiler diet for the destruction of 50%-70% of phytate. The inorganic P equivalent reported in the literature is between 0.3 g/kg-1.7 g/kg. Table 2.6 presents some P equivalent values, but the activity measurements may differ significantly due to the methods of analysis (Dersjant-Li *et al.*, 2015). However, in the last several years higher doses of phytase (three to four times the standard dose) are being used in poultry diets, showing some positive results in terms of nutrient availability and performance. It has been suggested that high phytase doses produce the complete de-phosphorylation of phytate and also release inositol, which is considered a growth promotor. Inositol is also known to have important metabolic roles, such as in fat metabolism and cell function, as well as being combined with phosphorus at a cellular level to recreate phytate, which is a potent anti-oxidant. Some studies like the one conducted by Zyla *et al.* (2004) addressed the positive effect of inositol provision in broilers. These authors observed that the supplementation of 0.10% inositol improved about 6.4% of FCR from 1 to 21d of age. According to Adoela *et al.* (2014), there may be three main mechanisms whereby using large doses of phytase may elicit beneficial effects:

1. It restores the dCa: dP ratio,
2. Less residual phytate and destruction of the anti-nutritive effect and increased generation of more soluble lower esters,
3. Generation of myo-inositol with lipotropic effects.

Shirley and Edwards (2003) indicated that a 94.8% phytate P disappearance could be achieved using 12,000 units of phytase (Natuphos 5000)/kg diet. Coon and Manangi (2004) indicated a 99.5% phytate hydrolysis in broilers fed diets supplemented with 5,000 units of phytase (Phyzyme XP) per kg diet. In recent years, the use of higher levels of exogenous phytase, referred to as super dosing, has been promoted as a strategy to release more phytic P and to reduce the anti-nutritive effects of phytate (Cowieson *et al.*, 2011).

Table 2.6. Summary of microbial phytase phosphorus equivalency studies in poultry.

References	tP (g/kg)	PP (g/kg)	Ca:P ratio	Response Criteria	P equivalence FTU=g P	Phyate-P Released (%)	Phytase source
Schoner *et al.* (1991)	4.5	2.3	1.33	P retention	700 = 1.0	43.5	*A. niger*
Schoner *et al.* (1993)	3.5	2.3	1.71	Weight gain, P retention	850 = 1.0	43.5	
Denbow *et al.* (1995)	3.8	1.8	2.00	Weight gain, toe ash	821 = 1.0	55.6	*A. niger*
Kornegay *et al.* (1996)	4.4	2.4	2.00	Weight gain, toe ash	939 = 1.0	41.7	*A. niger*
Yi *et al.* (1996)	4.5	1.8	2.00	Weight gain, toe ash	1146 = 1.0	55.6	*A. niger*
Yonemochi *et al.* (2000)	6.0	3.0	1.50	Gain, intake, tibia ash and P, plasma P	500 = 1.17	39.1	*A. niger*
Augspurger *et al.* (2003)	3.6	2.6	2.08	Weight gain, tibia ash	500 = 1.25	48.1	*E. coli*
Adedokun *et al.* (2004)	3.9	2.7	1.95	Gain, feed intake, toe and tibia ash,	1000 = 1.03	38.2	*E. coli*

(Selle and Ravindran, 2007)

According to Liberet *et al.* (1993), 25%-50% of the supplemented phytase activity takes places in the crop, when 500 FTU/kg or 1000 FTU/kg were added to a maize-soybean meal diet for chicken of 3-5 weeks old, and in the proventriculus, 10%-25% of the added phytase activity was detected. Li *et al.* (2016) demonstrated that phytase addition improved $InsP_6$ degradation in all segments of the gastrointestinal tract examined (Crop, proventriculus, gizzard and ileum), being the most effective with crop (pH= 5.5), proventriculus and gizzard (pH=3). These results were observed by Yu *et al.* (2004), who concluded that the crop and proventriculus are the major sites of exogenous phytase activity. The different types of phytase may differ in their activity in the digestive tract. Onyango *et al.* (2005) studied phytase activity in the gastro-intestinal tract of broilers from 8 to 22 days old and fed mash diets with or without microbial phytase (*E. coli* or *P.lycii*) at 100 FTU/kg. It was observed that supplementation of *E. coli* phytase significantly increased phytase activity in the crop, proventriculus and gizzard, jejunum and ileum, whereas *P.lycii* phytase activity progressively declined along the small intestine, with no detectable activity in the ileum in broilers. The low activity in the lower part of the small intestine may be due to the activity of endogenous digestive protease, which is able to break down exogenous phytase, thereby making its

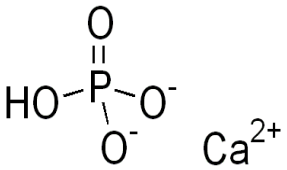
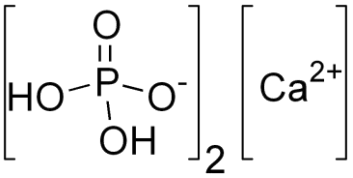
activity undetected in the ileum. However, different phytases differ in their resistance to endogenous protease (Dersjant-Li *et al.*, 2015).

2.4.2. Mineral sources of phosphorus

Phytases are widely used in the poultry industry, as they are critical for an efficient and sustainable use of vegetable sources. However, most of the diets need to be fortified with mineral sources of P.

Di-calcium, mono-calcium, mono-di-calcium phosphates and de-fluorinated rock phosphate are the most commonly used forms of inorganic feed phosphates. The terms mono- and di-calcium phosphate (Table 2.7) are commonly used in product descriptions; most commercial inorganic feed phosphates in the above categories are not pure products, but rather mixtures of MCP and DCP (Viljoen, 2001).

Table 2.7. Comparison between Di-calcium phosphate and Mono-calcium phosphate.

	Di-calcium phosphate	**Mono-calcium phosphate**
Molecular formula	$CaHPO_4$	$CaH_4P_2O_8$
Solubility in water	0.02 g/100 ml	2 g/100 ml
Molecule		

The ratio of these products in commercial, inorganic phosphates sources depends to a large extent on reaction conditions (heat, water and pressure) and on design conditions specific to the particular manufacturing plant. Baker (1989) described the typical commercial products; DCP ($CaHPO_4$) and MCP [Ca $(H_2PO_4)_2.H_2O$] contain mixtures of $CaHPO_4$, Ca $(H_2PO_4)_2.H_2O$, and $CaHPO_4.2H_2O$. Mono-calcium phosphate generally contains 13% $CaHPO_4$, and 61% Ca $(H_2PO_4)_2.H_2O$, with the remainder being small amounts of other phosphates and minerals. Commercial DCP generally contains about 14% Ca $(H_2PO_4)_2.H_2O$, 35% $CaHPO_4.2H_2O$ and 26% $CaHPO_4$ (Joseph and Scares, 1995). In general, DCP, which is less soluble than MCP, is the preferred source in poultry feeding. But the concentration and availability of P in commercial phosphates must receive proper attention in feed formulation, not only because absolute

concentrations of P may vary but also because differences in bioavailability of different commonly used phosphates have been well-documented.

2.5. Calcium sources and availability

2.5.1. Vegetable source of Calcium

Except for some vegetable ingredients, such as rapeseed meal, vegetable feedstuffs are very low in Ca content (Figure 2.6) and, therefore, the provision of adequate dietary Ca supply is almost entirely achieved through the use of animal-based and inorganic feedstuffs whose Ca bioavailability is > 66% (NRC, 2005).

Little is known about Ca availability in vegetable feedstuffs, perhaps as a consequence of their low Ca content (NRC, 2005). It is known that 20%-30% of Ca in plant tissues is bound to oxalate, which is relatively unavailable (NRC, 2001). Moreover, the high phytic content of some ingredients make Ca little available. Based on Ca digestibility in corn soy diets with no added inorganic Ca and P sources, Tamim and Angel (2003) calculated that availability of Ca in corn and SBM is 20% to 33%.

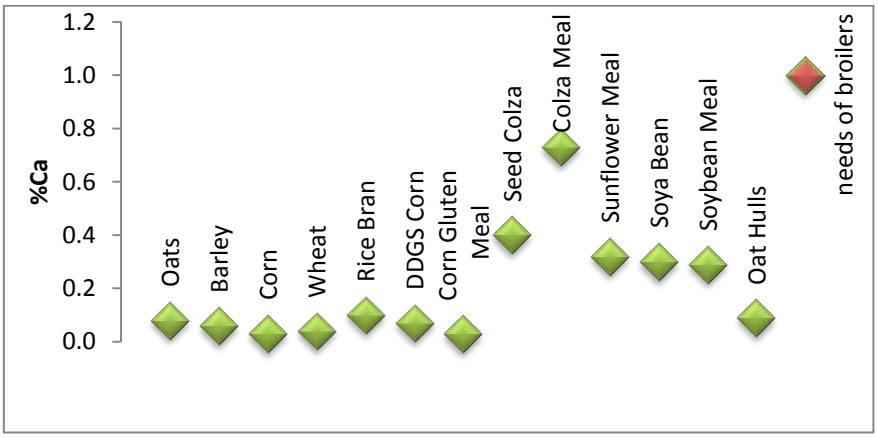


Figure 2.6. Calcium content in different cereal, vegetable protein and fibrous ingredients, as compared to total Ca requirements in broilers (FEDNA, 2016**).**

2.5.2. Mineral sources of Calcium

Principally used inorganic sources for Ca in the poultry industry are limestone, as well as di-calcium or mono-calcium phosphate (Walk *et al.*, 2012a). The primary source of Ca for diet supplementation is ground limestone (also known chemically as $CaCO_3$), because more than 80% of the Ca in the earth's crust exists as limestone. The bioavailability of Ca from these different sources has been extensively discussed (Shafey, 1993; Walk *et al.*, 2012a). In the diet, Ca can be present in a fine (e.g.,

limestone, which may contain a range of particle size) or coarse (e.g., oyster shell) form (Figure 2.7).

Limestone (large particles) Limestone (small particles) Oyster shell

Figure 2.7. Various sources of dietary calcium.

Oyster shell, a common source of Ca in laying-bird diets, also has highly (100%) relative available Ca. Marble dust and aragonite are considered less common sources of Ca for domestic animals (Peeler, 1972). Considerably more research on Ca bioavailability has been reported for poultry than for other animals, underscoring the importance of Ca to these species. However, as reported above, Ca bioavailability is highly dependent on different factors, such as the physical form of the ingredient and the dietary level, as well the animal age. Calcium requirements have usually been measured using limestone in the diet, which shows a reduced solubility during neutralization in the small intestine (Goss *et al.*, 2007) and few efforts have been made to optimize the use and availability of new Ca sources.

2.6. Calcium and Phosphorus homeostasis in chickens

The metabolism of Ca and phosphorus (P) is closely related, and a deficiency or an excess of either one will interfere with the utilization and metabolism of the other (Kebreab and Vitti, 2005).

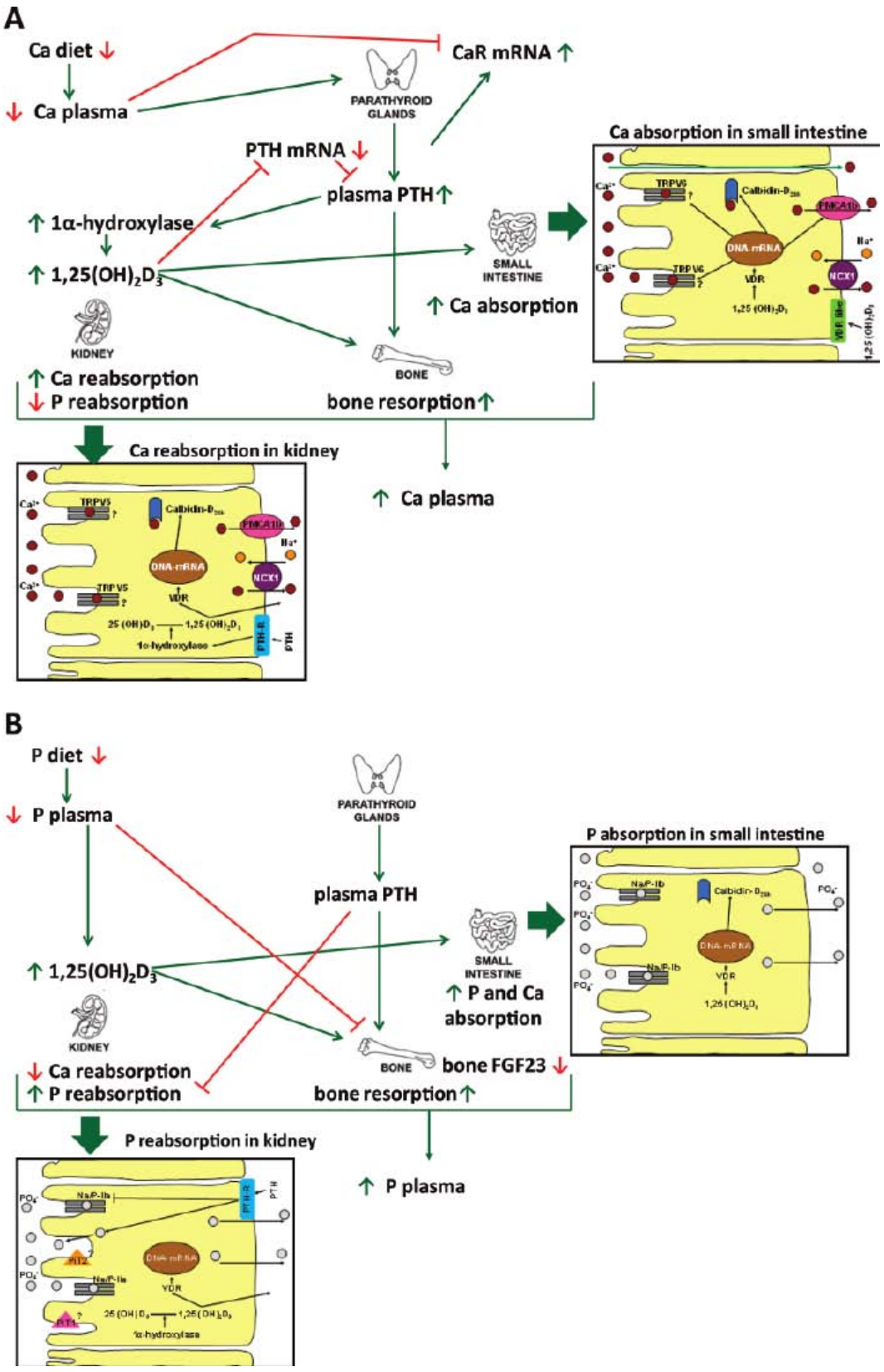
Absorbed P and Ca are rapidly transferred into skeletal and soft tissues of the body. Bones serve as storehouses of these two minerals, which can be mobilized when they are needed. There is a continuous turnover of P and Ca in the body, so resorption from bone and soft tissues takes place concurrently with synthesis. So, the metabolism of these two minerals involves not only deposition but also the processes of storage and mobilization (France *et al.*, 2010; and Veum, 2010). Calcium and P are ingested with the feed and absorbed primarily in the duodenum and jejunum in the small intestine (De Vries *et al.*, 2010). The extracellular pool contains 9 mg Ca/dl to 12 mg Ca/dl and from

4 mg P/dl to 9 mg P/dl. In mono-gastric animals, up to 50% of the dietary Ca^{2+} is absorbed by passive transport (diffusion), with active transport (energy-dependent transport) in situations of greater plasma Ca^{2+} deficiency.

Plasma Ca^{2+} and P concentrations are controlled by feedback mechanisms involving parathyroid hormone (PTH), vitamin D converted to the biologically active form ($1,25(OH)_2D_3$, also called di-hydroxycholecalciferol), calcitonin and their respective receptors in the small intestine, bone and kidney (France *et al.*, 2010; Veum, 2010).

Calcium and P homeostasis is maintained through a complex feedback system described by many authors (Proszkowiec-Weglarz and Angel, 2013), which is illustrated in Figure 2.8, when plasma Ca^{2+} and/or P are too low as a result of a Ca- or P-deficient diet in comparison to requirements. The parathyroid gland releases PTH which, in turn, stimulates the conversion of vitamin D_3 to the steroid hormone $1,25(OH)_2D_3$. Increased production of $1,25(OH)_2D_3$ in the kidney results in increased intestinal absorption of Ca and P and bone reabsorption and reduces Ca^{2+} and/or P excretion by the kidney (Proszkowiec-Weglarz and Angel, 2013; De Vries *et al.*, 2010) to maintain normal plasma Ca and P concentration.

Conversely, when plasma Ca^{2+} and/or P are too high, the peptide hormone calcitonin reduces the intestinal absorption and bone resorption of Ca^{2+} and P and increases excretion by the kidney. In conclusion, Ca and P homeostasis is maintained by feedback mechanisms regulated by plasma Ca^{2+} and P concentrations, which activate the release of hormones that affect intestinal absorption, bone apposition or resorption and kidney excretion of Ca^{2+} and P.



CaR = calcium sensing receptor; 1,25(OH)2D3 = active vitamin D3; FGF23 = fibroblast growth factor 23; PTH = parathyroid hormone;

Figure 2.8. Proposed model depicting Ca (A) and P (B) metabolism in broiler chickens (Proszkowiec-Weglarz and Angel, 2013).

2.7. Factors affecting calcium and phosphorus nutrition

2.7.1. Interactions between dietary calcium and phosphorus

2.7.1.1. Formation of calcium phytate complexes

On the one hand, calcium exists as mineral-phytate complexes in poultry diets; in fact, it is limited by the relatively low concentrations in feed ingredients and the inherent structure of phytate (Lott *et al.*, 2000; Selle *et al.*, 2009). On the other hand, the formation of Ca-phytate complexes along the gastrointestinal tract of birds is very important. Ca-phytate complex formation is influenced by molar ratios of their constituents, the gut pH, and their reduced solubility (Selle *et al.*, 2009). Poultry diets typically contain 10 g/kg of both Ca and phytate; if one phytate molecule binds five Ca atoms in the gastrointestinal tract, approximately one-third of dietary Ca would be present as Ca-phytate complexes. Instructively, Pang and Applegate (2007) found that Ca solubility was only 11% in the duodenum and jejunum of chickens offered diets containing 9 g/Kg of Ca and 9.22 g/Kg phytate. Mohammed *et al.* (1991) showed that decreasing Ca levels from 10 g/kg to 5 g/kg in low P diets resulted in a 15% increase in phytate-P digestibility in chicks. Plumstead *et al.* (2007) demonstrated that increasing dietary Ca from 4.7 g/kg to 11.6 g/kg linearly decreased ileal phytate-P digestibility by 71%.

2.7.1.2. Formation of calcium phosphate complexes

Calcium has the capacity to interact with inorganic P in the gut lumen, in addition to phytate-P (Selle *et al.*, 2009). Hurwitz and Bar (1971) concluded that an excess of Ca relative to inorganic P increased the formation of inorganic Ca-P precipitates, which decreased the concentration of soluble forms of P in the intestinal lumen and reduced P digestibility. Tamim *et al.* (2004) reported that 5 g Ca/Kg reduced ileal digestibility of phytate-P by 63%, but additional Ca also reduced the digestibility of total P by 57%. This can be due to the precipitation of Ca, which was binding with both phytate and inorganic P to form either Ca-phytate complexes and/or calcium phosphates (Selle *et al.*, 2009).

2.7.2. Intestinal pH

Several researchers have studied the solubility and stability of phytate-metal complexes and have observed that both are pH-dependent (Wise, 1983; Maenz *et al.*, 1999; Selle *et al.*, 2000).

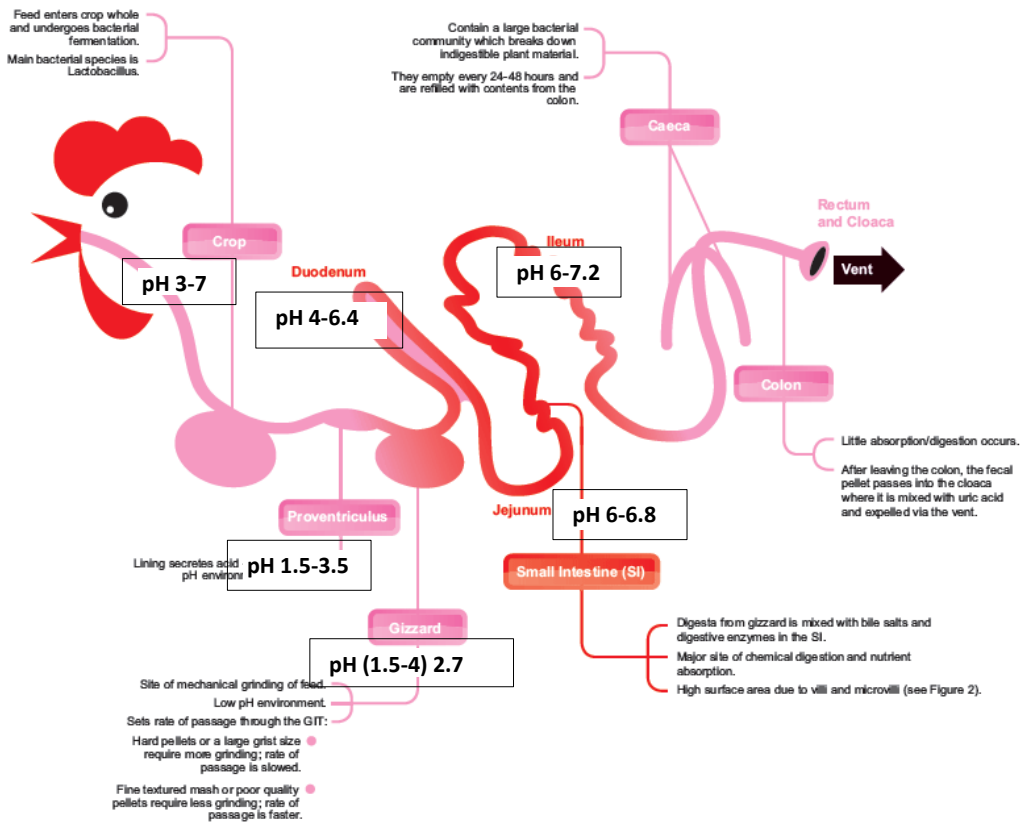


Figure 2.9. The pH value in the different parts of the broiler digestive tract (Aviagen, 2013).

Most phytate-mineral chelates are soluble at low pH (3.5), with decreasing solubility at higher values between pH 4.5 and pH 7. The approximate pH of the intestine, where absorption of minerals takes places, coincides with the pHs at which these complexes precipitate (Figure 2.9) (Tamim and Angel, 2003). At pH 6, the normal pH of the duodenum, maximum binding of phytic acid to minerals forming Zn-Ca-Cu - phytate and Cu-Ca-phytate complexes occurs (Oberleas, 1973).

The insoluble $InsP_6$- mineral complexes formed a higher pH, where plant phytases are most active, and may be resistant to hydrolysis by phytases of plant and microbial origin (Sooncharernying, 1993; Angel *et al.*, 2002).

An increase in gizzard pH significantly reduced Ca solubility in broilers (Guinotte *et al.*, 1995), and a higher pH (5) has been involved in Ca-phytate interactions in the gastrointestinal tract and interference with macro-mineral absorption (Simpson and Wise, 1990). Limestone, the dominant source of Ca in poultry diets, has a high acid-binding capacity (Lawlor *et al.*, 2005), so high dietary limestone may act as an anti acid in the distal portions of the gizzard and ileum. According to Guinotte *et al.* (1995), increasing dietary limestone increased gizzard pH of immature pullets and increased

crop and ileal pH in 12-d-old broilers. So the use of other Ca sources with lower ABC and higher solubility can limit the increase in pH values in the digestive tract. This will be one of our hypotheses in this thesis.

2.7.3. Calcium particle size

The solubility of Ca in the gastrointestinal tract may have a direct effect on the formation of phytic P-mineral complexes. Research usually neglects to describe the limestone particle size and Ca solubility in the mineral studies in poultry or studies aiming to evaluate exogenous phytase in broilers (Manangi and Coon, 2007). Despite its looking like a contradiction, broilers may gain more from feeding phytase by eating larger-particle $CaCO_3$ with lower solubility to minimize the solubility of $CaCO_3$ in the crop and in the anterior portion of the gastrointestinal tract. A low-solubility form of $CaCO_3$ may allow the phytase enzyme more access to phytic acid P in the gut and provide more available P from phytic acid hydrolysis in the broiler (Manangi and Coon, 2007). Phytic P hydrolysis was reduced 8% in an *in vitro* assay when the incubation mixture was pH 2.5 and contained the smallest particle size $CaCO_{3,}$ as compared to a mixture with the largest-particle size of $CaCO_3$ (Manangi and Coon, 2007).

Limestone with very small particles has high solubility and may pass through the gastrointestinal tract at a faster rate and decrease maximum retention. The highly soluble Ca from the small particles may also enhance the formation of a mineral-phytic complex that reduces the ability of added dietary phytase to hydrolyze phytic acid. These mechanisms may explain that feeding chicks a diet with $CaCO_3$ particle sizes between 137 μm and 388 μm increased the body weight gain of animals as compared to that obtained by feeding either smaller (28 μm) or larger particle (1306 μm) sizes (Manangi and Coon, 2007). An increased ash tibia content was also obtained for the chicks fed $CaCO_3$ particle sizes ranging from 137 μm -388 μm, as compared to the smallest (28 μm) or largest particle (1306 μm) sizes.

However, Walk *et al.*, (2012a) have presented the results on the influence of a highly soluble Ca source (from 0.45% to 0.9% Ca in the diet) on performance and bone mineralization. Their results showed that feeding broiler chicks with a higher soluble source of Ca with phytase allowed for reductions in dietary Ca while maintaining broiler performance and bone ash. Their results again suggest that the current recommendation of total Ca for broilers may be overestimated, as they have been

mostly defined using limestone-containing diets, which encourages the interest of moving forward to better know Ca requirements on a digestible basis.

2.7.4. Phytase interactions

It has been shown that some feed additives may have deleterious effects on phytase efficacy. For example, other mineral levels, in addition to Ca, in the diet may influence phytic phosphorus hydrolysis by phytase. According to Maenz *et al.* (1999), phytate can bind or chelate multivalent cations to form phytate-mineral complexes and limit phytase hydrolysis (Tamim and Angel, 2003). The order of stability of metal-phytate complexes was found to be Zn > Co > Mn > Fe > Ca. While Ca has one of the lowest affinities for phytate, it has the greatest impact, because it is the mineral at the highest level in the diet. In this thesis, we shall study only the effect of phytase on Ca and Cu-phytate complexes.

2.7.4.1. With Calcium

It was demonstrated by Applegate *et al.* (2003) that Ca levels used in broiler diets (0.9%) reduced intestinal phytase activity and apparent ileal PP hydrolysis, compared with a lower level of Ca (0.4%). In the same way, Sebastien *et al.* (1996) described that the best phytase efficacy supplemented to a corn-soy diet was seen with diets containing 0.6% Ca, as compared to a diet with 1% Ca. Tamim *et al*. (2004) also confirmed that the addition of Ca in broiler diets resulted in a decrease in PP hydrolysis. These authors attributed the effect of reduced PP utilization at higher Ca concentrations to one of three factors:

1. Precipitation of phytate by Ca through Ca-phytate complex formation, based on the findings of Wise (1983);
2. Increased intestinal pH caused by the addition of Ca, reduced mineral solubility and therefore availability, as reported by Shafey and McDonald (1991);
3. The direct effect of Ca on phytase competing for the active sites of phytase, as described by McGuaig *et al.* (1972).

2.7.4.2. With Copper

Copper is one of the essential trace minerals required by animals, for growth and for the prevention of a wide range of clinical and pathological disorders in all types of farm animals. It is also vital in the body as a component and cofactor for enzyme

systems involved in iron transport (caeruloplasmin) and metabolism, red blood cell formation and the immune function. Along with Fe, Cu is necessary for hemoglobin synthesis. Copper is not contained in hemoglobin, but a trace of it is necessary to serve as a catalyst before the body can utilize Fe for hemoglobin formation (McDowell, 1992).

Copper is an essential mineral required for proper bone growth and development as well as enzyme function (Banks *et al.*, 2004a). Rucker *et al*. (1975) found that Cu deprivation in the chick produces a normocytic and normochromic anemia, and low monoamine oxidase or cytochrome c oxidase activity in the bones may compromise osteoblastic activity leading to abnormal bone morphology. A deficiency of Cu was shown to decrease collagen crosslink formation and to lower mineralization (Osphal *et al.*, 1982). Reduction of collagen crosslinks through Cu deficiency can reduce calcification and, consequently, bone strength.

The copper requirements for broiler are 8 mg/kg NRC (1994). However, in some countries Cu is often added to poultry diets at prophylactic concentrations; it has also become a common practice in the United States and elsewhere to supplement broiler diets with 125 mg/kg to 250 mg/kg additional copper from Cu sulfate pentahydrate to enhance health and growth promoting effects (Pesti and Bakalli, 1996) as one alternative to antibiotics.

As described above, phytic acid is negatively charged over a wide pH range and chelate divalent and trivalent metal ions, such as Ca^{2+}, Cu^{2+}, Fe^{2+}, to form mineral-phytin complexes existing in soluble and insoluble forms (Pang and Applegate, 2006). Consequently, the high amount of Cu in diets has been shown to have negative influences in the availability of phytase to hydrolyze PP (Pang and Applegate, 2006). Similarly, Cu sources may have different chemical-property effects within the digestive tract, including different solubility, and a different chelation capacity for PP. Therefore, phytin can also bind to Ca as well as to other minerals at the same time. Multiple mineral complexes are assumed to be more stable than are single mineral complexes (Maenz *et al.*, 1999). The insoluble complexes formed will limit the hydrolysis of phytin-P, the mineral absorption (Banks *et al.*, 2004b) and reduce the bioavailability of the mineral due to decreased solubility at intestinal pH (Persson *et al.*, 1998). Thus, the likely effects of including different levels and sources of Cu in the diet on the P digestibility and broilers performance merits to be studied.

CHAPTER 3

Objectives and experimental design

The purpose of this thesis will be to **highlight dietary factors influencing Ca and P utilization by broilers chicks.** We hypothesize that:

1. A significant decrease in the level of Ca and P will improve broiler performance and bone mineralization by reducing digestive interactions and improving mineral retention.
2. High-soluble Ca sources with low acid-binding capacity can be used to improve the mineral digestibility and reduce the levels of Ca inclusion in the diet.
3. The use of different mineral sources of P will affect the dietary P absorption and overall productivity of broiler chickens.
4. Reducing the levels of Ca and P, and giving high doses of phytase, may help to increase P availability, performance, and bone mineralization in broilers.
5. The use of therapeutic doses of Cu in the form of di-copper oxide will improve PP hydrolysis and broiler performance as compared to copper sulfate.

Considering these hypotheses, the main objectives of this thesis are:

1. To establish optimum dietary Ca and NPP levels for starting broilers from their performance and bone mineralization responses to a factorial range of diets containing different levels of Ca and NPP plus a high dose of phytase. **Trial 1** proposes **to study** the interaction among three levels of Ca (0.5%, 0.7%, and 0.9%) and four levels of NPP (0.25%, 0.31%, 0.38%, and 0.45%) in the diet with a high dose of phytase, to establish the optimum dietary Ca and NPP for starting broilers from their performance, bone mineralization and whole-body fractional retention of Ca and P.

2. To evaluate the Ca *in vitro* solubility and ABC (acid-binding capacity) characteristics of different Ca sources, and explore how the incorporation of these sources may affect the ileal digestibility of Ca and P, animal performance, and bone mineralization in broiler chickens. **Trial 2** proposes to evaluate different Ca sources (limestone, Ca chloride and Lipocal, a fat-encapsulated tri-calcium phosphate, TCP) in conjunction with four dietary levels of NPP (0.3%, 0.35%, 0.4%, and 0.45%) in starting broiler chickens. Calcium sources were also evaluated *in vitro* to measure ABC and Ca solubility at different pH values.

3. To evaluate the effect of the P source provided at different levels in broiler diets on the availability of P and their effects on performance and bone mineralization for broilers. **Trial 3** was designed to compare P availability among MCP and different sources of DCP in broiler chickens up to d 21. **Trial 4** compared the inclusion of a source of TCP, as a single source of Ca and P, and MCP plus limestone in broiler diets, with respect to their effects on performance, bone mineralization and P retention in broilers up to d 35.

4. To evaluate the efficacy of increasing levels of a new microbial 3-phytase (FLF® 1000 FUT, liquid phytase produced by Fertinagro®) to improve performance and bone mineralization in broilers. In **Trial 5**, different dietary levels of Ca (0.7% and 0.9% in the starter phase; 0.63% and 0.85% in the grower phase) and NPP (0.32% and 0.47% in the starter phase; 0.29% and 0.43% in the grower phase) were used to test, in the first stage, the efficiency of a new microbial phytase at different levels of inclusion for broilers at 35 days.

5. To compare, in the second stage, the efficacy of the new phytase in the diet to three other commercial phytases for enhancing the utilization of low NPP diets with low and high Ca:aP ratios. **Trial 6** was designed to test the efficacy of different commercial phytase sources (OptiPhos®, Phyzyme®, Ronozyme®, FLF® 1000 FUT) for enhancing the utilization of low NPP diets with low and high Ca:aP ratios for broilers at 21 days.

6. To compare the effect of copper sulfate, the most commonly used Cu source for supplementation in poultry diets, and di-copper oxide (Cu_2O; CoRouge®) in the diets of broiler chickens. **Trial 7,** focused on the comparison of the effects of two sources of Cu at three levels of dietary Cu (15 ppm, 150 ppm, 300 ppm) in broiler chicken performance, mineral interaction in the digesta and mineral accumulation in organs and tissues. An *in vitro* test was also designed to compare the solubility of both sources and to identify likely interactions with the phytic phosphorus (PP) and phytase hydrolysis.

This thesis has been partially funded by the project Fertinagro FER -23-2013 and the Animal Nutrition and Welfare Service SNiBA.

This work also was possible with the intense collaboration of other research services of our University, such as the Animal Farm Facilities Services (SGiCE) and the the Service of Chemical Analyses (SAQ).

CHAPTER 4

The effect of different levels of calcium and phosphorus and their interaction on the performance of young broilers

The effect of different levels of calcium and phosphorus and their interaction on the performance of young broilers

M. Hamdi[1], S. López-Vergé[1], E. G. Manzanilla[2], A. C. Barroeta[1], and J. F. Pérez[1]

[1] Animal Nutrition and Welfare Service (SNiBA). Departament de Ciencia Animal i dels Aliments. Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain .

[2] Animal and Grassland Research and Innovation Centre, Teagasc; Moorepark, Fermoy, Co. Cork, Ireland.

Published in:

Poultry Science (September 2015) 94: 2144-2151 (Hamdi *et al.*, 2015a)

4.1. Abstract

A study was conducted to evaluate the interaction among 3 levels of Ca and 4 levels of non-phytate phosphorus (NPP) on broiler performance, bone ash and whole-body fractional retention of Ca and P. *Ross* male broiler-chicks (n = 420) were sorted by BW at d 1 post-hatch and assigned to 5 cages per diet with 7 birds per cage. Twelve diets were arranged in a 3 x 4 factorial of 3 levels of Ca (at 0.5%, 0.7%, or 0.9%) and 4 levels of NPP (at 0.25%, 0.31%, 0.38%, or 0.45%) with a high dose of phytase (1,150 U/kg) in all diets. On d 14, chickens were euthanized and the right tibia was collected from 3 birds per replicate; the rest of the animals were used to measure whole-body Ca and P retention. An interaction was observed between the level of Ca and NPP on feed intake (FI), tibia weight and bone-ash content ($P < 0.05$). Increasing the level of NPP from 0.25% to 0.38% increased FI ($P < 0.05$) on chickens fed the high-Ca diet (0.9%), but not with Ca at 0.5% or 0.7%. Broilers achieved their greatest weight gain (WG) and bone formation with 0.7% Ca and 0.38% of NPP. Increasing the dietary Ca decreased its fractional retention from 74% with dietary Ca at 0.5% to 46% with Ca at 0.9%. The increase in the levels of dietary P steadily increased the fractional retention of Ca from 53% to 61%, and increased the whole-body Ca content (g/kg BW). It can be concluded that a dietary level of 0.38% NPP/kg in diets containing a high dose of phytase (1,150 U/kg) and 0.7% Ca are adequate to ensure broiler performance and bone ash of broilers from d 1 to d 14 post-hatch.

4.2. Introduction

Chicken growth has been accelerated during recent decades, and leg problems in the barn, as well as bone fractures during processing of the carcass, are considered major threats in the poultry industry (Chen and Moran, 1995). Selected fast-growing strains have shown lower bone-ash content than slow-growing strains (Williams *et al.*, 2000), which may suggest that diets should be higher in Ca and P than current recommendations (10g Ca/kg and 4.5g non-phytate P (NPP)/kg at ages 1-21d, National Research Council, 1994) in order to reach skeletal integrity for modern strains.

It is generally accepted that an increase of Ca in the diet may increase bone-ash content when Ca is limiting bone mineralization (Driver *et al.*, 2005b; Létourneau-Montminy *et al.*, 2008). However, high dietary Ca has been also implicated in reduced animal performance (Sebastian *et al.*, 1996) and interference with macro-mineral absorption (Simpson and Wise, 1990). Calcium may form soap precipitates with free saturated fatty acids, thus decreasing the dietary energy digestibility (Pepper *et al.*, 1955; Edwards *et al.*, 1960), and has the capacity to interact with inorganic P in the gut (Hurwitz and Bar, 1971) as well as to form a mineral-phytate complex in excess of pH 5.0. The Ca-phytate complex may reduce Ca absorption (Lonnerdal *et al.*, 1989) but may also reduce the activity of endogenous and exogenous phytase (Tamim *et al.*, 2004). Decreasing dietary Ca may improve P utilization, while an excess of Ca may aggravate a P deficiency for ash criteria (Létourneau-Montminy *et al.*, 2008). Other factors, such as the high acid-binding capacity of limestone, have also been related to significant decreases in the protein and P solubility in the gizzard, and may affect N and P digestibility (Tamim and Angel, 2003; Selle *et al.*, 2009; Walk *et al.*, 2012b). Therefore, different authors have shown that a moderate reduction on dietary Ca had no deleterious effects on broiler performance (down to 0.6%, Driver *et al.*, 2005b; or 0.73%, Ziaei *et al.*, 2008) and bone ash (0.75%, Sing *et al.*, 2013).

On the other hand, the amount of P necessary to sustain broilers´ requirements can be provided with graded levels of inorganic P and/or phytase (Venäläine *et al.*, 2006; Adeola and Walk, 2013). Higher levels than the physiological threshold needed for maximum retention are eliminated through kidneys (Manangi and Coon, 2008), with the consequent environmental and economic threat.

The scenario becomes even more complex if we consider that Ca animal requirements are described on a total Ca basis and have usually been measured using

low soluble sources, such as limestone. Recently, high-soluble sources of Ca in the diets (Walk *et al.*, 2012c) or different limestone particle size (Manangi and Coon, 2007) have been explored, as well as the widespread use of overdoses of phytase in the diets to maximize phytate P utilization. For example, if it is assumed that 5.1 atoms of Ca are bound by one phytate molecule (Nelson, 1984), an overdose of dietary phytase with complete hydrolyses of phytate (1% in the diet) may liberate up to 0.36% Ca, allowing for significant reductions in dietary Ca without influencing broiler performance and bone ash. Therefore, redefining Ca and NPP requirements for broilers has become a major issue for the poultry industry, with economic, environmental and animal welfare implications.

Consequently, we tested the hypothesis that a significant decrease in the level of Ca may improve broiler performance and bone mineralization by reducing digestive interactions and improving mineral retention. The objective of this study is to establish optimum dietary Ca and NPP levels for starting broilers from their performance and bone mineralization responses to a factorial range of diets containing 3 levels of Ca and 4 levels of NPP plus a high dose of phytase.

4.3. Materials and methods

All study procedures were approved by the Animal Ethics Committee of the Universitat Autònoma de Barcelona and were in compliance with the European Union guidelines for the care and use of animals in research (European Parliament, 2010).

4.3.1. Bird Management, Husbandry, Experimental Design and Diets

In total, four hundred twenty 1-day-old male broilers (*Ross* 308) were obtained from a local hatchery, where they received *in ovo* vaccinations for Marek disease, Gumboro disease and Infectious Bronchitis. The birds were weighed, wing tagged and allotted to 12 dietary treatments in a completely randomized design. Each treatment was replicated 5 times in battery brooder cages with 7 chicks each. The brooder temperature was maintained at 35ºC from d 1 to d 4 post-hatch, and was progressively reduced to 25ºC on d 14. The light cycle was 24L:1D from d 1 to d 2, 23L:1D from d 3 to d 10, and 18L:6D from d 11 to d 14. Feed was provided ad libitum and water was freely available.

Three calculated levels of Ca at 0.5, 0.7, or 0.9% of the diet and 4 calculated non-phytate P (**NPP**) levels at 0.25%, 0.31%, 0.38% or 0.45% of the diet were used in a 3 x 4 factorial arrangement. With 5 replicates/ treatment. All diets met or exceeded the

nutrient requirements for broilers (Fundación Española Desarrollo Nutrición Animal, 2008), with the exception of Ca and available P, and fed in mash form. Diets contained 1,000 U *Escherichia coli* 6-phytase expressed in *Trichoderma reesei* (Quantum Blue, AB Vista Feed Ingredients; Marlborough, UK). The phytase activity analyzed in the diets was 1,150 FTU/kg. Not any release of Ca and/or P was attributed to the phytase addition during the diet formulation.

4.3.2. Growth Performance and Sampling

Birds were individually wing-tagged in order to monitor individual BW as well as the group BW at the start (1 d) and d 7 and d 14 posthatch. From these values the feed intake (FI), weight gain (WG), and G:F from d 1 to d 14 were calculated. On d 14, 3 birds with the closest BW to the average cage BW were killed by cervical dislocation. The pH of the gizzard and proventriculus were recorded by immersing the electrode of a digital pH meter into the center of the lumen. The right tibiotarsus was removed, boiled, and cleaned from adherent tissue for bone-ash determination. The rest of the chicks were fasted for 2h and killed by cervical dislocation to determine Ca and P content of the whole body. The 4 whole bodies were minced together and stored for ash, Ca and P content determination.

4.3.3. Laboratory Analyses

Diets were analyzed for DM, Ca and P. DM was determined by placing samples in a drying oven at 105ºC for 24 h. Dietary samples were digested in nitric perchloric and fluorhydric acid and subsequently analyzed for P and Ca by flame atomic absorption spectroscopy.

Table 4.1. Calculated composition of experimental diets.

Ca (%)	0.9				0.7				0.5			
NPP (%)	**0.25**	**0.31**	**0.38**	**0.45**	**0.25**	**0.31**	**0.38**	**0.45**	**0.25**	**0.31**	**0.38**	**0.45**
Ingredients, %												
Corn	23.87	23.87	23.87	23.87	23.87	23.87	23.87	23.87	23.87	23.87	23.87	23.87
Wheat	25.00	25.00	25.00	25.00	25.00	25.00	25.00	25.00	25.00	25.00	25.00	25.00
Soybean meal	27.15	27.15	27.15	27.15	27.15	27.15	27.15	27.15	27.15	27.15	27.15	27.15
Extruded full-fat soybean	13.27	13.27	13.27	13.27	13.27	13.27	13.27	13.27	13.27	13.27	13.27	13.27
Na phosphate	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29
L-Lysine	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29
DL-Methionine	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33
L-Threonine	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
Premix[1]	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Celite	1.10	0.93	0.77	0.61	1.60	1.45	1.29	1.13	2.13	1.97	1.81	1.65
Limestone[2]	1.90	1.76	1.63	1.49	1.38	1.24	1.10	0.97	0.85	0.72	0.58	0.44
Mono-calcium phosphate[3]	0.03	0.55	0.85	1.14	0.26	0.55	0.85	1.14	0.26	0.55	0.85	1.14
Calculated composition												
ME, kcal/kg	2,960	2,960	2,960	2,960	2,960	2,960	2,960	2,960	2,960	2,960	2,960	2,960
CP, %	22.0	22.0	22.0	22.0	22.0	22.0	22.0	22.0	22.0	22.0	22.0	22.0
Lys, %	1.38	1.38	1.38	1.38	1.38	1.38	1.38	1.38	1.38	1.38	1.38	1.38
TSAA, %	1.01	1.01	1.01	1.01	1.01	1.01	1.01	1.01	1.01	1.01	1.01	1.01
Thr, %	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.86
Na, %	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22
Ca, %	0.90	0.90	0.90	0.90	0.70	0.70	0.70	0.70	0.50	0.50	0.50	0.50
P, %	0.49	0.55	0.62	0.69	0.49	0.55	0.62	0.69	0.49	0.55	0.62	0.69
Available P, %	0.24	0.31	0.37	0.44	0.24	0.31	0.37	0.44	0.24	0.31	0.37	0.44
Analyzed composition												
Ca, %	0.96	0.96	0.96	0.96	0.79	0.79	0.79	0.79	0.62	0.62	0.62	0.62
P, %	0.64	0.68	0.78	0.84	0.66	0.70	0.80	0.84	0.64	0.68	0.76	0.84

[1]Provides per kg of feed: vitamin A (from retinol), 13,500 IU; vitamin D_3 (from cholecalciferol), 4,800 IU; vitamin E (from alfa-tocopherol), 49.5 IU; vitamin B_1, 3 mg; vitamin B_2, 9 mg; vitamin B_6, 4.5 mg; vitamin B_{12}, 16.5 µg; vitamin K_3, 3 mg; calcium pantothenate, 16.5 mg; nicotinic acid, 51 mg; folic acid, 1.8 mg; biotin, 30 µg; Fe (from $FeSO_4 \cdot 7H_2O$), 54 mg; I (from $Ca(I_2O_3)_2$), 1.2 mg; Co (from $2CoCO_3 \cdot 3Co(OH)_2 \cdot H_2O$), 0.6 mg; Cu (from $CuSO_4 \cdot 5H_2O$), 12 mg; Mn (from MnO), 90 mg; Zn (from ZnO), 66 mg; Se (from Na_2SeO_3), 0.18 mg; Mo (from $(NH_4)_6Mo_7O_{24}$); 1.2 mg. phytase, 1,000 FTU/kg; Endo-1,4-beta-xylanase EC 3.2.1.8, 150 FXU.[2] Limestone supplied 38% Ca.[3] Mono-calcium phosphate supplied 22.6% P and 17.8% Ca.

In-feed phytase activity (U/kg) was determined by the internal, validated method of the producer (Method B-074). One phytase unit is defined as the amount of enzyme required to release 1 μmol of inorganic P/min from sodium phytate at 37°C. The tibias were defatted for 48h in ethyl ether. They were then dried for 12h at 110ºC and then ashed overnight at 550ºC (Brenes *et al.*, 2003). Ash content in the BW mince was determined following incineration of samples (8 g) for 12 h at 550°C. Calcium and phosphorus content were analyzed using an atomic absorption and mass spectrophotometer in ash samples (0.5 g) that were digested in 5 ml nitric acid and 1 ml of hydrogen peroxide using microwave digestion.

4.3.4. Calculations and Statistical Analyses

Whole-body Ca and P retention was calculated using the following equation:

$$\textbf{Ca or P retention} = (G:F)\ ([N]_B / [N]_D)$$

where G:F is gain-to-feed ratio, $[N]_B$ is the Ca or P content in the whole body, and $[N]_D$ is the Ca or P content in the diet.

Data were analyzed by ANOVA using the GLM procedure of SAS 9.2 (Cary, NC, USA). The main factors used in the model were Ca level and NPP level and their interaction was also included. Multiple mean comparisons were done using Tukey´s correction. The experimental unit was the pen. The alpha level used for determination of significance was 0.05.

4.4. Results

The nutrients of the diets are presented in Table 4.1. It is worth noting that Ca in the diet (0.62, 0.79, and 0.96% for the 3 levels of Calcium) was higher than what was formulated as a consequence of the presence of Ca in some ingredients. The Ca content in celite (the ingredient used in the trial to pair the diets) was 5.6%, and in the vitamin and mineral premix it was 13.5%, likely due to limestone usually added as an inert carrier in the premix. The analyzed P levels in diets (from 0.64 to 0.84%), and phytase activity (1,150 FTU/kg) showed also higher values than it was calculated in the formula.

Table 4.2. Influence of Ca and NNP[1] levels in diets containing phytase at 1,150 FTU/kg on feed intake and growth performance of broilers from d 1 to d 14[2].

Treatment	NPP, %	BW d14, g	FI[3] d1-14, g/d	WG[4] d1-14, g/d	WG d 7-14, g/d	G:F d 1-14
0.5	0.25	428	37.6^{a}	27.4	34.7	0.732
	0.31	436	36.6^{ab}	28.0	35.5	0.766
	0.38	431	35.7^{ab}	27.7	35.3	0.776
	0.45	408	35.7^{ab}	25.9	34.0	0.736
0.7	0.25	428	37.7^{a}	27.4	35.4	0.731
	0.31	444	37.3^{a}	28.5	37.0	0.765
	0.38	460	39.2^{a}	29.7	39.0	0.770
	0.45	440	36.6^{ab}	28.3	36.0	0.771
0.9	0.25	391	30.3^{b}	24.7	30.9	0.821
	0.31	417	34.6^{ab}	26.6	33.5	0.770
	0.38	445	37.5^{a}	28.7	36.6	0.766
	0.45	446	35.3^{ab}	28.7	37.9	0.815
Ca level, %						
0.5		426	36.3	27.2	34.8^{ab}	0.752
0.7		443	37.7	28.4	36.8^{a}	0.759
0.9		425	34.4	27.1	34.7^{b}	0.793
NPP level, %						
	0.25	416^{b}	35.1	26.5^{b}	33.6^{b}	0.761
	0.31	432^{ab}	36.1	27.7^{ab}	35.3^{ab}	0.767
	0.38	446^{a}	37.4	28.7^{a}	36.9^{a}	0.770
	0.45	431^{ab}	35.8	27.6^{ab}	35.9^{ab}	0.774
SEM		12.7	1.3	0.9	1.3	0.033
P value[5]						
Ca level ×NPP level		0.122	0.048	0.107	0.068	0.614
Ca level		0.086	0.005	0.086	0.045	0.185
NPP level		0.050	0.242	0.045	0.025	0.969

[1] Non-phytate P
[2] Data are means of 5 pens with 7 chicks each.
[3] Average daily feed intake
[4] Average daily weight gain
[5] ^{abc} Values in the same column with different letters are significantly different ($P < 0.05$).

Bird Performance and Bone Mineralization

None of the birds presented any signs of rickets or died during the study. The FI and WG are presented in Table 4.2. An interaction (Ca × P level) was observed on FI from d 1to 14. Increasing the level of NPP from 0.25% to 0.38% increased ($P < 0.05$) the FI in chickens fed the high Ca diet (0.9%) but not in birds fed lower levels of dietary Ca (0.7% and 0.5%). A similar pattern was observed for growth performance from d 7 to d 14 (interaction, $P = 0.068$). The rest of the performance parameters did not show Ca × P level interactions. The chicks fed 0.7% Ca reach BW at d 14 close to the *Ross* standards and tended ($P = 0.086$) to show higher BW than birds fed 0.5% and 0.9% Ca. Added levels of P increased ($P < 0.05$) the growth performance, being higher for birds fed 0.38% NPP rather than it was for the 0.25% NPP diet.

The pH in the Gizzard and proventriculus was not affected by the level of Ca, the level of NPP or their interaction. The pH averaged 2.31 ± 0.06 (mean ± SEM) in the gizzard (ranging from 1.65 to 3.20) and 2.91 ± 0.15 in the proventriculus (ranging from 2.05 to 4.17).

The effects of the Ca and NPP levels on tibia weight and tibia ash content are presented in Table 4.3. Tibia weight and tibia ash content were influenced by the Ca level and the P level having a significant interaction ($P < 0.001$ and $P = 0.007$ respectively). Tibia weight was the greatest in birds fed on the 0.9% Ca and 0.38% NPP diet. The lowest tibia weight and ash content was observed in birds with the greatest Ca:P imbalance: 0.5% Ca with 0.45% NPP in the diet, and for 0.9% Ca with 0.25% NPP in the diet. The Ca and P whole-body content and retention are shown in Table 4.4. The increase on dietary Ca decreased ($P < 0.001$) its fractional retention from 74% with the 0.5% Ca diet to 46% with the 0.9% Ca diet. An increase in the levels of dietary Ca from 0.5% to 0.7% decreased ($P = 0.025$) the whole-body Ca content (g/kg BW) and tended ($P = 0.089$) to decrease the P content.

Added levels of P decreased ($P <0.001$) its fractional retention from 66% with the 0.25% NPP diet to 52% with the 0.45% diet. The increase in the levels of dietary P steadily increased ($P = 0.015$) the fractional retention of Ca from 53% to 61% with the 0.25 and 0.45% NPP, respectively, and increased ($P = 0.025$) the whole-body Ca content (g/kg BW), with higher values in birds fed 0.31%, 0.38% and 0.45% NPP diets than it was for birds fed the 0.25% NPP diet.

4.5. Discussion

The Ca level promoted differences on FI and WG, with 0.7% Ca (analyzed, 0.79%) promoting higher FI and WG than 0.9% Ca did with limiting values of NPP. Birds fed on 0.7% Ca also showed higher tibia ash and tibia weight than birds fed the 0.5% Ca diet. Then, birds exposed to diets with a medium level in Ca (0.7%) and 0.38% NPP performed the best, while higher Ca levels (0.9%) induced negative responses concerning FI and WG, which shows that a lower Ca concentration is desirable to reach better performance in starting broilers. These results agree with Driver *et al.* (2005a), who described BW and FCR optimized at 0.625% Ca in the diet. On other hand, Rama-Rao *et al.* (2006) did not find differences in WG on d 14 due to variation in the dietary Ca level.

There are different reasons which may explain the negative effects of high levels of Ca on the broiler performance. Calcium is known to form insoluble complexes with phytate phosphorus, which may hinder phytase activity (Angel *et al.*, 2002). Calcium also has the capacity to interact with inorganic P in the gut lumen to form insoluble Ca orthophosphate (Plumstead *et al.*, 2008), which may also make inorganic P less soluble and available for absorption in excess of pH 5.0. This effect could explain our results that the lowest performance was observed with high Ca diets containing limiting values of NPP (0.25% NPP). Some reserchers have reported increases on the intestinal pH and low apparent ileal P digestibility in broilers fed diets containing a high dietary level of Ca (Sebastian *et al.*, 1996; Adeola and Walk, 2013). Thus, high concentrations of limestone, which is the dominant source of Ca in poultry diets with phytase, may increase the pH in the proximal gastrointestinal tract due to its high acid-binding capacity. Shafey (1999) described a limestone-induced pH increase from 5.68 to 6.24 in small intestine digesta. An increase in the crop and gizzard pH may promote Ca, phytate, and P precipitation, and it may also reduce the Ca and P digestibility (Selle *et al.*, 2009; Walk *et al.*, 2012b). However, we were not able to observe differences in the pH in the gizzard and proventriculus. Gacs and Barltrop (1977) showed that some aggregations between minerals and dietary polymers in the digesta may also contribute to reduce the digestibility coefficients for protein and fat. Calcium is able to form insoluble soaps with free fatty acids and bile acids and there is some evidence that these soaps limit the absorption of fat *in vivo* (Gacs and Barltrop, 1977; Govers, *et al.*, 1996; Shahkalili *et al.*, 2001).

Table 4.3. Influence of Ca and NPP[1] levels in diets containing phytase at 1,150 FTU/kg on tíbia weight and ash of 14-day-old broilers[2].

Treatment	NPP, %	Tibia weight, g	Tibia weight, %BW^{-1}	Tibia ash, %	Tibia ash, mg/tibia
0.5	0.25	0.88^{bcd}	0.203 ^{bc}	50.17^{bc}	439^{bcd}
	0.31	0.87^{bcd}	0.199 ^{bc}	51.38^{ab}	451^{bc}
	0.38	0.87^{bcd}	0.201 ^{bc}	50.09^{bc}	437^{bcd}
	0.45	0.80^{cd}	0.196 ^{c}	49.55^{c}	395^{cd}
0.7	0.25	0.85^{bcd}	0.204 ^{bc}	51.44^{ab}	439^{bcd}
	0.31	0.92^{ab}	0.206 ^{abc}	51.97^{a}	479^{ab}
	0.38	0.93^{ab}	0.202 ^{bc}	51.87^{a}	484^{ab}
	0.45	0.94^{ab}	0.214 ^{abc}	51.38^{ab}	481^{ab}
0.9	0.25	0.77^{d}	0.194 ^{c}	49.65^{c}	381^{d}
	0.31	0.90^{abc}	0.214 ^{abc}	50.83^{abc}	460^{bc}
	0.38	1.00^{a}	0.225 ^{a}	51.86^{a}	522^{a}
	0.45	0.97^{ab}	0.218 ^{ab}	51.39^{ab}	500^{ab}
Ca level, %					
0.5		0.85	0.199	50.30	431
0.7		0.91	0.207	51.67	471
0.9		0.91	0.213	50.93	466
NPP level, %					
	0.25	0.83	0.200	50.42	420
	0.31	0.90	0.206	51.39	463
	0.38	0.93	0.209	51.28	481
	0.45	0.90	0.209	50.77	459
SEM		0.03	0.004	0.34	15.6
P value[3]					
Ca level × NPP level		< 0.001	< 0.001	0.007	< 0.001
Ca level		0.0032	< 0.001	< 0.001	0.001
NPP level		< 0.001	0.032	0.002	< 0.001

[1] Non-phytate P.
[2] Data are means of 5 pens with 3 chicks each.
[3] ^{abc} Values in the same column with different letters are significantly different ($P < 0.05$).

These soaps could lower the utilization of energy derived from lipids, particularly saturated fats, in broiler diets. Nevertheless, it is relevant that FI was reduced in the high-calcium diet, without affecting the G: F. This result could suggest that broilers may have detected these high levels of calcium, or they reduced FI in order to avoid a larger Ca and P imbalance. Some recent reports suggest that broilers are able to detect Ca in the diet (Wilkinson *et al.*, 2012).

Tibia weight and bone mineralization were also influenced by the level of Ca, with the low-calcium diet showing the lowest bone weight and ash content. This result agrees with the result obtained by Onyango *et al.* (2003), who found that bone-mineral content, bone-mineral density and percentage of ash increased linearly as the level of dietary Ca increased from 0.45% to 0.91%. However, the level of Ca interacted with the dietary level of NPP on the tibia ash percent, which confirms that high levels of Ca may

affect P availability for bone mineralization. Al Masri (1995) saw that the values of dietary Ca and its ratio with P may affect P retention, with lower values of P retention when the levels of Ca in the diet are higher. Nonetheless, we did not observe this difference in the P retention with the levels of Ca used in our study, which could reflect that changes on the levels of P promoted changes on FI and growth performance of broilers, rather than changes in the fractional retention of the dietary P.

The increase on dietary Ca decreased its fractional retention, which concur with those of Mitchell and Edwards (1996a) and Ziaei *et al*. (2008), who reported that the reduced mineral content of diets resulted in a higher apparent retention of Ca, leading to a reduction in mineral excretion. Browning *et al.* (2012) show that reducing dietary Ca/aP concentrations were associated with increased efficiency of Ca retention as compared to high Ca/available P diets, which indicates a physiological response by the chicken to overcome a Ca deficiency by up-regulating its nutrient transfer and deposition infrastructure.

A level of NPP at 0.38% improved the growth of chicks on d 14, with BW values close to the standard of the breed for this period (473 g BW on d 14). Added levels of NPP up to 0.38% in the 0.7 and 0.9% Ca treatments also increased bone mineralization. Ravindran *et al.* (1995) observed that the bone-mineralization criterion is a good, sensitive indicator of the P status of the birds. Despite phosphorus being largely contained in all of the tissue, bone is the main storage organ for P, containing 85% of the body's total P. Through its involvement in metabolic and structural processes, P is essential for animals to attain their optimum genetic potential in growth and feed efficiency as well as skeletal integrity (Applegate and Angel, 2008). Some authors have reported substantial differences in the NPP requirement of broilers, as compared with the 0.45% NPP level published by NRC (1994). Waldroup *et al.* (2000) reported that the NPP requirement for the starter phase ranges from 0.37% to 0.39%. The difference could be explained by the fact that NRC (1994) recommendations for NPP in diets for broilers are based on peer-reviewed research published between 1952 and 1983. However, modern commercial birds are very different from commercial birds prior to 1983, due, in part, to genetic selection, but also because management practice has changed (Havenstein *et al.*, 1994), as has occurred with the addition of phytase to feed. In the present trial, we incorporated a high dose of a commercial phytase (analyzed at 1,150 U/kg), which it may explain the good responses at lower NPP values in the diet.

Increasing the levels of NPP from 0.25% to 0.31% NPP allowed for increases in the fractional retention of Ca, likely reflecting how body growth and bone mineralization respond to an improved Ca:P in the diet. However, it is worth stating that increases in the NPP level in the diet reduced the fractional retention of phosphorus, which is a similar response to that observed previously for increasing levels of calcium.

Table 4.4. Influence of Ca and NPP[1] levels in diets containing phytase at 1,150 FTU/kg on whole-body ash of 14-day-old broilers[2].

Treatment	NPP, %	Whole-body composition			Retention	
		Ash, %	Ca, g/kg	P, g/kg	Ca	P
0.5	0.25	2.40	5.36	4.27	0.65	0.66
	0.31	2.63	6.28	4.75	0.77	0.65
	0.38	2.55	6.13	4.61	0.76	0.58
	0.45	2.58	6.12	4.68	0.77	0.53
0.7	0.25	2.33	5.23	4.17	0.51	0.65
	0.31	2.36	5.39	4.23	0.52	0.58
	0.38	2.39	5.40	4.26	0.56	0.57
	0.45	2.50	5.94	4.57	0.58	0.51
0.9	0.25	2.30	5.12	3.99	0.43	0.66
	0.31	2.60	6.13	4.56	0.49	0.63
	0.38	2.48	5.78	4.43	0.46	0.55
	0.45	2.43	5.65	4.35	0.48	0.52
Ca level, %						
0.5		2.54	5.97^{a}	4.57	0.74^{a}	0.60
0.7		2.39	5.49^{b}	4.30	0.54^{b}	0.57
0.9		2.45	5.66^{ab}	4.33	0.46^{c}	0.59
NPP level, %						
	0.25	2.34	5.23^{b}	4.14^{b}	0.53^{b}	0.658^{a}
	0.31	2.53	5.93^{a}	4.51^{a}	0.59^{a}	0.622^{a}
	0.38	2.47	5.76^{a}	4.43^{a}	0.60^{a}	0.564^{b}
	0.45	2.50	5.90^{a}	4.53^{a}	0.61^{a}	0.519^{b}
SEM		0.10	0.25	0.19	0.03	0.03
P value[3]						
Ca level × NPP level		0.833	0.394	0.772	0.543	0.939
Ca level		0.160	0.025	0.089	<0.001	0.507
NPP level		0.161	0.003	0.052	0.015	< 0.001

[1] Non-phytate P.
[2] Data are means of 5 pens with 4 chicks each.
[3] [abc] Values in the same column with different letters are significantly different ($P < 0.05$).

These results could reflect a decrease in P digestibility (not analyzed in this experiment), but more likely this reflects an increase on the endogenous excretion of P in the urine (Al Masri, 1995). When broilers receive P levels that are higher than the physiological threshold for maximum utilization and retention, there is the possibility that the additional P may most likely be eliminated through the kidney (Leske and

Coon, 2002). To know this threshold is important to integrators in order to avoid the wasting of P in the litter.

Our results confirm that young chicks respond to changes in the NPP levels in the diet in growth performance and bone mineralization. Nevertheless, the consequences of these changes in later performance and leg quality, or in the incidence of broken clavicles or bloody breast meat during processing of the carcass, were not studied. Powell *et al.* (2011) suggested that broilers fed lower levels of NPP in the starter phase are better able to adapt and grow at a low level of NPP in the growing phase than those fed a higher level of NPP in the starter phase. Then, it could be speculated that some of the differences observed on d 14 could be clearly reduced and mineral retention improved by feeding adequate diets during the growing and finishing periods. However, this hypothesis deserves further studies.

The results reflect that an increase in the dietary levels of calcium may decrease chick growth and also may affect bone formation during the early period of growth. The effect was modified by the level of NPP in the diet, which it may indicates the likely formation of insoluble calcium phosphate and Ca-phytate complex when gut pH and Ca: P are increased. The results of this study also emphasize the importance of formulating diets that meet or exceed P requirements of broilers, particularly when high-Ca diets are used.

CHAPTER 5

Calcium sources and their interaction with the level of non-phytate phosphorus affect performance and bone mineralization in broiler chickens

Calcium sources and their interaction with the level of non-phytate phosphorus affect performance and bone mineralization in broiler chickens

M. Hamdi, D. Solà-Oriol, R. Davin, and J.F. Pérez

Animal Nutrition and Welfare Service (SNiBA). Departament de Ciència Animal i dels Aliments. Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

Published in:

Poultry Science (September 2015) 94 : 2136-2143(Hamdi *et al.*, 2015b)

5.1. Abstract

An experiment was conducted to evaluate the influence of different Ca sources (limestone, Ca chloride, and Lipocal, a fat-encapsulated tri-calcium phosphate, TCP) in conjunction with 4 dietary levels of non-phytate P (NPP) on performance, ileal digestibility of Ca and P, and bone mineralization in broiler chickens. Calcium sources were also evaluated *in vitro* to measure acid-binding capacity (ABC) and Ca solubility at different pH values. Ca chloride showed the highest solubility of Ca, with TCP showing the highest ABC. *Ross* male broiler-chicks were sorted by BW at 1 d post-hatch and assigned to 5 cages per diet with 5 birds per cage. Twelve diets were arranged in a 3 × 4 factorial of the 3 Ca sources and 4 levels of NPP (0.3%, 0.35%, 0.4% or 0.45%) consisting of 4 added P levels ($Ca(H_2PO_4)_2$) with a high dose of phytase (1,150 U/kg) in all diets. On d 14 post-hatch, 3 birds were euthanized, and ileal digesta and the right tibia were collected to determine ileal Ca and P digestibility and bone mineralization, respectively. Feed intake (FI) and weight gain (WG) on d 14 was higher ($P < 0.01$) with TCP and limestone than with Ca chloride. Added P increased the tibia weight and tibia ash content in chicks fed TCP up to 0.4% NPP and limestone up to 0.35% NPP. Calcium ileal digestibility was higher ($P < 0.01$) with Ca chloride (73.7%) than with limestone (67.1%) or TCP (66.8%), and it increased ($P < 0.05$) with added levels of P from mono-calcium phosphate. Phosphorus ileal digestibility was not affected by the Ca source and increased ($P < 0.001$) with added levels of NPP. It can be concluded that starting broilers responded better to low-soluble rather than to high-soluble sources of Ca. A level of 0.35%-0.40% NPP with a high dose of phytase (1,150 U/kg) in diets including limestone or TCP is sufficient to guarantee performance and bone formation for broiler chickens from d 1 to d 14.

5.2. Introduction

Calcium (Ca) requirement for broilers is a major area of debate in the poultry industry, and contradictions persist regarding the optimum level required for growth and bone mineralization. In particular, Ca requirements are still described on a total Ca basis rather than on digestible or available Ca, and few efforts have been made to optimize the use and availability of new Ca sources. Moreover, Ca requirements have usually been measured using limestone in the diet, which shows a reduced solubility during neutralization in the small intestine, where most absorption takes place (Goss *et al.*, 2007).

The immature gastrointestinal tract young chicks could be more sensitive to the level and properties of Ca in the diet, unlike that of adult birds. Different sources and forms of dietary Ca have been evaluated in broilers. For example, Manangi and Coon (2007) described that broilers increased their weight gain (WG) with limestone particle sizes of between 137 µm and 388 µm, compared to BW gains obtained by feeding either smaller (28 µm) and more soluble or larger (1,306 µm) and less soluble particle sizes. Recently, Walk *et al.* (2012a) also showed that when diets were formulated (0.9% Ca) with a highly soluble source of Ca (calcified seaweed, Vistacal, AB Vista Feed Ingredients), broiler growth was lower than was the performance of animals fed limestone diets. Soluble sources of Ca may increase the acid-binding capacity (ABC) of digesta and decrease energy and protein digestibility (Tamim and Angel, 2003). They may also form a complex with phytin (Selle *et al.*, 2009) and phosphates (De Kort *et al.*, 2009), which interfere with the availability of P and Zn (Wise, 1983; Tamim *et al.*, 2004; Lonnerdal, 2000). Supplementing the diet with phytase and reducing dietary Ca from 0.9% to 0.45% (Walk *et al.*, 2012a) and from 0.6% to 0.5% (Adeola and Walk, 2013) to balance the soluble Ca and P in the small intestine improved broiler growth performance and tibia ash weight when using a high-soluble source of Ca in the diet. An overdose of phytase (1,000 U/kg) allowed for the release of 1.70 g and 1.56 g of phytic P for absorption when Ca was 0.5% and 0.6%, respectively (Adeola and Walk, 2013).

The objective of the current work is to evaluate the Ca *in vitro* solubility and ABC characteristics of different Ca sources, and explore how the incorporation of these sources may affect the ileal digestibility of Ca and P, animal performance, and bone mineralization in broiler chickens. Diets were formulated to contain a low-calcium level, a high dose of phytase and 4 graded levels of non-phytate P (NPP) to identify and

better characterize the interactions between Ca and P, and their effects on animal performance.

5.3. Materials and methods

5.3.1. Calcium Sources

Three main sources of Ca were used to design the experimental treatments: Ca chloride, limestone and tri-calcium phosphate (TCP, Lipocal (Lipofoods; Barcelona, Spain)). Lipocal is a tri-calcium phosphate powder which has been treated with lecithin to reduce its interactions with other minerals and feed ingredients, especially in aqueous media. Limestone, Ca chloride and mono-calcium phosphate were obtained from a local feed manufacturer.

5.3.2. Calcium Solubility and Acid-Binding Capacity (*in vitro*)

The *in vitro* solubility of added Ca from limestone, Ca chloride and TCP was measured in a phosphate-citrate buffer at a pH ranging from 2.96 to 6.52, with or without phytic P (0.2475 g, phytic acid solution 40%, Fluka Analytical; Buchs, Switzerland). For calcium chloride, 75 mL of buffer and 90 mg of Ca-shaped calcium chloride (250 mg anhydro Calcium chloride) was added in each tube. For the limestone and TCP tubes, 224.7 mg limestone and 250 mg TCP were added. Seven phosphate-citrate buffer solutions were prepared at pH 2.96, 3.53, 4.18, 4.77, 5.27, 6.01, and 6.52 from solutions of 0.2 M dibasic sodium phosphate (Na_2HPO_4) and 0.1 M citric acid, according to Pearse (1980). All buffers were prepared and pH-adjusted before the addition of phytic acid. The tubes were vortexed and incubated for 60 min at 37ºC to observe the amount of mineral precipitate and to obtain supernatant of the samples that were subsequently analyzed for soluble Ca content. Ca was analyzed using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES model Optima 4300DV, PerkinElmer Inc.; Waltham, MA).

The ABC of the 3 Ca sources was measured in batch solutions with increasing amounts of Ca to simulate a dietary Ca level of 0.2%, 0.4%, 0.6%, 0.8%, 1.0% and 1.2% and a water-to-feed ratio of 2:1. The buffering capacity was measured by the procedure of Lawlor *et al.*, (2005) in duplicate tubes by titration dripping increasing amounts of HCl (0.1 N), and measuring the pH continuously until reaching pH = 3 (ABC-3).

5.3.3. Bird Management, Husbandry, Experimental Design and Diets (in vivo)

All experimental procedures were approved by the Animal Ethics Committee of the Universitat Autònoma de Barcelona and were in compliance with the European Union guidelines for the care and use of animals in research (European Parliament, 2010).

One-day-old male broilers (*Ross* 308) were obtained from a local hatchery, weighed, wing-tagged, and allotted to 12 dietary treatments in a completely randomized design. Each treatment was replicated 5 times in battery brooder cages with 5 chicks each. The brooder temperature was maintained at temperatures of 35ºC from d 1 to d 4 post-hatch, and was progressively reduced to 25ºC on d 14. The light cycle was 24h/day from d 1 to d 2, 23h/d from d 3 to d 10 and 18h/d from d 11 to d 14. Feed and water was provided ad libitum.

Sources of Ca (limestone, Ca chloride and TCP) and NPP at 0.3%, 0.35%, 0.4% and 0.45% of the diet, consisting of 4 added levels of mono-calcium phosphate, were used in a 3 × 4 factorial arrangement (Table 5.1). All diets met or exceeded the nutrient requirements for broilers (Fundación Española Desarrollo Nutrición Animal, 2008), with the exception of Ca and available P. Diets contained 1,000 units of *Escherichia coli* 6-phytase expressed in *Trichoderma reesei* (Quantum Blue, AB Vista Feed Ingredients; Marlborough, UK). The phytase activity analyzed in the diets was 1,150 FTU/kg. Diets were fed in mash form and contained 0.3% titanium dioxide as an indigestible marker.

5.3.4. Experimental procedures

Birds were individually wing-tagged in order to monitor individual BW as well as the group BW at the start (1 d) and d 7 and d 14 post-hatch. From these values the feed intake (FI), weight gain (WG), and G:F from d 1 to d 14 were calculated. On d 14, 3 birds with the closest BW to the average cage BW were killed by cervical dislocation.

The pH of the gizzard and proventriculus were recorded by immersing the electrode of a digital pH meter into the center of the lumen. Ileal digesta were collected in the region from Meckel's diverticulum to about 2 cm anterior to the ileo-cecal junction and stored at -20ºC. The right tibias were collected from the same animals, boiled, and cleaned of adherent tissue for bone-ash determination.

Table 5.1. Ingredient and nutrient composition (% as fed-basis, unless otherwise indicated) of the experimental diets.

	Calcium chloride				Limestone				TCP			
NPP (%)	**0.3**	**0.35**	**0.4**	**0.45**	**0.3**	**0.35**	**0.4**	**0.45**	**0.3**	**0.35**	**0.4**	**0.45**
Ingredients, %												
Corn	23.87	23.87	23.87	23.87	23.87	23.87	23.87	23.87	23.87	23.87	23.87	23.87
Wheat	25.00	25.00	25.00	25.00	25.00	25.00	25.00	25.00	25.00	25.00	25.00	25.00
Soybean meal	27.15	27.15	27.15	27.15	27.15	27.15	27.15	27.15	27.15	27.15	27.15	27.15
Extruded soybean	13.27	13.27	13.27	13.27	13.27	13.27	13.27	13.27	13.27	13.27	13.27	13.27
Na phosphate	0.48	0.48	0.48	0.48	0	0	0	0	0	0	0	0
L-Lysine	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29
DL-Methionine	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33
L-Threonine	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
Soy oil	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00
Premix[1]	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
$CaCl_2$[2]	0.70	0.70	0.70	0.70	0	0	0	0	0	0	0	0
Limestone[3]	0.34	0.23	0.13	0.03	0.66	0.56	0.46	0.36	0.17	0.17	0.17	0.17
TCP [4]	0	0	0	0	0	0	0	0	0.93	0.74	0.54	0.35
MCP[5]	0.32	0.55	0.77	0.99	0.72	0.94	1.16	1.38	0	0.37	0.74	1.11
Titanium dioxide	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Calculated Composition (%)												
ME(kcal/kg)	2,960	2,960	2,960	2,960	2,960	2,960	2,960	2,960	2,960	2,960	2,960	2,960
CP	22.0	22.0	22.0	22.0	22.0	22.0	22.0	22.0	22.0	22.0	22.0	22.0
Ca	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55
Total P	0.54	0.59	0.64	0.69	0.54	0.59	0.64	0.69	0.54	0.59	0.64	0.69
PP	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
NPP	0.30	0.35	0.40	0.45	0.30	0.35	0.40	0.45	0.30	0.35	0.40	0.45
Analyzed composition (%)												
Ca	0.60	0.57	0.64	0.63	0.76	0.81	0.85	0.81	0.74	0.78	0.68	0.68
Total P	0.67	0.67	0.78	0.83	0.71	0.76	0.89	0.95	0.72	0.75	0.81	0.86

[1]Provided per kg of feed: vitamin A (from retinol), 13,500 IU; vitamin D_3 (from cholecalciferol), 4,800 IU; vitamin E (from alfa-tocopherol), 49.5 IU; vitamin B_1, 3 mg; vitamin B_2, 9 mg; vitamin B_6, 4.5 mg; vitamin B_{12}, 16.5 µg; vitamin K_3, 3 mg; calcium pantothenate, 16.5 mg; nicotinic acid, 51 mg; folic acid, 1.8 mg; biotin, 30 µg; Fe (from $FeSO_4 \cdot 7H_2O$), 54 mg; I (from $Ca(I_2O_3)_2$), 1.2 mg; Co (from $2CoCO_3 \cdot 3Co(OH)_2 \cdot H_2O$), 0.6 mg; Cu (from $CuSO_4 \cdot 5H_2O$), 12 mg; Mn (from MnO), 90 mg; Zn (from ZnO), 66 mg; Se (from Na_2SeO_3), 0.18 mg; Mo (from $(NH_4)_6Mo_7O_{24}$); 1.2 mg. phytase, 1,150 FTU/kg; Endo-1,4-beta-xylanase EC 3.2.1.8, 150 FXU.
[2] Calcium chloride supplied 27.8% Ca. [3] Limestone supplied 38% Ca. [4] Lipocal supplied 34% Ca and 17.4% P (Lipofoods; Barcelona, Spain). [5]Mono-calcium phosphate supplied 17.8% Ca and 22.6% P.

5.3.5. Laboratory analyses

Diets and ileal digesta were analyzed for DM, Ti, Ca and P. Dry matter was determined by placing samples in a drying oven at 105ºC for 24h. Samples were digested in nitric perchloric and fluorhydric acid and subsequently analyzed for P, Ca, and Ti by flame atomic-absorption spectroscopy. The tibias were defatted for 48h in ethyl ether. They were then dried for 12h at 110ºC and then ashed overnight at 550ºC (Brenes *et al.*, 2003).

5.3.6. Calculations and statistical analyses

Apparent ileal digestibility of Ca and P (%) was calculated by the index method using the following equation:

$$\textbf{Ileal Ca or P Digestibility} = 1 - ([Ti]_D / [N]_D / [Ti]_M / [N]_M)$$

where $[Ti]_D$ is the concentration of Ti in the diet, $[N]_D$ is the Ca or P content in the diet, $[Ti]_M$ is the concentration of Ti in ileal digesta and $[N]_M$ is the Ca or P content in ileal digesta.

Data were analyzed as a completely randomized design using the GLM procedure of SAS software (SAS, 2008), version 9.2. The dietary treatments were arranged in a 2-way factorial design and Tukey´s multiple comparisons were used to determine main effects of the Ca source, NPP level, and the interaction between the two. The pen of the chicks served as the experimental unit. The alpha level used for significant differences was 0.05.

5.4. Results

5.4.1. Calcium solubility and Acid-Binding Capacity

Soluble calcium from limestone, Ca chloride and TCP at different pHs, with or without phytic acid is shown in Figure 5.1. Limestone showed a low solubility at pH > 4, but the solubility increased as pH decreased. Calcium chloride showed a higher Ca solubility in the pH range between 2.96 and 6.0. However, an increase in the pH above 6 drastically reduced soluble Ca from calcium chloride. TCP showed a linear increase in the solubility of Ca as pH decreased, from 6.52 to 2.69, showing lower values of soluble Ca than did limestone and calcium chloride at a pH above 5. No relevant differences were observed in the two lines derived from phytic acid incorporation. With the

exception of calcium chloride, the rank order of calcium solubility changed depending on pH. At a pH above 4.77, the Ca solubility order was: TCP (Lipocal) < limestone < calcium chloride. At a pH below 4.77, the order was: limestone < TCP < calcium chloride.

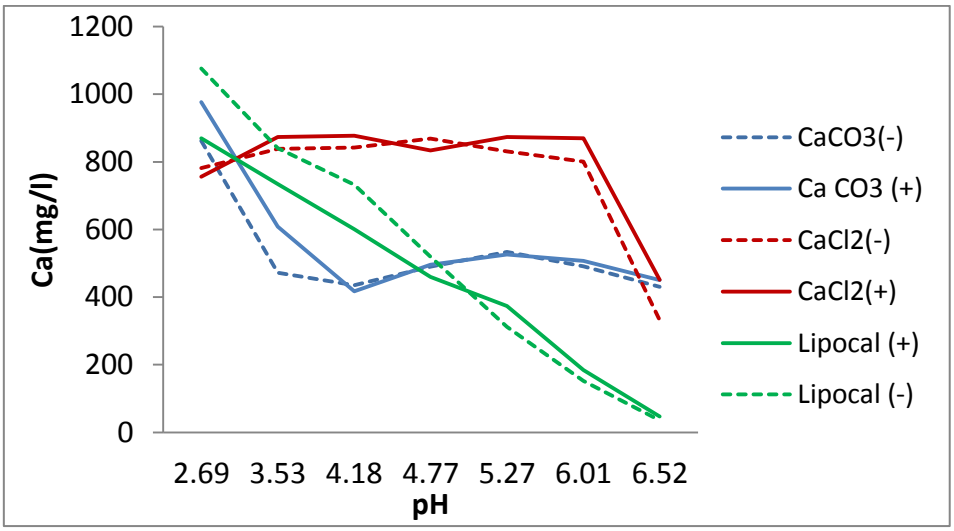


Figure 5.1. Concentration of Ca (mg/L) in the supernatant of Ca chloride, limestone and TCP solutions at different pH (from left to right, 2.69, 3.53, 4.18, 4.77, 5.27, 6.01, 6.52) and with the addition or not of phytic acid (+ vs -).

ABC was affected by the Ca source and concentration (Table 5.2). There was a linear increase in ABC with the dietary dose of the 3 Ca sources, but values were lower with calcium chloride than they were with limestone and TCP. The ABC-3 estimated for a 1.2% Ca supplementation of Ca sources in feed was 2.4 mEq/kg feed, 244 mEq/kg feed and 420.3 mEq/kg feed, respectively, for Ca chloride, limestone and TCP.

Table 5.2. Acid-binding capacity (ABC, mEq/kg feed requried to reach pH=3) of different sources and dietary Calcium levels.

	Sources of calcium		
%Ca	Ca Chloride	Limestone	TCP
0.2	1.6	46.9	72.8
0.4	1.9	94.1	140.3
0.6	1.9	141.1	219.2
0.8	2.1	167.7	280.8
1	2.1	190.4	343.2
1.2	2.4	244.0	420.3

5.4.2. Bird performance and bone mineralization

The feed intake and growth performance responses of broiler chickens to different sources of Ca and NPP levels are presented in Table 5.3. There was no interaction between the calcium source and NPP levels. Feed intake was higher ($P < 0.05$) in birds fed the TCP diet than it was in birds fed the Ca chloride diet, showing intermediate values birds fed on limestone.

Table 5.3. Influence of Ca source and NPP[1] levels on feed intake and growth performance of broilers from 1d to 14d .

Treatment	NPP, %	BW d14, g	FI d 1-14, g/d	WG d 1-14, g/d	G:F d 1-14
Ca chloride	0.30	413.3	32.1	26.5	0.826
	0.35	410.1	32	26.3	0.825
	0.40	402.9	31	25.8	0.828
	0.45	416.7	31.7	26.8	0.844
Limestone	0.30	442.2	33.6	28.4	0.847
	0.35	436.4	31.9	28.2	0.886
	0.40	441.2	34.3	28.5	0.828
	0.45	431.1	33.4	27.8	0.832
TCP[3]	0.30	424.5	33	27.3	0.828
	0.35	432.1	33.4	27.8	0.833
	0.40	460.7	35	29.9	0.858
	0.45	447.5	34.5	28.9	0.840
Calcium Source					
Ca Chloride		410.7[b]	31.6[b]	26.3[b]	0.831
Limestone		437.7[a]	33.3[ab]	28.1[a]	0.848
TCP		441.2[a]	33.9[a]	28.4[a]	0.840
NPP level,%					
	0.30	426.7	32.9	27.4	0.834
	0.35	426.2	32.4	27.4	0.848
	0.40	435.0	33.5	28.0	0.838
	0.45	431.8	33.2	27.8	0.839
SEM		15.59	1.43	1.11	0.019
P value					
Ca Source × NPP Level[4]		0.686	0.855	0.692	0.351
Ca Source		0.006	0.045	0.007	0.430
NPP Level		0.849	0.778	0.819	0.829

[1]Non-phytate P. [2] Data are means of 5 pens with 5 chicks each. [3]Lipocal (Lipofoods; Barcelona, Spain).
[4 abc] Values in the same column with different letters are significantly different ($P < 0.05$).

The chicks fed TCP and limestone reach BW at d 14 close to the *Ross* standards, showing a higher ($P < 0.01$) WG than birds fed calcium chloride. The feed efficiency was not significantly affected by the source of Ca, the level of NPP or their interaction ($P > 0.05$). The pH in the Gizzard and proventriculus was not significantly affected by

the source of Ca, the NPP level or their interaction. The pH averaged 2.09 ± 0.06 (mean ± SEM) in the gizzard (ranging from 1.24 to 3.89) and 2.18 ± 0.10 in the proventriculus (ranging from 1.02 to 3.95).

Table 5.4. Influence of Ca and NPP[1] levels on tibia weight and tibia ash of 14-day-old broilers[2].

Treatment	NPP, %	Tibia weight, g	Tibia weight, %BW^{-1}	Tibia ash , %	Tibia ash, mg/tibia
Ca Chloride	0.30	0.77^{ab}	0.185	50.61	393
	0.35	0.74^{b}	0.183	50.46	377
	0.40	0.70^{b}	0.175	50.68	359
	0.45	0.78^{ab}	0.184	51.24	404
Limestone	0.30	0.78^{ab}	0.181	50.51	398
	0.35	0.83^{a}	0.185	50.35	418
	0.40	0.79^{ab}	0.180	50.65	405
	0.45	0.80^{ab}	0.185	50.77	407
TCP	0.30	0.77^{ab}	0.182	50.96	394
	0.35	0.78^{ab}	0.185	51.81	406
	0.40	0.89^{a}	0.183	51.08	450
	0.45	0.80^{ab}	0.179	51.27	409
Ca source					
Ca Chloride		0.75^{b}	0.182	50.75^{b}	383^{b}
Limestone		0.80^{a}	0.183	50.57^{b}	407^{ab}
TCP[3]		0.81^{a}	0.182	51.29^{a}	415^{a}
NPP level,%					
	0.30	0.78	0.183	50.69	50.69
	0.35	0.79	0.184	50.88	50.88
	0.40	0.79	0.179	50.80	50.80
	0.45	0.80	0.183	51.10	51.10
SEM		0.03	0.004	0.32	16.95
P value					
Ca Source × NPP[4]		0.029	0.763	0.291	0.058
Ca Source		0.011	0.976	0.002	0.011
NPP level		0.883	0.559	0.382	0.800

[1]Non-phytate P;[2] Data are means of 5 pens with 5 chicks each;[3]Lipocal (Lipofoods; Barcelona, Spain);[4 abc] Values in the same column with different letters are significantly different ($P < 0.05$).

The effects of the Ca source and NPP levels on tibia weight and tibia ash content are presented in Table 5.4. Tibia weight was influenced by the significant interaction of the Ca source and the P level. Added levels of P increased the tibia weight with limestone and TCP but not with calcium chloride. Tibia weight was higher in birds fed TCP at 0.4% NPP, and limestone at 0.35% NPP, while it was lower for treatments including Ca chloride in the diet with 0.35% and 0.4% NPP. Tibia weight was higher ($P < 0.05$) for birds fed TCP and limestone than it was for Ca chloride.

However, the tibia-ash percentage was higher ($P < 0.01$) for TCP than it was for limestone and Ca chloride. As a consequence, the ash content per tibia was the greatest for birds fed TCP, and it was the lowest for birds fed the Ca chloride diet, with birds fed limestone showing intermediate results.

The ileal digestibility of Ca and P responses to different sources of Ca and NPP levels are presented in Table 5.5. There was no interaction between the Ca source and NPP level. Birds fed Ca chloride showed higher ($P < 0.01$) ileal digestibility of Ca (73.7%) than did birds fed limestone (67.1%) or TCP (66.8%). Added levels of P increased ($P < 0.05$) the ileal digestibility of Ca and the ileal digestibility of P ($P < 0.01$), being higher for the 0.45% NPP diet than it was for the 0.3% NPP diet.

5.5. Discussion

5.5.1. Calcium solubility and Acid-Binding Capacity

Maintaining soluble calcium in the gastrointestinal tract is essential for intestinal absorption (Bronner, 2003). Nevertheless, many Ca salts have a pH-dependent solubility and may have limited availability in the small intestine. In particular, the pH of the broiler gut changes according to the region, from the acidic environment of the proventriculus and gizzard, where the pH is governed by the secretion of hydrochloric acid, to the less acidic, nearly neutral environment throughout the intestine, where the pH is governed by sodium bicarbonate. In the present *in vitro* study, calcium solubility was determined at different pH values and in the presence of excess solids to mimic the environment each salt may encounter in the GI tract. Calcium chloride showed the highest solubility among the 3 Ca sources at a pH above 3.5 until neutrality. Differences in Ca solubility were consistent with Selle *et al.*, (2000), who suggested that most mineral complexes were soluble at low pH (less than 3.5), with maximum insolubility occurring between 4 and 7. *In vitro* research in corn-based diets by Walk *et al.*, (2012a) also described that limestone is approximately 80% soluble in the acidic medium of the gastrointestinal tract, but that solubility decreased to 77% in the neutral conditions of the intestine, suggesting no further dissolution of Ca in the intestinal phase. Moreover, greater concentrations of ionized Ca dissolved by acid in the stomach may precipitate in the more neutral intestine before absorption occurs.

Table 5.5. Influence of Ca and NPP[1] levels on Ca and P ileal digestibility in 14-day-old broilers[2].

Treatment	NPP, %	Ca ileal digestibility, (%)	P ileal digestibility, (%)
Ca Chloride	0.30	70.4	78.8
	0.35	72.8	80.0
	0.40	74.6	84.0
	0.45	77.0	89.2
Limestone	0.30	64.8	78.8
	0.35	68.8	81.0
	0.40	64.6	83.0
	0.45	70.2	86.0
TCP[3]	0.30	62.25	72.75
	0.35	67.4	83.0
	0.40	67.2	79.8
	0.45	70.4	83.2
Ca source			
Ca Chloride		73.7[a]	83.0
Limestone		67.1[b]	82.2
TCP		66.8[b]	79.7
NPP level,%			
	0.30	65.8[b]	76.8[b]
	0.35	69.7[ab]	81.3[ab]
	0.40	68.8[ab]	82.3[ab]
	0.45	72.5[a]	86.1[a]
SEM		1.67	1.55
P value			
Ca Source× NPP[4]		0.948	0.575
Ca Source		0.001	0.188
NPP		0.047	0.001

[1]Non-phytate P.
[2] Data are means of 5 pens with 5 chicks each.
[3]Lipocal (Lipofoods; Barcelona, Spain).
[4 abc] Values in the same column with different letters are significantly different ($P < 0.05$).

Therefore, due to the secretion of HCO_3^-, the free Ca^{2+} in the intestine may precipitate out of the solution as carbonate and phosphate. Goss *et al.*, (2007) evaluated the concentration of soluble Ca using the *in vivo* concentration of calcium and bicarbonate, and showed that soluble amounts decrease by an order of magnitude with each increasing pH unit. Goss *et al.*, (2010) also reported that calcium salt selection (Ca chloride vs. Ca citrate), rate of neutralization, and the presence of other digesta compounds (phosphates, amino acids and bile components) impact the concentration of total soluble calcium and may modify the physiological components affecting calcium absorption. In this respect, Champagne (1988) reported that Ca-phytate complexes may precipitate at pH between 4 and 6, which is the approximate pH of the intestine where

Ca ions should be absorbed. Taylor (1965) and Manangi and Coon (2008) suggested that the primary factor affecting phytic P utilization is the Ca ion concentration in the small intestine, where insoluble Ca-phytate complexes form. However, we were not able to identify changes in the soluble calcium concentration in the presence of phytic acid.

Walk *et al.* (2012a) have also stated that high soluble sources of Ca may increase the buffering capacity of digesta. In our *in vitro* study, Ca chloride showed the highest Ca solubility, but a lower buffering capacity, when compared to TCP and limestone. The Ca supplementation required to reach 1% Ca in the diet when using limestone or TCP caused an increase in the ABC-3 of 190 mEq/kg and 343 mEq/kg, respectively, as compared to the basal diet, practically doubling the ABC of a maize-soybean diet (Lawlor, 2005). Jasaitis *et al.* (1987) found that carbonates and dibasic or tribasic mineral additives had the highest ABC.

5.5.2. Broiler performance and bone mineralization

The Ca source promoted significant differences in FI, with TCP promoting higher FI than did calcium chloride. Birds fed limestone and TCP also showed higher WG than did birds fed Ca chloride. Based on these results, it could be suggested that sources of Ca with a lower solubility may allow for better performance than might a high-soluble Ca source. Walk *et al.*, (2012a) observed that broiler chickens fed 0.90% Ca from limestone ate more and were heavier than were birds fed 0.90% Ca from a high-soluble Ca source. Due to the soluble nature of the highly soluble Ca source, the authors suggested that feeding 0.90% Ca from this source may have reduced broiler performance as a result of a high Ca:P ratio and an increase in calcium-phosphate or calcium phytate precipitation. The authors also demonstrated that N digestibility was reduced as the highly soluble Ca source increased, and suggested that the buffering capacity of high dietary levels of high-soluble Ca may reduce pepsin efficacy in the proventriculus and gizzard (pH optimum at 2.8; Bohak, 1969). Both *in vivo* and *in vitro* studies (Manangi and Coon, 2007) indicated that solubility of limestone depends on particle size, with the lowest particle size (28 μm) showing a higher Ca solubility and a reduced phytate hydrolysis. Therefore, it could be proposed that the effects of soluble Ca limiting phytate hydrolysis could be counteracted by a higher dose of phytase or by adding NPP in the diets. Walk *et al.*, (2012a) demonstrated that a high dose of phytase in feed to reach 2,000 U/kg-2,500 U/kg was able to increase the performance of broilers

fed 0.9% Ca of a high-soluble Ca source to values close to those presented by a limestone source without phytase. Similar performances were observed with lower levels of Ca, which suggest that reductions in dietary Ca may be obtained with high-soluble sources of Ca while maintaining broiler performance and bone mineralization. However, in the present study, all diets were supplemented with a high dose of phytase to reach an analyzed phytase activity of 1,150 U/kg, and Ca was included at a low dose of 0.55% in order to reduce the likely negative interactions among nutrients in the digestive tract, allowing chicks to respond to different sources of soluble Ca. The results showed that Ca digestibility was higher for Ca chloride than it was for limestone and TCP, but no differences were observed among Ca sources in P ileal digestibility. These results make the explanation of limited P digestibility with the highly soluble Ca sources questionable. Additionally, ABC was the lowest for calcium chloride, without undermining any negative effect of soluble sources on protein digestibility. On the other hand, results showed a consistent effect of Ca chloride on FI and WG. It could be speculated that feed palatability is likely affected in the Ca chloride diets if broilers were able to detect the highly soluble Ca and P sources in the beak and crop (such as Ca chloride and Na phosphate). Some recent reports suggest that broilers are able to detect calcium in the diet (Wilkinson *et al.*, 2012).

Increasing the level of NPP increased apparent ileal P digestibility, which is in accordance with the results reported by Rodehutscord and Dieckmann (2005). Al Masri (1995) showed that the values of dietary Ca and its ratio with P may affect P absorption. Lower values of P absorption were recorded when higher ratios between Ca and P were added in the diet. Nevertheless, this result could also reflect the fact that mineral sources used to increase NPP have a higher P digestibility than do vegetable sources, even when a high dose of phytase is added to the diet. In this respect, when using the 0.3% NPP diet to compare P digestibility among diets, the TCP diet with a main content of tri-calcium phosphate showed lower ileal P digestibility (72.7%) than did the limestone diet (78.8%) and calcium chloride diet (78.8%), which contained higher amounts of mono-calcium and sodium phosphate, respectively. The results appear to confirm a higher P digestibility for mono-calcium and sodium phosphate than for the tri-calcium phosphate, likely reflecting the lower solubility of this source at the neutral pH of the small intestine.

It is also relevant that higher levels of NPP also promoted an increase in Ca digestibility, which could reflect the changes in the main mineral ingredients used in

each diet. Angel (2013) has recently described that true digestibility of Ca for limestone (34.1%) is lower than it is for mono-calcium phosphate (67.9%). The increase in ileal Ca digestibility with increasing levels of NPP in the TCP treatments also suggests that Ca digestibility is also lower for tri-calcium phosphate than it is for mono-calcium phosphate. Thus, a change in the levels of included mineral ingredients, such as those promoted when phytase is included in the diet, is also expected to promote changes in Ca digestibility.

Tibia weight and ash content were influenced by changes in the Ca source, with calcium chloride showing the lowest tibia weight, with TCP showing the highest tibia weight and ash content. Moreover, the significant interaction observed between the Ca source and level of NPP on tibia weight and ash content is worth stating. In 0.4% NPP diets, broilers fed TCP had an average of 450 mg of ash in the tibia, while values were 405 mg and 359 mg in limestone and Ca chloride broilers, respectively. The results are coherent with differences observed in FI and WG, but not with the results concerning Ca and P digestibility (Ca chloride > limestone > TCP). It is difficult to find an explanation for this effect on bone mineralization, which appears to be greater than are the effects observed in FI and performance. The results could suggest that bone mineralization will depend on the daily amount of absorbed Ca and P, but also on the way and rate in which minerals are released and absorbed in the intestinal tract, reaching the highest bone mineralization when derived from less soluble sources. However, this hypothesis deserves further study and may pose difficulties to formulate diets on a digestible or available Ca basis.

In conclusion, the results confirm that ileal Ca and P digestibility may vary according to the source of minerals used in the diet, being higher for calcium chloride, mono-calcium phosphate, and sodium phosphate than for limestone and TCP. However, growth performance and bone mineralization reach the highest values in animals fed low-soluble sources. A level of 0.35%-0.40% NPP with a high dose of phytase in diets including limestone or TCP is sufficient to guarantee performance and bone formation for broiler chickens from d 1 to d 14.

CHAPTER 6

Comparison of how different mineral phosphorus sources affect performance, bone mineralization and phosphorus retention in broilers

Comparison of how different mineral phosphorus sources affect performance, bone mineralization and phosphorus retention in broilers

M. Hamdi [1,] D. Solà-Oriol[1], R. Franco-Rosselló [1], R. Aligue [2], and J.F. Pérez [1]

[1] Animal Nutrition and Welfare Service (SNiBA). Departament de Ciència Animal i dels Aliments. Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain.

[2] Department of Biomedical Sciences. University of Barcelona, IDIBAPS, 08036 Barcelona, Spain.

Submitted to *British Poultry Science*

6.1. Abstract

1. Two experiments were designed to evaluate the influence of different sources of phosphate on animal performance, bones mineralization and mineral retention in broilers.

2. In Experiment 1, twenty mash diets were prepared including five mineral sources of P to supplement non phytic P (NPP) levels at 3.0, 3.5, 4.0 and 4.5 g/kg in diets without phytase for boilers (n=500).

3. In Experiment 2, three treatments were used: the low MCP (mono-calcium phosphate) diet was deficient in NPP (3.1 g/kg for the starter phase and 2.8 g/kg for the grower phase) and adequate in Ca (9.0 g/kg and 8.5 g/kg, respectively); the high MCP diet and the high TCP (tri-calcium phosphate) diet included adequate levels of NPP and Ca.

4. In experiment 1, an increase in the level of NPP in the diet from 3.0 to 4.0 g/kg increased WG (weight gain) and FI (Feed intake) between d 1 and d 21. Alternatively, tibial weight and ash percentage at d 21 responded up to the level of 4.5 g/kg and showed significant difference with birds of the 4.0 g/kg NPP group.

5. In experiment 2, chickens fed with the high MCP and high TCP showed an improvement in animal performance and bone mineralization. A higher P retention was also observed for birds fed high MCP (1.28 g/day) and TCP (1.51 g/day) as compared to broilers feds low MCP (0.88 g/day).

6. The results did not show differences on the availability of P from the different mineral P sources. A level of 4.5 g/kg NPP is recommended when phytase is not include to maximize both performance and bone mineralization in broiler chickens up to d 21.

6.2. Introduction

In addition to environmental concerns regarding excessive Phosphorus (P) in ground water, P is one of the most expensive nutrients in poultry diets. The broad development of phytase has allowed for the reduction of dietary P and the inclusion of mineral sourced P. However, mineral sources of P are still needed in the diet to meet the requirement of poultry and prevent P deficiencies. With increases in prices for mineral sources, the poultry and commercial feed industry have become increasingly interested in detailed information about the variation in availability of P among different raw materials, as well as batches of the same raw material. The bio-availability for each P source is pivotal to formulate diets at a higher precision and avoid excessive P. However, a limited amount of information exists regarding the biological availability or retention of different types of mineral P sources. Phosphorus is also a nutrient with a direct linear effect on growth response and bone characteristics when levels below P requirements are applied (Hamdi *et al.*, 2015a). Different methodologies exist to measure *in vivo* bio-availability of P or to compare P sources including bio-assays based on growth, bone weight and bone ash weight (Ravindran *et al.*, 1995; Lima *et al.*, 1997). These procedures are useful relative measurements for the comparison of different sources of Ca and P, but they don´t provide a quantitative value for formulation purposes (Leske and Coon, 2002).

Different sources have been used as a P reference in bio-assay studies, mono-sodium phosphates (Shastak *et al.*, 2012), mono-calcium phosphates (MCP) (Groote and Huyghebaert, 1997) or di-calcium phosphates (DCP) (Coon and Manangi, 2007, Fernandez *et al.*, 1999; Ravindran *et al.*, 1995). The MCP has a higher concentration of P than Ca, namely 22.6% P and 17.8% Ca. In contrast, P and Ca content in DCP depend on the degree of hydration. The DCP dihydrate contains typically 17.7% P and 24% Ca and DCP anhydrous contains 20.1% P and 27% Ca (FEDNA, 2010).

It is believed that P in MCP is more digestible than P in DCP (Grimbergen *et al.*, 1985; Eekhout and De Paepe, 1997). However, because most feed phosphates designated as MCP or DCP are mixtures of both, differences within a source may exist (Peterson *et al.*, 2011). Commercial products labeled DCP are industrial products resulting from the acidulation of rock phosphate, frequently with sulfuric acid, yielding phosphoric acid, which is neutralized with Ca carbonate after purification (Lima *et al.*, 1997). In contrast, tri-calcium phosphate (TCP) provides a Ca:aP ratio close to the

recommended dietary ratios (TCP phosphate supplied 17.4% P, and 34% Ca). Therefore, TCP could be used as a unique source to satisfy poultry Ca and P requirements without the need of Ca from limestone.

Consequently, in this study we tested the hypothesis that different mineral sources of P, varying in the amount of P and Ca, will affect the dietary P absorption and overall productivity of broiler chickens. Two experiments were conducted to evaluate the effect of the P source provided at different levels in broiler diets on the availability of P and their effects on performance and bone mineralization for broilers. In the first experiment, the P availability was compared between MCP and the different sources of DCP in broilers chickens up to d 21. In the second experiment, the inclusion of a source of TCP as a single source of Ca and P was compared to MCP in broiler diets with respect to their effects on performance, bone mineralization and P retention in broilers up to d 35.

6.3. Materials and methods

All the animal experimentation procedures used in the two experiments were approved by the animal Ethics Committee of the Universitat Autònoma de Barcelona and were in compliance with the European Union guidelines for the care and use of animals in research (European Parliament, 2010).

6.3.1. Birds managment, husbandry, study design and diets

6.3.1.1. Experiment 1

Five hundred broiler male chickens (*Ross* 308) were included in the study. Animals were randomly distributed into twenty experimental groups according to the experimental treatment and continuously controlled for 21 days. Birds were individually weighed and distributed in 100 battery brooders cages (5 chicks per cage) in order to get a similar initial average body weight for each cage. Chicks were individually wing-tagged in order to record individual body weight as well the group body weight during the experimental period. The brooder temperature was maintained at 35ºC during the 4 first days post-hatch, and then was progressively reduced to 25ºC on day 14 to d 21 day. The light cycle was 24h/d from d 1 to d 2, 23h/d from d 3 to d 10 and 18h/d from d 11 to 21.

Table 6.1. Composition of the experimental diet (Experiment 1).

	MCP[1]				DCP[2]1				DCP[2]2				DCP3				DCP4			
	3	**3.5**	**4**	**4.5**	**3**	**3.5**	**4**	**4.5**	**3**	**3.5**	**4**	**4.5**	**3**	**3.5**	**4**	**4.5**	**3**	**3.5**	**4**	**4.5**
Ingredients (%)																				
Corn	28.7	28.7	28.7	28.7	28.7	28.7	28.7	28.7	28.7	28.7	28.7	28.7	28.7	28.7	28.7	28.7	28.7	28.7	28.7	28.7
Wheat	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0
Soybean meal	24.8	24.8	24.8	24.8	24.8	24.8	24.8	24.8	24.8	24.8	24.8	24.8	24.8	24.8	24.8	24.8	24.8	24.8	24.8	24.8
Extruded soybean	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0
L-Lysine	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29
DL-Methionine	0.27	0.27	0.27	0.27	0.27	0.27	0.27	0.27	0.27	0.27	0.27	0.27	0.27	0.27	0.27	0.27	0.27	0.27	0.27	0.27
L-threonine	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Premix[*]	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Dicalite	0.46	0.34	0.23	0.11	0.51	0.41	0.31	0.21	0.56	0.48	0.39	0.31	0.54	0.45	0.35	0.26	0.57	0.49	0.40	0.32
P source inclusion	0.70	0.92	1.14	1.36	0.86	1.13	1.39	1.66	0.86	1.13	1.39	1.66	0.90	1.18	1.45	1.73	0.90	1.18	1.46	1.74
Limestone inclusion	1.57	1.47	1.36	1.26	1.36	1.19	1.02	0.86	1.31	1.13	0.94	0.76	1.29	1.11	0.92	0.73	1.26	1.07	0.87	0.67
Calculated Composition (g/kg diet)																				
Total P	5.5	6.0	6.5	7.0	5.5	6.0	6.5	7.0	5.5	6.0	6.5	7.0	5.5	6.0	6.5	7.0	5.5	6.0	6.5	7.0
PP	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Available P	2.8	3.3	3.7	4.2	0.27	0.31	0.35	0.40	0.27	0.31	0.35	0.40	0.27	0.31	0.35	0.40	0.27	0.31	0.35	0.40
Ca	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0
Analyzed composition (g/kg diet)																				
DM	896	894	895	894	897	897	897	896	896	898	897	899	895	895	896	898	896	896	897	898
CP	226	222	219	217	218	221	218	221	222	218	216	220	210	213	220	211	210	220	212	218
Total P	6.5	6.9	7.3	7.4	6.4	6.7	7.2	7.9	6.5	6.6	7.3	8.0	5.7	6.7	7.4	7.9	6.1	6.9	6.9	7.7
Ca	13.1	13.0	13.4	12.2	15.1	13.1	12.5	14.5	13.2	13.0	12.9	13.4	11.7	11.9	13.3	12.1	12.3	13.1	12.2	12.8

(*) Provided per kg of feed: Vitamin A (retinil acetate) (UI) 10000; Vitamin D (Vitamin D3) (Colecalciferol) (UI) 2000; Vitamina D (25- hidroxicolecalciferol. 25 mcg equivalent at 1000 UI) (UI) 1500; Vitamin E/acetate de tot-rac-3- tocopheril) (mg) 75; Vitamin K3 (MNB Menadiona nicotinamida bisulfit)(mg) 5; Vitamin B1 (Tiamin mononitrat) (mg) 2; Vitamin B2 (Riboflavin) (mg) 5; Vitamin B6 (Piridoxin Chlorhidrate) (mg) 4; Vitamin B12 (cyanocobalamine) (mg) 0.015; Biotin (D-(+)-biotin) (mg) 0.15; Folic Acid (mg) 1; Iron (Iron sulfate monohydrate) (mg) 46; Zinc (Zn, zinc oxide) (mg) 125; Manganes (Mn, Manganes oxide) (mg) 150; Iodine (I, Calcium Iondine Anhidre) (mg) 2; Selenium (Se, Sodium Selenate) (mg) 0.3; Copper (Cu, Copper Sulfate pentahydrate) (mg) 20; Endo-1,3(4)-betaglucanase EC 3.2.1.6 (FBG) 10; Endo-1,4-beta-xylanase EC 3.2.1.8 (FXU) 150; Malic acid (mg) 60; Fumari acid (mg) 75; Sepiolite (mg) 400; Calcium Carbonate (g) 4. [1] Mono-calcium phosphate [2] Di-calcium phosphate.

An identical basic mixture of ingredients (wheat, 250 g/kg; corn, 287.2 g/kg; soybean meal, 248.8 g/kg; and soybean oil, 42.5 g/kg) was formulated and prepared to contain adequate levels of nutrients (ME, 2,960 Kcal/kg; DM, 882 g/kg; and CP, 220 g/kg) to meet and exceed nutritional requirements in broilers (FEDNA, 2008), except for P. All the diets were presented in mash form. A total of 20 experimental treatments were prepared adding 4 increasing levels of non-phytic phosphorus (3.0, 3.5, 4.0 and 4.5 g NPP/kg) depending on the inorganic P source under study and maintaining a level of 9.0 g Ca/kg. Phytase was not added in the experimental diets. Five main sources of P were used to design the experimental treatments, which were MCP- monohydrate (22.3% P, 16.8% Ca), DCP1-dihydrate (18.7% P, 23.9% Ca), DCP2-dihydrate (18.7% P, 26.1% Ca), DCP3-dihydrate (17.9% P, 25.6% Ca) and DCP4-dihydrate (17.9% P, 26.9% Ca). Table 6.1 shows the P concentration and the levels of incorporation of each source of P. All P sources were obtained from independent distributors rather than manufacturers of the products. The broilers chicken had free access to drinking water served via a nipple system and to feeders containing the feed in a mash form.

6.3.1.2. Experiment 2

Ninety-six broilers male chickens (*Ross* 308) were included in the study. Animals were randomly distributed into three experimental groups according to the experimental treatment and continuously controlled for 35 days. Birds were individually weighed and distributed in 24 battery brooder cages (4 chicks per cage) in order to get a similar initial average body weight for each cage. Chicks were individually wing-tagged in order to monitor individual BW as well as the group BW along the experimental period. The brooder temperature was maintained at 35ºC during the 4 first days post-hatch, and was progressively reduced to 25-22ºC on d 14 to d 35 day. The light cycle was 24h/d from d 1 to d 2, 23h/d from d 3 to d 10 and 18h/d from d 11 to d 35. All diets met or exceeded the nutrient requirements for broilers (FEDNA, 2008), with the exception of P (Table 6.2).

All birds received two experimental diets, the first one in the starter phase (from d 1 to d 21) and the second diet during the grower phase (from d 21 to d 35). The low MCP treatment included limestone (38% Ca) and MCP (22.6% P and 17.8% Ca) as sources of Ca and P and was formulated to be adequate in Ca (9.0 g/kg and 8.5 g/kg for

the starter and grower phase, respectively), but deficient in NPP (3.1 g/kg and 2.8 g/kg for the starter and grower phase, respectively).

Table 6.2. Day one to day 35 broiler starter and grower experimental diets (Experiment 2).

	Starter phase			Grower phase		
	Low MCP[1]	**High MCP**	**High TCP[2]**	**Low MCP**	**High MCP**	**High TCP**
Ingredients (g/kg)						
Corn	191.1	184.7	200.5	254.6	248.4	263.8
Wheat	350.0	350.0	350.0	350.0	350.0	350.0
Soybean meal	212.6	213.8	210.7	151.0	152.0	149.6
Extruded soybean	150.0	150.0	150.0	180.0	180.0	180.0
Limestone	15.8	13.1	0	15.3	12.7	0
Lipocal	0	0	18.9	0	0	17.1
Mono-calcium phosphate	7.7	13.4	0	6.4	12.1	0
Salt	4.3	4.2	4.3	4.0	4.0	4.0
Premix*	3.0	3.0	3.0	3.0	3.0	3.0
Calculated Composition (g/kg)						
DM	889.0	889.0	889.0	885.0	885.0	885.0
M.E(Kcal/Kg)	3,000	3,000	3,000	3,140	3,140	3,140
CP	210.0	210.0	210.0	198.0	198.0	198.0
Ca	9.0	9.0	8.0	8.5	8.5	7.3
Total P	5.6	6.9	7.2	5.3	6.6	6.8
Available P	3.0	4.2	4.2	2.7	3.9	3.9
PP	2.5	2.5	2.5	2.5	2.5	2.5
NPP	3.1	4.4	4.7	2.8	4.2	4.3
Analyzed composition(g/kg)						
Ca	9.4	10.0	9.5	11.4	11.2	10.5
Total P	5.7	7.2	7.0	5.4	6.5	7.0

(*) Provided per kg of feed: Vitamin A (retinil acetate) 10000UI; Vitamin D (Vitamin D3) (Colecalciferol) 2000 UI; Vitamina D (25- hidroxicolecalciferol. 25 mcg equivalent at 1000 UI) 1500 UI; Vitamin E/acetate de tot-rac-3-tocopheril) 75 mg; Vitamin K3 (MNB Menadiona nicotinamida bisulfit)5 mg; Vitamin B1 (Tiamin mononitrat) 2 mg; Vitamin B2 (Riboflavin) 5 mg; Vitamin B6 (Piridoxin Chlorhidrate) 4 mg; Vitamin B12 (cyanocobalamine) 0.015 mg; Nicotinic Acid (Nicotinic Acid) (Niacin) 25 mg; Pantotenic Acid (Calcium D-pantotenate) 10 mg; Biotin (D-(+)-biotin) 0.15 mg; Folic Acid 1mg; Iron ($FeSO_4$) 46 mg; Zinc (ZnO) 125 mg; Manganes (MnO) (150 mg); Iodine ($Ca(IO_3)_2$) 2 mg; Selenium (Na_2SeO_3) 0.3 mg; Cobalt (Co, $CoCO_3$) 0.5 mg; Copper ($CuSO_4$) 20 mg;DL-Methionin 500 mg; Etoxiquin 0.1332 mg; Endo-1,3(4)-betaglucanase EC 3.2.1.6 (10 FBG); Endo-1,4-beta-xylanase EC 3.2.1.8 (150 FXU); Malic acid 60 mg; Fumaric acid 75 mg; Sepiolite 400 mg; Calcium Carbonate 4 g.

[1] Mono-calcium phosphate

[2] Tri-calcium phosphate

The high MCP treatment included also limestone and MCP as sources of Ca and P and was formulated to be adequate in NPP (4.4 g/kg and 4.2 g/kg for the starter phase and grower phase). The high TCP treatment was formulated without any other mineral Ca or P source to be adequate in all nutrients and energy. The Ca content was 8.0 g/kg for the starter phase and 7.3 g/kg for grower phase. The NPP was 4.7 g/kg for the starter phase and 4.3 g/kg in growth phase with the use of Lipocal® (Lipofoods; Barcelona,

Spain) (a fat-encapsulated tri-calcium phosphate, supplied 17.4% P and 34% Ca) as a source of Ca and P. Phytase was not incorporated in the experimental diets.

6.3.2. Sample collection and processing

6.3.2.1. Experiment 1

The feed intake (FI), weight gain (WG) and gain feed ratio (G:F) were calculated between d 1 and d 21. On d 21, three birds were euthanized using the cervical dislocation and the left tibia was collected for bone-ash determination.

6.3.2.2. Experiment 2

Individual BW as well as the group BW was monitored at the start (d 1) and d 7, d 14, d 21, d 28 and d 35 post-hatch. From these values the FI, WG and G: F ratio were determined. Feed intake was registered and excreta were collected from d 19 to d 21 and from d 33 to d 35 in order to determine the retention of Ca and P. At d 35, three chickens per cage were euthanized using the cervical dislocation and the left tibia was collected for bone-ash determination.

6.3.3. Laboratory analyses

Representative samples of diets and excreta were analyzed.

Analytical determinations of feeds were performed according to the methods of AOAC International (2005): dry matter (Method 934.01), crude protein with Dumas Method (Method 968.06).

Diets and excreta samples were digested in nitric perchloric and fluorhydric acid mixture and subsequently concentration of P and Ca were determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES) using optical emission spectrometer optimal 4300DV Perkin-Elmer.

Bone mineralization was determined by the procedure of Brenes *et al*., (2003). The tibias were defatted by a 48h extraction in ethyl ether. They were then dried for 12h at 110ºC and then ashed overnight at 550ºC to determine ash content.

6.3.4. Calculations

The total tract retention coefficients were calculated according to the procedure outlined using the following equation (Adeola, 2001): The apparent total tract retention coefficients of Ca or P (ATTRC)

ATTRC (%) = [(total P or Ca ingested − total P or Ca excreted)/ (total Por Ca ingested)] x 100.

P or Ca retention (g/day) = total P or Ca ingested x ATTRC

6.3.5. Statistical analyses

Data were analyzed as a completely randomized design using the GLM procedure of SAS software (SAS, 2008), version 9.2. In experiment 1, the main factors used in the model were P level (4 levels), sources (5 sources) and their interaction. In experiment 2, the dietary treatments and the source of P were taking into account. The pen of the chicks served as the experimental unit. The results are presented as least square means. Probability was considered significant when $P \leq 0.05$.

6.4. Results

6.4.1. Experiment 1 (comparison between MCP and DCP)

6.4.1.1. Growth performance and bone mineralization

Table 6.3. Effect of different P sources and levels on feed intake and growth performance of broilers between d 1 to d 21 of age (Experiment 1).

	BW[5]d 21, g	WG[6]d 1-21, g/d	FI[7]d 1-21, g/d	G: F[8]d 1-21
Source				
MCP[1]	728	32.6	46.4	0.70
DCP[2]1	733	32.9	46.8	0.70
DCP 2	737	33.1	46.7	0.71
DCP 3	744	33.5	47.5	0.70
DCP 4	737	33.0	46.4	0.71
NPP (g/kg)				
3.0	670[b]	30.0[c]	43.8[c]	0.69[b]
3.5	714[b]	32.1[b]	46.1[b]	0.70[b]
4.0	771[a]	34.7[a]	48.1[a]	0.72[a]
4.5	788[a]	35.4[a]	49.1[a]	0.72[a]
SEM[3]	21.4	0.04	1.13	0.018
P value[4]				
Source	0.879	0.818	0.638	0.930
NPP	<.0001	<.0001	<.0001	0.002
Source × NPP	0.461	0.617	0.803	0.630

[1]Mono-calcium phosphate; [2] Di-calcium phosphate; [3] SEM: Standard error of the mean; [4 a,b,c] Values in the same column with different letters are significantly different (P<0.05); [5] Body weight; [6]Average daily weight gain; [7] Average daily feed intake; [8] Gain: Feed ratio.

Animal performances along the experimental period are presented in Table 6.3 as least square means. Productive performance was not significantly affected by the source of phosphorus or their interaction with NPP levels. However, higher BW at d 21,

WG, FI and G: F were observed from the broilers in the 4.0 and 4.5 g NPP/kg group as compared to the broilers in the 3.0 and 3.5 g NPP /kg group.

The effect of different sources and levels of P on bone mineralization (tibial weight and ash) and tibial weight/BW is reported in Table 6.4. The results are presented as least square means. No significant interaction effect was observed between P sources and NPP levels. On d 21, tibial weight and bone ash percentage were increased (P< 0.05) by the dietary P supplementation, regardless of the source of P utilized. Broilers in the 4.5 g NPP /kg level showed a greater tibial weight than broilers in the 4.0 g NPP /kg group. Broiler chickens fed diets supplemented with 4.0 and 4.5 g NPP /kg showed a higher tibial ash (%) and tibial weight/BW than those fed diets supplemented with 3.0 and 3.5 g NPP /kg.

Table 6.4. Effect of different P sources and level on tibial weight and ash content in birds from d1 to d 21 of age (Experiment 1).

	Tibial weight, g	**Tibial ash, %**	**Tibial weight, %/BW**	**Tibial ash, mg/tibia**
Source				
MCP	1.57	50.83	0.215	0.798
DCP[1]1	1.62	51.07	0.216	0.831
DCP[2]2	1.60	51.05	0.215	0.816
DCP 3	1.59	51.53	0.214	0.821
DCP 4	1.58	50.63	0.213	0.800
NPP (g/kg)				
3.0	1.41[c]	48.29[c]	0.207[c]	0.682[c]
3.5	1.52[d]	50.80[b]	0.211[b]	0.770[d]
4.0	1.66[b]	52.11[a]	0.217[a]	0.868[b]
4.5	1.76[a]	52.88[a]	0.223[a]	0.932[a]
SEM[3]	0.053	0.530	0.0038	0.028
P value[4]				
Source	0.619	0.179	0.875	0.409
NPP	<.0001	<.0001	<.0001	<.0001
Source × NPP	0.809	0.234	0.570	0.611

[1]Mono-calcium phosphate ; [2] Di-calcium phosphate; [3] SEM: Standard error of the mean; [4 a,b] Values in the same column with different letters are significantly different (P<0.05).

6.4.2. Experiment 2 (comparison between TCP and MCP)

6.4.2.1. Growth Performance and Bone Mineralization

The feed intake and growth performance responses of broilers chickens to different levels and sources of Ca and P are presented in Table 6.5. Higher BW was observed ($P < 0.05$) at d 21 and d 35 for broilers fed the high MCP and high TCP compared to the birds fed the low MCP group. Higher WG and FI (P< 0.05) from d 1 to

21 and from d 21 to 35 was also observed for birds of the high MCP and high TCP. As a consequence, G: F was also greater for broilers chicken of the high MCP and high TCP compared to those birds of the low MCP.

Table 6.5. Effect of MCP and TCP diets on feed intake and growth performance in broilers between d1 and d35 (Experiment 2).

Treatments	BW[5],g		WG[6],g/d		FI[7],g/d		G: F[8]	
	d 21	d 35	d 1-21	d 21-35	d 1-21	d 21-35	d 1-21	d 21-35
Low MCP[1]	714[b]	1680[b]	32.1[b]	70.9[b]	48.2[b]	118.7[b]	0.665[b]	0.554[b]
High MCP	824[a]	2108[a]	37.3[a]	91.1[a]	51.4[ab]	146.9[a]	0.725[a]	0.620[a]
High TCP[2]	832[a]	2087[a]	37.7[a]	89.6[a]	52.8[a]	145.8[a]	0.713[a]	0.615[a]
SEM [3]	20.9	41.5	0.99	2.67	1.18	3.48	0.0105	0.0106
P value[4]	0.001	<.0001	0.001	<.0001	0.034	<.0001	0.001	0.0003

[1]Mono-calcium phosphate [2] Tri-calcium phosphate [3] SEM: Standard error of the mean. [4 a,b] Values in the same column with different letters are significantly different (P<0.05).[5] Body weight. [6]Average daily weight gain. [7] Average daily feed intake. [8]Gain: Feed ratio.

The effect of different treatments on bone mineralization (ash content, %), tibial weight (g, and % as total BW) are presented in Table 6.6. Tibial weight (g and %/BW) showed significantly higher values (P< 0.001) for broilers of the high MCP and TCP diets than in birds fed the low MCP diet. Tibial ash (%, g/tibia) was significantly lower in the low MCP group than in the high MCP and TCP groups. The amount of tibial ash (%, and g/tibia) for birds fed the high MCP was similar to those fed high TCP.

Table 6.6. Effect of MCP and TCP on tibial weight and ash content in 35-day-old broilers (Experiment 2).

Treatments	Tibial weight, g	Tibial ash, %	Tibial weight, %/BW	Tibial ash, (g/tibia)
Low MCP[1]	3.41[b]	48.1[b]	0.203[b]	1.64[b]
High MCP	4.82[a]	52.9[a]	0.228[a]	2.55[a]
High TCP[2]	4.57[a]	52.3[a]	0.219[a]	2.39[a]
SEM[3]	0.151	0.47	0.0057	0.079
P value[4]	<.0001	<.0001	0.013	<.0001

[1] Mono-calcium phosphate; [2] Tri-calcium phosphate; [3] SEM: Standard error of the mean; [4 a,b] Values in the same column with different letters are significantly different (P<0.05).

6.4.2.2. Apparent retention of calcium and phosphorus

The retention of P and Ca expressed on a percent basis and g/day is shown in Table 6.7. On days 19 to 21, P retention (%) was significantly ($P < 0.05$) affected by the

dietary treatment, with greater values observed for birds in the low MCP (59.46%) diet compared to the high TCP (44.57%) diet. Intermediate values were observed for chicks of the high MCP treatment (50.66%). Ca retention was not affected by the dietary treatment. Furthermore, during the finishing phase (d 33 to d 35), no significant differences were observed on the P retention (%). However, broilers in the high MCP (1.28 g/day) and TCP (1.51 g/day) groups showed a higher daily retention of P than the low MCP (0.88 g/day) group.

Table 6.7. Effect of MCP and TCP on P retention, Ca and P digestibility (%) and Ca and P retention (g/day) in 35-day-old broilers (experiment 2).

	P retention coefficient (%)		Ca retention coefficient (%)		P retention (g/day)		Ca retention (g/day)	
	d 19- 21	**d 33-35**	**d 19- 21**	**d 33-35**	**d 19- 21**	**d 33-35**	**d 19- 21**	**d 33-35**
Low MCP[1]	59.46[a]	55.43	35.24	37.05	0.64	0.88[b]	0.63	1.24
High MCP	50.66[ab]	50.16	28.19	38.65	0.73	1.28[a]	0.56	1.73
High TCP[2]	44.57[b]	53.07	29.08	37.96	0.60	1.51[a]	0.56	1.66
SEM[3]	3.05	1.88	4.52	4.06	0.05	0.09	0.08	0.20
P value[4]	0.005	0.166	0.50	0.962	0.252	0.0003	0.811	0.199

[1] Mono-calcium phosphate; [2] Tri-calcium phosphate; [3]SEM: Standard error of the mean.; [4] [a,b] Values in the same column with different letters are significantly different (P<0.05).

6.5. Discussion

Our study focused on the comparison of P availability from either MCP, DCP or TCP sources. In addition, our study focused on the hypothesis of existing differences among a number of DCP sources. Results showed that MCP, DCP and TCP are highly available P sources; and no differences were observed among P sources with respect to productive performance and bone mineralization of broiler chicken at ages of 21 and 35 days. Lima *et al.*, (1997) showed the same results on broiler performance when they evaluated seven di-calcium phosphate sources from different origins. In the same way, Shastak *et al.*, (2012) didn't find significant effects of the P source on feed intake and BW gain for broilers at 3 and 5 weeks of age, when comparing the effects of mono-sodium phosphate and di-calcium phosphate anhydrous. In contrast, Gillis *et al.*, (1962) reported higher P availability in purified grade mono-calcium phosphate than in di-calcium phosphate. The authors suggested the reason for this was the difference in hydration degree of P source. Specifically, P in the anhydrous di-calcium phosphate

form is less available for poultry than the hydrated salt. It is noteworthy to describe that during the DCP manufacturing, conditions including temperature are responsible for the formation of the dihydrate or anhydrate product. Grimbergen *et al.*, (1985) showed that the growth response was lower when anhydrous-DCP was included in the diet as compared to MCP or hydrated-DCP. In addition, Grimbergen *et al.* (1985), didn´t detect any difference in the growth response between MCP and hydrated DCP. According to Rucker *et al.*, (1968), the dihydrate form of DCP dissolved more rapidly in an acid environment than the anhydrous form. In addition, Lima *et al.*, (1997) also suggested that the particle size of phosphate may also affect P availability. Specifically, the larger particle size phosphate sources are retained longer in the gizzard than smaller particles, likely allowing a higher solubility and availability (Brunell *et al.*, 1990; Lima *et al.*, 1997). This criterion of particle size was not registered or evaluated in this study.

No significant difference was observed between high MCP and TCP in animal performance and bone mineralization. However, Wilcox *et al.*, (1954) reported that the P in tri-calcium phosphate was poorly utilized by the turkey poults. The results of these two experiments demonstrated a significant effect of NPP level in animal performance. Lower animal BW gain and feed intake were observed for animals fed diets with low levels of NPP (3.0 to 3.5 g/kg) as compared to 4.0 and 4.5 g /kg at d 21 and 35. However, increasing NPP from 4.0 to 4.5 g /kg increased both the tibial weight and tibial ash content (mg/tibia). Hamdi *et al.*, (2015a) concluded that a level of 3.8 g NPP /kg improved the growth of chicks and increased bone mineralization on d 14, but no further increases were observed with 4.5 g NPP /kg in diets including the addition of high levels of phytase. The lower required levels observed for this experiment as compared to the actual results may be due to the effect of phytase inclusion in the diet and also the duration of the experiment. According to Applegate *et al.* (2008), typical removal amounts of P for 500 FTU of phytase in the diets can vary from 0.06% to 0.10% for broilers. In our experiment, no differences in animal performance were observed between the broilers fed the 4.0 and 4.5 g NPP /kg diets. However, increasing the NPP from 4.0 to 4.5 g/kg increased tibial weight and tibial ash (mg/tibia). Yan *et al.*, (2001) also reported that NPP requirements for BW gain and feed conversion were considerably less than required for tibial ash for broilers of 3 to 6 weeks of age in the absence of phytase. They suggested NPP levels of 0.330%; 0.186% and 0.163% to optimize tibial ash, BW gain and G: F ratio, when no phytase was included in the diet.

However, with 800 FTU phytase diets, the suggested NPP were lowered to 0.240%; 0.151% and 0.109% respectively in order to optimize tibia ash, BW Gain and BW G: F ratio.

Increasing the P level in the diet decreased P retention (%) in experiment 2. However, the results were not affected by the P source (MCP, TCP). This result is consistent with Leske and Coon (2002) who demonstrated that the retention of P from different P sources depends on the amount of the inorganic P included. They found that P retention from MCP declined from 98% to 59% when NPP was increased from 1.6 to 4.5 g/kg. Wasserman and Taylor (1973) suggested the existence of a saturable component in P absorption, which can be responsible for the decrease in P absorption in the intestine. The use of TCP for young broilers decreased the P retention. However, no difference was observed between high MCP and TCP in the grower phase. Hamdi *et al.*, (2015b) showed that the supplementation of broilers diet with TCP, improved FI and WG at d 14 but ileal digestibility of Ca and P was lower for diets including TCP and limestone as compared to diets including Ca chloride, mono-calcium phosphate and sodium phosphate. This difference could be associated with the solubility of the P sources in birds of different ages.

Despite the highly different physical structure and chemical properties among P sources, the results observed in the present study did not provide evidence which suggests that there are any differences in the in vivo availability of P in young broilers. Higher NPP levels in the broiler diets are required to optimize bone mineralization than to optimize growth performance.

CHAPTER 7

Effect of microbial phytases and the dietary calcium and phosphorus level on the productive performance, and bone mineralization of Broilers

Effect of microbial phytases and the dietary calcium and phosphorus level on the productive performance, and bone mineralization of Broilers

M. Hamdi [1], D. Solà-Oriol[1], R. Franco-Rosselló [1], R. Aligue [2], and J.F. Pérez [1]

[1]Animal Nutrition and Welfare Service (SNiBA). Departament de Ciència Animal i dels Aliments. Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain.

[2]Department of Biomedical Sciences. University of Barcelona, IDIBAPS, 08036 Barcelona, Spain.

Submitted to *British Poultry Science*

7.1. Abstract

1. Two trials were conducted to evaluate the effect of phytase and different levels of Ca in the diet limited in available P on performance, bone mineralization, and Ca and P retention of broilers from d 1 to d 35 of age.

2. In Trial 1, 160 one-d-old male broilers were placed into 40 cages and allocated to 5 corn-wheat-soybean meal-based dietary treatments, which were a positive control [PC, 4.7 and 4.3 g/kg non-phytate P (NPP) and 9.0 and 8.5 g/kg Ca, from 1 to 21 d, and from 21 to 35 d of age, respectively] and 4 negative control [NC, 3.2 and 2.9 g/kg non-phytate P (NPP) and 7.0 and 6.3 g/kg Ca for the starter and grower diet] groups consisting of 4 concentrations of phytase (0, 250, 500 and 1,000 FTU/kg). In trial 2, 300 one-day-old broilers were place into 60 cages and allocated to 10 corn-wheat-soybean meal-based dietary treatments, which were two NC diets (3.2 g/kg NPP) with 7.0 g/kg Ca (Low Ca) or 9.0 g/kg Ca (High Ca), respectively, which were supplemented (0 or 500 FTU/kg) with 4 different commercial phytases (phytase A, B, C and D) in a 2 x 5 factorial arrangement from 1 to 14 d of age.

3. In trial 1, PC and the high dose of phytase (1,000 FTU/kg) had significantly higher average daily gain from 1 to 21 d of age, and a higher tibia weight and tibia ash content than the NC, 250, 500 FTU/kg groups. In trial 2, the low Ca diets improved feed efficiency from d 1 to 7, ileal digestibility of Ca and P, and bone mineralization on d 14. Supplementation with any of the 4 phytase increased bone mineralization at 14 d of age, but not differences were observed on performance.

5. Briefly, this research showed that high levels of phytase (1,000 FTU/kg) may be required to promote performance and bone mineralization responses in growing broilers. The results showed that lowering Ca:aP ratio may have a higher effect on performance and P ileum digestibility than low levels (500 FTU/kg) of phytase. The results don´t confirm the detrimental effect of high dietary Ca on phytase activity and subsequent growth and bone performance of starting broilers (d 14). In contrast, lowering Ca:aP ratio and including phytase (500 FTU/kg) improved bone mineralization in an additive way.

7.2. Introduction

Phosphorus (P) content of grain-based feeds is in general sufficient to support adequate animal growth and bone development. However, vegetable ingredients mostly contain P in phytate molecules and thus low available to birds. Commercial phytases are phosphatase enzymes from fungal or bacterial origin which are obtained from genetically modified organisms and capable to release organic bound phosphate. They allow significant reductions on the supplementation of feed with calcium phosphate, and consequently a decrease on total P excretion. Phytases used for animal feed application differ on their enzymatic properties, such as the position of the phosphate group they hydrolyze first, on how many phosphates are able to remove, their pH profile (Selle *et al*., 2000), stability under digestive tract conditions (ie. pH stability or pepsin cleavage), kinetic constants, or substrate specificity (Meneze-Blackburn *et al.*, 2015). The pH profile of supplementary phytases is especially important because generally determine their ability to develop catalytic activity in the gastrointestinal compartments (high in the acid conditions of crop, gizzard and proventriculus, and negligible in small intestine). Some enzymes may exhibit a higher or lower activity at pH 3 than pH 5.5. However the bio-efficacy of supplementary phytase is standardized at defined conditions (pH 5.5, 37 ºC, and 5 mmol/L sodium phytate). Therefore, it is virtually impossible to compare the different commercialized phytase products by the properties reported by their producers. Although there have been efforts to compare the biochemical properties of commercially available phytase products in *in vitro* simulations of the digestive tract (Menezes-Blackburn *et al.*, 2015), their comparative biological efficacy depends on the conjunction of all properties, and can be fully determined only by direct feeding trials (Chung *et al.*, 2013).

Phytases are now extensively used in most poultry diets due to the declining inclusion costs of phytases coupled with the increasing prices of mineral phosphorus. In general, supplementation of animal feed with 250-1000 FTU is recommended by the phytase suppliers but, phytase overdoses are also evaluated in order to maximize phytate P utilization (Walk *et al.*, 2014). However, the capacity of birds to utilize phytate-P is limited, and may depend on the level of exogenous phytase, but also on the large number of digestive interactions evolving along the gastrointestinal tract of broilers among Ca, non-phytate P (NPP) and phytase (Akter *et al.*, 2016). Calcium has the capacity to interact with inorganic P in the gut (Hurwitz and Bar, 1971; Plumstead *et*

al., 2008) as well as to form a mineral-phytate complex in pH higher than 5.0 (Taylor, 1965; Lei *et al*., 1994). It has been reported that the incorporation of high levels of limestone in the diet as Ca source may limit the capacity of exogenous phytases to hydrolyze phytate (Qian *et al*., 1996; Korenegay, 1999), and decrease performance and bone mineralization in broilers (Sebastian *et al.*, 1996; Hamdi *et al*., 2015). In contrast, Powell *et al.*, (2011) reported that increasing the dietary Ca level from 6.7 to 13.3 g/kg had no effect on phytase activity. In practice, it has been suggested that reducing the levels of Ca (Driver *et al*., 2005; Rama-Rao *et al*., 2006) and giving high doses of phytase (Selle and Ravindran, 2007) may help to increase P availability of low NPP diets, performance, and bone mineralization in broilers. However, the reported magnitude, additivity or synergy of the birds´ response to changes on the level of dietary Ca and phytase supplementation has not been consistent. In a recent report, Akter *et al.*, (2016) confirmed the detrimental effect of high dietary Ca on performance and bone mineralization in broilers up to d 24 of age; as well as the positive bird's response of supplying 500 phytase units or 4 g NPP/kg as compared to 3 g NPP/kg. However, a Ca x NPP x phytase interaction was observed for the feed intake, performance, and bone mineralization. The greatest response to phytase was observed in High Ca-Low NPP diets, and with Low Ca-High NPP diets, which it likely indicates phytase is active to release digestible P and digestible Ca from the mineral-phytate complex, respectively.

In this context, the present study was conducted through two trials designed to determine the efficacy of increasing levels of a new microbial 3-phytase (FLF® 1000 FUT, Fertinagro, Spain) in a low NPP diet, and to explore the interactions of the ratio of Ca:aP on phytase activity and its impact on broilers performance and bone mineralization.

7.3. Materials and methods

7.3.1. Animal ethics

All animal experimentation procedures used in the 2 experiments were approved by the animal Ethics Committee of the Universitat Autònoma de Barcelona and were in compliance with the European Union guidelines for the care and use of animals in research (European Parliament, 2010).

7.3.2. Phytases

Phytase A: is a preparation of 6-phytase produced by a genetically modified strain of Pichia pastoris (OptiPhos® 5000 CT); **Phytase B**: bacterial 6-phytase is sourced from an E.coli species bacterium and is expressed in a Saccharomyces pombe (Phyzyme® XP 10000 TPT); **Phytase C**: is a preparation of 6-phytase fungi produced by a genetically modified strain of Aspergillus oryzae (Ronozyme® NP), and **Phytase D**: an exogenous microbial 3-phytase produced by Pichia pastoris by the expression of a cloned gene from Serratia odorifera FLF® 1000 FUT.

7.3.3. Dietary treatments

7.3.3.1. Experiment 1

The experiment was conducted with 5 dietary treatments. Diets were formulated to be iso-energetic and iso-nitrogenous to meet or exceeded the nutrient requirements for broilers (FEDNA, 2008), with the exception of Ca and available P in the negative control and phytase-supplemented diets. The positive control diet (PC), without supplemental phytase, was formulated to be adequate in energy and all nutrients, including NPP (4.7 g/kg for the starter phase and 4.3 g/kg in the growth phase) and Ca (9.0 g/Kg in the starter phase and 8.5 g/Kg in the growth phase). The negative control diet (NC), without supplemental phytase, was formulated to be adequate in nutrients and energy content, while it was deficient in NPP (3.2 g/Kg for the starter phase and 2.9 g/Kg in the growth phase) and Ca (7.0 g/Kg for the starter phase and 6.3 g/Kg the growth phase). The rest of the diets were prepared by adding four levels of exogenous phytase D (0, 250, 500, and 1,000 FTU/kg). The ingredient composition and nutrient specifications of diets are presented in Table 7.1. Phytase was mixed in premix and then into the diets. Titanium dioxide (5 g/kg) was added to the diets as indigestible marker for nutrient digestibility analysis. All the diets were fed in mash form as starter (1 - 21d) and grower (21- 35 d). Feed was provided ad libitum and water was freely available.

Table 7.1. Composition of the basal diet for starter and Grower phase for broilers from 1 to 35 days (Experiment 1).

	Starter		Grower	
	NC[1]	PC[2]	NC	PC
Ingredients, g/kg diet				
Corn	204.3	186.6	250.0	250.0
Wheat	350.0	350.0	385.0	363.8
Soybean meal	210.2	213.6	140.6	146.4
Extruded full-fat soybean	150.0	150.0	180.0	180.0
L-Lysine	3.4	3.3	2.8	2.7
DL-Methionine	3.1	3.1	2.6	2.6
L-Threonine	1.2	1.2	0.9	0.9
Limestone	7.7	8.1	7.4	8.2
Soy oil	51.6	57.5	14.5	21.1
Di-calcium Phosphate	10.7	18.8	8.7	16.9
Salt	2.8	2.8	2.7	2.7
Premix*	3.0	3.0	3.0	3.0
Sodium bicarbonate	2.0	1.9	1.7	1.7
Calculated composition, g/kg diet				
DM	889	890	883	885
ME, Kcal/kg	3,000	3,000	3,100	3,100
CP	210	210	197	197
Ca	7.0	9.0	6.3	8.5
Total P	5.8	7.1	5.4	6.8
Available P	3.0	4.2	2.7	3.9
PP	2.5	2.5	2.5	2.5
NPP	3.2	4.7	2.9	4.3
Ca:aP	2.33	2.14	2.33	2.18
Analyzed composition, g/kg diet				
DM	893	895	892	899
CP	204	205	197	200
Ca	7.8	10.0	8.6	10.0
Total P	6.0	8.0	6.2	8.0

(*) Provided per kg of feed: Vitamin A (retinil acetate) 10000UI; Vitamin D (Vitamin D3) (Cholecalciferol) 2000UI; Vitamin D (25- hidroxicolecalciferol. 25 mcg equivalent at 1000 UI) 1500UI; Vitamin E/acetate de tot-rac-3-tocopheril) 75mg;Vitamin B1 (Tiamin mononitrate) (2 mg) ; Vitamin B2 (Riboflavin) (5 mg) ; Vitamin B6 (Piridoxin Chlorhidrate) (4 mg; Vitamin B12 (cyanocobalamine) 0.015 mg; Nicotinic Acid (Nicotinic Acid) (Niacin) 25 mg ; Pantotenic Acid (Calcium D-pantotenate) 10 mg; Biotin (D-(+)-biotin) (0.15 mg); Folic Acid (1 mg); Iron ($FeSO_4$) (46 mg); Zinc (ZnO) 125 mg; Manganese (MnO) 150 mg; Iodine ($Ca(IO_3)_2$) 2 mg; Selenium (Na_2SeO_3) 0.3 mg; Cobalt (Co, $CoCO_3$) 0.5 mg; Copper ($CuSO_4$) 20 mg; DL-Methionin 500 mg; Etoxiquin (0.1332 mg); Endo-1,3(4)-betaglucanase EC 3.2.1.6 (10 FBG); Endo-1,4-beta-xylanase EC 3.2.1.8 (150 FXU); Malic acid (60 mg); Fumaric acid (75 mg); Sepiolite (400 mg); Calcium Carbonate (4 g).

[1]Negative control; [2]Positive control.

7.3.3.2. Experiment 2

A single diet was offered to the broilers during the whole experimental phase (d 1 to d14). Diets were formulated and prepared to contain adequate levels of nutrients (Table 2) to meet and exceed nutritional requirements except for calcium, total and NPP (FEDNA, 2008). Two basal diets (NC) were prepared (one with a high Ca:aP ratio and the other with a low Ca:aP ratio), which were supplemented with (0 or 500 FTU/kg)

from 4 different commercial phytases (phytase A, B, C and D) in a 2 x 5 factorial arrangement from d 1 to 14 d of age. The ingredient composition and nutrient specifications of diets are presented in Table 7.2. To formulate the precise phytase activities in feed, the commercial products were previously analyzed for their enzymatic activities (pH 5.5, 37 ºC, and 5 mmol/L sodium phytate) and the doses to be included were calculated to reach 500 FTU/kg. All of the diets were presented in mash form. Diets were supplemented with titanium dioxide (TiO_2) as an indigestible marker. Feed was provided ad libitum and water was freely available.

Table 7.2. Composition of the basal diet for broilers from 1 to 14 days (Experiment 2).

Treatment	Low Ca:aP	High Ca:aP
Ingredients, g/kg diet		
Corn	204.3	193.4
Wheat	350.0	350.0
Soybean meal	210.2	212.3
Extruded full-fat soybean	150.0	150.0
L-Lysine	3.4	3.4
DL-Methionine	3.1	3.1
L-Threonine	1.2	1.2
Limestone	7.7	12.9
Soybean oil	51.6	55.2
Di-calcium Phosphate	10.7	10.7
Salt	2.8	2.8
Premix*	3.0	3.0
Sodium bicarbonate	2.0	1.9
Calculated composition, g/kg diet		
DM	888.5	880.7
ME, (Kcal/kg)	3,000	3,000
CP	210.0	210.0
Ca	7.0	9.0
Total P	5.8	5.7
Available P	3.0	3.0
PP	2.5	2.5
NPP	3.3	3.2
Ca:aP	2.33	3.00
Analyzed composition , g/kg diet		
DM	894.0	895.0
CP	201.0	201.0
Ca	8.5	9.4
Total P	6.3	6.8

(*)Provided per kg of feed: Vitamin A (retinil acetate) 10000UI; Vitamin D (Vitamin D3) (Cholecalciferol) 2000UI; Vitamin D (25- hidroxicolecalciferol. 25 mcg equivalent at 1000 UI) 1500UI; Vitamin E/acetate de tot-rac-3-tocopheril) 75mg;Vitamin B1 (Tiamin mononitrate) (2 mg) ; Vitamin B2 (Riboflavin) (5 mg) ; Vitamin B6 (Piridoxin Chlorhidrate) (4 mg; Vitamin B12 (cyanocobalamine) 0.015 mg; Nicotinic Acid (Nicotinic Acid) (Niacin) 25 mg ; Pantotenic Acid (Calcium D-pantotenate) 10 mg; Biotin (D-(+)-biotin) (0.15 mg); Folic Acid (1 mg); Iron ($FeSO_4$) (46 mg); Zinc (ZnO) 125 mg; Manganese (MnO) 150 mg; Iodine ($Ca(IO_3)_2$) 2 mg; Selenium (Na_2SeO_3) 0.3 mg; Cobalt (Co, $CoCO_3$) 0.5 mg; Copper ($CuSO_4$) 20 mg; DL-Methionin 500 mg; Etoxiquin (0.1332 mg); Endo-1,3(4)-betaglucanase EC 3.2.1.6 (10 FBG); Endo-1,4-beta-xylanase EC 3.2.1.8 (150 FXU); Malic acid (60 mg); Fumaric acid (75 mg); Sepiolite (400 mg); Calcium Carbonate (4 g).

7.3.4. Management of birds

7.3.4.1. Experiment 1

A total of 160 one-day-old *Cobb 500* male broilers were obtained from a commercial hatchery. The birds were individually weighed and distributed to 40 cages with 4 chicks each in a 3 floor battery brooder unit located in the same environmental controlled room. The five dietary treatments were replicated 8 times. The brooder temperature was maintained at 35ºC from d 1 to d 4 post-hatch, and was progressively reduced to 25ºC from d 14 to d 35. The light cycle was 24h/d from d 1 to d 2, 23h/d from d 3 to d 10, and 18h/day from d 11 to d 35.

7.3.4.2. Experiment 2

A total of 300 one-day-old *Cobb* 500 broiler male chickens were individually weighed and randomly distributed into 10 experimental groups with 6 replicate cages and 5 birds per replicate according to the experimental treatment. These birds share the same light cycle protocol. The brooder temperature was maintained at a temperature of 35ºC from d 1 to d 4 post-hatch, and was progressively reduced to 25ºC on d 14.

7.3.5. Sample collection and processing

7.3.5.1. Experiment 1

Birds were individually wing-tagged in order to monitor individual BW as well as the group BW at the start (d1) and d 7, 14, 21, 28 and 35 post-hatch. From these values the feed intake (FI), weight gain (WG), and gain: feed ratio (G: F) from d 1 to d 21 and from d 21 to d 35 were calculated. On d 35, three birds were euthanized by cervical dislocation, and the left tibia was collected for bone-ash determination, ileal samples were collected to determine P ileal digestibility and total excreta were also collected from d 33 to 35 d to determine P retention.

7.3.5.2. Experiment 2

Birds were individually wing-tagged in order to monitor individual BW as well as the group BW at the start (d1) and d 7 and 14 post-hatch. From these values the feed intake (FI), weight gain (WG), and gain: feed ratio (G: F) from d 1 to d 7 and from d 7 to d 14 were calculated. At the end of the experiment (d 14), the chicks were euthanized by using the cervical dislocation. Left tibia bones of three animals per cage were collected for bone-ash determination and ileal samples were also collected to determine Ca and P ileal digestibility.

7.3.6. Laboratory analyses

Representative samples of diets, ileal digesta content and excreta were analysed. Analytical determinations of feeds were performed according to the methods of AOAC International (2005): dry matter (Method 934.01), crude protein with Dumas Method (Method 968.06).

Diets, excreta samples and ileal digesta contents were digested in nitric perchloric and fluorhydric acid mixture and subsequently concentration of P, Ca and Ti were determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES) using optical emission spectrometer optimal 4300DV Perkin-Elmer.

Apparent ileal digestibility coefficients of Ca and P (%) were calculated using the titanium ratio in the diet and digesta:

$$\textbf{Ileal Ca or P Digestibility} = 1 - ([Ti]_D/[N]_D/[Ti]_M/[N]_M)$$

where $[Ti]_D$ is the Ti concentration of in the diet, $[N]_D$ is the Ca or P concentration in the diet, $[Ti]_M$ is the concentration of Ti in the ileal digesta and $[N]_M$ is the Ca or P content in the ileal digesta.

In feed phytase activity (FTU/Kg) was determined with international standard method of animal feeding stuffs-determination of phytase activity (ISO 30024).

Bone mineralization was determined by the procedure of Brenes *et al.*, (2003). The tibias were defatted by a 48h extraction in ethyl ether. They were then dried for 12h at 110ºC and then ashed overnight at 550ºC to determine ash content.

7.3.7. Statistical Analyses

Data were analyzed as a completely randomized design using the GLM procedure of SAS software, version 9.2. The statistical model in experiment 1 included the NC, PC and the level of phytase inclusion in the diet. In experiment 2, data were analyzed as a 5 x 2 factorial arrangement and included the Ca level, source of phytase and their interaction. The pen of 5 chicks was considered as the experimental unit. The results are presented as least square means. Probability was considered significant when $P \leq 0.05$.

7.4. Results

7.4.1. Experiment 1

7.4.1.1. Growth performance

Average body weight (BW), feed intake (FI), weight gain (WG) and Gain:Feed ratio (G:F) along the experimental period are presented in Table 7.3. Male broiler receiving the PC diet showed a slightly lower performance than the Cobb standards on d 35 (2,139 g as compared to 2,191). Higher BW and weight gain was observed ($P < 0.05$) for birds of the PC diet and 1,000 FTU/kg diet as compared to those birds of the NC supplemented with lower level of phytase (250 FTU/kg and 500 FTU/kg), on d 21 and 35.

Table 7.3. Effect of different levels of dietary phytase on feed intake and growth performance of broilers from d1 to 35[1] (Experiment 1).

Treatment	BW[2](g)		FI[3] (g/d)		WG[4] (g/d)		G: F[5]	
	d 21	d 35	d1-21	d 21-35	d1-21	d 21-35	d1-21	d 21-35
NC[6]	810^{ab}	2055^{ab}	50.4^{ab}	138.7^{bc}	36.7^{ab}	88.9	0.730	0.641
NC+250 FTU/kg	779^{b}	2030^{b}	46.5^{c}	137.2^{c}	35.1^{b}	89.4	0.756	0.652
NC+500 FTU/kg	771^{b}	1998^{b}	48.0^{bc}	136.7^{c}	34.8^{b}	87.6	0.727	0.641
NC+1,000FTU/kg	842^{a}	2108^{a}	49.5^{abc}	143.9^{ab}	38.2^{a}	90.4	0.770	0.628
PC[7]	842^{a}	2139^{a}	51.7^{a}	148.6^{a}	38.2^{a}	92.6	0.737	0.624
SEM [8]	20.6	32.4	1.23	2.15	0.98	1.70	0.0127	0.0093
P value [9]	0.047	0.026	0.041	0.001	0.047	0.315	0.096	0.227

[1] Data are means of 8 pens with 4 chicks each. [2] Body weight. [3] Average daily feed intake. [4]Average daily weight gain. [5] Gain:Feed ratio. [6] NC, Negative control. [7]PC, Positive control [8] SEM: Standard error of the mean. [9] a,b,c Values in the same column with different letters are significantly different (P<0.05).

7.4.1.2. Bone mineralization and Mineral retention

The effect of phytase supplementation on bone mineralization (tibia weight, and ash content) and P ileum digestibility on d 35 and P retention from d 33 to 35 is reported in Table 7.4. Higher tibia weights were observed for the PC and the 1,000 FTU treatments than the NC, 250 FTU/kg, and 500 FTU/kg treatments. A dose response ($P < 0.05$) was observed for the relative contribution of tibia weight to total BW. Birds of the PC and 1,000 FTU groups showed also higher tibia ash (g/tibia) content than birds of the NC, 250 FTU/kg, and 500 FTU/kg treatments. However, no differences were observed among treatments in terms of tibia ash concentration (%). A higher P retention (P< 0.001) was observed for animals fed diets with phytase in comparison with PC and NC diets.

Table 7.4. Effect of different levels of dietary phytase on bone mineralization and P digestibility of 35-day-old broilers[1] (Experiment 1).

Treatment	**Tibia weight, g**	**Tibia weight, %/BW**	**Tibia ash, %**	**Tibia ash, g/tibia**	**P retention, (%), 33 - 35 d**	**P ileal digestibility (%)**
NC[2]	4.37[b]	0.208[c]	51.5	2.24[b]	53.0[b]	49.9
NC+250FTU	4.34[b]	0.213[bc]	51.4	2.23[b]	58.4[a]	54.2
NC+500FTU	4.35[b]	0.218[ab]	51.5	2.20[b]	58.9[a]	53.4
NC+1,000FTU	4.77[a]	0.224[a]	51.6	2.47[a]	57.7[a]	55.7
PC[2]	4.82[a]	0.225[a]	52.2	2.52[a]	46.0[c]	50.3
SEM [4]	0.104	0.0035	0.35	0.059	0.84	2.40
P value [5]	0.002	0.006	0.490	0.0005	<0.001	0.283

[1] Data are means of 8 pens with 4 chicks each. [2] NC, Negative control. [3]PC, Positive control. [4] SEM: Standard error of the mean. [5 a,b,c] Values in the same column with different letters are significantly different (P<0.05).

7.4.2. Experiment 2

7.4.2.1. Growth Performance

Average body weight (BW), weight gain (WG), feed intake (FI) and gain:feed ratio (G:F) along the experimental period are presented in Table 7.5. No different BW, WG and FI was observed among the experimental treatments due to the source of phytase or the interaction between the dietary Ca:aP ratio and phytase source (P > 0.05).

However, a tendency to an interaction Ca x Phytase was observed on the weight gain from d 7 to 14 of age, with broilers of the high Ca diets showing a higher response to phytase. Birds fed a diet with a low Ca:aP ratio showed greater BW ($P < 0.1$) and gain:feed efficiency ($P < 0.01$) on d 14 than broilers fed diets with a high Ca:aP ratio.

Table 7.5. Influence of Ca: aP Ratio and types of phytase on feed intake and growth performance of broilers from d 1 to 14[1](Experiment 2).

Treatment		BW[2](g)		FI[3](g/d)		WG[4] (g/d)		G:F[5]	
		d7	d14	d1-7	d7 -14	d1-7	d7 -14	d1-7	d7 -14
Ca:aP									
High	NC	146	371	16.5	41.8	14.8	32.2	0.896	0.771
	A	146	388	16.4	45.8	14.7	34.6	0.895	0.756
	B	153	395	17.5	44.5	15.7	34.5	0.901	0.777
	C	147	387	16.2	43.9	14.7	34.3	0.911	0.783
	D	147	392	16.2	45.8	14.8	35.1	0.915	0.766
Low	NC	149	403	16.2	45.4	15.1	36.3	0.936	0.800
	A	150	391	16.8	44.3	15.2	34.4	0.908	0.776
	B	154	404	16.9	45.1	15.9	35.7	0.945	0.793
	C	147	386	15.8	45.4	14.9	34.1	0.942	0.753
	D	154	396	16.7	45.2	15.9	34.6	0.951	0.765
Ca:aP	High	148	387	16.6	44.4	14.9	34.1	0.904	0.771
	Low	151	396	16.5	45.1	15.4	35	0.936	0.777
Phytases	NC[6]	148	387	16.3	43.6	14.9	34.2	0.916	0.785
	A	148	389	16.6	45.1	14.9	34.5	0.902	0.766
	B	154	399	17.2	44.8	15.8	35.1	0.923	0.785
	C	147	386	16	44.6	14.8	34.2	0.927	0.768
	D	151	394	16.4	45.5	15.4	34.8	0.933	0.766
SEM [7]		4.6	7.6	0.7	1.04	0.65	0.9	0.0178	0.019
P value									
Ca:aP		0.288	0.059	0.84	0.29	0.267	0.128	0.006	0.585
Phytases		0.55	0.412	0.566	0.473	0.534	0.848	0.472	0.677
Ca:aP × phytases		0.931	0.227	0.904	0.137	0.936	0.072	0.92	0.578

[1] Data are means of 6 pens with 5 chicks each; [2] Body weight; [3]Average daily feed intake; [4] Average daily weight gain. [5] Gain:Feed; [6] Negative control, without phytase; [7] SEM: Standard error of the mean.

7.4.2.2. Bone Mineralization and Mineral Retention

Table 7.6 shows the effects of the Ca:aP ratio and phytase source on bone mineralization (tibia weight and tibia ash content) of broilers on d 14 of age, and ileal Ca and P digestibility. The tibia ash weight was greater with low Ca:aP ratio diets as compared to high Ca:aP ratios. The incorporation of phytase increased the tibia ash weight as compared to the NC groups. However, no differences were observed among phytases or on the interaction between Ca and phytase supplementation.

Low Ca:aP ratio diets resulted in an increase of ($P < 0.05$) ileal digestibility for Ca and P as compared to diets with high Ca:aP ratios. No significant effects were

observed associated to phytase source or the interaction between the Ca:aP ratio and phytase supplemented sources.

7.5. Discussion

The broiler response to phytase and NPP

It is noteworthy that growth performance of broilers in experiment 1 was lower than the breed standards for Cobb broilers (Cobb, 2015), and small differences on performance were observed between NC and PC groups. These responses were likely due to the birds being housed in cages rather than in floor pens, and in conditions which could have limited bird response to the studied dietary factors.

However, there was an effect of dietary phytase on growth performance and bone mineralization when phytase was added at 1,000 FTU/kg but, not at lower doses. Other authors have also reported a dose response to the release of P from 31% to 58% (Denbow *et al.*, 1995) and from 14% to 37% (Yi *et al.*, 1996) by the addition of phytase from 250 FTU/kg to 1,000 FTU/kg. Until recently, a standard phytase dose (500 FTU) aimed to release 0.15% P (0.12% digestible P for poultry) and a maximum phytase destruction of 50%-70% (Masey O'Neil *et al.*, 2014). However, nowadays, a new phytase generation and very high doses (three to four times the standard dose) aim to attain up to a 90% destruction of phytate (González-Ortiz *et al.*, 2015).

In our trial, a similar response was observed with the novel dietary phytase (1,000 FTU) and the PC (with a 0.18-0.2% higher analyzed total P content) on BW gain, tibia weight and P retention. However, feed efficiency was not altered by the dietary phytase or by the supplementation with inorganic P (positive Control). Similar response on performance and bone mineralization between both treatments could indicate an increase on digestible P with the high dose of phytase as reported by Ravindran *et al.*, (1995). Angel *et al.* (2001) also reported an equivalency or sparing effect of 0.09% NPP, when using mono-calcium phosphate as standard, for 500 FTU/kg of 3-phytase. Mitchell and Edwards (1996) reported that 600 FTU from the same phytase are equivalent to 0.20% inorganic P from dicalcium phosphate.

Weight gain was also increased (24.2%) and feed efficiency improved (5.9%) in young broiler chickens at 21 days of age fed diets supplemented with 1,000 FTU/kg (Olukosi *et al.* 2013). Previously, Cabahug *et al.* (1999) also reported that with lower

levels of phytase addition (400 FTU/kg and 800 FTU/kg) to 2.3g/kg NPP diets was enough for a weight gain increase of 18.8%, 9.0% of feed intake and 7.9% in feed efficiency in broiler chicks from d 7 to 25 of age. Phytase has been described to increase the apparent metabolizable energy of diets (Ravindran *et al.*, 2001) and the digestibility of the dietary protein (Ravindran *et al.*, 1999) while limited the negative impact of phytic acid, which can form complexes with proteins, cause a reduction in proteolytic digestion (Hill and Tyler, 1954) and increase endogenous losses (Selle *et al.*, 2007). It has recently reported that high levels of phytase inclusion promoted three to four points of improvement in the feed conversion rate (Walk *et al.*, 2014), probably due to the broke and reduction of phytic acid structure and the provision of inositol which is considered a growth promoter in broiler chickens. The present study doesn´t provide evidences of a liley extra-phosphoric effect, nor provide results about the digestibility of energy or protein. Further studies with specific objectives should clarify the quantitative contribution of phytase to a likely phosphoric an extra phospohoric effect.

Comparison among Phytases at Different Ca:aP ratio

Lower Ca:aP ratio diets increased bird performance and bone mineralization, which is in accordance with our previous study (Hamdi *et al.*, 2015), where young broilers fed diets with a medium level of Ca (7.9 g/kg) and 3.8 g/kg NPP performed better during the two first week of life than broilers with higher Ca levels (9.6 g/kg) did. It has been reported that calcium is able to form insoluble soaps with free fatty acids and bile acids, and there is some evidence that these soaps limit the absorption of fat in vivo (Gacs and Barltrop, 1977; Govers *et al.*, 1996; Shahkalili *et al.*, 2001). These soaps could lower the utilization of energy derived from lipids, particularly saturated fats, in broiler diets. Soluble Ca in the diet may also precipitate phytate by forming Ca-phytate complexes (Wise, 1983; Tamim *et al.*, 2004), and interact with inorganic P in the gut (Hurwitz and Bar, 1971), limiting mineral Ca and P absorption (Lonnerdal *et al.*, 1989). Soluble Ca may also increase intestinal pH and reduce mineral solubility and availability as reported by Shafey and McDonald (1991). Ca-phytate complexes are known to precipitate at pHs between 4 and 6, which coincides with pHs of the intestine where absorption of metal ions takes places (Tamim *et al.*, 2003). Even though Ca has one of the lowest affinities for phytate ($Cu^{2+} > Zn^{2+} > Co^{2+} > Mn^{2+} > Fe^{2+} > Ca^{2+}$; Vohra *et al.*, 1985), it may have the greatest impact, because of its high concentration in the diet (Tamim *et al.*, 2003). Increasing the level of Ca in broiler diets from 6 to 15 g/kg

reduced intestinal phytase activity by 75% (McGuaig *et al.*, 1972), as we hypothesized in this study. Applegate *et al.* (2003) reported that 0.90% dietary Ca reduced intestinal phytase activity by 9% and phytate P hydrolysis by 11.9%, compared with 0.40% Ca (Powell *et al.*, 2011). Ballam *et al.* (1985) found that total tract degradation of phytate by mucosal and microfloral phytases ranged from 0.06 to 0.57, depending on dietary levels of Ca and NPP. However, Powell *et al.*, (2011) reported that no effects were observed on phytase activity by increasing the dietary Ca level from 6.7 to 13.3 g/kg.

Table 7.6. Effect of different types of phytase and Ca:aP ratio inclusion on bone mineralization and mineral digestibility of 14-day-old broilers [1](Experiment 2).

		Tibia Weight, g	Tibia Weight, %/BW	Tibia ash, g/Tibia	Ca ileal digestibility, (%)	P ileal digestibility, (%)
Ca:aP						
High	NC	0.63	0.170	0.292	64.4	53.3
	A	0.67	0.173	0.322	61.8	52.7
	B	0.67	0.172	0.325	57.3	53.5
	C	0.67	0.174	0.319	58.9	54.2
	D	0.67	0.172	0.318	54.3	51.6
Low	NC	0.67	0.168	0.324	63.8	54.2
	A	0.67	0.173	0.332	64.3	61.2
	B	0.70	0.176	0.351	62.9	54.6
	C	0.68	0.172	0.337	62.6	54.7
	D	0.69	0.172	0.337	63.9	56.7
Ca:aP	High	0.66	0.172	0.315	59.4	53.1
	Low	0.68	0.172	0.336	63.5	56.3
Phytases	NC[2]	0.65	0.169	0.308[b]	64.1	53.8
	A	0.67	0.173	0.326[a]	63.1	57
	B	0.69	0.174	0.338[a]	60.1	54
	C	0.67	0.173	0.328[a]	60.8	54.5
	D	0.68	0.172	0.327[a]	59.1	54.2
SEM [3]		0.015	0.0030	0.0072	2.77	2.23
P value [4]						
Ca:aP		0.068	0.986	<.0001	0.015	0.037
Phytases		0.156	0.451	0.003	0.281	0.688
Ca:aP × phytases		0.626	0.906	0.635	0.385	0.409

[1] Data are means of 6 pens with 5 chicks each. [2] Negative control, without phytase. [3] SEM: Standard error of the mean. [4 a,b,c] Values in the same column with different letters are significantly different (P<0.05).

In the present study, no interactions were observed between the Ca:aP ratio and dietary phytase. In contrast, additive responses were achieved on bone mineralization after a supplementation with lower levels of Ca and a phytase supplementation. A likely explanation could be the short period of study and the small differences tested on the two levels of Ca (7.0 g/Kg and 9.0 g/Kg Ca). However, the nature of this relationship is

still unclear (Powell *et al.*, 2011). Some authors, such as Qian *et al.* (1997), reported that increasing Ca decreases microbial phytase activity and Sebastian *et al.* (1996) reported the best phytase efficacy when supplemented in corn-soy diets containing 0.6% Ca, as compared with those containing 1% Ca. In contrast, Driver *et al.* (2005) observed that growth and bone response to phytase was greatest with high Ca levels (from 0.38% to 0.98%), and these responses decreased when Ca was reduced and NPP was increased.

Powell *et al.* (2011) also concluded that the use of 6-Phytase increased growth and bone responses more as the levels of Ca were higher in the diet, indicating that high levels of dietary Ca (1.0% and 1.33%) did not have a negative impact on 6-phytase efficacy. Recently, Akter *et al.*, 2016 provided results which could rise the existence of a triple interaction, Ca (6, 8, 10 g/kg) x NPP (3, 4 g/kg) x phytase (0, 500 U). While with high Ca diets, phytase gave the highest response in low NPP diets (3 g NPP/kg), which it indicates an increase on P availability; the response in low Ca diets to phytase was higher with the high NPP content (4 g NPP/kg), which it likely indicates also a release of Ca from the mineral Ca complex. It could be suggested that although reducing dietary Ca may promote growth performance and P availability, such a reduction must be carried out with care given that Ca reduction could in turn limit bone mineralization. It has been assumed that 5.1 atoms Ca are bound by one phytate molecule (Nelson, 1984), then a complete hydrolyses of phytate (1% in the diet) may liberate up to 3.6 g/kg Ca, allowing for significant reductions in dietary Ca without influencing broiler performance and bone ash.

In our experiment, growth performance for broilers from d 1 to 14 were not influenced by the type of phytase; A, B, and C being 6-phytases, and phytase D was a 3-phytase. Four Phytase from bacterial or fungal origin were used in this study. On the basis of the carbon in the myo-inositol ring of phytate at which hydrolyses initiate, three commercial phytases were 6-phytase (A, B and C) and one was 3-phytase (D).

Differences among phytases were also declared in relation to pH range of optimal activity or temperature optima. However, not many studies are available in the literature comparing different types of phytases, in direct feeding studies. According to Payne *et al.* (2005), 6-phytases would completely dephosphorylate the phytate molecule, while 3-phytases would not do it due to their respective initiation sites (Wodzinski and Ullah, 1996). Thus, Sands *et al.* (2003) reported that while broilers fed diets supplemented with 250 FTU/kg of 6-phytase achieved similar BWG and FCE as

did those fed a PC diet, a higher dose of 3-phytase (750 FTU/kg) was required to achieve a similar response. However, Juin *et al.* (2001) reported no differences in body weight of male turkeys fed a 3- vs 6-phytase added at 250 and 500 FTU/Kg. Payne *et al.* (2005) also compared two commercial phytases, 3-phytase and 6-phytase supplemented from 100 to 300 FTU, and found that WG and FI increased linearly in broilers regardless the phytase source. We were not able to observe differences among phytase products at low levels of phytase (500 FTU/kg), however further studies should be performed to evaluate differences at higher concentrations, including overdoses of phytase in the diets.

It can be concluded that high levels of phytase (1,000 FTU/kg) may be required to enhance performance and bone mineralization in growing broilers fed on low NPP diets. The results showed that lowering Ca level may have a higher effect on performance and P ileum digestibility than low levels of phytase (500 FTU/kg). Consequently, caution is recommended to keep dietary Ca at moderate levels at least during the first weeks of growth. The results don´t confirm the detrimental effect of high dietary Ca:aP ratio on phytase activity and subsequent growth and bone performance of starting broilers (d 14). In contrast, lowering Ca levels and phytase (500 FTU/kg) improved bone mineralization in an additive way. Further studies should be performed in order to decide the best dietary levels incorporation of calcium and phytase in the diets to improve growth performance and bone mineralization in broilers.

CHAPTER 8

Including copper sulfate or dicopper oxide in the diet up to 300 mg Cu/kg affects performance and copper accumulation in broiler chickens

Including copper sulfate or dicopper oxide in the diet up to 300 mg Cu/kg affects performance and copper accumulation in broiler chickens

M. Hamdi[1], D. Solà-Oriol[1], R. Franco-Rosselló[1], S. Durosoy[2], A. Roméo[2] and J.F. Pérez[1]

[1]Animal Nutrition and Welfare Service (SNiBA). Departament de Ciència Animal i dels Aliments. Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

[2] ANIMINE, 74330 Sillingy, France

Submitted to *Poultry Science*

8.1. Abstract

Copper supplementation (125 to 250 ppm) in poultry diets is a common practice in many non-EU countries to enhance bird health, but high amounts of Cu may interact with phytate and affect animal performance. The effects could depend on the nature of the Cu source. Thus, the objective of this trial is to compare the effects of two sources of Cu, copper sulfate ($CuSO_4$) and dicopper oxide (Cu_2O, CoRouge®), at three levels of dietary Cu (15, 150, 300 ppm). A total of 576 one-day-old male broiler (*Ross* 308) were distributed into 6 experimental groups (8 pens/treatment, with 12 birds/pen). Body weight (BW) and feed intake (FI) were recorded weekly. On d 35 post-hatch, one bird per replicate was euthanized, the skin fat and breast muscle were sampled, and the liver and kidneys were collected. The two Cu sources were also evaluated *in vitro* to measure Cu and phytic phosphorus (PP) solubility, and PP hydrolysis by phytase at pH 2.5, 4.5 and 6.5. The use of 300 ppm of $CuSO_4$ decreased (P = 0.001) BW on d 14, 28 and 35 and increased (P = 0.04) liver Cu content in comparison with the use of 300 ppm of Cu_2O. The feed-conversion ratio increased for broilers of the 300 ppm $CuSO_4$ group in comparison to the 300 ppm Cu_2O group (2.19 vs. 1.84, P < 0.001). The use of the highest level of Cu (300 ppm), either of Cu_2O or $CuSO_4$, also increased (P < 0.001) Cu concentration in kidney and breast muscle in comparison, to 15 and 150 ppm. In the *in vitro* trial, including a level of 300 ppm of $CuSO_4$, reduced PP solubility (68.66%) in comparison to Cu_2O (97.41%), and reduced PP hydrolysis by phytase at pH 4.5 and 6.5 with both sources. It can be concluded that dietary levels of 150 and 300 ppm Cu of Cu_2O are adequate to ensure broiler growth performance and limit organ accumulation in comparison to $CuSO_4$.

8.2. Introduction

Broiler chickens need copper for iron transport and metabolism, red-blood-cell formation, enzyme-coenzyme catalytic reactions, immune and connective tissue maturation, especially in the cardiovascular system (Jegede *et al.*, 2011) and bones (Banks *et al.*, 2004a). Copper is also part of the linkage between elastin and collagen, which gives the bone its tensile strength (Carlton and Henderson, 1964). Cu requirements for broilers chickens at different ages were reported as being 5-8 mg/Kg according to NRC (1994) and 3-10 mg/Kg according to FEDNA (2008). In the European Union (EU), dietary copper is supplied for poultry up to a maximum of 25 mg Cu/Kg (EFSA, 2012). However, in other areas of the world, including USA, the poultry industry includes 125 ppm to 250 ppm Cu in the diets as growth promoters (Pesti and Bakalli, 1996). The mechanisms behind these effects are attributed to the bactericidal and bacteriostatic effects of Cu on the gastrointestinal tract´s microbiota (*Hawbaker et al.*, 1961; Bunch *et al.*, 1965; Pang and Applegate, 2007) and growth-promoting effects (Pesti and Bakalli, 1996). Copper has become especially useful since the use of antibiotics as growth promotors has been prohibited over the last 50 years.

However, therapeutic doses of copper, which are usually, included in poultry feeds as inorganic mineral salts (copper sulfate pentahydrate), are mostly excreted in the faeces and are a cause of environmental concerns. The high doses of Cu may also easily chelate phytate (Cheryan, 1980), the major storage form of phosphorus in plant seeds (Tamim and Angel, 2003). The solubility of these complexes depends on pH (Selle and Ravindran, 2007), the complexes being precipitated at pH 6.5 (approximate pH of intestine) and non-accessible for hydrolysis by phytase or absorption in the intestine (Pang and Applegate, 2006).

Copper sulfate pentahydrate ($CuSO_45H_2O$) is the most commonly used source of Cu as a dietary supplement for poultry (Pesti and Bakalli, 1996; Pang and Applegate, 2006). It is very soluble in both water and acidic solvents, and has normally been used as a reference point to compare the bio-availability of various Cu sources (Pang and Applegate, 2006). Other Cu sources are being used and considered for use by poultry producers. They have different relative bio-availability and solubility, so they might differently affect intestinal microbiota (Pang *et al.*, 2009) and PP hydrolysis (Banks *et al.*, 2004b). The term “copper oxide” is used to refer to either cupric oxide (CuO) or cuprous oxide (Cu_2O), both oxides occur in nature, but the industrial production of

Cu_2O requires an extra step of furnace reduction (The Merck Index, 1990; Aoyagi and Baker, 1993). Oxides of Cu are used to supply chicks feed because smaller inclusion rates are needed with CuO (80% Cu) and Cu_2O (89%Cu), as compared to $CuSO_45H_2O$ (25% Cu, Baker, 1991). There is a difference in copper bio-availability due to its valence form. Cupric oxide (CuO) has zero bio-availability, when compared with cuprous oxide (dicopper oxide; Cu_2O), which is 100% available in animals (Baker, 1999).

In the present study we have hypothesized that therapeutic doses of dicopper oxide can be included in the diet without affecting PP hydrolysis and broiler performance. Thus, the objective of the current work is to compare the effect of copper sulfate , the most commonly used Cu source for supplementation in poultry diet, and dicopper oxide (Cu_2O; CoRouge®) at 3 levels of dietary Cu (15 ppm, 150 ppm, 300 ppm) in the diets on broiler chicken performance, mineral interactions in the digesta, and mineral accumulation in organs and tissues. An *in vitro* trial has also been designed to compare the solubility of both sources and to identify likely interactions with the phytic phosphorus (PP) and phytase hydrolysis.

8.3. Materials and methods

The experimental products under study were two different Cu sources: Copper Sulfate ($CuSO_4$) containing 24.1% Cu, and Copper oxide (Cu_2O, CoRouge®, produced by ANIMINE) containing 75.4% Cu.

8.3.1. *In vivo* Trial

8.3.1.1. Bird Management and Husbandry

All animal experimentation procedures used in the experiments were approved by the animal Ethics Committee of the Universitat Autònoma de Barcelona and were in compliance with the European Union guidelines for the care and use of animals in research (European Parliament, 2010).

The study was carried out at a commercial growing poultry unit (Vila-rodona, Tarragona, Spain). The room was provided with 48 floor pens (4 lines of 12 pens each, divided by a central feeding aisle). A total of 576 one-day-old broiler male chickens (*Ross* 308) were randomly distributed into 6 experimental groups/treatments (8

pens/treatment, 12 birds/pen, 10.6 birds/m^2) according to initial body weight and continuously controlled over a period of 35 days.

Brooder temperature was maintained at 35ºC from d 1 to d 4 post-hatch, and was progressively reduced to 25ºC from d 14 to d 35. The light cycle was 24h/d from d 1 to d 2, 23h/d from d 3 to d 10, and 18h/day from d 11 to d 35.

8.3.1.2. Experimental design and diets

Three different diets (starter, growing and finishing) were formulated to meet the requirements for maintenance and growth (FEDNA, 2008) and offered to the broilers from d 1 to 14, from d 14 to 28, and from d 28 to d 35. The six experimental treatments were prepared according to two different Cu sources (Cu_2O and $CuSO_4$) at 3 levels of inclusion (15 ppm, 150 ppm, 300 ppm Cu). Feed and water were offered *ad libitum*. All of the diets were presented in mash form. Diets were sampled and stored for their subsequent analysis.

Body weight (BW) was individually monitored at the start and at the end of each phase (d 1, d14, d 28 and d 35) and feed disappearance was registered by pen in order to calculate average daily feed intake (FI), weight gain (WG) and feed conversion ratio (FCR). Mortality rate was also monitored. At the end of the experiment (d 35), one bird/pen (n=8) was euthanized by cervical dislocation, tissue (fat and breast muscle) and organ samples (liver and kidney) were collected and weighed to determine Cu content. Ileal digesta were collected in the region from Meckel's diverticulum to about 2 cm anterior to the ileo-cecal junction and stored at -20ºC to determine mineral and micro-mineral content.

8.3.1.3. Laboratory Analyses

The Cu content was analyzed in the basal diet, in both Cu sources, and before and after the experimental diets´ preparation in order to confirm the adequacy of Cu dosage. Mineral concentration (Ca, P, Fe, Cu, Zn and Mn) was also analyzed in the supernatant and precipitate of ileum digesta centrifuged at 6,000 x g for 15 min, and in tissues and organs (liver, kidney, and muscle (breast muscle)). All mineral contents in samples were analyzed using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES, model Optima 4300DV, PerkinElmer Inc.; Waltham, MA).

Table 8.1. Composition of the basal diet for different phases for broilers from 0 to 35 days.

	Starter	Growing	Finishing
Ingredients (%)			
Corn	39.90	29.20	17.10
Wheat	15.00	30.00	45.00
Soybean meal	26.10	26.40	24.60
Extruded full-fat soybean	9.80	7.00	5.00
L-Lysine	0.29	0.23	0.24
DL-Methionine	0.16	0.15	0.14
L-Threonine	0.09	0.08	0.08
Limestone	1.15	1.09	1.16
Soy oil	5.00	0.60	0.50
Mono-calcium phosphate	1.59	1.46	1.35
Salt	0.24	0.21	0.21
Trace mineral-vitamin premix*	0.40	0.40	0.40
Sodium bicarbonate	0.30	0.30	0.30
Lard	0.00	3.00	4.00
Calculated composition (%)			
DM	87.70	87.90	88.50
ME, Kcal/kg	3,000	3,100	3,140
CP	21.00	20.90	20.10
Ca	0.90	0.85	0.85
Total P	0.74	0.71	0.68
Available P	0.45	0.44	0.43
PP	0.25	0.25	0.25
NPP	0.50	0.47	0.44
Analyzed composition (%)			
DM	90.00	90.00	90.10
GE, Kcal/kg	4,223	4,139	4,140
CP	19.90	20.90	20.00
E.E	8.50	7.93	7.45
Ca	0.97	1.03	1.08
Total P	0.69	0.71	0.68

(*) Provided per kg of feed: Vitamin A (retinil acetate) (UI) 13500; Vitamin D (Vitamin D3) (Colecalciferol) (UI) 4800; Vitamin E/acetate de tot-rac-3- tocopheril) (mg) 45; Vitamin K3 (MNB Menadione nicotinamide bisulfit)(mg) 3; Vitamin B1 (Tiamin mononitrate) (mg) 3; Vitamin B2 (Riboflavin) (mg) 9; Vitamin B6 (Piridoxin Chlorhidrate) (mg) 4.5; Vitamin B12 (cyanocobalamine) (mg) 0.04; Nicotinamide (mg) 51; Pantotenic Acid (Calcium D-pantotenate) (mg) 16.5; Biotin (D-(+)-biotin) (mg) 0.15; Folic Acid (mg) 1.8; Choline chloride (mg) 350; Iron (Iron sulfate monohydrate) (mg) 54; Zinc (Zn. zinc oxide) (mg) 66; Manganese (Mn. Manganese oxide) (mg) 90; Iodine (I. Calcium Iondine Anhidre) (mg) 1.2; Selenium (Se. Sodium Selenate) (mg) 0.18; Etoxiquin (mg) 5.332; Endo-1.3(4)-betaglucanase EC 3.2.1.6 (U) 70; Endo-1.4-beta-xylanase EC 3.2.1.8 (U) 270; Endo-1.4-betaglucanase EC 3.2.1.4 (U) 80; 6-phytase EC 3.1.3.26 (FTU) 500; D.L-Malic acid (mg) 60; Fumaric acid (mg) 75; Sepiolite (mg) 1007.36; Vermiculite (mg) 2.668; Colloidal silica (mg) 45.

Table 8.2. Experimental diet calculated and Cu concentration analyzed.

Treatment		Cu (mg/kg)	
		Calculated	Analyzed
T1	$CuSO_4$	15	19
T2		150	162
T3		300	390
T4	Cu_2O	15	19
T5		150	135
T6		300	349

8.3.1.4. Statistical Analyses

Body weight (BW), average daily weight gain (WG), average daily feed intake (FI) and feed conversion ratio (FCR) were analyzed with ANOVA using the GLM procedure of the statistical package SAS®. The main factors used in the model were the Cu source and Cu level, and their interaction. The statistical unit was the pen of 12 birds (N=8) for all production measurements. The alpha level used for the determination of significance was 0.05.

8.3.2. *In vitro* trial

8.3.2.1. Solubility of copper sulfate or dicopper oxide with and without Phytate

The solubility of copper sulfate or dicopper oxide was measured at concentrations of 0 mg Cu/L, 15 mg Cu/L, 150 mg Cu/L and 300 mg Cu/L in 200 mM glycine buffer (pH 2.5) and 200 mM sodium acetate buffer (pH 4.5 and 6.5). Each Cu source was mixed with 20 ml of buffer with and without 2.9 mM phytate and incubated at 41ºC in a shaking water bath for 1h and filtered through 42 µm whatman filter paper for Cu and P analysis by atomic absorption spectroscopy. The solubility of P at different values of pH and with Cu inclusion (soluble Cu x 100 / total Cu) was expressed as a percentage relative to the P soluble when no copper was added to the solution.

8.3.2.2. Effects of copper source and level on PP hydrolysis by Phytase

The effect of copper on PP hydrolysis was studied following the general procedures of Pang and Applegate (2006), with some modifications. Three buffer solutions were prepared: 200 mM glycine buffer (pH 2.5) and 200 mM sodium acetate

buffer (pH 4.5 and 6.5). At the same time, four Cu solutions were prepared to represent dietary Cu concentrations (0 mg/L, 15 mg/L, 150 mg/L and 300 mg/L) from each Cu source (Cu_2O and $CuSO_4$). Substrate solutions were prepared by mixing Cu solution with phytate 2.9 mM (538 mg P/L) from sodium phytate solution (dodecasodium salt from rice, Sigma-Aldrich) in buffer solution and adjusting the pH to 2.5 (simulating gastric pH), 4.5 and 6.5 (simulating small intestinal pH). The phytase enzyme was diluted, then suspended in a buffer such that 0.1 ml would contain 500 FTU/kg diet (equivalent to phytase activity in the *in vivo* experiment) when added to the substrate solutions.

Substrate solution (3ml) and phytase solution (0.1ml at the same pH of the substrate solution) were added to test tubes in duplicate and incubated at 41ºC for 60 min. A 2.07 mL amount of ammonium molybdate-metavandate reagent was added to stop the reaction (ISO, 2009), and liberated P was then measured calorimetrically at 410 nm, using inorganic P as a standard. The P released was expressed as a percentage relative to the P released when no copper was added to the mixtures.

8.4. Results

The nutrients of the diet are presented in Table 8.1. It is worth noting that Ca in the 3 diets (0.97%, 1.03% and 1.08%) was higher than was formulated, likely as a consequence of the presence of Ca in some ingredients (Table 8.2).

8.4.1. *In vitro* trial

8.4.1.1. Bird Performance

Total mortality rate (%) for the entire trial was 0.69%, corresponding to 4 random birds from T2, T3, T4 and T5, respectively. The animals´ performances are presented in Table 8.3. An interaction (Cu level x Cu source) was observed for BW, WG, FI and FCR.

Lower WG was observed ($P<0.001$) for broilers of the 300 ppm Cu as $CuSO_4$ group, as compared to birds offered lower levels of $CuSO_4$ (15 ppm and 150 ppm) or those of the 15 ppm, 150 ppm and 300 ppm Cu_2O group for the period between d 14 and d 28. In the growing period (between d 28 and d 35), birds fed 300 ppm Cu as $CuSO_4$ showed a lower WG ($P<0.05$) than did those of the 150 ppm Cu as $CuSO_4$ group. No differences were observed among the other treatments.

Increasing the level of Cu in the diet from 15 ppm to 300 ppm with $CuSO_4$ increased (P<0.05) FI from d 28 to d 35, a result which was not observed with Cu_2O. The broilers fed the 300 ppm $CuSO_4$ diet showed a higher (P<0.001) FCR (2.19) than did broilers from the 300 ppm Cu_2O group (FCR=1.84), and higher than the FCR observed in broilers of the 15 ppm and 150 ppm $CuSO_4$ group (1.86).

Table 8.3. Effect of different copper sources and level of inclusion on feed intake and growth performance of broilers from d1 to 35[1] (Trial *in vivo*).

Treatment		BW[2](g)			FI[3] (g/d)		WG[4] (g/d)		FCR[5]	
Source	Cu (ppm)	**d 14**	**d 28**	**d 35**	**d14-28**	**d 28-35**	**d14-28**	**d 28-35**	**d 14-28**	**d 28-35**
$CuSO_4$	15	481^{a}	1672^{a}	2444^{ab}	131.6	204.5^{b}	85.1^{a}	110.3^{ab}	1.55^{b}	1.86^{b}
	150	463^{a}	1634^{a}	2448^{ab}	128.9	213.7^{ab}	83.7^{a}	116.2^{a}	1.54^{b}	1.86^{b}
	300	429^{b}	1481^{b}	2221^{c}	140.1	231.1^{a}	75.1^{b}	105.7^{b}	1.87^{a}	2.19^{a}
Cu_2O	15	458^{a}	1614^{a}	2361^{b}	130.7	217.1^{ab}	82.1^{a}	106.7^{ab}	1.60^{b}	2.03^{ab}
	150	481^{a}	1704^{a}	2512^{a}	131.1	208.4^{b}	87.3^{a}	115.4^{ab}	1.50^{b}	1.81^{b}
	300	464^{a}	1655^{a}	2463^{ab}	133.5	212.6^{ab}	85.1^{a}	115.5^{ab}	1.57^{b}	1.84^{b}
Source										
$CuSO_4$		458	1596	2371	133.5	216.4	81.3	110.7	1.65	1.97
Cu_2O		468	1658	2446	131.7	212.7	84.9	112.6	1.56	1.90
Dose (ppm)										
	15	470^{a}	1643^{a}	2403^{b}	131.1^{b}	210.8	83.6^{a}	108.5^{b}	1.57^{b}	1.95^{ab}
	150	472^{a}	1669^{a}	2480^{a}	130.0^{b}	211.0	85.5^{a}	115.8^{a}	1.52^{b}	1.83^{b}
	300	446^{b}	1568^{b}	2342^{b}	136.8^{a}	221.8	80.1^{b}	110.6^{ab}	1.72^{a}	2.02^{a}
SEM[6]		6.6	22.6	31.1	2.34	5.27	1.40	2.37	0.039	0.060
P value[7]										
Source		0.074	0.001	0.005	0.352	0.387	0.003	0.355	0.004	0.135
Dose		0.0005	0.0002	0.0003	0.013	0.068	0.001	0.011	<.0001	0.011
Source × Dose		0.0003	<.0001	<.0001	0.173	0.019	0.0002	0.019	0.0002	0.0004

[1] Data are means of 8 pens with 12 chicks each; [2] Body weight; [3] Average daily feed intake; [4]Average daily weight gain; [5] feed conversion ratio ; [6] SEM: Standard error of the mean; [7] [a.b.c] Values in the same column with different letters are significantly different (P<0.05).

8.4.1.2. Organ weights and copper content in organs and tissue samples

No differences were observed on the organ weights for liver (64.6 g), kidney (15.7 g) or breast (211.1 g) due to the different experimental treatments used. The effects of different Cu sources and levels of inclusion on Cu concentration in serum, and in organs and tissue samples (liver, kidney, breast and fat), at the end of the experimental period (35 days of life) are reported in Table 8.4.

Copper content in the liver was influenced by the Cu source and level, showing a significant interaction ($P < 0.05$). Indeed, higher levels of Cu in the liver were observed for birds fed 300 ppm Cu as $CuSO_4$ (7.91 µg/g), as compared to the rest of the

experimental treatments (< 4.63 µg/g). No source x dose interactions were observed in the rest of organs and tissues. High doses (300ppm) of copper with both sources increased (P < 0.01) Cu concentration in the kidney (2.17 µg/g) and breast (0.50 µg/g) in comparison to broilers fed the 15 ppm (1.95 µg/g and 0.32 µg/g, respectively) and 150 ppm (1.98 µg/g and 0.37 µg/g, respectively) amounts. Copper also tended (P = 0.074) to increase the concentration in the fat of chicks fed 300 ppm Cu, compared to broilers of the 15 ppm and 150 ppm Cu groups.

Table 8.4. Effect of different copper sources and level of inclusion on copper content in organs and the concentration of copper and zinc in serum at 35 d[1] (Trial *in vivo*).

Treatment		Cu				
		Serum (mg/L)	**Liver (µg/g)**	**Kidney (µg/g)**	**Breast muscle (µg/g)**	**Fat (µg/g)**
Source	Cu(ppm)					
$CuSO_4$	15	0.16	2.69^{b}	1.96	0.31	0.31
	150	0.14	3.41^{b}	2.06	0.40	0.48
	300	0.16	7.91^{a}	2.18	0.51	0.73
Cu_2O	15	0.14	2.71^{b}	1.94	0.33	0.35
	150	0.15	2.97^{b}	1.90	0.34	0.21
	300	0.16	4.63^{b}	2.16	0.48	0.50
Source						
$CuSO_4$		0.15	4.67	2.07	0.41	0.51
Cu_2O		0.15	3.44	2.00	0.38	0.35
Dose (ppm)	15	0.15	2.70^{b}	1.95^{b}	0.32^{b}	0.33
	150	0.15	3.19^{b}	1.98^{b}	0.37^{b}	0.35
	300	0.16	6.27^{a}	2.17^{a}	0.50^{a}	0.62
SEM[2]		0.013	0.759	0.064	0.032	0.145
P value[3]						
Source		0.560	0.033	0.195	0.400	0.179
Dose		0.395	<.0001	0.001	<.0001	0.074
Source × Dose		0.839	0.046	0.454	0.287	0.483

[1] Data are means of 8 pens with 12 chicks each. [2] SEM: Standard error of the mean. [3 a.b.c] Values in the same column with different letters are significantly different (P<0.05).

Table 8.5. Effect of different copper sources and level of inclusion on mineral content (Cu, P, Fe, Zn and Mn) in ileum digesta (soluble and insoluble) at 35 d[1] (Trial *in vivo*).

Treatment source	Cu (ppm)	Cu (µg /g)		P (µg /g)		Fe (µg /g)		Zn (µg /g)		Mn (µg /g)	
		insoluble	soluble	insoluble	soluble	insoluble	soluble	insoluble	soluble	insoluble	soluble
$CuSO_4$	15	12.8^{d}	9.2^{d}	2517.1	1612.7^{a}	148.4	7.4	71.6	20.3	75.0	29.4^{a}
	150	96.8^{c}	67.3^{c}	3353.1	547.7^{cd}	172.5	6.1	92.4	6.3	104.8	9.1^{b}
	300	223.8^{a}	163.4^{a}	3191.8	419^{d}	173.9	9.0	92.0	5.8	101.5	5.3^{b}
Cu_2O	15	11.8^{d}	7.9^{d}	2346.5	1077.5^{b}	136.0	5.5	63.5	12.7	67.6	17.5^{ab}
	150	92.0^{c}	74.0^{c}	2812.5	970.9^{b}	155.1	6.0	79.6	8.1	94.5	15.3^{b}
	300	169.0^{b}	129.1^{b}	2975.8	792.9^{bc}	161.0	8.0	80.5	6.1	85.4	6.6^{b}
Cu source											
$CuSO_4$		111.1	79.9	3020.7	859.8	164.9	7.5	85.3	10.8	93.8	14.6
Cu_2O		90.9	70.3	2711.6	947.1	150.7	6.5	74.5	9.0	82.5	13.1
Dose(ppm)											
	15	12.3^{c}	8.5^{c}	2431.8^{b}	1345.1^{a}	142.2	6.4^{ab}	67.6^{b}	16.5^{a}	71.3^{b}	23.5^{a}
	150	94.4^{b}	70.6^{b}	3082.8^{a}	759.3^{b}	163.8	6.1^{b}	86 ^{a}	7.2^{b}	99.6^{a}	12.2^{b}
	300	196.4^{a}	146.3^{a}	3083.8^{a}	605.9^{b}	167.4	8.5^{a}	86.3^{a}	5.9^{b}	93.4^{a}	5.9^{b}
SEM[2]		10.84	7.69	201.96	76.51	12.47	0.92	5.76	2.60	7.84	3.21
P value[3]											
Source		0.028	0.135	0.054	0.341	0.171	0.182	0.027	0.399	0.087	0.582
Dose		<.0001	<.0001	0.001	<.0001	0.104	0.024	0.003	0.0004	0.002	<.0001
Source × Dose		0.030	0.026	0.570	<.0001	0.976	0.620	0.92	0.164	0.851	0.021

[1] Data are means of 8 pens with 12 chicks each. [2] SEM: Standard error of the mean. [3 a.b.c] Values in the same column with different letters are significantly different (P<0.05).

8.4.1.3. Mineral concentration in ileum digesta

The results obtained from the analysis of mineral in the ileum digesta (soluble and insoluble) are presented in Table 8.5. A significant interaction was observed between Cu source and level on the Cu, P, and Mn content in soluble and insoluble fractions.

A higher copper level in ileum digesta was observed ($P < 0.05$) for birds of the 300 ppm $CuSO_4$ group in both soluble (163.4 µg/g) and insoluble (223 µg/g) fractions, as compared to birds of the 300 ppm Cu_2O group (129.1 µg/g and 169 µg/g, respectively for soluble and insoluble fractions).

The P content in ileum digesta was also affected by the interaction between dose and source. The use of $CuSO_4$ at a high level (300 ppm) decreased ($P < 0.001$) the P content in the soluble ileum fraction (419 µg P/g), in comparison to the use of the same Cu source at 15 ppm (1612.7 µg P/g). There is no significant difference among groups of birds supplemented with Cu_2O at different levels.

Fe and Zn content in soluble and insoluble fractions of the ileum was not affected by the interaction between Cu sources and doses. Increasing the Cu level in the diet from 15 ppm to 150 ppm and 300 ppm increased ($P<0.01$) the Zn and Mn content in the insoluble fraction and decreased their content in the soluble fraction. In the other hand, increasing the Cu doses ($CuSO_4$ and Cu_2O) in the bird's diet increased ($P<0.05$) Fe content in the soluble fraction of ileum digesta.

8.4.2. *In vitro* trial

8.4.2.1. Copper solubility with and without phytate

The results of this trial are presented in Figure 8.1 and 8.2. At pH 2.5, no difference in the solubility of Cu was observed between the two Cu sources ($CuSO_4$ and Cu_2O) with and without phytic phosphorus (Figure 8.1 and 8.2). The solubility of Cu_2O at pH 4.5 and 6.5 decreased when the Cu level increased to 150 ppm and 300 ppm, and independently of phytic acid presence. The solubility of $CuSO_4$ at pH 4.5 and 6.5 showed a clear response depending on the presence of phytic acid in the digesta. Without phytate in the sample, $CuSO_4$ showed high solubility, while with phytate the increase of Cu (from 15 ppm to 300 ppm) reduced its solubility.

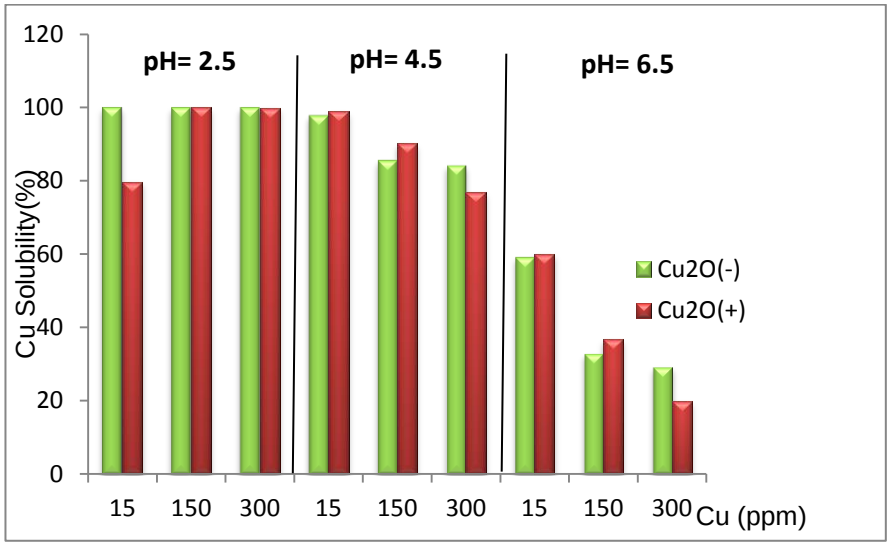


Figure 8.1. Effects of Cu concentration on the solubility of Cu_2O with (+) and without phytate (-) at pH 2.5; 4.5 and 6.5.

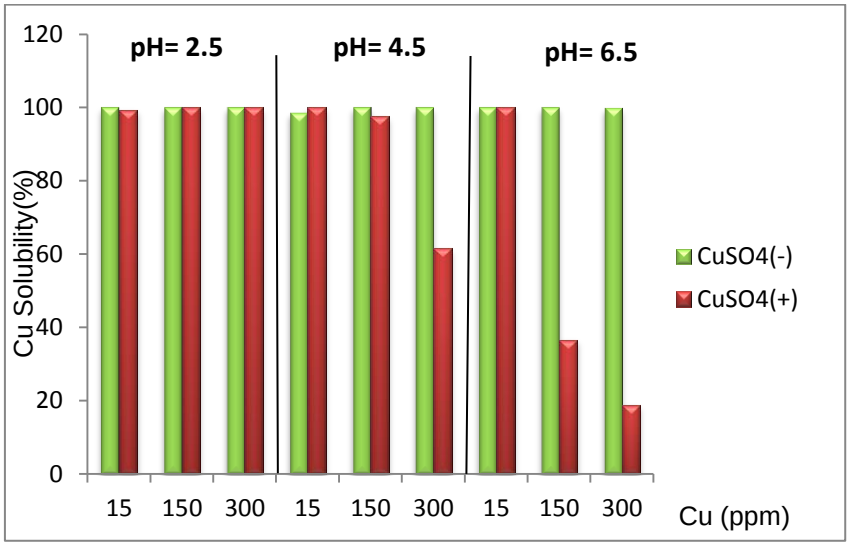


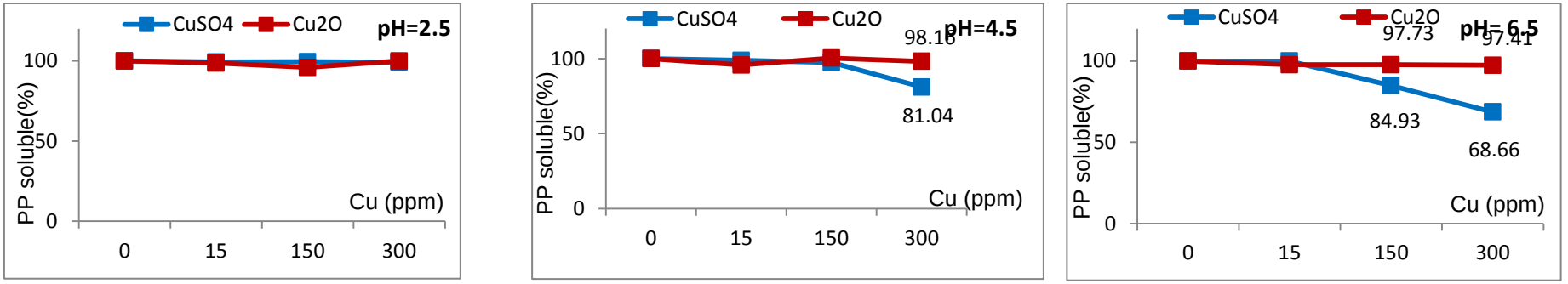
Figure 8.2. Effects of Cu concentration on the solubility of $CuSO_4$ with (+) and without phytate (-) at pH 2.5; 4.5 and 6.5.

8.4.2.2. Phytic phosphorus solubility and PP hydrolysis by phytase

Solubility of phytic phosphorus (Figure 8.3) was high and unaffected by the presence of Cu_2O at different levels and pH. On the other hand, increasing levels of $CuSO_4$ reduce PP solubility at 150 ppm when pH was 6.5 and at 300 ppm when pH was 4.5 and 6.5. The efficacy of phytase is described in Figure 8.4 as being affected by the pH and level of Cu supplementation. Increasing the levels of Cu with both sources did not affect the phytase activity at pH 2.5. However, increasing the levels of Cu decreased phytase activity at pH 4.5 and 6.5. Average values of phosphorus liberated were higher at pH 4.5 when Cu_2O was included in the solution, as compared to $CuSO_4$.

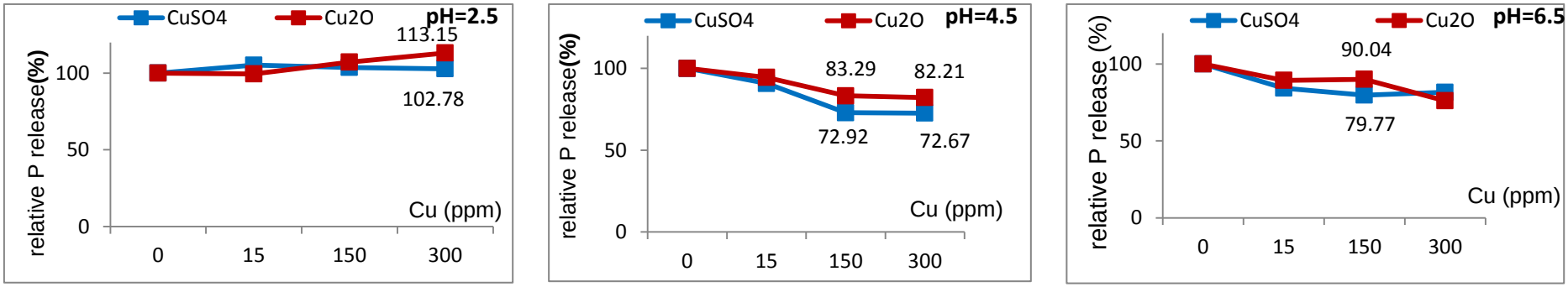
8.5. Discussion

The results of this study show that Cu supplementation at therapeutic doses (150 ppm Cu) increase performance when Cu_2O is used as the source, but does not affect (150 ppm) or reduce (300 ppm) the BW of the animals or gain:feed ratios when Cu is supplied as $CuSO_4$. The growth stimulating action of dietary Cu has been attributed to different mechanisms (Zhou *et al.*, 1994) in which soluble Cu 1) may affect microbiota; 2) increases serum mitogenic activity; 3) increases pituitary growth hormone expression (La Bella *et al.*, 1973); 4) promotes a posttranslational modification of regulatory peptides (Eipper and Mains, 1988); and 5) is a component of the growth factor Iamin (Parkart, 1987). An increase in feed intake and growth was observed by Jenkins *et al.* (1970) when birds were fed 250 mg Cu/kg of Cu. Pesti and Bakalli (1996) also concluded that a 250 ppm of $CuSO_4$ improved the growth and feed-conversion ratio of broilers. However, the response may depend on the Cu source; Lu *et al.* (2010) described that chicks fed 200 mg Cu /kg as TBCC (Tribasic Copper Chloride) had higher ADG than those fed a lower level (0 mg/Cu/kg to 150 mg/Cu/kg) as TBCC or the same level (200 mg Cu /kg) as $CuSO_4$.



Soluble PP is expressed as a percentage relative to the soluble PP when Cu was not added to the mixture.

Figure 8.3. Effects of Cu concentration on the PP solubility of Cu_2O and $CuSO_4$ at pH 2.5, 4.5 and 6.5.



P released is expressed as a percentage relative to the P when Cu is not added to the mixture.

Figure 8.4. Effects of Cu source and concentration on phytase efficacy at pH 2.5, 4.5 and 6.5.

On the other hand, Arias and Koutsos (2006) showed that at d 45, chicks supplemented with 188 mg Cu/kg as $CuSO_4$ and TBCC had higher carcass weight, compared with those fed negative controls (8 mg Cu/kg), and they were not different from positive control supplemented with sub-therapeutic antibiotics (AGP) under immune-challenging conditions (recycled vs. fresh litter). It could be suggested that the animal growth response to high dietary Cu supplementation may depend on the sanitary status of the farm.

In contrast, the results of our experiment showed that a high Cu level in the diet, such as copper sulfate , decreased BW gain and the feed efficiency. Pesti and Bakalli, (1996) also indicated that supplementation of either 125 mg/kg or 250 mg/kg of Cu as copper sulfate improved growth and the feed-conversion rate, but higher levels at 375 mg/kg provided no further beneficial effect. Banks *et al.* (2004b) also described that supplementation with 250 mg/Cu/kg of copper sulfate had linear reductions in performance (BW gain, feed consumption and feed conversion efficiency) and pointed out that excesses of Cu might cause a possible toxic or corrosive response. In fact, one of the roles of the liver is to regulate the amount of copper in the body, as the primary copper storage organ. When the liver reaches its storage limit, copper is liberated in the bloodstream and then accumulates in different organs. Reece *et al.* (2015) also suggest that the presence of free unbound copper in the blood acts as a strong oxidizing agent and causes hemolysis of red blood cells. Moreover, some studies with broilers have indicated that supplementation with a 250 mg/Cu/Kg diet as copper sulfate can cause irritation and erosions in the gizzard (Robbins and Baker, 1980), proventriculus (Wideman *et al.*, 1996) and oral cavity, tongue and pharynx (Chiou *et al.*, 1999).

On the other hand, Milers *et al.*, (1998) proved that during the storage period of feed, ″painty″ odors in samples containing high levels of Cu were detected. The ″painty″ odors originate from hexanal, which is a product of linoleic acid oxidation (Frankel, 1985; Milles, 1998) likely promoted by the proxidant effect of Cu. Milers *et al.*, (1998) also proved that rates of oxidation can be greater when the copper source is highly water-soluble, and Lu *et al.*, (2010) revealed that TBCC was less active than was Cu sulfate in promoting the oxidation of vitamin E in feeds and in reducing vitamin E content in plasma and the liver. We can speculate that dicopper oxide promoted a lower oxidation effect than did copper sulfate. However, we did not measure oxidation

parameters in feed or in the animals. Thus, a decrease in growth performance for animals fed high levels of Cu can be the result of the reduction in feed intake due to the rancid odor in the diet. Nevertheless, in our experiment the high level of Cu sulfate (300 ppm Cu) decreased BW and WG but did not affect or even increased feed intake.

The difference in feed efficiency with copper sulfate being incorporated at high doses could reflect the toxicity of a copper excess in the body. Copper is preferably stored in the liver, the other storage organ is the kidney, but to a lesser extent. Lower levels are observed in muscle and fat whatever the level used in the feed (E.C., 2003). In this experiment, kidney and breast-muscle Cu content increased with the dietary Cu level independently of the mineral source used. Samanta *et al.*, (2011) and Cromwell *et al.*, (1989) opined that gradual increases in the concentration of dietary Cu until 300 ppm have a direct influence on tissue and organ accumulation of Cu. Absorption and organ accumulation of Cu appeared to be highly related to the solubility of the copper source, the accumulation of Cu in the liver was greater for birds fed 300 ppm as $CuSO_4$ than in those given the same Cu level as Cu_2O. This increase in liver content of Cu might be due to a higher solubility of copper sulfate, in comparison to dicopper oxide.

Soluble sources of Cu in digesta could also interact with phytate and phytase activity. High levels of Cu of copper sulfate are known to interfere with phytate at intestinal pH, and the resulting complexes tend to be resistant to the hydrolytic activity of phytases (Persson *et al.*, 1998). We have also revealed in *in vitro* trial that the addition of 150-300 mg/Cu/Kg as $CuSO_4$ at pH=4.5 reduced Cu solubility in the presence of phytic phosphorus, coupled with the reduction in PP solubility and phytase hydrolysis. This inhibitory effect was greater at pH=6.5 (intestinal pH). This result is in accordance with Pang and Applegate (2006), who showed that increasing doses of Cu inhibited PP hydrolysis at pH 5.5 and pH 6.5, and the effect of Cu on phytase activity was dependent on the Cu source. In fact, they found that TBCC and copper lysinate inhibited PP hydrolysis much less than did copper sulfate and copper citrate. This result was also described by Banks *et al.*, (2004a) in an *in vivo* experiment, where they observed that supplementation with 250 mg/Cu/Kg diet from copper citrate or copper sulfate decreased apparent P retention; however, supplementation with a 250 mg/kg diet of copper lysinate did not affect apparent P retention. Furthermore, Champagne *et al.*, (1990) confirmed that, at pH similar to intestinal pH, insoluble complexes with more than one cation per phytate molecule are formed. Therefore, phytin can bind to Cu as well as to other minerals concurrently. The resulting insoluble complexes do not allow

the hydrolysis of phytin-P by endogenous and exogenous phytases or the absorption of the minerals within the complex (Banks *et al.*, 2004b). In fact, the phytate precipitated with different minerals present in the diet, and the order of stability of metal-phytate complexes was found to be Cu > Zn > Co > Mn > Fe > Ca (Tamim and Angel, 2003). In this experiment, higher insoluble Cu in the ileum digesta was accompanied by lower soluble P when high $CuSO_4$ was added to the diet. This result may indicate that $CuSO_4$ precipitated with phytic phosphorus at high pH (intestinal pH) and may limit the bio-availibility of phytic phosphorus to the phytase. Moreover, a high copper dose decreased Mn and Zn solubility in the ileum, which may also reflect the high affinity of phytate to Cu, Zn and Mn. Maenz *et al.*, (1999) described that multiple mineral complexes such as Ca-Zn-phytate are thought to be more stable than are single mineral complexes such as Ca phytate or Zn phytate (Banks *et al.*, 2004a).

Based on the results acquired in this experiment, we can conclude that a high dose of Cu as dicopper oxide (150 ppm) improved BW of the animals. However, high doses of $CuSO_4$ (300 mg/Cu/kg) may decrease performance, either as a result of decreased PP hydrolysis by phytase or as a consequence of the toxicity of copper accumulated in different organs. Further research may be warranted to determine the possible oxidative effects of these two copper sources and also the fate of the released anion after Cu dissociation.

CHAPTER 9

General discussion

In the present study the effect of administering lower levels of Ca and P in the broiler diets as compared to requirements proposed by FEDNA, NRC or INRA was investigated. The outcomes derived from the use of different mineral Ca and P sources based on *in vitro* and *in vivo* trials have also been compared. In general, positive effects have been observed in animal performance and mineral retention after reducing Ca and P levels in diets supplemented with phytase. The use of different calcium sources affects growth performance and bone mineralization, but the phosphorus source used did not affect the studied parameters.

Results have been discussed in the different chapters of the present thesis. Therefore, this general discussion will bring the results together to highlight the most interesting findings and gain new insights.

9.1. Ca and P requirements

Calcium and P requirements were evaluated based on a multifactorial approach, as determined by animal performance or bone mineralization at different age periods.

9.1.1. Animal performance

In **Chapter 4**, birds exposed to diets with a medium level of Ca (7g/kg) and 3.8 g/kg NPP with high doses of phytase (1,150 FTU) performed the best in the starting phase, while higher Ca levels (0.9%) induced negative responses concerning FI and WG. Similarly, in **Chapter 7** also, the use of lower Ca:aP ratio (2.33:1) increased bird performance in comparison to a higher Ca:aP ratio (3:1) for broilers at 14 days of life. Rama-Rao *et al.* (2006) described that weight gain, feed intake and leg abnormality scores were not affected by increasing the dietary Ca from 6 g/kg to 9 g/kg, and NPP levels from 3 g/kg to 4.5 g/kg for commercial broilers up to 42 days of age. It has been reported that Ca may form insoluble soaps with free fatty acids and bile acids, and there is some evidence that these soaps reduce the absorption of fat (Gacs and Barltrop, 1977; Govers *et al.*, 1996; Shahkalili *et al.*, 2001), particularly saturated fatty acids in broiler diets. Soluble Ca may also increase intestinal pH and reduce mineral solubility and availability, as reported by Shafey and McDonald (1991). Soluble Ca in the diet may also insolubilize phytic acid by forming Ca-phytate complexes (Wise, 1983; Tamim *et al.*, 2004) and interact with inorganic P in the gut lumen to form insoluble Ca orthophosphate (Plumstead *et al.*, 2008), an insoluble complex in the chicken gut

resulting in reduced absorption (Underwood, 1981; Georgievskii *et al.*, 1982, Rama-Rao *et al.*, 2006).

In particular, the negative effects of higher levels of Ca in performance are clearly observed when diets are formulated with lower levels of P. The results obtained in **Chapter 4** confirmed that with Ca levels at 9 g/kg, the use of 4 g NPP/kg and 4.5 g NPP/kg, which corresponded to the lower Ca:NPP ratio (2-2.25:1), increased animal performance, as compared to those broilers fed with 3 g NPP/kg and 3.5 g NPP/kg, which correspond to the wider Ca:NPP ratio (2.57-3:1). Higher Ca levels and Ca:aP ratios may also reduce exogenous phytase efficacy. At least two mechanisms have been proposed to explain the detrimental effect of increased concentrations of dietary Ca on P utilization: 1.- excess dietary Ca tends to form insoluble complexes with phytate (*Lei et al.*, 1994; Angel *et al.*, 2002), rendering the phytate unavailable for hydrolysis; extra Ca in the diet may also compete for the active site of phytase and thereby reduce the efficacy of phytase in hydrolyzing phytate (Wise, 1983; Pointillart *et al.*, 1985; Qian *et al.*, 1996); 2.- high dietary Ca favors an increase in digestive tract pH, which, in turn, decreases phytase activity and phytate solubility (Selle and Ravindran, 2007). We explored this interaction between the Ca:aP ratio and phytase inclusion in the diet in **Chapter 7**, but no interactions were observed for starting broilers during the first 14 days of life. A likely explanation could be the short period of study.

9.1.2. Bone mineralization

In addition to growth performance, bone mineralization is also a good sensitive criterion of P status in growing birds (Ravindran *et al.*, 1995). In **Chapter 4**, tibia weight and bone mineralization were also influenced by the level of Ca, with the low-Ca diet (5 g/Kg) showing the lowest bone weight and ash content. However, the lowest tibia ash content was observed in chicks of the highest unbalanced diets, either 4.5 g NPP/kg with 5g Ca/diet, or 2.5 g NPP /kg with 9 g Ca / kg in the diet. This depression observed with the Ca and P unbalanced diets reflect the improper utilization of these minerals at wider Ca and NPP ratios (Rama-Rao *et al.*, 2006). If diets with a low Ca:P ratio are fed, most of the P is excreted in the urine due to a lack of Ca for bone-tissue synthesis. The same decrease in bone growth may occur when high levels of Ca are administered with low levels of dietary P. A low concentration of one mineral will prevent bone synthesis and cause excretion of the other mineral in the urine

In **Chapter 6**, increasing NPP from 4.0 g/kg to 4.5 g /kg increased the tibia weight and ash content (mg/tibia) in diets without phytase at 21 d of age. In contrast, in **Chapter 4**, with diets including high levels of phytase, 3.8 g NPP /kg improved the growth of chicks and increased bone mineralization on d 14, but no further increases were observed with 4.5 g NPP /kg in the diet. The difference between the two experiments may reflect the effect of high doses (1,150 FTU) of phytase inclusion in the diet, but also the length of the experiment.

Furthermore, in **Chapter 6**, increasing the NPP from 4.0 g/kg to 4.5 g/kg increased tibia weight and tibia ash (mg/tibia), but no further increases were observed for growth performance at d 21. This result confirms that NPP requirements for BW gain and feed conversion are lower than those required for bone mineralization for broilers of 3 to 6 weeks of age (Yan *et al.*, 2001).

Several authors have demonstrated that the addition of phytase has positive effects on bone ash content and bone mineralization in broilers fed low available P diets. Moreover, the productive efficacy of phytase is higher as the level of dietary P is lower. Higher tibia ash and body weight was observed for broilers supplemented with 1,000 FTU of a new phytase in **Chapter 7**. Therefore, Ca and P in the diet can be reduced without affecting bone mineralization because phytase supplementation increased the release of available P. Growth performance of broilers fed with Ca level (7 g/kg and 6.3 g/K/kg) and NPP (3.2 g/kg and 2.9 g/K/kg) levels, respectively, for starter and growth phases supplemented with 1,000 FTU/Kg diet were not significantly different from the positive control with a 0.18%-0.2% higher analyzed total P content. No differences were observed either among different phytases used in this thesis.

9.1.3. Mineral retention and digestibility

The metabolism of Ca and P is closely related, and a deficiency or an excess of one will interfere with the utilization and metabolism of the other. It has been observed in **Chapters 4, 5, 6 and 7** that the increase in dietary Ca decreased its fractional retention. On the other hand, increasing the levels of NPP from 0.25% to 0.31% allowed for increases in the fractional retention of Ca, likely allowing a higher retention of Ca in the bones.

However, increases in the NPP level in the diet reduced the fractional retention of P, which is a similar response to that observed previously for increasing levels of Ca.

Mitchell and Edwards (1996a) and Ziaei *et al.* (2008) have stated that reducing mineral content of diets resulted in a higher apparent retention of Ca and P, leading to a reduction in mineral excretion. Al Masri (1995) showed that the values of dietary Ca and its ratio with P may affect P absorption, with lower values of P absorption when higher ratios between Ca and P were added in the diet. Higher ileal digestibility of Ca and P were observed for birds fed a low Ca:aP ratio than for those fed higher Ca:aP ratios.

Several studies have shown that phytase inclusion in the diet improved ileal digestibility and retention of P in broilers fed low Ca and available P diet (Ravindran *et al.*, 2000; Woyengo *et al.*, 2010). These effects were also observed in **Chapter 7**, when higher P retention and ileal digestibility was observed for animals fed diets supplemented with phytase. Phytase may liberate P from phytate-P and prevents the formation of insoluble Ca-phytate complexes in poultry diets (Woyengo *et al.*, 2010).

Nevertheless, the incorporation of high levels of other divalent cations in addition to Ca may also affect phytase activity. It has been demonstrated that Cu and Ca may interact with phytic acid, because phytin has chemical characteristics enabling it to bind divalent and trivalent minerals within the digesta and render the minerals less available for absorption (Maenz *et al.*, 1999; Banks *et al*., 2004b).

9.2. Phytase interaction with copper and pH

The addition of dietary Cu in excess of the nutritional requirements to poultry diets has been a common practice for many years in non-European countries. The excess supplemental Cu has been reported to have growth-promoting effects, which have been attributed to the antibacterial activity of Cu (Ward *et al.*, 1994). However, high levels of Cu are known to interfere with phytic acid in the intestinal pH and the resulting complexes tend to be resistant to the hydrolytic activity of phytases (Persson *et al.*, 1998). Most research trials carried out to study these effects have used copper sulfate ($CuSO_4$) as a source of Cu. In **Chapter 8**, two different sources of Cu were compared, either of a high ($CuSO_4$) or low (Cu_2O) solubility, concerning their effects on phytase hydrolysis and efficacy *in vitro* and *in vivo*. In the *in vitro* test, we confirmed that the efficacy of phytase was affected by the interaction of the pH and Cu level supplementation. Increasing the levels of Cu with both sources did not affect the phytase activity at pH 2.5. However, increasing the levels of Cu decreased phytase

activity at pHs 4.5 and 6.5. Average values of phosphorus released by phytase were higher at pH 4.5 when Cu_2O was included in the solution, as compared to $CuSO_4$.

This result is in accordance with Pang and Applegate (2006), who showed that increasing doses of Cu inhibited PP hydrolysis at pH 5.5 and pH 6.5, and the effect of Cu on phytase activity was dependent on the Cu source. Phytin can bind to Cu as well as to other minerals concurrently at a different pH. The resulting insoluble complexes do not allow for the hydrolysis of phytin-P by endogenous and exogenous phytases or for the absorption of the minerals within the complex (Banks *et al.*, 2004a).

The *in vitro* results help to explain the results observed *in vivo*. Indeed, higher insoluble Cu in the ileum digesta was accompanied by lower soluble P when high doses of $CuSO_4$ were included in the diet, as compared to high doses of Cu_2O. This result may indicate that $CuSO_4$ may have reduced the bio-availibility of phytic phosphorus to phytase.

Moreover, a high Cu dose, regardless of the source, decreased soluble Mn and Zn content in ileum digesta, which may also reflect the formation of complex phytate to Cu, Zn and Mn. Maenz *et al.* (1999) stated that multiple mineral complexes such as Ca-Zn-phytate are thought to be more stable than are single mineral complexes such as Ca phytate or Zn phytate (Banks *et al.*, 2004a). Higher insoluble Cu in ileum digesta was accompanied by lower soluble P when high $CuSO_4$ was added to the diet. This result may indicate that $CuSO_4$ precipitated with phytic phosphorus at high pH and limits the availability of phytic phosphorus to the phytase. High doses of $CuSO_4$ may decrease animal performance, either as a result of decreased PP hydrolysis by phytase or as a consequence of the toxicity of copper which accumulated in different organs, specially the liver.

9.3. Mineral sources

9.3.1. Phosphorus sources

With the increases in prices of mineral sources, the poultry and feed industries have become increasingly interested in detailed information about the variation in the availability of P among different inorganic sources. Di-calcium phosphate is often utilized as the standard P source in poultry feed (Lima *et al.*, 1995). However, the characteristics of di-calcium phosphate obtained from different manufactures may vary

according to the origin of the rock phosphate, phosphoric acid and limestone used during the industrial processing. In another experiment in this thesis, different phosphorous sources in the broilers diets were compared, but contrary to what was expected no differences were observed among MCP, DCP and TCP. In previous studies, a higher bioavailability has been described of hydrated P sources, as compared to the anhydrous form (Lima *et al.*, 1995). In the present study, all of the selected sources were mono- or di-hydrate, and none of them was anhydrous.

9.3.2. Calcium sources

Calcium requirements have usually been measured using limestone in the diet, which shows a lower solubility, as compared to other calcium sources, during neutralization in the small intestine. Indeed, limestone is approximately 80% soluble in the acidic medium of the gastrointestinal tract, but that solubility decreased to 77% in the neutral conditions of the intestine (Walk *et al.*, 2012a), where most absorption takes place. Although diets are usually formulated on a total Ca basis, there has recently been an interest to know the availability of Ca from different sources. To verify the importance of Ca sources in mineral availability for young broilers, an *in vitro* and *in vivo* study was carried out in **Chapter 5**. The results of this experiment refute the hypothesis that the use of high-soluble Ca sources with a low acid-binding capacity can improve mineral digestibility and reduce the level of Ca in the diet. Indeed, Ca chloride showed the lowest buffering capacity and the highest solubility among the three sources (Limestone, Ca Chloride and Lipocal (TCP)) at pH above 3.5 until neutrality. Nevertheless, Champagne (1988) reported that Ca-phytate complexes may precipitate at pHs between 4 and 6. So the free Ca^{2+} derived from the high-soluble Ca (Ca chloride) in the intestine may precipitate with inorganic P and/or phytic P and form phytate-mineral complexes in the intestine that are more resistant to phytase action (Tamim *et al.*, 2004) and reduce Ca and P absorption (Lonnerdal *et al.*, 1989). This process could explain our results of higher WG observed for birds fed limestone and TCP than for birds fed Ca chloride. The results are in agreement with those observed by Walk *et al.* (2012a), who showed that broiler growth was lower when diets were formulated with a highly soluble source of Ca, as compared to birds fed on limestone. So, based on these observations, it could be suggested that Ca sources with lower solubility may allow for better performances than with high-soluble Ca sources.

CHAPTER 10

Conclusions

From the results presented in this dissertation, the following conclusions can be drawn:

1) Calcium and phosphorus dietary recommendations should be considered together because both macro-minerals show clear interactions in the digestive tract as well as on bird performance and bone formation. Increasing the NPP levels increases the fractional retention of Ca. The use of a lower Ca:aP ratio increases bird performance in comparison to a higher Ca:aP.

2) A dietary level of 3.8 g NPP /Kg in a pre-starter diet, simultaneously overdosed with phytase (1,150 FTU), was adequate to ensure the best growth and bone formation of broilers from d 1 to 14 of age. Increasing NPP to 4.5 g/Kg without phytase supplementation increased tibia weight and ash for broilers at 21 days of age.
3) A calculated dietary level of 7 g Ca/kg (analysed at 7.9 g Ca/kg) may optimize performance and bone mineralization of chicks during the first two weeks of life; this value is below those currently used by the industry. Higher values of Ca promote significant decreases on feed intake and body-weight gain and on the Ca fractional retention during the first 14 days of life.
4) Including a novel phytase (1,000 FTU/kg) in low NPP diets (3.2 g NPP/kg) enhanced growth performance, P retention and ileal digestibility, as well as bone mineralization in broilers at d 35, to reach values similar to those broilers fed a diet with a 2 g/kg higher total P content.
5) No differences were observed among different commercial phytases and no interactions were observed between the dietary Ca:aP ratio and the different phytases.
6) Several mineral Ca and P sources show clear differences in their *in vitro* solubility at a wide pH range, and they promote differences in Ca and P ileum digestibility in 14-d-old broilers, which encourages the interest of using digestible Ca values on feed formulation.
7) However, Calcium chloride, which was the calcium source with the highest solubility and ileum digestibility, depressed feed intake and affected bone mineralization of the young chicks. No differences were observed among the studied sources of NPP, mono-calcium phosphate, di-calcium phosphate, and tri-calcium phosphate.

8) A high dose of Cu as di-copper oxide (150 ppm) improved BW of the animals. Nonetheless, high doses of $CuSO_4$ (300 mg/Cu/kg) may decrease performance (not observed with the use of Cu_2O), either as a result of decreased PP hydrolysis by phytase or as a consequence of the toxicity of Cu highly accumulatedin different organs. Further research may be warranted to determine the possible oxidative effects of these two copper sources and also the fate of the released anion after Cu dissociation.

CHAPTER 11

References

Abudabos, A.M. 2012. Intestinal phytase activity in chickens (Gallus Domesticus). *African Journal of Microbiology Research*, **6**: 4932-4938.

Adedokun, S. A., J. S. Sands, and A. Adeola. 2004. Determining the equivalent phosphorus released by an Escherichia coli-derived phytase in broiler chicks. *Canadian Journal of Animal Science*, **84**:437-444.

Adeola O., and A. J. Cowieson. 2011. Opportunities and challenges in using exogenous enzymes to improve nonruminant animal production. *Journal of Animal Science*, **89**:3189-3218.

Adeola O., N. D. Ryan, E. M. Onyango, and A. J. Jendza. 2005. Utilización del fósforo en aves y ganado porcino. XXI curso de especializacion FEDNA. pp 243-265.

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

Akter, M., H. Graham, and P. A. IJI. 2016. Response of broiler chickens to different levels of calcium, non-phytate phosphorus and phytase. *British Poultry Science*, DOI: 10.1080/00071668.2016.1216943.

Al Masri, M. R. 1995. Absorption and endogenous excretion of phosphorus in growing broiler chicks, as influenced by calcium and phosphorus ratios in feed. *British Journal of Nutrition*, **74**:407-415.

Angel, R. 2013. Calcium to phosphorus ratios in broilers. *Australian Poultry Science Symposium*, **24**:10-13. (Abstr.).

Angel, R., A.S. Dhandu, T. J. Applegate, and M. Christman, 2001. Phosphorus sparing effect of phytase, 25-hydroxycholecalciferol, and citric acid when fed to broiler chicks. *Poultry Science*, **80**:133-134.

Angel, R., E. Jimenez-Moreno, W. Li, S-W. Kim, M. Proszkowiec-Weglarz, and F. Escribano. 2015. Dietary calcium, phosphorus and phytase interactions. XXXI curso especialización FEDNA, pp 111-120.

Angel, R., N. Tamim, T. Applegate, A. Dhandu, and J. Ellestad. 2002. Phytic acid chemistry: influence on phytin-phosphorus availability and phytase efficacy. *The Journal of Applied Poultry Research*, **11**: 471-480.

Angel, R., T. J. Applegate, M. Christman, A. D.and Mitchell. 2000. Effect of dietary nonphytate phosphorus level on broiler performance and bone measurements in the starter and grower phase. *Poultry Science*, **79**:21-22.

Anwar M.N., V. Ravindran, P.C.H. Morel, G. Ravindran, and A.J. Cowieson. 2016. Effect of limestone particle size and calcium to non-phytate phosphorus ratio on true ileal calcium digestibility of limestone for broiler chickens. *British Poultry Science*, published online DOI: 10.1080/00071668.2016.1196341.

Aoyagi, S., and D. H. Baker. 1993. Estimates of Copper Bioavailability from Liver of Different Animal Species and from Feed Ingredients Derived from Plants and Animals. *Poultry Science*, **72**:1746-1755.

Applegate, T. J, D. M. Webel, and X. G. Lei. 2003. Efficacy of a Phytase Derived from Escherichia coli and Expressed in Yeast on Phosphorus Utilization and Bone Mineralization in Turkey Poults. *Poultry Science*, **82**:1726-1732.

Applegate, T. J., and R. Angel. 2004. Phytase: Basics of Enzyme Function, Purdue University Department of Animal Sciences.

Applegate, T. J., and R. Angel. 2008. Phosphorus requirements for poultry. A key ingredient in livestock and poultry nutrient management. USDA, NRCS CIG program.

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

Applegate, T. J., Richert, B. and Angel, R. 2008. Phytase and other phosphorus reducing feed ingredients.

Arias, V. J., and Koutsos E. A. 2006. Effects of copper source and level in intestinal physiology and growth of broiler chickens. *Poultry Science*, **85**: 999-1007.

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

Baker, D. H. 1989. Phosphorus supplements for poultry. Multistate Poult. Exten. Res. Newsletter. University Illinois 1(5):5.

Baker, D.H. 1999. Cupric Oxide Should Not Be Used As a Copper Supplement for Either Animals or Humans. *Journal of Nutrition*, **129**: 2278-2279.

Baker, D.H., J. Odle, M. A. Funk, and T. M. Wieland. 1991. Bioavailability of Cu in cupric oxide, cuprous and in a Cu- lysine complex. *Poultry Science,* **70**: 177-79.

Ballam, G.C., T. S. Nelson, and L. K. Kirby. 1985. Effect of different levels of calcium and phosphorus on phytate hydrolysis by chicks. *Nutrition Reports International Journal*, **32**: 909-913.

Banks K. M., K. L. Thompson, P. Jaynes, and T. J. Applegate. 2004b. The effects of Copper on the efficacy of phytase, growth, and phosphorus retention in Broiler Chicks. *Poultry Science*, **83**:1335-1341.

Banks, K. M., K. L. Thompson, J. K. Rush, and T. J. Applegate. 2004a. Effects of copper source on phosphorus retention in broiler chicks and laying hens. *Poultry Science*, **83**: 990-996.

Barrier-Guillot, B., P. Casado, P. Maupetit, C. Jondreville, and F. Gatel. 1996. Wheat phosphorus availability: 1-*In vitro* study; Factors affecting endogenous phytasic activity and phytic phosphorus content. *Journal of the Science of Food and Agriculture*, **70**:62-68.

Bernstein A. M., B. Titgemeier, K. Kirkpatrick, M. Golubic, and M. F. Roizen. 2013. Major cereal grain fibers and psyllium in relation to cardiovascular health. *Nutrients*, **5**: 1471-1487.

Biehl, R. R., and D. H. Baker. 1997. 1α-hydroxycholecalciferol does not increase the specific activity of intestinal phytase but does improve phosphorus utilization in both cecectomized and sham-operated chicks fed cholecalciferol-adequate diets. *Journal of Nutrition*, **127**: 2054-2059.

Bitar, K., and J.G. Reinhold. 1972. Phytase and alkaline phosphatase activities in intestinal mucosa of rat, chicken, calf and men. *Biochimica et Biophysica Acta*, **268**: 442-452.

Blaabjerg, K., N. G. Carlsson, J. Hansen-Moller, and H. D. Poulsen. 2010. Effect of heat-treatment, phytase, xylanase and soaking time on inositol phosphate degradation *in vitro* in wheat, soybean meal and rapeseed cake. *Animal Feed Science and Technology*, **162**:123-134.

Blake, G. M., and I. Fogelman. 2002. Methods and clinical issues in bone densitometry and quantitative ultra-sonometry. *Principles of Bone Biology*, **2**:1573-1585.

Bohak, Z. 1969. Purification and characterization of chicken pepsinogen and chicken pepsin. *The Journal of Biological Chemistry*, **244**:4638-4648.

Bradbury, E. J., S. J. Wilkinson, G. M. Cronin, C.L. Walk, and A. J. Cowieson. 2012. The effect of marine calcium source on broiler leg integrity. *Australian Poultry Science Symposium*, 85-88.

Brenes, A., A. Viveros, I. Arija, C. Centeno, M. Pizarro, and C. Bravo. 2003. The effect of citric acid and microbial phytase on mineral utilization in broiler chicks. *Animal Feed Science and Technology*, **110**:201-219.

Bronner, F. 2003. Mechanisms of intestinal calcium absorption. *Journal of Cellular Biochemistry*, **88**:387-393.

Brown, E. M. 2002. Biology of the extracellular Ca^{2+} sensing receptor. *Principles of Bone Biology*. Vol. **1**:371-387.

Browning, L. C., C. Antipatis, and A. J. Cowieson. 2012. The interactive effects of vitamin D, phytase, calcium, and phosphorus in broiler performance and skeletal integrity. *Australian Poultry Science Symposium*, **23**:81-84.

Bunch, R. J., J. T. Mccall, V. C. Speer, and V. W. Hays. 1965. Copper supplementation for weanling pigs. *Journal of Animal Science*, **24**:995-1000.

Burnell, T. W., G. L. Cromwell, and T. S. Stahly. 1990. Effects of particle size on the biological availability of calcium and phosphorus in defluorinated phosphate for chicks. *Poultry Science*, **69**:1110-1117.

Cabahug, S., Y. Ravindran, P. H. Selle, and W.Bryden. 1999. Response of broiler chicken to microbial phytase supplementation as influenced by dietary phytic acid and non-phytate phosphorus contents. 1. Effect on the bird performance and toe ash. *British Poultry Science*, **40**:660-666.

Carlton, W. W., and W. Henderson. 1964. Skeletal lesions in experimental copper deficiency in chickens. *Avian Diseases*, **8**:48-55.

Catala -Gregori, P., V. Garcıa, F. Hernandez, J. Madrid, and J.J. Ceron. 2006. Response of broilers to feeding low-calcium and phosphorus diets plus phytase under different environmental conditions: body weight and tibiotarsus mineralization. *Poultry Science*, **85**:1923-1931.

Champagne, E. T. 1988. Effects of pH on mineral phytate, protein-mineral-phyate and mineral-fiber interactions. Possible consequences of atrophic gastritis on mineral bioavailability from high-fiber foods. *The Journal of the American College of Nutrition*, **7**:499-508.

Champagne, E. T., M. S. Fisher, and O. Hinojosa. 1990. NMR and ESR studies of interactions among divalent cations, phytic acid, and N-acetyl-amino acids. *Journal of Inorganic Biochemistry*, **38**:199-215.

Chen, X., and E. T. Moran. 1995. The withdrawal feed of broilers: Carcass responses to dietary phosphorus. *The Journal of Applied Poultry Research*, **4**:69-82.

Cheryan, M. 1980. Phytic acid interactions in food systems. *Critical Reviews in Food Science and Nutrition*, **13**:297-335.

Chiou, P.W.S., C.L. Chen, and C.P. Wu. 1999. Effects of high dietary copper on the morphology of gastro-intestinal tract in broiler chickens. *Asian-Austalasian Journal of Animal Science,* **12**: 548-53.

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

Cobb. 2015**.** Broiler performance and nutrition supplement. cobb-vantress.com

Coon, C. N., and M. K. Manangi. 2004. The effect of a novel phytase on retainable phosphorus in broilers. pp 56-68

Coon, C. N., S. Seo, and M. K. Manangi. 2007. The determination of retainable phosphorus, relative biological availability, and relative biological value of phosphorus sources for broilers. *Poultry Science,* **86**:857-868

Coon, C., and K. Leske. 1998. Retainable phosphorus requirements for broilers. *Maryland Nutrition Conference*, p 18-31.

Cooper, R. J., and H. S. Gowing. 1983. Mammalian small intestine phytase (EC 3.1.3.8). *British Journal of Nutrition*, **50**:429-435.

Cosgrove, D.J. 1980. Inositolhexakis phosphates, Inositol Phosphates: Their Chemistry, Biochemistry and Physiology, pp 26-43.

Cowieson, A. J., and O. Adeola. 2005. Carbohydrase, protease, and phytase have an additive beneficial effect in nutritionally marginal diets for broiler chicks. *Poultry Science*, **84**:1860-1867.

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

Cromwell, G. L., 1980. Biological availability of phosphorus in feedstuffs for swine. *Feedstuffs,* **52**:38-12.

Cromwell, T. E., T. S. Stahly, and H. J. Monegue. 1989. Effect of source and level of Cu on performance and liver Cu stores in weanling pigs. *Journal of Animal Science*, **67**: 2996.

De Groote, G., and G. Huyghebaert. 1997. The bioavailability of phosphorus from feed phosphates for broilers as influenced by bioassay method, dietary Ca-level and feed form. *Animal Feed Science and Technology*, **69**:329-340.

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

De Vries, S., R. P. Kwakkel, and J. Dijkstra. 2010. Dynamics of Calcium and Phosphorus Metabolism in Laying Hensin. Phosphorus and calcium utilization and requirements in farm animals. CAB International. pp133-150.

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

Dersjant-Li, Y., A. Awati, H. Schulze, and G. Partridge. 2015. Phytase in non-ruminant animal nutrition: a critical review on phytase activities in the gastrointestinal tract and influencing factors. *Journal of the Science of Food and Agriculture*, **95**: 878-896.

Driver, J. P., G. M. Pesti, R. I. Bakalli, and H. M. Edwards. 2005a. Effects of calcium and nonphytate phosphorus concentrations on phytase efficacy in broiler chicks. *Poultry Science*, **84**:1406-1417.

Driver, J. P., G. M. Pesti, R. I. Bakalli, and H. M. Edwards. 2005b. Calcium requirements of modern broiler chicken as influenced by dietary protein and age. *Poultry Science*, **84**:1629-1639.

E.C. 2003. Opinion of the scientific committee for animal nutrition on the use of copper in feeding stuffs. European Commission health and consumer protection directorate-general.

Edwards, H. M. 2000. Nutrition and skeletal problems in poultry. *Poultry Science*, **79**:1018-1020.

Edwards, H. M., W. S. Dunahoo, J. L. Carmon, and H. L. Fuller. 1960. Effect of protein, energy and fat content of the ration on calcium utilization. *Poultry Science*, **39**:1389-1394.

Eeckhout, W., and M. De Paepe. 1994. Total phosphorus, phytate-phosphorus and phytase activity in plant feedstuffs. *Animal Feed Science and Technology*, **47**:19-29.

Eekhout, W., and M. De Paepe. 1997. The digestibility of three calcium phosphates for pigs as measured by difference and by slope-ratio assay. *Journal of Animal Physiology and Animal Nutrition*, **77**:53-60.

EFSA, 2012. Cu legal UE : *EFSA Journal*, 10(12):2969.

Eipper, B. A., and R. E. Mains. 1988. Peptide α-amidation. *Annual Review of Physiology*, **50**:333.

Emery, P. 2005. Metabolic changes in malnutrition Eye19, Department of Nutrition and Dietetics, King's College London, UK. pp 1029-1034.

European Parliament, 2010: Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

FEDNA. Fundación Española Desarrollo Nutrición Animal. 2008. Necesidades Nutricionales para Avicultura: Pollos de Carne y Aves de Puesta. R. Lázaro and G. G. Mateos, ed. Fundación Española para el Desarrollo de la Nutrición Animal, Madrid, Spain.

Fernandes, J. I. M., F. R. Lima, C. X. Mendonc, I. Mabe, R. Albuquerque, and P. M. Leal. 1999. Relative Bioavailability of Phosphorus in Feed and Agricultural Phosphates for Poultry. *Poultry Science,* **78**:1729-1736.

France, J., R. S. Dias, E. Kebreab, D. M. S. S. Vitti, L. A. Crompton, and S. Lopez. 2010. Kinetic models for the study of phosphorus metabolism in ruminants and monogastrics. Phosphorus and calcium utilization and requirements in farm animals. CAB International. pp 18-42.

Frandson, R. D., and T. L. Spurgeon. 1992. Anatomy and Physiology of Farm Animal, 5th Edition. Lea and Febiger, Philadelphia. pp 503-505.

Frankel, E. N., 1985. Pages 1-37 in: Flavor Chemistry of Fats and Oils. D. B. Min and T. H. Smouse, ed. American Oil Chemists' Society, Champaign, IL.

Gacs, G. and D. Baltrop. 1977. Significance of Ca-soap formation for calcium absorption in the rat. Gut **18**:64-68.

Georgievskii, V. I., B. N. Annenkov, and V. I. Samokhin. 1982. Mineral Nutrition of Animals. Butterworths, London.

Gibson, D. M., and A. B. J. Ullah. 1990. Inositol metabolism in plants. Eds. D.J. Morre, W.F.Boss y F.A. Loewus. Wiley-Liss, New York. pp: 77-92.

Gillis, M. B., H. M. Jr. Edwards, and R. J. Young. 1962. Studies on the availability of calcium orthophosphates to chickens and turkeys. *Journal of Nutrition*, **78**:155-161.

Gonzalez-Ortiz, G., W. Mirza, V. Pirgozliev, and M. Bedford. 2015. Efecto de la adicion de xilansasa y fitasa en pollos broiler alimentados con dietas en bsae a trigo. Page 241-245 in LII Simposio Científico de Avicultura 2015.

Goss, S. L., J. Prushko, and R. H. Bogner. 2010. Factors affecting calcium precipitation during neutralization in a simulated intestinal environment. *Journal of Pharmaceutical Sciences*, **99**:4183-4191.

Goss, S. L., K. A. Lemons, J. E. Kerstetter, and R. H. Bogner. 2007. Determination of calcium salt solubility with changes in pH and P(CO_2), simulating varying gastrointestinal environments. *Journal of Pharmacy and Pharmacology*, **59**:1485-1492.

Govers, M. J., D. S. Termont, J. A. Lapre, J. H. Kleibeuker, R. J. Vonk, and R. Van der Meer. 1996. Calcium in milk products precipitates intestinal fatty acids and secondary bile acids and this inhibits colonic cytotoxicity in humans. *Cancer research journal,* **56**:3270-3275.

Gregory, N. G., and L. J. Wilkins. 1990. Broken bones in chickens: Effect of stunning and processing in broilers. *British Poultry Science*, **31**:53-58.

Gregory, N. G., and L. J. Wilkins. 1992. Skeletal damage and bone defects during catching and processing. *Bone Biology and Skeletal Disorders in Poultry*. C. C. Whitehead, ed. Carfax Publishing Co., UK. pp 313-328.

Grimbergen, A. H. M., J. P. Cornelissen, and H. P. Stappers. 1985. The relative bioavailability of phosphorus in inorganic feed phosphates for young turkey and pigs. *Animal Feed Science and Technology,* **13**:117-130.

Guinotte, F., J. Gautron, Y. Nys, and A. Soumarmon. 1995. Calcium solubilization and retention in the gastrointestinal tract in chicks (Gallus domesticus) as a function of gastric acid secretion inhibition and of calcium carbonate particle size. *British Journal of Nutrition,* **73**:125-139.

Hamdi, M., R. Davin, D. Solà-Oriol, and J. F. Pérez. 2015b. Calcium sources and their interaction with the level of inorganic phosphorus affect performance and bone mineralization in broiler chickens. *Poultry Science,* **94**:2136-2143.

Hamdi, M., S. López-Vergé, E. G. Manzanilla, A. C. Barroeta, and J. F. Pérez. 2015a. Effect of different levels of calcium and phosphorus and their interaction on the performance of young broilers. *Poultry Science*, **94**: 2144-2151.

Harland, F. B., and E. R. Morris. 1995. Phytin: A good or a bad food component. *Nutrition Research,* **15**:733-754.

Havenstein, G. B., P. R. Ferket, S. E. Scheidler, and B. T. Larson. 1994. Growth, livability, and feed conversion of 1991 vs 1957 broilers when fed "typical" 1957 and 1991 broiler diets. *Poultry Science,* **73**:1785-1794.

Hawbaker, J. A., V. C. Speer, V. W. Hayes, and D. V. Catron. 1961. Effects of copper sulfate and other chemoprophylactics in growing swine rations. *Journal of Animal Science*, **20**:163-167.

Hegeman, C. E., and E. A. Grabau. 2001. A Novel Phytase with Sequence Similarity to Purple Acid Phosphatases Is Expressed in Cotyledons of Germinating Soybean Seedlings. *Journal of Plant Physiology*, **126**:1598-1608.

Hill, R. and C. Tyler. 1954a. The reaction between phytate and protein. *Journal of Agricultural Science*, **44**:324-326.

Hill, R. and C. Tyler. 1954b. The influence of time, temperature, pH and calcium carbonate on the activity of the phytase of certain cereals. *Journal of Agricultural Science*, **44**:306-310.
http://dx.doi.org/10.1080/00071668.2016.1196341.

Humer, E., C. Schwarz, and K. Schedle. 2014. Phytate in pig and poultry nutrition. *Journal of Animal Physiology and Animal Nutrition*, DOI: 10.1111/jpn.12258

Hurwitz, S. and A. Bar. 1971. Calcium and phosphorus interrelationships in the intestine of the fowl. *Journal of Nutrition*, **101**:677-686.

INRA.1989. Institut national de la recherche agronomique, France.

ISO. 2009. International standard, animal feeding stuffs determination of phytase activity, ISO30024:2009(E).

Jasaitis, D. K., J. E. Wohlt, and J. L. Evans. 1987. Influence of feed ion content on buffering capacity of ruminant feedstuffs *in vitro*. *Journal of Dairy Science*, **70**:1391-1403.

Jegede, A. V., O. O. Oduguwa, A. M. Bamgbose, A. O. Fanimo and L. Nollet. 2011. Growth response, blood characteristics and copper accumulation in organs of broilers fed on diets supplemented with organic and inorganic dietary copper sources. *British Poultry Science*, **52**:133-139.

Jenkins, N. K., T. R. Morris, and D. Valamotis. 1970. The effect of diet and copper supplementation on chick growth. *British Poultry Science*, **11**:241-248.

Jongbloed, A. W., and P. A. Kemme. 1990. Effect of pelleting mixed feeds on phytase activity and the apparent absorbability of phosphorus and calcium in pigs. *Animal Feed Science and Technology*, **28**: 233-242.

Joseph, H., and J. Scares. 1995. Phosphorus bioavailability, bioavailability of nutrients for animals; amino acids. *Minerals and vitamins*, pp 257-294.

Juin, H., Y. Nys, and J. Broz. 2001. Comparative evaluation of two phytase preparations in young turkeys fed a wheat-based diet. *Arch Geflügel* , **65**:231-35.

Julian, R. J. 1998. Rapid growth problems: ascites and skeletal deformities in broilers. *Poultry Science*, **77**:1773-1780.

Kebreab, E., and D. Vitti. 2005. Mineral metabolism. In Quantitative aspects of ruminant digestion and metabolism (ed. J Dijkstra, JM Forbes and J France), pp. 469-486. CAB International, Wallingford, UK.

Kerr, M. J., H. L Classen, and R. W. Newkirk. 2000. The effects of gastrointestinal tract micro-flora and dietary phytase on inositol hexaphosphate hydrolysis in the chicken. *Poultry Science*, **79** (Suppl. 1): 11 (Abstr.).

Kies, A. K., K. H. F. Van Hemert, and W. C. Sauer. 2001. Effect of phytase on protein and amino acid digestibility and energy utilization. *World's Poultry Science Journal*, **57**:110-124.

Knowles, T. G., and L. J. Wilkins. 1998. The problems of broken bones during handling of laying hens. *Poultry Science*, **77**:1798-1802.

Kornegay, E. T. 2001. Digestion of phosphorus and other nutrients: The role of phytases and factors influencing their activity. Enzymes in Farm Animal Nutrition. M. R. Bedford and G. G. Partridge, ed. CABI Publishing, Wallingford, UK. pp 239.

Kornegay, E. T., D. M. Denbow, Z. Yi, and V. Ravindran. 1996. Response of broilers to graded levels of microbial phytase added to maize-soyabean-meal-based diets containing three levels of non-phytate phosphorus. *British Journal of Nutrition*, **75**:839-852.

Kornegay, E.T. 1999. En: Biotechnology in the feed industry. Proc. Alltech's 15th Annual Symposium. Ed. T.P. Lyons y K.A. Jacques. Nottingham University Press. Reino Unido. pp: 461-490.

Krieger, C. H. 1938. Phytase and other phosphatases of the gastrointestinal tract. Master Thesis, University of Wisconsin, Madison, Wisconsin, U.S.A.

LaBella, F., T. Dular, S. Vivian, and G. Queen. 1973. Pituitary hormone releasing or inhibiting activity of metal ions present in hypothalamic extracts. *Biochemical and Biophysical Research Communications*, **52**: 786.

Lan, G.Q., N. Abdullah, S. Jalaludin, and Y. W. Ho. 2002. Efficacy of Supplementation of a Phytase-Producing Bacterial Culture on the Performance and Nutrient Use of Broiler Chickens Fed Corn-Soybean Meal Diets. *Poultry Science,* **81**:1522-1532.

Larbier, M., and B. Leclercq. 1992. Nutrition et alimentation des volailles par INRA Editions.

Lawlor, P. G., P. B. Lynch, P. J. Caffrey, J. J. O'Reilly, and M. K. O'Connell. 2005. Measurements of the acid-binding capacity of ingredients used in pig diets. *Irish Veterinary Journal*, **58**:447-452.

Lei, X.G, P. k. Ku, E. R. Millar, M. T. Yokoyama, and D. E. Ullrey. 1994. Calcium level affects the efficacy of supplemental microbial phytase in corn-soybean diets of weanling pigs. *Journal of Animal Science,* **72**:139-143.

Leske, K. L. and C. N. Coon. 2002. The development of feedstuff retainable phosphorus values for broilers. *Poultry Science,* **81**:1681-1693.

Létourneau-Montminy, M. P., C. Jondreville, C. Pomar, M. Magnin, D. Sauvant, J. Bernier, Y. Nys, and P. Lescoat. 2007. *16ème Symposium Européen de Nutrition des Volailles*, Strasbourg, pp 117-120.

Létourneau-Montminy, M. P., P. Lescoat, A. Narcy, D. Sauvant, J. F. Bernier, M. Magnin, C. Pomar, Y. Nys, and C. Jondreville. 2008. Effects of reduced dietary calcium and phytase supplementation on calcium and phosphorus utilization in broilers with modified mineral status. *British Poultry Science*, **49**:705-715.

Li, W., R. Angel, S. W. Kim, E. Jimenez-Moreno, M. Proszkowiec-Weglarz, and P. W. Plumstead. 2014. Age and adaptation effects to Ca and P deficiencies: Effect on P digestibility. *Poultry Science*, 93:84 (Abstr).

Li, W., R. Angel, S. W. Kim, K. Brady, S. Yu, and P. W. Plumstead. 2016. Impacts of dietary calcium, phytate, and nonphytate phosphorus concentrations in the presence or absence of phytase on inositol hexakisphosphate (IP6) degradation in different segments of broilers digestive tract. *Poultry Science,* **95**:581-589.

Liebert, F., C. Wecke, and F. J. Schöner. 1993. En: Proc. *1st Symposium on* Enzymes in Animal Nutrition. Ed. C. Wenk y M. Boessinger. pp: 202-205.

Lima, F. R., C. X. Mendonc, J. C. Alvarez, H. J. M. F. Garzillo, E. Ghion, and P. M. Leali. 1997. Biological evaluations of commercial dicalcium phosphates as sources of available phosphorus for broiler chicks. *Poultry Science,* **76**:1707-1713.

Lima, F. R., C. X. Mendonca, J. C. Alvarez, G. Ratti, S. L. R. Lenharo, H. Kahn, and J. M. F. Garzillo, 1995. Chemical and physical evaluations of commercial dicalcium phosphates as sources of phosphorus in animal nutrition. *Poultry Science*, **74**:1659-1670.

Lönnerdal, B. 2000. Dietary factors influencing zinc absorption. *Journal of Nutrition*, 130:1378S-1383S.

Lonnerdal, B., A. S. Sandberg, B. Sandstrom, and C. Kunz. 1989. Inhibitory effects of phytic acid and other inositol phosphates on zinc and calcium absorption in suckling rats. *Journal of Nutrition*, **119**:211-214.

Lott, J. N. A., I. Ockenden, V. Raboy, and G.D. Batten. 2000. Phytic acid and phosphorus in crop seed and fruits: a global estimate. *Seed Science Research Journal*, **10**: 11-33.

Lott, J. N. A., I. Ockenden, V. Raboy, and G.D. Batten. 2002. Phytic acid and phosphorus in crop grains, seeds and fruits. In NR Reddy, SK Sathe, eds, Food Phytate. CRC Press, Boca Raton, FL, pp 7-24.

Lu, L., R. L. Wang, Z. J. Zhang, F. A. Steward, and B. Liu. 2010. Effect of Dietary Supplementation with Copper Sulfate or Tri-basic Copper Chloride on the Growth Performance, Liver Copper Concentrations of Broilers Fed in Floor Pens, and Stabilities of Vitamin E and Phytase in Feeds. *Biological Trace Element Research*, **138**: 181-189.

Lundy, M. W., J. E. Russell, J. Avery, J. E. Wergedal, and D. J. Baylink. 1992. Effect of sodium fluoride on bone density in chickens. *Calcified Tissue International*, **50**:420-426.

Maddaiah, V. T., A. A. Kurnich, B. J. Hulett, and B. L. Reid. 1969. Nature of intestinal phytase activity. *Proceedings of The Society for Experimental Biology and Medicine*, **115**: 1054-1056.

Maenz, D. D. 2001. Enzymatic characteristics of phytases as they relate to their use in animal feeds. Enzymes in Farm Animal Nutrition. M. R. Bedford and G. G. Partridge. CABI Publishing, Wallingford, UK. pp 72-76.

Maenz, D. D., and H. L. Classen. 1998. Phytase Activity in the Small Intestinal Brush Border Membrane of the Chicken. *Poultry Science*, **77**:557-563

Maenz, D. D., C. M. Engele-Schaan, R. W. Newkirk, and H. L. Classen. 1999. The effect of minerals and mineral chelators on the formation of phytase-resistant and phytase-susceptible forms of phytic acid in solution and in a slurry of canola meal. *Animal Feed Science and Technology*, **81**:177-192.

Manangi, M. K., and C. N. Coon. 2007. The effect of calcium carbonate particle size and solubility on the utilization of phosphorus from phytase for broilers. *International Journal of Poultry Science*, **6**:85-90.

Manangi, M. K., and C. N. Coon. 2008. Phytate phosphorus hydrolysis in broilers in response to dietary phytase, calcium, and phosphorus concentrations. *Poultry Science*, **87**:1577-1586.

Marounek, M., M. Skřivan, O. Rosero, and O. Rop. 2010. Intestinal and total tract phytate digestibility and phytase activity in the digestive tract of hens fed a wheat-maize-soyabean diet. *Animal Feed Science and Technology*, **19**: 430- 439

Masey- O'neill, H.V., M. R. Bedford, and N. Walker. 2014. Recent developments in feed enzyme technology. *Recent Advances in Animal Nutrition*, 97-106.

McDowell, L. R. 1992. Copper and molybdenum. In: T. J. Cunha (ed.) Minerals in Animal and Human Nutrition. pp 235-276. Academic Press, San Diego, CA.

McGuaig, L. W., M. I. Davies, and I. Motzok. 1972. Intestinal alkaline phosphatase and phytase of chicks: Effect of dietary magnesium, calcium, phosphorus and thyroactive casein. *Poultry Science*, **51**:526-530.

Menezes-Blackburn, D., S. Gabler, and R. Greiner. 2015. Performance of Seven Commercial Phytases in an in Vitro Simulation of Poultry Digestive Tract. *Journal of Agricultural and Food Chemistry*, **63**: 6142-6149.

Miles, R. D., S. F. O'Keefe, P. R. Henry, C. B. Ammerman, and X. G. Luo. 1998. The effect of dietary supplementation with copper sulfate or tribasic copper chloride on broiler performance, relative copper bioavailability, and dietary prooxidant activity. *Poultry Science*, **77**:416-425.

Mitchell, R. D., and H. M. Edwards. 1996a. Additive effects of 1,25 dihydroxy cholecalciferol and phytase on phytate phosphorus utilization and related parameters in broiler chickens. *Poultry Science*, **75**:111-119.

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

Mohammed, A., M. J. Gibney, and T. G. Taylor. 1991. The effects of dietary levels of inorganic phosphorus, calcium and cholecalciferol on the digestibility of phytate-P by the chick. *British Journal of Nutrition*, **66**: 251-259.

Moran, E. T., and M. C. Todd. 1994. Continous submarginal phosphorus with broilers and the effect of preslaughter transportation: Carcass defects, further-processing yields, and tibia-femur integrity. *Poultry Science*, **73**:1448-1457.

Nelson, T. S. 1984. Available calcium for poultry. Proc. Florida Nutr. Conf. Feed Manuf, pp 1-7. Orlando, Florida.

NRC. 1994. Nutritional Requirements of Poultry. 9th rev. ed. National Academy Press, Washington, DC.

Oatway, L., T. Vasanthan, and J. H. Helm. 2001.Phytic acid. *Food Reviews International*, **17**:419-431.

Oberleas. 1973. Oberleas, D. Phytates. In: Toxicants occurring naturally in foods, National Academy of Sciences, Washington, DC, p 363.

Olukosi, O. A., C. Kong, F. Fru-Nji, K. M. Ajuwon, and O. Adeola. 2013. Assessment of a bacterial 6-phytase in the diets of broiler chickens. *Poultry Science*, **92**: 2101-2108.

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

Onyango, E. M., P. Y. Hester, R. Stroshine, and O. Adeola. 2003. Bone densitometry as an indicator of percentage tibia ash in broiler chicks fed varying dietary calcium and phosphorus levels. *Poultry Science*, **82**:1787-1791.

Osphal, W., H. Zeronian, M. Ellison, D. Lewis, R. S. Rucker, and R. Riggins. 1982. Role of copper in collagen-linking and its influence on selected mechanical properties of chick bone and tendon. *Journal of Nutrition*, **12**: 708-716.

Pallauf, J., and G. Rimbach. 1997. Effect of supplemental phytase on mineral and trace element bioavailability and heavy metal accumulation in pigs with different type diets. In *Phytase in Animal Nutrition and Waste Management*. M. B. Coehlbo and E. T. Kornegay, ed. BASF Publication DC9601. BASF Corporation, Mount Olive, NJ.

Pang, Y. and T. J. Applegate. 2007. Effects of Dietary Copper Supplementation and Copper Source on Digesta pH, Calcium, Zinc, and Copper Complex Size in the Gastrointestinal Tract of the Broiler Chicken. 2007. *Poultry Science*, **86**:531-537.

Pang, Y., and T. J. Applegate. 2006. Effects of copper source and concentration on *in vitro* phytate phosphorus hydrolysis by phytase. *Journal of Agricultural and Food Chemistry*, **54**:1792-1796.

Pang, Y., J. A. Patterson, and T. J. Applegate. 2009. The influence of copper concentration and source on ileal microbiota. *Poultry Science,* 88:586-592.

Parkart, L. 1987. Iamin: A human growth factor with multiple wound healing properties. In: J.R.J. Sorenson (Ed.) Biology of Copper Complexes. p 273. Humana Press, Clifton, NJ.

Payne, R. L., T. K. Lavergne, and L. L. Southern. 2005. Comparison of Two Sources of Phytase in Liquid and Dry Forms in Broilers. *Poultry Science,* **84**:265-272.

Pearse, A. G. E. 1980. Histochemistry. Theoretical and Applied. Vol. 1. Preparative and optical technology. Ed. P. J. Stoward, Churchill Livingstone, Edinburgh, Scotland.

Peeler, H.T. 1972. Biological availability of nutrients in feeds: availability of major mineral ions. *Journal of Animal Science,* **35**: 695-712.

Peers, F. G. 1953. The phytase of wheat. *Biochemical Journal*, **53**: 102-110.

Pepper, W. F., S. J. Slinger, and I. Motzok. 1955. Effect of animal fat on the calcium and phosphorus requirements of chicks. *Poultry Science,* 34:1216 (Abstr.).

Perry, R. W., G. N. Rowland, T. L. Foutz, and J. R. Glisson. 1991. Poultry malabsorption syndrome. III. Skeletal lesions in market age turkeys. *Avian Diseases*, **35**:707-713.

Persson, H., M. Turk, M. Nyman, and A. S. Sandberg. 1998. Binding of Cu^{2+}, Zn^{2+}, and Cd^{2+} to inositol tri-, tetra-, penta, and hexaphosphates. *Journal of Agricultural and Food Chemistry*, **46**:3194-3200.

Pesti, G. M., and R. I. Bakalli. 1996. Studies on the feeding of cupric sulfate pentahydrate and cupric citrate to broiler chickens. *Poultry Science,* **75**:1086-1091.

Petersen, G. I., C. Pedersen, M. D. Lindemann, and H. H. Stein. 2011. Relative bioavailability of phosphorus in inorganic phosphorus sources fed to growing pigs. *Journal of Animal Science*, **89**:460-466.

Phillippy, B. Q. 1999. Susceptibility of wheat and Aspergillus niger phytases to inactivation by gastrointestinal enzymes. *Journal of Agricultural and Food Chemistry,* **47**: 1385-1388.

Phillips, R., R. Angel, E. Jimenez-Moreno, S. W. Kim, S. L. Vieira, M. DeBeer, N. Ward, and F. Fru. 2012. Calcium and phosphorus requirements for two strains of broilers from hatch to 10 days of age. *Poultry Science,* 91(Suppl. 1):23 (abst).

Plumstead, P. W., A. B. Leytem, R. O. Maguire, J. W. Spears, P. Kwanyuen, and J. Brake. 2008. Interaction of calcium and phytate in broiler diets: 1. Effects on apparent prececal digestibility and retention of phosphorus. *Poultry Science*, **87**: 449-458.

Pointillart, A. 1994. Phytates, phytases: leur importance dans l'alimentation des monogastriques. Inra Productions Animales, **7**: 29-39.

Pointillart, A., N. Fontaine, M. Thomasset, and M. E. Jay. 1985. Phosphorus utilization, intestinal phosphatases and hormonal control of calcium metabolism in pigs fed phytic phosphorus: soyabean or rapeseed diets. *Nutrition reports international*, **32**:155-167.

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

Proszkowiec-Weglarz, M., and R. Angel. 2013. Calcium and phosphorus metabolism in broilers: Effect of homeostatic mechanism on calcium and phosphorus digestibility.*The Journal of Applied Poultry Research*, **22**: 609-627.

Qian, H., E. T. Kornegay, and D. M. Denbow. 1997. Utilization of phosphorus and calcium as influenced by microbial phytase, cholecalciferol, and the calcium/total phosphorus ratio in broiler diets. *Poultry Science,* **76**:37-46.

Qian, H., H. P. Veit, E. T. Kornegay, V. Ravindran, and D. M. Denbow. 1996. Effects of supplemental phytase and phosphorus on histological and other tibial bone characteristics and performances of broilers fed semi-purified diets. *Poultry Science*, **75**: 618-626.

Raboy, V. 2009. Approaches and challenges to engineering seed phytate and total phosphorus. *Plant Science*, **177**: 281-296.

Raboy, V., and D. B. Dickinson. 1987. The timing and rate of phytic acid accumulation in developing soybean seeds. *Plant Physiology*, **85**:841-844.

Rama-Rao, S. V., M. V. L. N. Raju, and M. R. Reddy. 1999. Non-phytin requirements of comercial broilers and white leghorn layers. *Animal Feed Science and Technology*, **80**:1-10.

Rama-Rao, S. V., M. V. L. N. Raju, M. R. Reddy, and P. Pavani. 2006. Interaction between dietary calcium and non-phytate phosphorus levels on growth, bone mineralization and mineral excretion in commercial broilers. *Animal Feed Science and Technology,* **131**:133-148.

Rama-Rao, S.V., A. K. Panda, M. V. L. N. Raju, G. Shyam Sunder, and N. K. Praharaj. 2003. Requirement of calcium for commercial broilers and White Leghorn layers at low dietary phosphorus levels. *Animal Feed Science and Technology*, **106**: 199-208.

Rath, N. C., G. R. Huff, W. E. Huff, and J. M. Balog. 2000. Factors Regulating Bone Maturity and Strength in Poultry. *Poultry Science,* **79**:1024-1032.

Rath, N. C., J. M. Balog, W. E. Huff, G. R. Huff, G. B. Kulkarni, and J. F. Tierce. 1999. Comparative difference in the composition and biomechanical properties of tibiae of seven- and seventy-two-week-old male and female broiler breeder chickens. *Poultry Science*, **78**:1232-1239.

Ravindran, V., E. T. Kornegay, L. M. Potter, B. O.Ogunabameru, M. K.Welten, J. H. Wilson, and M. Potchanakorn. 1995. An evaluation of various response criteria in assessing biological availability of Phosphorus for Broilers. *Poultry Science,* **74**:1820-1830.

Ravindran, V., P. H. Selle, G. Ravindran, P. C. H. Morel, A. K. Kies, and W. L. Bryden. 2001. Microbial phytase improves performance, apparent metabolizable energy, and ileal amino acid digestibility of broilers fed a lysine-deficient diet. *Poultry Science,* **80**: 338-344.

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

Ravindran, V., S. Cabahug, G. Ravindran, W. L. Bryden, and P. H. Selle. 2000. Response of broilers to microbial phytase supplementation as influenced by dietary phytic acid and non-phytate phosphorus levels. II. Effects on nutrient digestibility and retention. *British Poultry Science*, **41**:193-200.

Ravindran, V., W. L. Bryden, and E. T. Kornegay. 1995. Phytates: Occurrence, bioavailability, and implications in poultry nutrition. *Poultry and Avian Biology Reviews*, **6**:125-143.

Rebollar, P. G., and G. G. Mateos. 1999. El fósforo en nutrición animal necesidades, valoración de materias primas y mejora de la disponibilidad. XV Curso de Especialización: avances en nutrición y alimentación animal.

Reece, W. O., H. H. Erickson, J. P. Goff, and E. E. Uemura .2015. Dukes' Physiology of Domestic Animals, 13th Edition. Wiley-Blackwell.

Reichmann, K. G., and J. K. Connor. 1977. Influence of dietary calcium and phosphorus on metabolism and production in laying hens. *British Poultry Science,* **18**:633-640.

Rennie, J. S., R. H. Flemming, H. A. McCormack, C. C. McCorquodale, and C. C. Whitehead. 1997. Studies on effects of nutritional factors on bone structure and osteoporosis in laying hens. *British Poultry Science,* **38**:417- 424.

Robbins, K. R., and D. H. Baker. 1980. Effect of sulfur amino acid level and source on the performance of chicks fed high levels of copper. *Poultry Science,* **59**:1246-1253.

Rodehutscord, M., and A. Dieckmann. 2005. Comparative studies with three-week-old chickens, turkeys, ducks, and quails on the response in phosphorus utilization to a supplementation of monobasic calcium phosphate. *Poultry Science,* **84**:1252-1260.

Rucker, R. B., R. S. Riggins, R. Laughlin, M. M. Chan, and M. T. Chen. 1975. Effects of nutritional copper deficiency on the biomechanical properties of bone and arterial elastin metabolism in the chick. *Journal of Nutrition* **105**:1062-1070.

Rucker, R., H. Parker, and J. C. Rogler. 1968. Utilization of calcium and phosphorus from hydrous and anhydrous dicalcium phosphates. *Journal of Nutrition*, **96**: 513-518.

Rutherfurd, S. M., T. K. Chung, and P. J. Moughan. 2002. The effect of microbial phytase on ileal phosphorus and amino acid digestibility in the broiler chicken. *British Poultry Science,* **44**:598-606.

Samanta, B., A. Biswas and P. R. Ghosh. 2011. Effects of dietary copper supplementation on production performance and plasma biochemical parameters in broiler chickens. *British Poultry Science,* **52**:573-577.

Sandberg, A. S. and T. Andlid. 2002. Phytogenic and microbial phytases in human nutrition. *International Journal of Food Science and Technology,* **37**: 823-833.

Sands, S., R. Stilborn, J. Berg, and R. E. Salmon. 2003. Comparative Efficacy of two Microbial Phytases for improving performance in Broilers Fed Low-P Diets. Concurrent Meetings of International Poultry Scientific Forum. pp 30. (Abstr.)

Sauveur, B. 1989. Phosphore phytique et phytases dans l'alimentation des volailles. *Inra Productions Animales*, **2**: 343-351.

Schoner, F-J., P. P. Hoppe, and G. Schwartz. 1991. Comparative effects of microbial phytase and inorganic phosphorus on performance and retentions of phosphorus,

calcium and crude ash in broilers. *Journal of Animal Physiology and Animal Nutrition*, **66**:248-255.

Schoner, F-J., P. P. Hoppe, G. Schwartz, and H. Weische. 1993. Comparison of microbial phytase and inorganic phosphate in male chickens: the influence on performance data, mineral retention and dietary calcium. *Journal of Animal Physiology and Animal Nutrition*, **69**:235-244.

Scott, M. L., M. C. Nesheim, and R. J. Young. 1982. Essential inorganic nutrients. Nutrition of the Chicken, 3rd Edition. M.L. Scott and Associates, Ithaca, New York, pp 288-304.

Sebastian, S., S. P. Touchburn, E. R. Chavez, and P. C. Lague. 1996. Efficacy of supplemental microbial phytase at different dietary calcium levels on growth performance and mineral utilization on broilers chickens. *Poultry Science*, **75**:1516-1523.

Selle, P. H., A. J. Cowieson, and V. Ravindran. 2009. Consequences of calcium interactions with phytate and phytase for poultry and pigs. *Livestock Science*, **124**:126-141.

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

Selle, P. H., V. Ravindran, G. Ravindran, and W. L. Bryden. 2007. Effects of dietary lysine and microbial phytase on growth performance and nutrient utilisation of broiler chickens. *Asian-Australian Journal of Animal Science*, **20**: 1100-1107.

Selle, P. H., V. Ravindran, R. A. Caldwell, and W. L. Bryden. 2000. Phytate and phytase: Consequences for protein utilisation. *Nutrition Research Reviews*, **13**:255-278.

Shafey, T. M. 1993. Calcium tolerance of growing chickens: effect of ratio of dietary calcium to available phosphorus. *Worlds Poultry Science Journal*, **49**: 5-18.

Shafey, T. M. 1999. Effects of high dietary calcium and fat levels on the performance, intestinal pH, body composition and size and weight of organs in growing chickens. *Asian-Australian Journal of Animal Science*, **12**:49-55.

Shafey, T. M. and M. W. Mcdonald. 1991. The effects of dietary calcium, phosphorus and protein on the performance and nutrient utilization of broiler chickens. *Poultry Science*, **70**:548-553.

Shafey, T. M., M. W. McDonald, and J. G. Dingle. 1991. Effects of dietary calcium and available phosphorus concentration on digesta pH and on the availability of Ca,

Fe, Mg and Zn from the intestinal content of meat chickens. *British Poultry Science*, **32**:185-194.

Shahkalili, Y., C. Murset, I. Meirim, E. Duruz, S. Guinchard, C. Cavadini, and K. Acheson. 2001. Calcium supplementation of chocolate: effect on cocoa butter digestibility and blood lipids in humans. *The American Journal of Clinical Nutrition,* **73**:246-252.

Shang, Y., A. Rogiewicz, R. Patterson, B. A. Slominski, and W. K. Kim. 2015. The effect of phytase and fructooligosaccharide supplementation on growth performance, bone quality, and phosphorus utilization in broiler chickens. *Poultry Science*, **94**:955-964.

Shastak, Y., M. Witzig, K. Hartung, and M. Rodehutscord. 2012. Comparison of retention and prececal digestibility measurements in evaluating mineral phosphorus sources in broilers. *Poultry Science,* **91**:2201-2209.

Shirley, R. B. and H. M. Edwards. 2003. Graded levels of phytase past industry standards improve broiler performance. *Poultry Science,* 82:67-680.

Simons, P. C. M., H. A. J. Versteegh, A. W. Jongbloed, P. A. Kemme, P. Slump, K. D. Bos, M. G. E. Wolters, R. F. Beudeker, and G. J. Verschoor. 1990. Improvement of phosphorus availability by microbial phytase in broilers. *British Journal of Nutrition,* **64**:525-540.

Simpson, C. J., and A. Wise. 1990. Binding of zinc and calcium to inositol phosphates (phytate) *in vitro*. *British Journal of Nutrition*, **64**:225-232.

Smith, A. K., and J. J. Rackis. 1957. Phytin elimination in soybean protein isolation. *Journal of the American Chemical Society*, **79**:633-637.

Sooncharernying, S., and H. M. Edwards. 1993. Phytate content of excreta and phytate retention in the gastrointestinal tract of young chickens. *Poultry Science*, **72**:1906-1916.

Suttle, N. F. 2010. Mineral Nutrition of Livestock, 4th Edition.

Tamim N. M., and R. Angel. 2003. Phytate Phosphorus Hydrolysis as Influenced by Dietary Calcium and Micro-Mineral Source in Broiler Diets. *Journal of Agricultural and Food Chemistry,* **51**:4687-4693.

Tamim, N. M., R. Angel, and M. Christman. 2004. Influence of dietary calcium and phytase on phytate phosphorus hydrolysis in broiler chickens. *Poultry Science,* **83**:1358-1367.

Taylor, T. G. 1965. The availability of the calcium and phosphorus of plant materials for animals. *Proceedings of the Nutrition Society*, **24**:105-112.

The international Union of Biochemists International Union of Biochemists (IUB). 1979. Enzyme nomenclature. Page 247 in Recommendations of the Nomenclature Committee of the International Union of Biochemistry. Academic Press, New York.

The Merck Index, 1990. 10th ed. Merck and Co., Inc. Rahway, NJ.

Toru, I., M. Takahashi, K. Oda, Y. Terada, and K. T. Yoshida. 2012. Dynamic Changes in the Distribution of Minerals in Relation to Phytic Acid Accumulation during Rice Seed Development. *Plant Physiology*, **160**: 2007-2014.

Underwood, E. J. 1981. The Mineral Nutrition of Livestock, 2nd ed. Commonwealth Agricultural Bureaux.

Venäläinen, E., J. Valaja, and T. Jalava. 2006. Effects of dietary metabolisable energy, calcium and phosphorus on bone mineralization, leg weakness and performance of broiler chickens. *British Poultry Science*, **47**:301-310.

Veum, T. L. 2010. Phosphorus and Calcium Nutrition and Metabolism. Phosphorus and calcium utilization and requirements in farm animals. CAB International 2010. pp 94-111.

Viljoen. 2001. Quality of feed phosphate supplements for animal nutrition. SA-ANIM SCI. vol 2: http://www.sasas.co.za/Popular/Popular.html

Viveros, A., A. Brenes, I. Arija, and C. Centeno. 2002. Effects of Microbial Phytase Supplementation on Mineral Utilization and Serum Enzyme Activities in Broiler Chicks Fed Different Levels of Phosphorus . *Poultry Science,* **81**:1172-1183.

Vohra, P., G. A. Gray, and F. H. Kratzer. 1965. Phytic acid-metal complexes. *Proceedings of the Society for Experimental Biology and Medicine,* **120**:447-449.

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

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

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

Walk, C. L., M. R. Bedford, and A. P. McElroy. 2012c. *In vitro* evaluation of limestone, dicalcium phosphate, and phytase on calcium and phosphorus solubility of corn and soybean meal. *Poultry Science*, **91**:674-682.

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

Ward T. L., K. L. Watkins and L. L. Southern. 1994. Interactive effects of dietary copper and water copper level on growth, water intake, and plasma and liver copper concentrations of poults. *Poultry Science,* **73**:1306-1311.

Wasserman, R. H., and A. N. Taylor. 1973. Intestinal absorption of phosphate in the chick. Effect of vitamin D_3 and other parameters. *Journal of Nutrition*, **103**:586-599.

Wilcox, R. A., C. W. Carlson, W. Kohlmeyer, and G. F. Gastler. 1954. The availability of phosphorus from different sources for poults fed purified diets. *Poultry Science,* **33**: 1010-1014.

Wilkinson, S. J., P. H. Selle, M. R. Bedford, and A. J. Cowieson. 2012. Exploiting the calcium specific appetite of broilers. *Australian Poultry Science Symposium*, **23**:48-51(Abstr.).

Williams, B., D. Waddington, S. Solomon, and C. Farquharson. 2000. Dietary effects on bone quality and turnover and Ca and P metabolism in chickens. *Research in Veterinary Science*, **69**:81-87.

Wise, A. 1983. Dietary factors determining the biological activities of phytate. *Nutrition Abstracts and Reviews*, **53**:791-806.

Wise, A., and D. J. Gilburt. 1982. Binding of cadmium and lead to the calcium–phytate complex *in vitro*. *Toxicology Letters,* **9**:45-50.

Wodzinski, R. J., and A. H. J. Ullah. 1996. Phytase. *Advances in Applied Microbiology*, **42**: 263-302.

Woyengo, B. A. Slominski, and R. O. Jones. 2010. Growth performance and nutrient utilization of broiler chickens fed diets supplemented with phytase alone or in combination with citric acid and multicarbohydrase. *Poultry Science,* **89**:2221-2229.

Yan, F., J. H. Kersey, and P. W. Waldroup. 2001. Phosphorus Requirements of Broiler Chicks Three to Six Weeks of Age as Influenced by Phytase Supplementation. *Poultry Science,* **80**:455-459.

Yi, Z., E. T. Kornegay, V. Ravindran, and D. M. Denbow. 1996. Improving phytate phosphorus availability in corn and soybean meal for broilers using microbial phytase and calculation of phosphorus equivalency values for phytase. *Poultry Science*, **75**:240-249.

Yonemochi, C., H. Takagi, M. Hanazumi, S. Hijikuro, K. Koide, T. Ina, and T. Okada. 2000. Improvement of phosphorus availability by dietary supplement of phytase in broiler chicks. *Japanese poultry science*, **37**:154-161.

Yu, B., Y. C. Jan, T. K. Chung, T. T. Lee, and P. W. S. Chiou. 2004. Exogenous phytase activity in the gastrointestinal tract of broiler chickens. *Animal Feed Science and Technology*, **117**:295-303.

Zhou, W., E. T. Kornegay, M. D. Lindemann, J. W. Swinkels, M. K. Welten, and E. A. Wong. 1994. Stimulation of growth by intravenous injection of copper in weanling pigs. *Journal of Animal Science*, **72**:2395-2403.

Ziaei, N., J. H. Guy, S. A. Edwards, P. J. Blanchard, J. Ward, and D. Feuerstein. 2008. Effect of reducing dietary mineral content on growth performance, water intake, excreta dry matter content and blood parameters of broilers. *British Poultry Science*, **49**:195-201.

Zyla K., M. Mika, B. Stodolak, A. Wikiera, J. Koreleski, and S. Swiatkiewicz. 2004. Towards Complete Dephosphorylation and Total Conversion of Phytates in Poultry Feeds. *Poultry Science*, **83**:1175-1186.

Zyla, K., D. R. Leudoux, and T. L.Veum. 1995. Complete enzymic de phosphorylation of corn soybean meal feed under simulated intestinal conditions of the turkey. *Journal of Agricultural and Food Chemistry*, **43**:288-294.

Curriculum Vitae

Curriculum vitae

Personal information

Surname, Name: Hamdi, Manel Nationality: Tunisian

E-mail: manelhamdi@ymail.com Date of birth: 21/12/1987

Phone**: +34654965062 / +21621313656**

Education

2013-present **Ph.D. Student in Animal Science**

Universitat Autònoma de Barcelona

2011-2013 **M.Sc. in Research in Animal Nutrition**

The Mediterranean Agronomic Institute of Zaragoza (IAMZ)

2011-2012 **Postgraduate Specialisation in Animal Nutrition**

The Mediterranean Agronomic Institute of Zaragoza (IAMZ)

2006-2011 **Engineer in animal production and feed sciences**

University of Carthage Tunisia

Post-graduate courses

2016 **Training Course for Researchers on Laboratory Animal Management**

Universitat Autònoma de Barcelona

2016 **Mendeley Institucional basic**

Universitat Autònoma de Barcelona

2015 **Writing and Presenting Scientific Papers Wiley Workshop**

Universitat Autònoma de Barcelona

2014 **Biological Agents: Risk and Preventive Measures**

Universitat Autònoma de Barcelona

2013 **Workshop Rapid Methods and Automation in Food Microbiology**

Universitat Autònoma de Barcelona

Professional experience

March-June 2016 **Practical training in Schothorst Feed Research (SFR),**

Netherlands

2013-present **Member of the Animal Nutrition and Welfare Service (SNiBA)**

Technical Department Assistant

Universitat Autònoma de Barcelona (Bellaterra)

- Collaboration in several research projects (experimental design, farm controls, laboratory analyses, and statistical analyses)
- Collaboration in practical sessions of the subject Animal Production and Management (Veterinary Degree)
- Write technical report related to poultry experiments

2013 **Practical training (1 month)**

"Laboratory Agro-Ambiental De Aragon" in Zaragoza

2011 **Engineering in practice (6 Months)**

Higher School of Agriculture Mateur (Tunisia)

2010 Improvement practices at a farm of dairy cows 'Amila' located northern Tunisia

2009 Practices at the Office Regional Agricultural Development Bizerte, at the Department of Animal Production.

Fellowships

2013-present **Pre-doctoral research grant of department of animal and feed sciences**

Universitat Autònoma de Barcelona (Bellaterra)

2011-2013 **Scholarship to attend the Master of sciences in animal nutrition**

International Centre for Advanced Mediterranean Agronomic Studies (CIHEAM)

Scientific publications

Manel Hamdi, David Solà Oriol, Rosa Franco Rosselló, Stéphane Durosoy, José Francisco Pérez .Including copper sulfate or dicopper oxide in the diet up to 300 ppm of feed affects performance and Cu accumulation in broiler chickens. *Poultry sciences annual meeting*, 2016, New Orleans.

M. Hamdi, D. Solà-Oriol, R. Franco-Rosselló, S. Durosoy, A. Roméo, and J.F. Pérez (2016). Including copper sulfate or dicopper oxide in the diet up to 300 mg Cu/kg affects performance and copper accumulation in broiler chickens. *Poultry Science*: submitted.

M. Hamdi, D. Solà-Oriol, R. Franco-Rosselló, R. Aligue , and J.F. Pérez (2016). Effect of different microbial phytases and dietary Calcium: Phosphorus ratio on the productive performance, mineral retention and bone mineralization of Broilers. *British Poultry Science*: submitted.

M. Hamdi, D. Solà-Oriol, R. Franco-Rosselló, R. Aligue, and J.F. Pérez (2016). Comparison of how different mineral phosphorus sources affect performance, bone mineralization and phosphorus retention in broilers. *British Poultry Science*: submitted.

M.Hamdi, R.Davin, D.Solà-Oriol and J.F. Pérez (2015). Calcium sources and their interaction with the level of inorganic phosphorus affect performance and bone mineralization in broiler chickens. *Poultry Science* 94:2136–2143

M.Hamdi, S. López-Vergé, E. G. Manzanilla, A. C. Barroeta, and J. F. Pérez (2015). The effect of different levels of calcium and phosphorus and their interaction on the performance of young broilers. *Poultry Science* 94:2144–2151.

M.Hamdi, R., Solà-Oriol, D. y Pérez, JF (2015). Estudio del efecto de la fuente mineral del fosforo y su iteraccion con la relación Ca:Pdis en el rendimiento, digestibilidad y mineralización ósea en pollos broilers de 0 a 21 dias .2015. Poultry Science Conference 52, Malaga-AECA WPSA Symposium: 251-256.

M.Hamdi, R., D. Solà-Oriol, J.F. Pérez (2015).Efecto de la relación Ca:Pdis y el tipo de fitasa sobre los parámetros productivos, la digestibilidad y la mineralización ósea en pollos de carne de 0 a 14 dias. Poultry Science Conference 52, Malaga-AECA WPSA Symposium: 257-263.

M.Hamdi, Franco-Rosselló, R., Solà-Oriol, D. y Pérez, JF (2015). Efecto de la variación de la relación calcio:fósforo sobre los resultados productivos y formación ósea de pollos broiler de 0 a 35 días. XVI jornadas sobre producción animal AIDA (2015),Tomo I,263-265.

M.Hamdi , E. G. Manzanilla , S. López-Vergé y J.F. Pérez (2014) .Evaluación de extractos de plantas en el control de infección experimental por coccidios Poultry Science Conference 51, Valencia-AECA WPSA Symposium .

M.Hamdi, A.C.Barroeta and J. F. Perez (2013), The study of the interaction between calcium and phosphorus and its effect on the performance of broiler chicks, Poultry Science Conference 50, Lleida-AECA WPSA Symposium: 277-285.

Conference proceedings

- Participation and presentation of two communications, the 52 Scientific AECA WPSA Poultry Congress, Symposium, held in Malaga, on 28, 29 and 30 October 2015.

- Participation and presentation of communication, in the XVI conference on animal production AIDA, held in Zaragoza, on 19, 20 May 2015.

- Participation and presentation of communication, in the Conférence international sur l'agriculture et la biotechnologie Tunisie 2015, held in Tunisia, on 2 and 3 November 2015.

-Participation and presentation of communication, the 51 Scientific AECA WPSA Poultry Congress, Symposium, held in Valencia, on 2, 3 and 4 October 2014.

-Participation and presentation of communication in format poster, the 50 Scientific AECA WPSA Poultry Congress, Symposium, held in Lleida, on 2, 3 and 4 October 2013.

-Participation to Scientific the 49- AECA WPSA Poultry Congress, Symposium held at the Faculty of Veterinary Medicine, University Autonoma of Barcelona, on 4 and 5 October 2012.

Languages

Arabic • • • • •

French • • • • •

Spanish • • • • •

English • • • • •

Softwares

Microsoft Office • • • • •

SAS • • • • •