



LONG STORY FROM PAST TO PRESENT: CALCIUM, PHOSPHORUS, AND PHYTASE – A REVIEW

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Abstract

Calcium (Ca) and phosphorus (P), two main elements, have vital physiological and metabolic roles in animal bodies. Accurate comprehension of the interaction of these two elements and their value in various resources helps to obtain their optimal formulation in poultry diets. Hence, in previous studies, the hormonal axes controlling Ca and P homeostasis have been primarily investigated. However, to estimate Ca and P requirements in modern broiler chickens, in addition to growth performance, other parameters such as Ca and P digestibility, bone strength, and excretion into the environment should also be considered. Since a large amount of P in poultry feed ingredients is bound to phytate, phytases are added to poultry diets to release the P from phytate. However, many nutritionists need clarification on what dose of dietary phytase is required to release the maximum phytate P and how phytase activity will be optimized. Therefore, the present review study has attempted to explore the factors that affect the digestibility of different sources of Ca and P. In addition, the effect of excess dietary Ca on phytase activity and studies related to superdosing of phytase in broilers are provided. Finally, the values of phytate P in standard poultry feed ingredients and the latest update of the studies on determining Ca and P requirements are summarized.

Key words: broiler, calcium, nutrition, phosphorus, phytase

It is well established that calcium (Ca) and phosphorus (P) are essential nutrients with crucial structural and physiological functions in human and animal bodies. Many biological processes, such as blood clotting, enzyme activation, intracellular signaling, muscle contraction, acid-base balance, protein synthesis, cell adhesion, and nerve impulse transmission, require adequate levels of Ca in the body (Bai et al., 2022; David et al., 2021 a; Yang et al., 2020). In addition, Ca is the main element in the structure of bone and egg (Yang et al., 2020). As the second most abundant mineral in the animal body, P is important for skeletal health. About 80% of P is found in the bones (Kaiza et al., 2023; Mello et al., 2012). The remaining P is involved in the structure of nucleic acids, nucleotides, adenosine 5'-triphosphate (ATP), phospholipids, and phosphorylated proteins, which plays an important role in the expression of specific genes, maintenance of cell integrity, fluidity and electrolyte balance, and cellular metabolism of carbohydrates, lipids, and proteins (Proszkowiec-Weglarz and Angel, 2013; Suttle, 2010; McDowell, 2003). The interaction of Ca, P, and vitamin D and the effect of it on determining the required levels of these elements in different stages have been recently reviewed (Li et al., 2017). Vitamin D, Ca, and P deficiency or an improper Ca:P ratio affects bone strength, mineralization, egg production, and growth performance (Xu et al., 2021). In addition, since about 40

to 60% of the total P in main ingredients of poultry diets such as corn and soybean meal are in the form of phytate P, it is necessary to supplement inorganic sources of P and/or phytase to poultry diets (Cufadar et al., 2024; Li et al., 2016). Some studies have argued that when there is a balance between Ca and P, endogenous phytase is sufficient for breaking down phytate (Tamim et al., 2004). However, monogastric animals, including poultry, are unable to utilize P from phytic acid (*myo*-inositol hexakisphosphate, IP6) due to a lack of endogenous phytase in their intestine (Cowieson et al., 2016).

In recent decades, extraordinary progress in the poultry industry in achieving goals such as increasing body weight (BW) in broilers and egg production in laying hens, has drawn more attention towards leg problems in the barn and bone fractures during the processing of the carcass (Raei et al., 2021; Chen and Moran, 1995). Insufficient bone growth, leg problems such as rickets, and low eggshell quality in poultry are identified as the first indications of Ca and P deficiency (Yang et al., 2020; Valable et al., 2018). In broiler chickens, at early ages, P deficiency impairs skeletal health by decreasing bone mineral density, bone-breaking strength, and bone ash content (Yang et al., 2020; Liu et al., 2017). In addition, feeding birds with low levels of Ca leads to incomplete bone mineralization and high P excretion. On the other hand, very high dietary Ca reduces the performance of

the animal (Mello et al., 2012). In general, overfeeding the birds with Ca decreases dietary energy by forming soap precipitates with free saturated fatty acids, which in turn reduces nutrient availability (Ceylan et al., 2020; Hamdi et al., 2015; Tamim et al., 2004). It is assumed that excess dietary Ca sources increase the pH of the gastrointestinal tract (GIT). On the other hand, phytase has optimal activity at low pH (less than 4). Hence, excess dietary Ca decreases the efficiency of endogenous and exogenous phytase by forming the mineral-phytate complex (Hamdi et al., 2015; Tamim et al., 2004). The presence of high levels of dietary Ca leads to a decrease in the digestibility of lipids through an increase in the pH of the digestive system (Imari et al., 2020). It has been demonstrated that lower lipid digestibility through unbalancing or impairing gut microflora causes loss of gut integrity (Zhao et al., 2013).

Limestone, as one of the primary sources of Ca, has a negative effect on P and nitrogen utilization as it reduces the solubility of P and protein in the gizzard (Ceylan et al., 2020; Walk et al., 2012). Based on this, P excretion may increase environmental pollution in the poultry industry (Abd El-Hack et al., 2018). Besides, dietary inorganic P sources are limited natural resources that are one of the expensive components of the diet; for this reason, poultry diets need to be formulated according to the minimum requirement of Ca and P (Valable et al., 2018). The current review does not intend to discuss the details of Ca and P metabolism, compare methods of measuring phytate, or determine the accurate Ca and P requirements as they have already been reviewed (Zaefarian et al., 2021; Walk et al., 2021 b; Li et al., 2017; Cowieson et al., 2016). But, on the other hand, knowing the relation among Ca, P, and vitamin D, having a better estimation of birds' Ca and available P requirement, and exact determination of total P and phytate fraction of plant-based raw materials can help the nutritionists feed the birds with a balanced diet in terms of Ca and P and more accurate phytase use. Since more than two decades ago many studies are carried out in this regards and in the current review, we have attempted to summarize this valuable information. Also, recent reports related to estimating Ca and P requirements in broilers are provided.

Methodology

The objective of the present study is to provide a comprehensive review of Ca, avP (available phosphorus), and phytase in poultry nutrition, encompassing historical perspectives, current practices, and future trends. Furthermore, the authors present original data on the solubility of various limestone samples, heavy metal levels in limestone, and the total P and phytate levels in different raw materials. Limestone samples were procured from 20 disparate geographical regions, including farms and limestone mines, while 35 samples of raw materials were obtained from various farms.

The *in vitro* solubility of the limestone utilized in this investigation was ascertained in accordance with

the methodology delineated by Zhang and Coon (1997). In summary, 2 grams of limestone were introduced to a 400-mL beaker containing 200 mL of a 0.2 N HCl solution. The mixture was heated to 42°C in a water bath with oscillating agitation at 80 Hz until a stable temperature was reached, which took approximately 15 minutes. Following a 10-minute reaction period, the undissolved limestone was filtered through a preweighed Whatman ashless filter paper (No. 41). Subsequently, the filter paper with the residue was subjected to drying in an oven maintained at a temperature of 60°C for a period of 20 hours. Thereafter, the *in vitro* solubility of the limestone was calculated as the percentage of weight loss.

In order to create the mineral solution, 2 grams of finely ground limestone were dissolved in hydrochloric acid (HCl) in a 3:1 ratio. Following digestion, the solutions were cooled and transferred to acid-cleaned volumetric flasks. Thereafter, they were diluted with deionized water. Mineral concentrations were measured using Vista MPX type inductively coupled plasma-optical emission spectrometry, in accordance with the requirements set out in EN 15510:2007 (Golzar Adabi et al., 2019). Phytate levels in various raw materials were determined employing the methodology outlined by Haug and Lantzsich (1983).

Ca digestibility from different sources

Generally, dietary Ca and P can be supplied from plant-based ingredients, animal by-products, and inorganic mineral sources. Only in the last few years, more attention is devoted to Ca digestibility due to the low cost and abundance of Ca sources, such that Ca requirements are still formulated as total Ca (David et al., 2019). However, considering the close relationship between Ca and P, notable efforts have been made to measure Ca digestibility in different sources in poultry (Walk et al., 2022 a, 2021 a; David et al., 2021 a, 2019). However, several factors may influence the formulation of diets based on digestible Ca. The absence of suitable values for raw materials, differences in methods employed, *in vitro* solubility and particle size, differences in dietary Ca:P ratios, differences in nutrient composition, the presence or absence of phytate, age of the birds, and adaptation period may affect the measurement of Ca digestibility in poultry (Walk et al., 2021 a, b). Recently, Walk et al. (2021 b) reviewed the digestibility of Ca in various sources based on different digestibility methods, ingredient sources, and particle sizes. The authors concluded that digestible Ca formulation systems could be practical for nutrient recommendations to achieve appropriate experimental methods, characterizing the ingredients, and exogenous phytase doses. Concerning the methodology assay, similar to the determination of ileal amino acid digestibility, three methods, including direct, regression, and difference, for determining the digestibility of Ca and P in feed ingredients have been reported in previous studies (Anwar et al., 2018, 2017, 2016 a, b, c, 2015). For example, the Ca digestibility coefficient of dicalcium phosphate

(DCP) is found to be higher in direct method compared to other methods (Anwar et al., 2018).

The composition of diet has been shown to be effective in Ca digestibility. In support of this statement David et al. (2019) reported higher digestibility level of Ca in corn-based diets compared to corn starch-based purified ones in broilers. They related the reasons to particle sizes of the diet which changes the rate of the passage by creating more contact time for greater solubility and digestibility. The authors also documented significant differences in apparent ileal digestibility (AID), standard ileal digestibility (SID), and true ileal digestibility (TID) of Ca from different sources. Accordingly, limestone had a higher AID and TID than meat and bone meal, monocalcium phosphate (MCP), and DCP (Walk et al., 2021 a; David et al., 2019). David et al. (2021 b) reported that the TID of Ca was higher in canola meal than in soybean meal in the presence of 2000 FTU/kg dietary phytase. Finally, they concluded that the composition of the diet and age of the birds can affect Ca digestibility. In another study, David et al. (2021 c) compared two sources of limestone with different particle sizes and amounts of Ca:P ratio in broilers and laying hens and concluded that limestone with a coarser size resulted in higher AID of Ca for broilers and laying hens. These authors also indicated that laying hens had a higher ability to absorb Ca from limestone than broilers. Diana et al. (2021) compared the effect of age and Ca sources on the digestibility coefficient of Ca in laying hens. They found that DCP led to higher digestibility coefficient of Ca than limestone. The authors also reported lower digestibility coefficient of Ca in older birds compared to the younger ones. The effect of age on the digestibility and retention of Ca has been also researched in other studies. David et al. (2020) registered a linear decrease in retention of Ca in limestone from 56% to 31% with advancing age (starting at day 7 to day 42). It was explained that in younger ages in order to meet the requirements of rapid bone formation during the first two weeks of growth Ca absorption is more efficient. Moreover, the presence of residual yolk sac provides a rich source of Ca and improves Ca absorption. In contrast, in later ages higher endogenous Ca losses may negatively affect the AID of Ca values.

As mentioned, the particle size of Ca sources is one of the factors that may affect the solubility and digestibility of Ca in the GIT. In this regard research has demonstrated that *in vivo* and *in vitro* solubility of Ca sources, especially limestone, has inverse relationship with each other. So that, coarse particles that have low *in vitro* solubility increase Ca availability in laying hens by a long-term stay in the digestive tract (Anwar et al., 2016 a). In spite of higher *in vitro* solubility of fine particle size limestone (0.06 and 0.075 mm) to coarse particle size (0.6 and 0.402 mm) from the same source, it has lower AID of Ca (Kim et al., 2018, 2019). Anwar et al. (2016 a, 2017) reported similar results researching the AID of Ca from limestone and oyster shells with two different particle sizes (<0.5 and 1–2 mm).

Kim et al. (2018) evaluated the effect of different dietary Ca levels (0.6, 0.8, and 1.0%) and limestone particle size (fine < 0.075 mm and coarse = 0.402 mm) with or without phytase (0 and 1000 FTU/kg) on P digestibility in broiler chickens. The researchers concluded that the AID of P in broilers fed on fine particle size limestone had a negative relation with the inclusion level of Ca (from 0.6 to 1%). It was reported that fine particle size limestone (< 0.075 mm) that has higher solubility was more detrimental to P digestibility as compared to the same limestone with coarse particle size (0.402 mm) (Kim et al., 2018). It was also revealed that the addition of exogenous phytase improved the AID of Ca from limestone sources by 15%. Another important issue about the particle size of Ca source is that small particle size limestone solubilizes fast and makes chelate with IP6 which then cannot be easily degraded, this also reduces the efficiency of phytase (Kim et al., 2018). Limestone that is used as an ingredient in poultry diets is locally supplied in three forms (Figure 1, unpublished data). The results of sieve and *in vitro* solubility tests on three limestone samples (fine, medium, and coarse) are presented in Figure 1 (unpublished data). As it is also shown in the Figure 1, similar to the reports, the *in vitro* solubility of limestone decreases as the particle size increases.

Ca:non-phytate P (NPP) ratio is another factor affecting Ca and P digestibility, the details of which will be discussed in Ca to P ratio: P digestibility and phytase activity section of the current review. Considering the factors affecting the measurement of digestibility, the digestibility of Ca and P in various sources is summarized in Tables 1 and 2, respectively.

Estimating phytate P in plant-based feed ingredients

The concentration and source of phytate are effective factors in deciding dietary phytase levels. Hence, the method of determining phytate P in feed ingredients is an important point that should be addressed. Various methods such as high-performance liquid chromatography (HPLC), near-infrared (NIR) spectroscopy, and wet chemistry have been introduced by researchers to measure the amount of phytate P in ingredients and feeds (Aureli et al., 2017; de P Naves et al., 2014; Tahir et al., 2012). The HPLC is currently the most widely used method for phytate analysis which requires expensive and time consuming anion-exchange purification. In addition, the levels of phytate in the processed feed may be overestimated due to the eluting of lower *myo*-inositol phosphate forms along with phytate (Morgan, 2014). NIR is so susceptible to errors and may lack precision in analysis of low levels. Wet chemistry is also time-consuming but cheaper though, experienced lab technicians can obtain reasonable data by decreasing the analytical errors. However, nutritionists may calculate the value of phytate P by referring to feedstuff databases. Though diets formulated based on feedstuff databases such as NRC (1990), CVB (2021), and INRA (2002) may not always

be accurate for all local situations and in this case phytate P is not an exception (Zaefarian et al., 2021). The total P and phytate P of the main feed ingredients from various feedstuff databases, as well as the results from chemical analysis of feed ingredients' samples collected from broiler integrations (unpublished data) are summarized in Table 3. Table 4 shows the minimum, maximum, and average values for total P and phytate levels of different common raw materials in poultry nutrition (unpublished data).

In conclusion, the solubility and particle size of calcium sources, such as limestone, are also crucial criteria

that should be considered to enhance the efficiency of phytase and to meet the P requirements of the birds. It is also noteworthy that the range of phytate levels observed in the raw materials under consideration (Table 4) is quite pronounced. In such instances, the nutritionist has two potential courses of action to make an appropriate and accurate decision regarding dosage. The first possibility entails the analysis and determination of the level of phytate in raw materials. This approach, however, is both time-consuming and costly. The second alternative involves the use of high doses of phytase, which is capable of eliminating the antinutritional effects of phytate.

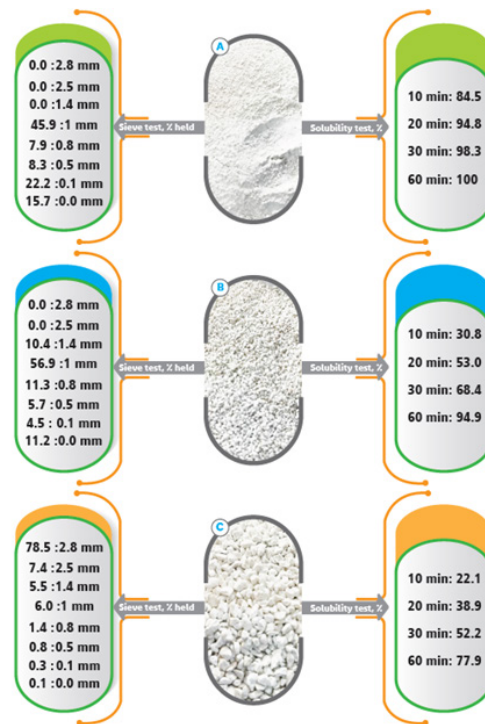


Figure 1. Solubility (right hand) and sieve tests (left hand) in different types of limestone based on particle size: A, fine; B, medium; C, coarse. Values are expressed as mean of 20 samples with 3 replicates per each (The photos by Shahram G. Adabi)

Table 1. The values of total calcium (Ca), Ca digestibility coefficient, and Ca digestibility in various sources tested in broilers

Source	Age	Method ¹	Total Ca, g/kg	Ca digestibility coefficient	Ca digestibility, g/kg	Reference
Plant-based ingredients						
corn	10 d	SID	0.20	0.50	0.10	David et al., 2021 a
wheat	NA	SID	0.40	0.70	0.28	Walk et al., 2021 a
canola meal	21 d	SID	7.20	0.48	3.46	Anwar et al., 2018
soybean meal	21 d	SID	3.50	0.54	1.89	David et al., 2021 a
Animal by-product						
meat and bone meal	31 d	AID	96.00	0.48	46.08	Anwar et al., 2016 c
fish meal	24 d	AID	67.00	0.23	15.41	Anwar et al., 2018
poultry by-product meal	24 d	AID	40.00	0.28	11.20	Anwar et al., 2018
Inorganic sources						
limestone	24 d	AID	420.00	0.49	205.80	Anwar et al., 2017
monocalcium phosphate	24 d	AID	174.00	0.41	71.34	David et al., 2019
dicalcium phosphate	24 d	AID	260.00	0.30	78.00	David et al., 2019
oyster shell	24 d	AID	370.00	0.43	159.10	Anwar et al., 2017

¹SID; standard ileal digestibility, AID; apparent ileal digestibility.

Table 2. The values of total phosphorus (P), P digestibility coefficient and P digestibility in various sources tested in broilers

Source	Age	Method ¹	Total P, g/kg	P digestibility coefficient	P digestibility, g/kg	Reference
Plant-based ingredients						
corn	28 d	SID	2.30	0.70	1.61	Mutucumarana et al., 2015
wheat	28 d	TID	3.22	0.46	1.49	Mutucumarana, 2014
canola meal	21 d	TID	9.70	0.66	6.40	David et al., 2021 a
soybean meal	21 d	SID	5.90	0.75	4.43	Mutucumarana et al., 2015
Animal by-product						
fish meal	24 d	TID	44.30	0.49	21.71	Dilelis et al., 2021 a
poultry by-product meal	22 d	SID	13.10	0.95	12.45	Dilelis et al., 2021 b
Inorganic sources						
monocalcium phosphate	19 d	AID	223.00	0.87	194.01	An et al., 2020
monodicalcium phosphate	19 d	AID	214.00	0.86	184.04	An et al., 2020
dicalcium phosphate	19 d	AID	185.00	0.76	140.60	An et al., 2020
tricalcium phosphate	19 d	AID	201.00	0.54	108.54	An et al., 2020

¹SID; standard ileal digestibility, TID; true ileal digestibility, AID; apparent ileal digestibility.

Table 3. Comparison of total P and phytate P of poultry feed ingredients among NRC (1994), CVB (2021), and INRA (2002), and chemically analyzed feed ingredients samples¹

Feed ingredients	NRC (1994), %	CVB (2021), %	INRA (2002), %	Analyzed results ¹ , %
Corn				
Total P	0.310	0.250	0.250	0.251
Phytate P	0.220	0.210	0.190	0.201
Wheat				
Total P	0.370	0.300	0.340	0.220
Phytate P	0.240	0.190	0.220	0.198
Sunflower meal				
Total P	0.93	0.970	0.990	1.094
Phytate P	0.79	0.770	0.840	0.811
DDGS corn				
Total P	0.774	0.820	0.840	0.782
Phytate P	0.355	0.250	0.210	0.102
Soybean meal				
Total P	0.688	0.590	0.620	0.569
Phytate P	0.244	0.410	0.370	0.346
Wheat bran				
Total P	1.150	1.060	0.950	1.061
Phytate P	0.950	0.900	0.760	0.900
Barley				
Total P	0.360	0.320	0.340	0.323
Phytate P	0.190	0.240	0.190	0.212

¹Values are expressed as a mean of 35 samples per each (unpublished data).

Phytase superdosing

Structurally, phytic acid has a *myo*-inositol nucleus bound (by ester bonds) to six phosphate groups. Phytate refers to the salts formed by phytic acid with Mg, Ca, K, and Na. Phytase enzymes belonging to phosphatases are widely added to the diet of monogastric animals to utilize phytate P (Cowieson et al., 2016). Based on the carbon site of hydrolysis in the phytate molecule, these enzymes are categorized as 3-phytases or 6-phytases (Hernandez et al., 2022). Phytase can break the inositol ring into 5

lower *myo*-inositol phosphates (IP2, IP3, IP4, IP5, and IP6) but the dephosphorylation of IP1 needs alkaline phosphatase (ALP; Zaghari et al., 2015). Alkaline phosphatase as a phosphomonoesterase (similar to phytase) catalyzes the hydrolysis of organic phosphate at alkaline pH (pH 8–9) in the presence of cations such as Mg, Mn, and Zn. Regarding the effect of dietary Ca on ALP activity, it has been determined that high levels of Ca could reduce the activity of ALP by impacting hormonal balance (Morgan, 2014).

Table 4. Maximum, minimum, and average of total P and phytate levels of common raw materials used in poultry nutrition (unpublished data)¹

Variable	Minimum (%)	Maximum (%)	Average (%)
Corn phytate	0.176	0.224	0.201
Corn total P	0.228	0.271	0.251
Wheat phytate	0.175	0.231	0.198
Wheat total P	0.195	0.248	0.220
Sunflower meal phytate	0.782	0.873	0.811
Sunflower meal total P	1.040	1.160	1.095
Full fat soy phytate	0.348	0.427	0.396
Full fat soy total P	0.541	0.621	0.592
Corn DDGS phytate	0.072	0.166	0.103
Corn DDGS total P	0.740	0.897	0.782
Soybean meal phytate	0.191	0.494	0.346
Soybean meal total P	0.416	0.679	0.569
Wheat bran phytate	0.811	1.061	0.900
Wheat bran total P	0.976	1.234	1.062
Barley phytate	0.173	0.302	0.212
Barley total P	0.284	0.411	0.323

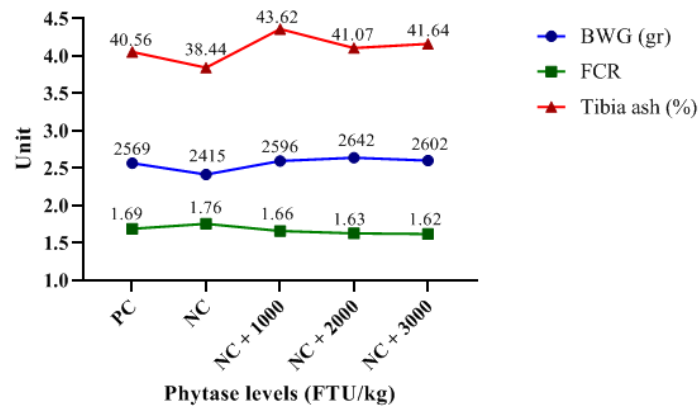
¹Values are expressed as a mean of 35 samples per each.

Figure 2. Effect of dietary graded phytase on BWG, FCR, and tibia ash in broilers at whole period (0–42 d). PC: a positive control diet with normal Ca and P content. NC: the negative control diet with nutritional reduction of 0.12% of Ca and 0.14% of P; NC + 1000, 2000, and 3000: NC diet with the addition of 1000, 2000, and 3000 FTU/kg phytase, respectively. To show the differences, the BWG and tibia ash data have been divided by 1000 and 10, respectively (Broch et al., 2018)

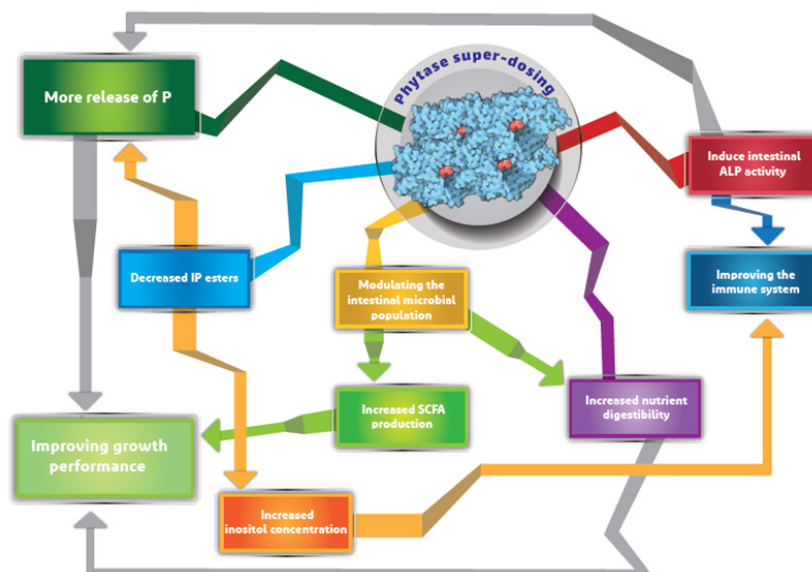


Figure 3. A summary of the extra-phosphoric effects of phytase superdosing in poultry

Today, it is clear that the bioavailability of phytate P can increase by including exogenous phytase in the diet, but the important point to be attentive to is the dose of dietary phytase. Some commercial phytase producers have suggested 500 FTU/kg and 250–300 FTU/kg of phytase for broilers and laying hens, respectively. However, several studies have reported that the application of high levels of phytase leads to an increase in the degradation of phytate and thereby improves the P matrix values (refers to an indication of the P release from phytate P) (Cufadar et al., 2024; Lee et al., 2017; Kim et al., 2017). In a review study, Cowieson et al. (2011) addressed the inclusion levels higher than 2500 FTU/kg in the diet and defined superdosing concept in feed formulation. However, in studies, levels above 1500 FTU/kg may also be considered as phytase superdose (Beeson et al., 2017). Walk et al. (2014, 2013) observed significant improvement in growth performance and tibia characteristics of broiler chickens when 1500 FTU/kg dietary phytase was included and concluded that the application of phytase in a superdose may increase final BW by increasing phytate destruction and the provision of inositol. Kriseldi et al. (2021) noted that the inclusion of graded levels of phytase (from 500 to 40500 FTU/kg) linearly decreased IP esters in broiler chickens so that the highest level of phytase had the highest concentration of inositol in the gizzard and ileal digesta at 15, 29, and 43 d of age. For a better understanding, a summary of the effects of graded phytase levels (from 0 to 3000 FTU/kg) on body weight gain (BWG), feed conversion ratio (FCR), and tibia ash in broilers is shown in Figure 2. Fernandes et al. (2019) provided data on the effects of superdosing phytase with simultaneously decreasing P levels on nutrient digestibility and bone development of broilers and concluded that phytase levels higher than 1000 FTU/kg had a positive effect on these parameters through increasing phytate degradation and *myo*-inositol availability. Dersjant-Li et al. (2022 a) examined the effect of various levels of phytase (0, 500, 1000, 2000, and 4000 FTU/kg) and phytate (as one of the factors affecting phytase activity) on the AID of IP and the concentration of IP esters in broilers and found that increasing the dietary phytate content led to a significant decrease in the AID of IP6 and IP 3–5, though supplementing 4000 FTU/kg dietary phytase, regardless of phytate levels, increased the AID of IP6 and IP 3–5 and decreased IP (6, 5, 4) esters concentration. Besides increasing P availability, superdosing of phytase may also have extra-phosphoric effects (Figure 3). These effects in total may improve the birds' zootechnical performance through beneficial impacts on the digestive and immune systems. As mentioned above, ALP is required to dephosphorylate IP1 from the inositol ring, which requires feeding birds with high levels of phytase to induce intestinal ALP activity. It has been indicated that ALP, in addition to dephosphorylating IP1, has a critical role in improving the immune system and preserving gut integrity by dephosphorylating bacterial lipopolysaccharide and preventing transepithelial passage by bacteria (Bed-

ford and Apajalahti, 2022). Furthermore, the term extra-phosphoric effects of superdosing phytase refers to the improvement of the digestibility of amino acids and the beneficial effects of inositol released under the influence of high levels of phytase (Selle et al., 2023). For example, Babatunde et al. (2021, 2022) investigated the response of broiler chickens to various phytate concentrations and phytase levels in two starter and grower phases and concluded that regardless of dietary phytate levels, increasing the level of phytase from 500 to 4000 FTU/kg caused a linear increase in AID coefficients of amino acids in both phases. As mentioned by Cowieson et al. (2016), the formation of the phytate-protein complex is one of the antinutritional effects of phytate in monogastric nutrition. Phytate reduces protein availability by decreasing protein digestibility and solubility, as well as impairing the activity and accessibility of enzymes such as proteases. So, the inclusion of high levels of phytase in the diet improves the digestibility of protein and amino acids (Cufadar et al., 2024; Novotny et al., 2023; Gulizia et al., 2023; Martínez-Vallespín et al., 2022; Lima et al., 2021). With regards to the major role of *myo*-inositol in cellular processes, bone formation, skeletal muscle metabolism, reproduction, nervous system, and the immune system, phytase superdosing has been shown to be beneficial by releasing more *myo*-inositols (Gonzalez-Uarquin et al., 2020 a; Li et al., 2018). Several studies have indicated that phytase superdosing increases plasma concentration of *myo*-inositol and improves animal zootechnical performance through the extra-phosphoric effects (Kriseldi et al., 2021; Gonzalez-Uarquin et al., 2020 b; Lu et al., 2019; Beeson et al., 2017; Walk et al., 2014). Another extra-phosphoric effect of phytase is modulating the intestinal microbial population by increasing the lactic acid bacteria enumeration and reducing coliforms. On the other hand, it has been shown that the change in the microbial population following the inclusion of phytase increases the production of short-chain fatty acids (SCFA) such as propionate, acetate, and butyrate. Short-chain fatty acids, in turn, maintain the balance of the gut microbiological environment, improve the gut barrier function, and nutrient digestibility and absorption (Cufadar et al., 2024; Farrokhi et al., 2021; Liu et al., 2021; Ptak et al., 2015). For instance, butyric acid provides energy for enterocytes and as a result stimulates villi growth (Khatibjoo et al., 2018). Cufadar et al. (2024) supplemented laying hens' diets with 500, 750, or 1000 FTU/kg, which provided 0.176, 0.188, and 0.200% of avP, respectively. Dietary supplementation of phytase significantly increased villus surface area by 15, 36, and 40% in 500, 750, and 1000 FTU/kg birds, respectively, compared to positive control birds. In addition, a significant increase in *Lactobacillus* count was observed in line with increasing the level of phytase. Furthermore, phytase supplementation significantly increased the concentrations of total cecal SCFA. Cufadar et al. (2024) concluded that along with improving performance parameters, the inclusion of phytase in laying hens' diet could ameliorate intestinal

morphology, influence the cecal microflora composition, and increase the cecal concentration of SCFA. Improving the GIT environment in terms of villus histomorphometry, microflora, and SCFA production by the inclusion of high dietary phytase has also been reported by other researchers (Moita et al., 2021; Nari et al., 2020; Ptak et al., 2015; Smulikowska et al., 2010).

Cowieson et al. (2016) reviewed the factors that may influence phytase superdosing strategy. Some of these factors are phytate concentration and source, *myo*-inositol delivery, nutrient density such as energy and amino acids, dietary anion/cation balance, age, and genetic effects of the animal species.

To sum up, the analysis of phytate in plant sources is frequently a challenging endeavor due to the constraints of time and financial resources involved. Moreover, the administration of elevated doses of phytase in the diet does not impair the performance of birds and may, in fact, prove advantageous. Recent studies have demonstrated that the incorporation of dietary phytase at 2000–3000 FTU/kg in broilers and 1000 FTU/kg in laying hens enhances gut integrity and, subsequently, the overall zootechnical performance of the birds. Therefore, the administration of an additional superdose of phytase in the formulation represents a cost-effective strategy in poultry nutrition. It is evident that the local price of the phytase enzyme, the price of the final feed, and the level of improvement in performance in a specific field are crucial factors in the implementation of such a strategy.

Ca to P ratio: P digestibility and phytase activity

The improving effect of dietary fungal and bacterial phytase on utilizing plant originated P in poultry species has been approved since many decades ago (Oliveira et al., 2018; Abd El-Hack et al., 2018; Li et al., 2016; Cowieson et al., 2016; Walk et al., 2012; Tamim et al., 2004). The content of Ca and P, phytate concentration, pH of diet and GIT, age and species of bird, proteases in GIT, origin and dosage of phytase, thermostability of phytase, and the level of vitamin D₃ are factors that affect phytase activity *in vivo* (Hossain et al., 2022; Bavaresco et al., 2020; Oliveira et al., 2018; Amerah et al., 2014).

Researchers have used various terms such as digestible P, available P, and retainable P to evaluate the bioavailability of P (Li et al., 2017). Until a few years ago, the bioavailability of NPP was assumed to be 100% for the birds. Though birds are not able to use all NPP, they may utilize some of phytate P. Previously, the total required Ca:NPP ratio in the broiler's diet was 2:1 (NRC, 1990). However, in an ileal digestibility study the ratio was changed to 1:1 (Anwar et al., 2016 a). In a study designed based on the ratio of 2:1 of Ca:NPP and inclusion of 20 kg/ton of MCP the results showed that regardless of the source of dietary limestone AID of Ca increased from 0.56 to 0.60 in broiler chickens (Anwar et al., 2016 b). On the other hand, Anwar et al. (2016 a) reported that increasing the ratio of Ca:NPP from 1.5:1 to 2.5:1 decreased the AID coefficient of Ca from 0.63 to 0.48.

High Ca digestibility in low Ca:NPP ratios may be due to the upregulation of Ca transporter genes (Li et al., 2012). In broilers, the Ca:NPP ratio has a massive effect on the AID of Ca and P; any increase in the ratio decreases the digestibility of both minerals (Oliveira et al., 2018), phytase activity (Oliveira et al., 2018), and parathyroid hormone (Sebastian et al., 1996).

In recent years, there has been a notable increase in the focus on Ca nutrition in poultry. The primary reasons for the neglect of Ca were its affordability, abundance, and reduced environmental polluting impact compared to P. Recent studies have posited that poultry, including broilers, are provided with dietary Ca in quantities exceeding their requirements. It is paramount to bear in mind that balancing the diets based on a proper Ca:NPP ratio is a vital nutritional strategy because limestone as an important source of calcium has a high buffering or acid binding capacity (Stas et al., 2022; Selle et al., 2009; Lawlor et al., 2005). Moreover, today limestone is also used as flow agents/carriers and/or diluents in premixes, enzymes, and ionophores in the poultry feed industry. But, unfortunately, it is occasionally ignored to be included in balancing the diet which in turn is another reason that increases Ca beyond the sufficient level (Kim et al., 2018). Inclusion of high dietary Ca:NPP level elevates the pH of GIT (Walk et al., 2021 b; Kim et al., 2018; Shafey et al., 1991). Such environment is not suitable for exogenous phytase and endogenous enzymes activities and would ultimately reduce the digestibility of nutrients such as P and protein. Excess dietary Ca attaches to phytate in GIT and forms a Ca-phytate chelate which reduces P solubility and availability of it for phytase; that in turn decreases phytate degradation (Bavaresco et al., 2020; Li et al., 2018; Nolan et al., 1987). In this regard, the formation of the Ca-IP6 complex/chelate increases when the pH of GIT is higher than 5 (Grynspan and Cheryan, 1983). David et al. (2021 a) estimated Ca and P requirements based on SID in broiler chickens in the starter period (0–10 days old). Accordingly, the performance and bone parameters were negatively affected by increasing SID Ca concentrations (from 3.3 to 5.5 g/kg) and decreasing SID P concentrations (from 6 to 4 g/kg), which was in accordance with the results of Amerah et al. (2014), who noted that increasing dietary Ca from 0.4 to 1% causes a linearly reduced positive effect of phytase on broilers' growth performance and nutrient digestibility. Li et al. (2018) reported significant increases in the concentration of IP6 at 1% Ca compared to 0.7%. The same authors demonstrated that adding phytase had a positive effect on IP6 disappearance in the GIT, although the impact of the enzyme depended on the amount of dietary Ca and Ca:NPP ratio (Li et al., 2018). Similarly, Tamim et al. (2004) showed that increasing dietary Ca to 0.5% decreased phytate P utilization from 69.2% to 25.4%. In this case, adding 500 FTU/kg phytase can ameliorate the negative effects of high Ca:NPP ratio (Tamim et al., 2004). Moreover, it has been reported that supplementing CaCO₃-containing diets with formic acid increases

phytase activity by reducing pH and changing the microbial community of GIT (Krieg et al., 2021).

In addition to the negative effect of high Ca levels, heavy metals in inorganic sources of Ca and P decrease phytase activity. Heavy metals such as fluorine, arsenic, cadmium, and mercury have toxic effects on animals (Viljoen, 2001). One of the sources of Ca in poultry diets is dolomitic limestone which contains at least 10% Mg. This element easily causes Ca deficiency by complexing with Ca or competing with Ca for absorption sites, leading to poor skeletal growth or eggshell quality in poultry. Based on this, the application of dolomitic limestone in poultry diets is never recommended (Leeson and Summers, 2005). The minimum, maximum, and average of heavy metals and some other minerals in limestone are summarized in Table 5 (unpublished data).

A review of recently published data on the feeding of Ca and P in broilers

Based on the knowledge noted in the aforementioned, several factors impact the estimation of Ca and P requirements of modern broiler chickens, all of which may not have been considered in references (according to strain nutrient recommendations and previous literature). Evidence from Ceylan et al.'s (2020) study on 11,200 Ross 308 broiler chickens demonstrated significant positive effects on BWG and FCR when Ca and NPP were 6 (starter), 14 (grower), and 23% (finisher) below Aviagen recommended levels (Aviagen, 2014). In another study, Ca with 20% lower than recommended level of the Brazilian Tables for Poultry and Swine had no significant impact on performance and carcass characteristics of broiler chickens (Tizziani et al., 2019). Furthermore, among different levels of Ca from 0.60 to 1.20%, the lowest one was determined to provide the best growth performance in the broiler chickens (Bai et al., 2022). Therefore, it could be concluded that the requirement level of Ca and P in modern broilers is lower than the recommended levels by NRC (1994) and Aviagen (2014, 2019).

Broiler chickens may adapt to early dietary restrictions of Ca and P. For example, Rousseau et al. (2016) noted that the BW of chickens fed on 0.48% Ca and

0.24% NPP from 10–35 days was higher than those fed on 0.90% Ca and 0.35% NPP. It was concluded that it resulted from upregulation in Ca and P transporting genes that could increase the digestibility of Ca and P and consequently improve growth performance. Data presented by Imari et al. (2020) suggests that compensatory pathways can be induced efficiently in broilers through early and late Ca and P deficiencies. Valable et al. (2018) also showed that growth performance and bone strength were not negatively affected in chickens fed on a diet based on suggested levels of Ca and P in the starter phase and lower Ca and P in the grower (0.60% Ca and 0.30% NPP) and finisher (0.48% Ca and 0.24% NPP) phases.

In some studies, it is indicated that reducing the content of Ca and P in the diet simultaneously with the administration of phytase or vitamin D₃ improved the broilers' performance. In a study, the reduction of Ca at three levels (1.6, 1.9, and 2.3 g/kg of Ca compared to breeder's recommendations) along with inclusion of supplementing phytase at 500 and 1000 FTU/kg in broilers was investigated. The results showed that performance, AID of Ca and P, and energy efficiency were improved in the birds fed on diets containing moderate and high Ca levels and phytase (Dersjant-Li et al., 2018). This was confirmed in Bavaresco et al.'s (2020) study who reported improvement in the performance of broiler chickens and in research by Moradi et al. (2023), Hamdi et al. (2015), and Delezie et al. (2015) who did not observe any adverse effects on performance, nutrient digestibility, and bone mineralization. However, reducing other nutrients such as energy, protein, and amino acids along with Ca and P can result in unfavorable performance in broiler chickens. This contention is supported by recent research by Dersjant-Li et al. (2022 b) who found that decreasing 68 kcal/kg ME, 10 g/kg CP, 0.1 to 0.4 g/kg digestible amino acids, 2 g/kg Ca, 2.2 g/kg digestible P, and 0.4 g/kg Na over 10 days (5–15 d) impaired performance, P retention, and nutrient digestibility. Though, dietary inclusion of phytase (Dersjant-Li et al., 2022 b) or 65 µg/kg 25-hydroxycholecalciferol (Zhang et al., 2020) may mitigate the negative effects.

Table 5. Concentration of heavy metals in limestone samples (unpublished data)¹

Variable	Hg, mg/kg	As, mg/kg	Cd, mg/kg	Chr, mg/kg	Pb, mg/kg	Ni, mg/kg	Cu, mg/kg	Mg, %	Na, %	K, %
Samples' number	20	20	20	20	20	20	20	20	20	20
Mean	< 0.02	< 0.50	0.24	1.27	0.69	1.07	4	0.30	< 0.02	0.11
Lowest	< 0.02	< 0.50	< 0.20	0.00	< 0.50	< 0.50	2	0.28	–	0.03
Highest	< 0.02	< 0.50	0.47	3	0.93	2.01	19	0.89	0.02	0.13

¹Values are expressed as a mean of 20 samples with 3 replicates per each.

Table 6. A summary of the effect of dietary Ca, P, and phytase levels on various parameters in broilers

Strain	Ca level	P level	Phytase level (FTU/kg)	Phytase origin	Age	Effects	Reference
Ross 308	Low	Low	500–1500	<i>A. oryzae</i>	1–42 d	Improved performance, ↑ plasma inositol concentration, ↓ fecal excretion of nutrient	Lim et al., 2024
Cobb 500	Low	Low	500 and 1500	<i>Buttiauxella</i>	14–23 d	Improved body composition (bone mineral density, bone mineral content, total body fat, and muscle), ↑ bone health	Shi et al., 2024
Ross 308	Normal	Low	1000–3000	-	1–33 d	Improved performance, improved carcass quality, ↑ nutrient digestibility, ↑ bone mineralization	Iqbal et al., 2023
Cobb 500	Low	Low	1000 and 2000	<i>Trichoderma reesei</i>	10–42 d	Improved performance, ↑ nutrient digestibility, ↑ bone mineralization	Cozannet et al., 2023
Cobb 500	Normal	Low	500, 1000, and 3000	<i>A. niger</i>	1–21 d	Improved performance, ↑ nutrient digestibility, improved bone (bone weight, ash, and Ca and P contents) and intestinal histomorphometric parameters	Martinez-Vallespin et al., 2022
Yield Plus × Ross 708	Low	Low	500–40500	<i>E. coli</i>	1–43 d	↑ IP2-6 disappearance, ↑ plasma inositol concentration	Kriseldi et al., 2021
Ross 308	Low	Low	500–6000	<i>E. coli</i>	1–23 d	↑ Ca, P, Zn, and Fe solubilities in digesta	Farhadi et al., 2019
Cobb 500	Normal	Low	250–3000	<i>A. niger</i>	1–28 d	Improved performance, ↑ nutrient digestibility, ↑ bone mineralization	Walters et al., 2019
Cobb 500	Normal	Low	500, 1500, and 3000	<i>E. coli</i>	1–44 d	Improved performance, no effect on processing yields	Smith et al., 2019
Cobb 500	Low	Low	1000–3000	<i>A. oryzae</i>	1–42 d	Improved performance, ↑ bone quality, ↑ mineral content in bones and blood	Broch et al., 2018
Ross 308	Low	Low	500 and 1000	<i>Buttiauxella</i>	1–42 d	Improved performance, ↑ nutrient digestibility, ↑ mineral content in bones	Dersjant-Li et al., 2018
Ross 308	Low	Low	500 or 1500	<i>E. coli</i>	1–21 d	Improved performance, ↑ ileal IP5-6 disappearance, ↑ nutrient digestibility	Beeson et al., 2017
Cobb 500	Low	Low	500, 1000, and 1500	<i>E. coli</i>	1–21 d	Improved performance, ↑ IP3-6 disappearance in gizzard	Walk et al., 2014

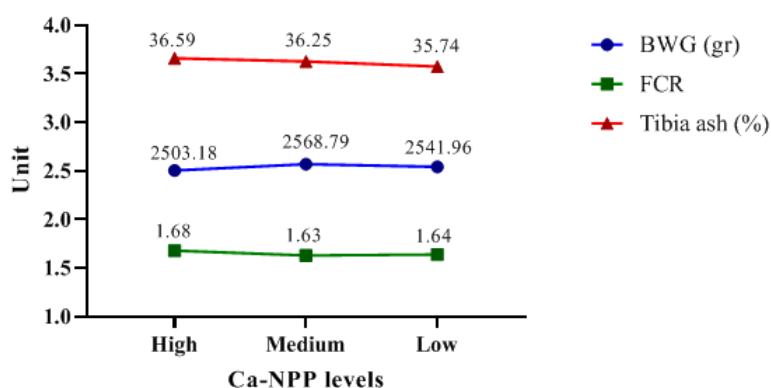


Figure 4. Effect of dietary Ca and NPP levels on BWG, FCR, and tibia ash in broiler Ross 308 at whole period (1–41 d). To show the differences, the BWG and tibia ash data have been divided by 1000 and 10, respectively. Ca and avP levels: high: control diet was formulated to meet suggested Ca and NPP nutrient specifications (Aviagen, 2014); medium: 94% for starter, 91% for grower, and 83% for finisher Ca and NPP as percentage of recommended levels of Aviagen; low: 94% for starter, 86% for grower, and 77% for finisher Ca and NPP as percentage of recommended levels of Aviagen (Ceylan et al., 2020)

More recent studies suggest that formulating Ca and P based on SID coefficients can improve growth performance, preserve resources, and reduce nutrient excretion to the environment (Anwar, 2015, 2016 a, b, c, 2017, 2018; David et al., 2019, 2020, 2021 c). David et al. (2021 a, 2022) examined the requirements of digestible Ca and P in the starter and grower phases in broiler chickens. In the starter phase, at a concentration of 5 g/kg SID P the required SID Ca for maximum BW and bone mineralization was estimated at 3.32 and 4.36 to 4.78 g/kg, respectively (David et al., 2021 a); and during the grower phase, at a concentration of 3.05 g/kg SID P it was 3.5 g/kg (David et al., 2022). Following the investigations, Walk et al. (2021 a, 2022 a, b) ran a series of studies on male Arbor Acres Plus broilers in three phases (1–10 d), (11–24 d), and (25–42 d). They concluded that birds fed on SID Ca performed better than birds fed on total Ca in terms of growth performance, Ca and P digestibility and retention. Moreover, Walk et al. (2022 c) compared the growth performance of broiler chickens fed on two systems of total Ca and SID Ca. The results showed that diets formulated based on SID Ca improved the performance of the birds including BWG and FCR but reduced AID of Ca and P from day 17 to 37. In SID system, the ratio of Ca:avP is also important. Accordingly, SID Ca:avP ratio was optimized at 1.1 to 1.02 for starter, 0.86 to 1.17 for grower, and 0.90 to 0.95 for finisher. The effects of various levels of Ca and NPP on BWG, FCR, and tibia ash at 1–42 days in broiler chicken are included in Figure 4. As mentioned, the reduction of Ca and P in the diet not only did not have any negative effect on the performance of broiler chickens but also improved BWG and FCR.

To sum up, the ratio of dietary Ca to avP has a marked impact on phytase activity, with elevated Ca levels in the diet leading to a reduction in phytase efficacy within the GIT. Aviagen (2022) has recently updated its broiler

chicken nutrition specifications, providing more precise recommendations for Ca and avP requirements. Ongoing studies are aimed at providing a more accurate estimation of the digestible Ca requirements of birds and the digestible Ca levels present in feed ingredients.

Conclusion

In addition to the optimal ratio of dietary Ca to avP, the absolute levels of these elements in the diet are of critical importance. The inclusion of excessive Ca in poultry diets has been demonstrated to compromise GIT integrity and overall body homeostasis. This is achieved through several mechanisms, including an increase in GIT pH, the formation of Ca-phytate chelates, and a reduction in the solubility of IP6. Such alterations have the potential to negatively impact the zootechnical performance of the birds. The most recent updates to Aviagen's broiler nutrition specifications (2022) advise reducing the levels of dietary Ca and avP across various stages of broiler production.

Another crucial factor is the appropriate dosing of dietary phytase. The objective of superdosing phytase is to facilitate the release of additional P and to leverage its extra-phosphoric effects. This approach has been demonstrated to enhance nutrient digestibility, increase inositol concentration, stimulate intestinal ALP and SCFA production, and positively influence the microbial population in the GIT. The cost of the enzyme and its beneficial effect on poultry performance are essential considerations when determining whether superdosing should be incorporated into feed formulations.

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