THESIS

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THE EFFECT OF PHYTASE SUPPLEMENTATION ON NUTRIENT UTILIZATION IN LAYING HENS

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ABSTRACT OF THESIS

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This study examined the impact of phytase supplementation at levels of 500 and 700 FTU/kg on nutrient (dry matter, crude protein, Ca, P, Na, and energy) digestibility and retention in laying hens. A total of 120 Lohmann Brown hens at 35 weeks old were used in this experiment. Four dietary treatments were applied, with the positive control formulated based on recommendations. 10 birds and 30 birds per treatment were used for retention and digestibility studies, respectively. Data were analyzed using a one-way ANOVA and Significance was set at $P<0.05$; $P<0.10$ Results indicated improvements in the digestive efficiency of essential nutrients, phosphorus (74.79% and 73.82%), nitrogen (N) (82.73% and 83.16%), and energy (73.64% and 74.36%), in phytase-supplemented diets at phy500 FTU/kg and phy700 FTU/kg, respectively, compared to the positive control and negative control diets. Additionally, relative retention of phosphorus (40.4% and 45.9%) and calcium (55.4% and 55.7%) was enhanced in phytase-supplemented diets at phy500 FTU/kg and phy700 FTU/kg, respectively, compared to P (31.7% and 39.9%) and C (49.9% and 49.8%) in PC and NC treatments, respectively, suggesting increased utilization of dietary phosphorus and calcium.

These findings highlight the efficacy of phytase in enhancing nutrient utilization and retention in laying hens, particularly for phosphorus, while also indicating a positive impact on calcium retention. Incorporating phytase supplementation at appropriate levels could be a valuable strategy to optimize nutrient utilization and improve the overall productivity and health of laying hens.

The results of this work enable us to conclude that the efficiency of nutrient utilization can be improved by enzymes only if the nutrients are below the requirements.

Keywords: phytase, efficiency, nutrients, retention, supplementation

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1. 0 INTRODUCTION

1.1 Background of the study

The animal feed industry holds great importance to ensure quality and continuous food supply for the ever-increasing world population. It plays a significant role in the food chain, as it directly influences the quality of products like meat, milk, fish, and eggs that people consume. The dependence on farm animals for human nutrition is huge around the globe; hence, animal feed should be highly nutritious and able to be absorbed by the animal effectively to produce the animal's source of foods with wholesome nutrition (El Enshasy et al., 2018). In contemporary poultry production, optimizing nutrient utilization is paramount for both economic and ecological sustainability. Phytase has been the focus of research for a long time; it could have economic benefits but also plays a significant role in reducing P excretion in monogastric animals.

In cereal grain-based diets, 50%–85% of P is known to be present in a phytate form (phytic acid) (Selle & Ravindran, 2007). Its presence in poultry feed ingredients limits the effective use of organic P, among other nutrients such as calcium, energy, and amino acids, in the digestive tract. This phenomenon has been explained as a result of the chelation of Ca, amino acids, and starch (Liu et al., 2007) by phytate. Consequently, phytate passes largely undigested through the digestive system. To compensate for the lost phytate P and to fulfill the P requirement of monogastric farm animals, bio-available phosphate sources such as dicalcium phosphate (DCP) and monocalcium phosphate (MCP) are being integrated into feed formulations (Lei et al., 2007). However, this practice is not sustainable or environmentally friendly.

The phytase enzyme catalyzes the release of P from the phytate complex, which increases P utilization and availability in diets. These enzymes sequentially cleave orthophosphate groups from the inositol core of phytate, the major chemical form of phosphorus in plants (Lei et al., 2007). Recent studies have demonstrated that phytase hydrolyzes phytate and increases the digestion of P, thereby reducing the excretion of P and lowering environmental pollution. Although the application of phytase to conventional diets improved the digestion of energy and amino acids in broilers (Cowieson et al., 2006b; Ravindran et al., 1999), some studies have shown that phytase supplementation did not affect hen performance or amino acid digestibility, although an interaction between feed type and phytase on the digestibility of amino acids was detected (Snow et al., 2003; Liu et al., 2007).

The use of higher levels of phytase in animal diets has recently gained increasing attention, especially for poultry diets. Cowieson et al. (2006) reported that more than 1,000 FTU/kg phytase improved P and other nutrient utilization in diets fed to broiler chickens as compared to the standard levels. Furthermore, Cowieson et al. (2009) suggested that the recommended inclusion level of phytase in poultry diets is 500 phytase units (FTU)/kg in broilers. However, limited information on laying hens fed diets containing very high doses of phytase has been available, although Ca and P utilization in laying hens is likely more important than in broiler chickens (Kim et al., 2017; Javadi et al., 2021).

The efficacy of phytase on performance and Ca and P digestibility in layers at different levels of phytase treatment demonstrated varying responses. This study therefore intends to examine the impact of phytase supplementation at two different levels, 500 and 700 FTU/kg, on nutrient digestibility and retention in laying hens.

1.2 The specific objectives of the study

- Assess the impact of phytase supplementation on the retention of essential nutrients, such as phosphorus and calcium, in laying hen diets.
- Determine the influence of phytase supplementation at levels of 500 and 700 FTU/kg on the ileal digestibility of crude protein, sodium, Ca, P, and energy in laying hens

2.0 LITERATURE REVIEW

The chapter presents an overview of previously published works on the topic under study. The literature review of this study assessed phytase enzyme, its role in poultry nutrition, industrial application, the effect of phytase supplementation on performance, egg quality traits as well as nutrient digestibility and availability.

2.1 Phytase enzyme and its roles

Phytase is an enzyme that breaks down phytic acid, a substance found in plant feeds such as corn and soybeans, among others. In plants, phytate is stored as phytic acid (myoinositol 1,2,3,4,5,6-hexakis dihydrogen phosphate) and is an important component of phytonutrients. According to Gahlawat et al. (2018), the molecular formula of phytic acid is $C_6H_{18}O_{24}P_6$ with a molecular weight of 659.86, as given by Posternak in 1965 (see Fig. 1). Wodzinski and Ullah (1996) defined phytate as the primary source of inositol and phosphorus storage in plant seeds used as animal feed

materials (oilseed meal, cereals, and legumes). Phytase enzymes work in many ways in animal nutrition, benefiting not only the animal but also the environment and the feed producer.

In animal nutrition, phytase helps release phosphate from phytate, which represents approximately 60–90% of the total phosphate that would otherwise be wasted and pollute the environment through animal feces (Nissar et al., 2017). Phytase (Myo-inositol hexakisphosphate phosphohydrolase) was redefined by Eck (2012) as a type of phosphatase enzyme that catalyzes the hydrolysis of phytic acid (Myo-inositol hexakisphosphate), a type of phosphorus found in grains and oil-based seeds that releases inorganic phosphorus upon hydrolysis. Moreover, phytase initiates the removal of phosphate groups from myo-inositol hexakisphosphate. Kumar and Sinha (2018) also reported that the enzyme can remove orthophosphate groups from the inositol ring of phytic acid to form phytase 3 and produce free inorganic P and low-chain sub-phosphate (inositol pentaphosphate to inositol monophosphate) as intermediaries.

Ruminant animals maintain a microflora that facilitates the release of inorganic phosphorus acid from phytic acid. However, phytic acid is known to be unavailable to monogastric animals. They produce little or no phytase in the intestine, so phytin-P is almost always excreted entirely. Therefore, sufficient external phosphorus sources should be provided to meet the daily mineral needs of an animal. When phytase supplements are added to the feed of broilers, their general growth and development increase (Eltahan et al., 2023). When phytase is added to feed during processing and production, it reduces production costs while improving the quality of the final product.

This anti-nutrient inhibits the absorption of many minerals, such as zinc, iron, calcium, magnesium, manganese, and copper, and also reduces protein digestion (Nissar et al., 2017). Phytate can be removed by the phytase enzyme through hydrolysis to inositol, phosphate, and other minerals such as calcium, iron, and zinc (El Enshasy et al., 2018). Once the substrate is bound, the enzyme catalyzes the hydrolysis of phytic acid, removing the phosphate group (Lei et al., 2007). This process releases free phosphorus and activates inositol, making it a bioavailable mineral. Enzymatic degradation not only increases phosphorus levels and its absorption but also releases other bound minerals such as calcium, magnesium, iron, and zinc.

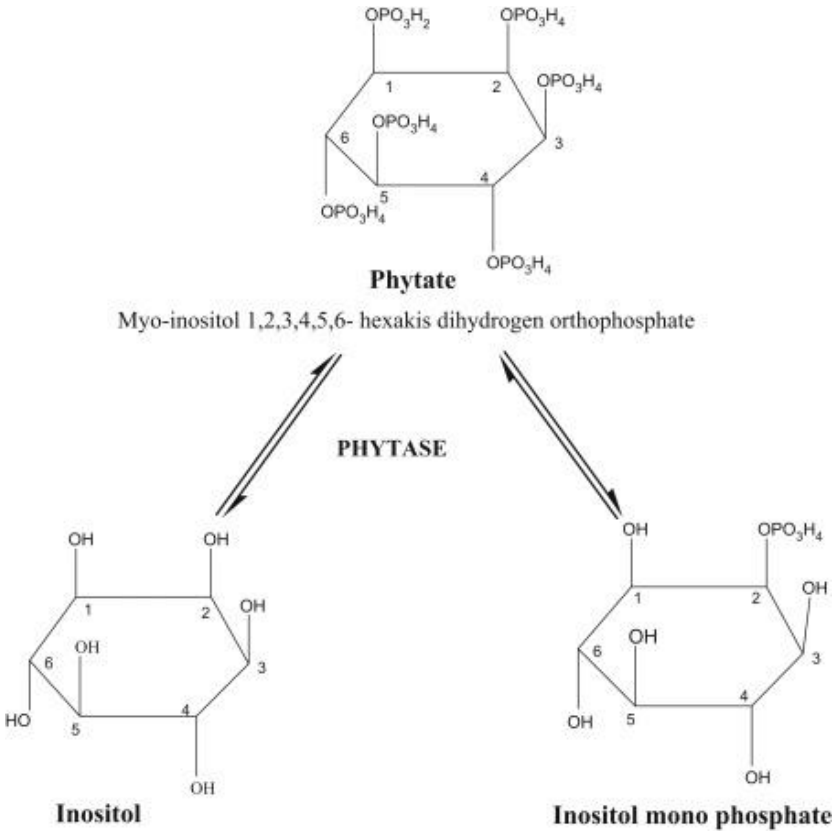
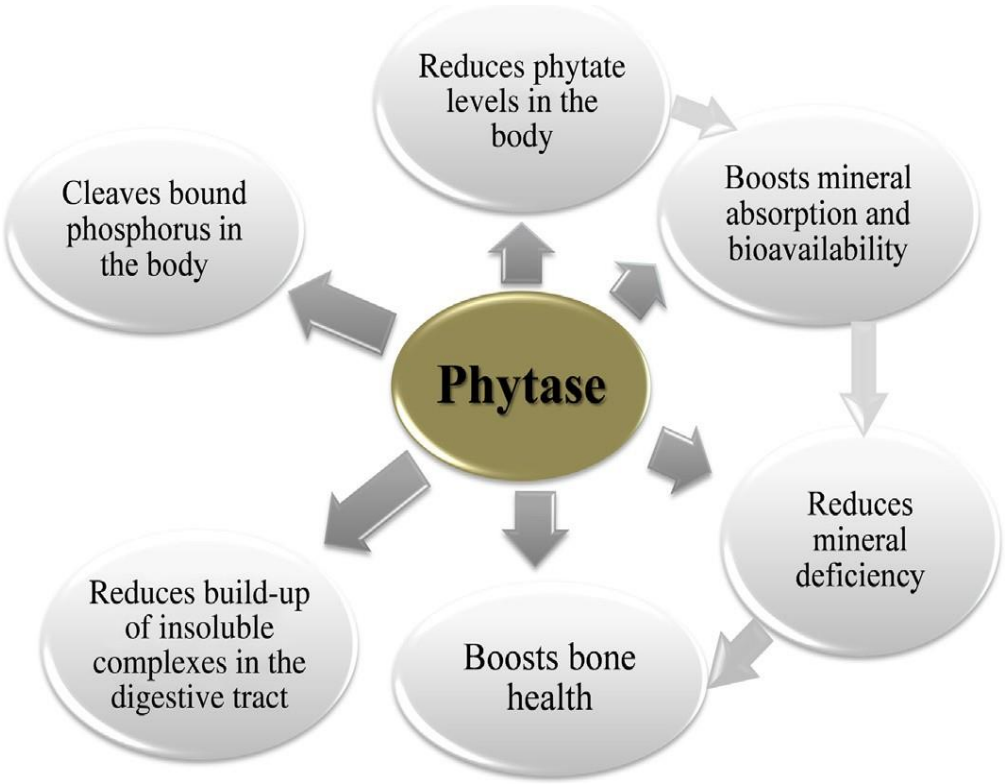


Figure 1. Scheme of phytase action

Studies have reported that phytic acid binds $Ca^{2+,}$ Zn2+8, $Mg^{2+,}$ and Fe^{3+} and trace minerals such as Mn2+, $Cu^{2+,}$ and Mo^{+}, and also binds and forms complexes with proteins, thus rendering them unavailable to the animal (Wodzinski & Ullah, 1996). However, Kumar & Sinha (2018) reported that supplementing the basal diet with phytase can significantly increase the body's ability to absorb and assimilate important minerals such as Ca, Mg, Zn, and Fe, among other health benefits (Figure 2**).** Phytic acid or its intermediates (inositol) have been reported to play a role in starch digestibility and other blood functions (Thompson, 1986) and, as an antioxidant (Graf et al., 1987), in reducing cholesterol and triglycerides (Jariwalla et al., 1990).

Figure 2.The health benefits of supplementing phytase in the diet



Adopted from; Kumar, (2018),

2.2 Types of phytates

Currently, there are four classes of phosphatase enzymes that are known to degrade phytate and they include representatives of: (1) Histidine Acid Phosphatase (HAP), (2) Purple Acid Phosphatase (PAP), (3) Cysteine Phosphatase (CP), and (4) b-Propellar Phytase (BPP). According to Greiner & Konietzny (2006), phytases have been divided into acid and alkaline phytases - depending on their pH optima - and also on the carbon ring in the myo-inositol of phytate where dephosphorylation into 3-phytases (E.C. 3.1.3.8), 5-phytases (E.C. 3.1.3.72) and 6-phytases (E.C. 3.1.3.26) is initiated. However, all classes cannot effectively utilize phytate substrate. Each phytase type has unique structural features due to its distinct catalytic apparatus that allows it to utilize phytate as a substrate in various environments. Only phytases belonging to the PAP and HAP groups have recently been identified and recognized and this current review focuses on the major features of such phosphatases.

2.3 Purple acid phosphatase phytases (PAPhys)

These are a specific class of enzymes found in various organisms, renowned for their ability to hydrolyze phytic acid, a major storage form of phosphorus in plant seeds and grains. These enzymes have gained significant attention due to their crucial roles in nutrient release, making phosphorus more accessible for plants, animals, and various industrial applications. All members of the purple acid phosphatase metal-lophosphoesterases contain a unique set of seven amino acid residues (D, D, Y, N, H, H, H) vital for metallic chelation. These seven metal ligands are contained in a shared pattern of five common consensus motifs (DxG/GDx2Y/GNH(E, D)/Vx2H/GHxH) (Brinch-Pedersen et al., 2014).

PAPhys are prevalent in the plant kingdom. They are notably found in seeds and grains, such as wheat, barley, soybeans, and maize. According to Brinch-Pedersen et al. (2014), the wheat TaPAPhy is glycosylated with different site-specific N-glycans. Phylogenetic evaluation of various plant PAPs identified 5 types of PAPs where the PAPhy group. In addition to the five PAP metal coordinating motifs, contained a consensus of four consensus motifs (Dionisio et al., 2011b). Furthermore, Dionisio et al. (2011), stated that the PAPhys possessing motifs all have a high specific phytase activity.

2.4 Histidine acid phosphatase phytases (HAPhys)

Among the very large group of HAPs, HAPhys represents only a small subgroup of enzymes within the broader category of acid phosphatase phytases. However, all members of HAP share a common catalytic mechanism and site function. The RHGXRXP is an N-terminal active site motif and HD is the C-terminal motif (Wodzinski and Ullah, 1996). HAPhys are particularly characterized by the presence of histidine residues within their active sites, which are essential for their enzymatic function. The distant RHGXRXP and HD sequences link to form a single catalytic center that initiates a two-step reaction that hydrolyzes phosphomonoesters (Brinch-Pedersen et al., 2014). Glycosylation is an important factor in determining and maintaining the structure of HAPhys, while the disulfide bridges perform an important role in maintaining the correct 3-dimensional structure to allow for catalytic activity in phytase (Wang et al., 2004).

2.5 Sources of Phytases

Phytase is widely distributed in various life forms. The most important source of phytase is microorganisms, followed by plants. Broadly, there are four possible sources of phytase and these include; plant phytase, microbial phytase, phytase generated by the small intestinal mucosa, and

gut-associated microfloral phytases (Kumar & Sinha, 2018). In plants, phytases are usually observed in oilseeds and nuts, legumes, and in cereal pollen grains. Specifically, during seed germination, phytase activity increases to promote fast plant growth (Greiner & Konietzny, 2006). Reports suggest that phytases can also be found in plant roots, but with lower hydrolysis activity. Many soil microorganisms, especially rhizosphere microorganism species produce phytase as one of the mechanisms to increase phosphorus availability for plant growth and, hence are used in biofertilizers (El Enshasy et al., 2018). For this study report, however, the focus has been put on only two sources; Microbial and fungal phytases.

2.5.1 Microbial phytase

Bacteria and fungi comprise the most important sources of phytase. The most commonly used strains of yeast for the commercial production of phytases are A. niger, Aspergillus ficuum, A.fumigatus, and S. cerevisie (Kumar & Sinha, 2018). At present several commercial microbial phytases are available on the market with different trade names, (see **Table 1)**. In addition, a group of marine yeasts producing phytase is capable of producing alkaline phytase (Kumar & Sinha, 2018).

Microbial phytases have been isolated from fungi, yeast, bacteria, and protozoa. Most of these enzymes belong to the histidine acid phosphatase or alkaline phytase sub-families and exhibit considerable variations in kinetics, stereospecificities, and biochemical properties. Several microbial phytases have been produced and commercially used as animal feed supplements in recent years.

Table 1. Commercial production information of microbial phytases (source; Coa et al., 2007)

Company	Country	Phytase source	Production strain	Trademark
AB Enzymes	Germany	*Aspergillus awamori*	*Trichoderma reesei*	Finase
Alko Biotechnology	Finland	*A. oryzae*	*A. oryzae*	SP, TP, SF
Alltech	USA	*A. niger*	*A. niger*	Allzyme phytase
BASF	Germany	*A. niger*	*A. niger*	Natuphos
BioZyme	USA	*A. oryzae*	*A. oryzae*	AMAFERM
DSM	USA	*P. lycii*	*A. oryzae*	Bio-Feed Phytase
Fermic	Mexico	*A. oryzae*	*A. oryzae*	Phyzyme
Finnfeeds International	Finland	*A. awamori*	*T. reesei*	Avizyme
Genencor International	USA	*P. simplicissimum*	*Penicillium funiculosum*	ROVABIO
Roal	Finland	*Aspergillus awamori*	*T. reesei*	Finase
Novozymes	Denmark	*A. oryzae*	*A. oryzae*	Ronozyme® Roxazyme®

2.5.2 Fungal and yeast phytases

Usually classified as 3-phytases, most phytases isolated from fungi and yeast are histidine acid phosphatases, glycosylated, and active for a wide variety of substrates (Wyss et al., 1999). According to Lei et al. (2007) *Aspergillus niger*, PhyA was the first well-characterized and commercialized phytase. It is encoded by a 1.4 kb DNA fragment, and the enzyme is encoded by a monomer with an approximate molecular weight of 80 kDa, a bi-hump pH profile with two optimal pH at 2.5 and 5.0–5.5. It has an optimal temperature of 55–60^{O}C, and a high affinity for phytic acid.

Aspergillus fumigatus phytase shares 66% sequence identity with A. *niger* PhyA phytase but displays better thermo-tolerance. Thermal tolerance has been associated with better post-thermal recovery and can be modified by specific buffers used for thermal treatment (Lei et al., 2007). Characterization studies by El Enshasy et al., (2018) revealed that phytases from soil micromycetes, yeast, Bacillus sp., and Enterobacter sp. exhibit high specific activity and show a broad range of optimum pH (pH 3.5 to 7.5) and temperature (37˚C - 70˚C). Amongst yeast, extracellular phytase has been reported in *Schwanniomyces castellii, Arxula adeninivorans,* and *Pichia anomala* and characterized (Gahlawat et al., 2018)

2.5.3 Plant phytases

In small grains, approximately 90% of phytate accumulates in the aleurone layer and approximately 10% in the embryo (O'Dell et al., 1972)., in wheat, for example, the phytic P constitutes 70-75 % of the total P of the grain (Peers, 1953) (**Table 2**). Most phytates exist as phytin, a mixed salt (usually with K +, Ca 2+, Mg 2+, or Zn 2+) that is embedded as globoid crystals in the same membrane with proteins (Lott, 1984). Phytase is present in the purified aleurone vacuole of wheat grains, and phytase de novo is synthesized during germination in purified wheat grains after 6 days of germination. (Dionisio et al., 2011).

Phytase enzymes have been isolated and characterized from several plant sources—wheat, rice, rape seed, soybean, maize, and rye (Table 2). Most of these phytases catalyze the hydrolysis of phytate at the C6 position of the myo-inositol hexaphosphate ring, so they are considered type 6 phytases. However, in soybean, major InsP5 is DL-Ins (1,2,4,5,6) P5 and thus soybean phytase is a 3-phytase (Kumar & Sinha, 2018). Two enzymes, Phy1 and Phy2, have previously been purified from wheat bran (Lim & Tate, 1973) and two isozymes with the N-terminal amino acid have also been identified.

Table 2. Phytase contents in plants or plant products

	Total P (g/kg)	Phytate-P (g/kg)	Proportion (%)
Cereals Wheat grain	3.07	2.19	71.6
Oat	3.60	2.10	59.0
Corn grain	2.62	1.88	71.6
Barley grain	3.21	1.96	61.0
Sorghum grain	3.01	2.18	72.6
Rye	3.05	1.95	63.9
Oilseed meals Canola meal	9.72	6.45	66.4
Cottonseed meal	10.02	7.72	77.1
Corn glutton meal	4.24	2.67	63.0
Rapeseed meal	9.60	6.34	66.0
Soybean meal	6.49	3.88	59.9
By-products			
Rice bran	17.82	14.17	79.5
Wheat bran	10.96	8.36	76.3

Source: Cao et al., 2007

The proportion of bounded P in grains varies among different cereal grains and represents the fraction of phosphorus tightly bound to phytate or other compounds within grains, which animals struggle to digest efficiently (**Table 3**). Ravindran et al. (1995) stated that understanding this proportion is critical in formulation and allows the use of appropriate processing techniques to enhance phosphorus availability in cereal grains.

Table 3. Bounded P in grains

Grain	Phytic acid [(1)] g/kg	Bounded P g/100g DM. [(2)]	Bounded P in % of total P [(2)]	Endogenous phytase activity (FTU/kg)
Maize	2.22	0.24	72	15
Wheat	0.4-0.9	0.27	69	1193
Barley	0.99	0.27	64	582
Rye	0.68-1.4	0.24	66	5130
Rice	0.1-0.2	0.27	77	Na
Pea	1.1	0.24	50	Na
Soybean meal	0.3-1.7	0.39	60	8
Rape seed meal	na	0.70	59	16
Sunflower meal	1.9	0.89	77	na

Adopted from Ravindran et al. (1995).

In most cereal grains, enzymes (referred to as pre-formed enzymes) are of fundamental biological importance as they ensure the early progression of germination (Brinch-Pedersen et al., 2014). The level of pre-formed phytase, constituting the mature grain phytase activity (MGPA), varies considerably between cereal species (Brinch-Pedersen et al., 2014), (see **Table 4).** However**,** according to Ram et al. (2010), this variation also occurs within species, with up to two-fold differences between cultivars of wheat. Non-Triticeae cereals such as maize (*Zea mays* L.), rice (*Oryza sativa* L.), and oats (*Avena sativa* L.) by contrast, however, have very little MGPA.

The choice of cereal species and also the cultivar has significant implications on the efficiency of phytate hydrolysis and whether soaking or germination of the grain is necessary for initiating the hydrolysis (Brinch-Pedersen et al., 2014).

Table 4. Mature grain phytase activity of the most important cereals

Species	Phytase activity (mean ± SD) FTU/Kg^{-1}[a]
Rye (*Secale cereale* L.)	5147 ± 649
Triticale (*Triticosecale*)	1688 ± 227
Wheat (*Triticum aestivum* L.)	1637 ± 275
Barley (*Hordeum vulgare* L.)	1016 ± 330
Millet (*Pennisetum typhoides* L.)	56 ± 0.6
Oat (*Avena sativa* L.)	84 ± 39
Maize (*Zea mays* L.)	70 ± 5
Rice (*Oryza sativa* L.)	190 ± 14
Sorghum (*Sorghum sudanensis* L.)	110 ± 12

Source: Brinch-Pedersen et al., 2014

[a] 1 FTU is the amount of enzyme that liberates 1 mmol orthophosphate/min from phytate.

2.5.4 Animal phytases

In animals, there is evidence of phytase activity in the stomach and intestine of pigs and broiler, (Humer et al., 2015), however, it is not significant. Low stomach mucosal phytase activity has been discovered in pigs (El Enshasy et al., 2018) and broilers (Tamim et al., 2004). Phytase was partially purified from rat, calf, chicken, and human intestines, first phytate hydrolysis was observed in the rat intestine (Gahlawat et al., 2018). It is reported that the human intestine shows about 30 times lower phytase activity than rats. Maximum phytase activity was found in the duodenum and minimum in the ileum, but humans have limited capacity to digest undegraded phytases (Iqbal et al., 1994).

2.6 Condition for phytase activity

Under favorable conditions, endogenous phytase can play an important role in hydrolyzing phyta te in food and feed. In general, hydration and breakdown of the plant material are required to facilitate the contact between phytase and substrate. Furthermore, the kinetic parameters such as maximum velocity (Vmax), Michaelis constant (Km), pH, temperature, and other potential inhibitors or activators must be within ranges where the endogenous phytase is active and stable (See **Table 5**). These conditions must be maintained for sufficient time for the hydrolysis to proceed. For instance, a lower Km value indicates a higher affinity between the enzyme and

substrate. This incubation can occur in the stomach. A positive effect on endogenous phytase activity in the gut has been demonstrated by experimental feeding of corn (low phytase activity) and triticale (high phytase activity) in pigs (Pointillart et al., 1987).

Table 5. Properties of biological purified cereal phytase**.**

	*V*max (mmol∗min^{-1} mg^{-1})		*K*$_m$ (mM)	*k*cat (s^{-1})	Temp opt (^{o}C)	Monomer MW (Dalton)	pH optimum	Reference
Wheat PHY1(bran)	127	0.48		150	45	71,000	6.0	(Nakano et al., 1999)
Wheat PHY2 (bran)	242	0.77		266	50	66,000	5.5	(Nakano et al., 1999)
Wheat phytase (bran)	230		830	238	45	62,000	6.0	(Bohn et al., 2007)
Spelt D21 PhyI (germinating)	262		400	297	45	68,000	6.0	(Konietzny et al., 1995)
Rye PhyI (mature grains)	517		300	586	45	68,000	6.0	(Greiner et al., 1998)
Rice F1 (bran)	51		170	187	45	66,000	4.4	(Hayakawa et al., 1989)
Rice F2 (bran)	58	90		102	45	68,000	4.6	(Hayakawa et al., 1989)
Oat PhyI (germinating)	307	30		348	38	68,000	5.0	(Greiner and Alminger, 1999)
Barley P1(germinating)	117	72		131	45	67,000	5.0	(Greiner et al., 2000)
Barley P2 (dry seeds)	43		190	48	55	67,000	6.0	(Greiner et al., 2000)

Source: Brinch-Pedersen et al., 2014

2.7 Production of phytase enzyme

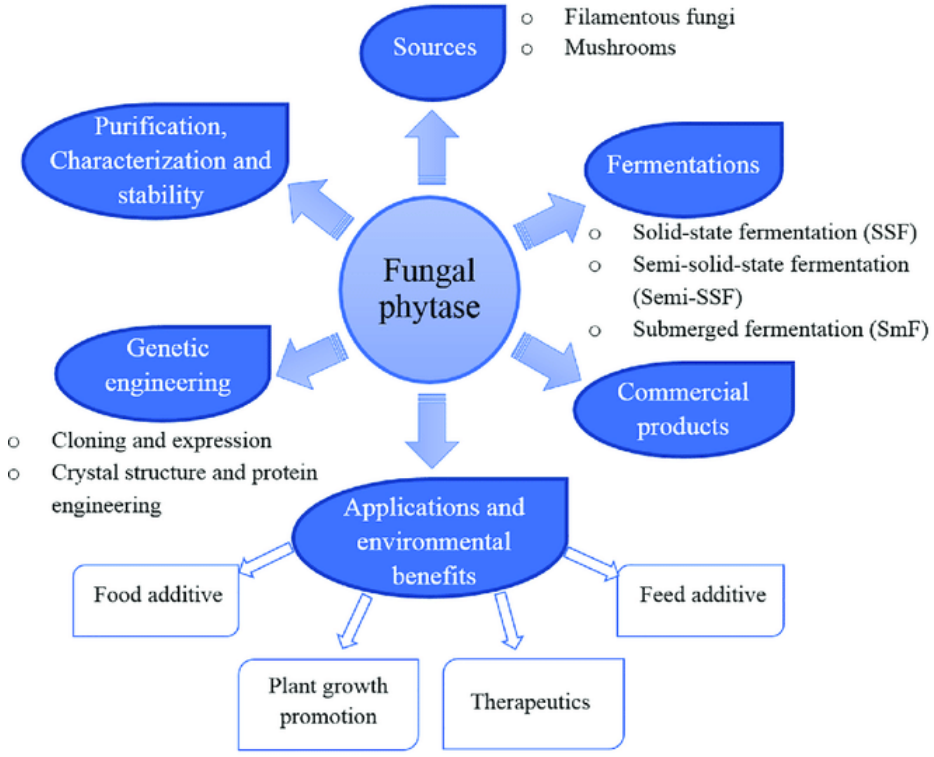
Fermentation is the dominant and sustainable method for fungal phytase production. Both solid-state and submerged fermentation have been employed to manufacture phytase by filamentous fungus (Rizwanuddin et al., 2023). The production of fungal phytases has been achieved under three different fermentation methods including solid-state, semi-solid-state, and submerged fermentation (Han & Wilfred, 1988). Moreover, exogenous phytases that are commercially accessible are frequently produced from microbes employing solid-state fermentation (SSF), semi-

solid-state fermentation (SSSF), and submerged fermentation (SMF) (See Fig 3). Jatuwong et al. (2020) described *Aspergillus ficuum* NRRL 3135 as the most active fungal phytase producer and most commonly employed at the commercial level.

Fungal phytases are commonly produced using solid-state fermentation (SSF) methods (**Figure 3**), here agricultural wastes and other valuable by-products are used as substrates in the SSF process.

According to Jatuwong et al. (2020), solid-state fermentation is a process through which microorganisms are grown on a solid material surface with the absence or near absence of free water. However, the process must include sufficient moisture to support microbial growth**.** During the fermentation process, agricultural residues and other waste materials have been used as substrates for the evaluation of enzyme production. Other nutrients, physical conditions such as pH and temperature, and protease resistance are important factors for increasing phytase production.

Figure 3. The summarization of fungal phytase production



Adopted from; (Jatuwong et al., 2020)

In this way, enzymes can be easily extracted from water and the process is known to be cheap, easy to use, and time-consuming. The process is widely used in fermentation processes, especially in the production of enzymes**.** However, not only SSF has been investigated for phytase production, but several research studies have also investigated phytase production involving SmF and semi-solid fermentation methods (Han & Wilfred, 1988; Jatuwong et al., 2020).

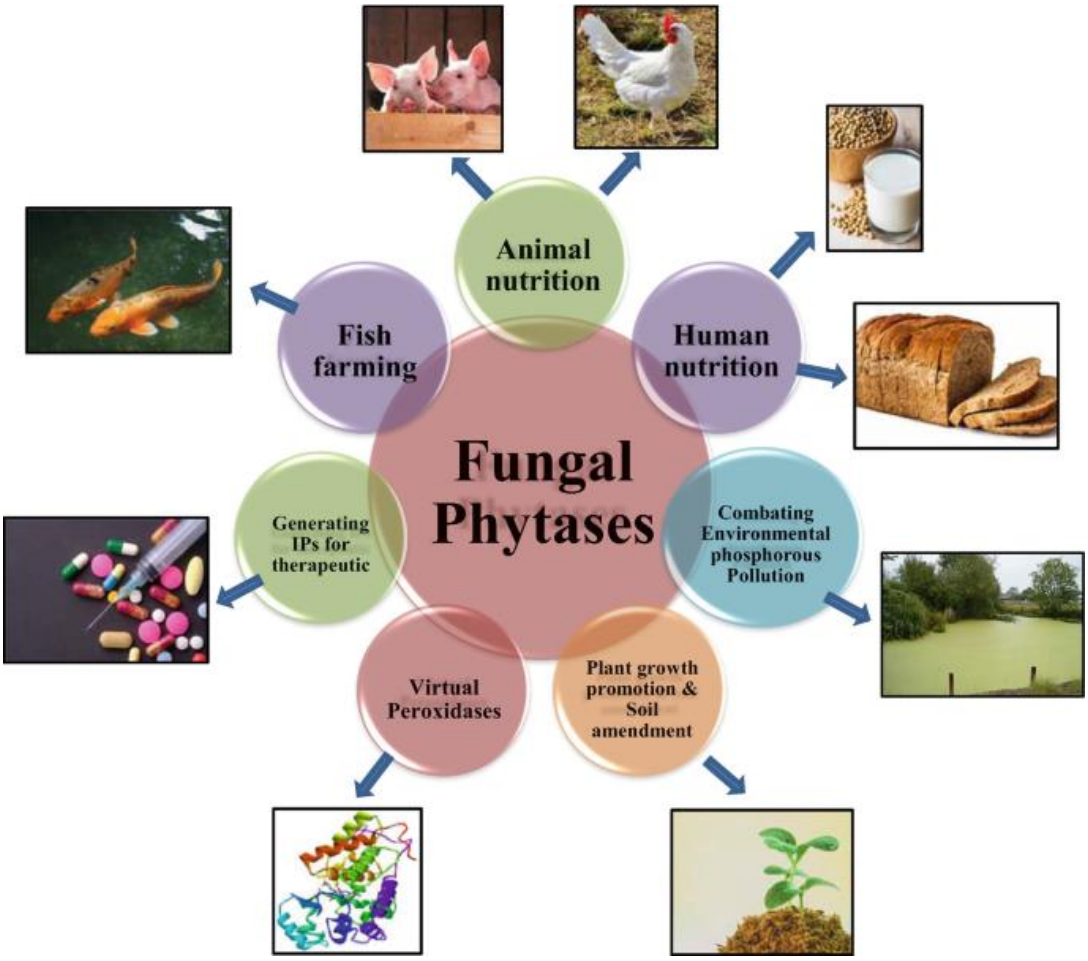
2.8 Industrial Application of Phytase in Animal Feeds

To date, phytase has been used in various biological processes related to the processing and prod uction of animal and human foods. About, total enzyme market production, 60% of phytase is used for the production and manufacturing of animal feed and food supplements (**Figure 4)** that have a market capitalization of 350 million USD annually. The main application of animal feed supplements is to increase phosphorus availability and also reduce the phosphorus burden in the ecosystem. About 70% of animal feed contains phytase as an additive.

According to Cao et al. (2007), Natuphos was the first commercially available phytase enzyme developed in 1991 by a Germany-based company BASF from a genetically modified A. niger strain. Since then, Natuphos and other phytase products have become integral components of animal feed formulations, contributing to improved feed efficiency and reduced environmental impact associated with phosphorus excretion in animal waste. Later, a commercial product 6-phytase (Ronozyme P) was derived from *Peniophora lycii.*

Currently, on a commercial scale, phytase production is either carried out using phytate-producing fungi or recombinant DNA technology. Phytase activity is defined as phytase units (FTU or U), where one FTU is defined as the quantity of enzyme that liberates 1 micro-mol of inorganic-P per minute from 0.0015 mol/l sodium phytate at pH 5.5, and 37℃ (Cao et al., 2007).

Figure 4. Application of fungal Phytase



Adopted from Kaur et al. (2021)

2.9 Phytase Market trends and manufacture

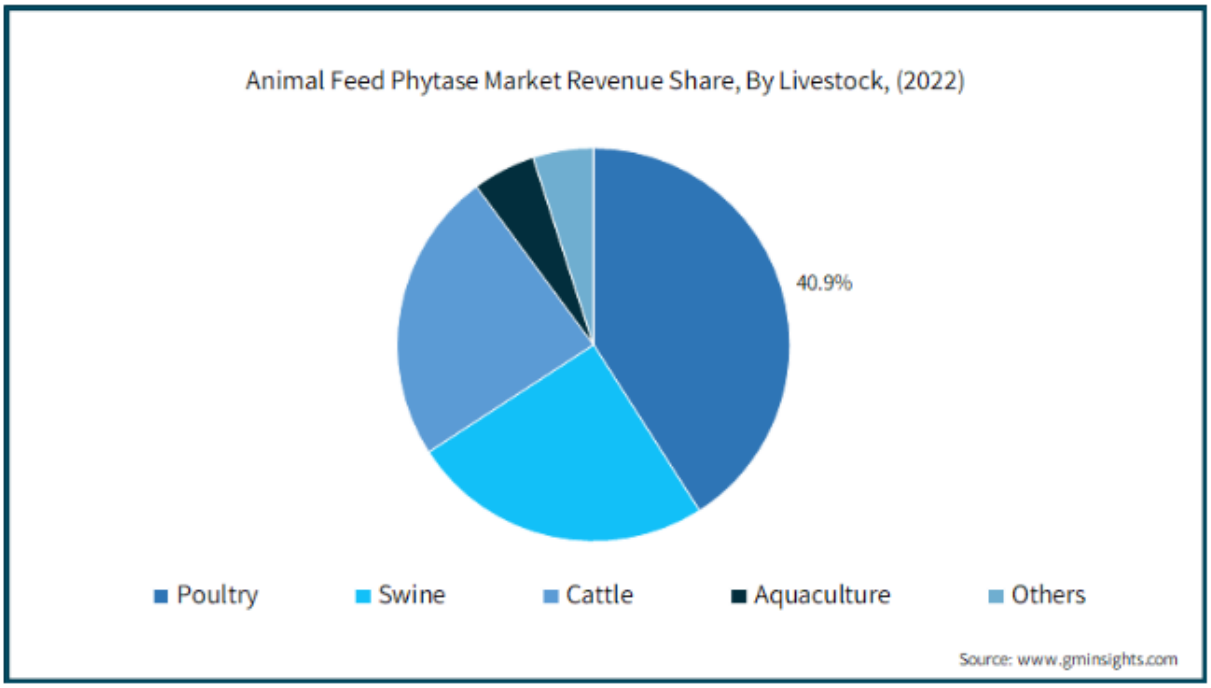
The phytase enzymes have come into view as big feed augments in animal nutrition across the world. With the EU authorizing its use in poultry and pig feed as EFSA declared it safe as an enzyme for aquafeed, accelerating the usage of 6-phytase in swine and poultry feed application. According to the Global Market Insight Report (2022), The animal feed phytase market size was worth USD 510 million in 2021, the industry is estimated to depict a CAGR of 6.5% from 2022 to 2030. The report suggested that the trends were being influenced by overlapping demands for food to feed the ever-growing population.

The European pet food market is expected to reach US$394.26 million by 2030 at a CAGR of 4. 54% and is currently expected to reach US$26 million by 2030 at a CAGR of 4.54%. (http://www.minsights.com)

The animal feed phytase is divided into 3-phytase, 6-phytase, and among others. 6- The phytase segment is expected to reach a valuation of USD 390 million by 2030. Jatuwong et al. (2020) reported that 3-phytase and 6-phytase held the highest revenue share of 83.6% of the total industry in 2015 and accounted for annual sales of US$ 350 million. 6- phytase is manufactured by a genetically modified strain of *Aspergillus aryzae* and *Komagataela phaffii*. On the other hand, the animal feed phytase market from the plant source segment is slated to grow at a CAGR of 7% by 2030 according to a global market insight report (2022).

The third category of animal feed phytase market is the liquid and granules or powder. This segment is estimated to grow at a CAGR of 6.5% due to its extensive use in the post-pelleting process, free from turbidity or suspended particles thus guaranteeing optimum post-pelleting system application to cooled pellets. In terms of livestock, the poultry segment was valued at USD210 million in 2021 and is forecast to progress at a CAGR of 6% by 2030 (**Fig 5**). Additionally, this surging demand for poultry meat in the food and beverage industry is amplifying the demand for phytase additives in poultry feed production.

Figure 5. Animal feed phytase Market revenue



2.10 Effect of phytase supplementation on the performance of laying hen

Phytate is abundant in feed ingredients and is not easily broken down in the digestive system of chickens due to the lack of endogenous phytase. It comprises approximately two-thirds of the total P in feed items of plant origin (cereal grains, beans, and oilseed meals).

Its presence in poultry feed reduces the efficient use of P and other nutrients, including Ca, energy, and amino acids, in the digestive tract. This is due to the chelation of Ca, amino acids, and starch by phytate (Liu et al., 2007). Phosphorus is a crucial nutrient for metabolic processes and one of the most important minerals. Calcium and P are critical to the formation and maintenance of skeletal function and eggshells (Eltahan et al., 2023).

2.10.1 Effect of phytase on performance and egg quality traits.

Several studies have indicated that supplementing laying diets with dietary phytase results in improved performance (Javadi et al., 2021), particularly when dietary levels of non-phytate P (NPP) are low (Gordon & Roland, 1998). For this reason, it is largely agreed that the influence of phytase on egg production is particularly notable. Furthermore, laying hens supplemented with phytase often exhibit increased egg production rates and improved persistency in laying over their productive life. Eltahan et al. (2023) reported that the addition of phytase to the diet containing 0.20, 0.25, and 0 0.30% NPP increased egg production by 1.50, 1.64, and 0.97%, respectively, and improved eggshell thickness. Furthermore, in the same study, NPP at 0.25% and 0.30% increased the plasma concentration of albumin (ALB), high-density lipoprotein (HDL), and phosphorus (P). Yolk color, expressed as lightness (L*), redness (a*), and yellowness (b*), increased significantly in the treatment groups supplemented with phytase (Dersjant-Li et al., 2018).

Supplementing phytases in the negative control diet improved the egg production, egg mass, and eggshell quality to the levels of birds fed the positive control diet (Liu et al., 2007), significantly improved the performance of birds and the eggshell thickness ($P < 0.05$), and also increased feed intake, egg mass, eggshell hardness. Taylor et al. (2018) further reported that the addition of phytase increased daily egg mass ($P < 0.05$), and again, and hen-day production ($P < 0.05$) irrespective of phytase level. This improvement was observed at both phytase levels with no additional benefit at the super-dose level. A study by Javadi et al. (2021) reported that phytase inclusion linearly increased the yolk color ($P < 0.05$). Tibia of laying hens fed with PC had significantly higher ash content than those on the NC diet ($P < 0.05$).

2.10.2 Effect of phytase on digestibility and availability of nutrients

Phytase plays a pivotal role in improving the digestibility and availability of nutrients in animal diets. By enhancing phosphorus availability, phytase positively influences the utilization of other essential nutrients (Dersjant-Li et al., 2018). Phosphorus is a key mineral involved in various physiological processes, including bone development, energy metabolism, and nutrient utilization. When phytic acid is degraded, not only is inorganic phosphorus released, but the enzyme also improves the bioavailability of other minerals bound to phytic acid, such as calcium, magnesium, iron, and zinc. It is well established that a high dietary level of calcium (Ca) reduces the activity of phytase (Ravindran et al., 2000).

Furthermore, P plays an important role in Ca metabolism. Part of the phytase benefit observed in poultry-fed low-NPP diets may be due not only to P but also to the effect of P on efficient Ca utilization (Gordon & Roland, 1998). However, the number of studies on the use of exogenous phytases in laying hens is smaller, and some authors affirm that the benefits of supplementing layer diets with phytases are still under discussion (Javadi et al., 2021). Although some authors have indicated that phytase inclusion in the diet at 250–500 FTU units can improve dietary P absorption, there is no consensus on its possible effect on improving dietary energy and protein utilization, and therefore, on laying hens' performance and bone mineralization (Javadi et al., 2021). To improve the utilization of these nutrients, some authors mention that super-dosing these exogenous phytases (1000 FTU or more) could eliminate phytates from the diet, contributing to an improvement in the nutritional value of the diet (Cowieson et al., 2011; Javadi et al., 2021).

The earliest report on the possible energy effect of phytase was by Rojas and Scott (1969), and Miles and Nelson (1974) who found that AME yields for chicks from cottonseed meal and soybean meal were significantly improved following treatment with a crude phytase preparation from *Aspergillus ficuum* (Ravindran et al., 2000). However, early research reported that there were no significant differences between 0.2 to 0.5% NPP diets and supplementation of phytase gave no further improvement in performance (Abudabos, 2012).

In some study by Liu et al. (2007), phytase supplementation numerically improved the digestibility of Lys (3.9%), Arg (2.5%), His (5.2%), Phe (4.2%), Leu (3.8%), Ile (16.8%), Thr (7.1%), and Val (6.5%), and improved the digestibility of P and Ca in the negative control diet (**See Table 6)** respectively, whereas it improved the digestibility of amino acids by 2 to 8% ($P < 0.05$).

Table 6. Effect of phytase on ileal digestibility of nutrients in brown layers from 23 to 28 weeks of age (adopted from Liu et al., (2007)

Item	Digestible energy [Mcal/kg (MJ/kg)]	N (%)	Ca (%)	P (%)
Positive control	2.65 (11.07)[a]	74.62[a]	45.37[ab]	50.68[a]
Negative control	2.44 (10.20)[b]	67.84[c]	33.25[b]	30.59[c]
Negative control + A[1]	2.39 (9.97)[b]	71.93[ab]	—	39.29[b]
Negative control + B[1]	2.32 (9.69)[b]	70.27[bc]	37.79[ab]	41.61[ab]
Negative control + C[1]	2.56 (10.69)[ab]	72.36[ab]	48.33[a]	44.12[ab]
SEM	0.03	0.61	2.34	1.79

[a–c]Mean values within columns not sharing the same superscript are different ($P < 0.05$). [1]A, B, and C are phytases, each at 300 phytase units/kg of feed. A is derived from *Aspergillus niger, and* B and C are derived from *Escherichia coli* [2] A dash (—) indicates a missing value.

In a similar study by Juanpere et al. (2005), phytase supplementation to low-P diets significantly improved the apparent retention of minerals, and the apparent P retention coefficients increased from 0.68 to 0.70. Phytase addition in a diet increased P digestibility at 300 FTU/kg and further increased digestibility at 1500 FTU/kg phytase ($P < 0.001$) and increased Mg digestibility ($P = 0.061$) in laying hens (Taylor et al., 2018). Dietary treatment with phytase had significant effects on the P percentage content in the excreta and fecal P excretion measured at 53 weeks of age compared with the control group (Rizwanuddin et al., 2023). The availability of, Ca, P, and energy has increased in laying hens when phytase is supplemented to the diets.

2.10.3 Summary of literature

Phytase enhances phosphorus availability, benefiting nutrient utilization and bone health in poultry diets. Its efficacy in improving mineral absorption, such as phosphorous and calcium, and potentially enhancing amino acid digestibility. While some researchers advocate for higher phytase dosages to maximize nutrient release, its impact on energy utilization remains debated. Nevertheless, phytase supplementation shows promise in enhancing laying hen performance and bone mineralization, particularly in diets deficient in non-phytate phosphorus. Overall, phytase offers a multifaceted approach to improving nutrient utilization and may play a crucial role in optimizing dietary formulations for poultry production. However, it is still not clear how the nutrients' ileal digestibility, particularly protein, sodium, and energy changes when phytase is added to feeds.

3.0 MATERIALS AND METHODS

All trial procedures (including pen size) were in line with local welfare regulations, approved by the Animal Use and Care Administrative Advisory Committee, and approved by the Agricultural Administrative Authority, Hungary (permission ID: SO/31/01324-3/2020).

3.1 Experimental design and dietary treatments

In the trial, 4 dietary treatments were used as shown in Table 7. Positive control feed (PC) was formulated according to the recommendation, and Negative control feed (NC) was under-formulated for P and Ca. Those feeds were not supplemented with any dietary phytase enzyme. In diets fed in treatment Phy500 and Phy700, phytase was added at 500 and 700 FTU/kg levels to the negative control feed, respectively.

The experimental feeds were based on wheat, corn, soybean meal, and sunflower meal. Diet composition and the analyzed nutrient content are found in Table 7.

Diets were manufactured by the Department of Farm Animal Nutrition (Hungarian University of Agriculture and Life Sciences, Kaposvár Campus).

Proximate analysis of each diet (treatment) was undertaken for TiO_2, ether extract, protein, ash, fiber, GE, Ca, P, and Na (see later).

Table 7. Analyzed the nutrient content of the diet.

Treatment	DM %	Ca g/kg	Pg/kg	CP%	Na g/kg	Tio2 %	GE J/g
PC	91.6	35.60	6.00	16.4	2.2	0.59	15084
NC	91.5	34.70	4.45	16.5	2.1	0.55	15368
Phy500	91.5	33.70	4.69	16.8	2.0	0.55	15518
Phy700	91.6	34.10	4.66	16.5	2.1	0.61	15013

Table 8. Analyzed enzyme activity of feeds in different treatments

Treatments	Phytase (FTU/kg diet)
PC	ND
NC	ND
Phy500	490
Phy700	635

ND: Not detected.

Table 9. Feed composition

	PC	NC	Phy500	Phy700
Wheat	48,185	50,145	50,140	50,135
Corn	15,000	15,000	15,000	15,000
Soybean meal (CP 44 %)	12,270	11,720	11,720	11,720
Sunflower meal (CP 34 %)	10,000	10,000	10,000	10,000
Sunflower oil	3,000	2,460	2,460	2,460
Limestone	3,200	3,150	3,150	3,150
MCP	1,180	0,400	0,400	0,400
Corse Ca	6,120	6,070	6,070	6,070
Salt	0,230	0,230	0,230	0,230
L-Lysine HCL	0,190	0,200	0,200	0,200
DL-Methionine 99%	0,160	0,160	0,160	0,160
L-Threonine	0,050	0,050	0,050	0,050
Phytase	0,000	0,000	0,005	0,010
Others*	0,415	0,415	0,415	0,415

** Vitamin and mineral premix, choline chloride, sodium bicarbonate.*

3.2 Birds and Housing

A total of 120 Lohmann Brown hens, 35 weeks old at arrival were used in this experiment. The retention study started 4 weeks later when the flock was 39 weeks old. Birds were allocated at random to 4 treatments, with 2 birds/cage and 10 cages/treatment (760 cm^2/hen). The layers were in their peak production, the laying intensity was 96% when the retention study started.

Feed and water were available *ad libitum* throughout the experiment. Lighting and ventilation followed the standard breed recommendations. Birds received standard commercial rearing and housing before placement on trial.

3.3 Digestibility and Retention Study

A retention study with 10 birds per treatment and an ileal digestibility study with 30 birds per treatment was performed.

The retention study was performed by collecting the total excreta produced per cage. In advance of the collection period trial feeds contained the marker (Ti-dioxide) too. The adaptation period lasted for 4 days as well as the collection period. Feed consumption was measured daily in both the pre-collection and the collection period as the difference between the offered feed in each cage and the feed residue.

The digestibility of nutrients was determined right after the retention study. In the *post-mortem* digesta collection, 30 hens per treatment were used. Nutrient (DM, P, N, GE, Ca, Na) digestibility assessment was conducted using standard protocols to determine apparent ileal digestibility *post-mortem* at the end of the trial. All birds were kept in the same room and environmental conditions and received identical feeds according to their experimental treatment group. Digesta samples used for chemical analysis consisted of pooled ileal digesta of 3 birds for each sample. At the end of the trial, birds were asphyxiated with CO_2, and the intestine section beginning at Meckel's diverticulum up to 2 cm anterior to the *ileocaecocolonic* junction was immediately removed and the digesta was collected.

3.4 Chemical analysis

The sample preparation in retention and digestibility studies involved the following: ileal digesta was freeze-dried at -51ºC for 24 hours, while the excreta was dried at 65 ºC for 48 hours, and samples were ground. The laboratory analysis of dry matter, N, Ca, P, and Na was performed according to AOAC 934.01, 968.01, 927.02, and 995.11 in feed, ileal digesta, and excreta samples.

The gross energy content of the feed, dried and milled ileal digesta, and excreta samples was determined by IKA-Calorimeter C6000 adiabatic bomb calorimeter with benzoic acid used as a standard. Titanium was measured using a colorimetric method based on Short et al. (1996). Half a gram of each dried sample was weighed and ashed for this procedure. Following ashing, each sample was titrated with 10 mL of sulfuric acid (7.4 M) and then boiled at 200 ˚C for 2 hr until dissolved. Samples were then titrated with 20 mL of 30% hydrogen peroxide and brought to 100 mL using distilled water. Samples were then analyzed for absorption using a spectrophotometer at 410 nm.

3.5 Statistical analysis

Outliers were identified using a recognized method and excluded from statistical analysis. An outlier data was considered to be out of the range of 2 times of standard deviation. Data were analyzed, using one-way ANOVA. In case of significant difference confirmed by ANOVA, post hoc Tukey's test, a recognized means separation method was performed to compare the means. Significance was set at $P<0.05$; $P<0.10$.

4.0 RESULTS AND DISCUSSION

4.1 Body weight of laying hens

The results for body weight of hens are indicated in Table 9. Weight loss was observed in all the treatment groups irrespective of supplementation of phytase at different levels, and the results exhibited no statistically significant differences among the treatments.

Table 9. The effect of dietary treatments on the body weight of laying hens

Treatments*	Trial start	Start of excreta collection	End of excreta collection
PC	2189	2079	2085
NC	2132	2037	2042
Phy500	2160	2011	2027
Phy700	2166	2032	2036
P-value	*0.6675*	*0.7899*	*0.8705*

PC=Positive control, NC=Negative control, Phy500=Phytase 500FTU/kg, Phy700=Phytase 700FTU/kg

P-values comparisons, namely PC vs. NC (0.6675), PC vs. Phy500 (0.7899), and PC vs. Phy700 (0.8705), exceed the conventional significance threshold of 0.05, suggesting a lack of substantial treatment effects. However, the initial body weights tracked weight changes until the end of the trial, revealing minimal variations at the end of excreta collection (2085, 2042, 2027, and 2036g) respectively. There was no difference in the body weight of laying hens between the positive control and the negative control diets. This suggests that the low phosphorous content in the diet had no negative impact on the body weight gain of laying hens during the study period.

The results of this study agree with the findings of a study conducted by Keshavarz & Austic (2004), who reported that BW change of laying hens in treatment groups was not different from those of the positive control and negative control. Furthermore, Jing et al. (2021) reported no significant differences in performance (BW and feed conversion ratio, egg weight, egg production, feed intake), while in a study by Boling et al. (2000), a 0.10% available Phosphorous (AP) diet resulted in significantly depressed body weight by 28 wk of age compared to the 0.45% AP. However, Gordon & Roland (1998), reported that the body weights of laying hens increased by 4.4% with phytase inclusion, also, Javadi et al. (2021) reported that hens fed with P500 diets had the greatest body weight at the end of the trial at 500 FTU/kg inclusion and improved FCR at 1000 FTU/kg. Javadi et al (2021) further suggested that the extra-phosphoric effects of phytase inclusion allowed greater availability of other nutrients, especially when phytase is overdosed, which could slightly contribute to improving laying hens' performance. Additionally, Lan et al. (2002) reported that supplementation of *Mitsuokella jalaludinii* culture (AMJC) to the low-NPP diet (equivalent to 250 to 1,000 U phytase/kg of feed, significantly increased the body weight gain by 14.9 to 18.3% during the whole experimental period (days 1 to 42 of the experiment in broilers). The low body weight in the current study could probably be due to other factors such as low feed intake associated with the temperature of the surroundings since the experiment was conducted during the summer.

4.2 Apparent digestibility of nutrients

The results for the apparent ileal digestibility of nutrients of hens are given in Table 10. The apparent ileal digestibility of dry matter, phosphorous, and energy showed a significant difference ($P < 0.0001$). The reduction of P and Ca in the negative control group did not affect the digestibility of energy, N, dry matter, and Ca compared with the positive control group.

The apparent ileal digestibility of nutrients was significantly higher in dietary treatments with Phy500 than in other dietary treatments. The PC diet exhibited the lowest apparent ileal energy digestibility among the treatments (70.03%).

Table 10. Effect of dietary treatments on the apparent ileal digestibility of selected nutrients and energy

	PARAMETERS					
Treatments*	**Dry matter**	**Crude protein**	**Sodium**	**Calcium**	**Phosphorus**	**Energy**
			%			
PC	66.07[b]	80.99	-47.37	40.67	55.05[c]	70.31[b]
NC	70.03[a]	81.98	-34.21	48.70	53.70[c]	72.93[ab]
Phy500	71.37[a]	82.73	-36.67	51.21	74.79[a]	73.64[ab]
Phy700	70.90[a]	83.16	-28.06	40.23	73.82[ab]	74.36[a]
P-value	***<0.0001***	*<0.10*	*<0.10*	*NS*	***<0.0001***	***<0.0001***

PC=Positive control, NC=Negative control, Phy500=Phytase 500FTU/kg, Phy700=Phytase 700FTU/kg

Phy500 and Phy700 treatments showed an improvement in the AID of P compared to the NC, suggesting that the inclusion of phytase enhanced the absorption of nutrients in the ileum. The results agree with the findings of (Javadi et al., 2021), who observed an increase in P digestibility in hens fed with P-deficient diets compared to the positive control, both at the fecal and ileal levels. Lan et al (2002) reported that the dry matter concentration was also significantly higher ($P< 0.05$) and was induced by supplementing the diet with an active culture of Mitsuokella jalaludinii (AMJC), which produces more phytase enzyme than other dietary treatments. Francesch et al. (2005) reported that the digestibility of P was increased from 0.250 to 0.513 in maize diets and from 0.335 to 0.583 in barley diets when phytase was added ($P<0.01$). Lan et al. (2002) further noted that DM digestibility was significantly improved by supplementation to medium to high levels of AMJC (equivalent to 500 to 1,000 U phytase/kg of feed). As observed, phytase increased the digestibility of P-deficient diets until the requirement was met, but further increments in phytase could not offer extra effect.

Regarding crude protein, the results show that the differences in AID were statistically significant ($P<0.10$), the PC exhibited slightly lower nitrogen and calcium digestibility (80.99%) than NC (81.98), respectively. Based on the observed data, there is no conclusive evidence to ascertain that dietary supplementation had a significant impact on calcium digestibility in laying hens. The

results of this present study contradict the findings of Walk et al. (2024) who reported that phytase supplementation increased the AID of Ca at 8, 12, or 24 h. However, Walk et al (2024) further observed that phytase decreased the AID of Ca at 48 h, which tends to relate to the decrease in calcium digestibility at Phy700 in the present study.

The ileal digestibility of energy and N was higher in the phytase-supplemented diets compared with the PC and NC diets, these results are in agreement with the finding of Liu et al. (2007), Cowieson et al. (2006b) who reported that phytase supplementation in laying broiler hens improved the ileal digestibility of energy, P, N, Ca, and amino acids compared with the negative control diet. In this study, a higher dose of phytase (phy700) significantly improved the digestibility of energy in Ca and P-deficient diets, and this could be attributed to an increase in the digestibility of organic nutrients, including protein, fat, and starch.

4.3 Intake, excretion, absolute retention, and relative retention of nutrients (Ca, DM, and P).

The results for intake, excretion, absolute and relative retention of dry matter, calcium, and phosphorous are indicated in Table 11. The phytase supplementation did not improve dry matter intake (g/d), output (g/d), absolute retention (g/d), and relative retention. However, for calcium and phosphorous, there is a significant difference in calcium output (g/d), phosphorous intake (g/d), output (g/d), and relative retention (%).

From Table 11, the observed variations in phosphorus intake, output, and relative retention are statistically significant ($P<0.0001$). Phytase (Phy500 and Phy700) supplementation significantly improved the relative retention of phosphorous during the study period. The highest phosphorus intake was observed in the PC treatment (705.9 mg/d), which further aligns with the highest output in phosphorous compared to other treatment

Table 101. Effect of dietary treatments on intake, excretion, absolute retention, and relative retention of dry matter, Ca and P

Treatments*	P A R A M E T E R S											
	Dry matter				**Calcium**				**Phosphorus**			
	Intake	**Output**	**Retention**	**Rel. ret**	**Intake**	**Output**	**Retention**	**Rel. ret**	**Intake**	**Output**	**Retention**	**Rel. ret**
	g/d	**g/d**	**g/d**	**%**	**mg/d**	**mg/d**	**mg/d**	**%**	**mg/d**	**mg/d**	**mg/d**	**%**
PC	107.6	27.8	79.8	74.1	4017.5	1972.5^{a}	2003.6	49.9	705.9^{a}	477.5^{a}	224.0	31.7c
NC	106.5	28.3	78.2	73.7	3974.8	2056.7^{a}	2000.0	49.8	556.8^{b}	338.7^{b}	225.1	39.9^{bc}
Phy500	104.1	28.0	76.1	73.2	3887.3	1771.6^{ab}	2183.6	55.4	544.5^{b}	318.1^{b}	222.5	40.4^{ab}
Phy700	100.1	26.5	73.5	73.4	3735.3	1627.6^{b}	2107.7	55.7	523.2^{b}	283.8^{b}	239.4	45.9^{a}
P-value	*NS*	*NS*	*NS*	*NS*	*NS*	***<0.0001***	*NS*	*NS*	***<0.0001***	***<0.0001***	*NS*	***<0.0001***

PC=Positive control, NC=Negative control, Phy500=Phytase 500FTU/kg, Phy700=Phytase 700FTU/kg, NS=Non-significant, Rel. ret= Relative retention

The relative phosphorus retention exhibited significant changes, particularly with the supplementation of Phytase at 700/FTU. The improvement in relative retention suggests that phytase supplementation positively influenced phosphorus utilization by laying hens and reduced phosphorus excretion in the feces, indicating that a greater proportion of the absorbed phosphorus was retained by the laying hens for metabolic processes and egg production.

The results of this study agree with another previous study by Javadi et al. (2021), who reported that the P digestibility and retention were higher and P excretion was lower with the diet including phytase at 500 FTU/kg compared to the NC diet.

Similarly, Lan et al. (2002) phytase supplementation increased the AID of Ca at 8, 12, or 24 h. Chickens fed the normal diet or a low NPP diet with different levels of AJMC had significantly higher plasma P concentrations than those fed with the low-NPP diet without AMJC supplementation. Additionally, Javadi et al. (2021) reported that animals fed with the P500 diet at 25 weeks of age and P1000 at 31 weeks of age showed higher CTTAD ($P<0.05$) and retention ($P<0.05$), but lower excretion of phosphorus ($P<0.05$) than those fed with NC diet.

A study by Francesch et al. (2005), reported that dietary P reduction decreased ($P<0.001$) excreta P content by 34% at week 27, by 47% at week 36, and by 46% at week 46 from hens fed on maize diets. Similarly, Jing et al. (2021) reported that total P excretion of the birds fed on the 3- phytase-supplemented diets was reduced, on average, by 40.4 mg/hen per day (12.2%; $P < 0.01$) compared with that of the birds fed on the non-phytase supplemented diets. Keshavarz & Austic (2004) also reported that absolute daily P excretion was higher for birds of treatment T1, T2, T3, and T4 than for those of treatment T5, T6, T7, and T8. While Lim et al. (2003) reported an increased P retention by phytase resulted in a reduction of P excretion by about 13% in laying hens. It can be suggested that improved retention may be explained by the fact that phytate complexes were, to some extent, cleaved by phytase (Nair et al., 1991; Lim et al. (2003).

Regarding calcium and dry matter, the results indicate a significant variation ($p<0.0001$) in calcium output, particularly with Phy700 compared to both positive and negative controls. However, no significant variations across treatments were observed in calcium and dry matter intake, retention, and relative retention in laying hens. The high, but not significant relative calcium retention observed in phy500 and phy700, could be attributed to slightly enhanced Ca utilization associated with the phytate P liberation. Although some studies observed a decrease in Ca digestibility when dietary inorganic P was overdosed (Javadi et al., 2021), others have observed the opposite behavior, decreasing Ca digestibility in Ca-deficient diets. Francesch et al. (2005) observed that calcium absorption was not significantly affected by phytase addition or by dietary NPP content in either treatment. Still, the type of cereal affected dietary calcium absorption ($P<0.05$). It was higher in barley diets than in maize diets (0.622 vs 0.527). The effects of phytase supplementation were significantly modified by the levels of Ca and NPP (Lim et al., 2003). Furthermore, Lan et al. (2002) reported that supplementation of AMJC to the low-NPP diet significantly increased the Ca retention in chickens by 9.1 to 10.6 and 9.7 to 16.6 percentage units from 11 to 13 and 18 to 20 d of age, while plasma Ca concentrations were not influenced by the dietary treatments with

phytase supplementation. Javadi et al. (2021) pointed out that dietary inclusion of the 3-phytase at 700FTU did not affect Ca ileal digestibility or Ca and P blood concentration at 31 weeks of age. The lowest dry matter output was observed in the Phy700 treatment, suggesting efficient utilization or potentially altered digestive processes due to higher phytase levels. However, in an experiment by Lim et al. (2003) phytase supplementation at 300 U/kg increased the availability of DM, fiber, and phosphorus in laying hens.

4.4 Intake, output, absolute retention and relative retention of Nitrogen and Sodium, and utilization of dietary energy.

The results for intake, output, absolute retention and relative retention of crude protein and sodium, and utilization of dietary energy are indicated in Table 12.

Analyzing the results, the P-values indicate non-significant differences ($P>0.10$) across all parameters, suggesting that the treatments did not result in statistically significant changes in nitrogen, sodium, and energy parameters availability.

Table 11. Effect of dietary treatments on the intake, output, absolute retention and relative retention of Nitrogen and Sodium as well as Utilization of dietary energy.

Treatments*	P A R A M E T E R S											
	Crude protein				Sodium				Energy			
	Intake	Output	Retention	Rel. ret	Intake	Output	Retention	Rel. ret	Intake	Output	Balance	Efficiency
	mg/d	mg/d	mg/d	%	mg/d	mg/d	mg/d	%	MJ/d	MJ/d	MJ/d	%
PC	3060	1495	1572	51.4	259.4	90.2	169.7	65.5	1.79	0.39	1.38	77.6
NC	3028	1554	1473	49.4	256.7	85.2	168.7	66.5	1.77	0.38	1.38	78.1
Phy500	2961	1480	1481	50.9	251.0	96.0	154.9	62.6	1.73	0.39	1.34	77.4
Phy700	2845	1503	1342	47.0	241.2	88.7	152.5	63.0	1.67	0.37	1.29	77.1
P-value	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*

PC=Positive control, NC=Negative control, Phy500=Phytase 500FTU/kg, Phy700=Phytase 700FTU/kg

In terms of nitrogen metabolism, no significant differences were observed in nitrogen intake, output, retention, or relative retention among the treatments. Numerically, the daily N intake observed was higher for hens of the PC than for the other treatments. The percentage of relative N retention was lower for the hens of the Phy700 and NC than for other dietary treatments and, as a result of this, the percentages of N outputs were higher than for hens in the other dietary treatments. The high daily N intake in the PC could be associated with the higher dietary protein of this treatment. While absolute daily N retention was higher for hens of the positive control group than for hens on other dietary treatments. The results of this study correspond with the findings of Keshavarz & Austic, (2004)

who also reported that the daily N intake and absolute daily N excretion were higher for hens of the positive control group than for hens on other dietary treatments.

Regarding energy, there were no significant differences in energy intake, output, balance, and efficiency. The results indicate a gradual decrease in energy intake from 1.79 MJ/d in the PC group to 1.67 MJ/d in the Phy700 group.

Whereas there is a relatively consistent energy output across treatments, with values ranging from 0.37 to 0.39 MJ/d. Phy500 and Phy700 exhibited lower energy balances as compared to the PC and NC treatments. Regarding energy efficiency, we observed a stable energy efficiency, ranging from 77.1% to 78.1% across all treatments. From the results above it can be said that phytase supplementation did not influence energy metabolism among laying hens.

The results obtained from the current experiment contradict with previous finding by Lan et al. (2002), who reported that AMJC supplementation significantly increased the AME of the low-NPP diet as compared to the normal-NPP. The authors also emphasized that AME levels were not affected by increasing amounts of AMJC supplementation, except in diets supplemented with AMJC equivalent to 750 U phytase/kg. This may explain the low energy output observed in this current study between Phy500 and Phy700. Whereas Dersjant-Li (2018) reported a linear increase in both apparent metabolizable energy and ileal digestibility of total amino acids.

Reviewing the main mechanisms proposed by the literature for the extra phosphoric effects, proposed that phytate could reduce the digestive utilization of dietary energy and protein by binding to amino acids, increasing mucin, and then the loss of endogenous protein and compromising the Na+-dependent transport of starch, glucose and amino acids in the gut. Lei et al. (2011) stated that the AME and CP content of laying hens' diets could be slightly reduced by the extra phosphoric consequences of phytase supplementation without penalties.

Similarly, sodium exhibited non-significant variations, encompassing intake, output, retention, and relative retention. This suggests that the treatments did not induce statistically significant changes in sodium metabolism in the laying hens.

5.0 CONCLUSION

It can be concluded that higher doses of phytase supplementation beyond the standard may offer improved nutrient digestibility and relative retention, especially for energy and phosphorous respectively. The improvement in ileal digestibility of P in supplemented groups suggests that a further reduction in dietary P is possible while the phytase enzyme is supplemented. Furthermore, the efficiency of nutrient utilization can be enhanced by enzymes only if the nutrients are below the requirement and the higher digestibility does not result in higher nutrient excretion via urine. The results suggest that NC feed without supplementation was sufficient for optimal P retention since absolute P retention was the same in the NC group as that in hens fed with the PC feed.

6.0 SUMMARY

This study examined the impact of phytase supplementation at levels of 500 and 700 FTU/kg on nutrient (dry matter, crude protein, Ca, P, Na, and energy) digestibility and retention in laying hens. A total of 120 Lohmann Brown hens at 35 weeks old were used in this experiment. Four dietary treatments were applied, with the positive control formulated based on recommendations. 10 birds and 30 birds per treatment were used for retention and digestibility studies, respectively. Data were analyzed using a one-way ANOVA and Significance was set at $P<0.05$; $P<0.10$

Results indicated improvements in the digestive efficiency of essential nutrients, phosphorus (74.79% and 73.82%), nitrogen (N) (82.73% and 83.16%), and energy (73.64% and 74.36%), in phytase-supplemented diets at phy500 FTU/kg and phy700 FTU/kg, respectively, compared to the positive control and negative control diets. Additionally, relative retention of phosphorus (40.4% and 45.9%) and calcium (55.4% and 55.7%) was enhanced in phytase-supplemented diets at phy500 FTU/kg and phy700 FTU/kg, respectively, compared to P (31.7% and 39.9%) and C (49.9% and 49.8%) in PC and NC treatments, respectively, suggesting increased utilization of dietary phosphorus and calcium.

These findings highlight the efficacy of phytase in enhancing nutrient utilization and retention in laying hens, particularly for phosphorus, while also indicating a positive impact on calcium retention. Incorporating phytase supplementation at appropriate levels could be a valuable strategy to optimize nutrient utilization and improve the overall productivity and health of laying hens.

The results of this work enable us to conclude that the efficiency of nutrient utilization can be improved by enzymes only if the nutrients are below the requirements.

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9.0 APPENDIXES

9.1 DECLARATION

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9.2 STATEMENT ON CONSULTATION PRACTICES

As a supervisor of TUKAMUSHABA SILVER (KI9F3E), I here declare that the final essay/thesis/master's thesis/portfolio[4] has been reviewed by me, the student was informed about the requirements of literary sources management and its legal and ethical rules.

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