THE EFFECTS *OF ESCHERICHIA COLI* PHYTASE ON NUTRIENT METABOLIZABILITY AND ILEAL DIGESTIBILITY IN LAYING HENS

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Summary. The aim of this experiment was to determine the efficacy of different inclusion levels of *Escherichia coli* phytase (ECP) in diets based on corn meal and soybean meal, fed to laying hens. Phytase efficacy was determined based on the metabolizability and ileal digestibility of phosphorus, calcium and crude protein. The experiment was conducted during the first 24 weeks of the laying period, and it involved Lohmann Brown hens kept individually in cages. Two hundred and twenty birds were randomly assigned to five treatments, providing 44 replicates per treatment, as follows: positive control (I), negative control (II) and three treatments where diets were supplemented with different amounts of ECP - 125 FTU (III), 250 FTU (IV) and 500 FTU/kg diet (V). Phytase supplementation numerically improved the metabolizability of Ca, P and crude protein. The effect was dose-independent in most cases. The values noted in the positive control treatment were not achieved. The effect of phytase on the ileal digestibility of the analyzed nutrients was more pronounced. The addition of Optiphos phytase to the negative control diet resulted in a significant improvement in the ileal digestibility of phosphorus, calcium and nitrogen, compared with the negative control group. This effect was observed at all phytase doses. Again, there was no dose-dependent effect.

Keywords: laying hens, phytase, *Escherichia coli*, metabolizability, ileal digestibility, phosphorus, calcium, crude protein.

FITAZĖS, GAUTOS IŠ *ESCHERICHIA COLI,* POVEIKIS VIŠTŲ DEDEKLIŲ MAISTINIŲ MEDŽIAGŲ METABOLIZMUI IR VIRŠKINAMUMUI KLUBINĖJE ŽARNOJE

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Santrauka. Bandymo tikslas buvo nustatyti įvairių dozių *Escherichia coli* fitazės (ECP), esančios sudėtyje lesalo, kurio pagrindas kviečių ir sojų miltai, poveikį dedeklėms vištoms. Fitazės veiksmingumas nustatytas pagal fosforo, kalcio ir žalių proteinų metabolizmą ir virškinamumą klubinėje žarnoje. Bandymas atliktas pirmąsias 24 kiaušinių dėjimo savaites su „Lohmann Brown“ vištomis, laikytomis skirtinguose narveliuose. Analogu principu 220 vištų suskirstytos į penkias tiriamąsias grupes, po 44 vištas šia tvarka: teigiamos kontrolės (I), neigiamos kontrolės (II) ir tris poveikio tyrimo grupes, kur lesalai buvo papildyti trimis skirtingais ECP kiekiais – 125 FTU (III), 250 FTU (IV) ir 500 FTU kg lesalo (V). Fitazės priedas pagerino Ca, P ir žalių proteinų metabolizmą. Daugeliu atveju šis poveikis nepriklausė nuo priedo dozės. Teigiamos kontrolės grupėje nustatytos vertės nebuvo pasiektos. Fitazės poveikis analizuojamų maistinių medžiagų virškinamumui klubinėje žarnoje buvo daug ryškesnis. „Optiphos“ fitazės priedas neigiamos kontrolės grupės lesaluose reikšmingai veikė fosforo, kalcio ir azoto virškinamumą klubinėje žarnoje palyginti su neigiamos kontrolės grupe. Ši poveikio tendencija pastebėta visose grupėse vištų, gavusių fitazės. Nustatyto poveikio reikšmė nuo dozės nepriklausė.

Raktažodžiai: vištos dedeklės, fitazė, metabolizmas, virškinamumas klubinėje žarnoje, fosforas, kalcis, žali proteinai.

Introduction. Today diets for laying hens are based on components of plant origin, including cereal grains, legume seeds, fat-rich seeds and the by-products of plant food processing. Phytate phosphorus accounts for 50% to 80% of the total phosphorus content of the above raw materials (Oloffs et al., 2000a, Johri, 2010; Ravindran et al., 1995, Singh, 2008). Organically bound phosphorus is available to laying hens or other birds only if the enzyme phytase is present in their digestive tract. Laying hens produce very small amounts of phytase, and phytase production by microorganisms in the gastrointestinal tract is also limited (Maenz and Classen 1998, Abudabos et al., 2000, Kerr et al., 2000, Marounek et al., 2008). Only the grains of selected cereal species (barley, wheat, triticale, rye) contain phytase whose activity is sufficient to hydrolyze phytin (Eeckout and De Pappe, 1994; Johri, 2010; Oloffs et al., 2000b). Corn, milo corn, oat, legume seeds, oil-rich seeds and their by-products show minimal activity levels of phytase (Cossa et al., 1997; Selle and Ravindran, 2007; Johri, 2010). Total phosphorus contained in wheat is available to laying hens in nearly 50%, whereas the respective value for corn is as low as 20% (Oloffs et al., 2000a). Due to the breeding of early-maturing and high-yielding varieties, the area under corn cultivation has increased recently also in countries (including Poland) and regions where it had been produced on a very small scale before. Corn is a widely used ingredient in poultry diets. The utilization of phosphorus contained in corn may be increased if diets for laying hens are supplemented with microbial *E. coli* phytase (Wu et al., 2006; Augspurger et al., 2007; Liu et al., 2007; Hughes et al., 2009; Kozłowski et al., 2009; Kozłowski et al., 2010a, b; Kozłowski and Jeroch, 2010).

The aim of the present experiment was to determine the effect of different inclusion levels of *Escherichia coli* phytase in corn-soybean meal diets fed to laying hens on the metabolizability and ileal digestibility of phosphorus, calcium and crude protein. Such diets contain approximately 50% of the recommended available phosphorus level (Smulikowska and Rutkowski, 2005).

Materials and Methods. The experimental materials comprised a total of 220 Lohmann Brown hens from a commercial pullet grower house, grown in accordance with the breeder's recommendations (LTZ, 2010), aged 18 weeks. The hens were randomly divided into five treatments (44 replicates in each, 1 hen = 1 replicate) and they were kept in individual cages (30 x 60 cm). The replicates were equally distributed among three cage levels to minimize the cage level effect. Environmental conditions (temperature, lighting program, ventilation) followed the recommendations of the breeding material producer (LTZ, 2010). The birds had free access to feed and tap water. All experimental procedures involving animals were approved by the local Animal Experimentation Ethics Committee (decision no. 12/2007) at the University of Warmia and Mazury in Olsztyn.

The hens received experimental diets *ad libitum* during the first 24 weeks of the laying period (from 21 to 44 weeks of age). The diets, provided as meal, were formulated so as to meet the nutrient and energy requirements of laying hens (Smulikowska and Rutkowski, 2005). Diet composition is presented in Table 2. Two corn-soybean based diets were produced as follows: a positive control diet followed by a negative control diet divided into four identical parts. Diets for treatments III, IV and V were supplemented with different concentrations of *Escherichia coli*-derived 6-phytase expressed in *Pichia pastoris* (Optiphos, HUVEPHARMA NV, Belgium). The experimental design is shown in Table 1.

Table 1. **Experimental design**

Group	aP-content, g/kg	Ca-content, g/kg	Phytase, FTU/kg
I (positive control)	2.5	35.5	-
II (negative control, NC)	1.3	33.0	-
III (NC + Phytase)	1.3	33.0	125
IV (NC + Phytase)	1.3	33.0	250
V (NC + Phytase)	1.3	33.0	500

Nutrient metabolizability was determined on eight birds selected randomly from each treatment, 40 birds in total. Excreta samples were collected three times: in the 8^{th}, 16^{th} and 24^{th} week of the laying period, from the same laying hens. There was a collection period of four days during which excreta samples were collected from eight birds from each treatment. Excreta samples were collected two times daily, at 8am and 8pm, they were frozen (-18ºC) and stored for later analysis. The metabolizability of Ca, P, N was calculated as follow:

Metabolizability (%) = 100 x (nutrient intake - nutrient excretion)/ nutrient intake x 100

Apparent ileal digestibility (AID) was determined on the same birds which were used in the previous digestibility trial. Samples were collected from 40 birds (5 groups x 8 birds) in the 24^{th} week of the laying period. Before this part of the experiment, all diets were removed from the feeders and replaced with respective diets containing the indigestible dietary marker TiO_2. Four days later, eight birds from each group were euthanized, their body cavities were opened immediately, and the section between Meckel's diverticulum and 2 cm anterior to the ICCJ (ileo-caeco-colonic junction) was isolated. The ileal contents of each bird were gently flushed out with distilled water and immediately frozen. Subsequently, samples were freeze-dried for later analysis.

In order to determine TiO_2, Ca and P content, samples of freeze-dried excreta and digesta were digested in a microwave system (Multiwave 3000, Anton Paar) at a temperature of 260ºC and a pressure of 60 bar. TiO_2, Ca and P were determined using optical emission spectrometry with inductively coupled plasma (ICP-OES, Inductively Coupled Plasma - Optical Emission Spectrometry) on the Ultima 2 sequencial apparatus with vertical plasma (Horiba Jobin Yvon). Experimental basal diets for the PC and NC groups were assayed for the content of DM, crude protein, crude fat and crude fiber (Naumann and Bassler, 1993). Phytase activity in all experimental diets was analyzed in triplicate according to the assay procedure outlined in Han et al. (1999). The trial began after the confirmation that the actual concentrations of the experimental diets were in compliance with the target levels for each treatment group. Based on the results of nutrient and marker (TiO_2) analyses in digesta and diets, nutrient digestibility (Y) was calculated based on the following formula:

AID (%) = 100 - [100 x (TiO_2 in diet/ TiO_2 in ileal digesta) x (nutrient in ileal digesta/nutrient in diet)]

The results of the experiment were analyzed using a one-way analysis of variance (ANOVA), and significant differences between treatments were determined with Duncan's multiple range test. The Statistica software package version 9.0 (StatSoft Inc., 2009) was used for statistical calculations. Data in tables are given as means and standard deviations.

Results and Discussion. The results of a performance study with the same experimental design are presented in another paper (Kozłowski et al., 2011). In this experiment the phytase supplementation significantly affected, vs. negative control, the performance results of hens over the entire experimental period (number of eggs laid, egg mass and FCR), and egg quality parameters - egg yolk color (significantly in week 24 of the laying period) and shell thickness (significantly in week 12 of the laying period). Therefore the available P content of layer diets based on corn meal and soybean meal can be reduced by 1.2 g/kg provided that the diets are supplemented with ECP in the amount of 125-250 FTU (Kozłowski et al., 2011).

The present experiment proceeded normally, therefore the data from all hens could be included in the evaluation. A proximate feed analysis showed that the actual nutrient concentrations were consistent with the calculated values listed in Table 1. The results of an analysis of phytase activity in the diets are shown in Table 2. They were satisfactory and corresponded with the target values.

Table 2. **Composition of basal diet** (%)

Components	PC (I)	Treatments II-V
Corn	59.99	61.88
Sunflower meal	5.00	5.00
Soybean meal 48% CP	22.50	22.20
Soybean oil	0.50	0.50
Animal fat	1.50	0.85
$NaHCO_3$	0.15	0.15
Salt	0.22	0.22
Limestone	9.00	8.60
Monocalcium phosphate	0.54	-
DL-methionine	0.10	0.10
Premix[1]	0.50	0.50
Energy and nutrient content		
AME_N (MJ/kg)	11.45[2]	11.45[2]
Crude protein (g/kg)	171.1[2]/176.8[3]	171.4[2]/176.0[3]
Crude fat (g/kg)	47.0[2]/51.2[3]	41.3[2]/43.6[3]
Crude fibre (g/kg)	33.0[2]/33.9[3]	33.3[2]/34.3[3]
Na	1.5[2]	1.5[2]
Ca	35.5[2]/37.5[3]	33.0[2]/35.0[3]
P (total)	4.6[2]/4.9[3]	3.5[2]/3.9[3]
P (av)	2.5[2]	1.3[2]

[1] Provided per kilogram of diet: vitamin A, 8,000 IU; vitamin D_3, 2,000 IU; vitamin E, 20 mg; vitamin K_3, 1 mg; thiamine, 1.5 mg; riboflavin, 4 mg; pyridoxine, 1 mg; biotin, 0.1 mg; pantothenic acid, 6 mg; folic acid, 0.5 mg; niacin, 20 mg; vitamin B_{12}, 0.02 mg; I, 0.7 mg; Se, 0.15 mg; Cu, 6 mg; Mn, 65 mg; Zn, 52 mg; Fe, 45 mg.
[2] Calculated (Smulikowska and Rutkowski, 2005)
[3] Analyzed (Naumann and Bassler, 1993)

Table 3. **The activity of *Escherichia coli* phytase in experimental diets** (FTU/kg feed)

Item	Treatments				
	PC (I)	NC (II)	NC+125FTU (III)	NC+250FTU (IV)	NC+500FTU (V)
Expected values	0	0	125	250	500
Analyzed values	20	9	114	236	483

Nutrient metabolizability

Phosphorus metabolizability was highest in positive control group (I) hens, due to inorganic P supplementation (Table 4). As expected, the lowest values were noted in negative control group (II) hens, because phosphorus contained in their diet had a high proportion of phytin-P, while plant-specific phytase was almost absent. In an experiment conducted by Oloffs et al. (2000) on laying hens, P metabolizability reached 19% for maize grain and 46 – 49% for wheat grain with high intrinsic phytase activity levels. In another experiment performed by the same working group (Oloffs and Jerome, 2000) phytase contained in wheat was inactivated by grain extrusion, which resulted in P metabolizability of 24% for wheat and 26% for wheat-rich layer rations. Different levels of phytase supplementation in the negative control diet improved total P metabolizability. However, the effects were low to moderate and significant only at the highest phytase supplementation level (group V) in the 1st experimental period (8th week of the laying period). The results show no or an insignificant dose-response effect.

The experimental design had no significant effect on calcium and crude protein metabolizability, yet in all cases the lowest values were obtained in the negative control group (II), and the outcome of all experimental groups (III-V) was higher in comparison with this group (Table 4). The lack of significant effects of phytase supplementation on the above parameters have also been reported by other authors (Liebert et al., 2005).

Table 4. **The effect of phytase on the metabolizability of nutrients** (%)

Nutrients	Week of laying period	Groups ($\overline{x}$ ± SD)					*P value*
		PC (I)	NC (II)	NC+125FTU (III)	NC+250FTU (IV)	NC+500FTU (V)	
P	8th	$42.7^a \pm 6.2$	$25.2^c \pm 6.7$	$32.1^{bc} \pm 9.1$	$31.5^{bc} \pm 5.0$	$35.6^b \pm 5.4$	*0.000*
	16th	$38.0^a \pm 9.9$	$25.6^b \pm 6.4$	$29.6^b \pm 7.3$	$28.8^b \pm 6.0$	$27.3^b \pm 5.8$	*0.017*
	24th	33.4 ± 8.5	24.1 ± 8.5	24.7 ± 5.7	26.7 ± 5.0	26.8 ± 6.5	*0.088*
Ca	8th	75.1 ± 5.4	67.6 ± 10.6	67.5 ± 6.1	73.6 ± 8.1	72.3 ± 7.2	*0.186*
	16th	64.1 ± 10.1	58.4 ± 12.9	64.6 ± 12.8	64.2 ± 6.7	64.9 ± 14.3	*0.783*
	24th	71.4 ± 4.5	68.9 ± 6.1	70.4 ± 3.5	73.8 ± 5.7	72.9 ± 4.6	*0.244*
CP	8th	48.6 ± 4.6	40.4 ± 6.1	45.4 ± 10.3	47.4 ± 3.7	47.9 ± 3.8	*0.079*
	16th	38.8 ± 3.8	35.3 ± 9.1	39.0 ± 6.3	37.8 ± 5.0	40.0 ± 10.1	*0.749*
	24th	40.9 ± 6.2	36.4 ± 4.7	37.8 ± 4.6	40.4 ± 5.7	37.8 ± 4.5	*0.373*

Values with the different letters differ significantly; abc - $P \leq 0.05$

Ileal digestibility of nutrients

The ileal digestibility of phosphorus, calcium and crude protein was affected by phytase (Table 5), yet the noted increase was not always significant due to the considerable variation in individual data. No dose-response relationship was observed with respect to phytase supplementation. Three different phytase doses do not differ in their effects. The pre-cecal digestibility of calcium and phosphorus noted in the positive control group was not reached in phytase-supplemented groups. The only exception was crude protein. Liu et al. (2007), who applied a highly similar experimental design in their study, obtained similar results to those reported here with regard to the pre-cecal digestibility of all nutrients (P, Ca, crude protein) in the positive control, negative control and phytase-supplemented groups, and the effectiveness of microbial phytase addition to the negative control diet. Besides an improvement in crude protein digestibility, the cited authors also reported an increase in pre-cecal amino acid digestibility. In another study on the subject (Kozłowski and Jeroch, 2011), we observed no effect of Optiphos phytase supplementation to layer diets on the ileal digestibility of dry matter and calcium. However, dietary phytase supplementation at 125 FTU to 500 FTU/kg had a positive dose-response effect on ileal phosphorus digestibility, in comparison with the negative control group.

Table 5. **The effect of phytase on apparent ileal digestibility of nutrients** (%)

Nutrients	Groups ($\overline{x}$ ± SD)					*P value*
	I	II	III	IV	V	
P	$48.48^a \pm 8.05$	$31.01^b \pm 5.98$	$39.38^{ab} \pm 7.39$	$40.18^{ab} \pm 14.78$	$40.92^{ab} \pm 9.96$	*0.041*
Ca	$46.19^a \pm 4.93$	$36.46^b \pm 8.13$	$41.53^{ab} \pm 3.57$	$42.10^{ab} \pm 5.54$	$40.59^{ab} \pm 2.60$	*0.033*
CP	$72.47^a \pm 2.24$	$66.63^b \pm 2.99$	$70.93^a \pm 3.85$	$71.48^a \pm 1.93$	$69.93^a \pm 1.87$	*0.004*

Values with the different letters differ significantly; abc - $P \leq 0.05$

Conclusions. It can be concluded that the supplementation of diets based on corn and soybean meal with *Escherichia coli*-derived 6-phytase expressed in *Pichia pastoris* (Optiphos) improved the metabolizability and ileal digestibility of Ca, P and crude protein in laying hens. Dietary phytase supplementation increased the utilization of the analyzed nutrients, reduced nutrient excretion in feces, and enabled to decrease the addition of inorganic phosphorus to rations for laying hens.

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