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RESEARCH ARTICLE

The effect of enzyme and protein source on laying hens performance, eggshell and bone traits

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ABSTRACT

The aim of the study was to investigate the interaction between exogenous phytase with protease and protein source in laying hens diet. A completely randomised design study with a 2 × 4 factorial arrangement was conducted to observe effects of protein source (soybean vs. yellow lupine seeds diet) and enzyme addition (no enzyme vs. phytase or protease or both) on laying hens performance, bone mineralization and some egg traits. One hundred sixty Hy-Line Brown hens at the age of 18 weeks were weighed and randomly assigned to 10 treatments, each with 16 birds. According to the producer recommendation in diets containing enzymes reduced the level of available phosphorus (50% reduction) and digestible amino acids - 5% reduction. The egg production during the entire experiment was similar in all groups without significant differences. The egg weight was also on an equal level in all treatments and it was about 58 g. Birds fed different protein source were characterized by similar feed intake and feed conversion ratio during the whole experiment. The feed conversion ratio (FCR) was about 1.9-2.0 in all groups. Tibia ash was affected by enzymes supplementation. There was an interaction between protein source and enzymes addition. Birds from treatments fed with yellow lupine meal (YLM) diets reached highest tibia ash level in a group with phytase addition and in groups with SMB in a group with phytase and protease mix. The inclusion of enzymes improved the thickness of the shell. There was an interaction between protein source and enzyme inclusion. It was an interaction between experimental factors in shell breaking strength. Stronger eggshell was laid by hens from groups fed with the inclusion of yellow lupine meal. Addition of phytase had a positive impact on increasing the strength of eggshell. Only inclusion of enzymes affected significantly on shell elastic deformation in part I of eggs. Eggs from hens fed diets with inclusion of protease and phytase mix were most resistant to elastic deformation.

Keywords: Phytase; Lupines; Protease; Eggs; Tibia

INTRODUCTION

Soybean meal (SBM) is the best vegetable protein source used in livestock feed in the world. More than 50% of the world's protein meals is SBM. This is due to the relatively high content of crude protein (CP) and very good amino acid (AA) profile and high digestibility of them (Ravindran et al., 2014). SBM has also ani-nutritional factors (ANF) like protease and trypsin inhibitors, that affects adversely on protein digestibility and AA availability (Jahanian and Rasouli, 2016). SBM is mostly genetically modified and many of customers all over the world does not accept the GMO products used in feed industry (Rutkowski et al., 2015). It is expected that in 2020 on the earth will live more than 10 billion people. This means increased demand for food which is associated with an increased demand for feed for livestock. For all this reasons it became necessary to find new and alternative protein source that could make up or replace SBM in poultry diets (Krawczyk et al., 2015; Mikulski et al., 2012) In recent years interest in use of homegrown legume like yellow lupine increased (Smulikowska et al., 2014; Viveros et al., 2007).

In the past use of yellow lupine in poultry diets was limited by the high alkaloid and non-starch polysaccharides (NSP) content which negatively affected on growth and production performance of birds (Kocher et al., 2000; Olkowski et al., 2001; Olkowski et al., 2005). Due to progress in plant

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breeding, the level of antinutrients in lupine seeds had decreased, resulting in the development sweet lupines. Seeds of new Polish cultivars of yellow lupine characterized by high protein content (38% CP), a low alkaloid content and high NSP concentration (Kaczmarek et al., 2014; Rutkowski et al., 2015). In recent years the use of lupine seed in the broiler (Hammershoj and Steenfeldt, 2005; Olkowski et al., 2005) and laying hens (Krawczyk et al., 2015; Liebert et al., 2005; Zdunczyk et al., 2014) has become popular. Studies are not conclusive about inclusion rate of lupine seed in poultry diets. Smulikowska et al. (2014) suggested that 15% is optimum inclusion rate in broiler chicken diets. On the other hand, Orda et al. (2004) and van Barneveld (1999) used 25-30% without a negative effect on broiler chickens performance. Krawczyk et al. (2015) used 30% in laying hens diets without any deterioration in egg production.

A variable but large proportion of the phosphorus (P) in plant material is in form of phytate-P (Sandberg, 2002). The capacity of poultry to utilize phytate-P by endogenous enzymes is very limited (Selle et al., 2012). Approximately 2/3 of total P in legume seeds are bonded to phytate (Steiner et al., 2007). Phytic acid forming insoluble slats of many mineral ions at intestinal pH, reducing the availability of mineral absorption (Sandberg, 2002; Selle et al., 2000). The presence of phytate also influenced to the reduction of availability of dietary AA and energy (Selle et al., 2000). There are several modes of action to explain the interaction between phytate and AA digestibility (Kies et al., 2001). The binary protein-phytate complex does not undergo enzymatic degradation in the digestive system and excreted in faeces. For the feed industry the most important is phytate interaction with specific amino acids. It has been sown that the most sensitive to the adverse effect of phytate are sulfur-containing AA (Selle et al., 2000).

Whole lupine seeds contain significant content of phytate P witch limiting the use of lupine in poultry diets (Birk, 1994). In 1991 it was introduced the use of the enzyme phytase in poultry diets to reduce P concentrations in waste from intensive poultry production (Selle et al., 2012). The addition of microbial phytase significantly improves the utilization of phytic phosphorus by poultry, which has been proved in many studies (Simons et al., 1990; Snow et al., 2004). This enzyme has also a positive effect on the digestibility of protein and AA in broilers (Snow et al., 2004) and laying hens (Liebert et al., 2005). The usefulness of exogenous phytate diets for laying hens are still subject to discussion. Studies have shown that supplementation with phytase in the corn-SMB diets improved feed intake, FCR, the mass of eggs and had a positive impact on the digestibility of Ca and P (Jalal and Scheideler, 2001). Later reports suggest that the addition of enzyme did not affect ileal digestibility of AA in hens (Snow et al., 2003).

For the reason of increasing costs of protein feedstuff and increased public concern of environmental pollution making by animal production, there was a need to reduce the amount of nutrient in the waste generated by animals (Angel et al., 2011; Nir et al., 1993). However, studies show that the feed components use in poultry diets are not completely digested in birds organism (Parsons et al., 1997). Protease is used to complement the action of digestive enzymes and improve productive results. Studies show that the addition of protease to diets for broilers resulted in improvement of production performance and better use of energy and nitrogen form feed material (Ghazi et al., 2003). Earlier studies in which the protease was used in diets for broiler chickens showed improvement in FCR and the digestibility of protein and AA (Freitas et al., 2011). The impact of exogenous protease on birds performance are often inconsistent. Differences in the type of the protease and frequent use of multi-enzyme complexes may partially explain the different results (Cowieson, 2008).

The aim of the study was to investigate the interaction between exogenous phytase with protease and protein source in hens diet.

MATERIAL AND METHODS

Lupin seeds and enzymes

Seeds of yellow lupine (*Lupinus luteus* L., Mister cv.) were obtained from the Plant Breeding Stations in Wiatrowo (Poland). The chemical composition of seeds is presented in Table 1. In trial was used phytase (HiPhos) and protease (Ronozyme ProAct) produced by DSM Nutritional Products, Kaiseraugst, Switzerland.

Table 1: Chemical composition of SBM and yellow lupine seeds used in experiment

	SBM (g/kg)	YLM
Dry matter	909	890
Crude protein (N · 6.25)	438	415
Crude fat	30.1	52.5
ADF	6.42	24.24
NDF	9.71	28.24
Ca	3.0	2.95
P	6.1	7
P-phytic	3.7 (60%)	5.25 (75%)
RFO		
Raffinose	11.4	11
Stachyose	44.5	449.4
Verbaskose	0.32 (mg/kg)	25.3
Total alkaloids		
Lupinine	n.d.	170.89
Sparteine	n.d.	90.72

SBM - soybean meal, YLM - yellow lupine meal, ADF - acid detergent fibre, NDF - neutral detergent fibre, RFO - raffinose family oligosaccharides, n.d. - not detected

Birds management

One hundred sixty Hy-Line Brown hens at the age of 18 weeks were weighed and randomly assigned to 10 treatments, each with 16 birds. Next hens were placed in individual cages with free access to drinking water and fed. The lighting program was 14 h of light and 10 h of darkness. All animal procedures were conducted in accordance with the guidelines of the Polish Council of Animal Care. The protocol for this study was approved by the Local Animal Care Committee of the Poznan University of Life Sciences. After 18 weeks of experiment, birds were weighed again. On last day of the experiment, 8 hens from each group were sacrificed by CO_2 gas, according to the recommendations for euthanasia of experimental animals (Close et al., 1997) and left tibiae was taken from them. Tibiae were kept frozen until analysis.

Table 2: Nutrition value of the diets

Components	T1 i T6	T2-T5 T7-T10
Metabolizable enrgy (MJ/kg)	11.63	11.05
Crude protein (%)	15.4	15.3
P-available (%)	0.39	0.35
Ca (%)	3.54	3.19
Digestible AA (%)		
Lys	0.75	0.71
Met+Cys	0.64	0.60
Tryp	0.16	0.15
Treo	0.53	0.50
General AA (%)		
Arg	1.18	1.18
Val	0.6	0.6
Iso	0.55	0.55
Leu	1.33	1.33
Na (%)	0.16	0.16
Cl (%)	0.16	0.16
Phytic-P (g/kg)	2.35	2.35

Experimental diets

Experimental diets were isoenergetic and isonitrogenous and containing about 15% of crude protein and 11, 5MJ ME/kg feed (Table 2). First, five groups contained only soybean meal (SBM) as a protein source. The diets for treatments from 6 to 10 consisted SBM and 20% of yellow lupine meal (YLM). In six groups used feed enzymes: phytase, protease or both. According to the producer recommendation in diets containing enzymes reduced the level of available phosphorus (50% reduction) and digestible amino acids - 5% reduction (Table 3).

Chemical analyses

For chemical analysis, the representative samples of yellow lupine seeds were ground to pass through a 0.5 mm sieve. Seeds were analyzed in duplicate for dry matter (DM), crude protein (CP), ether extract (EE), crude fiber (CF), crude ash (CA), acid detergent fiber (ADF) and neutral detergent fiber (NDF) using methods 934.01, 976.05, 920.39, 978.10, 942.05, 973.18 respectively, according to AOAC (2007). The amino acid content was determined using a type AAA-400 Automatic Amino Acid Analyzer employing ninhydrin for post-column derivatization. Before the analysis, samples were hydrolyzed (procedure 994.12; AOAC (2007). Sugars were analyzed according to PN-R-64784 method. Gross energy was determined using an adiabatic bomb calorimeter

Table 3: Composition of the experimental diets (g/kg as fed)

Components (%)	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
	PC	NC	NC+PRO	NC+PHY	NC+PRO+PHY	PC	NC	NC+PRO	NC+PHY	NC+PRO+PHY
Maize	65.55	67.23	67.21	67.22	67.2	59.41	58.22	58.2	58.21	58.19
SBM (CP 44%)	21.25			21.9		5.4			8.79	
Yellow lupine (37%)	-			-		20			20	
Limestone 2-4 mm	4.4			4.4		4.4			4.4	
Limestone pon. 2 mm	4.09			3.96		4.05			3.94	
Soybean oil	1.88			0.76		3.81			3.02	
Monocalcium phosphate	1.33			0.38		1.4			0.41	
Vitamin-mineral premix[1]	0.5			0.5		0.5			0.5	
NaHCO3	0.38			0.46		0.38			0.31	
DL-Methionine	0.21			0.17		0.21			0.14	
NaCl	0.11			0.13		0.1			0.15	
L-Lysine	0.17			0.09		0.24			0.08	
L-Valine	0.07			0.01		-			0.04	
L-Tryptophan	0.02			0.01		0.04			0.01	
L-Treonine	0.04			-		0.08			-	
Protease (RonozymeProAct)	-	-	0.02	-	0.02	-	-	0.02	-	0.02
Phytase (HiPhose)	-	-	-	0.02	0.02	-	-	-	0.02	0.02

PC - positive control, NC - negative control, PRO - protease, PHY - phytase

[1]Provided per kg diet: vit. A 10 000 IU, vit. D3 2000 IU, vit. E 20 mg, vit. K3 1.5 mg, vit. B1 1 mg, vit. B2 4 mg, vit. B3 20 mg, vit. B5 8 mg, vit. B6 1.5 mg, vit. B9 0.8 mg, cholin 200 mg, Fe 45 mg, Mn 90 mg, Cu 8 mg, Zn 60 mg, I 1 mg, Co 0.5 mg, Se 0.25 mg, antioxidant 15 mg, vit. B12 3300 mg, biotin 50 mg

(KL 12 Mn, Precyzja-Bit PPHU, Poland) standardized with benzoic acid. Mineral composition (Ca, P, Na, K, Zn, Mg, Cu, Mn, Fe) was analyzed by ICP-OES (P.10I35-ICP method) after microwave mineralization. Lupine alkaloids were extracted from flour by trichloroacetic acid and methylene chloride. The determination was provided by gas chromatography method (Shimadzu GC17A) with a capillary column (Phenomenex). Raffinose family oligosaccharides (RFO) were extracted and analyzed by high-resolution gas chromatography as described previously by Zalewski et al. (2001). Phytate content was analyzed according to AOAC (2007) (methods 986.11). The absorbance was measured with Spectrophotometer Marcel at a wavelength of 519 nm (Table 1).

After defrosting of the left tibia (8 birds per each treatment) and removal of the muscle tissue percentage of tibia ash was determined on a fat-free dry-weight basis, in accordance with AOAC (2005).

Performance and egg quality

The laying performance was recorded weekly in the period of 18 weeks of an experiment for each of 160 hens. The feed consumption was registered in the same way.

The average egg weight was determined also weekly on the basis of collected all eggs from every hen. In the ninth and seventeenth weeks of the experiment, 30 eggs from each treatment were randomly selected (eggs were selected from all laid during all week). The eggshell quality was determined by taking into consideration the following parameters: thickness, elastic deformation and breaking strength. Eggshell thickness (μm) together with shell membranes at the sharp, blunt and equatorial part of the egg using a screw micrometer for this purpose. Examination of the eggshell elastic deformation and the breaking strength were performed using a TA.XT Plus Texture Analyzer (Stable Micro Systems) with a set of suitable starters. Measurement of elastic deformation (μm) was carried out with an accuracy of 0.1 μm in a three-point measurement of egg after application of three different loads, i.e. 0.50 kg, 1.00 kg and 1.50 kg. This study allowed to determine the degree of elastic deformation of the eggshell (microns) under the influence of the applied pressure. Evaluation of the breaking strength of eggshell was carried out applying pressure that was gradually increased until eggshell was cracked. This measurement allowed to define force (N), which cause the eggshell break, crush or puncture.

Calculations and statistical analyses

Two-way analysis of variance was performed using the R environment (R Development Core Team 2014) and using the "agricolae" package (De Mendiburu 2014) according to the following general model:

$$Y_{ij}=\mu+\alpha_i+\beta_j+(\alpha\beta)_{ij}+\varepsilon_{ij},$$

where Yij is the measured dependent variable, μ is overall mean, αi is the effect of diet βj is the effect of the enzyme $(a\beta)$ is the interaction between diet and enzyme and εij is the random error. Means were compared pairwise using Duncan's multiple range test. In the event of the absence of interactions significances, main effects were discussed. All data are presented as means with a pooled standard error of the mean (SEM).

RESULTS AND DISCUSSION

The diets used in this experiment had similar crude protein content. Fed with yellow lupine characterized by higher protein content (about 1.3%). The diets supplemented with YLM contained 1.5% more crude fat, 2.5 g/kg more ADF and 3.4 g/kg NDF. Level of Ca was very similar in all groups and was higher than expected when formulating diets (Table 4).

The egg production during the entire experiment was similar in all groups without significant differences. The egg weight was also on an equal level in all treatments and it was about 58 g. In these two parameters, there was no interaction between the source of protein and inclusion of enzymes. Results in experimental treatments were similar to control groups witch shows that reduced level of available phosphorus and digestible amino acids do not affect laying rate and egg weight. Birds fed different protein source were characterized by similar feed intake and feed conversion ratio during the whole experiment. The inclusion of enzymes also had no effect on these parameters. The FCR was about 1.9-2.0 in all groups (Table 4).

Protein source had no effect of tibia bone ash. Tibia ash was affected by enzymes supplementation. There was an interaction between protein source and enzymes addition. Birds from treatments fed with YLM diets reached highest tibia ash level in a group with phytase addition and in groups with SMB in a group with phytase and protease mix (Table 5). Reducing the level of available phosphorus and amino acids caused a decrease in ash content of the tibia bones of birds from group 7 compared to the control group.

Shell thickness in eggs from part I was significantly different between groups. The inclusion of enzymes improved the thickness of the shell. There was an interaction between protein source and enzyme inclusion. Thickest shell characterized eggs from group fed diets with a mix of phytase and protease (Table 5). It was an interaction between experimental factors in shell breaking strength. Stronger

eggshell was laid by hens from groups fed with the inclusion of YLM. Addition of phytase had a positive impact on increasing the strength of eggshell (Table 5). Only inclusion of enzymes affected significantly on shell elastic deformation in part I of eggs. Eggs from hens fed diets with inclusion of protease and phytase mix were most resistant to elastic deformation (Table 5). In eggs, there were no significant differences between groups in all egg parameter (Table 6).

The obtained results suggest that a 20% addition of yellow lupine did not affect egg laying performance and egg weight which is in agreement with previous research (Krawczyk et al., 2015; van Barneveld, 1999). Alternative research has reported that the application of 25% sweet lupine seeds to diets had a significant impact on reducing the weight of the eggs (Hammershoj and Steenfeldt, 2005). Above authors suggested that decrees in egg weight were caused by methionine deficiency. However, in this study, the methionine content was corrected after the addition of lupine. In the present study, the levels of amino acids in diets containing SMB and those with the addition of yellow lupine have been aligned. Besides, these differences may result from the use of different cultivar of lupine (Nalle et al., 2011). It could be speculated that the reduction in egg weight after YLM addition was caused by a reduction in AME of diets. Kaczmarek et al. (2015) found that 20% inclusion of YLM for broilers chickens, reduces dietary AME_N for 0.8 MJ/kg of diet. Above authors speculated, that lupin cultivars and species may differ with regard to their oligosaccharide and NSP content and may be characterized by different water extract viscosity, which may undesirably affect AME_N, crude fat digestibility and, subsequently, birds performance.

A previous study (Kaczmarek et al., 2015) shown that effectiveness of phytase does not only depend on used plant protein. Results of our study seem to confirm this thesis. Almost in all group with low P level diets inclusion

Table 4: Chemical composition of diets

Treatment	Protein source	Enzyme	Crude protein (%)	Crude fat (%)	ADF (g/kg)	NDF (g/kg)	Ca (%)	Phytic-P
PC	SBM	NO	15.8	2.78	3.72	6.69	4.43	2.84
2		NO	16.38	2.11	3.9	7.34	4.12	3.33
3		PROT	16.14	2.17	3.73	7.4	4.23	3.19
4		PHY	15.84	2.34	3.3	7.29	3.75	2.68
5		PROT+PHY	16.68	2.07	3.28	6.88	4.41	2.67
PC	YLM	NO	16.54	4.12	6.16	9.85	4.66	2.18
7		NO	17.9	4.04	7.27	10.35	4.17	2.79
8		PROT	17.74	3,0	7.31	10.84	3.81	3.25
9		PHY	17.61	3.5	6.38	10.1	4.17	3.32
10		PROT+PHY	17.4	3.78	6.24	11.7	4.21	2.6

Table 5: Laying rate, egg weight, FI, FCR and bone ash of birds fed with experimental diets

Treatment	Protein source	Enzyme	Laying rate (%)	Egg weight (g)	FI (g)	FCR	Bone ash (%)
PC	SBM	NO	91.2	60.12	78.4	1.92	30.35
2		NO	90.6	58.87	112	1.98	28.73^{abc}
3		PROT	90.32	59.68	112.6	1.96	28.72^{abc}
4		PHY	89.31	59	109.3	1.95	27.41^{bc}
5		PROT+PHY	90.15	58.33	112.8	2.04	30.46^{a}
PC	YLM	NO	89.63	57.89	77.95	2.09	33.24*
7		NO	91.6	58.45	114.9	2.02	26.71^{c*}
8		PROT	88.51	57.6	109.2	2	28.69^{abc}
9		PHY	90	58.21	112.2	1.97	30.4^{a}
10		PROT+PHY	90.68	57.41	112.6	2.05	30.33^{ab}
		SEM	0.36	0.3035	0.7054	0.01	0.3295
	SBM		90.1^{a}	58.97^{a}	111.7^{a}	1.98^{a}	28.84^{a}
	YLM		90.2^{a}	57.92^{a}	112.2^{a}	2.01^{a}	28.97^{a}
		NO	91.1^{a}	58.66^{a}	113.5^{a}	1.99^{a}	27.66^{b}
		PROT	89.41^{a}	58.64^{a}	110.0^{a}	1.98^{a}	28.7^{ab}
		PHY	89.66^{a}	58.61^{a}	110.7^{a}	1.96^{a}	28.81^{ab}
		PROT+PHY	90.42^{a}	57.87^{a}	112.7^{a}	2.04^{a}	30.34^{a}
	PROTEIN		0.89	0.09	0.7	0.3	0.78
		ENZYMES	0.35	0.75	0.44	0.23	<0.05
INTERACTION			0.5	0.79	0.33	0.98	<0.05

FI - feed intake, FCR - feed conversion ratio, SBM - soybean meal, YLM - yellow lupine meal, NO - no enzyme added, PROT - protease, PHY - phytase, PC - positive control

Table 6: Shell thickness, shell breaking strength and elastic deformation of eggs from birds fed experimental diets. I and II part

Treatment	Protein source	Enzyme	Shell thickness (µm)		Shell breaking strength (N)		Shell elastic deformation (µ)	
			I	II	I	II	I	II
PC	SBM	NO	0.36*	0.37	4.07*	4.34	0.35*	0.37
2	SBM	NO	0.35^{de}*	0.35	3.89^{c}*	4.21	0.35^{de}*	0.35
3		PROT	0.36^{cd}	0.34	4.94^{ab}	4.28	0.36^{cd}	0.34
4		PHY	0.37^{bc}	0.32	4.83^{ab}	4.58	0.37^{bc}	0.32
5		PROT+PHY	0.39^{a}	0.34	3.61^{c}	4.47	0.39^{a}	0.34
PC	YLM	NO	0.39*	0.34	3.61	4.47*	0.41	0.35
7	YLM	NO	0.37^{bc}*	0.33	4.72^{b}	4.3*	0.37^{bc}	0.33
8		PROT	0.35^{e}	0.34	3.89^{c}	4.58	0.34^{e}	0.34
9		PHY	0.37^{bc}	0.34	4.79^{ab}	4.57	0.37^{bc}	0.34
10		PROT+PHY	0.38^{ab}	0.34	5.36^{a}	4.48	0.38^{ab}	0.34
		SEM	0.002	0.003	0.07	0.003	0.002	0.003
	SBM		0.37^{a}	0.34^{a}	4.32^{b}	4.37^{a}	0.37^{a}	0.34^{a}
	YLM		0.38^{a}	0.34^{a}	4.69^{a}	4.49^{a}	0.37^{a}	0.34^{a}
		NO	0.36^{c}	0.34^{a}	4.3^{b}	4.25^{a}	0.36^{c}	0.34^{a}
		PROT	0.35^{c}	0.34^{a}	4.41^{b}	4.44^{a}	0.35^{c}	0.34^{a}
		PHY	0.37^{b}	0.33^{a}	4.81^{a}	4.57^{a}	0.37^{b}	0.33^{a}
		PROT+PHY	0.39^{a}	0.34^{a}	4.48^{ab}	4.47^{a}	0.39^{a}	0.34^{a}
	PROTEIN		0.81	0.46	<0.0001	0.52	0.85	0.46
		ENZYMES	<0.0001	0.83	<0.0001	0.55	<0.0001	0.83
INTERACTION			<0.0001	0.10	<0.0001	0.89	<0.0001	0.10

SBM - soybean meal, YLM - yellow lupine meal, NO - no enzyme added, PROT - protease, PHY - phytase, PC - positive control

of enzymes improve the content of crude ash in tibia bone. Exogenous phytase caused dephosphorylation of inositol hexaphosphate, thanks to which minerals (P, Ca) previously bound in the phytate mineral complexes were released. Released minerals could be absorbed in the digestive tract and then participate in the bone mineralization process. (Qian et al., 1996). Reducing the level of available phosphorus in diets f in groups fed with the addition of YLM resulted in a significant decrease in ash content in the bones. This may be due to the fact that the yellow lupine seeds like other legumes contain 75% of the phosphorus bound in phytate mineral complex which is no available to the animal organism. After the addition of the phytase, the negative effects of phosphorus reduction subsided. The addition of phytase to the diet with SBM as the only source of protein did not affect the increase of the ash content in the tibia. This is confirmed in other studies (Hughes et al., 2009) in which the authors suggest that this is related to the length of the laying period (36 weeks of age and 18 weeks of laying period) and ability of the bird to utilize dietary P. In these studies, significant differences in bone ash content was observed only in the 61 weeks of age. Eggs laid by hens fed with the addition of yellow lupine characterized by a higher mechanical strength of the eggshell. According to Jalal and Scheideler (2001) source of protein have no effect on this parameter. The addition of phytase had a positive influence on the mechanical strength of the shell, shell thickness and elastic deformation of eggshells. This may be related to the breakdown of phytate-mineral complexes, which resulted in the higher availability of minerals that affect the construction of the shell (Selle et al., 2006). Use of enzymes improved shell thickness and shell breaking strength, but the enzymes addition was more pronounce in SBM than YLM diets which resulted in significant protein source x enzyme interaction ($P<0.05$). The location of phytate salts in raw materials varies among sources (Steiner et al. 2007). In cereals such as maize, around 90% of phytate is located within the germ portion of the kernel, but in soybeans, phytate-P is associated with protein bodies, usually distributed throughout the seed (O'Dell et al. 1972; O'Dell and De Boland 1976). Based on above could be hypothesized that use of protease alone or in combination with phytase, degraded protein bodies and released some part of encapsulated phytate. According to Selle et al. (2006), proteases could degrade protein bodies and in that way improve the availability of phytate for hydrolysis by phytase. Additionally, Kies et al. (2006), showed that exogenous phytase prevents the creation of phytate-protein complexes. Insoluble protein-phytate complexes are formed at low pH, as present in the gizzard of birds and this may affect protein digestibility significantly.

It is unknown why there were no differences in shell quality parameter in the seventeenth weeks of the experiment. It could be speculated that with the age of the bird's diet was utilized better, resulting in higher P and Ca absorption because of higher digestive tract capacity.

Authors' contributions

S. A. K and A. R. designed and directed the project; M. K., L. L., S. N., M. H. performed the experiments; M.K and S. A. K. wrote the article.

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