Polish Journal of Veterinary Sciences Vol. 24, No. 1 (2021), 127–133

DOI 10.24425/pjvs.2021.136801

Original article

The effect of Artichoke (*Cynara scolymus L.*) on the expression of calcium-binding proteins in the eggshell gland of laying hens

D. Abadjieva[1], D. Ankova[1], S. Grigorova[2], E. Kistanova[1]

[1] Institute of Biology and Immunology of Reproduction – Bulgarian Academy of Sciences 1113 Sofia, Tzarigradsko shosse 73, Bulgaria
[2] Institute of animal science - AA, 2232 Kostinbrod, Bulgaria

Abstract

The purpose of the present work is to investigate the effect of dietary-supplemented artichoke (*Cynara scolymus L.*) on the mRNA expression of calbindin 1 (*Calb1*), osteopontin (*Spp1*), albumin (*Alb*) and CALB1 protein in the eggshell gland (ESG) of laying hens. A total of 80 ISA Brown hens (each at 40 weeks of age) were randomly divided into two groups: a control and a treated group. All poultry received 130 g/day of compound feed for laying hens but the treated hens' diet was also supplemented with 3g/kg of dried and milled artichoke (*Cynara scoly*mus L.). The increase of the Ca content in blood of the treated hens was established. Significantly decrease of *Spp1* mRNA transcripts was found in the eggshell gland of the treated hens, while the mRNA level of *Alb* was increased. The relative expression of *Calb*1 mRNA tended to increase in the treated group. The expression of calbindin protein in the cytoplasm of glandular cells of the shell gland was defined by immunohistochemical method. Very strong signals of calbindin were observed in the treated group.

The supplementation of the laying hens' diet with dried artichoke (*C. scolymus* L.) led to a significant increase of Ca content in blood that was reflected in the changes of expression of the eggshell gland genes involved in the mineralization of eggshell.

Key words: hens, egg shell gland, genes, artichoke

Introduction

Calcium (Ca2+), the most abundant chemical element in the body, plays a vital role in necessary physiological and biochemical processes such as bone mineralization, muscle contractions, neuronal excitability, blood coagulation, cell adhesion and apoptosis (Payne et al. 2009). Intestinal Ca2+ absorption, the main process used by the body to extract Ca2+ from nutrients, is facilitated through the use of two mechanisms. The first one, called the paracellular pathway, is a passive process driven by an electrochemical gradient across the epithelium. This typically occurs when dietary Ca2+ levels increase (Eckermann-Ross 2008). The second one is an active vitamin D-dependent transcellular transport, which occurs in conditions where

Correspondence to: D. Abadjieva, e-mail: dessi_l@abv.bg

Table 1. Composition and calculated nutritive value of the compound feed for laying hens.

Ingredients, g/kg^{-1}	Control group	Experimental group
Wheat	643.4	640.4
Sunflower meal	140	140
Soybean meal	90	90
Sunflower oil	20	20
Artichoke	-	3
Limestone	90	90
Monocalcium phosphate	4	4
Complex premix 6015*	12.5	12.5
Antioxidant (paradigmoks)	0.1	0.1
Nutritive value: Metabolizable energy, Kcal/kg	2710.23	2710.23
Crude protein	164.76	164.43
Crude fat	33.19	33.01
Crude fiber	45.81	46.18
Lysine	7.94	7.90
Methionine	4.39	4.40
Ca	37.27	37.33
total P	4.93	4.89

* Complex premix contains: sodium bicarbonate; lysine; methionine; threonine; choline chloride; 120 mg/kg Mn (MnO); 110 mg/kg Zn(ZnO); 140 mg/kg Fe ($FeSO_4$); 18 mg/kg Cu(Cu SO_4); 1.80 mg/kg I ($Ca(IO_3)_2$; 0.35 mg/kg Se (Na_2SeO_3); 9900 UI Vitamin A (retinyl acetate); 3000 UI Vitamin D_3 (cholecalciferol); 30 mg/kg Vitamin E (dl alpha-tocopherol). It does not contain nutritive antibiotics, synthetic dyes and carotenoids or other stimulants.

Ca2+ is scarcer (Bagur and Hajnóczky 2017). Wasserman et al. (2004) have reported that an active form of vitamin D (1,25-dihydroxycalciferol) regulates the transcription of transport factors as calbindin-D9k, osteopontin and other proteins.

Chicken eggshell is composed of 95% calcite (calcium carbonate) mineral and 3.5% organic matrix proteins (Marie et al. 2015). More than 500 proteins were found in the matrix proteome of the eggshell, some uniquely involved in the creation of eggshell (Mann et al. 2006). According to Marie et al. (2015), about 216 eggshell matrix proteins, including calcium-binding proteins, participate in the key stages of shell mineralization. The elucidation of the regulatory mechanisms and functions of these proteins is critical for the improvement of eggshell quality in the poultry industry, and will ultimately help ensure the economic efficiency of egg production. Most studies regarding the effect of nutrition on eggshell quality have focused on the dietary sources and doses of microelements (Lukić et al. 2009, An et al. 2016). Recently, an interest in the use of feed additives to improve intestinal health and mineral availabilities also grown (Światkiewicz et al. 2015). This inspired the purpose of our present work: to investigate the effect of dietary-supplemented artichoke (*Cynara scolymus L.*) on the mRNA expression of calbindin 1 (*Calb1*), osteopontin (*Spp1*), albumin (*Alb*) and CALB1 protein expression in the eggshell gland (ESG) of laying hens.

The experiment was carried out in the Experimental Poultry Breeding Center of the Institute of Animal Sciences, Kostinbrod, Bulgaria. This experiment and its procedures were approved by the Bulgarian Animal Ethics Committee in accordance with Bulgarian Veterinary Law (2011) on the protection of animals used for experimental and other scientific purposes and relevant provisions of Council Directive 86/609/EEC (Permission for using the agricultural animals for scientific purpose, N177, expire data 18.06.2020 obtained on the base of Protocol N33/18.06.2015). A total of 80 ISA Brown laying hens at the initial age of 40 weeks were randomly divided in two groups: control (n=40) and treated (n=40). Layers were raised on a deep litter pen on a 16 h lighting schedule. Water was supplied via nipple watering trough. The experiment lasted 50 days (10 days adaptation period and 40 days experimental period). All hens stayed healthy during the experimental period. The poultry received 130 g/day/hen compound feed for layers. Crude protein, crude fat and crude fiber were determined by the conventional Weende analysis. The metabolizable energy was calculated according to WPSA (1989). Composition and nutritive value of laying hens compound feed are shown in Table 1.

Table 2. Chemical composition and total antioxidant activity of ground and dried artichoke.

Chemical composition	Artishoke (C. scolumus L.)
Dry matter, g/100g product	89.56
Crude protein g/100g product	10.77
Crude fats, g/100g product	1.99
Crude fibers, g/100g product	14.58
Ca, g/100g product	1.01
P, g/100g product	0.18
Total polyphenols, mg GAE/100g product	546.6
TE/100g antioxidant activity	2928.7

Table 3. Sequences for primers.

genes	Sequences	bp	T°
CALB1	fw: TTG GCA CTG AAA TCC CAC TGA rev: CAT GCC AAG ACC AAC AAG GCTGA	116	60
SPP1	fw: CCA GCT CTG AAG AAA AAT ACG ACC rev: TTG GCT CTT GCT AGG AAT GTC AG	200	60
ALB	fw: CCT GGA CAC CAA CAA GGA AAT rev: TGT GGA CGC CGA TAG AAT	197	60
TBP	fw: TAG CCC GAT GAT GCC GTAT rev: GTT CCC TGT GTC GCT TGC	147	60
ß-actin	fw: AGC AAG CAG GAGG TAC GAT GAATC rev: ACAGTCCGGTTTAGAAGCATTTG	161	60

The diet of experimental group was prepared by reducing the quantity of wheat by 0.3%, after that remixed with 3 g/kg of dried and milled artichoke (*Cynara scolymus L.*). The supplemental product being tested (Profeed, Poland) was a dry mass of the above-ground part of the plant *Cynara scolymus L*. The following analysis of this product was made: crude protein, crude fat and crude fiber (by Weende analysis); the content of both Ca (BSS 11 374-86, 1990) and P (BSS 4336-73, 1990); total polyphenols content (by the Folin-Ciocalteu method described by Blainski et al. 2013) and total antioxidant activity (by the DPPH method described by Petrova et al. 2016). The results are presented in Table 2.

Collection and biochemical analysis of samples

At the end of the experimental period, fifteen poultry per group were randomly selected and weighed 12 hours after their last feeding. Blood samples were taken from each bird's vena cutanea (3 ml/hen) using sterilized syringes and needles. A serum was isolated from these samples through the use of centrifugation at 3000 x g 10 minutes at 4 °C and stored at -80°C until further analysis. Calcium (Ca) and phosphorus (P) concentrations were measured using an Eppendorf D30 biochemical analyzer (San Diego C.A.), according to the manufacturer's recommendations (BioSystems S.A., Costa Brava, Spain). After that, the birds were humanely euthanized. Their shell glands were then dissected, weighed and divided for immunohistochemistry analysis and real-time PCR analysis.

At the end of the experiment, ten eggs from each group, laid on the same day, were broken and of the egg white and yolk were carefully removed. The eggshell of each egg was ground and a feed (2 g) and eggshell (0.2 g) samples were put in a furnace (Tammann) at 600°C to ash overnight, according to procedures described by method 942.05 of AOAC (1990). After that, the Ca and P contents were determined by colorimetric methods according to BSS 11 374-86 (1990) and BSS 4336-73 (1990), respectively.

Real-Time PCR Analysis

Total RNAs from approximately 100 mg of tissue per sample (of eggshell glands) were isolated using the TRIsure reagent according to the manufacture's recommendations (Bioline, Australia). RNA was assessed for quality and quantity using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA). Reverse transcription (RT) was performed on 1µg of RNA template with a SuperScript II RNase H Reverse Transcriptase kit (Invitrogen, USA). The qPCR reactions were performed in triplicate using a cycler real-time PCR instrument (Agilent Stratagene Mx3005P). Each reaction involved a total volume of 20 µL, comprised of 2 µL cDNA, 500 nM primers (Table 3) and 12.5 µL SYBR

Table 4. Effect of dietary artichoke on Ca and P levels in blood serum and eggshell.

Parameters	Calcium		Phosphorus	
	blood serum (mmol/L)	egg shell (%)	blood serum (mmol/L)	egg shell (%)
Control gr. (n=10)	1.22±0.17	35.95±0.38	4.37±0.38	0.109±0.08
Treated gr. (n=10)	3.0±0.19	36.28±0.38	4.44±0.35	0.107±0.06
p-value	0.0001	0.880	0.894	0.984

Table 5. Relative expression of the candidate target genes in the shell gland of laying hens.

Genes	Control group	Treated group	p-value
Calb1	4.73	4.96	0.89
Spp1	0.23	0.07	0.02
Alb	0.15	2.82	0.004

Green Real-Time PCR Master Mixes (Thermo Scientific, USA). The primer sequences are presented in Table 2. Real-time PCR reactions were processed at 95°C for 5 min, 40 cycles of 95°C for 15s, 60°C for 1min, 95°C for 15s, 60 for 1min and 95°C for 15s. "No" template control (NTC) and "no" reverse transcriptase (-RT) control was used to detect possible contamination.

TATA-box binding protein (*Tbp*) and ß-actin were used as referent genes according to the recommendations of MIQE guidelines, as available literature on reference gene stability in human and various other animal species show variable results (Bustin et al. 2009). Mean and standard deviation (SD) of the triplicate RT-PCR reactions were calculated for each reaction plate. The expression levels of genes were evaluated using the $2^{-\Delta CT}$ method (Livak and Schmittgen 2001). The geometric means of two mentioned above referent genes were used for the evaluation of expression of target genes.

Immunohistochemical analysis

One portion of the collected eggshell gland samples was fixed in 10% buffered formalin solution for 48 h. After fixation, the tissues were dehydrated, embedded in paraffin and sectioned at 5 μm. Immunohistochemical detection of calbindin was acomplished at room temperature using the avidin-biotin complex (ABC) method. The primary antibody, mouse monoclonal IgG antibody (dilution 1: 2,000) against bovine CaBP-D28K (Sigma), was applied overnight. The sections were washed in the phosphate buffer (PBS), and then a secondary antibody - biotinylated goat polyclonal IgG antibodies against mouse IgG (Sigma) - was applied for 1 hour. The reaction product was developed using 3,3′-diaminobenzidine tetrahydrochloride (Santa Cruz Biot.). The treated sections were rinsed in distilled water and dehydrated before being placed on a coverslip for evaluation via light microscope (Olympus, Japan).

Immunohistochemical staining intensity of calbindin was classified into one of two categories: strong and weak. The "strong" category indicated the highest density of immunohistochemical reactions within the cytoplasm, and the "weak" indicated the lowest density of these reactions.

Statistics

Statistical analysis was conducted using the software program StatSoft, version 10 (StatSoft Inc., Tusla, USA). Data are presented as means ± SD. The differences between means in the control and treated groups were analyzed using the Student's t-test. Results were considered statistically significant at $p<0.05$.

Results

Dietary supplementation with the bioactive compounds found in artichokes (*C. scolymus L.*) did not affect the level of P in the blood and eggshells of laying hens. Total Ca was significantly increased in treated group's serum ($p<0.05$). This corresponded to the slightly increased levels of Ca in the same group's eggshells (Table 4).

Two reference genes, *Tbp and ß*-actin (Table 5), were used to assess candidate target genes *Calb1, Spp1 and Alb* in laying hens' shell glands. The relative expression of *Calb1* mRNA was higher in the treated group compared to the control, but the difference was not significant. RT-PCR analysis revealed that mRNA expression of *Spp1* was down-regulated in the eggshell gland of the treated hens. It should be noted that in the samples of treated hens was observed the most significant increase of *Alb* mRNA transcripts.

Using immunohistochemical analysis, the location of calbindin in the cytoplasm of glandular cells' of the shell gland of both groups was revealed. In the treated animals, the signal was found to be very strong

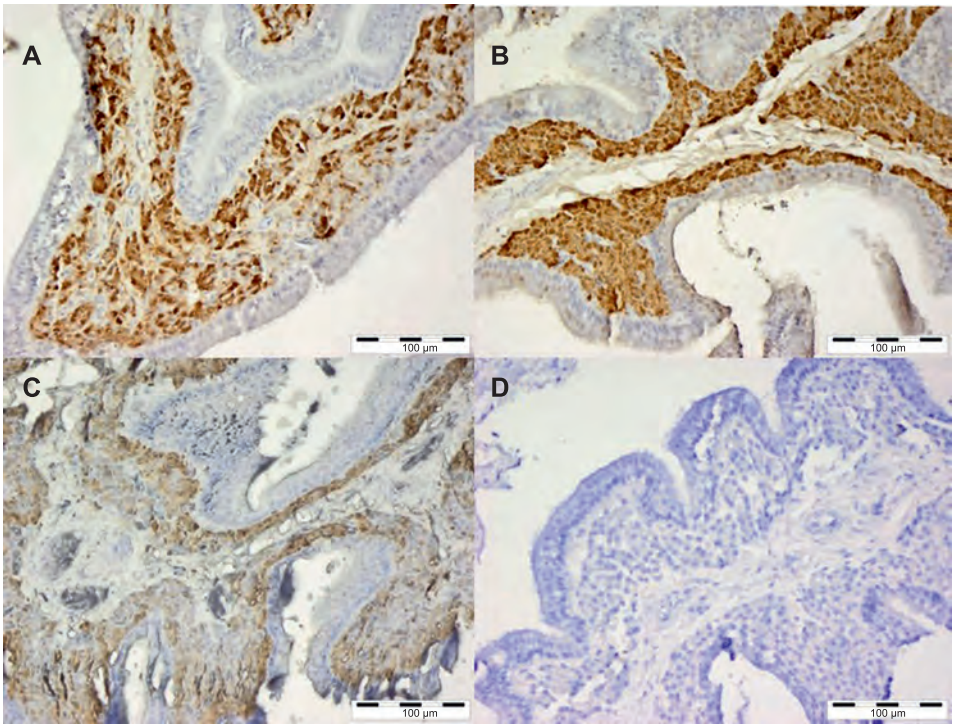

Fig. 1. Immunohistochemical localization of 28-kD calcium-binding protein (calbindin) in eggshell gland of laying hens: A. and B. treated group; C. control group; D. negative control of antibody; x40.

(Fig. 1 A, B). In the eggshell gland of control group, the intensity of the immunohistochemical signal was notably diminished.

Discussion

The present study investigated the effect of bioactive feed substance *Cynara scolymus L*. on both Ca content in blood and eggshells and on the expression of the genes in the eggshell gland, involved in the mineralization process, in lying hens. We observed significantly enhanced Ca level in the blood of treated hens, along with a tendency to slightly increase the Ca content in their eggshells. There are no data in the scientific literature dealing with the influence of *Cynara scolymus L*. extract on Ca levels in hens, but based upon the data on rodents and humans, it would appear that there is a potential effect (Fintelmann 1996). Our results confirmed that this effect also applies to laying hens. The increased Ca in the blood of the experimental hens allowed us to deduce that the nutritional additive was well-absorbed. *C. scolymus L.* is known to be a bountiful source of Ca. Salem et al. (2017) have reported that 100 g of artichoke leaves contain 1,359.3 mg of Ca. These data are close to our results obtained from biochemical analysis of used additive (1,010.0 mg of Ca per 100g). It is important to note that laying hens' intestinal capacity for Ca absorption does not reach a true maximum, but gradually increases during early laying periods (Scott and Balnave 1991). This is also supported by the high positive P balance at the beginning of the production cycle, with significantly decreased P balances in the later periods, signifying that P was released as Ca was removed for shell formation (Ahmad and Balander 2004).

The analyzing of genes expression in the shell gland was done with two reference genes. Using more than one reference gene allows for the achievement of more robust, accurate and reliable normalization of gene expression data (Vandesompele et al. 2002). Our choice of *Tbp* and ß-actin was based on the data of Khan et al. (2017), who characterized the main appropriative reference genes for gene expression study in the shell gland of laying hens challenged by viral infection and defined the *Tbp* as the most stable and ß-actin as a middle stable reference gene.

The selected target genes in the current study have demonstrated a clear association with eggshell calcification processes (Bar 2009, Marie et al. 2015, Khan et al. 2019). Despite the strong expression of the calbindin protein in the eggshell glands of experimental hens, the mRNA expression of this gene did not change significantly. In the eggshell gland calbindin is known as a facilitator of calcium diffusion (Li et al. 2012). The presence of it in the shell gland mucosa of laying hens increases with the onset of egg production and decreases as egg production ceases (Nys et al. 1989).

In our study, sample collection was conducted post-laying, so it is expected that the expression of *Calb1* would remain equally low in all hens. In support of this suggestion we did not define significant difference in *Calb1* mRNA level between control and treated group. The large accumulation of the calbindin protein in the eggshell gland of treated hens, probably, was a result of higher level of the *Calb1* mRNA transcripts, when shell calcification took place (Nys et al. 1989, Jeong et al. 2012, Khan et al. 2019).

The decrease of mRNA level of osteopontin (*Spp1)* was observed in the hens that received the feed additive. *Spp1* gene regulates eggshell formation by inhibiting calcite growth (Chien et al. 2009). A normal phenomenon in the hen: the expression of *Spp1* mRNA gets up-regulated by the entry of the egg into the eggshell gland for calcification (Lavelin et al. 2000, Jeong et al. 2012). The other factor, responsible for stimulating *Spp1* expression, is an enhanced level of bioavailable P in blood (Katsumata et al. 2014, Shet et al. 2018). Significant down-regulation of *Spp1* in our treated group compared to our control may be a result of post-laying sampling, as well as the likely low level of bioavailable P in blood resulting from increased levels of Ca.

The mRNA expression of *Alb* was most significantly affected by the dietary supplementation of artichoke. Albumin binds calcium (Kraghhansen and Vorum 1993) and is one of the major eggshell matrix proteins utilized at all stages of shell formation (Marie et al. 2015). It is highly likely that the remarkable increase of *Alb* mRNA expression observed in the treated group has relationship with the enhanced level of Ca in blood, provoked by *C. scolymus* L.

In conclusion, the supplementation of the laying hens' diet with 3 g/kg of dried artichoke (*Cynara scolymus L.*) led to a significant increase of Ca content in blood that reflected on the expression of the eggshell gland genes involved in the mineralization of eggshell, but with low effect on the Ca content in the eggshells. The further study needs to elucidate the exact genetic pathways regulating the mineralization machinery of eggshell in dependence on changes of Ca content in blood provoked by feed additives.

Acknowledgements

This research was financed by the Ministry of Education and Science of Republic of Bulgaria, project DM 16/4.

References

Ahmad HA, Balander RJ (**2004**) Physiological response of layers to alternative feeding regimen of calcium source and phosphorus level. Inte J Poultry Sci 3: 100-111.

An SH, Kim DW, An BK (**2016**) Effects of Dietary Calcium Levels on Productive Performance, Eggshell Quality and Overall Calcium Status in Aged Laying Hens. Asian-Australas J Anim Sc 29: 1477-1482.

AOAC (**1990**) Official Methods of Analysis. 15th ed. AOAC, Washington, DC. https://archive.org/stream/gov.law.aoac.methods.1.1990/aoac.methods.1.1990_djvu.txt

Bagur R, Hajnóczky G (**2017**) Intracellular Ca2+ sensing: Its role in calcium homeostasis and signaling. Mol Cell 66: 780-788.

Bar A (**2009**) Calcium transport in strongly calcifying laying birds: mechanisms and regulation. Comp Biochem Physiol A Mol Integr Physiol 152: 447-469.

Blainski A, Lopes GC, de Mello JC (**2013**) Application and analysis of the Folin Ciocalteu Method for the determination of the total phenolic content from Limonium Brasiliense L. Molekules 18: 6852-6865.

BSS 11374-86 **(1990**) Determination of calcium and phosphorus. Poultry and their products standartization, Sofia, pp 27-33. https://www.lex.bg/laws/ldoc/2135512321

BSS 4336-73 (**1990**) Eggs and eggs' products. In: Rules on sampling for survey in: Bulgarian State Standarts Food Industry. Poultry and their products standartization, Sofia, pp 117-180. https://www.lex.bg/laws/ldoc/2135512321

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (**2009**) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55: 611-622.

Chien YC, Hincke MT, McKee MD (**2009**) Ultrastructure of avian eggshell during resorption following egg fertilization. J Struct Biol 168: 527-538.

Eckermann-Ross C (**2008**) Hormonal regulation and calcium metabolism in the rabbit. Vet Clin North Am Exot Anim Pract 11: 139-152. Fintelmann V (**1996**) Klinische Bedeutung der lipidsenkenden und antioxidativen Wirkung von *Cynara scolymus* (Artischocke). In: Loew D, Rietbrock N (eds) Phytopharmaka II. Steinkopff Publishers, Deutschland, pp 145-159.

Jeong W, Lim W, Kim J, Ahn SE, Lee HC, Jeong JW, Han JY, Song G, Bazer FW (**2012**) Cell-Specific and Temporal Aspects of Gene Expression in the Chicken Oviduct at Different Stages of the Laying Cycle. Biol Reprod 86: 172.

Katsumata S, Matsuzaki H, Katsumata-Tsuboi R, Uehara M, Suzuki K (**2014**) Effects of high phosphorus diet on bone metabolism-related gene expression in young and aged mice. J Nutr Metab 2014: 575932.

Khan S, Roberts J, Wu SB (**2017**) Reference gene selection for gene expression study in shell gland and spleen of laying hens challenged with infectious bronchitis virus. Sci Rep 7: 14271.

Khan S, Wu SB, Roberts J (**2019**) RNA-sequencing analysis of shell gland shows differences in gene expression profile at two time-points of eggshell formation in laying chickens. BMC Genomics 20: 89.

Kragh-Hansen U, Vorum H (**1993**) Quantitative-analyses

of the interaction between calcium-ions and human serum-albumin. Clin Chem 39: 202-208.

Lavelin I, Meiri N, Pines M (**2000**) New insight in eggshell formation. Poult Sci 79: 1014-1017.

Li J, Yuan J, Guo Y, Sun Q, Hu X (**2012**) The Influence of Dietary Calcium and Phosphorus Imbalance on Intestinal NaPi-IIb and Calbindin mRNA Expression and Tibia Parameters of Broilers. Asian- Australas J Anim Sci 25(4): 552-558.

Livak KJ, Schmittgen TD (**2001**) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408.

Lukić M, Pavlovski Z, Škrbić Z (**2009**) Mineral nutrition of modern poultry genotypes. Biotechnol Anim Husb 25: 399-409.

Mann K, Macek B, Olsen JV (**2006**) Proteomic analysis of the acid-soluble organic matrix of the chicken calcified eggshell layer. Proteomics 6: 3801-3810.

Marie P, Labas V, Brionne A, Harichaux G, Hennequet-Antier C, Rodriguez-Navarro AB, Nys Y, Gautron J (**2015**) Quantitative proteomics provides new insights into chicken eggshell matrix protein functions during the primary events of mineralisation and the active calcification phase. J Proteomics 126: 140-154.

NRC (**1994**) Nutrient requirements of poultry. National Academy Press, Washington, USA. https://www.researchgate.net/publication/275313473_National_Research_Council_Nutrient_Requirements_of_Poultry_-_Ninth_Revised_Edition_1994

Nys Y, Mayel-Afshar S, Bouillon R, Van Baelen H, Lawson DE (**1989**) Increases in calbindin D 28K mRNA in the uterus of the domestic fowl induced by sexual maturity and shell formation. Gen Comp Endocrinol 76: 322-329.

Payne AM, Jimenez-Moreno R, Wang ZM, Messi ML, Delbono O (**2009**) Role of Ca2+, Membrane Excitability, and Ca2+ Stores in Failing Muscle Contraction with Aging. Exp Gerontol 44: 261-273.

Petrova I, Petkova N, Ivanov I (**2016**) Five edible flowers: Valuable sourse of antooxidants in human nutrition. Int J Pharmacog Phytochem Res 8: 604-610.

Salem MB, Kolsi BA, Dhouibi R, Ksouda K, Charfi S, Yaich M, Hammami S, Sahnoun Z, Zeghal KM, Jamoussi K, Affes H (**2017**). Protective effects of Cynara scolymus leaves extract on metabolic disorders and oxidative stress in alloxan-diabetic rats. BMC Complement Altern Med 17: 328.

Scott TA, Balnave D (**1991**) Influence of temperature, dietary energy, nutrient concentration, and self- selection feeding on the retention of dietary energy, protein, and calcium by sexually-maturing egg-laying pullets. Br Poult Sci 32: 1005-1016.

Shet D, Ghosh J, Ajith S, Awachat VB, Elangovana AV (**2008**) Efficacy of dietary phytase supplementation on laying performance and expression of osteopontin and calbindin genes in eggshell gland. Anim Nutr 4: 52-58.

Świątkiewicz S, Arczewska-Wlosek A, Krawczyk J, Puchala M, Jozefiak D, (**2015**) Dietary factors improving eggshell quality: An updated review with special emphasis on microelements and feed additives. World's Poult Sci J 71: 83-94.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (**2002**) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3: RESEARCH0034.

Wasserman RH (**2004**) Vitamin D and the dual processes of intestinal calcium absorption. JNutr 134: 3137-3139.

WPSA (**1989**) European table of energy values for poultry feedstuffs, 3rd ed., Beekbergen, Netherlands, pp 83-104.