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Research article

Supplementation of different sources of selenium on laying performance, egg quality traits, and shell calcification expressions in 50 weeks Lohmann Brown layer hen reproductive tract

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Abstract

Massive consumption and exportation of eggs in Malaysia are possible through improving layer productive performances and egg quality through antioxidant supplementation such as selenium. Preferential supplementation of organic sele-nium in poultry diets was due to its bioavailability. The present study assesses the effects of feeding 50 weeks layer hens with sodium selenite, selenised yeast, and *Stenotrophomonas maltophilia*, ADS18 as a source of selenium and vitamin E on laying integrity, egg qualities, and reproductive gene expressions. The four different treatments (diets) used in the experiment were tagged as follows: Control: a basal diet containing 100 mg/kg vitamin E, SS: basal diet plus 0.3 mg/kg sodium selenite, Se-yeast: basal diet plus 0.3 mg/kg selenised yeast, and VADS18: basal diet plus 0.3 mg/kg ADS18. Productive performances were analysed throughout a 90-days of production. Eggs were collected biweekly, and egg qualities were measured. The uterine and magnum tissues were examined for the genes *ovocleidin* and *ovocalyxin*; OCX32, OCX36, OC17 and OC116. The results showed that Se-yeast significantly ($P < 0.05$) increased laying integrity, while VADS18 supplementation significantly ($P < 0.05$) increased egg weight. Supplementation of different selenium sources significantly ($P < 0.05$) improved egg production and Haugh quality. Additionally, uterine gene expression was significantly ($P < 0.05$) increased by VADS18 supplementation. Therefore, adding organic selenium to the diet maintained various laying performance indicators, egg qualities, and upregulated gene expression. Further evaluations are required to determine the optimum inclusion level of ADS18 poultry.

Keywords: : ADS18, Egg quality traits, Organic selenium, Laying performance, Shell calcification

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INTRODUCTION

Organic trace minerals supplementation has been linked to improve eggshell quality in poultry (Wang et al., 2019) and improve ruminant productive performances (Khanthusaeng et al., 2022). Se is an important microelement used in the diet of poultry that is used to maintain laying integrity (LI) and egg production (Elpawati et al., 2018). Previously, inorganic Se causes an increased Se excretion rate in faecal due to its low absorption rate in body tissues. The use of the organic source of Se (*Stenotrophomonas maltophilia*, ADS18 (ADS18)) in layer diets have greatly enhanced layer performances and physiological functions (Muhammad et al., 2021c) due to its higher bioavailability and less toxicity compared to inorganic Se (Surai, 2018). Hens, given sufficient antioxidants, such as Se, regardless of their sources, could reach their full potential in terms of LI and feed conversion ratio (FCR) (Zhao et al., 2021).

Egg quality, haugh unit (HU) evaluation can be used to determine the bioavailability of Se when fed to hens, as the quality of the eggs degrades with the reduction in the supply of available antioxidants (Asadi et al., 2017). Aryee et al., (2020) discussed that the egg's weight was highly influenced by the egg's albumen height, which in turn affected HU positively. This was also agreed by Muhammad et al., (2021c) that ADS18 improved egg quality immensely compared to inorganic and selenised yeast-sourced groups.

Cracked shells account for 80% of the total rejected eggs and 8% – 11% of total eggs produced daily (Hamilton and Bryden, 2021). According to Hamilton and Bryden, (2021), layer houses' shell breakage occurrence can be reduced by 1% – 2% by improving the shell quality of chicken eggs through the feed. Measurements of shell weight, strength, and thickness, which were discovered to be deteriorating in older laying hens, can be used to measure the quality of the shell (Gan et al., 2020). These qualities can be related to the *ovocleidin* (OC) and *ovocalyxin* (OCX) genes, which are involved in the production of the shell matrix in the uterine and magnum (Sah and Mishra, 2018). Previously, expressions of OC and OCX genes were found highly upregulated when hens were fed with organic sources of Se and subsequently, the shell qualities of layer hens were improved (Muhammad et al., 2021c). However, further evaluation of the correlation between eggshell qualities and the OC and OCX expressions in the uterus and magnum of laying hens supplemented with different sources of Se and VE is important in alleviating laying production.

Organic Se has been explored to be better source of Se compared to inorganic Se. New source of Se, ADS18 was previously researched in supplementation on broilers (Dalia et al., 2017b) and 24 weeks old layer hens (Muhammad et al., 2021c) concerning productive performance, egg production, egg qualities, and shell calcification regulation in magnum and uterus has shown ADS18 in the poultry diet is an efficient Se source of antioxidants by alleviating oxidative status in hens. However, there were no reported work has been done on 50 weeks old layer hens taking into consideration of the physiological differences between production purpose and age of the layer. The present study aimed to determine the efficacy of ADS18 and VE on laying performance, egg quality, and genes involved in shell calcification expressions in the uterus and magnum.

MATERIALS AND METHODS

Bacterial Se, ADS18 preparation

The bacterial Se used in the study were sourced from *Stenotrophomonas maltophilia*, ADS18 (ADS18), which was obtained by isolating the bacteria from Selayang hot spring in Selangor (Dalia et al., 2017a). Following the procedure described by the author, the stock culture was cultivated in the Laboratory of Microbiology, Department of Animal Science, Faculty of Agriculture, Universiti Putra Malaysia (UPM). In brief, the bacteria were inoculated in a nutrient broth containing 10 µg/mL sodium selenite solution. Bacteria rich with organic Se were obtained by centrifuging and ultra-sonicating the culture. The pellet collected was then lyophilized and stored in a -20 °C freezer.

Animal Ethics

The Universiti Putra Malaysia's Institutional Animal Care and Use Committee (UPM/IACUC/AUP-R063/2018) approved this study to be conducted. All operations were conducted in accordance with the established rules and regulations for the management of experimental animals.

Experimental design and diet

One hundred and twenty Lohmann Brown Classic laying hens (n = 120) aged 50 weeks, weighing 1500 g – 2000 g, were used. Each treatment consisted of 6 replicates with 5 hens in each. The hens were subjected to treatment diets for 12 weeks as follows: Control: basal diet includes 100 mg/kg VE, SS: basal diet + 0.3 mg/kg sodium selenite, Se-yeast: basal diet + 0.3 mg/kg selenised yeast (*Saccharomyces cerevisiae* yeast, Sel-Plex, Altech Inc, Lexington, KY, USA), and VADS18: basal diet + 0.3 mg/kg ADS18, Table 1. Basal diets were formulated in accordance with the guidelines for Lohmann Brown Classic hens by Lohmann, (2018) using the FeedLIVE Software (v.1 52, Live informatics Co., Ltd, Thailand). Hens were given *ad-libitum* drinking water and fed 120 g daily to maintain uniform body weight and diminish the feed-selection behaviour in hens. In the experimental phase, the ambient temperature was approximately 24 – 32 °C. The hens were reared individually (30 cm width x 50 cm depth x 40 cm height) in A-shape two-tier stainless-steel cages housed in an open ventilated facility at the Poultry Unit, UPM, Serdang. Starting at 07:00 local time and following the Lohmann Brown Classic hen management guidelines, the lighting schedule was adjusted with 16 hours of light and 8 hours of darkness (Lohmann, 2018).

Table 1 Ingredient composition and calculated nutrients levels of the basal diet

Ingredients	Basal diet (%)
Corn	47.60
Soybean meal	30.50
Wheat pollard	2.87
Refined palm oil	6.10
DL-methionine	0.18
Dicalcium phosphate (18%)	2.10
Calcium carbonate	8.30
Choline chloride	0.10
Salt	0.35
Mineral mix*	0.60
Vitamin mix**	0.60
Antioxidant	0.30
Toxin binder***	0.30
VE	0.01
Total	100
Calculated Composition	
Metabolizable Energy Kcal/kg	2808
Crude Protein (%)	18.20
Crude Fat (%)	7.00
Calcium (%)	4.30
Total Phosphorus (%)	0.54
Average Phosphorus for Poultry (%)	0.38
Analyzed Se (mg/kg)	
Basal diet	0.031
Sodium selenite	0.321
Selenised yeast	0.320
ADS18 Se	0.339

* Mineral premix supplied (per kg of premix): copper 15 mg, zinc 120 mg, iron 120 mg, manganese 150 mg, iodine 1.5 mg, and cobalt 0.4 mg.
** Vitamin premix supplied (per kg of premix): Vitamin A (retinyl acetate) 10.32 mg, vitamin E (DL-tocopherol acetate) 90 mg, cholecalciferol 0.250 mg, vitamin K 6 mg, cobalamin 0.07 mg, thiamine 7 mg, riboflavin 22 mg, niacin 120 mg, folic acid 3 mg, biotin 0.04 mg, pantothenic acid 35 mg, and pyridoxine 12 mg.
*** Antioxidant contains butylated hydroxy anisole (BHA).
**** Toxin binder contains naturally hydrated sodium calcium aluminium silicates to reduce feed exposure to mycotoxins. Feed Live International Software (FeedLIVE) (Nonthaburi, Thailand) was used to formulate the diets.

Laying performance

Laying performance parameters were measured according to Muhammad et al., (2021c). Eggs' weight was recorded daily for 12 weeks. FI and feed refusal were recorded weekly. FCR was calculated using the ratio between FI and the EW produced according to treatment groups (g feed intake/g egg mass). LI was calculated based on the eggs produced daily and the number of hens available (egg number/ hen number). The LI indicated the efficiency of hens in laying eggs daily. Egg mass (EM) was calculated using LI and EW (LI x egg weight/100). EM was used to determine the uniformity of egg production and EW produced in a flock.

Internal egg qualities

Internal egg qualities such as albumen height, HU and yolk colour were determined using the Automated EggAnalyzer® (2006, ORKA Food Technology Ltd, USA). HU was used as an indicator for internal egg quality and is calculated based on the albumen height. Yolk colour was determined by the Automated Egg Analyzer® according to the DSM yolk colour fan that measures the yolk intensity from 1 – 16.

External egg qualities

The same egg from the internal egg quality analysis was used. Shells of the broken eggs were dried in a 60 ℃ oven for 24 hours. Shell weights were calculated after drying the shells at 60 ℃ for 24 hours. Shell thickness was measured in millimetres (mm) using a Vernier calliper (Digimatic 0–25 mm 0.001 mm, Mitutoyo Inc., Kawasaki, Japan) after weighing the shell. Shell thickness was measured in three points; the blunt end, the sharp end, and the middle. The average of the shell thickness was calculated. Surface area (cm^2) was calculated using the formula (3.155 − 0.0136L + 0.0115B)LB, in which both L and B are taken in mm. Shell strengths were determined using an Instron 100 kN Universal Testing Machine (Model 5542, Instron, Norwood, MA, USA).

Determination of shell calcification gene in uterus and magnum

Sample collection

Hens were fasted before the slaughtering process. Six hens were randomly selected at day 90 from each treatment diet and slaughtered. The hens were eviscerated, and the uterine and magnum tissue samples were collected for RT-PCR. Tissues were collected in a 5 mL capped tube, instantly transferred to liquid nitrogen to preserve the tissue integrity and stored at -80 ℃ for further sample preparation for RNA extraction.

RNA isolation and cDNA conversion

Samples stored after the slaughtering process were then further processed by crushing, aided by liquid nitrogen to reduce surface area and provide better homogenization of samples. Rneasy© Mini Kit (Cat. No. 74104, Qiagen, Hilden, Germany) was used to extract total RNA from tissues. The kit was equipped with procedures according to the manufacturer's instructions. The purity and concentration of total RNA were analysed using Thermo Multiskan® GO (Thermo Fisher, USA). Samples with purity 1.8 – 2.2 and a concentration of more than 100 were reverse transcripted using QuantiNova Rev Transcription Kit (cat. No. 205413, Qiagen, Hilden, Germany). Samples were then converted into cDNA using Thermocycler (Personal T20, Biometra, Germany) and stored in a -80 ℃ freezer.

Quantitative real-time RT-PCR (qPCR)

Obtained cDNA were used to determine expressions of OC and OCX genes. Six replicates from each treatment were analysed. Primers were sequenced (HuaGene™, MyTacg Bioscience Malaysia) in accordance with published layer hen sequences in Table 2. Briefly, 1 µL of each reverse and forward primer, 1 µL cDNA, 7 µL nuclease-free water, and 10 µL SYBR Green Master Mix make up the reaction to 20 µL. Targeted genes were compared with *glyceraldehyde 3-phosphate dehydrogenase* (GAPDH) as the housekeeping gene. The protocol for housekeeping gene suitability was followed in accordance to Muhammad et al., (2021a) which determined the most stable housekeeping gene as GAPDH compared to Beta-actin and TATA-Box binding protein. Protocols for RT-PCR following the protocols in the kit box to amplify the gene of interest. The thermal cycling programmed was computed in the qPCR system Bio-Rad CFX Manager™ 3.1 (Bio-Rad Laboratories, Hercules,

CA, USA): reverse transcription at 95 °C for 10 minutes, initial denaturation at 95 °C for 2 minutes, then 40 cycles of denaturation at 95 °C for 5 seconds, and primer annealing/extension combination at 60 °C for 10 seconds. Fluorescence data were taken at the end of each annealing step during PCR cycles using a melting curve to assess the PCR amplification. Cycle threshold (Ct) values were obtained and then calculated using the Livak method to determine tissue gene regulation (Pfaffl, 2001). In brief, Livak calculation use reference gene as a normalization to the relative expressions of targeted gene using the mathematical delta-delta calculation, ratio = $2^{-(\Delta Ct \text{ sample} - \Delta Ct \text{ control})}$. The value calculated would be further analysed using statistical analysis software.

Table 2 Sequence of targeted gene and primer nucleotides

Target genes*	Nucleotide sequence (5' - 3')	Fragment size (bp)	Reference(s)
Housekeeping genes			
β-actin	F: ACACACGGACACTTCAAGGG R: TACTCAGCACCTGCATCTGC	128	(Dalia et al., 2017b,Muhammad et al., 2021b)
GAPDH	F: AATGAGAGGTTCAGGTGCCC R: ACCAGACAGCACTGTGTTGG	150	(Dalia et al., 2017b,Muhammad et al., 2021b)
Shell calcification genes			
OCX32	F: GGACAGCACTGCACTACATCAA R: GGAATTTCGTGGAGCAAGACAA	514	(Gautron et al., 2001)
OCX36	F: TTGGAATGGTCGTCTTCTGTGG R: CGGTCTGAATGATGGCATCG	121	(Gautron et al., 2007)
OC17	F: CGTTCTGCCGCCGTTGGG R: CCCGCGACGCGTTGAGGA	96	(Zhang et al., 2014)
OC116	F: AGAGCCAACATCCAAGTGGGTGAGAAT R: CAGTGACCACATGGCTCCCTTTCCT	424	(Horvat-Gordon et al., 2008)

**Beta actin*: β-actin, *Glyceraldehyde-3-phosphate dehydrogenase*: GAPDH, *Ovocalyxin* 32: OCX32, *Ovocalyxin* 36: OCX36, *Ovocleidin* 17: OC17, *Ovocleidin* 116: OC116
** F: forward primer, R: reverse primer

Statistical analysis

The data were analysed using one-way analysis (ANOVA) using the Proc GLM procedure of the statistical analysis software (Statistical Analysis System, Version 9.4). The means of each study were compared using Tukey's studentized range (HSD) at $P < 0.05$. In all tables, the results were presented as mean ± SEM.

RESULTS

Laying performance

There was a significant ($P < 0.05$) difference in FI between the control and Se-supplemented (VADS18) groups in Table 3. VADS18 fed group had the highest FI compared to other Se-supplemented and control groups. In comparison to other treatment groups, the VADS18 group significantly ($P < 0.05$) produced 2.5% heavier eggs. The FCR was 7.42% lower in organic Se-supplemented groups, which was significantly ($P < 0.05$) different compared to the inorganic Se and control group. Similar patterns were observed in the LI, with the Se-supplemented groups significantly ($P < 0.05$) outperforming the control group. In comparison to inorganic Se source, organic Se-yeast had the highest LI. In comparison to inorganic SS, the organic Se-yeast supplemented group was found to increase the EM in hens significantly ($P < 0.05$). Results of the present study showed that the supplementation of organic Se significantly enhanced laying performance in various indicators, including EW, FCR, LI and EM.

Table 3 Effect of dietary supplementation of different Se sources combined with VE on the production performances of Lohmann Classic Brown layer hen.

Parameters**	Treatments*				P-value
	Control	SS	Se-yeast	VADS18	
FI (g)	105.26 ± 0.09 ^{a}	100.53 ± 0.09 ^{b}	99.46 ± 0.09 ^{b}	104.50 ± 0.09 ^{a}	<0.05
EW (g)	61.77 ± 0.33 ^{b}	62.25 ± 0.38 ^{b}	62.29 ± 0.38 ^{b}	63.33 ± 0.46 ^{a}	<0.05
FCR	2.02 ± 0.03 ^{a}	2.01 ± 0.03 ^{a}	1.87 ± 0.03 ^{c}	1.93 ± 0.03 ^{b}	<0.05
LI (%)	83.42 ± 1.12 ^{c}	87.33 ± 0.76 ^{b}	91.39 ± 0.34 ^{a}	87.30 ± 0.79 ^{b}	<0.05
EM	51.49 ± 0.72 ^{d}	53.71 ± 0.47 ^{c}	56.11 ± 0.23 ^{a}	54.73 ± 0.54 ^{b}	<0.05

Means in the same row with different superscripts ($^{a, b, c}$) are significantly different ($P < 0.05$).
*Control; Basal diet includes VE (100 mg/kg), SS; basal diet + sodium selenite (0.3 mg/kg), Se-yeast; basal diet + selenised yeast (0.3 mg/kg), and VADS18; Basal diet + ADS18 (0.3 mg/kg).
**FI; feed intake, EW; egg weight, FCR; feed conversion ratio, LI; laying integrity, EM; egg mass.

Internal egg qualities

Yolk colours were significantly ($P < 0.05$) different in the group receiving VE supplements along with SS in Table 4. In comparison to other treatment groups, the colour of the yolk fed with SS was 20% more yellow. Hens fed VE-supplemented diets of SS, Se-yeast, and VADS18 produced HU eggs that were 1.98%, 1.74% and 2.89% better, respectively, compared to the non-supplemented group. Comparing the VADS18 supplemented group to inorganic SS and organic Se-yeast-fed groups, the difference in albumen heights is significant ($P < 0.05$).

Table 4 effect of dietary supplementation of different Se sources combined with VE on the internal egg quality characteristics of laying hen

Parameters**	Treatment*				*P-value*
	Control	SS	Se-yeast	VADS18	
Colour	3.05 ± 0.12 [b]	3.68 ± 0.11 [a]	3.12 ± 0.14 [b]	3.23 ± 0.14 [b]	<0.05
HU	72.86 ± 0.43 [b]	74.30 ± 0.51 [a]	74.13 ± 0.51 [a]	74.97 ± 0.49 [a]	<0.05
Albumen Height (mm)	5.64 ± 0.08 [b]	5.73 ± 0.07 [b]	5.73 ± 0.08 [b]	6.06 ± 0.09 [a]	<0.05

Means in the same row with different superscripts ([a, b, c]) are significantly different ($P < 0.05$).
*Control; Basal diet includes VE (100 mg/kg), SS; basal diet + sodium selenite (0.3 mg/kg), Se-yeast; basal diet + selenised yeast (0.3 mg/kg), and VADS18; Basal diet + ADS18 (0.3 mg/kg).
**HU; haugh unit.

External egg qualities

Hens that were fed VADS18, their shell weight is significantly ($P < 0.05$) lower than when they were fed SS or Se-yeast, but not significantly ($P > 0.05$) different from the control group in Table 5. Moreover, the shell quality was 4% lighter and 9% thinner compared to other treatment groups. However, there was no significant ($P > 0.05$) differences in shell weight, strength, and surface area.

Table 5 Effect of dietary supplementation of different Se sources combined with VE on the external egg quality characteristics of laying hen

Parameters	Treatment*				P-value
	Control	SS	Se-yeast	VADS18	
Shell weight (g)	5.72 ± 0.17	5.81 ± 0.12	5.78 ± 0.15	5.55 ± 0.13	>0.05
Shell thickness (mm)	0.46 ± 1.42 [a]	0.48 ± 0.03 [a]	0.46 ± 0.02 [a]	0.43 ± 0.03 [b]	<0.05
Surface area (cm^2)	70.24 ± 0.92	70.89 ± 0.65	71.91 ± 1.02	68.69 ± 1.18	>0.05
Shell strength (N)	28.29 ± 3.28	25.54 ± 5.55	28.69 ± 3.39	26.87 ± 2.62	>0.05

Means in the same row with different superscripts ([a, b, c]) are significantly different ($P < 0.05$).
*Control; Basal diet includes VE (100 mg/kg), SS; basal diet + sodium selenite (0.3 mg/kg), Se-yeast; basal diet + selenised yeast (0.3 mg/kg), and VADS18; Basal diet + ADS18 (0.3 mg/kg).

Shell calcification gene in uterus and magnum

The VADS18-supplemented hens had significantly ($P < 0.05$) higher mRNA levels of the calcification gene expression in the uterus compared to other treatment groups in Figure 1. However, in hens fed with Se-yeast and SS, there was no significant difference ($P > 0.05$). OCX32 and OXC36 levels in the uterus of hens fed VADS18 were seven-fold and four-fold times higher, respectively than that of those hens exclusively fed with Se-yeast. OC116 gene expression was shown to be upregulated ($P < 0.05$) in VADS18 fed groups, as compared to other treatment groups. Figure 2 showed that when VADS18 was supplemented to hens, OC17 mRNA expression was highly upregulated ($P < 0.05$), resulting in a 50-fold higher expression of the targeted gene in uterine tissue compared to other treatment groups.

Figure 3 showed when hens were fed different Se sources along with VE, and there was a significant ($P < 0.05$) upregulation of mRNA expression in all eggshell matrix in magnum. When hens were fed with VADS18, the expression of OC17 was significantly ($P < 0.05$) higher, with a fifty-fold increase in expression. In comparison to Se-yeast and SS, OC116 was significantly ($P < 0.05$) upregulated seven-fold in VADS18-supplemented hens.

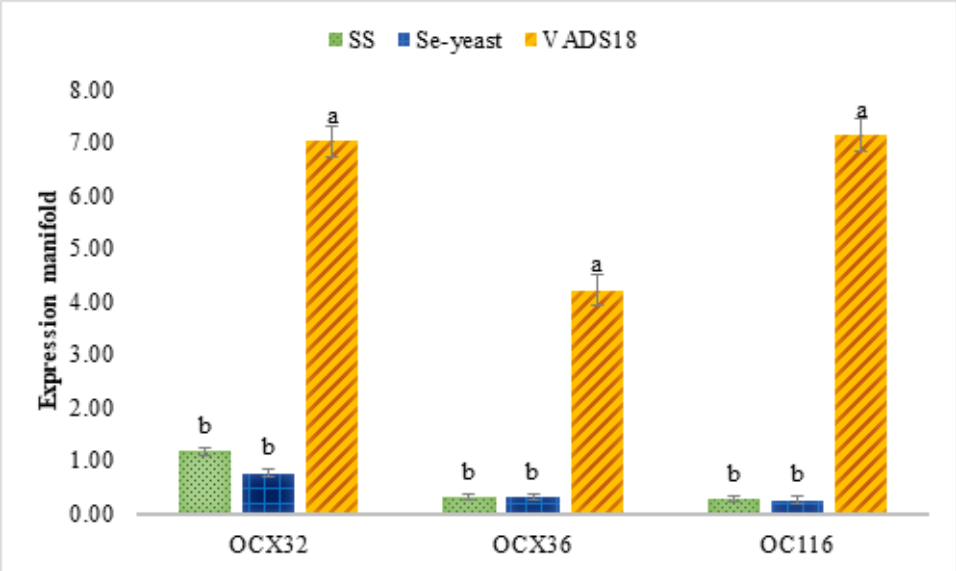

Figure 1 The expression manifold of the calculated target genes affected by different die-tary Se source in the uterus of laying hens. Bars with different superscripts (a, b) are significantly different ($P < 0.05$).

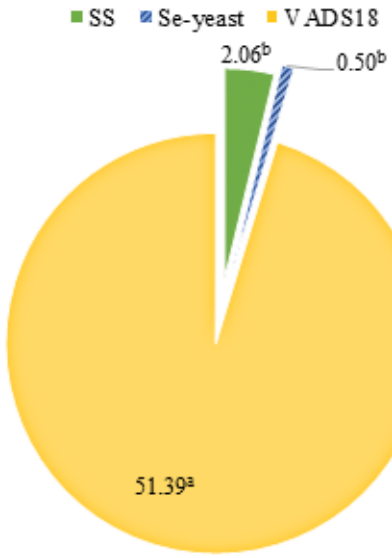

Figure 2 The expression manifold of the OC17 gene affected by different dietary Se sources in the uterus of laying hens. Means with different superscripts (a, b) are significantly different ($P < 0.05$).

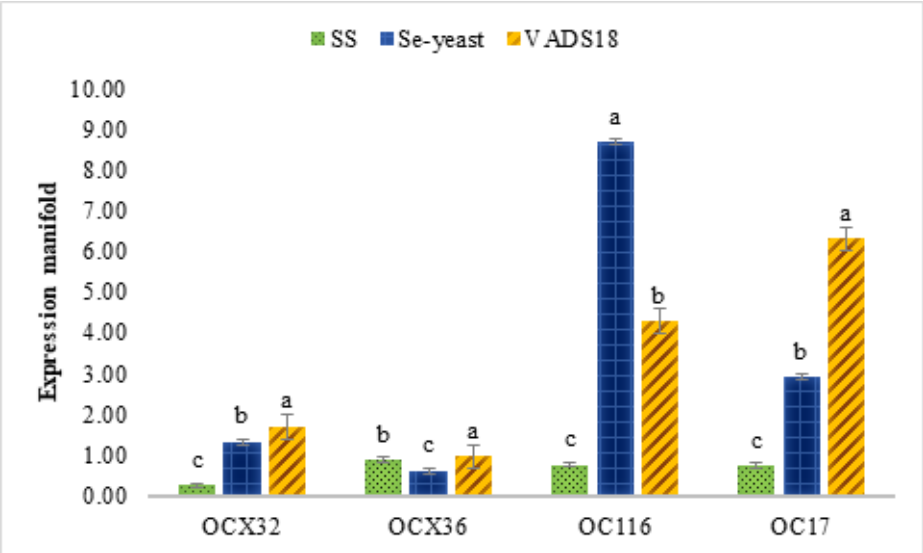

Figure 3 The expression manifold of the calculated target genes affected by different dietary Se sources in the laying hens' magnum. Bars with different superscripts (a, b) are significantly different ($P < 0.05$).

DISCUSSION

The FCR has been regarded as a crucial indicator for the table egg industry, which is thriving in the current market growth by including Se in laying hens' diets to maintain and improve FCR (Surai and Fisinin, 2014). In the present study, eggs from the VADS18-fed group were noticeably heavier than those from the other Se-treated groups. Albumen height was used to indicate egg quality because of the strong positive correlation between albumen height and egg weight (Aryee et al., 2020). The results of this study support the conclusion of the aforementioned author since VADS18-fed hens did produce eggs with higher albumen height and heavier eggs compared to other Se sources (Se-Yeast and SS). This result is consistent with research conducted by Ziaei and Pour, (2013), which found that adding VE and Se to layer hen feed greatly improved the FCR of laying hens. Nemati et al., (2020), in contrast, discovered no significant differences in the quail's overall laying performance throughout 8 weeks feeding regime. Although VADS18 supplementation did not reduce FCR in laying hens, it produced notably heavier eggs compared to other treatments. This study attributed the high FCR to the elevated FI in laying hens fed VADS18 Se. The hen's nutrition, housing, and age played a significant role in egg production percentage (LI) in laying hens. Lohmann Brown laying hens that are 50 weeks or older (87% of LI) to 60 weeks older (81% of LI), which is considered late laying stage, would show a declining trend of LI, according to Lohmann, (2018). At the late-laying stage, a reduction to 80% of LI was seen as the metabolic system's inability to transfer nutrients to maintain body functions. However, organic Se (Se-yeast) fed maintained their egg production at over 90% throughout the experimental period compared to inorganic Se-fed hens (Baylan et al., 2011).

Alagawany et al., (2021) discussed that trace mineral (Se) and vitamin (VE) capable of producing favourable result in improving egg production

and FCR in laying hens. According to previous studies, the FCR and LI were unaffected by the supplementation of different organic Se sources (Chantiratikul et al., 2018) or varying concentrations of organic Se (Lu et al., 2019). El-Deep et al., (2017) found that chickens with 0.3 mg/kg Nano Se had significant LI and FCR compared to non-supplemented groups. When compared to the control group, which received only VE and inorganic Se, the organic Se (Se-yeast) treated groups in the current study demonstrated higher laying performance. The results of this study were in agreement with Ziaei and Pour, (2013) study on the efficiency of Se and VE in FCR. Adding 0.75 mg/kg organic Se and 250 mg/kg of VE resulted in a high FCR and LI in 65-week-old hens compared to other treatments. The discrepancy in inclusion levels was caused by the age difference of the hens, which require higher doses of Se and VE to maintain high productivity. However, the results of both investigations disagreed with those of Mohiti-Asli et al., (2008), who found that supplementation of inorganic Se in combination with VE did not significantly increase LI compared to control. This might be as a result the difference breed of layer used in the study, whose genetic makeup may have an impact on intestine absorption efficiency.

HU is an important indicator for egg quality, which measures the height of thick albumen and EW. A good egg quality has HU ranges from 70 – 130 (Haugh, 1937). HU was significantly higher in hens fed with Se and VE compared to the control, HU in VADS18 treated group was notably firmer and had higher albumen height, as agreed by Jemiseye and Ogunwole, (2019). Aryee et al., (2020) discussed that the weight of the egg was highly influenced by the albumen height of the egg, which in turn affected HU positively. Muhammad et al., (2021c) also concurred that VADS18 significantly improved egg quality compared to inorganic and other organic Se sources. Although there were findings of significant egg quality improvement when Se and VE were supplemented in combination or individually, Asadi et al., (2017) observed minimal changes in HU and albumen height regardless of Se sources.

Yolk colour ranges from 1 – 15 according to the Roche Yolk Colour Fan® scale (RYCF), which considers the amount of pigmentation in an animal's diet. Yolk colour intensity was praised as a high-quality egg and the preferred choice for table eggs due to the vivid colour that attracts consumers (Saleh et al., 2021). Contrary to previous research on ADS18 Se supplementation, yolk colour intensity was not affected by the inclusion of dissimilar sources of Se (Muhammad et al., 2021c). This may be caused by the inclusion of VE in combination with ADS18 which was not the case in the previously stated research. Older laying hens may assimilate reduced carotenoid content in yolk without jeopardising the egg quality (Perić et al., 2017).

The eggshells in the VADS18-fed group were thinner compared to other groups. The age of the chicken, which has a significant impact on shell formation, might explain observed differences in shell thickness (Zhang et al., 2022). Although the VADS18-fed groups had thinner shells, there was no significant difference in shell strength among the treatment groups. The findings of this study were also confirmed previously by Pavlovic et al., (2010), who found no adverse effects of Se supplementation on the eggshell quality. Conserving shell strength is essential to reduce the prevalence of shell breakage. In contrast to the study of Invernizzi et al., (2016), no significant difference found in this study for eggshell weight. Further research on shell quality revealed that a hen's age has a substantial impact on the calcification

of the shell, and mineral segregations for shell deposition were decreasing with the increasing laying week (Perić et al., 2017). Thus, the decreasing shell thickness could be attributed to the layers' advanced age.

Uterine tissues invested 20 hours daily for shell formation, which was attributed to *ovocleidin* (OC) and *ovocalyxin* (OCX) genes that are responsible for shell thickness, egg shape, breaking strength, and elasticity (Sah and Mishra, 2018). Shell matrix proteins regulation is determined by the OCX32 and OC116 expressions, while the protein sequence of eggshell is determined through expressions of the OCX36 gene (Hincke et al., 2012). Calcium carbonate deposition in shells is regulated by OC17, which correlates to the calcite patterns in eggshells, which also play a role in shell strength determination, among others (Radwan, 2020). Shell-breaking strength was positively correlated with the microstructural build-up of eggshells, which encompasses shell thickness and weight (Fathi et al., 2019). Older laying hens' ability to maintain the quality of their shell deteriorates over time, which can be improved by supplementing antioxidants (Gan et al., 2020). Antioxidant supplementation has been previously linked to improved shell characteristics such as shell thickness and breaking strength (Invernizzi et al., 2016). It was hypothesized that the abundance of OC and OCX genes in the uterine tissues contributed to the improvement of these shell characteristics. Consequently, when supplemented with 0.3 mg/kg and 1.0 mg/kg of Se, high Se concentration were found in muscle and uterine tissues, the antioxidant supplementation also increased calcium modulation in uterine tissues of rats (Guo et al., 2013). Chicken genome sequencing found that mammals and avian share mineralization genes, the difference between the species is in the location at which the gene functions (Hincke et al., 2012). Thus the findings by Guo et al., (2013) might be an explanation for the significant upregulation of shell formation genes and improved shell characteristics in laying hens.

In the present study, VADS18 supplementation was observed to upregulate gene expression in the uterine and magnum tissues, which is consistent with findings in eggshell thickness. The OC116 and OCX32 gene, which was significantly upregulated in the VADS18-fed group, determined shell thicknesses. Additionally, these genes are also responsible for the mineralization process of egg shape (Sah and Mishra, 2018). Despite the abundance of OC116 production in VADS18-fed hens and appraised as the gene that regulates shell formation, it does not significantly affect the shell weight in this study. Similarly, OC117 and OC16 were upregulated considerably in VADS18-fed hens compared to other Se sources. These genes are responsible for the mineralization framework of protein cuticles in eggshells. Consistently, Muhammad et al., (2021b) found significant differences when supplemented with Se-yeast and ADS18 as dissimilar sources of organic Se on the expression of OC and OCX genes. Presently, laying hens aged 50 weeks were used compared to a younger hen at 23 weeks of age (Muhammad et al., 2021b). Recent studies' findings strengthen the notion that shell formation deterioration, which increases over time in older laying hens, can be reduced by antioxidant supplementation such as organic Se and VE (Fathi et al., 2019). Contradictory supplementation of antioxidants does not necessarily upregulate genes of interest, a study by Zhu et al., (2020) on cadmium supplementation demonstrated toxic and suppressed regulation of OC17, OCX32 and OCX36

genes. Based on the present study, it can be concluded that supplementation of VE and Se significantly upregulates the expressions of shell formation genes in older laying hens. Although bacterial selenium positively affects both uterine and magnum shell formation gene, uterine tissue have higher mRNA expression compared to magnum tissues due to the active calcification stage that occurs in the uterine tissue (Gautron et al., 2001).

CONCLUSIONS

Supplementation of Se-yeast in combination with VE significantly improved FCR, FI and LI in accordance with various studies using the same source of Se in comparison with other Se sources used in the study. Hens supplemented with or-ganic Se had improved EW and HU, which supported that organic ADS18 Se could perform considerably as a new source of organic Se. The findings of this study also highlighted the role of the tremendous upregulation of shell calcifica-tion genes OC17, OC116, OCX32, and OCX36 when hens fed with ADS18 bacterial Se. Thus, supplementation of 0.3 mg/kg of ADS18 with 100 mg/kg VE has the potential to maintain various laying performance indicators and upregu-lates shell calcification genes. Further evaluation on the inclusion level of ADS18 in laying hens is suggested to determine the suitable inclusion level according to laying week and environmental conditions.

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AUTHOR CONTRIBUTIONS

N.I.M.H. devised and conducted all of the laboratory analyses and animal experiments, analysed, and interpreted the results, and wrote the manuscript. **A.A.S.** designed, supervised and administrated the overall research project **N.N.Z, A.I.M., and L.T.C.** participated in the manuscript preparation. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declared no competing interests.

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