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Effects of supplementation with different levels of a multi strain probiotic on the performance of laying chicken

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Abstract

Use of antibiotic growth promoters in poultry production to enhance performance is not recommended due to accumulation of antibiotic residues in the end products which can confer antimicrobial resistance (AMR) to consumers. Inclusion of probiotics in layers diets has been reported to enhance laying performance and egg quality without the risks of AMR. A study to determine the effect of supplementation of a multi-strain probiotic on performance and egg quality was carried. A multi-strain probiotic (MolaPlus®) was purchased from a reputable supplier and administered to laying birds via drinking water at different levels; Prob0(control), Prob2.5(2.5ml/L), Prob5(5ml/L), Prob10(10ml/L) and Prob15(15ml/L). The birds were fed on mash feed which was purchased from a reputable feed manufacturer. One hundred and fifty (150) 65-weeks old ISA Brown were recruited from a laying flock and assigned to the five (5) treatments. The feed intake, body weight, egg weight, egg specific gravity, yolk colour, eggshell weight, and thickness was recorded weekly for 5 weeks. Hen day egg production and water intake were recorded daily while the mineral content of the eggshells (Ca & P) was determined during the 1st, 3rd and 5th week. The mean daily feed intake, feed conversion ratio (FCR), body weight, hen day egg production, water intake, yolk colour, egg weight, specific gravity, shell weight, shell thickness and eggshell % were not influenced significantly ($p > 0.05$) by probiotic inclusion levels. There was a significant ($p < 0.05$) increase in calcium content of the shells with inclusion of probiotics with Prob5 (52.8%) being the highest. The phosphorus content of the eggshells was significantly higher ($p < 0.05$) for Prob5 (0.5311) and Prob15 (0.5093) compared to control (Prob0), Prob2.5 and Prob10. From the findings, it can be concluded that a multi-strain probiotic (MolaPlus®) can be included in layers diet via drinking water to improve egg quality.

Keywords: *drinking water; egg quality; layers; Performance; Probiotics*

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Introduction

Poultry production has been a driver in uplifting the living standards of many people globally, thus improving their welfare (Mottet and Tempio, 2017). The global poultry egg production industry has grown tremendously estimated at 83 million tonnes in 2019, a 63% increase from 2000 (FAOSTAT, 2021). A significant proportion of players in the poultry industry within different countries have eliminated antibiotics usage to promote growth due to the rising concerns regarding the development of resistant microorganisms that were likely to affect humans consuming animals/animal products on prolonged use of antibiotics (Van *et al.*, 2020). Prior to the ban of antibiotic growth promoters, their use resulted in a positive impact in the poultry industry through enhancing growth performance by increasing feed efficiency, reduced mortality, and increase weight gain (Glasgow *et al.*, 2019). The ban has forced many farmers to seek alternative methods of enhancing layers performance and egg quality. Alternatives such as probiotics, prebiotics, synbiotics, essential oils and organic acids which are microbes that have beneficial effects on poultry are instead being used to achieve similar benefits as antibiotics (Abd el-hack *et al.,* 2020; Krysiak *et al.*, 2021). Multi-strain probiotics have shown promising effect in improving feed intake, reducing pathogenic load, improving egg quality and increasing nutrient digestibility in layers (Jha *et al.*, 2020). Probiotics supplements are microbes made from unique strains of bacteria that have the ability to contribute greatly to improving host's health benefits (Krysiak *et al.*, 2021). Probiotics properties and benefits depends on the specific strains of bacteria used in the manufacture, inclusion levels in the diet and age of birds (Yang *et al.*, 2009). Probiotics have been shown to affect eggshell quality parameters, mineral utilization rate and increase production of volatile fatty acids when bacteria such as *Lactobacillus*, *Pediococcus*, *Bacillus*, and *Enterococcus* are used either in single or multi-strain state (Sjofjan *et al.*, 2021). Probiotics in poultry used as feed supplements have positive impact on poultry through modification of gut microflora, improved digestive processes, enhance chicken health status, lessening of nitrogenous gas emissions, growth promotion and competing with other microbes for active sites (Hatab *et al.*, 2016; Deng *et al.*, 2020). Several studies have shown that dietary probiotics supplementation affects egg quality, increase egg production, feed intake and FCR (Mikulski *et al.*, 2020; Xiang *et al.*, 2019). Different types of bacteria have been included in different brands of probiotics some of which include; *Escherichia*, *Prevotella, Streptococcus*, *Clostridium*, *Enterococcus*, *Bacillus* and *Lactobacillus* species (Anee *et al.*, 2021). This study therefore focuses on supplementation of ISA Brown laying hens' diet with a commercially produced multi-strain probiotic MolaPlus® that has four bacterial strains i.e the *Bacillus megaterium*, *Bacillus subtilis*, *Cupriavidus metallidurans*, and *Bacillus safensis* (Atela *et al.*, 2019). The objective of these study was to determine the effect of supplementation of a multi-strain probiotic MolaPlus® on laying performance and egg quality of ISA Brown birds.

Materials and methods

Study site and animal ethics

The research was conducted at the Poultry unit of the department of Animal Production, Faculty of Veterinary Medicine, University of Nairobi located at Latitude 1°15'33.84''S and Longitude 36°43'30.828''E. The experimental procedure was approved by the faculty of Veterinary Medicine Biosafety, Animal Use and Ethics Committee. Ref: FVM BAUEC/2021/311.

Experimental layout, birds', diet, probiotic and management

In this study, 150 ISA Brown laying hens (65 wks) were assigned in a completely randomized design to 5 treatment groups each replicated 5 times. The 5 treatment groups consisted of a control (0ml/L) and 4 levels of probiotics in drinking water (2.5, 5, 10 and 15ml/L respectively). The hens were reared in metallic battery cage with fitted feeding and drinking troughs. Each cage held one bird and each replicate 6 birds. The hens were acclimatized to the experimental conditions for one week (week-64) before commencing data collection at week 65. The layers mash experimental feed was purchased from a reputable local feed manufacturer after confirming that it was formulated to meet nutritional standards (NRC, 1994) and KeBS, 2019 with a minimum of 2850Kcal/kg ME and 14-16% crude protein. A multi strain probiotic, MolaPlus® containing four bacterial strains i.e. Bacillus *subtilis*, *Bacillus safensis*, *Bacillus megaterium* and *Cupriavidus metallidurans* was obtained from a reputable supplier. The probiotic for each treatment was measured using a measuring cylinder and stirred homogenously. The hens were exposed to natural lighting for a relatively consistent period of 12hours daily and house temperature of 26°C in addition to proper ventilation. Additionally, to ensure biosafety, a footbath containing 10ml/L disinfectant (Norbrook®) was placed at the entrance.

Data collection

All the hens were weighed weekly from week 1 to 5. Hen day egg production and water intake were recorded daily for 5 weeks. Mean daily feed intake was calculated weekly. The eggs were collected twice daily from the hen house at 8:00h and 17:00h. Egg weight, egg specific gravity, egg yolk colour, eggshell weight and eggshell thickness were determined weekly. The mineral content of the eggshell was determined thrice (First week, 3rd week and 5th week) while the feed conversion ratio was calculated at the end of 5 weeks. The layers mash diet was sampled and analysed for entire proximate content (dry matter, moisture, crude protein, crude fibre, ether extract, ash) and for Ca and P. ME (metabolizable energy) was calculated using the predictive equation below;

$$ME = 9 \times E.E + 4 \times CP + 4 \times NFE$$

Where; $NFE = 100 - (CP + CF + EE + Ash)$ (AOAC, 2016).

Table 1. Mean chemical composition (%DM) of the layers mash fed to experimental birds

Components	Layer Mash (%)
Dry Matter	91.37 ± 0.61
Crude Protein	15.05 ± 0.43
Crude fibre	11.16 ± 1.44
Ether extract	4.79 ± 0.88
Ash	12.74 ± 1.32
Calcium	3.12 ± 0.48
Phosphorus	0.54 ± 0.04
ME (Kcal/kg) *	2772 ± 73.86

*Calculated metabolizable energy

The chemical composition of the layer diet is shown in Table 1. The layers mash had 91.37% dry matter (DM), 15.05% crude protein (CP), 11.16% crude fibre (CF), 4.79% ether extract (EE), 12.74% Ash, 3.12% calcium (Ca), 0.54% phosphorus (P) and 2772Kcal/kg metabolizable energy (ME).

Performance evaluation and egg quality determination

Hen day egg production data was obtained for the entire period by using the formula;

$$\%\ \text{Hen day Egg Production} = (\text{Number of eggs produced}) / (Number\ of\ live\ hens) \times 100$$

Each treatment was mixed in a bucket and 3L was provided to each replicate daily and intake was determined daily by subtracting refusal from initial and express for the entire period. Each replicate was allocated a bucket containing 9kg of layer mash diet per week and consumption was determined weekly by subtracting refusal from initial while feed conversion ratio was determined using the formula; Grams Feed intake/Grams Egg Weight. Seventy-five eggs (3 eggs from each replicate) were randomly sampled and weighed weekly using a 0.0001g precision analytical balance then used to determine the specific gravity, yolk colour, eggshell weight, eggshell thickness and mineral content of the eggshells. The 75 sampled eggs were used to determine the specific gravity (breaking strength) after a saline solution was prepared by dissolving specific amount of common salt (NaCl) in three litres of water with specific gravities ranging from 1.060 to 1.100g/cm^3 with gradient 0.005 (Butcher & Miles, 2017).

The 75 sampled eggs were broken carefully on a flat white plate and egg yolk pigmentation was determined visually using Roche Yolk Colour Fan with colour scores ranging from 1 (light yellow) to 15 (deep yellow). After breaking the egg carefully, and removing the yolk and albumen, the shells were carefully washed under running tap water to remove any remaining traces of albumen. They were then oven dried at 60°C for 12hours and left to cool at room temperature after which they were weighed using a 0.0001g precision analytical balance. Using a 0.001mm precision micrometre screw gauge, the shell thickness was measured at three locations on the egg (air cell, equator and sharp end) and the mean values represented the shell thickness. The eggshell percentage was calculated by dividing its weight over the egg weight and expressed as a percentage as shown in the formula below;

Eggshell % = [Weight of eggshell (g)/Egg weight(g)] × 100

The eggshells from sampled eggs were grounded into fine powder and ashed (to separate the organic and inorganic matter). The minerals were extracted via dry ashing, filtered into a volumetric flask and topped up with distilled water. Ca and P was then determined by atomic absorption spectrophotometer and UV-visible spectrophotometer respectively (AOAC, 2016).

Statistical analysis

All data obtained on performance and egg quality were subjected to a one-way Analysis of Variance (ANOVA) using GenStat Statistical package version 14. Significant treatment means were separated using Turkey's test and level of significance was set at P ≤ 0.05.

Results

Laying Performance

The effect of inclusion of probiotics on the feed intake, initial and final body weight, egg weight, hen/day/egg production, FCR, water intake and protein consumed are shown in Table 2.

Table 2. Effects of inclusion of different levels of a multi-strain probiotic on layers performance

Parameters	Prob0	Prob2.5	Prob5	Prob10	Prob15	SEM	P-value
FI[1](g/bird/day)	151.6	145.6	143.5	139.4	145.4	3.07	0.128
EW (g/egg)	67.82	68.61	68.63	67.99	68.30	0.866	0.948
IBW (g/bird)	2062.3	1922.3	1963.0	2057.6	2093.0	47.82	0.091
FBW (g/bird)	2088.7	2017.9	2006.6	1992.7	2087.9	37.97	0.247
HDEP (%)	94.48	92.38	94.76	88.38	95.43	2.357	0.246
FCR	2.24	2.12	2.09	2.05	2.13	0.054	0.193
WI (ml/bird/d)	341.7	341.2	337.9	341.6	347.5	13.59	0.992

Prob0: control, Prob2.5: 2.5ml/L, Prob5: 5ml/L, Prob10: 10ml/L, Prob15: 15ml/L, SEM- Standard Error of the Mean. Means with no superscripts within a row are not significantly different (P > 0.05)

[1]FI- Feed Intake (as fed basis), EW- Egg Weight, IBW-Initial Body Weight, FBW- Final Body Weight, HDEP- Hen Day egg production, FCR- Feed Conversion Ratio, WI- Water Intake

The average daily feed intake (FI) ranged from 139.4 to 151.6g/d and tended to be lower for the probiotic fed groups but the difference was non-significant (p = 0.128) compared to Prob0. The mean egg weight (EW) ranged from 67.99 to 68.63g for treatment groups compared to 67.82 for the control. However, the differences were not statistically significant (p = 0.948). The hen/day/egg production, feed conversion ratio (FCR) and water intake were not significantly affected by treatment. However, the FCR tended to decrease with inclusion of probiotic. The protein consumed (PC) was similar across all treatment, a reflection of similar feed intake of constant protein content.

Egg quality indices

The effect of inclusion of probiotic on specific gravity, yolk colour (YC), shell thickness, shell weight, eggshell %, and mineral (Ca & P) content of the eggshells is shown in Table 3.

Table 3. Effects of inclusion of different levels of a multi-strain probiotic on layers egg quality characteristics

Parameters	Prob0	Prob2.5	Prob5	Prob10	Prob15	SEM	P-value
SG^{1}(g/cm^{3})	1.089^{a}	1.089^{a}	1.087^{a}	1.088^{a}	1.087^{a}	0.001	0.513
YC	13.24^{a}	13.13^{a}	13.28^{a}	13.27^{a}	13.13^{a}	0.140	0.896
SW (g)	6.468^{a}	6.465^{a}	6.319^{a}	6.389^{a}	6.378^{a}	0.091	0.751
ST (mm)	0.45^{a}	0.45^{a}	0.44^{a}	0.44^{a}	0.44^{a}	0.004	0.150
Eggshell %	9.538^{a}	9.424^{a}	9.207^{a}	9.399^{a}	9.340^{a}	0.103	0.278
Ca %	49.523^{b}	50.793^{d}	52.827^{e}	49.789^{c}	49.389^{a}	0.018	<.001
P %	0.473^{a}	0.491^{ab}	0.531^{c}	0.487^{ab}	0.509^{bc}	0.006	<.001

Prob0: control, Prob2.5: 2.5ml/L, Prob5: 5ml/L, Prob10: 10ml/L, Prob15: 15ml/L, SEM- Standard Error of the Mean

^{abcde} *means having different superscripts within the same row differ significantly (P ≤ 0.05)*

^{1}*SG- Specific Gravity, YC- Yolk Colour, SW- Shell Weight, ST- Shell Thickness, Ca- Calcium, P- Phosphorus.*

The mean egg specific gravity ranged from 1.087 to 1.089 for birds receiving probiotic compared to 1.08920 in control, the difference was not significant (P = 0.513). The YC ranged from 13.13 to 13.28 for treatment groups compared to 13.24 for control and were not statistically significant (p = 0.896). The shell weight of eggs from layers supplemented with probiotic ranged from 6.3189 to 6.4647g compared to 6.4680g for Prob0. The difference was not statistically significant (P = 0.751). The shell thickness of eggs from the supplemented groups ranged from 0.44 to 0.45mm compared to 0.45mm in control, the difference not being significant (p = 0.15). The % eggshell ranged between 9.207 to 9.424 in treatment groups compared to 9.538 in control, with no significant difference between treatment and control (P = 0.278). The calcium content of the eggshell was significantly different (p < 0.001) across treatment groups compared to Prob0 with Prob5 recording the highest calcium content. Treatment Prob15 recorded a slightly but significantly lower Ca % compared to control (Prob0). The phosphorus content of the eggshells was highest for Prob5 (0.5311) followed by Prob15 (0.5093), Prob2.5 (0.4907), Prob10 (0.4865) and lowest for Prob0 (0.4731). Prob5 and Prob15 were significantly higher (p < 0.001) compared to Prob0, Prob2.5 and Prob10.

Discussion

The dry matter content was within the >90% range recommended for layers mash diet. High moisture feeds could lead to growth of fungus resulting in mycotoxins contamination (Mokubedi *et al.*, 2019). The moisture content was 8.63% which was lower than 10-12% reported by Singh *et al.*, (2019). The crude protein content was within 14-16% range recommended for layers mash (NRC, 1994; KeBS, 2019). Crude protein content in layers feed affects the egg production, egg weight and size and is necessary for both feather development and muscle growth (Van Emous *et al.*, 2015). The crude fibre was higher than the minimum 8% recommended by KeBS., (2019). The crude fibre content was also higher than 5.5% reported by Ekeocha *et al.*, (2021) and 4-6.25% reported by Olorunsogo *et al.*, (2018) in layers ration. This higher fibre content in the diet can be attributed to use of cereal milling by-products in feed formulation in the country (Zhang *et al.*, 2021). The higher fibre content in layers mash could also have a positive effect as has been reported to stabilize the chicken guts, reduce the concentration of ammonia in poultry houses and reduces the numbers of dirty eggs collected (Desbruslais *et al.*, 2021). The ether extract (representing the crude fat) was within the <6% recommended by KeBS., (2019).

The ash content of the ration was in the range of 11.9-17.6% reported by Ekeocha *et al.*, (2021). Layer diets have considerable high ash content due to the high requirement for calcium which is provided through inclusion of limestone in the rations. The calcium and phosphorus content were within the range of 3.0 - 4.20% and 0.40-0.64% respectively, reported by (Rizk *et al.*, 2019; Yan *et al.*, 2019). Ca and P are the key macro minerals that play a critical role in bone development, mineralization and eggshell formation with the former 94% and latter 1% in eggshell (Aditya *et al.*, 2021). The calculated metabolizable energy 2772Kcal/kg was slightly lower than (2850Kcal/kg) recommended by NRC., 1994.

Laying performance

Feed intake by layer birds is related to several factors including genotype, temperature, light, stocking density and feed additive used (Erensoy *et al.*, 2021). The mean daily feed intake in this study ranged from 139.4 to 151.6g/day (as fed) which is equivalent to 127 to 138DM/d (on DM basis) and was not affected by treatment. The DM intake in laying birds has been reported to range between 125 to 135g/day (Lee *et al.*, 2016; Wang *et al.*, 2017; Kim *et al.*, 2020) which is within the range observed in this study. Neijat *et al.*, (2019) observed an increase in feed intake when layers diet was supplemented with low, medium and high single strain Bacillus subtilis by 4.21%, 6.24% and 1.56% respectively at week-20. The researchers attributed the increase to probiotic ability to improve gut health that could mitigate adverse stress effect. Antara *et al.*, (2019) and Bidura *et al.*, (2019) observed no effect on feed intake with probiotic supplementation in layers diet. Fathi *et al.*, (2018) reported a decrease in feed intake in probiotic supplemented diets by 5.92% and 1.18% when 200 and 400ppm *Bacillus subtilis* respectively was supplemented compared to control (0ppm supplemented) that recorded an increase. Lack of significant effect on feed intake in this study could be as a result of probiotic not affecting the GI in a way that could increase rate of feed passage.

The mean egg weight in this study was 68.25g. Mean average egg weight for hybrid birds has been reported to range between 53.62 to 70.87g (Tang *et al.*, 2017; Aalaei *et al.*, 2019) which agree with 68.25g observed in this study. Egg weight tended to increase with probiotic supplementation though not significantly ($p>0.05$) different from control. Antara *et al.*, (2019) reported an increase in egg weight by 4.25% and 4.35% when layers diet was supplemented with (2% and 4% respectively) fermented extract of Moringa oleifera by probiotic Saccharomyces spp for 8 weeks. Ray *et al.*, (2022) also reported an increase ($p<0.05$) with both single strain (5.37%) and multi-strain probiotic (5.54%) supplementation in layers diet. The researchers postulated the increase was due to higher efficiency in nutrient utilization in probiotic supplemented groups. Yan *et al.*, (2019) reported a decrease in egg weight for all multi-strain probiotic supplemented groups. Others reported no effect on egg weight after probiotic supplementation (Aalaei *et al.*, 2018; Xiang *et al.*, 2019). Factors such as adhesion and replication of bacteria in the small intestine, the age of the birds, microbe species, single or multi-strain, amount used and method used can influence the positive effect of probiotics on egg weight (Mikulski *et al.*, 2012; Forte *et al.*, 2016).

The mean final body weight of the birds in this study ranged from 1992.94 to 2088.72g/bird. Final body weight of old hybrid layers has been reported to be between 1943 to 2035g/bird (Hossain *et al.*, 2016; Ray *et al.*, 2022) which is within the range observed in this study. Inclusion levels of probiotic had no effect on final body weight. Laying hens are however not expected to gain weight as the feed is used for egg production and maintenance.

The mean daily hen day egg production in this study was in the range 88.38 to 95.43% with a mean of 93.09%. The observed values fall within the range 83.9 to 98.5% for hybrid hens reported from other studies (Bozkurt *et al.*, 2011; Inatomi, 2016; Hameed *et al*., 2019). A number of factors affects the hen day egg production including feed intake, water intake, age of birds and light intensity (Philippe *et al*., 2020) which were kept constant in this study. Antara *et al*., (2019) reported an increase in egg production by 3.80% and 3.05% in treatment groups compared to control when layers diet was supplemented with 2 and 4% fermented extract of Moringa oleifera by probiotic Saccharomyces spp from week 70 to 78 of age. They attributed this to microbes' ability to survive through the digestion process, growth in the digestive tract and ability to increase digestibility of feed substances. In addition, Ray *et al*., (2022) fed ISA Brown layers with 0.75, 1.00 and 1.25g/kg multi-strain probiotic in feed and recorded a significant increase in egg production by 9.1% compared to single strain fed probiotic and control between 26 and 51 weeks of age. On the contrary, Yan *et al*., (2019) reported that 0.5 and 2.0g/kg inclusion level of probiotic tended to decrease hen day egg production though not significantly compared to control. Several studies have reported no effect on egg production for birds fed on probiotic supplemented diets (Aalaei *et al*.,2018; Fathi *et al*., 2018; Xiang *et al*., 2019). Lack of effect in the current study on HDEP could be attributed to similar feed intake and probably lack of any effect on feed utilization efficiency by the probiotic.

The ratio of grams of feed consumed to grams of egg weight was calculated to obtain feed conversion ratio (FCR). Since feed intake and egg production were not affected, it was not surprising that the FCR was not significantly different amongst supplemented groups compared to control. FCR in layers has been reported to vary between 1.60 to 2.45 (Inatomi., 2016; Lokapirnasari *et al*., 2019; Yenilmez *et al*., 2021) which agree with the mean (2.13) observed in this study. FCR in layers is influenced by several factors including feed quality and management practices. Mikulski *et al*., (2020) and Ray *et al*., (2022) reported an improved FCR ($p<0.05$) with probiotic supplemented groups while (Fathi *et al*., 2018; Yan *et al*., 2019) reported no effect. Layers fed on balanced diet that meet their nutritional requirement tended to convert the feed more efficiently than unbalanced diet (Thirumalaisamy *et al*., 2016). In addition, the management welfare of the birds such as protection from diseases influences the FCR (Tsiouris, 2016). However, in the current study, all factors were kept constant. The slight, though not significant increase in FCR which could be as a result of the probiotics competitive exclusion of pathogen through the production of lactic acid and enzymes hence improved intestinal epithelial barrier and nutrient absorption or the probiotic enhancing health status thus promoting metabolic processes of digestion and nutrient utilization (Macit *et al*., 2021).

The average water intake in this study ranged from 337.9 to 347.5ml/bird and was not significantly affected by treatment ($p = 0.992$). Several factors have been reported to affect water intake in layers including bird age, feed intake, dry matter content of feed and temperature/heat stress (Orakpoghenor *et al*., 2021). Pambuka *et al*., (2014) monitored water intake for birds fed rations containing 0.15% v/v, 0.30% v/v, and 0.45% v/v liquid probiotic mixed culture (LPMC) via drinking water and reported no effect of probiotic on water intake. Feed intake and dry matter content of the feed were all within the required limit and thus didn't affect water intake. Pambuka *et al*., (2014) reported water intake of 253 – 291ml/bird/day for layers birds which is lower than this study. The layers used in their study were younger (52-weeks old) compared to those in this study (65-weeks old) which could explain the difference. The lack of significance in water intake between the birds meant that the probiotic intake via water was at the calculated ratios for different diets.

Egg quality indices

Several factors have been reported to affect layers external egg quality including; bird age, induced moult, nutrition, heat stress, diseases and production system (Roberts., 2004) but none of these varied in the current study. There was no effect of treatment on the egg specific gravity in this study. The specific gravity of an egg gives an indication of eggshell quality with respect to its freshness (Malfatti *et al*., 2021). The observed mean in this study fell within the range 1.077 to 1.10g/cm^3 reported when probiotics were included in layer diets (Kurtoglu *et al*., 2004; Mikulski *et al*., 2012). Mikulski *et al*., (2012) reported a significantly higher egg specific gravity when dietary single strain probiotic (*Pediococcus acidilactici*) was supplemented in layers diet. The improvement was however attributed to bacteria improving the morphological structure of the small intestine mucosa thus increase absorption of nutrients. On the contrary, other studies where laying birds were fed on different dietary levels of probiotics reported no significant treatment effect on egg specific gravity (Yan *et al*., 2019; Mikulski *et al*., 2020). Lack of treatment effect in the current study could attributed to commercial diet sufficient in mineral elements (Ca &P) required for eggshell strength and probiotic not affecting the GI in a way that could increase the rate of feed passage.

Yolk colour in layers chicken eggs is affected primarily by the presence of carotenoids (xanthophylls, lutein and zeaxanthin) in their diets (Marounek and Pebriansyah, 2018; Kavtarashvili *et al*., 2019). Mean egg yolk colour score in this study range was 13.13 to 13.28. Yolk colour index in layers can range between 0 to 15 (Vuilleumier, J.P., 1969), the lighter colour being for diets deficient in carotenoids (will lead to a pale-yellow coloration in the yolk) while carotenoid rich diets will lead to a deep yellow coloration as in the case of this current study. Antara *et al*., (2019) and Macit *et al*., (2021) reported an increase in yolk colour score by 17.27% vs 19.24% and 8.44% vs 7.54% respectively in probiotic supplemented groups compared to control while (Aalaei *et al*., 2018; Fathi *et al*., 2018; Ray *et al*., 2022) reported no effect of probiotic supplementation on yolk colour. Neijat *et al*., (2019) reported a decrease in yolk colour score by 7.69% when high single strain Bacillus subtilis was supplemented in layers diet which was attributed to the temporal variation in the dosage of probiotic used in the study. Lack of effect in the current study could be attributed to sufficient carotenoids present in the diet.

The inclusion of probiotic had no significant effect on eggshell weight. The observed range of shell weight (6.3 to 6.5g) in this study fell within the range 4.7 to 6.5g reported by several authors when layer chickens were fed incremental levels of probiotics (Gnanadesigan *et al*., 2014; Fathi *et al*., 2018; Sarfo *et al*., 2019; Kinati *et al*., 2021). Fathi *et al*., (2018) reported an increase in eggshell weight by 4% when 200 and 400ppm probiotics were supplemented in layers diet. The increase was attributed to increase intestinal availability of calcium and eventual deposition in shells. Similar to the current study, several authors have reported no effect of probiotic supplementation on eggshell weight (Neijat *et al*., 2019; Macit *et al*., 2021; Yan *et al*., 2019; Yang *et al*., 2020). Lack of treatment effect on eggshell weight could be attributed to sufficient amount of mineral (Ca & P) in the commercial diet which was efficiently utilized for improved eggshell quality.

Inclusion of probiotic had no significant effect on eggshell thickness. The eggshell plays an important role in protecting the egg from physical and pathogenic damage and is affected primary by nutrition (Ca & P content in the diet) (Robert., 2004). The average eggshell thickness in this study ranged from 0.44 to 0.45mm and was within the reported range of 0.35 to 0.51mm (Aalaei *et al*., 2018; Fathi *et al*., 2018). Bidura *et al*., (2019) reported a significant increase in eggshell thickness by 18.26% and 23.53% when 0.20% and 0.30% Saccharomyces spp probiotic were incorporated in ducks' diet respectively. Ray *et al*., (2022) reported a significant increase in shell thickness in both single strain and multi-strain probiotic treated groups at week-37 of laying (11.11% vs 16.67% respectively) and week-49 of laying (5% vs 5% respectively) compared to control. The researchers attributed the increase to bacteria proliferation in the gut thus increasing rate of fermentation and fatty acid production that reduce luminal pH which improved calcium solubility and promoted absorption. In addition, Mikulski *et al*., (2020) and Fathi *et al*., (2018) reported a significant increase by 1.68% and 2.77% respectively on eggshell thickness in probiotic supplemented groups. They attributed it to probiotic ability to enhance calcium absorption and retention. Other studies have reported no effect of probiotic supplementation on eggshell thickness (Xu *et al*., 2006; Aalaei *et al*., 2018).

There was no significant effect of inclusion of probiotic on eggshell % (weight of eggshell relative to whole egg) as shown in Table 3. The eggshell % as with other eggshell qualities, is affected by age, nutrition, heat stress and diseases and serves as an indicator of sufficiency of mineral deposition in the shell relative to its weight. The average eggshell % in this study ranged between 9.21 to 9.54 and was within the range 9.79 to 11.86 for different studies where laying chicken were fed various diets (Shalaei *et al*., 2014; Sobczak & Kozlowski., 2015). Fathi *et al*., (2018) reported a significant increase in contribution of egg shell to egg weight (7.37%) when 200 and 400ppm probiotics were incorporated in layers diet. Milkuski *et al*., (2012) reported a significant higher shell % when dietary single strain probiotic (*Pediococcus acidilactici*) was supplemented in layers diet during layer phase 1. The increase in shell percent was attributed to probiotic ability to improve physiological condition of digestion and gut health (intestinal absorption). In addition, Ray *et al*., (2022) reported a significantly higher shell percent (7.36%) at week-37 in multi-strain fed birds while at week-49 both single and multi-strain improved shell percent (2.35 and 2.35% respectively). Several studies have reported no effect of probiotic supplementation on eggshell percent (Shalaei *et al*., 2014; Manafi *et al*., 2016; Yan *et al*., 2019).

The effect of inclusion of probiotic on mineral (Ca & P) content of the eggshells was significant and is shown in Table 3. The mean eggshell calcium content in this study was in the range of 49.39 to 52.83% which increased with inclusion of probiotics (except Prob15) while phosphorus was between 0.47 to 0.53% and also increased with inclusion of probiotic. Eggshell Ca and P content can be affected by principally by dietary content of calcium and phosphorus as they are important macro minerals for the shells. The observed values are however higher than 30.87 to 37.63% for calcium and 0.12 to 0.15% for phosphorus fed on probiotics (Abdelqader *et al*., 2013; Bidura *et al*., 2019; Wang *et al*., 2021). This could be attributed to the use of a single strain of bacteria in those studies while in the current study a multi-strain was used. Abdelqader *et al*., (2013) reported a significant increase in calcium content of the shells when probiotics (1g/kg vs 19.65% increase), prebiotics (1g/kg vs 36.99% increase) and synbiotics (1g/kg vs 38.73% increase) were fed to aged layers (64-weeks) compared to control. Wang *et al*., (2021) reported no effect on P content of the eggshell but Ca content of eggshell was significantly increased (8.25%) when *Bacillus subtilis* was supplemented to aged layers (79 weeks). In addition, Bidura *et al*., (2019) reported a significant increase of Ca content of eggshell by 17.28% and 16.85% when 0.20% and 0.30% Saccharomyces spp were incorporated in ducks' diet respectively. Increase mineral content of eggshell could be attributed to probiotic efficacy in increasing intestinal Ca and P availability, absorption and eventual deposition in eggshells (Zou *et al*., 2021). It has however been reported that calcium and phosphorus mineral salts require a low pH for solubility which was further enhanced by probiotic supplementation leading to ionization of the minerals and eventual absorption and deposition in eggshells (Soetan *et al*., 2010; Likittrakulwong *et al*., 2021). From this study and others, it can be concluded that dietary manipulation through probiotics supplementation is effective in improving mineralization of eggshells.

Conclusion

It was concluded that supplementation of laying birds with probiotics up to 15ml/L in drinking water had no significant effect on performance, and egg quality but increased Ca and P deposition in the eggshells.

Multi-strain probiotics (MolaPlus®) can be supplemented in layers diet via drinking water to improve mineralization of shells which is significant.

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