

The impact of multi-species bacteria as a probiotic on productive performance and egg quality of laying Japanese quail

Okasha M. Hamada^{1#}, Gafaar M. El-Gendi¹, K M. Eid² & Waleed Abdelmoez³

¹Animal Production Department, Faculty of Agriculture, Benha University, Moshtohor 13736, Egypt

² Animal Production Research Institute, Agriculture Research Centre, Giza, 12618 Egypt

³Animal Production Department, Faculty of Agriculture, Al-Azhar University, Nasr City 11751 Egypt

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Abstract

This study investigated the effect of individual and/ or a mixture multi-species bacteria as a probiotic (*Pediococcus acidilactici* (T1), *Enterococcus faecium* (m74) + *Pediococcus acidilactici* (T2), and *Bacillus licheniformis* + *B. subtilis* (T3)) supplementation at different levels on productive performance and egg quality characteristics in laying Japanese quail. At 42 d, 350 chicks were chosen with nearly similar live body weight and divided into ten groups of 35 chicks/group. Chicks were fed the basal diet as a control group (L₀) or basal diet supplemented with three levels L₁ (1.25), L₂ (1.75), and L₃ (2.25) g/kg diet, respectively. The bacteria-supplemented diets (T1 and T2) had an average feed intake that was the lowest and similar to control group. Better feed conversion was obtained in T1 birds in the first and third month and in the second month in T2. Age at sexual maturity was gradually decreased by dietary supplementation of T3 and L₁. Probiotics increased egg production (EP) and egg mass (EM). EP, egg weight (EW), and EM increased gradually due to T3 and L₂ supplementation. Bacteria-supplemented diets markedly increased absolute albumen and yolk weight as well as absolute and relative eggshell weight. In conclusion, the mixture of *E. faecium* (m74) + *P. acidilactici* and *B. licheniformis* + *B. subtilis* improved feed conversion ratio (FCR), egg production, and egg quality traits of laying Japanese quail.

Keywords: *Bacillus licheniformis*, *Bacillus subtilis*, *Enterococcus faecium* (m74), egg quality, performance, quail

Corresponding author: hamada.okasha@fagr.bu.edu.eg

Introduction

Due to the ban on in-feed antibiotics in the chicken sector, safe replacement-feed components are required. The use of antibiotics has made a substantial contribution to the development of animal husbandry because of the extraordinary improvement in animal production performance and animal health. However, the drawbacks of antibiotic feed additions have gradually been acknowledged, despite the considerable economic gains they provide (Abdel-Moneim *et al.*, 2020). It is vital to create a non-toxic, non-residual antibiotic replacement in feed additives to mitigate the hazards of antibiotic abuse in human health, while also maintaining efficiency in animal production (Yu *et al.*, 2020). In this context, feed additives that stimulate growth are continually being produced, with probiotics being one of the most effective supplements for poultry diets that have the capacity to enhance the immunological response, gastrointestinal health, conformation, and microbiota of poultry (Abd El-Moneim & Sabic 2019; Ebeid *et al.*, 2019; Fathi 2018). Probiotics (Rezaei-pour *et al.*, 2015), prebiotics (Hutsko *et al.*, 2016), antioxidant vitamins (Ghazi *et al.*, 2015), minerals (Oliveira *et al.*, 2014), and herbal essential oils (Peng *et al.*, 2016) have been shown to have therapeutic effects. Probiotics are live organisms or microbial feed supplements aimed at improving intestinal microbial balance (Salehimanesh *et al.*, 2016). Many studies have looked into the use of *Pediococcus acidilactici*, *Enterococcus faecium* (m74), *Bacillus licheniformis*, and *Bacillus subtilis* individually or as a mixture of two types in poultry production.

Utilizing a probiotic blend of types of bacteria may have beneficial, cumulative impacts on the growth, health, and welfare of birds. For example, it can improve poultry performance and growth promoters (Yang *et al.*, 2012; Ribeiro Jr *et al.*, 2014; Fathi 2018); product quality (Lei *et al.*, 2013; Youssef *et al.*, 2013; Zhou *et al.*, 2015; Abou-Kassem *et al.*, 2021); nutrient digestibility (Li *et al.*, 2014; Hossain *et al.*, 2015); intestinal morphology, microbial aspects, and blood metabolites (Hazrati *et al.*, 2020; Nour *et al.*, 2021); and immune function (Liao *et al.*, 2015). Furthermore, they have the potential to benefit the environment or minimize pollution (Zhang & Kim, 2013; 2014). Using a probiotic combination with multiple strains may have a synergistic effect on bird production. Jazi *et al.* (2018) discovered synergistic and complimentary impacts; the gains experienced were probably the result of the many beneficial pathways each supplement possesses. Probiotics have garnered a lot of attention as feed supplements for poultry diets, but little is known about their positive, synergistic, and cumulative effects in the diet of laying Japanese quail, particularly in egg production and incubation attributes. The present work aimed to evaluate the effect of dietary supplementation with different types of bacteria (*P. acidilactici*, *E. faecium* (m74) + *P. acidilactici*, and *B. licheniformis* + *B. subtilis*) on egg production performance, egg quality traits, and chemical composition of eggs in laying Japanese quail.

Materials and Methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Al-Azhar University. A total number of 600, one-day-old Japanese quail were randomly divided into three dietary groups with nearly similar live body weight (7.45 ± 0.0077 g). Chicks were kept under similar, standard hygienic and environmental conditions. Brooding cages with gas heaters were used for brooding chicks. The female to male ratio was 2:1 during the laying period. Chicks were divided into three groups of 180 chicks per group. Birds of the first, second, and third groups were fed a basal diet supplemented with either *P. acidilactici* (T1), *E. faecium* (m74) + *P. acidilactici* (T2), or *B. licheniformis* + *B. subtilis* (T3) as feed additives, respectively. Chicks of each group were subdivided into three subgroups each of 60 chicks receiving 1.25, 1.75, and 2.25 g/ kg of either T1, T2, or T3, respectively. The tenth group (60 chicks) was fed the basal diet only and was considered to be the control group. At 42 d, 350 chicks with nearly similar live body weight were randomly taken and divided into ten groups of 35 chicks/group. Birds were housed in cages constructed from 7-mm square welded wire mesh to provide secure footing, prevent leg injuries, and prevent chicks escaping through side walls.

Feed and clean fresh water were provided at all times and offered *ad libitum*. Birds were fed the basal diet, which was formulated according to the recommendations of the NRC (1994). The basic dietary composition is shown in Table 1.

Age at sexual maturity was determined as age in days when egg production for quail hens of each experimental group reached 10%. Egg production (number and weight) was recorded daily for three months after sexual maturity, and hen-day egg production was calculated. The monthly egg production average for three months after sexual maturity in each experimental group was calculated. Egg mass was calculated as the product of average egg weight and number of eggs laid.

Eggs were broken and yolk was separated from the albumen. The percentage of yolk, albumen, and eggshell (with its membranes) was expressed relative to EW. Eggshell thickness was measured from the sharp, blunt, and equatorial parts without a membrane with a 0.01-mm sensitive, electronic, digital micrometre. Eggshell percentage (ESP) was calculated using the equation of Aygun & Yetisir (2010):

$$\text{Eggshell percentage (\%)} = (\text{ESW (g)} / \text{EW (g)}) \times 100 \quad (1)$$

Only sound, clean eggs from each treatment were stored till incubation at 12.5 °C and 75–80% relative humidity for one week then incubated. Eggs were sprayed with Virkon disinfectant (15 g powder/litre of water) before being placed into the incubator. Eggs were allowed to warm overnight to room temperature prior to setting in order to reduce sweating of eggs. Incubation of eggs was carried out in a special automatic incubator (quail-egg-type incubator).

Table 1 Composition and calculated analysis of the basal quail layer diet

Ingredients (% as fed)	
Yellow corn	56
Soybean meal	29
Corn gluten	3.51
Vegetable oil	3.24
Calcium carbonate	5.3
Dicalcium phosphate	1.6
Sodium chloride	0.27
Sodium bicarbonate	0.11
DL-Methionine	0.15
Methionine*	0.10
L-Lysine	0.09
L-Threonine	0.08
Vitamin premix ^a	0.20
Mineral premix ^b	0.20
Washed sand	0.15
Total	100
<i>Calculated nutrients and energy</i>	
AME (kcal/kg)	2950
Crude protein (%)	20
L-Lysine (%)	10.3
DL-Methionine (%)	0.48
TSSA (%)	0.18
Calcium (%)	2.48
Non-phytate P (%)	0.45
Total P (%)	0.64
<i>Analysed values</i>	
AME, (kcal/kg)	2946
Crude protein (%)	19.94

^aVitamin mixture provided per kg of diet: 11000 IU of vitamin A, 1800 IU of vitamin D3, 11 mg of vitamin E, 2mg of vitamin K3, 4mg of vitamin B1, 5.7 mg of vitamin B2, 2mg of vitamin B6, 0.5 mg of folic acid, 2500 mg of choline chloride, 0.125 mg of antioxidants, 0.03 mg of biotin, and 0.024 mg of vitamin B12.

^bMineral mixture provided per kg of diet: 500 mg of FeSO₄, 65 mg of CuSO₄, 100 mg of MnSO₄, 0.5 mg of iodine, and 0.22 g of selenium.

CaCO₃: Calcium carbonate; AME: apparent metabolizable energy; TSSA: total sulphur amino acids

*quail diet as recommended by NRC (1994), which was used from sexual maturity to the end of the experimental period

The temperature and relative humidity in the incubator were maintained at 37.5–37.7 °C and 60%, respectively, for the first 15 d. The eggs were automatically turned through 45° every hour. On day 16, the eggs were transferred to the hatchery trays, at temperature and relative humidity averages of 37.0 ± 0.5 °C and 75%, respectively (Petek *et al.*, 2005). Hatching usually started on day 16 and was completed by the end of day 18. The quail chicks were removed and counted. The number of unhatched eggs was recorded for estimating the hatchability percentage. Hatchability percentage was calculated as the number of hatched chicks produced from fertile eggs.

Because the quail eggshell is coloured, tinted, and blotched, an accurate assessment of the development stage of the embryo by candling was not possible. Instead of candling, the unhatched eggs were broken for examination on day 18 and the number of eggs containing dead embryos and those free of embryos were counted and the percentage fertility was determined according to the method of Ozcelik *et al.* (2006). Fertility (%) was calculated as the number of hatched chicks plus number of eggs containing embryos from total incubated eggs set. Embryonic mortality was estimated for three intervals in the incubation period: from 1–6 d, from 7–14 d, and 15–17 d to determine the early, mid, and late embryonic mortality. This is known to depend on the cessation of embryonic development due to various factors that manifest in these periods.

The quail eggs were collected several times daily and stored at 15 °C for a period of not more than one week. Five eggs were taken from each experimental subgroup (total 50 eggs) to determine the following parameters: moisture content of egg yolks was determined using the AOAC 920.116 analytical method (AOAC, 2000). The moisture content of the samples was evaluated by drying them in a hot-air oven at 100 ± 5 °C until they reached a consistent weight. Fat content was determined using

Soxhlet's method (AOAC, 2000), and egg protein content was estimated by applying Kjeldahl's method (William, 2000). Egg ash content was determined by using five grams of sample placed in a dry, clean, weighed crucible. The sample and crucible were placed in muffle furnace at 550–600 °C for 6–8 h with a gradual increase in temperature, then cooled in the desiccator and weighed. Ash content was calculated according to the following equation:

$$\text{Egg ash weight percentage} = \frac{\text{Weight remaining (g)}}{\text{Weight of sample taken (g)}} \times 100 \quad (2)$$

Egg pH value was determined according to the method of Pearson *et al.* (1976). The pH value was determined using an electrical pH meter.

Analysis of variance was carried out using the SAS procedure guide (SAS, 2004) for the traits of age of sexual maturity, feed consumption, feed conversion, fertility, hatchability, and chemical analysis of quail eggs according to the following linear model:

$$X_{ijk} = \mu + T_i + L_j + TL_{ij} + e_{ijk} \quad (3)$$

where: X_{ijk} = the k^{th} observation; μ = overall mean; T_i = effect of the i^{th} treatment; L_j = effect of the j^{th} level applied; TL_{ij} = the interaction between the i^{th} treatment and j^{th} level applied; and e_{ijk} = the experimental error.

Responses in egg production, egg weight, egg mass, and egg quality were determined according to the following linear model:

$$X_{ijkl} = \mu + T_i + L_j + P_k + TL_{ij} + TP_{ik} + LP_{jk} + TPL_{ijk} + e_{ijkl} \quad (4)$$

where: TP_{ik} = the interaction between the i^{th} treatment and k^{th} period; LP_{jk} = the interaction between j^{th} level applied and k^{th} period; TPL_{ijk} = the interaction between i^{th} treatment and k^{th} level applied as well as k^{th} period; and e_{ijkl} = the experimental error.

Results and Discussion

Average amount of feed consumed in gram per bird per day was estimated for three months (Table 2). Analysis of variance indicated an effect of treatments and levels of added probiotics, as well as an interaction between the kind and level of probiotic on the average feed consumption in all periods ($P < 0.001$). The effect of the probiotic supplementation was statistically significant on feed conversion during the first month ($P < 0.01$) and second month ($P < 0.05$). The best feed conversion averages during the first month were obtained in laying pullets fed a mixture of *Enterococcus* and *Pediococcus*. Average feed conversion was affected by the level of probiotic supplemented ($P < 0.001$). The average feed conversion generally improved as the level of probiotic supplementation increased but without any characteristic trend. Supplementation with 1.75 and 2.25 kg probiotic per kg diet produced the best feed conversion during the 2nd and 3rd months, respectively. An interaction effect was found during the first month ($P < 0.05$) and during the second and third months ($P < 0.001$).

These results are scientifically feasible because the feed requirement and demand are known to increase with age to fulfil the increase in all biological reactions within the bird. The results concur with Abou-Kassem *et al.* (2021), who found marked increases in daily FI in the grower phases (1–21 d and 22–42 d) because of probiotic treatment. Moreover, Manafi *et al.* (2018) found substantial changes in overall FI. Variation in average feed consumption due to treatments applied and the time of estimation may be attributed to variation in the metabolic pathways within various experimental groups. Nutritive requirements differ according to the mode and rate of metabolism. Generally, applying probiotics at a level not exceeding 1.75 g/kg resulted in increasing feed consumption, compared to other two levels applied (Table 2). Changes occurred in average feed conversion according to bird's age and/or dietary additives applied and may be attributed to the state of metabolic activity in general and the changes in the balance that may occur between anabolic and catabolic processes. Premavalli *et al.* (2018) clarified that the improvement in FCR of quails fed diets supplemented with multispecies probiotics could be attributed to the probiotic's overall effects, which include changes in bacterial metabolism in the intestine, the maintenance of a favourable microbial population, and good feed digestion and absorption.

Data of age at sexual maturity (ASM) for birds in the experimental groups are listed in Table 2. It was found that ASM was affected by the kind of probiotic added to the diet as well as the level of supplementation ($P < 0.05$). Supplementing birds with probiotic preparations decreased ASM compared to birds fed the basal diet only (control group) and this decreased according to the kind of probiotic applied. Average ASM decreased with the 1.25, 1.75, and 2.25 g/kg treatments for birds fed the basal diet supplemented with T1, T2 and T3, respectively, when compared with control group.

Egg production (EP), egg weight (EW), egg mass (EM), and ANOVA results are detailed in Table 3. Treatments affected average egg production and egg mass ($P < 0.001$). Supplement level influenced average egg weight and egg mass ($P < 0.001$). Feeding 1.75 g probiotic/kg diet produced the highest average egg weight and egg mass; 2.25 g probiotic/kg diet produced the highest average egg production. Experimental period influenced the average egg weight, egg production, and egg mass ($P < 0.001$). Egg production and egg mass increased in older birds, with a maximum in the 2nd month of production, which decreased towards the end of the experiment. However, egg weight increased with age to reach a maximum at the end of the experiment (Table 3). All interaction effects on average egg production and egg mass were found to be significant ($P < 0.001$).

Table 2 Least square mean and standard error (LSM \pm S.E.) for feed intake, feed conversion ratio, and age at sexual maturity of quail fed individual and/or a mixture of multi-species probiotics at different concentrations

Variables and interaction	Average feed consumption (g/bird/day)			Average feed consumption (g feed /g egg)			Age at sexual maturity (day)	
	1 st month	2 nd month	3 rd month	1 st month	2 nd month	3 rd month		
Treatment (T)								
	T1	26.66 ^c	33.80 ^b	36.27 ^b	4.07 ^b	3.17 ^a	3.36	52.75 ^{ab}
	T2	28.44 ^b	31.89 ^c	35.15 ^c	5.13 ^a	3.04 ^{ab}	3.38	53.25 ^a
	T3	29.66 ^a	35.47 ^a	38.52 ^a	4.60 ^{ab}	2.88 ^b	3.50	51.75 ^b
SEM		± 0.32	± 0.47	± 0.31	± 0.19	± 0.08	± 0.07	± 0.408
level (L)								
	L = 0 control	24.45 ^c	33.92	34.97 ^c	4.27 ^{bc}	3.34 ^a	3.48 ^b	54.00 ^a
	L1 = 1.25 g/kg	25.16 ^c	33.81	36.18 ^b	4.03 ^c	3.12 ^a	3.27 ^{bc}	51.67 ^b
	L2 = 1.75 g/kg	32.65 ^a	34.42	39.19 ^a	4.80 ^{ab}	2.50 ^a	3.76 ^a	52.33 ^b
	L3 = 2.25 g/kg	30.76 ^b	32.72	36.24 ^b	5.30 ^a	3.16 ^b	3.14 ^c	52.33 ^b
SEM		± 0.36	± 0.54	± 0.36	± 0.22	± 0.09	± 0.08	± 0.47
Interaction (T x L)								
	T1x L0	23.89 ^f	34.21 ^c	34.64 ^{ef}	4.13 ^{cd}	3.24 ^{ab}	3.31 ^{cd}	54.00 ^{ab}
	T1x L1	26.01 ^{de}	34.39 ^{bc}	38.12 ^{bc}	3.66 ^d	3.12 ^{ab}	3.43 ^{bcd}	50.00 ^d
	T1x L2	27.54 ^{cd}	32.91 ^c	35.78 ^{de}	3.46 ^d	3.09 ^{ab}	3.15 ^{de}	54.00 ^{ab}
	T1x L3	29.20 ^c	33.68 ^c	36.52 ^{cde}	5.02 ^{bc}	3.22 ^{ab}	3.54 ^{bcd}	53.00 ^{abc}
	T2x L0	24.28 ^{ef}	32.56 ^c	34.54 ^{ef}	4.26 ^{cd}	3.39 ^a	3.80 ^b	54.00 ^{ab}
	T2x L1	24.09 ^{ef}	34.93 ^{abc}	35.05 ^{ef}	4.05 ^{cd}	3.48 ^a	3.18 ^{de}	55.00 ^a
	T2x L2	36.21 ^a	32.91 ^c	37.66 ^{bcd}	5.94 ^{ab}	2.84 ^{bc}	3.73 ^{bc}	51.00 ^{cd}
	T2x L3	29.18 ^c	27.15 ^d	33.34 ^f	6.26 ^a	2.46 ^c	2.79 ^e	53.00 ^{abc}
	T3x L0	25.19 ^{ef}	34.99 ^{abc}	35.73 ^{de}	4.41 ^{cd}	2.85 ^{bc}	3.33 ^{cd}	54.00 ^{ab}
	T3x L1	25.38 ^{ef}	32.12 ^c	35.36 ^e	4.38 ^{cd}	3.42 ^a	3.21 ^{de}	50.00 ^d
	T3x L2	34.19 ^b	37.44 ^a	44.13 ^a	4.99 ^{bc}	3.43 ^a	4.39 ^a	52.00 ^{cd}
	T3x L3	33.89 ^b	37.33 ^{ab}	38.87 ^b	4.62 ^{cd}	1.82 ^d	3.09 ^{de}	51.00 ^{cd}
SEM		± 0.63	± 0.94	± 0.63	± 0.38	± 0.16	± 0.14	± 0.81
	T	22.97 ^{***}	14.67 ^{***}	30.26 ^{***}	7.85 ^{**}	3.27 [*]	1.26	3.50 [*]
	L	124.96 ^{***}	1.75	24.75 ^{***}	6.77 ^{***}	15.74 ^{***}	10.93 ^{***}	4.46 [*]
F-value	TxL	17.00 ^{***}	9.25 ^{***}	15.69 ^{***}	3.09 [*]	8.04 ^{***}	9.96 ^{***}	4.83 ^{**}
	MSE	1.19	2.63	1.17	0.43	0.077	0.06	2.00

Means with the same letter in each column are not significantly different

T1 = *Pediococcus acidilactici*, T2 = *Enterococcus faecium* + *P. acidilactici*, T3 = *Bacillus licheniformis* + *B. subtilis*, * = significant at $P < 0.05$, ** = significant at $P < 0.01$, *** = significant at $P < 0.001$

Decreasing age at sexual maturity (ASM) may be attributed to the enhancement of growth rate and, concomitantly, the reproductive system, caused probiotic supplementation at an early age (a period of higher growth rate). Increasing levels of probiotics slightly increased ASM. As the level increased to 1.75 g/kg, average ASM increased from 51.67 d to 52.33 d. However average ASM was constant up to 2.25 g/kg of probiotics. Probiotics should therefore be applied at no more than 1.75 g/kg. This concurs with Ayasan *et al.* (2006), who found that ASM increased in birds fed supplementary probiotics (Protexin) at 0.5 and 1 g/kg diet, producing an ASM of 51–53 d and 51–57 d, respectively,

compared to the control (47–57 d). Various external and internal variables influence early sexual maturity. Lukanov & Pavlova (2020) confirmed that domestic Japanese quail reached sexual maturity at 4–5 w of age, and females begin laying eggs regularly at ~6 w of age when kept in ideal conditions and given a proper lighting schedule. The results of the current study may support the concept that ASM is a trait of high heritability and trying to improve it by improving environmental conditions (including nutrition) has limited success (El-Deen *et al.*, 2008; Kaye *et al.*, 2016).

The interaction between kind and level of probiotics was of statistically significant ($P < 0.01$). This indicates the importance of level for the kinds of probiotics applied. Probiotic application efficiency may be influenced by parameters such as microbial species composition (single or multi-strain), liveability, supplementary administration dose, application method and frequency, and food composition (Mikulski *et al.*, 2012). Zamanizadeh *et al.* (2021) reported variation in egg production of Japanese quail due to dietary supplementation of two probiotics, *Aspergillus oryzae* and *Saccharomyces cerevisiae* ($P < 0.001$). The highest egg productions were recorded in birds receiving *S. cerevisiae* at 200 mg/kg diet and/or a mixture of two probiotics at 100 and 200 mg/kg diet. Zamanizadeh *et al.* (2021) suggests that the improvement in the metabolic processes of nutrient absorption and utilization may be linked to increased egg production and egg weight as a result of the probiotics. Probiotics have been shown to change gut flora by lowering pH, boosting intestinal enzyme activity, and increasing food digestibility (Yang *et al.*, 2005). Shell formation and deposition are controlled hormonally and depend on the functional efficiency of the biological action of thyroid hormones and parathyroid hormones (calcitonin and parathormone), as well as the activity of the kidney in the conversion of vitamin D3 to its metabolic form (1,25(OH)2-D3). All of these regulators depend on genetic capacity rather than the nutritional regime (Molino *et al.*, 2015).

Table 3 Least square mean and standard error (LSM \pm S.E.) for egg production, egg weight, and egg mass of quail fed individual and/or a mixture of multi-species probiotics at different concentrations

Variables and interactions		Egg production (%/bird/day)	Egg weight (g)	Egg mass (g)/bird/day
Treatment (T)	T1	70.81 \pm 1.01 ^b	12.80 \pm 0.07	11.09 \pm 0.30 ^a
	T2	69.04 \pm 1.04 ^b	12.72 \pm 0.07	8.970 \pm 0.31 ^b
	T3	74.56 \pm 1.04 ^a	12.81 \pm 0.07	10.31 \pm 0.31 ^a
Level (L)	L0 = Control	70.16 \pm 1.20	12.45 \pm 0.08 ^c	8.92 \pm 0.36 ^c
	L1 = 1.25 g/kg	72.13 \pm 1.20	12.58 \pm 0.08 ^c	9.23 \pm 0.35 ^c
	L2 = 1.75 g/kg	71.30 \pm 1.20	13.18 \pm 0.08 ^b	11.77 \pm 0.36 ^a
Period (P)	L3 = 2.25 g/kg	72.28 \pm 1.20	13.10 \pm 0.08 ^a	10.56 \pm 0.36 ^b
	At first month	52.55 \pm 1.03 ^c	11.57 \pm 0.07 ^c	7.98 \pm 0.31 ^b
	At second month	82.71 \pm 1.03 ^a	13.19 \pm 0.07 ^b	11.54 \pm 0.31 ^a
<i>F</i> -value	At third month	79.15 \pm 1.03 ^b	13.58 \pm 0.07 ^a	10.86 \pm 0.31 ^a
	T	7.73 ^{***}	0.62	12.11 ^{***}
	L	0.66	18.76 ^{***}	13.49 ^{***}
	P	255.62 ^{***}	264.30 ^{***}	37.72 ^{***}
	P*T	0.51	2.01	17.28 ^{***}
	P*L	3.17 ^{***}	2.96 ^{**}	16.73 ^{***}
	T*L	3.37 ^{***}	4.61 ^{***}	18.28 ^{***}
MSE	337.19	0.16	30.11	

Means with the same letter in each column are not significantly different

T1 = *Pediococcus acidilactici*, T2 = *Enterococcus faecium* + *P. acidilactici*, T3 = *Bacillus licheniformis* + *B. subtilis*, * = significant at $P < 0.05$, ** = significant at $P < 0.01$, *** = significant at $P < 0.001$

The effect of treatments on average egg mass can therefore be attributed to their effect on egg number rather than on average egg weight. The highest average egg production and egg mass were found in groups of birds receiving T1 and T3, rather than T2. Absolute and proportional weights of egg components and shell thickness (EST) were estimated for three months after ASM to determine the effect of treatments applied and/or level of probiotics supplemented in laying quail (Table 4). T3 increased the absolute and relative weights of albumen and EST when compared to the other treatments. The concentration of probiotics added to the diet affected the absolute and relative weights of the eggshell and absolute albumen and yolk ($P < 0.001$). Feeding quail a diet supplemented with 1.75 g probiotic/kg ration had the highest average absolute weights of albumen and yolk and EST. In addition, variations were found in all estimated parameters due to bird age.

Table 4 Least square mean and standard error (LSM \pm S.E.) for absolute and relative weights of egg components and eggshell thickness (EST) in quail fed individual and/or a mixture of multi-species probiotics at different concentrations

Variables and interaction		Egg weight		Albumen		Yolk		Shell		EST (mm)
		(g)	(g)	(%)	(g)	(%)	(g)	(%)		
Treatment (T)	T1	12.22	7.02	57.42	4.16	34.01	1.05	8.56	23.33 ^{ab}	
	T2	12.11	6.93	57.24	4.13	34.07	1.05	8.68	23.62 ^b	
	T3	12.24	7.06	57.64	4.12	33.70	1.06	8.66	23.97 ^a	
SEM		± 0.07	± 0.04	± 0.2	± 0.04	± 0.2	± 0.01	± 0.04	± 0.27	
Level (L)	Control	11.87 ^c	6.81 ^b	57.41 ^a	3.98 ^b	33.50 ^b	1.08 ^a	9.08 ^a	23.77 ^{ab}	
	L1 = 1.25 g/kg	12.01 ^c	6.84 ^b	56.94 ^b	4.15 ^a	34.52 ^a	1.02 ^b	8.54 ^b	23.53 ^{ab}	
	L2 = 1.75 g/kg	12.53 ^a	7.23 ^a	57.70 ^a	4.22 ^a	33.70 ^b	1.08 ^a	8.59 ^b	24.25 ^a	
	L3 = 2.25g/kg	12.34 ^b	7.12 ^a	57.69 ^a	4.20 ^a	33.99 ^{ab}	1.03 ^b	8.32 ^c	23.00 ^b	
SEM		± 0.08	± 0.04	± 0.22	± 0.04	± 0.23	± 0.01	± 0.04	± 0.31	
Period (P)	First month	11.01 ^c	6.30 ^c	57.24 ^b	3.75 ^c	34.00 ^b	0.96 ^b	8.76 ^b	26.10 ^a	
	Second month	12.52 ^b	7.22 ^b	57.66 ^a	4.20 ^b	33.45 ^b	1.10 ^a	8.80 ^a	22.90 ^b	
	Third month	13.03 ^a	7.48 ^a	57.42 ^a	4.46 ^a	34.24 ^a	1.09 ^a	8.33 ^c	21.90 ^c	
SEM		± 0.069	± 0.04	± 0.2	± 0.04	± 0.2	± 0.2	± 0.04	± 0.27	
F- value	T	1.21	3.76 [*]	1.13	0.23	1.02	0.95	2.66	1.35	
	L	14.37 ^{***}	28.35 ^{***}	2.67 [*]	76.37 ^{***}	3.67 ^{**}	15.23 ^{***}	52.50 ^{***}	2.74 [*]	
	P	263.09 ^{***}	341.48 ^{***}	1.23	4.98 ^{**}	3.25 [*]	132.50 ^{***}	45.49 ^{***}	64.31 ^{***}	
	P*T	0.65	0.45	1.12	0.76	0.83	1.04	1.24	0.32	
	P*L	1.53	4.24 ^{***}	3.06 ^{**}	0.87	2.04	4.11 ^{***}	6.10 ^{***}	0.97	
	T*L	3.48	5.10 ^{***}	1.18	1.69	1.02	1.61	0.71	0.36	
	P*T*L	1.40 ^{**}	3.02 ^{***}	1.14	0.53	1.06	1.48	0.87	0.64	
	MSE	0.17	0.04	1.28	0.61	1.43	0.002	0.05	2.66	

Means with the same letter in each column are not significantly different

T1 = *Pedococcus acidilactici*, T2 = *Enterococcus faecium* + *P. acidilactici*, T3 = *Bacillus licheniformis* + *B. subtilis*, * = significant at $P < 0.05$, ** = significant at $P < 0.01$, *** = significant at $P < 0.001$

All averages increased up to the second month of the egg production period, with different magnitudes of increase, then tended to remain constant up to the third month (Figure 1). Different results were obtained in EST, which decreased over time, being higher until the second month. The findings concur with those of Zamanizadeh *et al.* (2021), who reported no changes between probiotic treatments in albumen %, all yolk parameters (yolk weight, yolk %, and yolk colour), or shell weight ($P > 0.05$). Decreased EST with age may be due to the increase in egg weight with an increase in surface area of the egg.

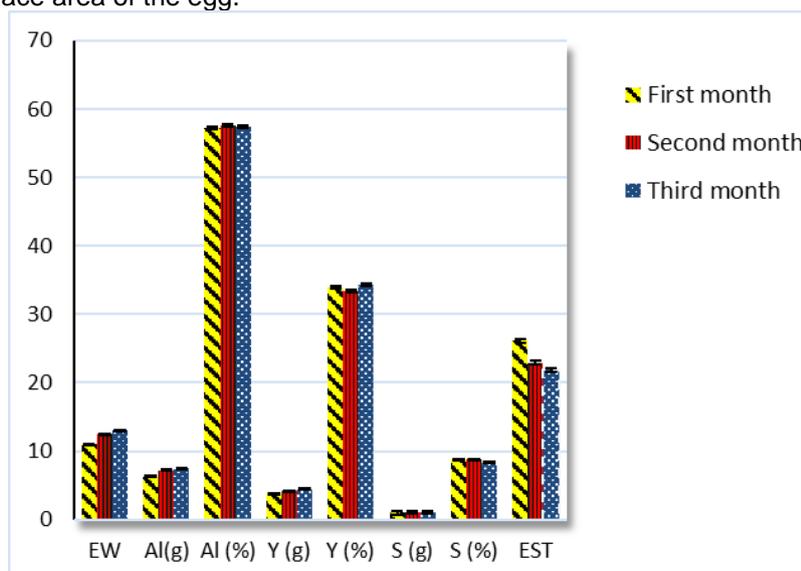


Figure 1. Absolute and relative weights and shell thickness in quail eggs in the first, second, and third months of egg production
EW, egg weight; Al, albumen; Y, yolk, S, shell; EST, eggshell thickness

Mikulski *et al.* (2020) noticed that in comparison to birds without probiotic supplementation, Hy-Line Brown hens fed probiotic-supplemented diets had thicker eggshells ($P < 0.002$) and heavier eggshells ($P < 0.008$). Without affecting yolk%, the increases in eggshell thickness and weight were accompanied by a drop in albumen percentage ($P = 0.043$).

Eggs laid by birds fed the basal diet alone or with probiotics at different levels were incubated when egg production reached more than 50% to elucidate the effect of probiotic treatments on hatching parameters (fertility-Fr, hatchability-H, and embryonic mortality; Table 5). There was no effect of the kind of probiotic or the level applied on either Fr (%), H (%) or embryonic mortality (early, mid, and late) during incubation. Regardless of the probiotic applied, feeding quail 1.75 and 1.25 g probiotic/kg diet produced higher fertility and hatchability. Feeding quail 1.25 g probiotic/kg ration produced the lowest mid- and late-embryonic mortality (Table 5). Dietary probiotics had no effect on hatchability, supporting the contention that nutritional factors have no effect on fertility, hatchability, or embryonic development. Many studies have found that the fertility and hatchability of viable Japanese quail eggs were 48–94% and 40.00–70.34%, respectively, from 8–52 w of age (Farooq *et al.*, 2001; Khurshid *et al.*, 2004; El-Hindawy *et al.*, 2021). The results concur with Güçlü (2011), who reported that probiotics and prebiotics raised the percentage of fertilized eggs and hatchability, but not statistically. Hajiaghapour & Rezaei-pour (2018) found that the dietary treatments including probiotics had no significant effect on hatchability percentage.

Table 5 Least square means and standard error (LSM \pm S.E.) for fertility (%) and hatchability (%) of quail fed individual and/or a mixture of multi-species probiotics at different concentrations

Variables and interaction		Fertility,%	Hatchability,%	Embryonic mortality rate%		
				Early %	Mid %	Late %
Treatment (T)	T1	88.64 \pm 2.50	83.29 \pm 1.50	4.97 \pm 0.84	2.12 \pm 0.60	7.76 \pm 0.96
	T2	86.50 \pm 2.50	82.93 \pm 1.50	5.53 \pm 0.89	3.36 \pm 0.60	8.20 \pm 1.02
	T3	87.75 \pm 2.50	83.61 \pm 1.50	4.63 \pm 0.82	2.88 \pm 0.60	8.93 \pm 0.93
Level (L)	L0 = Control	85.33 \pm 2.84	83.20 \pm 1.68	3.35 \pm 1.01	4.34 \pm 0.70	7.99 \pm 1.15
	L1 = 1.25 g/ kg	86.96 \pm 2.84	85.71 \pm 1.68	6.12 \pm 0.97	1.88 \pm 0.67	6.79 \pm 1.11
	L2 = 1.75 g/ kg	89.56 \pm 2.84	80.66 \pm 1.68	6.04 \pm 0.97	2.50 \pm 0.67	9.07 \pm 1.11
	L3 = 2.25g/ kg	88.67 \pm 2.84	83.54 \pm 1.68	4.66 \pm 0.97	2.41 \pm 0.67	9.29 \pm 1.11
	T1 \times L0	85.33 \pm 4.91	83.20 \pm 2.91	3.32 \pm 1.69	4.21 \pm 1.17 ^a	7.61 \pm 1.92
	T1 \times L1	88.56 \pm 4.91	86.73 \pm 2.91	4.91 \pm 1.69	0.80 \pm 1.17 ^b	6.28 \pm 1.92
	T1 \times L2	90.00 \pm 4.91	79.88 \pm 2.91	6.86 \pm 1.69	1.63 \pm 1.17 ^{ab}	7.15 \pm 1.92
Interaction (T \times L)	T1 \times L3	90.67 \pm 4.91	83.35 \pm 2.91	4.8 \pm 1.69	1.85 \pm 1.17 ^{ab}	9.99 \pm 1.92
	T2 \times L0	85.33 \pm 4.91	83.20 \pm 2.91	3.10 \pm 2.06	5.00 \pm 1.43	8.00 \pm 2.35
	T2 \times L1	87.33 \pm 4.91	85.05 \pm 2.91	6.84 \pm 1.69	2.60 \pm 1.17	6.86 \pm 1.92
	T2 \times L2	88.67 \pm 4.91	78.82 \pm 2.91	6.00 \pm 1.69	3.07 \pm 1.17	11.69 \pm 1.92
	T2 \times L3	84.67 \pm 4.91	84.66 \pm 2.91	6.17 \pm 1.69	2.76 \pm 1.17	6.13 \pm 1.92
	T3 \times L0	85.33 \pm 4.91	83.20 \pm 2.91	3.65 \pm 1.46	3.82 \pm 1.01	8.37 \pm 1.66
	T3 \times L1	85.00 \pm 4.91	85.35 \pm 2.91	6.61 \pm 1.69	2.25 \pm 1.17	7.24 \pm 1.92
	T3 \times L2	90.00 \pm 4.91	83.28 \pm 2.91	5.25 \pm 1.69	2.83 \pm 1.17	8.38 \pm 1.92
	T3 \times L3	90.67 \pm 4.91	82.61 \pm 2.91	3.01 \pm 1.69	2.61 \pm 1.17	11.74 \pm 1.92
	T	0.19	0.05	0.28	1.09	0.40
	L	0.44	1.52	1.73	2.40	1.07
F- value	T \times L	0.16	0.27	0.41	0.15	1.15
	MSE	72.38	25.41	8.53	4.07	11.07

Means with the same letter in each column are not significantly different

T1 = *Pediococcus acidilactici*, T2 = *Enterococcus faecium* + *P. acidilactici*, T3 = *Bacillus licheniformis* + *B. subtilis*, * = significant at $P < 0.05$, ** = significant at $P < 0.01$, *** = significant at $P < 0.001$

In contrast, El-Hindawy *et al.* (2021) observed that fertility and hatchability percentages (Fr% and H%) increased with increasing probiotics administration ($P < 0.01$ and $P < 0.05$, respectively). Mojgani *et al.* (2020) found that quail administered probiotics (108 CFU/ml *B. megaterium*) had an improvement in H% and a 10% reduction in embryonic mortality compared to the control ($P < 0.05$).

Moisture, ash, protein, and fat percentage, as well as pH value in egg contents were affected by adding probiotics to layer diets at different concentrations ($P < 0.001$). A higher moisture percentage was found in eggs laid by birds on T1, compared to the control. Eggs laid by birds on T3 had the highest average protein percentage and lower average fat percentage. However, birds that received T2 or T3 laid eggs with a similar ash% (2.06 and 2.08%, respectively), which was higher than the control and T1 (1.58%). No variation was found in average pH value due to treatments applied and ranged from 7.33

in the control to 7.50 on T2. The content of the probiotics didn't affect the protein, fat, and ash percentages, but moisture percentage ($P < 0.05$) and pH ($P < 0.001$) were different.

Moisture percentage ranged from 73.38% in eggs laid by birds treated with 1.75 g probiotic/kg and 73.87% in eggs laid by birds fed 1.25 g/kg. The pH was 7.50 and 7.54 in birds fed diets supplemented with 1.25 g/kg diet and 1.75 g/kg, respectively (Table 6). The chemical composition of quail eggs is comparable to that of chicken eggs, with ~88% water, 10% protein, and 1% ash (Tolik *et al.*, 2014). Genchev (2012) reported that quail eggs have a greater crude protein content (14.1–14.6%) in the albumen and a lower ash content (0.76–0.78%) in the albumen and yolk (1.1–1.3 percent) than was reported by Dudusola (2010). Sinanoglou *et al.* (2011) found that quail egg yolks had a lower fat and higher ash content (27.45% and 2.63%, respectively) than chicken egg yolks. However, results of hatchability must be re-evaluated in terms of the variability in chemical parameters of incubated eggs if increasing hatchability is being targeted.

Table 6 Least squares mean and standard error (LSM \pm S.E.) for the chemical analysis of eggs from quail fed individual and/or a mixture of multi-species probiotics at different concentrations

Variables and interaction		Chemical analysis of quail eggs				
		Moisture %	Protein %	Fat %	Ash %	pH
Treatment (T)	T1	74.24 \pm 0.10 ^a	11.40 \pm 0.10 ^a	9.70 \pm 0.07 ^a	1.58 \pm 0.05 ^b	7.46 \pm 0.02
	T2	73.59 \pm 0.10 ^b	12.80 \pm 0.10 ^b	9.20 \pm 0.07 ^b	2.06 \pm 0.05 ^a	7.50 \pm 0.02
	T3	73.06 \pm 0.10 ^c	13.10 \pm 0.10 ^a	9.00 \pm 0.07 ^b	2.08 \pm 0.05 ^a	7.46 \pm 0.02
	L0 = Control	73.65 \pm 0.10 ^{ab}	12.40 \pm 0.12	9.25 \pm 0.08	1.85 \pm 0.06	7.33 \pm 0.02 ^b
Level (L)	L1 = 1.25 g/kg	73.87 \pm 0.10 ^a	12.30 \pm 0.12	9.27 \pm 0.08	1.88 \pm 0.06	7.50 \pm 0.02 ^a
	L2 = 1.75 g/kg	73.38 \pm 0.10 ^b	12.30 \pm 0.12	9.30 \pm 0.08	1.90 \pm 0.06	7.54 \pm 0.02 ^a
	L3 = 2.25 g/kg	73.62 \pm 0.10 ^{ab}	12.60 \pm 0.12	9.22 \pm 0.08	1.98 \pm 0.06	7.52 \pm 0.02 ^a
	T1 \times L0	73.65 \pm 0.19 ^{cd}	12.40 \pm 0.21 ^{de}	9.25 \pm 0.13 ^c	1.85 \pm 0.10 ^{cd}	7.33 \pm 0.04 ^e
Interaction (T \times L)	T1 \times L1	74.85 \pm 0.19 ^a	10.70 \pm 0.21 ^g	10.00 \pm 0.13 ^a	1.25 \pm 0.10 ^e	7.45 \pm 0.04 ^{cd}
	T1 \times L2	74.05 \pm 0.19 ^{bc}	11.00 \pm 0.21 ^{fg}	9.85 \pm 0.13 ^{ab}	1.45 \pm 0.10 ^e	7.51 \pm 0.04 ^{bc}
	T1 \times L3	74.40 \pm 0.19 ^{ab}	11.50 \pm 0.21 ^f	9.50 \pm 0.13 ^{bc}	1.75 \pm 0.10 ^d	7.55 \pm 0.04 ^{abc}
	T2 \times L0	73.65 \pm 0.19 ^{cd}	12.40 \pm 0.21 ^{de}	9.25 \pm 0.13 ^{cd}	1.85 \pm 0.10 ^{cd}	7.33 \pm 0.04 ^e
	T2 \times L1	74.00 \pm 0.19 ^{bc}	12.15 \pm 0.21 ^e	9.25 \pm 0.13 ^{cd}	2.0 \pm 0.10 ^{bcd}	7.47 \pm 0.04 ^{bcd}
	T2 \times L2	73.55 \pm 0.19 ^{cd}	12.85 \pm 0.21 ^{cd}	9.1 \pm 0.13 ^{cd}	2.15 \pm 0.10 ^{abc}	7.56 \pm 0.04 ^{abc}
	T2 \times L3	73.15 \pm 0.19 ^{de}	13.65 \pm 0.21 ^{ab}	9.0 \pm 0.13 ^d	2.25 \pm 0.10 ^{ab}	7.65 \pm 0.04 ^a
	T3 \times L0	73.65 \pm 0.19 ^{cd}	12.40 \pm 0.21 ^{de}	9.25 \pm 0.13 ^{cd}	1.85 \pm 0.10 ^{cd}	7.33 \pm 0.04 ^e
	T3 \times L1	72.75 \pm 0.19 ^{ef}	13.95 \pm 0.21 ^a	8.55 \pm 0.13 ^e	2.40 \pm 0.10 ^a	7.59 \pm 0.04 ^{ab}
	T3 \times L2	72.55 \pm 0.19 ^f	13.15 \pm 0.21 ^{bc}	9.05 \pm 0.13 ^d	2.10 \pm 0.10 ^{abc}	7.55 \pm 0.04 ^{abc}
	T3 \times L3	73.30 \pm 0.19 ^{de}	12.70 \pm 0.21 ^{cde}	9.15 \pm 0.13 ^{cd}	1.95 \pm 0.10 ^{bcd}	7.38 \pm 0.04 ^{de}
	T	36.89 ^{***}	72.06 ^{***}	26.06 ^{***}	33.21 ^{***}	1.80
	L	3.13 [*]	1.60	00.41	00.99	21.70 ^{***}
F-value	T \times L	6.91 ^{***}	15.92 ^{***}	6.17 ^{***}	7.88 ^{***}	5.60 ^{***}
	MSE	0.113	0.13	0.05	0.03	0.004

Means with the same letter in each column are not significantly different

T1 = *Pediococcus acidilactici*, T2 = *Enterococcus faecium* + *P. acidilactici*, T3 = *Bacillus licheniformis* + *B. subtilis*, * = significant at $P < 0.05$, ** = significant at $P < 0.01$, *** = significant at $P < 0.001$

Conclusion

It is recommended that adding *Pediococcus acidilactici* bacteria as a probiotic (T1) to layer quail diets at 1.25 mg/kg will improve productive performance and egg quality characteristics.

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Faculty of Agriculture, Benha University, and Al-Azhar University, Egypt.

Author's contributions

OHM, WAM, and KME collected data. OHM, GME, and WAM conducted the statistical analysis. OHM, GME, KME, and WAM collaborated in interpreting the results, wrote the initial draft of this manuscript, and finalized the manuscript. OHM and GME developed the original hypothesis and designed the experiment. The authors have read and approved the manuscript.

Conflict of interest statement

There is absolutely no conflict of interest with any individual or organization regarding the writing, materials discussed in the manuscript or publishing.

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