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ENHANCING PRODUCTION, REPRODUCTION, AND HEALTH IN LAYING QUAILS THROUGH PROBIOTIC SUPPLEMENTATION (*BACILLUS TOYONENSIS* AND *BIFIDOBACTERIUM BIFIDUM*): EFFECTS ON PERFORMANCE, PHYSIOLOGICAL PARAMETERS, AND GUT MICROBIOTA

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Abstract

The objectives of this investigation were to evaluate the influence of dietary supplementation with different levels of *Bacillus toyonensis* ATCC 55050 (BT), *Bifidobacterium bifidum* ATCC 29521 (BB), and a combination of half the dose of BT and BB on the productive performance, carcass characteristics, physiological parameters, and gut microbiota of laying Japanese quails (*Coturnix coturnix japonica*). A total of 144 laying quail chicks (56 days old, Japanese quail) were assigned to four experimental groups in a fully randomized design experiment for 12 weeks. Twelve replicates, each consisting of two female and one male bird, were created for each group. The control group of birds received a corn-soybean basal diet from 56 to 140 days of the trial. In contrast, the groups receiving probiotics were given the same control diet supplemented with 0.1% *Bacillus toyonensis* (BT), 0.1% *Bifidobacterium bifidum* (BB), or a combination of 0.05% *Bacillus toyonensis* plus 0.05% *Bifidobacterium bifidum* (BT+BB). There was a significant enhancement (P<0.05) in performance traits during different weeks of age as a result of the treatments with *Bacillus toyonensis* (BT), *Bifidobacterium bifidum* (BB), and the combination of both (BT+BB), except for live body weight. The results demonstrated a significant influence (P<0.05) of various probiotic treatments on fertility (Fer) and hatchability (Ha) percentages. BT+BB showed the highest fertility (98.57%) and total embryonic mortality (15.95%) compared to the other treatments. Also, the digestive enzymes activity was significantly (P<0.05) enhanced by BT and BT+BB treatments; besides, the BT+BB treatment showed higher levels of all antioxidant traits. In summary, the addition of *Bacillus toyonensis* (BT), *Bifidobacterium bifidum* (BB), and their combination (BT+BB) to the diet of laying quails significantly improved performance, blood traits, and decreased total bacterial counts, coliform levels, total fungi, and the cecal microflora.

Key words: probiotic, laying, productivity, blood, microbial load

Recently, organic feed supplements have taken on the role of artificial growth stimulants and antibiotics in enhancing the growth parameters and carcass characteristics of poultry and livestock. Furthermore, natural feed additives, which improve immunity and productivity by regulating gut flora, have recently acquired popularity as alternatives to artificial growth promoters and antibiotics (Elsherbeni et al., 2024); additionally, these extracts were used to improve birds' immunity status and efficiency (Abd El-Hack et al., 2023 a; Kamal et al., 2023; Youssef et al., 2024). As of 2006, the European Union has banned the use of antibiotics as growth promoters (AGPs) in poultry diets due to antimicrobial resistance, the rise of foodborne illness-causing bacteria such as *Salmonella* and *Campylobacter*, and the increased risk of consumer infections (Chantziaras et al., 2014; Abd El-Hack et al., 2023 b). Additionally, emerging research suggests that certain natural feed additives, particularly bioactive compounds found in herbal and medicinal plants, may play a role in cancer prevention by reducing oxidative stress and modulating immune responses, thus enhancing animal health while potentially decreasing cancer-related risks (Elsherbeni et al., 2024). Consequently, utilizing natural products like synbiotics (Youssef et al., 2024), probiotics (Cufadar et al., 2024), prebiotics (Youssef et al., 2023 a, b, c), essential oils, herbal extracts, bioactive medicinal plants, and phytogenic mixes (Dosoky et al., 2024) became more popular as substitutes for growth stimulants

and pathogen resistance. Due to their high profitability and low cost, medicinal and herbal plants have been used extensively as natural antioxidants in recent years (Odubo et al., 2024).

The International Scientific Association of Probiotics and Prebiotics (ISAPP) outlines criteria for selecting probiotic bacteria, including their presence in animal gut flora, low pH survival, resistance to bile salts, and presence of vancomycin resistance genes (VRGs) (Salahi and Abd El-Ghany, 2024). The ability of *Bifidobacterium bifidum* (BB) to produce antimicrobial compounds, such as bacteriocins like bifidin and bifidocin B, makes it a useful alternative to chemotherapeutic agents in animal production, human food, and poultry (Abd El-Moneim et al., 2019). Additionally, *Bacillus toyonensis* (BT) is a fermentative, aerobic, not pathogenic (gram-positive) bacterium which forms spores. It was utilized as a source for probiotics in poultry diets (Roos et al., 2018).

Previous research has shown that probiotics can contribute to the synthesis of hydrogen peroxide (H_2O_2), a potent chemical that helps the body eliminate various species of harmful bacteria (Kumar al., 2023). This method is among the most effective ways to enhance chicken health. It reduces oxidative stress in the digestive system, promotes the synthesis of essential digestive enzymes and B vitamins, inhibits the growth of harmful amines and aerobic pathogens, and improves appetite and feed intake (Feng and Wang, 2020). Numerous variables, such as strain, dosage, age of the bird, manner of treatment, viability, and duration of storage, affect the benefits of probiotics; these variables could explain the differences that occurred in results (Aluwong et al., 2013). By secreting digestive enzymes, probiotics improve feed utilization, gastrointestinal function, reproductive performance, and birds' defense against pathogenic bacteria like *Campylobacter* and *Salmonella*; they also improve the intestinal microbiota (Abd El-Moneim et al., 2020). Numerous studies have shown that adding probiotics to the diet improves FCR and egg production rates (Youssef et al., 2013; Chung et al., 2015). Additionally, it has been discovered that providing probiotics to laying hens enhances the quality of eggshells and lowers the quantity of broken eggs (Zhang et al., 2012). Furthermore, Li et al. (2006) observed that laying hens fed diets supplemented with 500 mg of *Bacillus subtilis* culture per kilogram exhibited significant increases in egg production rates and egg quality. Abd El-Moneim et al. (2019) found that supplementing diets with probiotics boosted feed efficiency and egg production. Mikulski et al. (2012) demonstrated that supplementing laying hens with probiotics may also enhance the quality of their eggshells. Probiotic supplementation has been shown in numerous previous trials to reduce oxidative stress in birds (Abd El-Moneim et al., 2023). By competing with harmful bacteria and promoting healthy gut microbiota in broiler chickens' digestive tracts, these beneficial microorganisms increase host health (Yadav and Jha, 2019). Improving the health and productivity of chickens may be achieved through synergistic effects observed when using probiotic mixtures containing both aerobic and anaerobic bacterial strains.

Generally, employing probiotics in poultry is quite a fascinating development and a novel trend in the poultry industry; it confers health benefits to the host birds, such as enhanced gut health, boosted immunity, and acting as an alternative to antibiotics. Consequently, the objective of this study was to evaluate the effects of dietary dosages of BT and BB, or a combination of half the dose of BT and BB, on the growth performance, carcass characteristics, physiological parameters, and gut microbiota of laying Japanese quail from eight to twenty weeks of age.

Material and methods

Probiotic strains

The current research was organized in the Egyptian Atomic Energy Authority's (EAEA) Research Farms of Poultry, Biological Applications' Department, Division of Radioisotope Application, Nuclear Research Center, Inshas region. The experimental protocols were carried out in compliance with the Egyptian Atomic Energy Authority's (EAEA) Biological Applications Department, Center of Nuclear Research, and ethical norms.

Animals, strains, and diets

In this experiment, *Bifidobacterium bifidum* ATCC 29521 (BB) and *Bacillus toyonensis* ATCC 55050 (BT), two strains of pure probiotics, were obtained from the Egyptian Culture Collection MERCIN (Ain Shams University, Cairo, Egypt). BT and BB solutions had concentrations of 5×10^8 and 6×10^8 colony forming units/ml, in that order.

Design of the experiment

The local experimental animal care committee's guidelines were monitored when conducting the experiments. The ZU-IACUC/2/F/95/2018 ethical approval code was used. In a fully randomized experiment, 144 Japanese quail chicks, at 8 weeks of age with similar initial body weight, were divided into four groups at random. 12 replicates of 3 quail birds (two females plus one male), each in a single cage, comprised each group. The NRC (1994) guidelines were followed in the formulation of the essential diet (Table 1). The birds of the group received a corn-soybean diet only (control one) for the duration of the experiment (8 to 20 weeks). In contrast, the groups BT, BB, and BT+BB received the essential diet plus 0.1%, 0.1%, and 0.05% of *Bacillus toyonensis* combined with 0.05% of *Bifidobacterium*, respectively.

Management

The study was conducted in the Egyptian Atomic Energy Authority's Experimental Poultry Farm, which is located in Inshas City, Egypt. It was accomplished by the Unit of Poultry Research, Department of Biological Application, Division of Radioisotopes Applications, and

Center of Nuclear Research. Three birds (two females and one male) were housed in separate cages with a 16-hour light and an 8-hour dark cycle. The investigation was conducted in the fall using battery cages illuminated by white fluorescent bulbs. The ambient temperature ranged from 28 to 31 degrees Celsius. The experiment lasted from 8 to 20 weeks of age. The environmental and management circumstances for birds were the same. The birds had free access to both fresh water and food. Battery cages were supplied via stainless steel nipple drinkers.

Table 1. Ingredient and nutrient composition of experimental basal diet

Basal diet (%)	Ingredients (g/kg)
Maize corn	53.85
Soybean meal (44%)	34.5
Di-calcium phosphate	1.20
Limestone	5.70
Sodium chloride	0.30
Vitamin-mineral premix[1]	0.30
DL-methionine	0.15
Soybean oil	4.00
Total	100
Calculated analysis[B] (g/kg)	
CP	200.7
ME (MJ/kg)	12.196
CF	36.0
Ly	11.4
Me	4.80
Me + Cy	8.00
Ca	25.1
available phosphorus	3.60

Cp: crude protein, ME: metabolizable energy, CF: crude fiber, Ly: lysine, Me: methionine, Cy: cystine, Ca: calcium.

[1]The premix supplemented as follows per every kg of diet:

vitamin D_3, 1,200 IU; vitamin A, 4,500 IU; vitamin E, 2,500 IU; vitamin B_3, 45,000 mg; vitamin B_1, 5,000 mg;; vitamin K_1, 10,000 mg; folic acid, 3,000 mg; vitamin B_2, 20,000 mg; pantothenic acid, 35,000 mg; biotin, 1,500 mg; vitamin B_{12}, 40 mg; Se, 120 mg; Zn, 45 mg; Fe, 30 mg; Mn, 50 mg; I, 1 mg; Cu, 4 mg; Co, 120 mg.

Collecting samples and analyses

Laying performance

The birds were weighed (to the nearest 0.1 g) in the early morning before being supplied with food each day, in order to estimate changes in the birds' live weights. Egg weights (EW) were measured to the nearest 0.01 g throughout the duration of the experiment, and egg numbers (EN) were recorded daily for each replicate cage over the entire experimental period. Also, the egg mass (EM) in grams for each cage each day was calculated by multiplying the average egg weight (EW) by the number of eggs (EN). Daily feed intake (FI), measured in grams per bird, was calculated by subtracting the remaining feed in the feeders from the total feed consumed. Grams of feed per gram of egg were used to estimate the feed conversion ratio (FCR). At 140 days of age, six females were randomly selected from each treatment, fasted overnight, then weighed, and finally slaughtered. The empty hot carcasses, along with the liver, gizzard, and heart, were weighed and expressed as proportions of their pre-slaughter weight. Every carcass component was computed using the methodology outlined by Abd El-Moneim et al. (2019). To assess different productive organs and digestive tract traits, as well as the activity of digestive enzymes, samples from the duodenum were collected.

Fertility and hatchability percentages

At the age of 56 and 140 days, a number of 464 eggs (116 eggs/treatment) were selected randomly and then incubated. Before being put inside the incubator cabinet, the eggs selected were arranged on the platter according to their groups in different treatments. The eggs' temperature was kept at 37.5°C, and during the incubation period of 1 to 15 days, the humidity percentage in the incubator varied between 50 and 60%. The hatching room temperature was reduced by 1°C, and humidity was maintained at 80–90%. The ventilation and temperature were changed automatically. Also, eggs were turned around one time/2 hours every day automatically. The number of both unhatched eggs and hatched chicks was recorded. We counted the number of nonfertilized eggs by breaking unhatched eggs. The percentages of fertility (Fer) and hatchability (Ha) were studied as:

$$F\ \% = \text{number of fertile eggs/total eggs set} \times 100$$
$$H\ \% = \text{number of hatched chicks/numbers of fertile eggs} \times 100$$

Evaluation of blood biochemistry, activities of digestive enzymes, and antioxidant activity

When the quails were slaughtered at 140 days old, samples of blood (24 bird) were taken right away and placed in EDTA including tubes (ethylene diamine tetra acetic acid, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), an anticoagulant. All of the blood samples were centrifuged (4000 rpm for 10 minutes) in order to extract the blood plasma, as recognized by Zhou et al. (2023). Once the tube was thoroughly sealed and then kept at –20°C until analysis, serum samples were put into it. By an automated analyzer and a commercial kit (Biodiagnostic, Giza, Egypt), the levels of alanine aminotransferase (ALT), aspartate transaminase (AST), urea, creatinine, total cholesterol, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) were determined in accordance with the manufacturer's instructions (Wainstein et al., 2022; Zhou et al., 2023). Creatinine and urea levels were measured using commercial kits (Química Clínica Aplicada, Tarragona, Spain). The protease and amylase assays were carried out with the techniques established by Tietz and Fiereck (1966) and Najafi et al. (2005), respectively. Trypsin

was measured using the bovine trypsin enzyme-linked immunosorbent assay (ELISA) kit (Cosmo Bio USA, Carlsbad, CA, USA).

Microbiological analysis

As soon as the experiment ended, 25 grams of all experimental diet (diet samples) of the BT, BB, and BT+BB were examined microbiologically. An estimate was made of the number of microorganisms (total count of bacteria, coliforms, enterococci, and fungus) as illustrated by Feng et al. (2002). Briefly the experimental diet samples were put inside stomacher bags (Sewared, London, UK), and blended with 225 ml of sterile- saline peptone water (SPW: 1 g/l peptone, 8.5 g/l sodium chloride) for three minutes. Each sample was serially diluted ten times for quantitative microbiological analysis. A duplicate of one ml sample of the necessary dilutions was placed on plates of agar after sequential dilutions of sterile saline peptone water with samples were made. For 48 hours, at 35°C, the total viable bacterial count (TVC) was recorded on plate count agar (Merck, Darmstadt, Germany, #1.05463). For five days, total fungal counts (TFC) were measured at 25°C on rose Bengal chloramphenicol agar (Lab M Limited, Lancashire, UK, #36). Chloramphenicol was added to the agar. Using violet, red bile agar (VRB, Biolife Italiana, Milan, Italy), the population of the whole coliform group was discovered following a 24-hour incubation period at 37°C. From the same slaughtered birds at 140 days old (after carcass traits and blood samples were taken), five birds in each group were taken randomly in order to count the microbes in the cecum. Fresh samples of cecal digesta (1–2 g/bird) were placed in bottles with a CO_2 stream and directly submitted to the lab for microbiology analysis. The contents of ileum (10 cm anterior to the junction of caecum and rectum) were collected separately into sterile tubes for microbiological investigation. The stomacher bag (Sewared) was filled with approximately one gram of ileal digesta, and ten ml of sterile saline peptone water was utilized to homogenize it. Before being inoculated onto Petri dishes, the TVC, coliform, *E. coli, Bacillus toyonensis,* and *Bifidobacterium bifidum* were identified by sequential dilution. Counting agar plates (Merck, 1.05463) were used to measure the TVC following a 48-hour incubation period at 35°C. After incubating at 37°C for 24 hours, coliform was counted using VRB (Biolife Italiana). After 24 hours at a temperature of 37°C, *E. coli* was tallied on the agars of MacConkey (Thermo Scientific™ Oxoid, #CM0007, Kansas, USA). Using MRS agar (Merck, #110660), *B. bifidum* was counted under anaerobic conditions after 24 hours at 37°C. Utilizing nutrient agar plates (Merck, #11471), the dilution was pasteurized at 80°C for 15 minutes before Bacillus was counted after 24 hours at 37°C. To increase the feasible number up to 1×10^8 per one gram in the samples of diet, *B. toyonensis* (BT), *B. bifidum* (BB), or the mixture between them, was added to the essential diet treatment. The treated birds and the controls were housed in different rooms within the building with identical humidity and temperature levels to prevent spore cross-contamination.

Data statistical analysis

Data were statistically analyzed using the General Linear Models (GLM) procedure of SAS program (SAS, 2001). Means differences were compared using Tukey's test (5% significance level). The afterward model was followed:

$$Y_{ij} = \mu + T_i + e_{ij}$$

Where Y_{ij} = observed value of the concerned treatment, μ = observed mean for the concerned treatment, T_i = treatment effect and e_{ij} = the error related to individual observation.

Results

Performance traits

Live body weight

Data illustrated in Table 2 exhibits how the different additions of BT, BB, and BT+BB affect the laying quail hens' production performance. As shown there were insignificant differences between the initial weights of the live body (ILBW) and the final weights of the live body (FLBW) due to BT, BB, and BT+BB treatments; the highest FLBW was obtained from birds of the control group (300.58 g), followed by BT+BB (294.13 g), BT (290.42 g) and BB (274.17 g), respectively. Moreover, the data presented indicate that the different treatments have insignificant ($P>0.05$) effects on FI and FCR during the earliest stages of the experiment period. Only FCR was significantly ($P<0.05$) affected at the period 16–20 weeks of age and the overall experiment period (8–20 weeks); in addition, BT and BB treatments had caused a reduction in FI compared to BT+BB and the untreated group. While the control group and BT treatment gained the lowest FCR in overall the experiment period (8–20 weeks). Likewise, values of EN were statistically ($P<0.05$) affected by the probiotics treatments; the highest EN values were obtained from the control group at all periods of the experiment; In terms of the probiotics-treated groups, the BT and BT+BB groups exhibited the highest values of EN followed by BB group. In addition, EW was not affected by probiotics addition in all groups, the BT group showed the highest EW over all experiment period when compared to the other treatments and those of the control group. It is clear from the obtained data that egg mass values were significantly ($P<0.05$) affected during the experimental periods, except the period of 12–166 weeks; moreover, the control group displayed the maximum values of egg mass over all experiment periods compared to probiotics-treated groups, followed by BT, BT+BB, and BB, respectively.

Table 2. Effects of supplementing laying quail hens' diet with *B. toyonensis* (BT) and *Bifidobacterium bifidum* (BB) on production performance

Items	Treatments (ml/kg diet)				*SE*	P-value
	control	BT	BB	BT+BB		
Live body weight (g)						
initial	236.63	231.04	232.63	227.92	2.02	0.499
final	300.58	290.42	274.17	294.13	3.77	0.080
Feed intake (g/bird)						
8–12 weeks	34.09	33.05	33.11	33.42	0.74	0.970
12–16 weeks	34.32	35.39	32.79	38.77	1.88	0.773
16–20 weeks	42.61	38.61	42.27	44.12	1.03	0.304
8–20 weeks	37.01	35.69	36.06	38.77	0.70	0.462
FCR (g feed/g egg)						
8–12 weeks	4.56	4.19	5.92	5.26	0.27	0.069
12–16 weeks	4.27	5.01	5.34	5.60	0.29	0.470
16–20 weeks	5.64 b	5.62 b	6.53 ab	7.14 ab	0.23	0.020
8–20 weeks	4.82 b	4.94 b	5.93 a	6.00 a	0.18	0.002
Egg number/bird						
8–12 weeks	15.92 a	15.25 ab	12.67 c	13.17 bc	0.47	0.006
12–16 weeks	16.50 a	14.75 ab	12.33 b	14.08 ab	0.52	0.011
16–20 weeks	15.75 a	15.00 ab	13.25 b	13.17 b	0.38	0.006
8–20 weeks	16.06 a	15.00 ab	12.75 c	13.47 bc	0.43	0.002
Egg weight (g)						
8–12 weeks	13.25	13.66	13.30	12.47	0.18	0.094
12–16 weeks	13.65	14.05	13.39	13.30	0.12	0.094
16–20 weeks	13.49	13.58	13.87	13.30	0.15	0.659
8–20 weeks	13.46	13.76	13.52	13.02	0.11	0.124
Egg mass (g/bird)						
8–12 weeks	7.52 ab	8.05 a	5.87 c	6.38 bc	0.30	0.007
12–16 weeks	8.04	7.31	6.58	7.04	0.21	0.078
16–20 weeks	7.58 a	7.01 ab	6.52 ab	6.41 b	0.17	0.029
8–20 weeks	7.71 a	7.46 a	6.32 b	6.61 b	0.18	0.000

a, b, c – means in the same column with different letters are different significantly (P<0.05). SE: standard error of the means.

Table 3. Effects of supplementing laying quail hens' diet with *B. toyonensis* (BT) and *Bifidobacterium bifidum* (BB) on fertility, hatchability and total embryonic mortality

Items	Treatments (ml/kg diet)				*SE*	P-value
	control	BT	BB	BT+BB		
Fertility (%)	95.79 a	90.48 b	96.51 a	98.57 a	1.09	0.023
Hatchability (set eggs) (%)	87.54 a	72.62 b	83.41 a	82.62 a	2.11	0.044
Total embryonic mortality (%)	8.25	17.86	13.1	15.95	1.55	0.125

a, b, c – means in the same column with different letters are different significantly (P<0.05). SE: standard error of the means.

Percentage of fertility and hatchability

Fertility and hatchability data of set eggs were presented in Table 3. The obtained data showed a significant (P<0.05) influence of diverse probiotic treatments on fertility (Fer), and hatchability (Ha) percentages. The BT+BB showed the highest percentage of Fer (98.57%) and total embryonic mortality (15.95) in comparison with the other treatments. Besides, birds of the untreated group recorded a higher Ha percentage (87.54%) than other treatment groups.

Productive and digestive organ traits

The results presented in Table 4 illustrate that different probiotic treatments significantly (P<0.05) affected the various carcass productive and digestive organs characters, excluding each of the spleen, heart, ovary, and oviduct percentages. In comparison with other treatments, the highest live body weight and carcass percentage were shown by those of the untreated groups, additionally, the BB treatment recorded a higher percentage of the liver, intestine, and cecal length compared to the other different treatments; moreover,

the highest length of intestinal length, duodenum, and oviduct were showed by the BT+BB treatment.

Blood biochemistry

Data presented in Table 5 indicates how all treatments affect the different blood indices. Notably, the probiotics significantly (P<0.05) affected the triglycerides, the very low-density lipoprotein (VLDL), T_3, and T_4 levels only; in comparison to the other treatments, the highest levels of these traits were obtained from the control group, BB**,** and BT+BB treatments, respectively. Different probiotic treatments affect the hepatic and renal function biomarkers, as exposed from the obtained data that probiotics significantly (P<0.05) affected all the hepatic and renal function biomarkers except the urea, glucose, and aspartate aminotransferase (AST) levels. The BT treatment recorded the highest levels of total protein (TP), albumin (ALB), albumin to globulin (A/G) ratio, and alanine aminotransferase (ALT), while the highest levels of creatinine, uric acid (UA), and alkaline phosphatase (ALP) were obtained by BT+BB, BB and control groups, respectively.

Table 4. Effects of supplementing laying quail hens' diet with *B. toyonensis* (BT) and *Bifidobacterium bifidum* (BB) on different productive organs and digestive tract traits

Items	Treatments (ml/kg diet)				*SE*	P-value
	control	BT	BB	BT+BB		
LBW	305.00 a	293.00 b	289.00 b	302.50 a	2.10	<0.001
Carcass (%)	70.77 a	69.18 a	65.54 b	67.89 ab	0.73	0.039
Liver (%)	1.75 c	2.20 b	2.61 a	2.30 b	0.10	<0.001
Spleen (%)	0.10	0.10	0.07	0.10	0.01	0.319
Heart (%)	0.80	0.70	0.73	0.71	0.02	0.275
Intestine (%)	5.83 c	7.06 bc	9.38 a	8.49 ab	0.50	0.025
Ovary (%)	3.18	2.61	2.96	3.19	0.10	0.098
Oviduct (%)	3.24	3.04	3.20	3.55	0.10	0.333
Intestine length (cm)	81.75 b	90.00 ab	85.25 ab	94.00 a	1.82	0.054
Duodenum length (cm)	11.75 bc	12.50 ab	11.25 c	13.00 a	0.24	0.016
Oviduct length (cm)	31.50 b	32.50 b	34.00 b	38.50 a	0.99	0.027
Cecal length (cm)	9.00 b	9.50 b	12.50 a	10.50 ab	0.50	0.027

LBW: live body weight. a, b, c – means in the same column with different letters are different significantly (P<0.05). SE: standard error of the means.

Table 5. Effects of supplementing laying quail hens' diet with *B. toyonensis* (BT) and *Bifidobacterium bifidum* (BB) on different blood indices

Items	Treatments (ml/kg diet)				*SE*	P-value
	control	BT	BB	BT+BB		
Serum lipid and thyroid hormone						
TC (mg/dl)	534.85	265.9	306.51	509.75	62.56	0.335
TG (mg/dl)	1759.00 a	850.40 b	1351.85 a	594.95 b	148.32	0.002
HDL (mg/dl)	28.39	14.34	23.73	34.57	3.08	0.100
LDL (mg/dl)	240.76	81.49	159.13	223.39	36.32	0.447
VLDL (mg/dl)	351.80 a	170.08 b	270.37 a	118.99 b	29.66	0.002
glucose (mg/dl)	633.1	723.6	633.7	596.35	37.35	0.727
T3 (ng/ml)	0.56 b	0.73 b	0.99 a	1.14 a	0.07	0.001
T4 (ng/ml)	0.46 b	0.58 b	0.78 a	0.95 a	0.06	0.001
Serum hepatic and renal function biomarkers						
creatinine (mg/dl)	0.61 bc	0.47 d	0.72 ab	0.81 a	0.05	0.016
urea (mg/dl)	6.06	2.64	2.64	5.59	0.64	0.070
uric acid (mg/dl)	6.19 c	7.73 ab	11.12 a	8.97 b	0.61	0.004
TP (g/dl)	4.69 c	7.67 a	7.46 a	6.90 ab	0.46	0.057
ALB (g/dl)	1.64 b	3.01 a	2.34 a	2.36 a	0.17	0.012
G (g/dl)	3.05	4.66	5.12	4.54	0.32	0.089
A/G ratio	0.54 b	0.67 a	0.46 b	0.52 b	0.03	0.021
AST (U/L)	332.31	284.36	247.48	297.27	15.76	0.324
ALT (U/L)	14.44 b	19.14 a	14.72 b	11.02 b	1.00	0.008
ALP (U/L)	747.40 a	326.46 b	322.80 b	300.63 b	57.95	<0.001

TC: total cholesterol, TG: triglycerides, HDL: high density lipoprotein, LDL: low density lipoprotein, VLDL: very low-density lipoprotein, TP: total protein. ALB: albumin, G: globulin, A/G: albumin to globulin ratio, AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALP: alkaline phosphatase. a, b, c – means in the same column with different letters are different significantly (P<0.05). SE: standard error of the means.

Table 6. Effects of supplementing laying quail hens' diet with *B. toyonensis* (BT) and *Bifidobacterium bifidum* (BB) on digestive enzyme activities, and antioxidant status

Items	Treatments (ml/kg diet)				*SE*	P-value
	control	BT	BB	BT+BB		
Digestive enzyme activities						
AMS (U/L)	500.50 b	1445.00 a	738.50 b	828.50 b	119.05	0.005
LPS (U/L)	6.72 c	20.75 a	12.40 b	10.61 b	1.67	0.001
PRS (nmol/mg)	0.26 b	0.14 c	0.23 b	0.32 a	0.02	<0.001
Antioxidant status						
MDA (nmol/ml)	0.72 a	0.42 bc	0.57 ab	0.28 c	0.05	0.002
GSH (ng/ml)	0.10 d	0.19 c	0.21 b	0.27 a	0.02	<0.001
GPX (ng/ml)	0.11 c	0.18 b	0.19 b	0.26 a	0.02	<0.001
SOD (U/L)	0.08 c	0.15 b	0.18 b	0.25 a	0.02	<0.001
CAT (ng/ml)	0.09 c	0.17 b	0.18 b	0.24 a	0.02	<0.001
GSR (ng/ml)	0.09 c	0.17 b	0.18 b	0.25 a	0.02	<0.001
GST (pg/ml)	0.07 c	0.15 b	0.17 b	0.23 a	0.02	<0.001

AMS: amylase, LPS: lipase, PRS: protease, MDA: malondialdehyde, GSH: glutathione, GPx: glutathione peroxidase, SOD: superoxide dismutase, CAT: catalase, GSR: glutathione reductase, GST: glutathione S-transferase. a, b, c – means in the same column with different letters are different significantly (P<0.05). SE: standard error of the means.

Table 7. Effects of supplementing laying quail hens' diet with *B. toyonensis* (BT) and *Bifidobacterium bifidum* (BB) on total bacterial counts, coliform and total fungi (Log10 CFU/g) in the basal diet after four, eight and twelve weeks

Items	Treatments (ml/kg diet)				SE	P-value
	control	BT	BB	BT+BB		
Total bacterial count						
4 weeks	6.47 b	6.37 d	6.43 c	6.52 a	0.01	<0.001
8 weeks	6.54 a	6.42 de	6.47 cd	6.48 bc	0.01	<0.001
12 weeks	6.41 bc	6.33 d	6.44 bc	6.40 c	0.02	<0.001
Coliform						
4 weeks	4.92 a	4.86 a	4.86 a	4.85 a	0.03	<0.001
8 weeks	4.43 a	4.26 ab	3.30 c	4.15 b	0.06	<0.001
12 weeks	4.66 a	4.34 bc	4.43 b	4.42 b	0.03	<0.001
Total fungi						
4 weeks	3.52 a	2.85 bcd	2.66 cde	3.23 ab	0.09	<0.001
8 weeks	4.13 a	3.54 ab	2.57 d	2.69 cd	0.11	0.003
12 weeks	4.49 a	2.72 bc	2.50 c	2.86 bc	0.12	<0.001

a, b, c – means in the same column with different letters are different significantly (P<0.05). SE: standard error of the means.

Table 8. Effects of supplementing laying quail hens' diet with *Bacillus toyonensis* (BT) and *Bifidobacterium bifidum* (BB) on cecal microflora (Log10 CFU/g wet weight; total bacterial counts (TBC), probiotic bacteria, coliforms, and *E. coli*)

Items	Treatments (ml/kg diet)				*SEM*	P-value
	control	BT	BB	BT+BB		
TBC	8.80 a	8.64 bcd	8.53 de	8.54 de	0.02	<0.001
Probiotic	7.85	7.67	7.19	7.51	0.06	0.228
Coliform*s*	6.97 a	6.54 abcd	6.66 ab	6.23 bcd	0.06	0.019
E. coli	5.94 ab	5.58 abc	5.16 bcd	5.23 bcd	0.11	0.013

a, b, c – means in the same column with different letters are different significantly (P<0.05). SE: standard error of the means.

Digestive enzyme activities and antioxidant status

Table 6 summarizes the influences of probiotics on digestive enzymes and the antioxidant characteristics. The different levels of the digestive enzyme were affected significantly (P<0.05) by all treatments; both amylase (AMS) and lipase (LPS) recorded the highest levels due to the BT treatments, while the BT+BB treatments gave the higher protease (PRS) levels in comparison with other treatments and the untreated groups. Furthermore, it is clear that there was a significant (P<0.05) effect on all antioxidant characteristics due to the different treatments; the BT+BB treatments showed higher levels of all antioxidant traits compared with those of the control (untreated) groups which showed the lowest levels, and the other treatments, except the MDA levels which were higher in the control group compared to other treatments.

Microbiological findings

The data presented in Tables 7 and 8 indicates a significant (P<0.05 and P<0.001) modification of the proliferation of microorganisms in the diet, and cecal microflora by dietary supplementation with BT, BB, and BT+BB; from the obtained results it was noted that the control groups recorded the highest total bacterial counts and cecal microflora scores in comparison with those of other treatments. Consequently, it was shown that adding the probiotic (BT, BB, and their combination) to the quail's diet decreased the number of unuseful fungi and bacteria in the basal diet, as well as the cecal microflora.

Discussion

Although this study did not demonstrate a significant impact of probiotic treatments on final live body weight, probiotics are known to have various health benefits, including cholesterol reduction, immune system enhancement, and the production of antimicrobial agents like hydrogen peroxide, acetaldehyde, bacteriocins, lactone components, and organic acids. These agents may help inhibit the growth of harmful bacteria and improve overall gut health (Mukherjee et al., 2019; Abou-Kassem et al., 2021). Moreover, using of probiotics causes bacterial competition for colonization, rivalry for nutrition, and antagonism; these processes promote body weight by lowering harmful chemicals, enhancing the immunity system, and improving nutrient absorption (Applegate et al., 2010). Probiotics may also have an enhancing impact because of their capacity to increase digestive enzyme activities and decrease the formation of ammonia (Sugiharto, 2016). Increased carbohydrate and lipid metabolism was facilitated by higher lipolytic and amylolytic activities, which may link to the growth improvement in the recent study. Our findings conformed with Gupta et al. (2016) and Abdel-Moneim et al. (2020) who noticed that adding probiotics to the diet of Japanese quail caused an increase in live body weight. Microbe-produced metabolites can promote colonic mucosal differentiation and proliferation by causing apoptosis; They may also protect against colitis and colorectal cancer by regulating oncogene expression, these functions may be performed by multiple species, either separately or in combination (Abou-Kassem et al., 2021). Furthermore, probiotics released exogenous enzymes and the host's endogenous enzyme production may have contributed to the improvement in the live weight gain of broilers (Wang and Gu, 2010). Chickens treated with probiotics in drinking water at levels of 0.05, 0.10, and 0.25 g/liter exhibited significantly higher (P=0.027) average body weight gain (BWG) compared to the control group (Abramowicz et al., 2019). In contrast, some authors like Yıldırım et al. (2020) noticed an insignificant influence of the probiotic on the growth performance traits of quail; additionally, Hossain et al. (2015) indicated no changes in the broilers' body weights given diets provided with 0.10% of three probiotic strains (*B. subtilis*, *C. butyricum*, and *Lactobacillus acidophilus*); furthermore, they indicated that addition of 0.20% probiotic had significantly (P<0.05) raised the LBW compared to the negative treatment (control).

As shown from the data obtained in our study (Table 2), probiotics have substantial effects on FCR averages, only during the period 16–20 weeks of quails' age and over all the experiment period, the BT treatment tends to be the better treatment than other treatments. The improvement that occurred in FCR which was observed in quails provided via multispecies probiotics in diets, may have resulted from the probiotic's overall effects, which included modifications of intestinal bacterial metabolism, the preservation of a favorable microbial population, besides improving nutrient digestion, and absorption (Premavalli et al., 2018). Moreover, the enhancements in FCR values may be a reflection of the increased values of villus height, which may lead to a larger capacity for accessible nutrients (Jazi et al., 2018). Our findings are consistent with the results of Kumari et al. (2001), who reported an elevated FCR in quails supplemented with probiotics compared to the untreated group. Conversely, Manafi et al. (2016) observed a significant increase (P<0.05) in FCR rates in laying quails treated with *B. subtilis* and bacitracin relative to the control group. While, our findings disagree with those recorded by Zhang et al. (2013), Hossain et al. (2015), and Manafi et al. (2016) who noticed that the FI was insignificantly affected due to using different species of probiotics. Also, Hossain et al. (2015) found that the addition of 0.1% of tri-probiotics strains (*C. butyricum*, *B. subtilis*, and *L. acidophilus*) to broilers' diets did not modify the FCR; however, the addition of 0.2% had improved the FCR (P<0.05) in comparison with untreated one.

A significant (P<0.05) effect of probiotic supplementation on quail EN was observed (Table 2), especially during the latter stages of the experiment. Control groups exhibited the highest egg production, followed by those receiving 1 ml *Bacillus toyonensis*/kg diet. Quails on the 1 ml *Bacillus toyonensis*/kg diet tended to produce eggs with higher weight compared to other treatments. Sig-

nificant ($P<0.05$) impacts on egg mass were limited to weeks 8–9 and 10–11. Closely to our findings Zhou et al. (2020) noticed that adding *B. amyloliquefaciens* (2×10^{10} CFU/g) with levels of 0.01% or 0.06% to quails' diet caused a significant increase in egg production rates. In addition, among 20 and 44 weeks of age, layers fed diets enhanced by *Lactobacillus* showed a statistically significant ($P<0.05$) improvement in egg weight. Also, Jha et al. (2020) indicated that probiotic supplementing for laying hens' diet boosted FI, enhanced laying rate, and retained more calcium and nitrogen. The increases in egg production and weight seem to be related to the probiotics' ability to promote the metabolic processes involved in nutrient digestion and utilization (Zamanizadeh et al., 2021). Additionally, dietary supplementation with 1, 21, and 31×10^9 CFU/kg of *Lactobacillus acidophilus* (LA) improved growth performance, egg production, number, and weight in laying hens. This improvement may be attributed to LA's ability to enhance both humoral and cell-mediated immunity, reduce stress by lowering the H/L ratio, and ultimately lead to the observed benefits (Alaqil et al., 2020).

Some researchers, such as Xu et al. (2006), Yalcin et al. (2014), and Derakhshan et al. (2023), reported that diverse probiotic treatments had no significant influence on quail performance attributes. Differences in acquired results may be attributed to many reasons, such as the birds' age, trial period, and applied doses of probiotics. Probiotics are capable of synthesizing enzymes that facilitate the digestion of complex nutrients, including proteins, carbohydrates, and fats, thereby enhancing nutrient absorption. Furthermore, they produce vitamins, such as K and B vitamins, which are crucial for egg production and general health (Liu et al., 2023). Too, all previous motives may be attributed to a development in Fer and Ha percentages; as shown in our study the probiotics treatments caused a significant ($P<0.05$) improvement for both Fer and Ha percentages (Table 3), while the total embryonic mortality percentage was increased. Our results align with the findings of Nour et al. (2021), who demonstrated a significant ($P<0.01$ and $P<0.05$) positive correlation between probiotic supplementation levels and Fer and Ha rates in quails. Also, our findings were close to the results obtained by Nour et al. (2021) who found increasing rates of the probiotics in quails' diets caused a significant ($P<0.01$) and ($P<0.05$) increase in Fer and Ha percentages. Numerous studies found that in Japanese quails (from 8 to 52 weeks of age) the range of Fer percentage was about 48–94% while, the range of Ha percentage was about 40–70.34%, of fertile eggs (Seker et al., 2004). Gonadotropin-releasing (GR) hormone rates in layers were shown to be considerably elevated by dietary supplementation with *Bacillus subtilis*, according to Wang et al., 2017; additionally, Kim et al. (2018) indicated feeding Korean native heifers diet supplements containing *Bacillus subtilis* greatly increased the levels of progesterone and estradiol. Thus, improved hatching performance could be a result of increased reproductive hormones. Additionally, compared to the control group, quails administered probiotics (10^8 CFU/ml *Bacillus megaterium*) had a significant ($P<0.05$) increase in the Ha percentage by around 12%, and embryonic death was decreased by 10% (Mojgani et al., 2020). Our results differ from those reported by Ayasan (2013), who found no significant differences in Fer and Ha rates in Japanese quails fed diets supplemented with commercial probiotics at dosages of 0.05 and 0.10%/kg diet compared to the control group.

Even though, many previous studies indicated no effects on carcass characteristics due to different treatments with probiotics like the studies published by De-Souza et al. (2018), Abd El-Moneim et al. (2020 a), Abou-Kassem et al. (2021). Also, Tang et al. (2017) and Cufadar et al. (2024) indicated that lengths of both small intestine and cecum, and liver and gizzard weights were not affected when 1% (1×10^9 CFU/g) *B. megaterium* and *B. amyloliquefaciens/*kg was added to layer quail diets. In addition, in broiler chickens, the weight of the liver was not significantly altered by the inclusion of probiotics in the diet, as reported by Awad et al. (2009) and Chen et al. (2013). In contrast, the data of our research showed a significant ($P<0.05$) influence on most carcass traits due to the different treatments (Table 4); our findings conformed with Yu et al. (2020) who indicated that in layer chicks (Lohmann Brown strain), probiotic feeding augmented the length of both ileum and duodenum alongside a rise in the weights of liver, but weight of heart was not affected. The weights of the liver, heart, and gizzard have been demonstrated to increase by utilizing probiotic supplements in broilers' diets (Saiyed et al., 2015), and in quails (Bahrampour et al., 2020). The enhancement in carcass traits may be related to the various roles of probiotics in improving the live body weights and FCR. Probiotics can improve intestinal structure (the width, length, and depth of intestinal villi) and provide an ideal environment in the digestion tract that allows the small intestine to absorb nutrients more efficiently (Hidayat et al., 2016).

As shown in our study glucose levels were not affected by different probiotic treatments in this study, one possible explanation for blood glucose maintenance could be the anti-glucagon effect of probiotic microorganisms (Aluwong et al., 2013). Moreover, probiotics have increased T_3 and T_4 levels in quails' serum, which might be due to the ability of probiotics to motivate the thyroid stimulating hormone releasing hormone (TSH-RH) in the hypothalamus, which in turn prompts the anterior pituitary gland's release of thyroid stimulating hormone (Aluwong et al., 2013). Probiotics may increase the activities of corticotrophin-releasing factors, which stimulates the release of thyrotropin and, consequently, the secretion of T_4 (Klieverik et al., 2009).

In the extant study, probiotic-supplemented diets generally significantly ($P<0.05$) altered biomarkers of plasma hepatic and renal function (Table 5). Results indicated that probiotics increased total protein (TP) levels, albu-

min (ALB), and biomarkers of renal and hepatic function. These findings are consistent with previous studies (Ahmed et al., 2015; Pourakbari et al., 2016; Yazhini et al., 2018). By inhibiting the growth of pathogenic bacteria that decrease protein breakdown into nitrogen and increase nutrient absorption, lactic acid bacteria can enhance dietary protein utilization. This mechanism of inhibition and exclusion may account for the observed increases in total protein (TP) and albumin (AL) serum levels (Yazhini et al., 2018). Additionally, probiotic supplementation can lower serum transaminase levels by reducing the translocation of pathogenic microorganisms to the liver (Rishi et al., 2009). Moreover, the exclusion mechanism of inhibition, in which *Bacillus subtilis* prevents the growth of pathogens, may contribute to the increase in serum total protein and albumin levels. This mechanism reduces the breakdown of dietary protein into nitrogen and increases the surface area available for nutrient absorption (Yazhini et al., 2018; Abd El-Moneim et al., 2019).

Digestive enzymes are essential for the digestion of nutrients and eventually affect the health, performance, and retention of nutrients in poultry. According to results obtained in this study, the highest activities of AMS and LPS can be noticed in quails fed the BT, while 0.50 BT + 0.50 BB gave PRS the highest activity (Table 6); our findings are compatible with Wang and Gu (2010) and Abdel-Moneim et al. (2020) who claimed that probiotic therapy (*Lactobacillus* or *Bacillus*) increased the activity of LPS and AMS. The increase in digestive enzyme activity that has occurred could be the consequence of the combined influence of the exogenous enzymes released by the probiotic and the enzymes produced by the host (endogenous enzymes). On the other side, our obtained results disagreed with a previous study published by Rodjan et al. (2018) who suggested insignificant changes in PRS, AMS, and LPS activity when broilers were treated with probiotics.

Antioxidant enzyme activity like glutathione (GSH), glutathione peroxidase (GPx), catalase (CAT), and malondialdehyde (MDA) were used as a measurement of the ability of the body to remove the free radicals. The antioxidant status was significantly improved ($P<0.001$) by probiotic treatments, particularly the BT+BB combination, which yielded the best results compared to other treatments as displayed in Table 6. These findings align with previous studies (Popović et al., 2015). Additionally, dietary supplementation with *B. subtilis* or essential oils significantly increased the activity of glutathione peroxidase (GPx) and total antioxidant capacity (T-AOC), as well as the expression of related genes (Bai et al., 2017; Yu et al., 2018; Yang et al., 2019). The antioxidant properties of probiotics, such as their ability to generate compounds that neutralize free radicals and reduce oxidative stress, contribute to the improved health of Japanese quail (Lin and Yen, 1999). In addition, Zheng et al. (2016) found that *Enterococcus faecium* increases the antioxidant capacity of broilers by reacting with hydroxyl radicals, which in turn strengthens biological macromolecules' resistance to oxidation.

When food products like eggs, meat, and milk become contaminated with unintentional microbes, such as *Salmonella*, *E. coli*, *Shigella*, and *Listeria*, they can pose a serious health risk (U.S. FDA, 2016). *Lactobacillus* and *Bifidobacterium* species are considered to be unique microbiota in the animals and poultry intestinal tracts, among the numerous probiotic bacteria. Pathogenic bacteria can attach themselves to intestinal mucus and epithelial cells, which can lead to active growth and colonization. Bacterial adhesion to epithelial cells is a crucial first step in infection. Therefore, preventing bacterial adherence to the intestinal mucosa is considered an effective strategy for reducing the risk of foodborne illness (Kim et al., 2015). Acids like lactic acid, which have strong antibacterial and antifungal properties, are the primary active ingredients in *Bacillus* or probiotic bacteria, which generate antimicrobial components (Nour et al., 2021).

Our obtained results were close to Serafini et al. (2013) who discovered that *Bifidobacterium bifidum* exhibited inhibitory qualities against pathogenic bacteria, specifically *Cronobacter sakazakii* and *E. coli*, with respect to intestinal adaption through using monolayers of epithelial intestinal cells. Furthermore, Abd El-Moneim et al. (2020) and Abou-Kassem et al. (2021) observed a decrease in ileal total bacterial count (TBC) and total coliform counts, but an increase in lactic acid bacteria counts in broilers treated with BB. Probiotics can adhere to and colonize the intestinal epithelium, competing with pathogens for adhesion sites, increasing the complexity of enterocytes, and promoting interactions between various cell types, all of which enhance phagocytosis. Reduced concentration of hazardous bacteria is commonly referred to as a growth-stimulating factor, as it lessens the antagonism for nutrients among microbes and the host. Thus, in this investigation, enhanced productivity might be linked to the removal of pathogens species like *E. coli* as mentioned before by Guo et al. (2017). Cecal *Bifidobacterium* and *Lactobacilli* counts were observed to be considerably enhanced by supplementing with 4×10^{10} CFU/kg of *Bacillus cereus* and *Lactobacillus* (as a probiotic) according to Li et al. (2009). This development might be connected to *Bacillus subtilis*'s aerobic growth trait. Lower intestinal oxygen levels may promote the growth of *Bifidobacterium* and *Lactobacillus*; in addition, the reduction in cecal *E. coli* could be attributed to the increase in counts of *Bifidobacterium* and *Lactobacillus*.

Conclusion

Based on the findings of this study, it can be concluded that using probiotics such as *Bacillus subtilis*, *Bacillus bifidum* and a combination of half doses of both BT and BB in laying quail diets significantly improved some growth performance traits, especially, FCR, egg weight, egg number, fertility, hatchability and, most of productive

organs and digestive tract traits. Additionally, the probiotic supplements remarkably improved the microbiological measures, digestive enzyme activity, and antioxidant enzyme levels. Therefore, incorporating these probiotics into laying quail diets not only supports sustainable production but also contributes to improved physiological function and welfare, enhancing productivity and health outcomes.

Conflict of interests statement

The authors declare that they have no conflict of interests regarding the publication of this article.

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