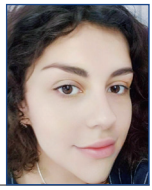
Effects of a probiotic containing *Bacillus subtilis* on the gut microflora, yolk quality and blood lipid concentrations of laying Pharaon quails



L. P. Ermakova, G. A. Nozdrin, S. N. Tishkov, Y. V. Novik, N. A. Gotovchikov and I. K. Mensh*

Abstract

The current literature is not comprehensive concerning the influence of *Bacillus subtilis* on lipid metabolism, egg chemical characteristics, and intestinal microbiota of Japanese quail. The aim of this study was to evaluate the effects of a *Bacillus subtilis* strain on yolk quality, gut bacterial populations, and total cholesterol and triglyceride concentrations in the plasma of organically farmed Pharaon quail layers. Forty-five-day-old female quails were randomly distributed into four groups of 10 birds each: a no-treatment control group and three test groups receiving powder of *Bacillus subtilis* DSM 32424 at a minimum rate of 1×10^6 colony forming units per gram, dissolved in drinking water at doses of 50, 75 and 100 mg per kg body weight, daily during 30 days. The use of the probiotic at doses of 50 and 100 mg resulted in significantly reduced faecal staphylococci count on day 30 ($P<0.05$), while no significant changes were detected in lactobacilli or coliform bacteria. This study gives the first evidence of the influence of *Bacillus subtilis* on acid value and carotenoid levels in egg yolks laid by quails. Carotenoids were significantly elevated in the 75 mg group on day 30 ($P<0.05$), but significantly decreased in the 50 and 100 mg groups ($P<0.05$). Acidity, triglyceride and total cholesterol concentrations in quail serum were not affected significantly by the treatment throughout the study, though their values were reduced. The inclusion of *Bacillus subtilis* DSM 32424 in drinking water for laying Pharaon quails inhibited faecal staphylococci proliferation and enhanced yolk carotenoid content. Therefore, it can be proposed that including this probiotic in laying quail diets may have beneficial outcomes for both layers and the second generation.

Key words: *quail; Bacillus subtilis; gut microflora; triglyceride; total cholesterol; yolk*

Lyudmila P. ERMAKOVA*, PhD-student, (Corresponding author, e-mail: ermakovansau@yahoo.com), Grigory A. NOZDRIN, DVM, PhD, Full Professor, Sergei N. TISHKOV, Undergraduate, the lab head, Yana V. NOVIK, Undergraduate, lecturer, Nikita A. GOTOVCHIKOV, Graduate student, Senior laboratory assistant, Irina K. MENSH, fifth-year student, Faculty of Veterinary Medicine, Novosibirsk State Agrarian University, Novosibirsk, Novosibirskaya oblast, Russia

Introduction

Poultry production diseases, such as clostridiosis or salpingoperitonitis, caused by different types of infectious agents, are responsible for considerable financial losses to commercial producers worldwide, and are thus among the ultimate challenges faced by the global poultry industry (Dittoe et al., 2018; Jones et al., 2019).

Apart from probiotics, effectiveness in the control of pathogenic bacteria has been reported for prebiotic substrates, antimicrobial peptides, and immunostimulants (Redondo et al., 2014; Yadav et al., 2016; Gadde et al., 2017; Denli and Demirel, 2018; Mohammadi Gheisar and Kim, 2018; Suresh et al., 2018). However, the latter may cause immunosuppression if overdosed (Mehana et al., 2015), while a multitude of antimicrobial peptides have low bioavailability and/or high toxicity (Radzishevsky et al., 2007). The pathogen antagonism of prebiotics is lower compared to probiotics (Lee and Salminen, 2009; Valpotić et al., 2016a,b, Valpotić et al., 2017; Valpotić et al., 2018).

Perhaps for these reasons, probiotics are utilized widely in poultry production. Among them, those containing various strains of *Bacillus subtilis* have been documented to suppress the potential of bacterial species such as *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus* by forming a biofilm, colonizing the digestive tract, and suppressing quorum sensing (Gonzalez et al., 2011; Algburi et al., 2017; Piewngam et al., 2018). Probiotic supplements containing *B. subtilis* improve the protective properties of the intestinal barrier and exert anti-inflammatory effects (Rhayat et al., 2019). Aliakbarpour et al. (2012) found that increased expression of the intestinal mucin-2 gene in broilers administered with *B. subtilis* could positively affect the interactions between gut microbial communities, along with mucosal cellular proliferation and, therefore, improve the efficiency of nutrient absorption.

Though many feeding trials involving probiotics in laying quails (*Coturnix coturnix japonica*) have been conducted, the current literature is not comprehensive concerning poultry health issues, such as lipid metabolism, which plays a crucial role in the formation of yolk and body fat deposition in birds. There is virtually no research addressing the influence of *B. subtilis* on intestinal staphylococci populations of Japanese quails. Moreover, to our knowledge, there are no reports of the effects of probiotic supplementation on egg chemical characteristics, such as acid value and carotenoid content. Therefore, the purpose of this study was to investigate the effects of *B. subtilis* strain DSM 32424 on the chemical characteristics of egg yolks (yolk acid value and carotenoid content), gut microflora (faecal coliform, staphylococci and lactobacilli count) and lipid metabolism (serum triglyceride (TG) and total cholesterol (T-CHOL) levels) of laying Pharaoh quails.

Materials and Methods

This experiment was performed on a farm in the Novosibirsk Region of Russia. All procedures involving the birds were conducted in compliance with Directive 2010/63/EU and were approved by the Ethics Committee of the Novosibirsk State Agrarian University (Novosibirsk, Russia). Forty-five-day-old unvaccinated female Pharaon quails were used in this study. They were randomly allocated into four groups of 10 birds each: a no-treatment control group (C) and three test groups receiving powder of *B. subtilis* strain DSM 32424 at a minimum rate of 1×10^6 colony forming units (CFU)/g, dissolved in drinking water at doses

of 50 (T1), 75 (T2) and 100 (T3) mg/kg bodyweight (BW), daily during 30 consecutive days. The composition of the basal diet (produced by the commercial food company) is indicated in Table 1. Quails had free access to feed and water throughout the experiment.

On the day prior to initiating treatment with *B. subtilis* (day -1) blood sampling (via the jugular vein) of five quails randomly selected from the entire flock was undertaken in the morning. On days 15 and 30, individual blood samples were collected from five birds per group, and samples were transferred into non-heparinized tubes and immediately sent to the university laboratory for measurement of serum concentrations of TG and T-CHOL on an automatic biochemical analyser (iMagic V7, Shenzhen iCubio Biomedical Technology, China), following the manufacturer's instructions. Given that ANOVA is robust to small sample sizes, blood sampling was performed on only five quails per group to minimise stress to birds.

On day -1, 10 eggs were collected from the breeding flock and delivered to a certified laboratory (Kraevaya veterinarnaya laboratoriya, Krasnoyarsk) for the analysis of yolk acidity and carotenoid content in accordance with the national standards. A total of 20 eggs (5 from each group) were obtained over 30 days of treatment for repeated evaluation. On the pre-experimental (day -1), and during treatment (on days 15 and 30), faecal samples (3–5 g/quail) were separately collected from each bird, placed in sterile tubes, and transported to a local laboratory (Issledovatel'skij centr, Novosibirsk) for microbial population

Table 1. Ingredients and nutrient composition of the basal diet of laying Pharaon quails (provided by the manufacturer)

Ingredient	%	Nutrient	
Maize	28.00	Metabolizable energy, kcal/kg	2,500
Wheat	23.60	Moisture, %	14.00
Wheat bran	15.00	Crude protein, %	16.00
Sunflower meal	14.07	Crude fibre, %	5.65
Soybean meal	4.60	Natrium, %	0.10
Fodder yeast	4.50	Sodium chloride, %	0.26
Chalk	3.49	Available phosphorus, %	0.70
Limestone powder	3.00	Calcium, %	2.57
Monocalcium phosphate	1.24	Lysine, %	0.69
Fish meal	1.00	Methionine + cysteine, %	0.65
Salt	0.20	Crude fat, %	3.22
Zeolite-A	0.10	–	–
Sodium bicarbonate	0.10	–	–
Methionine	0.10	–	–
Premix*	1.00	–	–

*Premix supplied the following per kg of diet (calculated values): Vitamin A, 1,500,000 international units (IU); vitamin D_3, 300,000 IU; vitamin E, 0.5 g; vitamin K_3, 0.2 g; vitamin B_1, 0.2 g; vitamin B_2, 0.8 g; vitamin B_3, 1.5 g; vitamin B_4, 100.0; vitamin B_5, 3.0 g; vitamin B_6, 0.4 g; vitamin B_{12}, 0.0025 g; vitamin H, 0.02 g; Fe, 2.5 g; Cu, 0.25 g; Zn, 7.0 g; Mn, 10.0 g; Co, 0.1 g; I, 0.07 g; Se, 0.02 g; antioxidant, 0.5 g

Table 2. Mean and standard deviation of serum triglyceride (TG, mmol/L) and total cholesterol (T-CHOL, mmol/L) levels in laying Pharaon quails treated with different levels of *Bacillus subtilis* DSM 32424 at doses: 0 mg/kg bodyweight (C), 50 mg/kg bodyweight (T1), 75 mg/kg bodyweight (T2) and 100 mg/kg bodyweight (T3)

Experimental day	Parameter	Treatment			
		C	T1	T2	T3
-1	TG	10.76 ± 0.03	10.76 ± 0.03	10.76 ± 0.03	10.76 ± 0.03
	T-CHOL	4.95 ± 1.50	4.95 ± 1.50	4.95 ± 1.50	4.95 ± 1.50
15	TG	10.18 ± 0.80	5.29 ± 4.42	5.94 ± 5.05	6,66 ± 5.80
	T-CHOL	4.61 ± 1.16	5.09 ± 3.71	3.99 ± 2.47	1.59 ± 1.98
30	TG	5.33 ± 4.39	9.39 ± 4.04	8.43 ± 5.62	3.08 ± 5.04
	T-CHOL	7.77 ± 5.31	7.17 ± 1.08	4.92 ± 3.37	2.16 ± 3.18

Table 3. Mean and standard deviation of logarithmically transformed colony forming units of faecal bacterial populations per g of faeces (log_{10} CFU/g) from laying Pharaon quails treated with different levels of *Bacillus subtilis* DSM 32424 at doses: 0 mg/kg bodyweight (C), 50 mg/kg bodyweight (T1), 75 mg/kg bodyweight (T2) and 100 mg/kg bodyweight (T3)

Experimental day	Parameter	Treatment			
		C	T1	T2	T3
-1	Coliforms	7.00 ± 0.00	7.07 ± 0.06	7.10 ± 0.00	7.10 ± 0.00
	Staphylococci	6.17 ± 0.15	5.97 ± 0.25	5.80 ± 0.00	5.93 ± 0.12
	Lactobacilli	5.33 ± 0.58	5.33 ± 0.58	5.67 ± 0.58	6.00 ± 0.00
15	Coliforms	7.06 ± 0.05	7.01 ± 0.10	7.03 ± 0.07	7.03 ± 0.07
	Staphylococci	6.26 ± 0.18	6.35 ± 0.14	6.33 ± 0.13	6.34 ± 0.13
	Lactobacilli	5.63 ± 0.52	5.50 ± 0.53	5.50 ± 0.53	5.50 ± 0.53
30	Coliforms	7.06 ± 0.09	7.04 ± 0.18	7.18 ± 0.14	7.08 ± 0.17
	Staphylococci	6.28 ± 0.20	2.34 ± 3.23*	3.94 ± 3.27	2.33 ± 3.21*
	Lactobacilli	5.63 ± 0.52	5.50 ± 0.53	5.50 ± 0.53	5.50 ± 0.53

* = Values differ (P<0.05) from control group

Table 4. Mean and standard deviation of yolk acid value (mg of potassium hydroxide per g yolk) and yolk carotenoid content (mcg/g yolk) in eggs of laying Pharaon quails treated with different levels of *Bacillus subtilis* DSM 32424 at doses: 0 mg/kg bodyweight (C), 50 mg/kg bodyweight (T1), 75 mg/kg bodyweight (T2) and 100 mg/kg bodyweight (T3)

Experimental day	Parameter	Treatment			
		C	T1	T2	T3
-1	Acid value	6.18 ± 0.66	6.18 ± 0.66	6.18 ± 0.66	6.18 ± 0.66
	Carotenoids	18.69 ± 0.23	18.69 ± 0.23	18.69 ± 0.23	18.69 ± 0.23
30	Acid value	5.44 ± 0.27	5.80 ± 0.48	5.88 ± 0.34	5.46 ± 0.42
	Carotenoids	30.18 ± 0.73	27.32 ± 0.77***	33.68 ± 0.82***	22.06 ± 0.38***

*** = Values differ (P<0.001) from control group

counts. Microbial populations were determined by serial dilutions (10^{-2} to 10^{-10} g/mL) of faecal samples in sterile buffer solution prior to inoculation onto Petri dishes. Plates for staphylococci (cultivated on yolk-salt agar), lactobacilli (cultivated on Blickfeldt medium), and coliforms (cultivated on 5% Sheep Blood Agar) were incubated for 24–72 h at 37°C, and bacterial colonies were quantified by enumerating colony forming units (CFUs). The results were expressed as logarithmic ($\log_{10}$) transformation per gram of faeces.

The data are presented as means with standard deviations. Differences between treatment means were subjected to one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test with 0.05 family-wise error rate ($P<0.05$) using GraphPad Prism 8.3.0 for Windows (GraphPad Software, San Diego, California, USA). When residuals were heteroscedastic or non-Gaussian, then Welch or Brown-Forsythe ANOVA tests were applied, respectively.

Results

The effects of the probiotic on lipids in quail plasma are summarized in Table 2. There were no significant differences relative to the control group. Nevertheless, quails that received the probiotic had numerically lessened TG values on day 15 and decreased T-CHOL concentrations on day 30, as opposed to non-treated birds.

The gut microbiome data of quails were not significantly affected by *B. subtilis*, except for those birds given the strain at the doses of 50 and 100 mg/kg BW (Table 3). Their faecal staphylococci counts were lowered ($P<0.05$) in comparison to controls at the completion of the experiment.

Yolk quality results are presented in Table 4. Regardless of dose, yolk acidity in eggs of probiotic-treated did not differ ($P>0.05$) from non-treated birds. As for yolk carotenoid content, there was a significant increase ($P<0.001$) on day 30 for quails that consumed the powder at the dose of 75 mg/kg BW, when compared with control birds. On the contrary, other test groups exhibited significantly lower ($P<0.001$) carotenoid levels in egg yolk, relative to controls.

Discussion

Oxidation of egg yolk lipids is the principal source of energy for embryonic growth and development (Şahan et al., 2014; Van Der Wagt et al., 2020). On the other hand, excessive peroxide formation in yolk during the prenatal period may have a detrimental impact on essential biological processes in a developing avian organism, with subsequent implications for its reproductive performance, immune response capability and survival (Parolini et al., 2017; Watson et al., 2018). Therefore, it is of vital importance to minimize oxidative damage during hatchling development. In the present study, the acid value was examined to indicate the degree of yolk lipid oxidation of quail eggs.

Although several studies have revealed the beneficial effects of *B. subtilis* on antioxidant enzyme activity in the serum, jejunum and liver of various bird species (Li et al., 2011b; Rajput et al., 2013; Abdel-Moneim et al., 2020), molecular mechanisms of an antioxidant response of *B. subtilis* have not yet been fully elucidated. According to Helmann et al. (2003), *B. subtilis* might elaborate resistance factors protecting cells from reactive oxygen species. In this regard, biosensor-based assays (Prazdnova et al., 2015) showed superoxide scavenging capacity of *B. subtilis* KATMIRA1933 supernatant. In this study, *B. subtilis* DSM 32424 had no significant effect on acidity in egg yolk, whereas carotenoid levels were

significantly higher or lower in yolks of birds receiving the probiotic. Yolk carotenoids are chain-breaking antioxidants with immunostimulatory and regulatory effects on the developing chick. Their transfer from dietary components into the embryonic tissues is believed to reduce the susceptibility of the embryo to lipid peroxidation (Surai et al., 2016).

The evidence suggests that some *Bacillus* species are carotenoid-producing (Hong et al., 2008; Crescenzo et al., 2017), though there are no literature reports of this for birds. However, Tang et al. (2015) observed no significant impact of a multistrain probiotic on the carotenoid levels in the yolks of laying hens. The authors expressed the view that this is owing to the prevalence of maize in the basal feed (57.78%), which provided birds with a sufficient source of carotenoids, thereby the surplus could not substantially affect the corresponding parameter. In this study, the basal diet consisted of 28.0% maize, which may have contributed to the statistically significant fluctuations in carotenoid levels. However, the effect direction of the studied strain on this indicator is unclear.

Thus, our data indicate that the influence of *B. subtilis* DSM 32424 supplementation to the diets of Pharaon quail layers on the carotenoid status and the oxidative resistance of egg yolks is inconsistent.

In line with Kalafova et al. (2018), this study did not revealed significant effects of *B. subtilis* supplementation on TG or T-CHOL content in the plasma of Japanese quails. Some differences between control and test groups were substantial but statistically insignificant, conceivably owing to the dependence of P values on sample sizes (Sullivan and Feinn, 2012), which were rather small in this study. However, there was a declining trend in the levels of T-CHOL and TG in the birds fed with a probiotic containing *B. subtilis*, which is consistent with the observations of Mahdavi et al. (2005), Li et al. (2011a) and Manafi et al. (2016).

Interaction between gut microflora and the metabolism of bile acids is well established (Dawson and Karpen, 2015). Sharifi et al. (2013) demonstrated how serum TG, T-CHOL, and low-density lipoprotein cholesterol levels could rise due to curtailed deconjugation of bile salts in virtue of declined populations of luminal bacteria.

The mechanisms underlying the cholesterol-lowering action of probiotics include assimilation or binding of cholesterol, and the enzymatic deconjugation of primary bile salts by bile salt hydrolases, with their consequential hydrolysis into secondary bile salt poorly reabsorbed by the intestines. This leads to intensified hepatic cholesterol mobilization, and with it a decrease in the enterohepatic circulation of bile salts and their faecal elimination (Begley et al., 2006; Cho and Kim, 2015).

Furthermore, probiotics may exhibit suppression against lipoprotein lipase, thus preventing hepatic uptake of chylomicron remnants, in turn reducing triglyceride absorption into plasma (Cho and Finocchiaro, 2009; McGuire et al., 2016).

Pathogenic bacteria, *inter alia* coliforms, can become detrimental for birds. Notably, pathogens consume carbon and energy from a host organism, catabolizing its macromolecules (Somerville and Proctor, 2009). In the present study, feeding quails with a *Bacillus* probiotic did not significantly influence the occurrence of coliform bacteria in faeces as compared to the control, which corroborates the literature data (Siriken et al., 2003; Bahrampour et al., 2020; Jazi et al., 2020). Nevertheless, Deniz et al. (2011) and Manafi et al. (2016) revealed diminishing levels of caecal coliforms in Japanese quails given probiotics containing *B. subtilis*.

Following germination, *B. subtilis* spores might generate an anaerobic environment for the growth and proliferation of lactobacilli species through intestinal oxygen uptake (Jazi et al., 2020). Lactobacilli, in turn, have been proven to reduce pathogen viability through competitive exclusion (Dittoe et al., 2018). Our results showed no considerable effect of the dietary inclusion of *B. subtilis* DSM 32424 on lactobacilli in the faeces of quails. This is in agreement with the reports of Siriken et al. (2003), Deniz et al. (2011) and Bahrampour et al. (2020).

Pathogenic staphylococci are characterised by having pronounced phenotypic plasticity, biofilm formation, high virulence potential and antibiotic and oxidative stress resistance (Becker et al., 2007). These bacteria are apt to incite acute and chronic infections in a variety of host species (Gaupp et al., 2012). Interestingly, Lubianskienė and Butkauskas (2000) detected the inhibitory activity of microorganisms in the gastrointestinal tract of Japanese quails against *S. aureus*. Literature data on the impact of *B. subtilis* on intestinal staphylococci numbers in quails are lacking. In this study, the dietary inclusion of *B. subtilis* DSM 32424 significantly lowered the staphylococci population ($P<0.05$) in the faeces of quails on day 30 of treatment. Parallel to our results, Li et al. (2016) found decreased caecal concentrations of *Staphylococcus* bacteria in response to the addition of *B. subtilis* CGMCC 1.1086 to broiler diets. This may imply the antagonistic activity of *B. subtilis* against staphylococci, though further evaluation of the topic is required.

Conclusions

In summary, the results show that supplementation of 1×10^6 CFU/g *B. subtilis* DSM 32424 in organically farmed Pharaon quail layers might significantly down-regulate faecal staphylococci proliferation and enhance yolk carotenoid levels, though the latter was affected in contradictory ways. Other evaluated parameters of quails were not influenced by the treatment in a statistically significant manner. Nonetheless, the probiotic may also be capable of improving TG and T-CHOL concentrations in quail serum. Given the positive changes in blood lipid counts, gastrointestinal bacteria colonization, and yolk quality, it can be proposed that the inclusion of *B. subtilis* DSM 32424 in laying quail diets may have beneficial outcomes for both layers and the second generation.

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Učinci probiotika koji sadrže *Bacillus subtilis* na crijevnu mikrofloru, kakvoću žutanjka i koncentracije lipida u krvi prepelica nesilica pasmine faraon

Lyudmila. P. ERMAKOVA*, doktorandica, Grigory A. NOZDRIN, dr. vet. med., dr. sc., redoviti profesor, Sergei N. TISHKOV, student preddiplomskog studija, voditelj laboratorija, Yana V. NOVIK, studentica preddiplomskog studija, lektor, Nikita A. GOTOVCHIKOV, studentica diplomskog studija, viši laboratorijski asistent, Irina K. MENSH, studentica pete godine, Veterinarski fakultet, Državno Poljoprivredno Sveučilište Novosibirsk, Novosibirsk, Novosibirskaya oblast, Rusija

Ovaj rad nije sveobuhvatan obzirom na utjecaj *Bacillus subtilis* na metabolizam lipida, kemijska svojstva jaja te crijevne mikroorganizme japanskih prepelica. Istraživanje je obavljeno u svrhu procjene učinaka *Bacillus subtilis* soja na kakvoću žutanjka, populacije bakterija u crijevima uz ukupne koncentracije kolesterola i triglicerida u krvi organski uzgajanih prepelica nesilica pasmine faraon. Četrdesetpetodnevne ženke prepelice nasumice su podijeljene u četiri skupine od po 10 ptica: kontrolnu skupinu koja nije primala dodatak prehrani i tri pokusne skupine koje su primale prašak *Bacillus subtilis* DSM 32424 pri najmanjem postotku od 1×10^6 jedinica koje stvaraju koloniju po gramu, otopljen u pitkoj vodi u dozama od 50, 75 i 100 mg po kilogramu tjelesne težine, svakodnevno tijekom 30 dana. Uporaba probiotika u dozama od 50 i 100 mg rezultirala je značajnim smanjenjem količine fekalnih stafilokoka na 30. dan (P<0,05), dok značajnije promjene laktobacila i koliformnih bakterija nisu otkrivene. Ovo je istraživanje prvi put pokazalo utjecaj *Bacillus subtilis* na vrijednost kiseline i razine karotenoida u žutanjcima koje nesu prepelice. Karotenoidi su se značajno podigli na 30. dan u skupini koja je primala 75 mg (P<0,05), ali su se i značajno smanjili u skupinama koje su primale 50 i 100 mg (P<0,05). Terapija nije statistički značajno djelovala na kiselinu. Pokusni dodatak nije značajno utjecao niti na koncentracije triglicerida i ukupni kolesterol u krvi prepelica tijekom ispitivanja, premda su te koncentracije dosta smanjene. Na temelju dobivenih rezultata, zaključeno je da je dodavanje *Bacillus subtilis* DSM 32424 u pitku vodu za prepelice nesilice pasmine Faraon gotovo na svim proučavanim razinama inhibiralo proliferaciju fekalnih stafilokoka i povećalo udio karotenoida u žutanjku. Stoga se može reći da uključivanje probiotika u prehranu prepelica nesilica može imati korisni učinak i na nesilice i na drugu generaciju.

Ključne riječi: *prepelica, Bacillus subtilis, crijevna mikroflora, trigliceridi, ukupni kolesterol, žutanjak*