Effects of lactobacilli cultures on egg quality of quails

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Abstract. This study investigates the effects of dietary supplementation with the probiotics on the egg quality parameters and cecum microbiota of quails. Quails were randomly divided into four groups, with nine birds in each group. The control group received a basal diet supplemented with a nutrient base for probiotics (10% clarified whey, 1% yeast extract, and 0.5% sucrose). The three experimental groups received the same diet, but supplemented with 1×10^{10} CFU/kg of *Limosilactobacillus fermentum* HFD1, *Lactiplantibacillus plantarum* FCa3L, or *Lactiplantibacillus plantarum* LS 4-4. The use of probiotic feed supplements led to an increase in shell strength, which improved the transportability of eggs. Dietary supplementation with *L. plantarum* FCa3L resulted in a statistically significant increase in eggshell hardness. In all experimental groups, serum calcium levels were within acceptable limits and did not differ significantly from the control group. The 16S ribosomal RNA-based sequencing technique was used to characterize the cecum microbiota of quails fed with different lactobacilli strains. We observed certain positive changes in the gut microbiota of quails that were fed with *L. fermentum* HFD1 and *L. plantarum* FCa3L. However, the supplementation of the diet with *L. plantarum* LS4-4 had little effect on the gut microbiota of quails.

1 Introduction

Quail egg market is a fast-growing sector in the poultry industry. Compared to chicken eggs, the leading product in this sector, quail eggs contain a higher proportion of yolk and a greater concentration of iron, selenium, vitamin B12, and riboflavin. Besides nutritional value, quail eggs are characterized by the culinary versatility. Their small size makes them ideal for garnishing salads, soups, and other dishes. They can also be pickled, marinated, added in baking or used as a quick snack when boiled or fried [1].

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Feeding is the main technological factor that determines not only the level of egg production, but also the commercial, nutritional and taste qualities of eggs. Probiotics are gaining increasing popularity in poultry industry as a feed additive and a good alternative to feed antibiotics. The mechanisms how probiotics positively influence egg production are different and not all are fully understood. They help maintain a healthy gut microbiota, enhance nutrient absorption, and boost the immune system. Probiotics can be administered through feed or drinking water. Different probiotic strains and concentrations can have varying effects [2].

The purpose of this work was to evaluate the effects of dietary supplementation with the biomass of *Limosilactobacillus fermentum* HFD1, *Lactiplantibacillus plantarum* FCa3L, or *Lactiplantibacillus plantarum* LS 4-4 on the egg quality parameters and cecum microbiota of quails.

2 Materials and Methods

2.1 Ethical approval

This study was conducted in strict compliance with the EU Directive 2010/63/EU for animal experiments. The experimental protocol was reviewed and approved by the Local Ethics Committee of Kazan Federal University (Protocol No. 40, date 9 March 2023). All procedures involving animals were carried out with the utmost care to minimize stress and discomfort, ensuring adherence to the principles of the 3Rs (Replacement, Reduction, and Refinement) in animal research.

2.2 Probiotic preparation

Limosilactobacillus fermentum HFD1, *Lactiplantibacillus plantarum* FCa3L, or *Lactiplantibacillus plantarum* LS 4-4 were cultured in De Man, Rogosa, and Sharpe (MRS) broth at 37 °C for 24 h under microaerophilic conditions. The bacterial cells were harvested by centrifugation at 5000 rpm for 10 min, washed twice with sterile physiological saline, and resuspended in a nutrient base of the probiotic (10% clarified whey, 1% yeast extract, and 0.5% sucrose) to achieve a final concentration of 1×10^9 CFU/mL. The probiotic suspension was mixed with the feed to ensure a total bacterial count of 1×10^{10} CFU/kg of the diet.

2.3 Experimental design and animal management

15-day-old Texas white quails were housed in three-tiered cages with a holding capacity 15 heads per cage, had 16 h of lighting daily, and had *ad libitum* access to feed and water. A 10-day period before starting the experiment was considered as an adaptation. The quails of all groups were fed with feedstuff PC5 (JSC Bogdanovichi Feed Mill, Russia) during the first month of bird life and DK-52 for quails (Glazovsky Feed Mill, Russia) starting with the fifth week (Table 1). The basal diets were calculated to meet the requirements of animals according to NRC (1994) standards [3].

The quails were randomly divided into four groups with eight quails per group. In the experimental groups, the probiotic suspensions were mixed with the feed to ensure a total bacterial count of 1×10^{10} CFU/kg of the diet. The quails of the control group received the nutrient base in the same concentration. The trial lasted for 63 days (nine weeks).

2.4 Egg analysis

During the laying phase (50–87 days) egg production was recorded. Eggs were collected and the following parameters were assessed: egg weight, yolk and white weight, yolk height and diameter, white height and diameter, albumen percentage, shell weight and percentage, shell thickness, membrane thickness, and yolk color. The yolk index (albumin) was calculated by dividing the height of the yolk (albumin) by the diameter of the yolk (albumin) of an egg broken onto a flat surface. To determine Haugh units, the egg weight and the height of the egg after cracking on a flat surface were determined. Haugh units were calculated as described by [4]. Eggshell breaking strength was measured using an eggshell force gauge (Robotmation Co. Ltd.). Eggshell thickness was measured using an ultrasonic thickness gauge (Robotmation Co. Ltd.). Eggs were broken, the internal contents were removed, and eggshells were dried at room temperature for 48 h to determine eggshell weight with a 0.001 g accuracy. Yolk color was measured using a CR20 Colorimeter (PRC) and expressed in units: *L*, black-white (0–100); *a*, green-red (–60 – +60); *b*, blue-yellow (–60 – +60).

Table 1. Ingredients and nutrient composition of the basal quail diet.

PK5 (JSC Bogdanovichi Feed Mill, Russia)		**DK52 for quails (Glazovsky Feed Mill, Russia)**	
Ingredients	%	Ingredients	%
Wheat		Wheat	
Barley		Barley	
Corn		Corn	
Corn gluten		Sunflower meal	
Soybean meal (46% CP)	48	Soy oil meal	
Soy full-fat extruded	37	Soy full-fat extruded	
Sunflower oil unrefined 1c		Meat flour	
Fish meal SP63		Sunflower oil	
Feed yeast		Limestone flour	
Lysine L monochlorohydrate 98/78%		Shell flour	
Methionine DL 99.0%,		Sodium carbonate	
Sodium chloride		Phosphates	
Sodium sulfate		Amino acids	
Monocalcium phosphate		Organic acids	
Limestone flour PS2		Enzyme complex	
		Vitamin-mineral premix[1]	
Calculated nutrients[2]		*Calculated nutrients*[2]	
Metabolizable energy (ME) kcal/kg	2944.3	Metabolizable energy (ME) kcal/kg	2880
Crude protein	22.30	Crude protein	18.05
Crude fiber	5,00	Raw fat	4.08
Calcium	0.93	Crude fiber	3.83
Available phosphorus	0.60	Linoleic acid	1.70
Lysine	1.17	Calcium	3.63
Sodium	0.18	Available phosphorus	0.77
Chlorine	0.20	Lysine	0.96
Moisture	0.00	Methionine	0.55
		Methionine + Cystine	0.82
		Threonine	0.58
		Tryptophan	0.20
		Sodium	0.24
		Chlorine	0.25

[1]Premix provided: vitamins A, D3, E, K3, B1, B2, B3, B4, B5, B6, B12, Bc, H, Fe, Cu, Zn, Mg, Co, I, and Se.

[2]According to NRC (1994) [3].

2.5 Blood sampling and laboratory analyses

Blood samples were collected post-mortem from the jugular vein. Serum was analyzed for calcium content using SPINREACT S.A. kits (Spain) at the ChemWell 2902 (Awareness Technology, USA).

2.6 Cecal microbiota analysis

The contents of the cecum of quails were used as samples for the analysis of the intestinal microbiota by sequencing of the 16S rRNA gene V3-V4 variable regions with an Illumina MiSeq sequencer at a 2 × 300 base pair mode. Software tools used for sequence analysis included QIIME v. 1.9.1 and GreenGenes v. 13.8.

2.7 Statistical analysis

Statistical analyses were performed using either a one-way ANOVA with Holm-Sidak post-hoc test or a Mann-Whitney test, both implemented in GraphPad Prism 6.0 for Windows (GraphPad Software). Data are expressed as the mean ± standard deviation (SD), unless otherwise specified. Differences were considered statistically significant at $p < 0.05$; n indicates the number of birds.

3 Results and Discussion

3.1 Effects of lactobacilli dietary supplementation on the egg quality parameters and blood calcium level of quails

Feeding is a key technological factor that determines not only egg production but also the marketability, nutritional quality, and taste of eggs. We assessed the effects of lactobacilli-containing probiotic feed additives on the egg quality parameters, like egg weight, albumen and yolk weight, yolk height and diameter, albumen height and diameter, albumen percentage, shell weight and percentage, shell thickness, membrane thickness, and yolk color (Table 2). Haugh units were used as a comprehensive indicator of egg white quality. This important industrial indicator of egg quality takes into account the egg weight and the height of the egg white. The higher the value, the better the quality of the egg [4]. According to the requirements for hatching quail eggs, Haugh units should exceed 87. The yolk index (albumin) is calculated by dividing the height of the yolk (albumin) by the diameter of the yolk (albumin) of an egg broken onto a flat surface. As egg quality deteriorates, the yolk index becomes lower, since the fibrous structure of the vitreous membrane weakens, and the strength of the membrane decreases over time. Of the protein quality indicators, Haugh units have the highest positive correlation with the albumen index (r = 0.85–0.95). Both indicators are determined based on measuring the height of the dense albumen. The difference is that Haugh units reflect the height of the dense albumen state adjusted for the egg weight, while the albumen index reflects the amount of dense albumen, measured indirectly by the diameter of the glass surface occupied by it. The similarity of these indicators is explained by the fact that the amount of dense albumen increases almost proportionally to the increase in egg weight.

Dietary supplementation of *L. plantarum* FCa3L and *L. plantarum* LS 4-4 resulted in the increase of the average egg weight compared to the control group. The egg shape index and Haugh units in the experimental groups ranged from 74.22±4.46 to 78.24±4.04 and from 103.71±0.38 to 104.16±0.29, respectively. These values were within acceptable limits for fresh eggs and did not differ significantly from the control values of 78.18±2.88 and 103.85±0.36, respectively.

In the group fed with *L. fermentum* HFD1 we detected low yolk weight (3.52±0.21), but the yolk proportion, conversely, was high, which is typical for small eggs. Indeed, the egg weight in this group was the smallest among the experimental groups (11.73±2.31) but comparable to that in the control (11.69±1.49). In proportion to the yolk parameters, the albumen weight and its proportion were lower than in the control and other experimental groups.

Yolk color may vary from pale yellow to deep orange. Although yolk color does not correspond to the nutritional value of the eggs, it is worth mentioning that diet supplementation with *L. plantarum* FCa3L resulted in lighter yolk color, as indicated by a higher *L* value, with greenish and blue components, indicated by lower values of *a* and *b*, respectively.

The thickness of the egg membrane in the experimental groups did not differ from the control values. The puncture resistance of the eggshells in all experimental groups was 1.06–1.89 times higher than in the control. *L. plantarum* FCa3L supplementation resulted in the statistically significant increase of the eggshell hardness. The proportion of shell and its thickness did not differ significantly from the control values, but in the group fed with *L. plantarum* LS 4-4, we noted a tendency towards a decrease in shell thickness (decreased by 20% compared to the control) (Table 2). The strength and marketability of the shell depend on the mineral nutrition of the poultry. The main mass of the shell (94%) is calcium carbonate. Calcium and sodium, as well as trace elements such as manganese and zinc, play an important role in shell formation. Blood analysis of quails revealed a higher level of total calcium in all experimental groups compared to the control samples (Table 2). The ability of probiotic lactobacilli to increase calcium absorption and its levels in the blood was well documented [5]. Thus, the use of a probiotic feed supplement resulted in increased shell strength without increasing shell weight in all experimental groups, which improves the transportability of such a fragile product as eggs and, consequently, reduces losses during transportation to the consumer.

Table 2. Effects of dietary addition of *Limosilactobacillus fermentum* HFD1, *Lactiplantibacillus plantarum* FCa3L, and *Lactiplantibacillus plantarum* LS 4-4 on the egg quality and serum calcium level of quails (mean ± SD).

	CON	**HFD1**	**FCa3L**	**LS 4-4**
Egg				
Weight, g	11.69±1.49	11.73±2.31	14.21±2.82	13.36±2.62
Egg shape index	78.18±2.88	77.12±2.45	78.24±4.04	74.22±4.46
Haugh units	103.85±0.36	104.16±0.29	103.75±0.28	103.71±0.38
Eggshell				
Eggshell, %	8.88±0.9	9.81±1.09	9.06±1.03	8.34±0.67
Eggshell thickness, mm	0.35±0.07	0.36±0.05	0.37±0.07	0.28±0.05
Eggshell breaking strength, kg	0.78±0.38	0.90±0.03	1.46±0.15*	0.88±0.27
Membrane thickness, mm	0.06 ± 0.03	0.08 ± 0.05	0.08 ± 0.03	0.06 ± 0.03
Yolk				
Yolk weight, g	3.39±0.75	3.52±0.21	4.67±1.31	4.79±1.07
Yolk fraction, %	30±3.68	37.07±14.75	31.74±3.38	34.23±3.28
Yolk Index	45.55±4.15	45.28±3.07	45.42±2.69	47.57±5.07
Yellowness	1.9 ± 0.1	1.5 ± 0.1	1.7 ± 0.2	1.5 ± 1.1
Redness	30.4 ± 0.8	28.0 ± 5.2	29.9 ± 1.2	30.4 ± 1.5
Yolk color				

L	55.28±0.89	55.33±0.48	56.22±0.98	54.94±1.34
a	5.47±1.09	5.23±1.2	4.64±0.62	4.86±0.95
b	21.19±0.48	21.38±1.14	20.99±0.65	21.16±0.77
Egg white				
Egg white weight, g	8.16±1.97	5.82±3.19	8.37±1.67	8.1±1.81
Albumin percentage, %	61.12±3.55	53.12±15.43	59.21±3.28	57.44±3.18
Egg white index, %	7.75±1.86	7.63±2.69	6.95±1.45	6.24±1.41
Blood calcium	9.7±2.2	11.2±1.6	10.9±2.1	10.4±1.5

* $p < 0.05$ compared to the control group.

3.2 Modulation of cecum microbiota of quails by lactobacilli supplementation

Poultry gut microbiota significantly impacts egg quality parameters like yolk and white characteristics, albumin quality, and shell strength by influencing nutrient utilization and and resistance to infections and stresses [6]. To study the effect of lactobacilli supplementation on quail gut microbiota, we analyzed the bacteria composition of their cecum using 16S rRNA gene sequencing on the Illumina MiSeq platform. The alpha-diversity indices, namely Shannon index, Faith's PD phylogenetic diversity index, and the number of ASV (amplicon sequence variants), did not differ significantly between the groups (Table 3). To assess beta-diversity, we calculated unweighted and weighted UniFrac distances with further visualization using principal coordinate analysis (PCoA). The results showed that the intestinal microbiota composition was more heterogeneous in the control group, while ehe microbiota samples of the three experimental groups were similar to each other.

In all tested groups, *Firmicutes* (also known as *Bacillota*) and *Bacteroidota* constituted the main bulk of cecal microbiota, which is consistent with literature data [7]. The ratio of *Firmicutes* to *Bacteroidota* (F/B) is often used as a characteristic of the gut microbiota health. Dysbiosis and other pathologies are frequently associated with the dominance of one phylum over another [8]. In the control group, the relative abundance of *Firmicutes* was 5.4 times higher than that of *Bacteroidota*. Diet supplementation with *L. fermentum* HFD1 and *L. plantarum* FCa3L had a positive effect on the intestinal bacterial community, leading to a balance between *Firmicutes* and *Bacteroidota* (F/B = 0.83 and F/B = 0.81, respectively) (Table 4).

The cecal microbiota of quails is mainly composed of the families *Bacteroidaceae, Lachnospiraceae, Ruminococcaceae,* and *Enterobacteriaceae*. *Bacteroides, Ruminococcus, Faecalibacterium, Enterococcus,* and *Clostridium* are usually the most prevalent genera in this area [7]. Indeed, these families were present in the studied cecum bacterial communities, but their abundances varied greatly (Table 4). In groups fed with standard diet without additives (control) and biomass of *L. plantarum* LS 4-4, *Bacteroidaceae, Lachnospiraceae*, and *Ruminococcaceae* constituted a total of 33.7% and 31.1%, respectively. In other groups receiving probiotic supplements, the proportion of these three families was significantly higher: 53.1% in the group, which received *L. fermentum* HFD1, and 57.3% in the group, which received *L. plantarum* FCa3L.

In the control group, the most numerous families in the cecum bacterial community, in descending order, were *Ruminococcaceae, Bacteroidaceae, Nanoperiomorbaceae, Lactobacillaceae,* and *Lachnospiraceae*. The use of the feed additives led to a significant increase in the proportion of *Bacteroidaceae* in the intestinal microbial community of quail. In contrast, probiotic intake induced reduction in the relative abundance of butyrate-producing *Ruminococcaceae*.

Surprisingly, the abundance of the *Lactobacillaceae* family in the ceca of quails fed *L. fermentum* HFD1 and *L. plantarum* FCa3L was lower than in the control. Contradictory information is available on the effect of lactobacilli-containing probiotics on the amount of lactobacilli in the gut. According to Siadati et al., consuming probiotics caused lactobacilli to colonize the small intestine and cecum; however, the quantity of these bacteria did not correspond with the dosages of lactobacilli that were given [9]. Additionally, Yu et al. found that the broilers provided probiotics had greater lactobacilli populations in their ileum, crop, and cecum than the control group [10]. Beck et al. showed that lactobacilli-containing probiotics did not enhance the amount of lactobacilli in the gut microbiota, which is consistent with our findings [11]. One may speculate that the supplemented lactobacilli did not impact the microbiota in the cecum because they directly colonized the colon.

For the first time, we found the families *Nanoperiomorphaceae, Acutalibacteraceae,* and *Atopobiaceae* in the ceca of quails from all groups. These taxa may have previously been categorized as unclassified. The most common genera within these families were *Nanoperiomorbus, Hydrogeniiclostridium*, and *Thermophilibacter*, which we also identified in quail gut for the first time (Table 4).

Table 3. Effects of dietary supplementation of *Limosilactobacillus fermentum* HFD1, *Lactiplantibacillus plantarum* FCa3L, and *Lactiplantibacillus plantarum* LS 4-4 on the alpha-diversity indices of quail cecum microbiota (mean±SD).

	CON	**HFD1**	**FCa3L**	**LS 4-4**
Raw reads	31290±7387	28692±5403	34726±12695	25677±6680
Non-chimeric reads	31176±7371	25351±6188	31286±12644	23992±7371
Alpha-diversity indices:				
Observed ASV (amplicon sequence variants)	125±45	151±25	140±53	123±48
Faith's PD	11.4±3.4	12.4±1.1	12.1±2.6	10.1±2.8
Shannon	4.8±0.9	5.3±0.5	4.7±0.8	4.6±1.3

Table 4. Effects of dietary addition of *Limosilactobacillus fermentum* HFD1, *Lactiplantibacillus plantarum* FCa3L, and *Lactiplantibacillus plantarum* LS 4-4 on the bacterial composition in the cecum of quails (median relative abundances, %).

Taxa	**CON**	**HFD1**	**FCa3L**	**LS 4-4**
Phylum *Actinobacteriota*	3.95	8.81	7.14	8.32
Family *Bifidobacteriaceae*	1.44	3.08	2.75	4.81
*Bifidobacterium*_388775	1.44	3.08	2.75	4.81
Family *Atopobiaceae*	0.76	1.72	2.32	0.65
Thermophilibacter	0.74	1.52	2.19	0.65
Family *Coriobacteriaceae*	0.59	2.12	0.93	1.47
Phylum *Bacteroidota*	10.19	41.94	39.93	18.37
Family *Bacteroidaceae*	8.42	36.83	35.98	16.69
*Bacteroides*_H	3.50	13.51	19.09	2.58
*Phocaeicola*_A_858004	1.66	9.16	8.97	1.86
Prevotella	1.25	5.26	1.43	0.90
Family *Rikenellaceae*	0.58	2.07	1.29	1.08
*Alistipes*_A_871400	0.19	1.28	0.37	0.51
Phylum *Campylobacterota*	0.15	6.20	0.15	0.83
Family *Helicobacteraceae*	0.15	6.20	0.15	0.83
Phylum *Firmicutes*	54.97	34.70	32.46	44.28
Family *Lachnospiraceae*	5.07	7.33	9.56	6.41
*Mediterraneibacter*_A_155507	1.83	3.68	2.99	2.12
Family *Acutalibacteraceae*	2.86	4.69	3.93	2.25
Acutalibacter	1.05	0.79	1.58	0.47
*Eubacterium*_R	0.19	0.96	0.77	0.78
Hydrogeniiclostridium	0.51	2.08	0.84	0.52

Family *Oscillospiraceae*_88309	1.25	2.44	1.39	1.89
Lawsonibacter	0.73	1.11	0.59	0.60
Family *Ruminococcaceae*	20.20	8.89	11.77	7.99
Faecalibacterium	7.58	2.36	7.16	1.69
*Gemmiger*_A_73129	1.48	1.61	2.54	0.45
Family *Lactobacillaceae*	5.82	0.31	0.30	15.59
*Weissella*_A_338544	5.33	0.04	0.09	10.31
Family CAG-826	0.03	3.00	3.82	0.19
Onthovivens	0.03	3.00	3.82	0.19
Phylum *Patescibacteria*	7.68	1.49	4.65	1.36
Family *Nanoperiomorbaceae*	7.67	1.49	4.65	1.36
Nanoperiomorbus	7.67	1.49	4.65	1.36

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