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Effects of spore-forming *Bacillus* probiotics on growth performance, intestinal morphology, and immune system of broilers housed on deep litter

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Primary Audience: Researchers, Microbiologists, Nutritionists, Veterinarians

SUMMARY

Aim of the Study: To evaluate the effects of feed supplementation of *Bacillus subtilis* KATMIRA1933 (0.1%), *B. subtilis* KB41 (0.1%), and *Bacillus amyloliquefaciens* KB54 (0.1%) probiotics on growth performance, biochemical blood parameters, intestinal morphology, and the immune system of Ross 308 broilers housed on deep litter. Methods and Results: A total of 160 newly hatched Ross 308 broilers were involved in the 42-day-long study with evaluation of growth performance at 7, 14, 21, 28, 35, and 42 d and biochemical blood analysis, histological investigation of jejunum tissues, and analysis of IL-6 and IL-10 gene expression of broilers at 42 d. As a result, probiotic KB41 significantly improved growth performance on the 42nd day of the experiment ($P < 0.01$), increased the number of *Lactobacillus* bacteria in the ceca ($P = 0.03$), and promoted IL-6 and IL-10 genes expression in the spleen of the chickens ($P < 0.01$). The results of this study generally correspond to our previous study, which included an evaluation of *Bacillus*-based KB41 and KB54 probiotics on production performance and the immune system of broilers housed in cages, although the effects are less prominent. Conclusions: Less prominent effects are probably linked to the colonization of the broilers' gastrointestinal tract with unknown bacilli of apparent environmental origin, as the

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broilers were housed in standard conditions. Significance and Impact of Study: This study provides more evidence of *B. subtilis* KB41 and *B. amyloliquefaciens* KB54 effectiveness in poultry.

Key words: *Bacillus*, probiotics, poultry, growth performance, gut microbiota

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DESCRIPTION OF PROBLEM

The use of probiotics as natural growth promotors is gaining more popularity amid the ban on the use of antibiotic growth promotors in the poultry industry (Popov et al., 2021a; Ruiz Sella et al., 2021). Among the most popular probiotics in poultry are lactic acid bacteria (**LAB**) due to their ability to inhibit pathogens and promote beneficial bacteria in the gut microbiota of animals (Patterson and Burkholder, 2003). However, as they are mostly anaerobic or microaerophilic, the biotechnological processes of probiotics development, manufacturing, and usage could have some limitations in creating the proper environment for the bacteria (Wang et al., 2021; Zoghi et al., 2021). On the other hand, spore-forming probiotics do not have these problems due to their unique ability for encapsulation, which grants them protection from the harshest conditions during probiotics manufacturing processes and results in better availability for host organisms because of their high survival rate in different regions of the gastrointestinal tract (**GIT**) of animals (Mazanko et al., 2022).

Spore-forming probiotics are able to improve poultry health and productivity through the inhibition of pathogenic bacteria in the gut microbiota (Tazehabadi et al., 2021), promotion of average daily weight gain (**ADWG**) (Ye et al., 2020), increased egg quality and production rates (Liu et al., 2019), meat and sperm quality (Duskaev et al., 2020), and a reduction in feed intake (**FI**) while improving the feed conversion ratio (**FCR**) (Rivera-Perez et al., 2021). However, some studies do not show a statistically significant effect due to the use of spore-forming probiotics in poultry (Bai et al., 2018; Oladokun et al., 2021). The effectiveness of spore-forming probiotics strongly depends on environmental conditions, especially the rearing conditions (Popov et al., 2021a).

The aim of this study is to assess the effects of *Bacillus subtilis* KATMIRA1933, *Bacillus subtilis* KB41, and *Bacillus amyloliquefaciens* KB54 spore-forming probiotics on growth performance, gastrointestinal colonization, intestinal morphology, and immune modulation in poultry housed on deep-litter.

MATERIALS AND METHODS

Probiotic Strains and Probiotics Preparation

B. subtilis KATMIRA1933, *B. subtilis* KB41, and *B. amyloliquefaciens* KB54 probiotics were tested in our previous studies (Chistyakov et al., 2015; Makarenko et al., 2019; Mazanko et al., 2022). These strains have been shown to have antioxidant and antimutagenic activity, and when used as a probiotic, they increase weight gain and egg production in birds.

We prepared a feed additive based on these strains by solid-state fermentation. The protocol for solid-state fermentation has been described in detail previously (Scanes et al., 2015). Briefly, soybeans were inoculated with an overnight culture of the studied *Bacillus* strains and grown for 2 d at 42˚C. The fermented substrate was then milled and dried. The final spore content in the poultry feed was 10^5 CFU/g.

Experimental Design

A total of 160 newly hatched Ross 300 broiler chickens were randomly divided into 4 groups, including the control group, with 4 replicates per group with 10 broilers in each replication. The 3 treatment groups were coded as T1, T2, and T3, and their feed was supplemented with *B. subtilis* KATMIRA1933 (0.1%), *B. subtilis* KB41 (0.1%), and *B. amyloliquefaciens* KB54 (0.1%), respectively. The control group was without probiotic supplementation to the diet (CON).

Birds, Diet, and Management

This study was carried out for 42 d at the poultry facility of the Stavropol State Agrarian University. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Don State Technical University, Rostov-on-Don, Russia (Protocol No. 5 31.05.2021). Each replicate group was housed separately on a deep litter made of cut wheat straw with a depth of 4 cm under the following light cycles: 24 h light for 1 d, 23 h light/1 h dark for 2 d, 18 h light/6 h dark for 3 to 9 d, 15 h light/9 h dark for 10 to 20 d, 12 h light/12 h dark for 21 to 35 d, 23 h light/1 h dark for 36 to 42 d. The initial room temperature was fixed at 32˚C on d 1 and gradually lowered until it reached 21˚C on d 21. For d 22 to 42, the temperature was held at 21˚C. The air humidity was 60 to 65% during all days of the experiment. The compound feed manufactured by the "Stavropol compound feed" company (Stavropol, Russia) was used for feeding in 3 phases: STARTER (1−14 d), GROWER (15−28 d), and FINISHER (29−42 d) (Table 1). The feed and feeding of the animals corresponded to the GOST P 51899-2002 "Granulated mixed feeds. General specifications." The composition of the compound feeds of all 3 phases included wheat, corn, soybean meal, wheat gluten, corn gluten, corn cake, soybean cake, sunflower cake, sunflower oil, tricalcium phosphate, lysine, methionine, threonine, protein-vitamin-mineral concentrates. The temperature, humidity, and light conditions in all groups were constant. Water and feed were provided ad libitum during the 42-day study period. No lethal cases were observed (Table 1).

Sampling

At the end of the study, all birds were euthanized by decapitation. Blood samples were collected from the axillar vein of 3 randomly chosen birds per replication into two 10 mL vacuum tubes containing EDTA K3 (InterVac-Technology, Narva, Estonia) per bird. The collected blood samples were aliquoted for plasma biochemical analyses. The plasma samples acquired after centrifugation (3,000 × g for 10 min) were examined for total protein (g/L), albumin (g/L), globulin (g/L), albumin/globulin ratio, AST (U/L), ALT (U/L), uric acid (μmol/L), cholesterol (mmol/L), Ca (mmol/L), and P (mmol/L) levels using an automatic biochemistry analyzer Accent 200 (Cormay Diagnostics Weterynaria, Warsaw, Poland).

Before euthanizing, the birds were not fed for 8 h according to the GOST 18292-2012 "Slaughter poultry. Specifications." The gut of 3 randomly chosen birds per replication was ligated and removed from the carcass. The cecal content was collected for studying bacterial composition. The content of the small intestines, ceca, and rectum was used for determining the survival of spore-forming bacteria in the GIT. The contents of each gastrointestinal section were collected into sterilized containers and immediately kept at 4˚C for further assays. Bacterial analysis was carried out within 24 h. For histological examination,

Table 1. Nutrient content of the experimental diets.

	Feeding phase		
Items	STARTER	GROWER	FINISHER
100 g of compound feed contains:			
Metabolic energy, kcal	300	302	312
Crude protein, %	23.09	20.52	19.59
Crude fat, %	2.5	3.5	3.5
Crude fiber, %	4.26	4.25	4.63
Lysine, %	1.34	1.06	0.93
Methionine + cystine, %	0.9	0.84	0.81
Ca, %	1.05−1.25	0.98−1.05	0.95
P, %	0.60−0.65	0.63−0.7	0.57
Na, %	0.15−0.19	0.17−0.23	0.19

jejunum tissues (1.5 cm long) were cut and fixed using 10% formalin. The spleen was aseptically collected into sterilized containers and immediately kept at −80°C for further experiments.

Measurement of Growth Performance

Growth performance parameters—Each chicken's body weight (**BW**) was measured at 7, 14, 21, 28, 35, and 42 d of age. ADWG was calculated from obtained data according to the following formula:

$$\text{ADWG} = \frac{\text{Final BW (g)} - \text{Initial BW (g)}}{\text{Time interval (d)}} \quad (1)$$

Bacterial Isolation

The small intestine, ceca, and rectum contents were plated on solid nutrient media. The appropriate selective media was used to determine the numbers of LAB (MRS, LenReactiv, Russia), *Bifidobacterium* (Bifidobacterium Broth, HiMedia, Maharashtra, India), *Enterococcus* (Enterococcus Confirmatory Agar, HiMedia, Maharashtra, India), *Escherichia coli*, and lactose-positive bacteria (Endo Agar, HiMedia, Maharashtra, India). Obtained samples were incubated in an anaerobic chamber Shell Lab Bactron (Sheldon Manufacturing, Cornelius) at a temperature of 42°C, which corresponds to the poultry body temperature (Scanes, 2015). After 48 h of incubation, the grown colonies were counted. When assessing bacterial growth on Petri dishes, in addition to direct growth on the medium, we also assessed the morphology of the grown colonies, and, in case of doubt, resorted to microscopy of individual colonies and Gram staining.

To determine the number of *Bacillus* spore forms, a second dilution was placed in a water bath following its heating to a temperature of 95°C for 5 min. During this time, all microorganisms in the sample died except for bacilli spores. Next, a series of successive dilutions were prepared and inoculated on nutrient agar (LenReaktiv, Saint-Petersburg, Russia). Then samples were incubated for 24 h at 42°C and then counted.

To determine the number of *Bacillus* vegetative cells were subjected to the same manipulations as described above, but after the samples were kept at +4°C for 48 h (before spore formation, see below). The difference between the first and second CFU/g was considered as the number of vegetative cells.

Study of the Probiotic Bacilli Sporulation Process at Low Temperatures

A 24 h suspension of each of the studied bacilli was prepared in liquid LB medium at a temperature of 42°C and with constant shaking in the New Brunswick Innova 40 shaker-incubator (Eppendorf, Hamburg, Germany). The 24 h suspension was then diluted to OD600 = 0.1 with sterile LB. The resulting suspension was placed in a refrigerator at +4°C. Samples were taken before cooling and 12, 24, and 48 h after cooling, each time with preshaking on a VXMNAL vortex (OHAUS, New Jersey) for 5 min. Decimal dilutions were prepared and inoculated 3 times on a solid LB medium. Thus, the total number of living cells, vegetative cells, and spores was determined. Then suspensions were heated to a temperature of 95°C for 5 min and reinoculated, which allowed us to obtain the number of cells only in the spore form.

Histomorphometric Evaluation of Small Intestine Morphology

After fixation, each sample of acquired jejunum tissues was cut with a disposable microtomy knife (Bio-Optica, Milan, Italy) into 3 pieces around the entire circumference (0.5 cm thick) and placed in disposable plastic containers (Bio-Optica, Milan, Italy), after which the material was passed through ethanol solutions of increasing concentration (50, 60, 70, 80, and 96%) and xylene. The resulting material was embedded in Histomix histological medium (BioVitrum, Saint-Petersburg, Russia) using a closed-type histological processor Tissue-Tek VIP 5 Jr and a Tissue-Tek TEC 5 paraffin embedding station (Sakura, Tokyo, Japan). Using a sledge microtome and a table for preparing histological sections (Bio-Optica, Milan, Italy), histological sections 5 μm thick were

Table 2. Sequences of the primers used in qPCR (Xu et al., 2019).

Genes	Forward primer (5′−3′)	Reverse primer (5′−3′)
IL-10	GGACTATTTTCAATCCAGGGACG	GGGCAGGACCTCATCTGTGTAG
IL-6	AAATCCCTCCTCGCCAATCTG	CCTCACGGTCTTCTCCATAAACG
β-actin	TATTGCTGCGCTCGTTGTTGAC	GATACCTCTTTTGCTCTGGGCTTC

made from the obtained blocks, which were stained with hematoxylin and eosin (Bio-Optica, Italy and BioVitrum, Russia) on an automatic multistainer Prisma (Sakura, Tokyo, Japan).

Microscopy of histological slides was carried out using an Olympus BX45 direct light microscope with a C 300 camera (Olympus, Tokyo, Japan). Morphometrical evaluation of i) entire intestinal wall height, ii) villus height and iii) thickness, iv) crypts height and v) thickness, vi) villi epithelium height, and vii) crypt epithelium height was carried out using VideoTest-Master Morphology 4.0 software (Zenit, Saint-Petersburg, Russia).

IL-6 and IL-10 Gene Expression Analysis

Total RNA was isolated from the spleen of 1 chicken from each replicate with 4 chickens in total per group (Schneider et al., 2001). Isolation of total RNA from the samples was carried out by the phenol-chloroform method using the ExtractRNA reagent (Evrogen, Moscow, Russia). Spleen samples (100 mg) were homogenized in a mortar with liquid nitrogen. Total RNA was purified using the CleanRNA Standard kit (Evrogen, Moscow, Russia) following manufacturer protocol. All actions were carried out following the instructions for the corresponding kits from the manufacturer.

Primers targeting (IL-10, IL-6) and reference (β-actin) genes were previously described in the literature (Table 2; Xu et al., 2019).

A reverse transcription reaction was carried out using the MMLV RT kit protocol (Evrogen, Moscow, Russia). qPCR from the obtained cDNA was performed using the qPCRmix-HS SYBR kit (Evrogen, Moscow, Russia) on a Bio-Rad CFX96 amplifier (Bio-Rad, Hercules). Experiments were conducted following the protocols of the kit manufacturers. The results were analyzed using the Bio-Rad CFX Manager software (Bio-Rad, Hercules).

Expression levels were normalized to β-actin, which was used as a reference gene. The change in the expression level of the target genes was calculated using the $\Delta\Delta$Ct method as a fold change in gene expression in experimental samples relative to the control sample. The difference was considered statistically significant at $P < 0.00625$, taking the Bonferroni correction into account (Ganger et al., 2017).

Statistical Analysis

IBM SPSS 26.0 (SPSS, Inc., Chicago, IL) was used for data analysis. Data were not normally distributed according to the Shapiro-Wilk test. The Kruskal-Wallis test, followed by the Dunn-Bonferroni post hoc test, was used to evaluate different means among treatments. Results were presented as mean ± standard error of the mean. The statistical significance was determined as $P < 0.05$.

RESULTS AND DISCUSSION

Biochemical Blood Plasma Analysis

There were no significant differences in total protein, albumin, globulin, albumin/globulin ratio, aspartate transaminase (**AST**), alanine transaminase (**ALT**), uric acid, cholesterol, Ca, and P levels among the groups (Figure 1).

Growth Performance

There are significant differences observed in BW and ADWG among the groups T1 and T2 on the seventh day of the experiment, with the highest values in the T1 group and the least in the T2 group (P value = 0.019). Also, there are significant differences on the 42nd day of the experiment between CON with the lowest

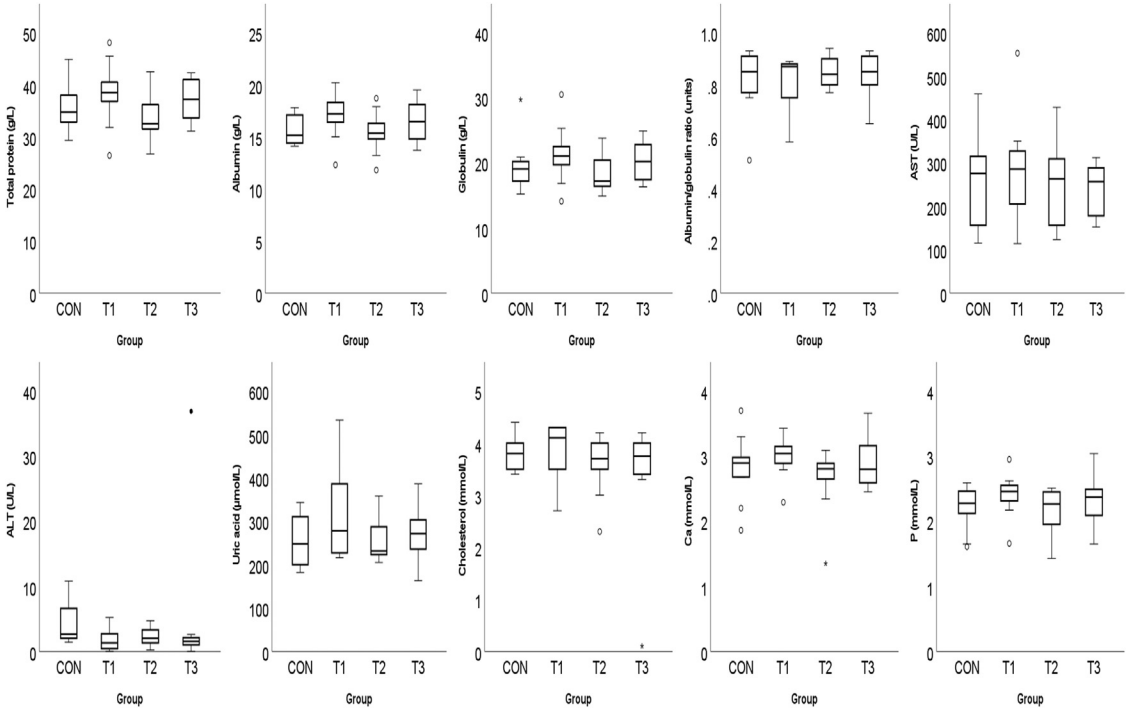

Figure 1. Biochemical blood analysis in broilers treated with *B. subtilis* KATMIRA1933 (T1), *B. subtilis* KB41 (T2), and *B. amyloliquefaciens* KB54 (T3), and control group (CON). There were no significant differences in studied variables among the groups.

Table 3. Effects of *Bacillus*-feed supplementation on the growth performance of broilers during the trial.

		Groups					
Period	Item	CON	T1	T2	T3	*P* value	SEM
7th day	BW	211.9 ± 28.1^{ab}	226.56 ± 20.8^{a}	208.85 ± 23.1^{b}	216.82 ± 22.3^{ab}	0.03	1.91
	ADWG	23.1 ± 4.1^{ab}	24.8 ± 2.9^{a}	22.8 ± 3.3^{b}	23.7 ± 3.3^{ab}		0.27
14th day	BW	567.1 ± 62.7	583.9 ± 61.4	576.6 ± 61.9	584.1 ± 57.2	0.57	4.82
	ADWG	36.9 ± 4.5	38.1 ± 4.4	37.7 ± 4.4	38.1 ± 4.1		0.34
21st day	BW	1015.8 ± 130.7	1034.3 ± 111.1	1042.8 ± 125.9	1041.8 ± 117.5	0.94	9.36
	ADWG	46.4 ± 5.7	46.9 ± 5.3	47.2 ± 6.1	47.2 ± 5.6		0.45
28th day	BW	1709.1 ± 213.6	1694.4 ± 188.6	1775.2 ± 186.2	1706.4 ± 207.1	0.33	15.55
	ADWG	59.8 ± 7.1	58.7 ± 6.7	61.3 ± 6.5	59.2 ± 7.4		0.56
35th day	BW	2368.6 ± 255.5	2295.3 ± 267.2	2392.1 ± 259.5	2324.8 ± 289.1	0.41	21.33
	ADWG	66.3 ± 7.3	64.2 ± 7.6	66.9 ± 7.4	64.6 ± 7.9		0.61
42nd day	BW	2806.8 ± 303.1^{a}	2901.8 ± 284.5^{ab}	3042.6 ± 336.1^{b}	2967.6 ± 354.7^{ab}	0.01	26.50
	ADWG	65.6 ± 7.2^{a}	67.9 ± 6.8^{ab}	71.25 ± 8.0^{b}	69.5 ± 8.4^{ab}		0.63

Values represent the arithmetic mean ± standard deviation. *P* values were presented according to the Kruskal-Wallis test. Means within the same row without common superscripts are significantly different ($P < 0.05$) according to the Dunn-Bonferroni test for multiple comparisons. Abbreviations: ADWG, average daily weight gain; BW, body weight; CON, control group; SEM, standard error of the mean; T1, group treated with *B. subtilis* KATMIRA1933; T2, group treated with *B. subtilis* KB41; T3, group treated with *B. amyloliquefaciens* KB54.

values of BW and ADWG and T3 with the highest values of these parameters (*P* value = 0.007) (Table 3).

Isolated Gut Bacteria Composition of Broilers Treated With Potential Probiotics

We isolated *Lactobacillus, Bifidobacterium, Enterococcus, E. coli*, and lactose-positive bacteria from the cecal contents of studied broilers and determined their concentrations within the cecum (log CFU/g). Significant differences were observed in the number of *Lactobacillus* with the highest values in groups treated with *B. subtilis* KB41 and *B. amyloliquefaciens* KB54 relative to the CON group; *E. coli* with the highest values in the group treated with *B. amyloliquefaciens* KB54 relative to the group treated with *B. subtilis* KATMIRA1933, and lactose-positive bacteria with the highest values in the group treated with *B. amyloliquefaciens* KB54 relative to the group treated with *B. subtilis* KB41 (Table 4).

Vegetative cells were detected already in the small intestine, as well as spore forms. The total number of vegetative and spore cells in the small intestine was lower than the number of bacillary cells in the feed (10^5 CFU/g). In the ceca, the number of vegetative cells increased, suggesting that the probiotic bacilli in the intestines not only survived but even grew. Moreover, the number of spore cells also increased.

Table 4. Effects of *Bacillus* probiotics on the ceca bacterial composition in broilers at the 42nd day of the trial.

	Number of microorganisms (lg CFU/g)					
Microorganisms	CON	T1	T2	T3	*P* value	SEM
Lactobacillus	8.28 ± 0.06^{a}	8.31 ± 0.05^{ab}	8.46 ± 0.01^{b}	8.43 ± 0.03^{b}	0.03	0.03
Bifidobacterium	8.16 ± 0.28	8.36 ± 0.10	8.10 ± 0.17	8.16 ± 0.28	0.49	0.06
Enterococcus	7.48 ± 0.04	7.15 ± 0.08	7.18 ± 0.08	7.05 ± 0.10	0.07	0.05
E. coli	6.25 ± 0.04^{ab}	5.99 ± 0.08^{a}	6.53 ± 0.03^{ab}	6.83 ± 0.17^{b}	0.02	0.09
Lactose-positive bacteria	6.78 ± 0.02^{ab}	6.23 ± 0.07^{ab}	6.05 ± 0.06^{a}	6.97 ± 0.02^{b}	0.02	0.16

Values represent the arithmetic mean ± standard deviation. *P* values are presented according to the Kruskal-Wallis test. Means within the same row without common superscripts are significantly different ($P < 0.05$) according to the Dunn-Bonferroni test for multiple comparisons. Abbreviations: CON, control group; SEM, standard error of the mean; T1, group treated with *B. subtilis* KATMIRA1933; T2, group treated with *B. subtilis* KB41; T3, group treated with *B. amyloliquefaciens* KB54.

In the cecum of birds treated with *B. subtilis* KB41, the number of vegetative cells exceeded the number of spores by about 20 times, while the number of spores of *B. amyloliquefaciens* KB54 was about 5 times higher than the number of vegetative cells.

In the large intestine of birds treated with *B. subtilis* KB41, the number of spores exceeded the number of vegetative cells by about 5 times and reached 5.04 ± 0.04 log CFU/g (Figure 2). The number of spores in groups treated with *B. amyloliquefaciens* KB54 did not significantly change in the cecum and colon, but the number of vegetative cells decreased significantly.

However, in the control group that did not receive any bacilli probiotics, a significant number of bacilli cells, both in spore and vegetative forms, were observed. Their number was significantly higher than normal in birds that did not receive the bacillary probiotics (Khan et al., 2020). In the colon, the number of vegetative cells reached 6.64 ± 0.02 log CFU/g, and in the cecum—5.17 ± 0.08 log CFU/g. In this case, the colonies were primarily monocultures with a few inclusions of other colonies with different morphology. Morphologically, the colonies of these bacilli differed significantly from all 3 of our probiotic strains. The same colonies were observed in the group that received a probiotic based on *B. subtilis* KATMIRA1933, however, no colonies morphologically corresponding to the *B. subtilis* KATMIRA1933 were found.

Morphometry of Jejunum Tissues

There were no significant differences (*P* value >0.05) in the entire intestinal wall height, villus height and thickness, crypt height and thickness, villi epithelium height, and crypt epithelium height in jejunum tissues obtained from the studied groups of animals (Figure 3). The examples of obtained histological slides are shown in Figure 4.

IL-6 and IL-10 Expression

Probiotic *B. subtilis* KATMIRA1933 (T1) had the least significant effect on the expression of IL-10 (*P* value = 0.04) and did not affect the expression of IL-6 compared to the control (*P*

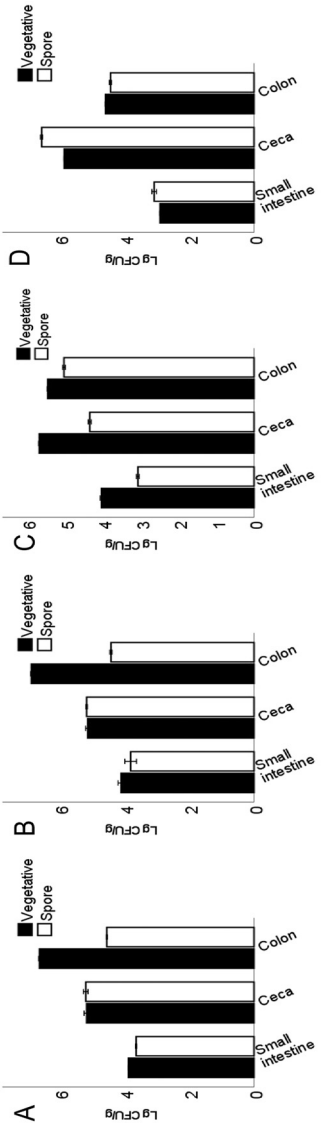

Figure 2. The number of *Bacillus* vegetative cells and spores in the different parts of the gastrointestinal tract in birds treated with: (A) Basal diet without probiotic supplementation; (B) *B. subtilis* KATMIRA1933; (C) *B. subtilis* KB41; (D) *B. amyloliquefaciens* KB54.

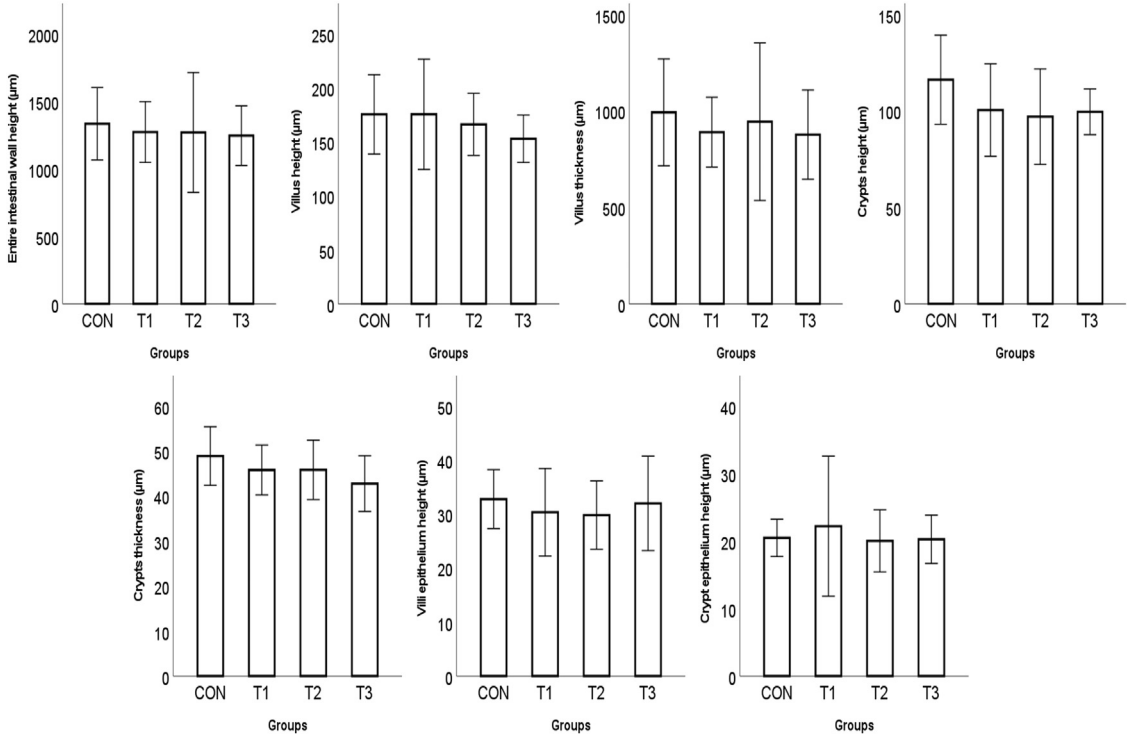

Figure 3. Entire intestinal wall height, villus height and thickness, crypts height and thickness, villi epithelium height, and crypt epithelium height in the jejunum of animals treated with *B. subtilis* KATMIRA1933 (T1), *B. subtilis* KB41 (T2), and *B. amyloliquefaciens* KB54 (T3), and control group (CON). There were no significant differences in studied variables among the groups.

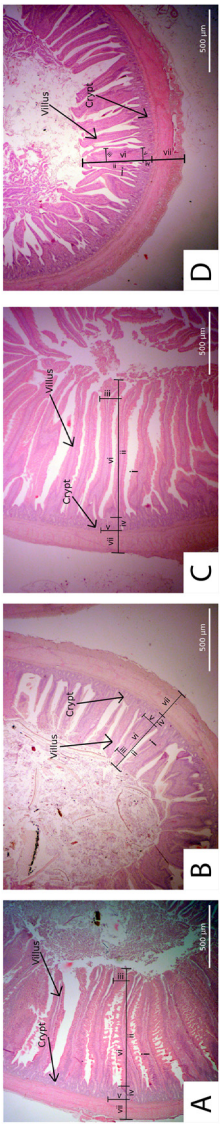

Figure 4. Representative images (4× magnification) of hematoxylin and eosin staining of jejunum tissues, obtained from broilers fed with (A) basal diet without probiotic supplementation; (B) *B. subtilis* KATMIRA1933; (C) *B. subtilis* KB41; (D) *B. amyloliquefaciens* KB54; i—entire intestinal wall height, ii—villus height, iii—villus thickness, iv—crypt height, v—crypt thickness, vi—villi epithelium height, vii—crypt epithelium height. The results of the morphometrical evaluation are shown in Figure 3.

value >0.05). Probiotics *B. subtilis* KB41 (T2) and *B. amyloliquefaciens* KB54 (T3) increased the expression of both pro- and anti-inflammatory cytokines. *B. subtilis* KB41 increased the expression of IL-10 by 12.54 times (*P* value = 0.0012), while IL-6 only increased by 1.97 times (*P* value = 0.0064). *B. amyloliquefaciens* KB54 showed a 5.66-fold increase in IL-6 expression (*P* value = 0.002) and a 6.06-fold increase in IL-10 expression (*P* value = 0.002). Probiotic *B. amyloliquefaciens* KB54 increases the production of both pro- and anti-inflammatory cytokines, while *B. subtilis* KB41 shifts the balance toward anti-inflammatory IL-10 (Figure 5).

Discussion

In this study, we evaluated the effect of 3 *Bacillus*-based probiotics on growth performance, biochemical blood parameters, gut bacteria composition, survival of probiotics in different GIT regions, intestinal morphology, and the immune system of Ross 308 broilers housed in deep litter.

Bacillus-based probiotics were chosen due to their high survival in the GIT due to their ability for encapsulation, which has been shown in artificial GIT studies (Keller et al., 2019; Ahire et al., 2020; Khalid et al., 2022). We suggest that the high survival rate of probiotics in the GIT results in better beneficial effects for the animals' health. In our previous studies, we determined the antimutagenic and antioxidant actions of *Bacillus* probiotics in vitro with *lux* biosensors, which is one of the criteria for probiotic candidate selection during the preclinical stage (Prazdnova et al., 2015; Chistyakov et al., 2018; Popov et al., 2021b). In this study, we used probiotics *B. subtilis* KB41 and *B. amyloliquefaciens* KB54, which also demonstrated high antimutagenic and antioxidant properties in vitro (Mazanko et al., 2022).

This is the second in vivo study of *B. subtilis* KB41 and *B. amyloliquefaciens* KB54 in poultry. In the first study, we conducted experiments on Cobb 500 broilers housed in cages (Mazanko et al., 2022). Both experiments mostly differ in the use of cross broiler breeds and housing conditions, which overall resulted in some differences in the outcomes of the

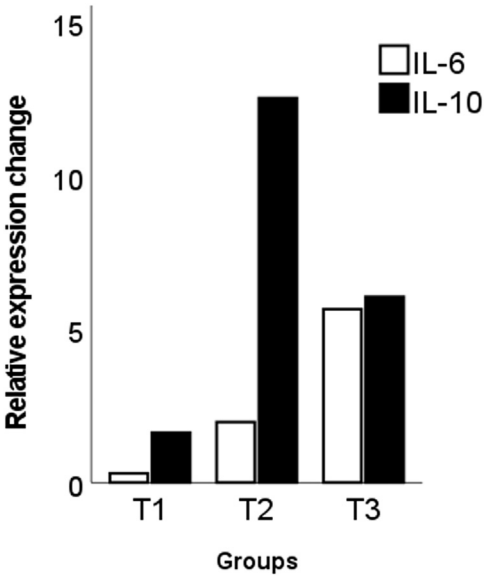

Figure 5. Fold expression difference of IL-6 and Il-10 in groups treated with probiotics relative to the control group. T1, group treated with *B. subtilis* KATMIRA1933; T2, group treated with *B. subtilis* KB41; T3, group treated with *B. amyloliquefaciens* KB54.

second experiment. First, we should acknowledge that we aimed to test our probiotics in different housing conditions, which include cages and deep litter. It is known that the type of housing directly affects the overall growth performance of broilers (Sekeroglu et al., 2009; Zhao et al., 2014). Averós et al. conducted a meta-analysis of the effects of different rearing environments on the production performance of broilers and found that the presence of bedding material significantly improves the growth performance of broilers (Averos and Estevez, 2018). In our studies, the growth performance of broilers housed with bedding material is also greater than in birds housed in cages (Mazanko et al., 2022). However, the beneficial effects of studied *Bacillus* probiotics are noticeably higher in the experiment with broilers housed in cages. We suggest that this could be due to the presence of other *Bacillus* bacteria in the deep litter, which could be delivered from other sources such as water, feces of studied animals, or soil, as the experiment was carried out in a standard poultry facility under standard conditions. This assumption is confirmed by the results of the bacteriological analysis: we identified bacilli in the small intestine, ceca, and colon of broilers fed with a basal diet without probiotic supplementation (Figure 2), while in the previous experiment, bacilli were not identified in these parts of the GIT in the CON group of broilers (Mazanko et al., 2022). This finding indicates that standard conditions of poultry facilities could alter the overall outcome of probiotic supplementation in broilers due to the conventional environment of broiler housing.

Table 5. Change in the number of spores from the total number of probiotic bacilli cells at low temperatures.

Item	T1	T2	T3	*P* value	SEM
Before cooling					
The total amount of cells, log CFU/mL	7.31 ± 0.02	7.73 ± 0.02	7.24 ± 0.03	0.027	0.077
Amount of spores, log CFU/mL	6.25 ± 0.04	6.82 ± 0.07	6.43 ± 0.03	0.027	0.086
Amount of spores, %	9%	12%	15%		
12 h after cooling					
The total amount of cells, log CFU/mL	7.33 ± 0.01	7.71 ± 0.02	7.23 ± 0.03	0.027	0.73
Amount of spores, log CFU/mL	6.54 ± 0.03	7.24 ± 0.01	6.68 ± 0.02	0.027	0.11
Amount of spores, %	16%	34%	28%		
24 h after cooling					
The total amount of cells, log CFU/mL	7.30 ± 0.02	7.73 ± 0.04	7.23 ± 0.02	0.027	0.078
Amount of spores, log CFU/mL	7.10 ± 0.06	7.59 ± 0.04	7.13 ± 0.03	0.061	0.081
Amount of spores, %	63%	73%	78%		
48 h after cooling					
The total amount of cells, log CFU/mL	7.33 ± 0.01	7.73 ± 0.04	7.27 ± 0.01	0.027	0.073
Amount of spores, log CFU/mL	7.29 ± 0.02	7.71 ± 0.001	7.24 ± 0.03	0.027	0.075
Amount of spores, %	91%	95%	63%		

Abbreviations: CON, control group; SEM, standard error of the mean; T1, group treated with *B. subtilis* KATMIRA1933; T2, group treated with *B. subtilis* KB41; T3, group treated with *B. amyloliquefaciens* KB54. *P* values represent Kruskal-Wallis test results.

In this study, we also performed a basic bacteriological analysis with the isolation of gut bacteria for the evaluation of their composition in the cecal contents. The number of gut microorganisms corresponded to normal microbiota values (Scanes et al., 2015). The bacillary probiotics we used did not disrupt the ratio of groups of microorganisms and did not reduce the total number of bacteria. A similar picture has been repeatedly observed in our studies of these bacillary probiotic effects, both within the framework of this project (Mazanko et al., 2022) and in previous projects (Prazdnova et al., 2019). However, the values could be confounded by the environmental features discussed above. Also, we should mention that the metagenomic and metabolomic analyses could reveal the effects of probiotics KB41 and KB54 on the gut microbiota of broilers in a much broader way than bacteriological analysis, the results of which are reported in this study. As this study adds more evidence of the effectiveness of *Bacillus*-based probiotics KB41 and KB54, various omics analyses could reveal some mechanisms behind it, such as the changes in the bacterial relative abundance or microbial metabolites production (Liu et al., 2021; Segura-Wang et al., 2021).

To assess the number of vegetative cells and bacilli spores in the intestine, we needed to develop a reliable method to distinguish between cells in different physiological states found in the same sample. We have shown (Table 5) that the probiotic bacilli we used pass from the vegetative to the spore form when cooled within 2 d. This allowed us to determine the number of bacilli spores in the intestinal contents immediately after sample collection, and the total number of bacilli after 2 d of cooling the sample. In the groups treated with *B. subtilis* KB41 and *B. amyloliquefaciens* KB54, we observed the following picture: in the intestinal content, bacteria with a colony morphology identical to the probiotic strain morphology were found in significant amounts. Colonies of other bacillary strains and species were visible but far fewer in number. Therefore, Figure 2 shows data only on the number of bacilli identical to the introduced probiotic. Apparently, the probiotic bacteria obtained with food began to germinate in the birds' crop and died in the acidic environment of the stomach, since in the small intestine the total number of bacilli was lower than in the food. However, in the cecum it increased and exceeded the number of bacilli in the feed, which suggests that the probiotic bacilli were able to multiply in the intestine and were therefore metabolically active. In the colon, the majority of the bacilli were again in spore form. Apparently, these are already secondary spores formed from vegetative cells after the growth cycle.

From the data obtained, it can be assumed that in the intestines, the population of these bacilli was divided into 2 subpopulations: one part actively multiplied, and the second part formed spores. We can observe a similar picture of bacilli community dynamics during the growth of biofilms (Asally et al., 2012).

However, in the control group that did not receive any bacilli probiotics, a significant number of bacilli cells, both in spore and vegetative forms, were observed. Their number was significantly higher than normal in birds that did not receive the bacillary probiotics (Khan et al., 2020). In the colon, the number of vegetative cells reached 6.64 ± 0.02 log CFU/g and in the cecum—5.17 ± 0.08 log CFU/g. In this case, the colonies were primarily monocultures with a few inclusions of other colonies with different morphology. Morphologically, the colonies of these bacilli differed significantly from all 3 of our probiotic strains. The origin of these bacilli is unknown. However, they could be delivered from sources such as water, feces of studied animals, or soil, as the experiment was carried out in a standard poultry facility under standard conditions.

It should be noted that the same colonies were observed in the group that received a probiotic based on *B. subtilis* KATMIRA1933. In our previous studies (Prazdnova et al., 2019; Mazanko et al., 2022), we noted that *B. subtilis* KATMIRA1933 cells do not have a high survival rate in the GIT, so we expected to see, at least in the colon, the absence of growth of the *B. subtilis* KATMIRA1933 strain. However, instead, we observed a monoculture of the same unknown strain in an amount close to that in the control group.

The studied *Bacillus* probiotics did not significantly affect the results of biochemical

blood analysis, which shows their relative safety for broilers and corresponds to our previous study (Mazanko et al., 2022). Also, these probiotics significantly affected growth performance at 2 time points of the experiment. On the seventh day of the experiment, the highest values of BW and ADWG were observed in the broilers treated with *B. subtilis* KATMIRA1933 and the smallest values in the broilers treated with *B. subtilis* KB41. This trend was seemingly reversed starting from the 21st day, as supplementation of the basal diet with *B. subtilis* KB41 resulted in higher BW and ADWG reaching statistically significant values on the 42nd day of the experiment. In our previous study, *B. subtilis* KB41 also showed beneficial effects on growth performance, but the treatment of broilers with *B. amyloliquefaciens* KB54 resulted in better overall BW and ADWG values (Mazanko et al., 2022). Some studies show no significant impact of *Bacillus*-based probiotics supplementation on the production performance at all (Bai et al., 2018; Oladokun et al., 2021). Also, we did not observe any significant effects of *Bacillus* probiotics supplementation on the intestinal morphology of the broilers, which could be explained by confounding factors associated with conventional housing conditions in poultry facilities. *Bacillus*-based probiotics are known for improving intestinal morphology by increasing the overall area of absorption in the small intestine, which results in better availability of nutrients following other beneficial effects on broilers' health and performance (Aliakbarpour et al., 2012). This effect is especially beneficial for broilers challenged with *Clostridium perfringens*, which induces necrotic colitis (Cheng et al., 2021). The outcomes of growth performance and morphometry of intestinal tissues could be affected by a confounding factor, which is the presence of unknown bacilli in the GIT of broilers that may originate from the poultry facility environment. These results indicate that we should conduct more experimental series excluding the risks of confounding factors, such as the presence of bacteria in deep litter. However, we should acknowledge that routine broiler rearing is also performed in conventional conditions, which includes the presence of various bacteria in the broilers' housing environment. According to the bacteriological analysis, bacilli found in high amounts within the GIT of broilers from T2 and T3 groups were the probiotics *B. subtilis* KB41 and *B. amyloliquefaciens* KB54, while in the T1 group treated with *B. subtilis* KATMIRA1933 colonies presented a variety of morphological features. This indicates that probiotics *B. subtilis* KB41 and *B. amyloliquefaciens* KB54 may have prevented environmental bacilli from getting into the broilers' GIT, which demonstrates their inhibitory activity relative to other bacteria. Also, IL-6 and IL-10 gene expression analysis showed similar results to the previous study with an improvement of IL-6 and IL-10 gene expression rates relative to the CON group but in lower overall values, which are probably confounded by previously discussed factors (Mazanko et al., 2022).

Since an increase in weight gain was observed in the experimental groups, especially closer to the 42nd day of the experiment, it can be postulated that the probiotic groups KB41 and KB54 exhibit a potent and favorable immunomodulatory effect on the host, as it can be expected from other similar findings (Wigley and Kaiser, 2003; Wu et al., 2016). The hypothesized underlying mechanism can be attributed to the physiological properties of the examined cytokines. Recent studies indicate that IL-6 not only functions as a proinflammatory cytokine but also as a "metabolic hormone." It augments lipolysis, enhances glucose utilization, and promotes mobilization of free fatty acids, ultimately leading to increased growth rates and improved feed conversion (Ghanemi and St-Arnand, 2018). Additionally, IL-6 has gained recognition as a primary anti-inflammatory myokine, reducing chronic muscle inflammation and muscle protein catabolism, thereby facilitating muscle building, which is particularly advantageous for animal husbandry (Brandt and Pedersen, 2010; Ghanemi and St-Arnand, 2018). The induction of IL-10 follows the principle of negative feedback, mitigating the proinflammatory effect of IL-6 while preserving its metabolic activity (Daftarian et al., 1996). Wu et al. (2019) reported a similar effect of probiotic bacteria, manifesting as enhanced chicken growth rates in conjunction with IL-6 and IL-10 induction (Wu et al., 2019).

It is important to note that probiotics are frequently considered as a substitute for antibiotic growth promoters due to their multifaceted action, encompassing antimicrobial effects and immunomodulation, without the associated risk of antibiotic resistance (Mingmongkolchai and Panbangred, 2018). Many authors attribute the action of antibiotic growth promoters to both pro- and anti-inflammatory effects, with a noticeable positive impact on IL-6 observed in both instances (Niewold, 2007; Kabploy et al., 2016; Oh et al., 2019). Therefore, it can be inferred that antibiotic growth promoters and probiotic strains KB41 and KB54 likely operate through a similar mechanism of action. Consequently, this research highlights the potential of the aforementioned probiotic formulations as a viable alternative to antibiotic growth promoters in the poultry industry.

In this study, we demonstrated the effects of *Bacillus*-based probiotics on the broilers housed in a deep litter. The results correspond to our previous studies, as *B. subtilis* KB41 and *B. amyloliquefaciens* KB54 showed similar effects. However, we should acknowledge the limitation of this study for our future research, which will include finding optimal housing conditions for the most effective beneficial action of *Bacillus* probiotics.

CONCLUSIONS AND APPLICATIONS

1. This preliminary study demonstrates the safety and potential beneficial effects of the investigated bacilli probiotics, *B. subtilis* KATMIRA1933, *B. subtilis* KB41, and *B. amyloliquefaciens* KB54, when given as nutritional supplements for poultry raised on deep bedding and highlights the potential impacts of endogenous environmental bacilli and other organisms on the effectiveness *Bacillus* probiotic supplementation
2. The obtained results highlight the need for future investigations into the compositional changes in the broiler GIT and bedding microbiota during the course of supplementation, and the identity and nature of endogenous bacilli in the poultry environment should be elucidated.
3. Continued efforts will focus on developing a tailored spore-forming *Bacillus* probiotic formulation that takes into account all environmental factors, especially those related to the rearing environment and associated microbial species, to deliver optimized probiotic function designed to meet the needs of specific rearing environments.

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DISCLOSURES

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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