3 Biotech (2021) 11:126
https://doi.org/10.1007/s13205-020-02634-2

ORIGINAL ARTICLE

Supplementation of *Bacillus subtilis* GM5 enhances broiler body weight gain and modulates cecal microbiota

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Received: 24 September 2020 / Accepted: 29 December 2020 / Published online: 14 February 2021

Abstract

We investigated the effect of the strain *Bacillus subtilis* GM5 on growth, feed conversion, and the composition of cecum microbiota in broiler chickens. Half of which received a control diet, while the other half was fed a diet supplemented with GM5 spores. Cecal contents on days 1, 10, and 42 were subjected to metataxonomic analysis. Principal Component Analysis showed that the control and probiotic groups formed three separate clusters, indicating changes, which occurred gradually in microbial communities. On day 1, *Firmicutes* (53.87–57.61%) and *Proteobacteria* (43.77–38.93%) were prevalent in both groups, whereas samples of days 10 and 42 were predominantly occupied by *Firmicutes* (54.55–81.79%) and *Bacteroidetes* (26.94–30.45%). In the group of chickens treated with probiotic, the average daily gain in body weight was higher, while feed conversion decreased by 1.44%. A surge in the presence of beneficial bacteria of the *Ruminococcaceae* family was observed. The introduction of the probiotic led to an elevated *Firmicutes*/*Bacteroidetes* ratio, which positively correlated with chickens' bodyweight (Spearman $\rho = 1.0$, $P < 0.05$). Supplementing broiler feed with *B. subtilis* GM5 spores leads to improved feed intake and digestibility, which is paramount in reducing the cost of the final product. Thus, the probiotic strain GM5 modulates the cecal microbiota of broiler chickens and increases microbial diversity, which is well exhibited on the 42nd day.

Keywords Broiler · Probiotics · GIT of chickens · Cecum · 16S rRNA gene · Metataxonomic

Introduction

Rapid growth in poultry production has been associated with the widespread use of antibiotic growth promoters (AGPs) aimed towards enhancing growth performance and inhibiting the spread of certain diseases (Musa et al. 2019). Side effects, such as the development and spread of antibiotic resistance and potentially harmful effects to the intestinal microbiota have stimulated the necessity in limiting the use of AGPs (Boeckel et al. 2015; Bai et al. 2017). Natural growth promoters (NGPs), such as prebiotics, phytobiotics, probiotics, or direct-fed microbial (DFM), are used as an alternative to antibiotics in animal husbandry (Huang et al. 2018; Musa et al. 2019).

The intestinal microbiota remains of great interest to researchers trying to improve the productivity and health of birds, as well as poultry food safety (Oakley et al. 2014a, b; Richards et al. 2019). The balanced dynamics of intestinal microbiota plays a vital role in the metabolic and immune processes of the organism of poultry birds (Rychlik 2020). The cecum of broilers shows the highest species diversity and this section of the GIT are still being investigated (Videnska et al. 2013; Medvecky et al. 2018; Hong et al. 2019). The primary function of the cecum involves the fermentation of nutrients, through which digestive processes in the cecum provide up to 10% of the metabolized energy in young and adult chicken (Clench and Mathias 1995; Jozefiak et al. 2004). In addition, the cecum is the hot site of colonization of pathogenic microorganisms (Crhanova et al. 2011). Monitoring the dynamics of microbiota helps to enhance the

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growth and productivity of birds, as well as adjust the diet using beneficial bacterial strains (Choi et al. 2015; Shang et al. 2018).

The formation of GI microbiota of commercially hatched birds occurs differently than in wild chickens since contact between parents and offspring are completely interrupted (Kubasova et al. 2019; Rychlik 2020). During the colonization of commercially hatched birds, the assembly of beneficial groups of bacteria could be delayed over time, which in turn could adversely affect the health and development of chickens. Furthermore, commercially hatched fowls are extremely sensitive to colonization by different pathogens such as *Salmonella* spp., *Escherichia coli* and *Campylobacter spp.* (Ranjitkar et al. 2016; Varmuzova et al. 2016). The composition of the intestinal microbiota is modulated by several external factors, including bird type and breed, sex, age, feed access, medication, antibiotics, stress, housing and other factors (Zhao et al. 2013; Antonissen et al. 2015; Schokker et al. 2015; Kers et al. 2018).

Bacillus spp. have gained increasing attention for industrial applications as probiotics. They form spores with resistance to the high temperatures, used in the modern production of feed for poultry, and show stability to low pH, bile and enzymes found in GIT of chickens (Ducatelle et al. 2015; Wealleans et al. 2017). Once in the intestines of the bird, *Bacillus* spores can germinate and produce secondary metabolites with potential health benefits for the host animal (Gao et al. 2017). Studies have indicated increased productivity in broiler and laying birds when introduced to *Bacillus*-based probiotics (Lee et al. 2015; Gadde et al. 2017; Rhayat et al. 2017; Neijat et al. 2018, 2019). Early stimulation of beneficial microbiota in broiler chickens is critical in enhancing productivity and health.

In previous studies, we showed that the *Bacillus subtilis* GM5 strain effectively inhibits the growth of pathogenic and opportunistic bacteria and phytopathogenic fungi, possesses probiotic properties, and can be used as a probiotic strain for broiler chickens (Mardanova et al. 2017; Khadieva et al. 2018). In addition, genes responsible for the synthesis of antimicrobial lipopeptides that can provide antagonistic activity against pathogens were annotated in the *B. subtilis* GM5 genome (Hadieva et al. 2019).

The objective of this study was to evaluate the effects of *B. subtilis* GM5 spores on the growth performance, nutrient utilization, and cecal microbial composition of broilers on the 1st, 10th and 42nd days of age.

Materials and methods

Bacterial strain

For bacterial spores acquisition, a 4-day old *B. subtilis* GM5 culture was incubated at 60 °C for 90 min to eliminate vegetative cells (Khadieva et al. 2018). The *Bacillus subtilis* GM5 strain is currently stored at − 80 °C and has been registered into the Collection of the Microbiology Department at the Institute of Fundamental Medicine and Biology of the Kazan Federal University (KFU) under the accession number GM RT 5. The probiotic was introduced into dry feed by spraying with a spray gun with constant manual stirring. The final product contained 1×10^7 spores/g of *B. subtilis* GM5.

Poultry farm, diets and experimental design

All experiments were carried out in compliance with bioethical standards. Animal housing, feeding, and care, as well as animal removal from the experiment, were carried out in accordance with: the requirements for the Care and Use of Experimental Animals of Kazan Federal University and of the experimental unit of the Z.I. Alimchueva commercial poultry farm (Medvedevsky District, Mari El, Russia). All animal management and experimental procedures for this study were approved by the Local Ethics Committee of KFU (Permit number: 22) and carried out in accordance with the Directive of the European Parliament and Council on the protection of animals used for scientific purposes dated September 22, 2010 (Directive 2010/63/UE on the protection of animals used of scientific purposes).

A total of 180 1-day-old chickens Cobb 500 were obtained from a Non-public joint stock company Mariskoe (Medvedevsky District, Mari El, Russia). Birds were neither vaccinated nor separated on a gender basis. For all aspects of the study, we involved both male and female birds at equal proportions. After weighing birds individually for equal weight distribution, an initial average body weight of 47.17 ± 3.13 g for all birds was recorded. The broilers were allocated to 12 battery cages and sorted into two primary groups (Control and Probiotic). Each group had six replicates, with each replicate (per cage) comprising 15 birds. The Control group (C group) was fed with a basal diet, whereas birds of the Probiotic group (P group) were fed the basal diet supplemented with 1×10^7 spores/g of *B. subtilis* GM5.

The chickens had free access to feed and water. At each diet switch, feeders were emptied, orts were weighed back and the feeders were filled with diets. The surrounding

temperature of birds was maintained at 35–36 °C from days 1 to 5, 30–32 °C for the next 6 to 10 days, 26–28 °C from the day 11 to 20, and finally at 20–24 °C from day 21 until slaughter. The experiment lasted 42 days. Photoperiod program was set up according to the European welfare regulation 43/2007 (Council Directive 2007/43/EC, laying down minimum rules for the protection of chickens kept for meat production).

Birds were raised on a three-phase diet. Starter diets were offered to the broilers from days 0 to 10, grower diets from days 11 to 20, and finisher diets from days 21 to 42. The chemical composition of the feed rations (Algorithm Investments LLC) is shown in Supplementary Tables S1a, S1b, S1c.

Observations on the general condition of the herd, temperature, light, water, feed, litter condition and mortality were recorded twice a day. Room temperature and relative humidity were also recorded daily and adjusted accordingly to avoid the influence of stressful conditions on broiler chickens.

Growth Performance indicators

Body weight (BW) of broilers was measured on days 0, 10, 20, and 42. Average daily weight gain (ADWG) was calculated at 42 days of age. Feed intake (FI) was evaluated weekly, and subsequently re-estimated for a single bird. Feed conversion ratio (FCR) was obtained on the 42nd day of age, and the European productivity index (EPI) of broilers was calculated by the formula:

$$\mathrm{EPI} = \frac{\text{Viability (\%)} \times \text{BW (kg)} \times 100}{\text{Age (d)} \times \text{FCR (kg feed/kg gain)}}$$

Sample collection, DNA extraction, and 16S rRNA gene sequencing

Since the feed rations of birds were altered on days 1, 10, and 42 based on their growth and physiological needs, six birds were randomly selected from each group for microbiome analysis on these days. They were euthanized with cervical dislocation followed by decapitation. Immediately after euthanasia, the abdominal cavity was opened, the ceca of each bird were incised, and the contents of both ceca were collected in a sterile 3 mL tube, frozen using liquid nitrogen, and transported to the laboratory on ice, then stored at − 80 °C until DNA extraction. Prior to total DNA isolation, the harvested cecal contents of two birds within each group were combined to obtain three replicates per treatment. In total, 18 samples for cecal contents were used for gut microbiota analysis. Chicken organs (spleen, liver, and heart) were collected and weighed immediately after euthanasia. The organ weight to whole body weight ratio was subsequently determined for each of the three organs.

Total genomic DNA was extracted from the 0.5 g cecal contents of each individual chicken using the commercially available QIAamp Fast DNA Stool Mini kit (QIAGEN, Germany) following the manufacturer's instructions. The quality and concentration of extracted DNA were measured using gel electrophoresis and Qubit 2.0 fluorometer (Life Technology, Carlsbad, USA). DNA was stored at − 20 °C until further processing.

PCR was carried out using Q5 Hot Start High-Fidelity 2X Master Mix (NEB, Great Britain) and universal primers 341F (5-CCT ACG GGN GGC WGC AG-3) and 805R (5-GAC TAC HVG GGT ATC TAA TCC-3) targeting V3–V4 variable regions of the bacterial 16S rRNA gene (Herlemann et al. 2011). The fragment distribution in the pooled library was evaluated using Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and computed using Qubit 3.0 fluorimeter (Thermo Fisher Scientific, USA). The libraries containing 16S rRNA genes were sequenced by 2 × 300 bp paired-end sequencing on the MiSeq platform using MiSeq v3 Reagent Kit (Illumina, USA) at Joint KFU-Riken Laboratory, Kazan Federal University (Kazan, Russia).

OTU clustering and statistical analysis

The Illumina paired-end raw reads of each sample were quality proven using the FastQC v0.11.9 program. Metataxonomic analysis was performed using QIIME2 software, version 2020.2. Poor reads were filtered at the limit of Q20, chimeric sequences were removed by the USEARCH v.10.0 method. After quality filtration and eliminating chimeric sequences from raw reads, clean sequences were clustered into Operational Taxonomic Units (OTUs) with 97% similarity cut off. OTU picking steps were performed using *pick_open_reference_otus.py* script with the default UCLUST approach. The minimum specified OTU size required to keep an OTU was 5, the any OTU which failed to meet this criterion was removed from the further analysis. For the taxonomic classification of the reads, the RDP database v. 2.9 was used. The minimum confidence to record an assignment for rdp classification was 0.8.

After assigning the taxonomy, we used OTUs with a minimum relative abundance of 0.01% of the OTUs per sample. The resulting biom file was summarized at the different taxonomic levels using *summarize_taxa.py*. For data visualization and statistical analysis of diversity metrics, the computing medium R, version 3.6.3 was used. Graphs were computed with biom file data and plotted using the R packages phyloseq v. 1.32.0 and vegan v. 2.5–6. Alpha diversity was assessed using rarefaction plots, Shannon and Chao1 indices. PCoA plots for beta-diversity analysis were

computed using unweighted UniFrac and Bray–Curtis distance matrices among samples.

Additional statistical processing of the results was performed in Graph Pad Prism, Graph Pad Software (LA Jolla, CA, USA) using one-way ANOVA and Tukey test for multiple pairwise comparisons of performance indicators. The results were presented as the mean ± SD, considering P value < 0.05 as significant. Data on relative organ mass-to-bodyweight of chickens were statistically analyzed using the Student t test.

Results

Overall Performance

The average bodyweight, daily weight gain, FI, and total feed conversion ratio were calculated at the indicated time points (1, 10, 20, and 42 days) for each of the two broiler groups (Table 1). The average body weight was higher ($P < 0.05$) in the probiotic group (P-group) than in the control group (C-group). After 10 days body weight gain (BWG) increased by 13.09% ($P > 0.05$) in the P-group in comparison with the C-group. FI was higher in the control group by 16.82% ($P > 0.05$). However, after 20 days, the BWG of the experimental group exceeded that of the control group by an average of 15.26% ($P < 0.0001$). In addition, feed intake was higher by 24.03% ($P < 0.0001$), respectively. On day 42, the increase in live weight of chickens of the experimental group exceeded the control by 12.97% ($P < 0.0001$), and the consumption of animal feed by 11.22% relative to the control ($P < 0.0001$). FCR in the probiotic group (days 1–42) was lower than in the control group by 1.44%. ADWG in the control and probiotic groups amounted to 49.71 g and 56.30 g ($P < 0.05$), respectively. The EPI in the control group was 244.43, as against 280.14 in the experimental group ($P < 0.0001$). Thus, the addition of *B. subtilis* GM5 spores to broiler feed leads to improved feed intake and digestibility, which is paramount in reducing the cost of the final product.

The ratio of organ mass to body weight for the heart ($P = 0.585$), liver ($P = 0.515$), and spleen ($P = 0.599$), was statistically indifferent among birds in the control and experimental groups on day 42 (Table 2)

Table 2 Relative organ to body weight of broilers at day 42

Organs	C-group	P-group	P-value
$Heart_a$	6.408 ± 0.533	6.678 ± 0.580	0.585
$Liver_a$	31.206 ± 2.148	29.573 ± 3.337	0.515
$Spleen_a$	1.477 ± 0.366	1.280 ± 0.474	0.599

Bacterial community structure and diversity of cecal microbiota

DNA sequencing data analysis

Cecal samples were collected, and DNA was sequenced. 116,119 raw paired-end reads were obtained on average for each sample, and following assembly, 75,620 raw spliced tags remained. The trimmed and merged sequences were clustered into OTUs at 97% similarity using uclust. A total of 242 ± 26 and 360 ± 30 OTUs (day 1), 1517 ± 78 и 1602 ± 60 (day 10), as well as 1362 ± 228 and 1628 ± 51 (day 42) were identified for C- and P-groups, respectively (Supplementary Fig. 1). Data saturation was achieved and evaluation of the OTU richness is illustrated via Rarefaction curves.

Microbial abundance and diversity analysis

Chao1 indices were selected to identify community richness, and the Shannon index was used to identify community diversity. Shannon–Wiener's Index of cecal bacterial

Table 1 Effect of *Bacillus subtilis* GM5 supplementation on the growth performance in broiler chickens

Parametrs	Day	Treatment		P value P vs. C
		C (mean ± SD)	P (mean ± SD)	
Body weight (kg)	10	0.275 ± 0.005	0.311 ± 0.006	n.s
	20	0.603 ± 0.024	0.695 ± 0.039	****
	42	2.135 ± 0.085	2.412 ± 0.106	****
Feed intake (kg)	10	0.333 ± 0.007	0.389 ± 0.007	n.s
	20	1.186 ± 0.051	1.471 ± 0.088	****
	42	4.349 ± 0.176	4.837 ± 0.216	****
Feed conversion ratio	1–42	2.08	2.05	
Daily weight gain (g)	42	49.71 ± 2.02	56.30 ± 2.52	**
European productivity index (g)	42	244.43 ± 9.71	280.14 ± 12.25	****

n.s. ** and **** correspond to not significant, significant ($P < 0.05$) and highly significant ($P < 0.0001$), respectively

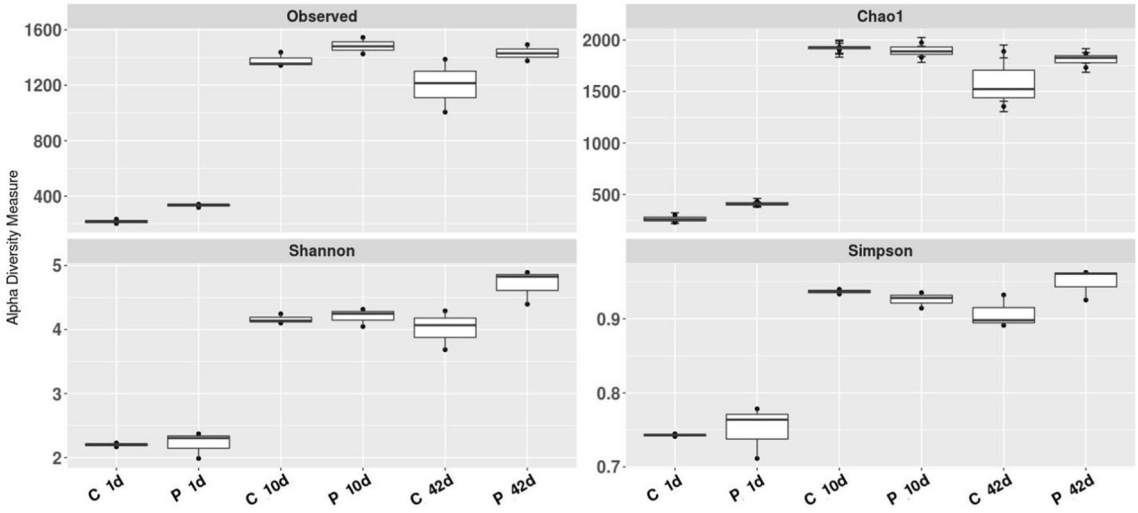

Fig. 1 Comparative differences in bacterial community diversity, richness, and cecal microfloral structure of the P-group and C-group based on Observed OTUs, Chao1, Shannon and Simpson indices. C 1d, C 10d and C 42d correspond to—control group birds on days 1, 10 and 42, respectively. P 1d, P 10d and P 42d refer to probiotic group birds on days 1, 10 and 42, accordingly

communities in the C-group was lower ($P < 0.05$), relative to that of the P-group. Shannon indices of 3.16 ± 0.04 and 3.20 ± 0.30 (day 1), 6.01 ± 0.11 and 6.06 ± 0.19 (day 10), along with 5.72 ± 0.35 and 6.81 ± 0.40 (day 42) were recorded for the C- and P- groups, respectively (Fig. 1, Supplementary Table 4). The species diversity in the probiotic group on the 42nd day was significantly higher than in the control group ($P < 0.05$), based on Shannon's diversity index. In the 1-day-old chicks, Chao1 Index considerably differed between the C-group and the P-group ($P < 0.05$), being 278.67 ± 35.92 and 415.00 ± 17.09, respectively. On days 10 and 42, the cecal composition richness (Chao1) was slightly but insignificantly greater in the probiotic group than in the control group (Fig. 1, Supplementary Table 4). Using Simpson's index, a higher diversity was established in the P-group on both days 1 and 42 in comparison to the C-group (Supplementary Table 4).

To assess the effect of probiotic treatment and growth stage on the gut microbiome, we calculated the unweighted UniFrac distance matrices. Principal coordinate analysis (PCoA) plots for unweighted UniFrac metrics were constructed to evaluate similarities between samples (Fig. 2). The coordinates of the PCoA plots explained 79.22% variation. As seen in Fig. 2, a significant clustering pattern is observed in the PCoA plots, presenting similarity in the microbiome of each growth stage (1-, 10- and 42-days of age). Bacterial communities formed comparatively a closer cluster on day 10, relative to the microbiota of the 1st and 42nd days. Significant differences in the structure of bacterial communities are confirmed by data on changes in the composition and structure of microbiota during its maturation. The intake of probiotics triggers a microbial shift, but

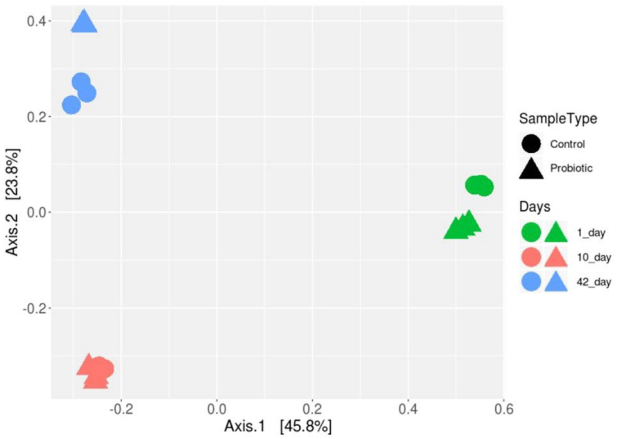

Fig. 2 Pairwise comparison based on unweighted Unifrac distances between cecal microbial communities in broilers supplemented with probiotic (P-group) and broilers fed without probiotics (C-group), on days 1, 10 and 42 of growth. The Principal Component Analysis plot (PCoA) based on Bray–Curtis dissimilarities confirmed bacterial community differences centered on bird's age. The unweighted UniFrac distance PCoA plots based on treatment did not unveil any significantly distinct clustering pattern between the C- and P-groups

not as significant as age-related changes (Fig. 2). Moreover, the most significant structural changes in the P-group microbiota were observed in broilers on days 1 and 42.

Analysis of dominant bacterial taxa

From the cecal samples of groups, 9 phyla, 17 classes, 20 orders, 38 families, and 68 genera were identified (Supplementary Table 5). *Firmicutes, Proteobacteria*, and

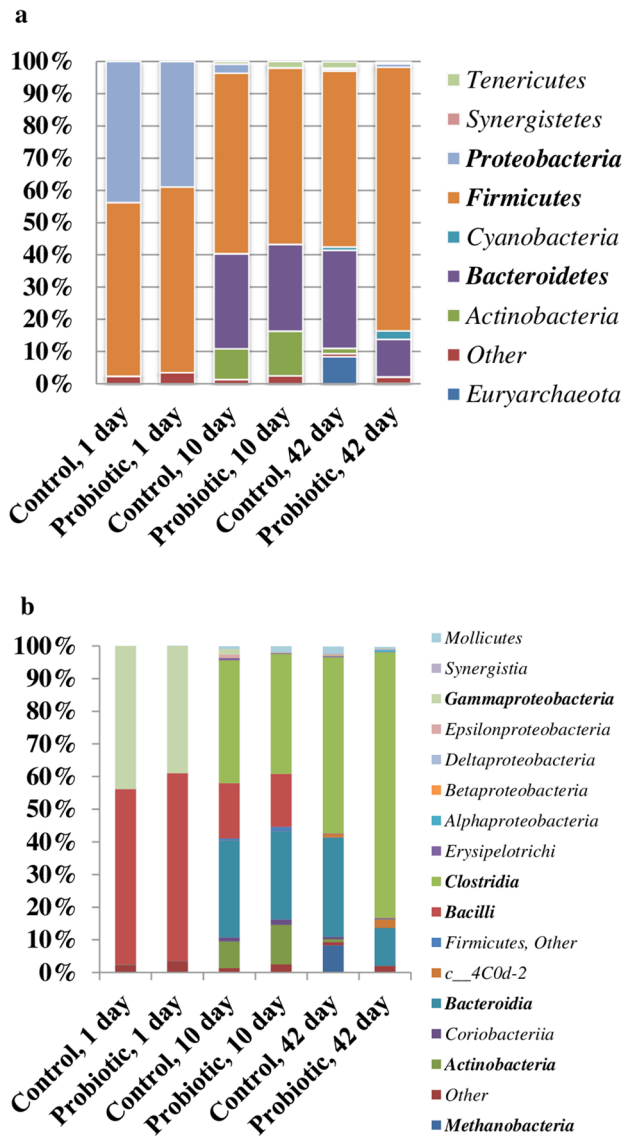

Fig. 3 Relative abundances (%) of bacterial phyla (**a**), class (**b**) in the cecum of broiler birds in control and treatment (probiotic) groups. Birds were euthanized on days 1, 10 and 42 of age. Treatments represent birds fed with the spores of *B. subtilis* GM5

Bacteroidetes were the predominant phyla (Fig. 3a). However, depending on the age of the chickens, changes in the representation of different phyla were observed. For instance, on day 1 *Firmicutes* and *Proteobacteria* were prevalent, while in samples of days 10 and 42—were occupied by *Firmicutes* and *Bacteroidetes* (Fig. 3a). On day 1 the proportion of *Firmicutes* was 53.87 ± 0.39% and 57.61% ± 1.38% in C-group and P-group, respectively. On days 10 and 42, the proportion of *Firmicutes* were 56.01 ± 3.65% and 54.55 ± 7.12% in C-group, along with 54.74 ± 1.75% and 81.79 ± 9.42% in P-group, respectively. The abundance of *Firmicutes* in cecal communities of P-group significantly increased on day 42 in comparison to day 10 ($P < 0.0001$).

Proteobacteria were only significantly present (43.77 ± 0.42% and 38.93 ± 1.44% for C- and P-groups, respectively) in the cecum for 1-day old chickens in both groups. Subsequently, *Proteobacteria* in the cecal samples decreased to 2.79 ± 1.25% and 0.094 ± 0.07% on day 10, to 0.54 ± 0.18% and 1.01 ± 0.68% on day 42 in C- and P-groups, respectively. The proportion of *Bacteroidetes* also varied significantly depending on the age of the broilers. Bacteria of this group were practically absent in cecal samples of 1-day old chicks, but on the 10th day, their proportion increases to 29.50 ± 2.73% (C-group) and 26.94 ± 2.22% (P-group). On day 42, the proportion of *Bacteroidetes* virtually remained unchanged in the C-group (30.45 ± 2.39%), but significantly decreased in the group of chickens treated with probiotic (11.65 ± 6.32%). It was interesting to note that representatives of the phylum *Actinobacteria* were significantly detected only in samples of 10-day old chickens and their share amounted to 9.48 ± 0.3% and 13.79 ± 5.71% in C- and P-groups, respectively (Fig. 3a). In cecal samples of 1 day-old chicks, their share was less than 0.1%. In 42 day-old birds, the proportion of these bacteria was as low as 1.63 ± 0.52% and 0.09 ± 0.02% in C- and P-groups, respectively. The members of phyla *Euryarchaeota, Cyanobacteria, Synergistetes*, and *Tenericutes* represented minor communities of the gut microbiota (Supplementary Table 5).

At the class level, *Firmicutes* were primarily represented by the classes *Bacilli* and *Clostridia* (Fig. 3b, 4a), but their proportions reduced sharply. On day 1, the abundance of *Bacilli* was 53.45–54.23% (C-group) and 56.21–58.95% (P-group) but significantly decreased to 16.89 ± 2.02% and 16.23 ± 2.57% on day 10, respectively ($P < 0.0001$). Conversely, the abundance of *Clostridia*, significantly increased on day 10, relative to day 1 samples and amounted to 37.66 ± 1.66% in C-group ($P < 0.0001$) and 36.71 ± 1.71% in P-group ($P < 0.0001$). On day 42 the cecal microbiota was predominantly inhabited by *Clostridia*, which occupied more than 53% in C-group and 81% in P-group, respectively.

Within *Firmicutes*, the majority belonged to the *Lachnospiraceae, Ruminococcaceae* families (order *Clostridiales*), and *Enterococcaceae*, *Lactobacillaceae, Streptococcaceae* families (order *Lactobacillales*) (Fig. 5a, b). The relative proportions of these bacteria varied depending on age and availability of feed additives. In particular, the cecum of 1-day old chickens was first colonized by different representatives of *Lactobacillales* (53.25 ± 0.35% and 57.49 ± 1.39% in C- and P-groups, respectively). Different families of the *Lactobacillales* order showed dominance in the control and experimental groups: in the C-group, the *Lactobacillales* order was represented chiefly by *Enterococcaceae* (51.92 ± 0.50%) and *Streptococcaceae* (0.69 ± 0.22%). The P-group presented an inverted picture, in which samples were predominantly occupied by *Streptococcaceae* (40.10 ± 4.75%) and *Enterococcaceae* (4.76 ± 1.72%).

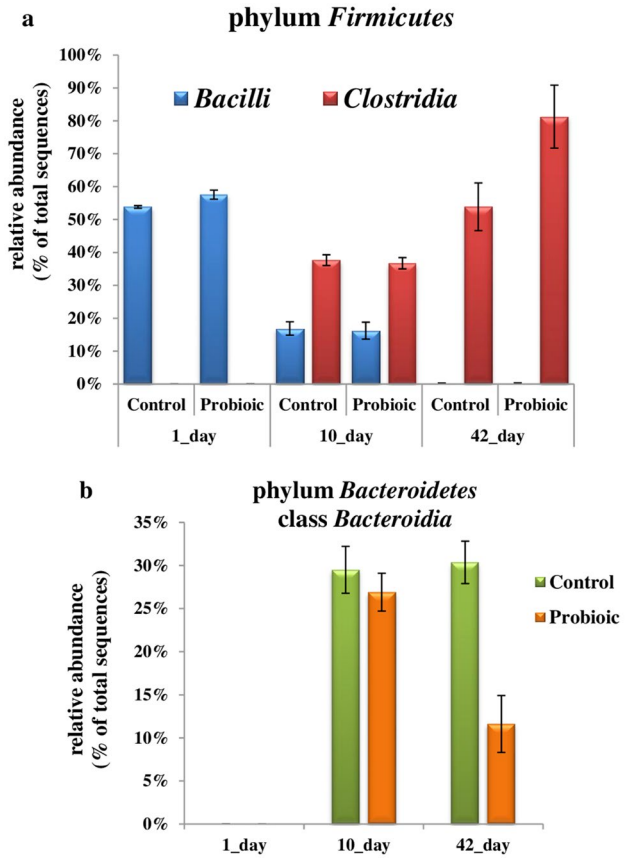

Fig. 4 Relative abundance of different classes within phylum *Firmicutes* (**a**) and phylum *Bacteroidetes* (**b**) in the cecum of the control and probiotic groups of broilers on days 1, 10 and 42 of age

The share of members of the *Lactobacillaceae* family in 1-day-old chickens varied considerably in the two groups and amounted to 0.06 ± 0.02% and 6.7 ± 2.49% in the control and probiotic group, respectively. It could be noted that in cecal samples of 10 day-old birds, the order of *Lactobacillales* is represented almost exclusively by *Lactobacillaceae*, the proportion of which were recorded at 16.64 ± 1.96% and 15.99 ± 2.52% in C- and P-groups, respectively. However, by the 42nd day, the abundance of *Lactobacillaceae* and other bacteria of this order had significantly decreased in both groups of the broiler birds ($P < 0.0001$). A decrease in the share of *Lactobacillales* on the 10th day correlated with an increase in the presence of *Lachnospiraceae* and *Ruminococcaceae* families of *Clostridiales* (C 37.65 ± 1.69%, P 36.68 ± 1.70%). The proportion of *Lachnospiraceae* in the control group (12.16 ± 0.45%) was higher than that of the experimental group (6.35 ± 0.52%) on the 10th day. By day 42, the proportion of these bacteria had decreased to 2.38 ± 0.4% (C) and 0.15 ± 0.002% (P).

On day 10 the proportion of *Ruminococcaceae* was 18.47 ± 0.74% in the C-group and 22.54 ± 3.07% in the P-group but significantly increased to 44.30 ± 5.45% and 64.67 ± 10.67% on day 42, respectively. Thus, the use of *B. subtilis* GM5 as probiotics led to a significant increase ($P < 0.0001$) in the number of *Ruminococcaceae* during the development of the cecal bacterial community.

At the genus level, different representatives exhibited dominance depending on the age of birds (Fig. 5). On day 1, *Firmicutes* were represented by *Enterococcus* (46.89 ± 0.38%) in the C-group and *Streptococcus* (38.70 ± 5.08%) in the P-group. On the 10th day, the cecal microbiota in the C- and P-groups was occupied mainly by *Lactobacillus* (16.37 ± 1.85% and 15.33 ± 2.55%) and *Faecalibacterium* (7.07 ± 0.8% and 14.16 ± 3.64%). Moreover, the proportion of *Faecalibacterium* in the probiotic group was significantly higher than that of the control group ($P < 0.0001$). On day 42, the two groups were highly populated with *Faecalibacterium* (25.44 ± 4.12% and 22.39 ± 5.92% for C-group and P-group, respectively), the proportion of which was significantly higher than on the 10th day ($P < 0.01$) (Table 3). In addition, *Firmicutes* were chiefly represented on day 42 by unclassified members *Ruminococcaceae* and *Oscillospira,* the relative abundances of which were two and three times higher in the P- and C-groups, respectively ($P < 0.0001$) (Fig. 5, Table 3).

The phylum *Proteobacteria* was predominantly represented by the class *Gammaproteobacteria,* representation of which covered 43.77 ± 0.42% and 38.93 ± 1.44% in C- and P-groups, respectively ($P < 0.01$) on day 1. However, on day 10 *Gammaproteobacteria* decreased significantly to 1.7 ± 0.61% in C-group and 0.05 ± 0.02% in P-group ($P < 0.0001$). Within the *Enterobacteriaceae* family, the majority corresponded to the genera *Escherichia* (C-group = 18.42 ± 2.48 and P-group = 37.77 ± 1.56%) and *Klebsiella* (C-group = 13.88 ± 2.94% and P-group = 0.04 ± 0.0%) (Supplementary Table 5).

The abundance of the dominant class *Bacteroidia* was 29.51 ± 2.73% (C-group) and 26.94 ± 2.22% (P-group) in the day 10 sample, but in 42-day-old chickens, they occupied 30.45 ± 2.39% in the control group but decreased to 11.65 ± 6.32% in the probiotic group (Fig. 4b). The principal genera were *Barnesiella* and *Bacteroides* (Table 3).

On day 10, the principal family and genus of *Actinobacteria* were *Bifidobacteriaceae* and *Bifidobacterium,* the abundance of which were, respectively, higher ($P < 0.05$) in the P-group (12.07 ± 5.16% and 10.14 ± 4.20%) than in the C-group (8.17 ± 0.49% and 7.40 ± 0.41%). However, their representation significantly decreased by day 42.

An important parameter is a ratio between the two dominant phyla, *Firmicutes* and *Bacteroides* (F/B ratio), the total share of which amounts to 81–85% in the cecum on the 10th day and 85–93% on the 42nd day. It is interesting to note that the use of the probiotic significantly increased the F/B ratio, especially in 42-day-old birds. To

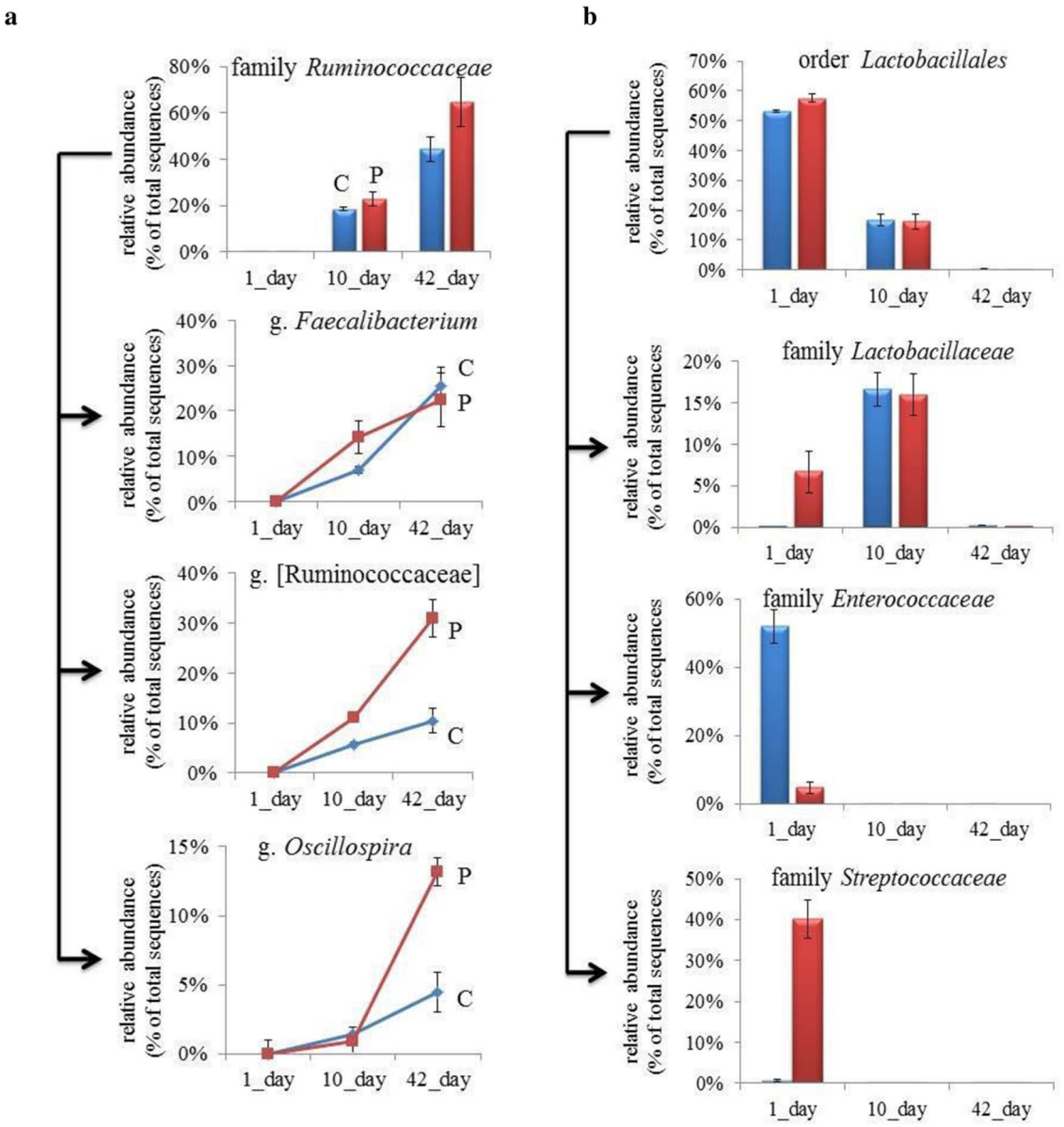

Fig. 5 Relative abundance of different genera within f. *Ruminococcaceae* (**a**) and different families of the order *Lactobacillales* (**b**) in the control (C) group and probiotic (P) group on days 1, 10 and 42 of age

be precise, the F/B ratio in the control group was approximately at the same level and amounted to 1.89 (10th day) and 1.79 (42nd day). On the other hand, the ratio value of the experimental group increased to 2.03 and 7.02 on days 10 and 42, respectively. The higher number of *Firmicutes* in the experimental group directly correlates with the higher weight gain of broiler birds in the experimental group on the 42nd day. Spearman's correlation analysis showed a positive correlation between the F/B ratio and chickens' body weight (Spearman $\rho = 1.0$, $P < 0.05$).

Discussion

The results of the present study demonstrate that supplementation with the spores of *B. subtilis* GM5 (1.0×10^7 CFU/g) as probiotic in broiler diets can promote broiler weight gain and daily weight gain while lowering FCR. The average body weight of the P-group chickens was higher than the mean body weight of the C-group by 15.26% ($P < 0.0001$) on day 20 and 12.97% ($P < 0.0001$) on day 42. FCR of broilers from the 1st to the 42nd day

Table 3 The relative abundance of the main bacterial taxa in the cecum of broiler chickens on day 42 and the effect of the probiotic *B. subtilis* GM5

Taxon	Relative abundance (% of total sequences)	
	Control	Probiotic
Phylum		
Firmicutes	54.55±7.12	81.79±9.42 ↑
Class		
Clostridia	53.88±7.24	81.28±9.56 ↑
Order		
Clostridiales	53.85±7.24	81.88±9.59 ↑
Family		
Ruminococcaceae	44.30±5.45	64.67±10.76 ↑
Genus		
Faecalibacterium	25.44±4.12	22.39±5.92
Oscillospira	4.48±1.41	13.16±0.42 ↑
Ruminococcus	2.34±2.01	4.12±0.69
Phylum		
Bacteroidetes	30.45±2.39	11.65±6.32 ↓
Class		
Bacteroidia	30.36±2.46	11.61±6.31 ↓
Order		
Bacteroidales	30.36±2.46	11.61±6.31 ↓
Family		
Bacteroidales; Other	3.40±1.94	0.23±0.06
Bacteroidaceae	11.83±8.41	3.24±2.50 ↓
Porphyromonadaceae	2.39±1.23	2.82±1.21
Prevotellaceae	2.95±2.10	1.79±0.67
Rikenellaceae	1.86±1.53	1.99±1.30
Genus		
Bacteroidales; Other; Other	3.40±1.94	0.23±0.06 ↓
Bacteroides	11.73±8.33	3.24±2.50
Parabacteroides	2.37±1.23	2.81±1.21
Prevotella	2.90±2.10	1.75±0.67

Mean percentages and standard error (S.E.) of bacterial abundance observed from 18 samples

Arrows indicate an increase (↑) or decrease (↓) in the relative abundance (% of total sequence)

was 1.44% lower in the P-group than in the control. It has been shown that dietary supplementation with *B. subtilis* CGMCC 1.1086, *B. subtilis* UBT-MO_2 and three other *B. subtilis* strains effectively improves the growth performance and FCR of broilers via the beneficial modulation of cecal microbiota (Amerah et al. 2013; Zhang et al. 2013;

Li et al. 2016). Some probiotics exert a minimal effect on the growth rates of broiler chickens (Mountzouris et al. 2007; Lee et al. 2010; Jerzsele et al. 2012). These discrepancies in results could be tied to differences in strains used as probiotics, the dosage of administration, preparation methods, poultry age, dietary composition and hygiene (Lee et al. 2010; Zhang et al. 2012; Li et al. 2019). The formation of the intestinal microbiota of broiler chickens depends on several factors and undergo regular age-related changes (Xu et al. 2016; Kers et al. 2018; Ngunjiri et al. 2019). Noteworthy differences in the composition of the cecal microbiota were found in chickens raised under wild conditions and those bred on farms (Pandit et al. 2018; Kubasova et al. 2019; Rychlik 2020).

Microbes inhabiting the intestines of a day-old chick are classified as variable microbiota, thus the colonization and composition of the original intestinal microbiota of hatched chickens are expected to vary significantly among individual birds from different incubators (Pedroso et al. 2005; Kers et al. 2018). This explains the divergence in results obtained by various researchers. For example, Ballou et al. (2016) showed that the dominant group in the cecum on the first day are *Enterobacteriaceae* (85%) (phylum *Proteobacteria*). In contrast, another study reported a relative dominance of *Pelotomaculum* (o. *Clostridiales*) and *Enterococcus* (o. *Lactobacillales*) in the cecum (Pedroso et al., 2016). Upon arrival, chickens are exposed to a more diversified microbial environment at the farm, consisting primarily of bacteria from litter, feed, and water. Succession emerges so rapidly that the microbiota of the intestines begins to differentiate from the third day of age in poultry birds (Lu et al. 2003). The cecal microbiota of a 3-day old chick is predominantly populated with *Firmicutes*, in particular members of the *Ruminococcaceae* family, as well as other representatives of the order *Clostridiales.* The prevalence of these groups of bacteria persists in birds even until the 28th day of age (Caporaso et al. 2012). It has been reported that the GI of a 7-day old bird is primarily inhabited by *Flavonifractor*, *Pseudoflavonifractor*, and *Lachnospiraceae* (o. *Clostridiales*), but with age (from 7 to 42 days), the diversity of the cecal microbiota steadily increases (Oakley et al. 2014a, b). In another study, it was shown that the displacement of *Enterobacteriaceae* bacteria with *Clostridiales* occurs on day 14 (Wise and Siragusa, 2007). Similar results were found in other investigations, confirming that the cecum is first colonized by *Enterobacteriaceae*, *Lactobacillus*, and *Bifidobacterium*, which are later replaced by *Clostridiales* (Zhu and Joerger 2003).

Our studies revealed that the cecum of broilers was initially colonized by gram-positive *Firmicutes* and gram-negative *Proteobacteria*, the proportion of which was approximately equal in both control and experimental birds. However, these phyla were represented by diverse dominant families in fowls, depending on their diet. To be precise, *Firmicutes* were represented mainly by *Enterococcaceae*, in the cecum of control chickens and *Streptococcaceae* in experimental chickens. The structure of representatives of *Enterobacteriaceae* likewise varied. The enterobacteria *Escherichia* and *Klebsiella* were present in the control birds, while the absence of *Klebsiella* was noted in chickens fed with the probiotic. Thus, the use of a probiotic led to changes in microbiota even at the very early stages of cecal colonization, causing a decrease in the representation of the bacterial groups *Enterococcaceae* and *Klebsiella*, among which many pathogenic strains are classified.

On the 10th day, the chickens showed significant changes in the cecum microbiota. These changes occurred primarily due to the maturation of the microbiota and correlated with probiotic use (Fig. 2). In both groups, the previously dominant *Bacilli* group is partially replaced by *Clostridia*. Subsequently, the emergence of *Bacteroidi*a and *Actinobacteria* (fam. *Bifidobacteriaceae*) in the community is observed, as the share of *Enterobacteriales* significantly decreases. The addition of a probiotic leads to a more significant decrease in the proportion of *Enterobacteriales*, which is a positive factor. But in general, it shows that on day 10, beta-diversity of the cecal microbiota in experimental and control chickens does not differ much in terms of bacterial composition. Data from a previous study suggested that gut the microbiome is differentially affected by age than treatment (Ballou et al. 2016). The relatively high representation of groups such as *Bifidobacteriaceae* and *Lactobacillaceae* in the cecum of chickens in the early stages of development seems to be a natural stage in gut microbiota development. Thus, as the cecal microbiota matures, *Enterobacteriales* is gradually replaced by *Bacteroidales* and *Bifidobacteriales*.

On day 42, besides significant age-related changes, substantial differences are revealed in the microbiota structure of the control and experimental groups (Fig. 2, 4, 5a). In both groups, clostridial members almost completely ousted *Lactobacilli*, which are usually present in early life. Moreover, the proportion of *Clostridiales* in experimental broilers (81.79%) was significantly higher as compared to the control (54.55%) (Table 3, Fig. 4), while the proportion of *Bacteroidales* was 2.6 times lower in experimental chickens than in the control.

The bacteria of the two dominant phyla, *Bacteroidetes* and *Firmicutes* are known to play a vital role in the digestion of nutrients. The genomes of *Bacteroides* contain many genes involved in the metabolism and degradation of complex polysaccharides and mono sugars. These genes are likewise known to actively produce organic acids, as well as encode proteins and enzymes that play a central role during interactions with their hosts (Magnusdottir et al. 2017; Medvecky et al. 2018). Many representatives of *Bacteroidetes* and *Firmicutes* are capable of synthesizing short-chain

fatty acids, especially butyrate (Anand et al. 2016; Medvecky et al. 2018). Several members of the family *Ruminococcaceae* can digest the cellulose in feed and produce short-chain fatty acids, as well as play essential roles in the digestions of lipids (Li et al. 2019; Medvecky et al. 2018).

It was shown that increased *Firmicutes/Bacteroidetes* (F/B) ratios were associated with growth promotion in chickens (Mancabelli et al. 2016; Salaheen et al. 2017). A high F/B ratio in the cecum leads to the active fermentation of volatile fatty acids, contributing to the deposition of fat (Mancabelli et al. 2016). In this study, the addition of *B. subtilis* GM5 facilitated bird growth and modulated the microbiota and, in particular, increased the F/B ratio in the experimental group relative to the control. The data obtained indicate that an increase in the level of *Firmicutes,* and *Clostridiales* in broiler ceca may be one of the many factors contributing to the enhanced growth of broiler chickens.

The *Firmicutes* phylum includes a wide range of beneficial bacteria, such as *Ruminococcaceae*, *Lactobacillaceae*, *Lachnospiraceae,* and *Streptococcaceae*. Probiotic strains of *Bacillus* are aerobic bacteria that consume large amounts of free oxygen, as well as secrete various metabolites when growing in the intestinal tract. As a result, they inhibit the growth of most pathogenic bacteria (*Escherichia, Salmonella*, etc.) and enhance the growth of beneficial anaerobic bacteria, such as *Lactobacillus* and *Bifidobacterium* (Wang et al. 2006; Gao et al. 2017). Being a transient member of the intestinal microbiota *B. subtilis* does not colonize the intestines. On the contrary, they can increase the relative prevalence of beneficial microbiota in the cecum and, apparently, contribute to faster maturation of the gut microbiota and increase its diversity.

Conclusion

The results of this study demonstrate that supplementing the diet of broilers with spores of *B. subtilis* strain GM5 improves productivity by increasing the weight gain of birds and reducing feed conversion, as well as increases diversity and the relative abundance of beneficial microbiota in the cecum. We conclude that the addition of the probiotic from the first days of life can regulate and stabilize the microbiota of the digestive tract of chickens, which is essential for growth and development.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13205-020-02634-2.

Acknowledgements The culturing of *Bacillus subtilis* GM5 and the obtainment of spores were supported by the research grant of Kazan Federal University. All other experiments were supported by a grant from the Russian Science Foundation (No. 16-16-04062).

Author contributions MR AM designed the research and supervised all research; GH, MT, NG, DP, YA carried out the experiments and analyzed the data and drafted; DP, GH prepared figures; GH, AM, YA wrote the manuscript; MS, AM, ES partook in the revision of the manuscript. All authors have read and approved the final version of the manuscript.

Availability of data All datasets generated for this study have been included in the article/Supplementary Material. Sequence data has been uploaded to the European Nucleotide Archive and can be accessed from the website https://www.ebi.ac.uk/ena using the accession number PRJEB37602.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interests regarding the publication of this paper.

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