

Effect of probiotic strains of *Bacillus subtilis* on the growth parameters of broiler chickens and caecal microbiota

Ayslu Mardanova¹, Anastasia Nikolaeva¹, Guzel Lutfullina^{1,*}, Aygul Ganeeva¹, and Margarita Sharipova¹

¹SRL "Microbial biotechnologies", Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan, Russia

Abstract. In this paper, the probiotic properties of *Bacillus subtilis* GM2 and GM5 strains were studied. It is shown that the use of probiotic additives based on the spores of these bacteria leads to an increase in the live weight gain of broiler chickens by 4.16% and 10.76% relative to the control. Metagenomic analysis showed that representatives of the phylum *Firmicutes* (54.55%) and *Bacteroidetes* (30.45%), mainly represented by the families *Ruminococcaceae* and *Bacteroidaceae*, predominate in the caecal microbiota of broiler chickens on day 42. It was found that a probiotic based on the *B. subtilis* GM5 strain leads to an increase in the proportion of *Firmicutes* in caecum by 27% and a decrease in *Bacteroidetes* by 19%. There was also a significant decrease in the number of representatives of opportunistic pathogenic bacteria of the *Enterobacteriaceae* family relative to the control group.

1 Introduction

Scientists estimate that by 2050, the World's population will reach a peak of 10 billion [1]. The dynamic growth of the population is accompanied by an increase in the need for food products and an increase in their quality and productivity standards [2]. One of the urgent problems on the way to intensification of food production is the exposure of farm animals to stress, including those associated with unsatisfactory conditions or unbalanced nutrition, which can lead to an imbalance of the intestinal microbiota and, as a result, to the risk of infection with pathogens that lead to reduced productivity and contamination of food products [3].

In the conditions of large poultry enterprises, broiler chickens are exposed to a number of adverse stress factors that pose a threat to poultry health, leading to a shift in the balance of normal gastrointestinal microflora and slowing down the development of immune-competent organs [4]. Certainly, it is currently impossible to completely abandon vaccination, the use of antibiotics, anthelmintics and coccidiostats in large farms. However, it is possible to limit them with mandatory restoration of microflora using probiotics [5].

* Corresponding author: g.h95@mail.ru

Most probiotics used in poultry farming are bacteria that exist in the digestive tract of animals and have the properties of modulators of intestinal cell signals, stabilizers of the bacterial community, or competitors of pathogenic bacterial species [6]. The mechanism of action of probiotics is not aimed at destroying, but at displacing potentially pathogenic and pathogenic microflora due to antagonistic activity. By colonizing the intestines of chickens at an early age, probiotic bacteria create a kind of biological barrier for opportunistic microorganisms, and also synthesize antimicrobial compounds that act selectively against pathogens during their metabolism [7].

Probiotics based on *Bacillus* have a number of advantages: viability in a wide temperature range, ease of cultivation, and low cost of production. Probiotic functions are performed not only by vegetative cells, but also by germinating bacillus spores [8], which in turn are able to withstand various external stresses. The basis of probiotic activity of *Bacillus* is their high antagonistic activity, which provides competition with pathogenic microflora for adhesion sites and nutrients, production of antimicrobial compounds, and maintenance of the immune status [9]. The ability of *Bacillus* to withstand temperature changes is necessary at certain stages of feed processing [10]. It is known that the intestinal microbiota is involved in the formation of the immune system. It was found that the immune system of the gastrointestinal tract of broiler chickens is stimulated by probiotic strains of *Bacillus* due to an increase in the expression of cytokinins and interleukins (interleukin-1B (IL-1B) and interferon- γ (IFN γ)) [11], as well as the synthesis of such cytokines as IL-2, IL-4, IL-10 and TNF- α in the mucosa of the caecum and ileum of broiler chickens [12].

The gastrointestinal microbiota of chickens takes an active part in the digestion processes. With the introduction of metagenomics methods, it has become obvious that only a smaller part of bacterial taxons living in the intestine can be identified using culture techniques, and knowledge about the composition and dynamics of microbial communities in different sections of the intestine is still in dearth [13]. The highest density of bacteria is found in the outgrowths of the caecum, where nutrients are found for the longest time in the digestive process (12-20 hours). It was found that about 10% of the energy produced during digestion is provided by the caecum, the microbial community of which is represented by the phylum *Firmicutes* (*Clostridiales*), *Bacteroides*, *Proteobacteria* and *Actinobacteria*. The dominant group remains the *Clostridiaceae* family (up to 85%) [14].

Due to the need to increase the production of poultry products and search for alternatives to the use of feed antibiotics, it is important to study the regularities of the formation of the intestine microbiota of chickens and establish the effect of probiotics based on different types of bacteria on this process. The aim of the work was to characterize the ability of two strains of *B. subtilis* to stimulate the growth of Cobb-500 cross broiler chickens and the effect of the GM5 strain on the formation of the microbiota of the caecum of chickens.

2 Materials and methods

2.1 Research objects

B. subtilis GM2 and GM5 strains with high antagonistic activity isolated from potato rhizosphere were used as probiotics [15], whose probiotic properties were previously characterized *in vitro* [10]. *B. subtilis* GM2 and GM5 spores were obtained according to the method described in [10].

2.2 Study of the effect of *B. subtilis* GM2 and GM5 on broiler chickens

The *in vivo* experiment was performed in the conditions of the "Lachyn" Agricultural Enterprise (AE). Ninety 1-day-old Cobb-500 cross chickens were selected with an average live weight of 47.17 ± 3.13 g, from which a control group and two experimental groups of 30 chickens were formed. The experimental groups received compound feed with the addition of a suspension of *B. subtilis* GM2 (experimental group 1) and *B. subtilis* GM5 (experimental group 2) spores at a concentration of 1×10^7 CFU/g of feed. From 0 to 10 days, chickens were fed with a compound "Starter" feed (LLC Algorithm Investitsiy) in the form of pellets, from 11 to 20 days – a "Grower" feed (LLC Algorithm Investitsiy) in the form of grits, and from 21 days until slaughter (42 days) – a "Finisher" feed (LLC Algorithm Investitsiy) in the form of pellets. The probiotic was added to the dry food by spraying with a spray gun with constant manual stirring. The chickens were kept in ventilated cell batteries at a temperature of 35-36°C with artificial lighting for 24 hours a day. All experiments were performed in compliance with bioethical standards. Content, nutrition, care of animals and removal of birds from the experiment was carried out in accordance with the requirements of the Ministry of Higher and Secondary Special Education of the USSR Order № 742 dated 13.11.1984, approved the "Rules of works using experimental animals", which still apply today, and a Directive of the European Parliament and the Council dated September 22, 2010 on the protection of animals used for scientific purposes (Directive 2010/63/UE on the protection of animals used of scientific purposes).

2.3 Determining growth parameters of broiler chickens

In the course of scientific and practical experience, daily weighing of control and experimental poultry groups was carried out, including determining live weight, average daily body weight gains and livestock safety. By measuring the remaining feed, the amount of feed consumed was determined. The amount of feed consumed was recalculated per chicken. The feed conversion rate was calculated by dividing the feed consumed by the body weight gain.

2.4 Sample selection, DNA isolation and 16S rRNA sequencing

On day 42, 3 broilers were randomly selected from the experimental (No. 2) and control groups. All chickens were euthanized, after which the abdominal cavity was opened. The caecum of each bird was cut and the contents were collected in a 3 ml sterile tube, frozen using liquid nitrogen, and transported to the laboratory in a dry ice bag, then stored at -80°C until DNA was isolated. A total of 6 samples of the contents of the caecum were collected.

Total genomic DNA was isolated from 0.5 g of the contents of the caecum of each individual chicken using the commercial QIAamp Fast DNA Stool Mini kit (QIAGEN, Germany), according to the protocol. The concentration and quality of DNA were evaluated using NanoDrop 2000 (Thermo, USA) and gel electrophoresis. The DNA was stored at -20°C until further processing.

PCR was performed using Q5 Hot Start High-Fidelity 2X Master Mix (NEB, UK) and universal primers 341F (5-CCT ACG GGN GGC WGC AG-3) and 805R (5-GAC TAC HVG GGT ATC TAA TCC-3) (variable regions of V3 - V4 gene of the bacterial 16S rRNA) [16]. The distribution of fragments in the library was evaluated using an Agilent 2100 bioanalyzer (Agilent Technologies, USA), and was calculated using a Qubit 3.0 fluorometer (Thermo Fisher Scientific, USA). Libraries containing 16S rRNA genes were

sequenced on the MiSeq platform using the MiSeq v3 panel (Illumina, USA) at the KFU-Riken Extreme Biology laboratory.

Sequencing data was analyzed using the QIIME program version 1.5.0. Statistical processing of the results included the calculation of the mean value (M) and the standard error of the mean (\pm SD) with a confidence value of $P < 0.05$.

3 Results and discussions

3.1 Effect of *B. subtilis* GM2 and GM5 on growth and feed consumption of Cobb-500 cross broiler chickens

The effect of a probiotic supplement based on the spores of *B. subtilis* GM2 (experimental group 1) and GM5 (experimental group 2) strains on the growth dynamics of COBB-500 cross broiler chickens was studied. The safety of livestock over the entire period of scientific and practical experience was 100% (table 1). From 21 to 42 days, the live weight gain of chickens from both experimental groups was significantly higher in the experimental groups, exceeding the control by 4.16% and 10.76%, $p < 0.05$, respectively. Weight gain in broilers of experimental groups may be associated with normalization of the microflora composition, including a decrease in the number of pathogenic bacteria.

The use of a probiotic based on *B. subtilis* GM5 spores increased feed digestibility, and the addition of *B. subtilis* GM2 spores slightly reduced feed conversion. The value of feed conversion in experimental group 2 significantly decreased by 11.74%, $p < 0.05$ relative to the control. Earlier in their works, Sen et al. (2012) demonstrated improved feed conversion and increased live weight gain when using probiotics based on the *B. subtilis* LS 1 - 2 strain. It was noted that the introduction of *Bacillus* leads to histomorphologic changes in the intestines of broilers, an increase in the height of the villi, which improves the absorption of nutrients through an increase in the absorption capacity of the small intestine [17]. The results of our scientific and practical experiment with GM2 and GM5 spores are consistent with data from other studies, which also found that different strains of *B. subtilis* can improve feed conversion [18-19].

Table 1. The effect of feed additives based on *B. subtilis* GM2 and GM5 spores on the growth and development of COBB-500 cross broiler chickens

Indicator	Control group	Experimental group 1, receiving compound feed+ <i>B. subtilis</i> GM2	Experimental group 2, receiving compound feed + <i>B. subtilis</i> GM5
Body weight gain (g) per 1 chicken			
Live weight for 42 days	2134.33 \pm 49.23	2265.67 \pm 48.52	2422.00 \pm 58.28
Compound feed consumption (g) per 1 chicken			
Total	4349.40	4724.50	4709.00
Feed conversion			
Total	2.08 \pm 0.047	2.13 \pm 0.041	1.98 \pm 0.022

Number of livestock (pcs)	30	30	30
Safety of livestock (%)	100	100	100
Average daily weight gain, (g)	49.69±1.12	52.82±1.00	56.54±1.31

Note: $p < 0.05$

Thus, the addition of a probiotic supplement based on the spores of *B. subtilis* GM2 and GM5 bacteria to chicken compound feed leads to an improvement in feed intake, which may be due to the maintenance of beneficial intestine microflora, improvement of digestive processes and increased activity of intestinal enzyme synthesis.

3.2 Effect of feed additives based on *B. subtilis* GM5 spores on the caecal microbiota of broiler chickens

Comparative metagenomic analysis of the structure of the caecal bacterial microbiota in broiler chickens of the control group on day 42 of growth showed the dominance of representatives of the phylum *Firmicutes* (54.55%) and *Bacteroidetes* (30.45%) (Table 2). The use of probiotics based on *B. subtilis* GM5 spores resulted in an increase in the proportion of *Firmicutes* to 81.79% and a decrease in the proportion of *Bacteroidetes* to 11.65% (table 2). There was a decrease in the representation of *Actinobacteria* and *Tenericutes*: in the experimental group, the proportions of these phyla were 0.09% and 0.49%, while that of the control group were 1.63% and 1.97%, respectively. The proportion of *Euryarchaeota* archaean phylum (0.06%) significantly decreased compared to the control group (8.36%). The number of *Cyanobacteria* and *Proteobacteria* representatives, on the contrary, increased by 2.5 and almost 2 times relative to the control (table 2).

An increase in the proportion of *Firmicutes* and a decrease in *Bacteroidetes* are also described in other works that investigated the effect of *B. subtilis* spores on the intestinal microflora. *Firmicutes* are known to play an important role in polysaccharide metabolism and energy use in the intestine due to the secretion of glycane-destroying enzymes. It was also previously established that animal growth indicators positively correlate with the abundance of *Firmicutes* in the intestine, especially in relation to the abundance of *Bacteroidetes* [20]. It is assumed that the high relative number of *Firmicutes* in birds may be associated with increased metabolic efficiency, which is accompanied by an increase in feed conversion [21]. However, other studies have shown that under the influence of *Bacillus*, on the contrary, the proportion of *Firmicutes* decreased relative to *Bacteroidetes*, and the share of *Proteobacteria* increased relative to the control [22].

Table 2. The effect of probiotic based on *B. subtilis* GM5 spores on the caecal microbiota of broiler chickens on 42 days of growth

Domain	Phylum	Proportion in microbiota, %	
		Control group	Experimental group receiving compound feed + <i>B. subtilis</i> GM5
<i>Archaea</i>	<i>Euryarchaeota</i>	8.36±5.11	0.06±0.06
<i>Bacteria</i>	<i>Other</i>	0.98±0.32	1.96±0.71

	<i>Synergistetes</i>	0.38±0.32	0.07±0.02
	<i>Bacteroidetes</i>	30.45±2.39	11.65±6.32
	<i>Firmicutes</i>	54.55±7.12	81.79±9.42
	<i>Proteobacteria</i>	0.54±0.18	1.01±0.68
	<i>Actinobacteria</i>	1.63±0.52	0.09±0.02
	<i>Tenericutes</i>	1.97±1.73	0.49±0.45
	<i>Cyanobacteria</i>	1.02±0.72	2.65±1.22

Note: $p < 0.05$

The *Firmicutes* phylum in the caecum of chickens is mainly represented by the *Clostridia* and *Bacilli* classes, but the proportion of *Clostridia* was significantly higher in the experimental group (81.28%) compared to the control group (53.88%). Similar to the structure at the phylum level, the number of representatives of the *Bacteroidia* class decreases from 30.36% in the control group to 11.61% in the group receiving the probiotic.

At the order level, the use of probiotics led to an increase in the number of *Clostridiales*, and a decrease in the representation of *Bacteroidales*. High proportion of *Clostridium* is normal for the microflora of the caecum. This group is known to be an indicator of chicken health due to its role in the metabolism of SCFA (short-chain fatty acids), which perform important regulatory, immunomodulatory, and nutritional functions [23].

Analysis of the microbiota composition at the family level showed that the representatives of *Ruminococcaceae* (phylum *Firmicutes*) are dominant in the intestines of both groups, but their proportion is higher in the experimental group (64.67%) compared to the control group (44.30%). Representatives of this family can produce SCFA through glucose metabolism and participate in cellulose decomposition, which confirms their importance for feed digestion [22].

According to other authors, the addition of probiotics based on *B. subtilis* also led to a significant increase in the representation of *Ruminococcaceae*. We also showed an increase in the proportion of the *Christensenellaceae* family within *Clostridiales* in the experimental group relative to the control (0.71-1.66%). The functional role of these bacteria in the intestine is yet to be determined, but a decrease in the proportion of *Christensenellaceae* during intestinal inflammation in animals was previously noted [20].

Among the positive effects of probiotics, we noted a 0.03% to 0.01% decrease in the population of *Enterobacteriaceae*, which often include opportunistic human and animal pathogens. The proportion of *Bifidobacteriaceae* (from 0.87% to 0.05%) and *Lactobacillaceae* (from 0.21% to 0.06%) also decreased compared to the control. A decrease in the number of lactobacilli was also observed in the studies of Ma *et al* using *B. Subtilis* spores [20]. In the phylum of proteobacteria, a two-fold increase in *Desulfovibrionaceae* was observed (from 0.13% to 0.24%). As sulfate reducers, members of this family are known to have the ability to remove hydrogen, which limits the production of SCFA. By acting as a hydrogen absorbant within the chicken caecum ecosystem, *Desulfovibrionaceae* improves energy recovery from feed [22].

Thus, it was shown that the *B. subtilis* GM5 strain is promising for use as a probiotic in poultry farming. Spores of the GM5 strain had a positive effect on poultry body weight gain, improved feed intake, and helped to normalize the composition of the caecal microflora.

4 Acknowledgment

This work was supported by the Kazan (Volga region) Federal University and RSF grant No. 16-16-04062

References

1. S.A. Huws, C.J. Creevey, L.B. Oyama, I. Mixrahi, S.E. Denmam, *Front Microbiol*, **9**, 21-61 (2018)
2. P. Markowiak, K. Śliżewska, *Gut Pathog*, **10**, 1-20 (2018)
3. M. Truszczyński, M. Z. Pejsak, *Med Weter*, **62**, 1339-1343 (2006)
4. A.I. Panin, *Ptitsa Ptitseprod*, **3**, 13-16 (2008)
5. N.V. Danilevskaya, *Veterinariya*, **11**, 6-9 (2005)
6. J. M. Peralta-Sánchez, A. M. Martín-Platero, J. J. Ariza-Romero, M. Rabelo-Ruiz, *Front Microbiol*, **10** (2019)
7. A.I. Panin, *Ptitsa Ptitseprod*, **3**, 13-16 (2008)
8. N.V. Feoktistova, A.M. Mardanova, G.F. Khadieva, M.R. Sharipova, *Scient. notes of the Kazan University. Natural Sciences Series*, **159**, 85-107 (2017)
9. J. Latorre, X. Hernandez-Velasco, *Front. Vet. Sci*, **3**, 1-9 (2016)
10. G.F. Khadieva, M.T. Lutfullin, N.K. Mochalova, O.A. Lenina, M.R. Sharipova, A.M. Mardanova, *Mikrobiologiya*, **87**, 463-471 (2018)
11. K. Lee, H.S. Lillehoj, S.I. Jang, S.H. Lee, D.A. Bautista, G.R. Siragusa, *Asian-Australasian J. of Animal Sc*, **26**, 1592-1597 (2013)
12. J. Li, W. Li, J. Li, Z. Wang, D. Xiao, Y. Wang, X. Ni, D. Zeng, D. Zhang, *PLoS One*, **14** (2019)
13. T. Mahmood, Y. Guo, *Anim Nutr*, **6**, 1-8 (2020)
14. J. Lu, U. Idris, B. Harmon, C. Hofacre, J. J. Maurer, M. D. Lee, **69** (2003)
15. A.M. Mardanova, G. F. Hadieva, M. T. Lutfullin, I. V. Khilyas, L. F. Minnullina, A. G. Gilyazeva, L. M. Bogomolnaya, M. R. Sharipova, *Agricul. Sc*, **8**, 1-20 (2017)
16. D.P. Herlemann, M. Labrenz, K. Jürgens, **10**, 1571-1579 (2011)
17. S. Sen, S. Ingale, Y. Kim, J. Kim, K. Kim, J. Lohakare, E. Kim, H. Kim, *Res Vet Sci*, **93** (2012)
18. K. Pelícia, A.A. Mendes, E.S.P.B. Saldanha, *Br. J. of Poultry Sci*, **6** (2004)
19. F.W. Edens, *Brazilian J. Poult. Sci*, **5** (2003)
20. Y. Ma, W. Wang, H. Zhang, J. Wang, W. Zhang, *Sci Rep*, **8** (2018)
21. P. Richards, J. Fothergill, M. Bernardeau, P. Wigley, *Front Vet Sci*, **6** (2019)
22. Y. Li, Q. Xu, Z. Huang, L. Lv, X. Liu, C. Yin, H. Yan, J. Yuan, *J Appl Microbiol*, **120** (2016)
23. K.Y. Choi, T.K. Lee, W.J. Sul, *Asian-Australas J Anim Sci*, **28** (2015)