ORIGINAL ARTICLE

Engineering and Development of Probiotics for Poultry Industry

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Abstract

Objective: This work discusses the development of dry three-strain probiotic food additive on the basis of thermophilic lactic bacteria strains (*Lactobacillus acidophilus* B-8634, *Lactobacillus delbrueckii* subsp. *bulgaricus* B-6543 and *Streptococcus thermophiles* B-2894) for the poultry industry. **Materials and Methods:** The pattern of interrelations between the strains in the composition of the food additive has been studied by their simultaneous inoculation on solid and liquid nutrient media, incubation and colony-forming unit count by limiting dilution method. On cultivation on solid nutrient media by-products of plant reprocessing were used as a nutrient medium carrier and mineral sorbent - as dilution agent. Antibacterial activity of the considered probiont strains in the additive composition was studied on *Escherichia coli* and *Staphylococcus aureus*. Acid forming activity of the probiont strains was studied by determination of variety and number of organic acids produced by the considered microflora. **Results:** Experimental results demonstrated that the dry probiotic food additive satisfied the safety requirements, and its maximum storage period was 6 months. **Conclusion:** Combined usage of probiont strains in the additive promoted an increase in total titer of lactic bacteria, increase in inhibition zone of *E. coli* and *S. aureus*, as well as secretion of organic acids, which jointly provided high antibacterial properties. On the basis of used microflora as well as products of plant reprocessing, three-strain food additive was engineered and developed with high antagonistic, sorbing, and antitoxic properties. The used microorganism strains as natural forms of the gastrointestinal tract were able to survive here to the highest extent.

Key words: Antagonistic properties, nutrient medium, organic acids, soy okara, technology of production, three-strain probiotic additive, titer of microorganisms, vermiculite

INTRODUCTION

Production and breeding of healthy poultry are an important task of the current commercial poultry industry, since its health influences on subsequent growth, development, adaptation to unfavorable environmental factors, and maximum implementation of the genetic potential of performance.[1-5]

Under conditions of intensive commercial poultry operation, when limited sites are used for breeding of large poultry stock, there is high risk of occurrence of commensal and pathogenic microorganisms. Prolonged uncontrolled application of feed antibiotics in commercial poultry operation resulted in widespread of gastrointestinal problems, which rank next after virus diseases and are the main cause of death of growing stock in poultry farms of the Russian Federation.[6,7]

These and other circumstances lead to the necessity of development of new generation of safe and efficient medications, oriented at correction of intestinal biocenosis, and improvement of colonization resistance of lining of

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intestines. Global experience evidences that substitution therapy becomes more and more important in a solution of these problems, which are aimed at recovery of intestinal biocenosis by addition of live microorganisms into gastroenteric tract with water or feeding.[8-12]

Taking of such medications results in the evolution of biologically active substances and functioning of microbial cells, exerting both direct impacts on pathogenic and commensal microorganisms and indirect one: By activation of specific and non-specific protective systems. At the same time, bacterial cells actively produce ferments, amino acids, antibiotics, and other physiologically active substances, which supplement the protective action of diets. The consequence is the increase in digestibility and consumption of nutritional substances, hence, increase in live weight. Medications and additives, containing such microorganisms, are known as probiotics.[13-17]

At present new Russian probiotic additives and medications are being developed, they should be studied in details and introduced into commercial production. Therefore, the use of probiotics is promising nowadays, and development of their new forms is an urgent task.

This work is aimed at engineering and development of laboratory production of dry three-strain probiotic food additive for sanitation and prevention of infectious diseases of poultry stock, which cause serious economical damages.

MATERIALS AND METHODS

Titer of microorganisms in samples was determined by consecutive 10-fold dilutions of considered material in sodium chloride with their subsequent inoculation onto solid agar mediums, incubations at the optimum growth temperature and counting of developed colonies on Petri dishes. The dishes with 150–250 colonies were counted.

On cultivation on solid nutrient media by-products of plant reprocessing were used as a nutrient medium carrier and mineral sorbent - as dilution agent.

Antibacterial activity of the considered probiont strains in the additive composition was studied *in vitro* according to Litvinov (1947) with modifications by Egorov (1965) - cup plate technique. *Escherichia coli* and *Staphylococcus aureus* were used as test microbes. Antagonistic activity was determined after 24 h by inhibition zone of test microbe under the action of single probiont strain and on their combined cultivation in probiotic.

The acid-forming ability of probiont strains in probiotic food additive as the main factor of antibiotic action of lactic acid microorganisms was studied *in vitro* by determination of variety and amount of organic acids produced by the considered microflora. The determination was carried out using a Kapel-105 semiautomatic instrument of capillary electrophoresis.

Viability of lactic acid microorganisms in probiotic food additive during storage was analyzed by determination of bacteria titer (Russian Standard GOST 10444.11-89) and by increase in titratable acidity on its activation (Russian Standard GOST 3624-92) at production date of the additive as well as in 1, 2, 3, 4, 5, 6, and 7 months.

RESULTS AND DISCUSSION

Probiotic food additive is a dry mixture comprised three thermophilic lactic acid cultures (*Lactobacillus acidophilus* B-8634, *Lactobacillus delbrueckii* subsp. *bulgaricus* B-6543 and *Streptococcus thermophiles* B-2894), grown on soy plant raw material with the mineral sorbent. At least 1.0×10^8 colony-forming unit (CFU) microorganisms are contained in 1 g of final product. Due to unique component composition, the probiotic food additive is characterized by high antibacterial, sorbing, and antitoxic properties, and its microflora is able to survive in the gastroenteric tract.

The components used in the probiotic food additive have certain functions. Since lactic acid flora is a natural representative of microbial biocenosis of the gastroenteric tract, it normalizes the balance of gut microflora in favor of useful microorganisms. In host organism lactic acid microorganisms produce organic acids, which in their turn reduce medium pH, thus inhibiting growth and development of pathogenic microflora. Soy products, okara, in particular, used as an additive contain fiber, protein, macro- and micro-elements, and vitamins.[18] Nutritive value of okara is determined by protein constituent, oligosaccharides, and polyunsaturated fatty acids. Experiments revealed bifidogenic properties in soy oligosaccharides, which influence positively on intestine microflora. It should be mentioned that soy okara contains both non-essential and essential amino acids, in terms of amino acids okara is close to FAO/WHO standards and is characterized by good accessibility.[16] Mineral sorbent presented in the additive (foamed vermiculite) is characterized by high ion-exchanging properties, which enables escaping of ions of magnesium, potassium, calcium, sodium, and others in digestible form, as well as provides removal of radionuclides and other toxic elements from the organism. Combination of all components of probiotic food additive demonstrates its high sanitation, protective, and nutritive properties.

The cultures in the probiotic food additive are used for dairy production in Russia, and abroad, they are included in the list of microorganisms with a documented history of safe use in food (Bulletin of the IDF No. 377/2002). According to sanitary regulations (SanPin 1.2.731-99), the considered cultures fall into the category of human non-pathogenic

microflora.

The production procedure of dry probiotic food additive includes the following stages.

Selection of probiont strains of probiotic food additive

The microflora of the additive is included into All-Russian Collection of Microorganisms approved by Russian governmental decree No. 725-47 dated June 24, 1996, for list of collections of microorganisms, cultivated plant cells, and vertebral somatic cells for state needs. Microorganisms are obtained from natural or industrial sources without the use of gene modifications; they are identified and certified as appropriate.

Streptococcus thermophiles B-2894 is a strong acid former of lactic acid ferments capable to the development and acid formation at the temperatures above 50°C. It is comprised thermophilic, homofermentative, facultative anaerobic, and Gram-positive coccus. Growth temperature range is 15.0–55.0°C; optimum - 40.0–46.0°C. Ultimate acidity in milk is 110.0–120.0°T. In Microslide the culture has round, oval, and spherical shape with the size of 0.5–0.7 × 0.7–1.0 μm, located alone, by two or by chains of various length - beads [Figure 1].

Lactobacillus delbrueckii subsp. bulgaricus B-6543 is a strong acid former of lactic acid ferments capable to the development and acid formation at the temperatures above 50°C. It is comprised thermophilic, homofermentative, facultative anaerobic, Gram-positive, and non-spore forming rods of regular shape. Growth temperature range is 20.0−55.0°C; optimum - 40.0−45.0°C. Ultimate acidity in milk is 200.0−350.0°T. In Microslide the culture is presented by large rods with rounded edges, with the sizes of 0.8−1.5 × 2.0−20.0 μm, located alone or in the form of chains of various lengths, often granular [Figure 2].

L. acidophilus B-8634 is a strong acid-former, antagonist of pathogenic and harmful microflora. It is comprised thermophilic, homofermentative, facultative anaerobic, Gram-positive, and non-spore forming rods of regular shape. Growth temperature range is 20.0−55.0°C; optimum - 37.0−45.0°C. Ultimate acidity in milk is 180.0−300.0°T. In Microslide the culture is presented by large straight rods with the sizes of 0.6−1.5 × 3.0−40.0 μm located alone, by two, or in the form of short chains [Figure 3].

Obtaining of soy products - plant base of probiotic food additive

Soy seeds for obtaining of soy milk and okara as a base for production of probiotic food additive were thoroughly washed in water, poured with 3–4 portions of water with

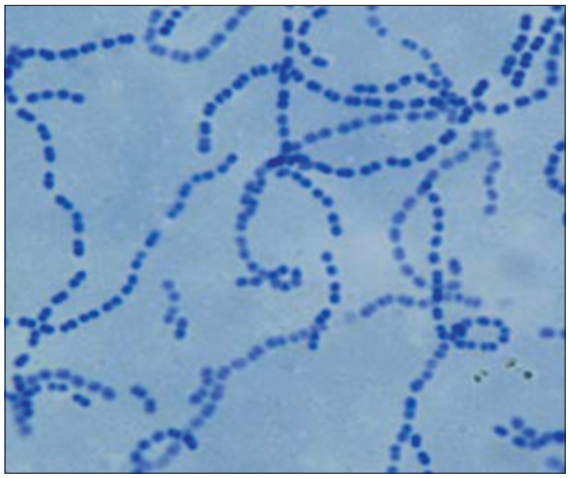

Figure 1: Microimage of pure strain *Streptococcus thermophilus* (1500-fold magnification)

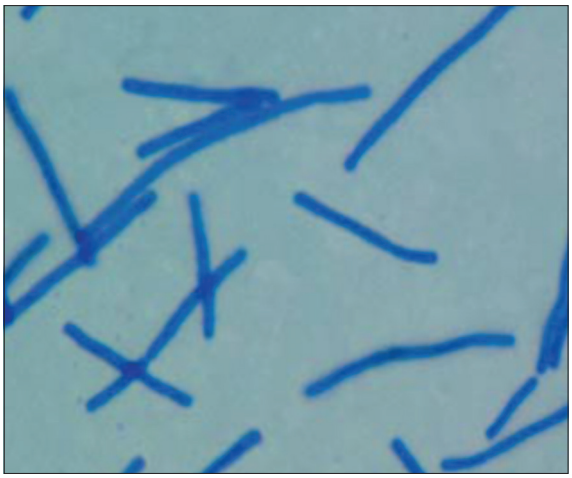

Figure 2: Microimage of pure strain *Lactobacillus delbrueckii* subsp. *bulgaricus* (1500-fold magnification).

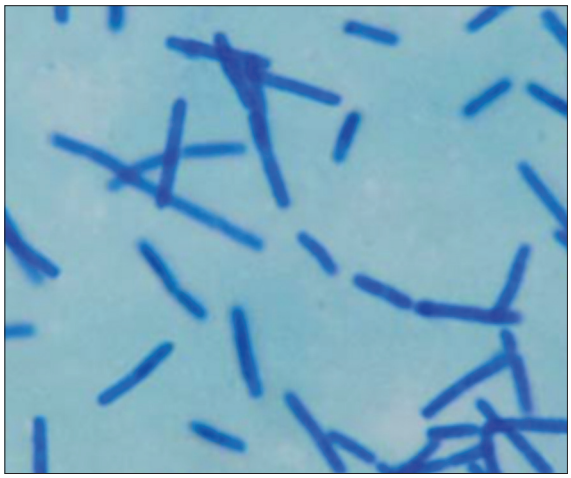

Figure 3: Microimage of pure strain *Lactobacillus acidophilus* (1500-fold magnification)

regard to dry seed weight, and stored at 16–17°C in 12–14 h. The swelled seeds were again washed in water, crushed in roller mill with addition of water (0.5 l per 1 kg of dry seeds). Then, the paste passed through rollers. The crushed paste was

mixed with water at 20°C, 6 portions of water per 1 portion of dry seeds. The paste was agitated and hold in 30 min; then, it was filtrated on 0.2 mm screen. Insoluble soy residue and slurry were obtained after filtration. Then, the soy milk and okara were treated in an autoclave for the destruction of antinutrient substances and further inoculation.

Mixing of components of probiotic food additive

Probiotic food additive was produced from dry bacterial concentrated products of three lactic acid microorganisms (*L. acidophilus* B-8634, *L. delbrueckii* subsp. *bulgaricus* B-6543 and *S. thermophiles* B-2894). Bacterial cultures of each microorganism containing 1 EA of viable cells were activated jointly in 1.0 L of soy milk in combination with milk whey in 1 day at 40–42°C. Then, at least 10.0% of activated culture was aseptically inoculated on sterile nutrient medium (soy okara).

Microflora was inoculated on the mentioned nutrient medium at 38–40°C, which decreased temperature shock of microorganisms. In the course of growing the substrate

Table 1: Properties of probiotic food additive and methods of their analysis

Description	Property and standard	Analytical method	Regulation
Organoleptic properties			
Appearance, color, and odor	Uniform loose bulk, straw color with faint odor	Determination of appearance, color, and odor	Russian Standard GOST 13496.13-75
Physicochemical properties			
Moisture content, wt%, not higher than	10.0	Moisture determination	Russian Standard GOST 24061-89
Toxic elements, mg/kg. not more than:			
Lead	1.0	Atomic absorption spectrometry	
Cadmium	0.2		
Arsenic	0.2		
Mercury	0.03		
Biological properties			
Amount of viable microorganisms, CFU/g, at least	1.0×10^8	Microbiological analysis (preparation of dilution for inoculation)	Russian Standard GOST 9225-84 (item 3.4.3)
		Determination of lactic acid microorganisms	Russian Standard GOST 10444.11-89 (item 4.2.2)
		Microbiological analysis (cell count)	Russian Standard GOST 9225-84 (item 4.5.3)
Coliforms, CFU/g, not more than	1.0	Determination of coliforms	Russian Standard GOST 9225-84 (item 4.6)
S. aureus, CFU/g, not more than	1.0	Determination of *S. aureus* (without preliminary enrichment)	Russian Standard GOST 30347-97
Salmonella bacteria, CFU/g, not more than	10.0	Immune concentration	Recommendations MR 11-3/278-09
Yeast and mold, CFU/g, not more than	5.0	Determination of yeast and mold fungi	Russian Standard GOST 10444.12-88
Identity	Obligatory existence of microorganisms, such as *Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus,* and *Lactobacillus acidophilus*	Bacteriological control in Gram-stained samples	Bergey's manual
Toxicity	Non-toxic	Determination of total toxicity	Russian Standard GOST P 52337-2005

S. aureus: Streptococcus aureus, CFU: Colony-forming unit

temperature was increased by 4–5°C/h, reaching at least 50–52°C. Cultivation at this temperature was carried out in 6–7 h, thus accelerating the growth of microorganisms, moreover, at the subsequent addition of microflora to host organism in the form of additive it provided their high viability. After termination of this time, aiming at the decrease in moisture content of substrate and provision of sorbing and antitoxic properties to required probiotic additive, 10.0% of mineral sorbent (foamed vermiculite and Specifications TU 5712-001-76685354-09) with regard to the nutrient medium was added by stepwise agitation, thus providing its uniform distribution in the paste. Then, to achieve the required moisture content, the mixture was dried in 24 h at 40–42°C, thus providing retention of viable cells. The dried bulk was disintegrated and packed.

Quality control of three-strain probiotic food additive

Quality of ready probiotic food additive was evaluated by organoleptic, physicochemical, and biological properties in accordance with specifications and standards summarized in Table 1.

Viability analysis of microflora in dry three-strain probiotic food additive

One of the main technological properties is a determination of viability variation of lactic acid microorganisms in probiotic food additive during its storage.

This analysis was based on the determination of bacteria titer as well as an increase in titratable acidity on its activation at production date as well as in various dates of its storage – in 1, 2, 3, 4, 5, 6, and 7 months.

Probiotic food additive was stored in moisture-proof containers, in the dry dark room at 20–24°C. Viability analysis of microorganisms in probiotic food additive as well as of titratable acidity during storage is summarized in Table 2.

It shows in Table 2 that the total titer of microorganisms in the food additive decreases in the course of storage period. However, it should be mentioned that minimum amount of microorganisms, which will provide the protective effect of the additive, has been detected at the 6th month of storage, which, in comparison with liquid probiotics with storage period of 1–3 months, is an advantageous property of this probiotic.

Acidity analysis of the used additive by Turner revealed that the titratable acidity at its production date was 88.33°T, which was optimum for the viability of microorganisms. At the end of the storage period of the additive, the acidity was 197.74–209.73°T, which was critical for the viability of lactic acid microflora.

Therefore, the experimental results demonstrated that maximum storage period of the probiotic food additive was 6 months. Its longer storage and use decreases the titter and will not provide the maximum protective effect.

Evaluation of antibacterial and acid forming activities of the strains

Exhibition of antibacterial activity with regard to pathogenic microorganisms is one of the main parameters providing curative and protective effect.

Antibacterial properties of the used probiont strains individually and in probiotic food additive were studied with

Table 2: Variation of total titer of microorganisms and titratable acidity of probiotic food additive during storage

Property	Storage period, months							
	Production date	1	2	3	4	5	6	7
Total titer of microflora, CFU/g	6.3×10^8	4.2×10^8	2.3×10^8	1.2×10^8	9.4×10^7	5.3×10^7	1.7×10^7	7.3×10^6
Titratable acidity, T	88.33	102.51	132.43	154.71	173.83	181.31	197.74	209.73

CFU: Colony-forming unit

Table 3: Antibacterial properties of microorganisms in three-strain probiotic food additive

Test microbe	Probiont strain			Three-strain probiotic
	Streptococcus thermophiles B-2894	*Lactobacillus delbrueckii* subsp. *bulgaricus* B-6543	*Lactobacillus acidophilus* B-8634	
Inhibition zone, mm				
E. coli	6.7±0.1	10.3±0.5	16.7±0.9	23.5±1.3
S. aureus	10.1±0.3	6.0±0.5	10.5±0.5	15.6±1.1

S. aureus: Streptococcus aureus, E. coli: Escherichia coli

Table 4: Quantitative and qualitative composition of organic acids, mg/L

Organic acid	Probiont strain			Three-strain probiotic
	***Streptococcus thermophiles* B-2894**	***Lactobacillus delbrueckii* subsp. *bulgaricus* B-6543**	***Lactobacillus acidophilus* B-8634**	
Lactic	1797.53	2001.12	2621.30	4765.57
Acetic	135.98	375.93	–	486.38
Propionic	–	583.75	364.86	606.18

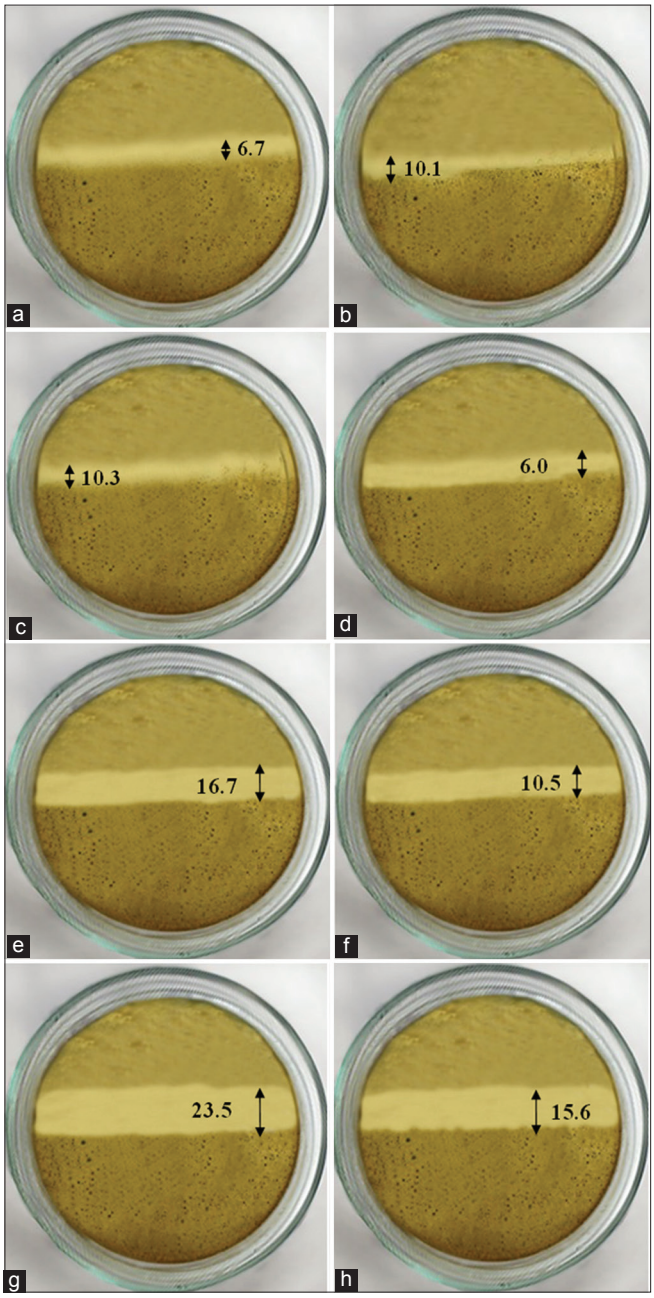

Figure 4: Antagonistic activity of probiont strains individually and in the composition of probiotic food additive (left - *Escherichia coli* and right - *Streptococcus aureus*): (a and b) *Streptococcus thermophiles*; (c and d) *Lactobacillus delbrueckii* subsp. *bulgaricus*; (e and f) *Lactobacillus acidophilus*; and (g and h) three-strain probiotic food additive

regard to *E. coli* and *S. aureus*. The result was accounted by inhibition zone of test microbe under the action of the considered probionts. Antibacterial properties of probiont strains are summarized in Table 3 and Figure 4.

It shows in Table 3 and Figure 4 that the used lactic acid microorganisms in the food additive have a high antibacterial property with regard to *E. coli* and *S. aureus*. It is established that on separate use of lactic acid bacteria (*L. acidophilus* B-8634, *L. delbrueckii* subsp. *bulgaricus* B-6543, and *S. thermophiles* B-2894) the coliform inhibition zone was 6.7, 10.3, and 16.7 mm, and that of *S. aureus*, respectively, 10.1, 6.0, and 10.5 mm. However, it should be mentioned that on joint use of these bacteria in probiotic food additive the antimicrobial activity with regard to *E. coli* was 23.5 mm, and the inhibition zone of *S. aureus* increased to 15.6 mm.

One of the main parameters of antibacterial activity of lactic acid microorganisms with regard to pathogenic microflora is their ability to generate such metabolism products as organic acids. In this regard, we studied variety and amount of acids produced by the used bacteria both individually and probiotic food additive. The acid forming properties are summarized in Table 4.

It shows in Table 4 that on the separate use of bacteria, production of organic acids is lower than on their use in the probiotic additive. Thus, the content of lactic acid on the use of probiotic food additive was 4765.57 mg/l, acetic acid - 486.38 mg/l, and propionic acid - 606.18 mg/l. The obtained results agree with those of other researchers (Donnik and Lebedeva, 2011), where production of organic acids by lactic acid microorganisms was also the main factor of antibacterial activity of useful microflora, probiotic additives and products with regard to pathogenic microflora.

Therefore, the three-strain probiotic additive is characterized by high antibacterial activity with regard to pathogenic microflora, which in its turn provides its high curative and protective effect.

CONCLUSION

Experimental results demonstrated that the applied collection strains – *L. acidophilus* B-8634, *L. delbrueckii* subsp. *bulgaricus* B-6543, and *S. thermophiles* B-2894 - were

promising for their inclusion into biological products. On their basis, a dry three-strain probiotic food additive was developed, which was a combination of these lactic bacteria, grown on soy plant raw materials with mineral sorbent, and at least 1.0×10^8 CFU/g of live microflora. According to sanitary and epidemiological standards, the additive met the quality requirements to this food raw material. The viability studies of microorganisms of the probiotic additive demonstrated that its maximum storage period was 6 months. Combined usage of probiont strains in the additive promotes an increase in total titer of lactic bacteria, increase in inhibition zone of *E. coli* to 23.5 mm, and *S. aureus* to 15.6 mm as well as secretion of organic acids, which jointly provide high antibacterial properties; due to mineral sorbent the probiotic is characterized by sorbing and antitoxic properties. The developed dry probiotic food additive can be recommended for use in commercial poultry industry.

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