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ORIGINAL ARTICLE

Probiotic effect of *Bacillus subtilis* B-2998D, B-3057D, and *Bacillus licheniformis* B-2999D complex on sheep and lambs

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ABSTRACT

Objectives: Probiotics are well documented for their health benefits by developing a balanced intestinal microbiota and boosting immunity. The present study was conducted to determine the effects of a probiotic preparation Enzimsporin™ (consisting of spore-forming bacteria *Bacillus subtilis* B-2998D, B-3057D, and *Bacillus licheniformis* B-2999D) on the biochemical, hematological, immunological parameters, intestinal microbiota, and growth dynamics of sheep and lambs.
Materials and methods: Enzimsporin was fed to lambs and sheep at different doses to determine the bacteria's probiotic effects. Sheep were divided into three groups (six each), which received 0, 1, and 3 gm of Enzimsporin/per head/day, respectively, and two groups of lambs (10 each), who received 0 gm and 1 gm of Enzimsporin/per head/day for 30 days in addition to their regular ration. On day 30, blood samples were collected, followed by the determination of biochemical, hematological, and natural resistance indicators. Fecal samples were examined to determine the intestinal microflora, and animals were weighed daily to determine their growth dynamics.
Results: Supplementation of probiotics (Enzimsporin™) improved the lambs' body weight gain by 18.8%. Analysis of the clinical parameters showed improvements in the levels of total protein, globulins, and urea by 5.3%, 10.8%, and 6.2%, respectively, in the blood of probiotic-supplemented lambs. Similarly, an increment in the total protein, albumins, and globulins was observed in the sheep with Enzimsporin™ supplementation. The decrease in bilirubin and cholesterol levels in the blood and increased bactericidal and phagocytic index in the sheep and lambs with probiotic supplementation indicated a positive influence of Enzimsporin™ on the liver function and natural resistance. Furthermore, an increase in *Lactobacillus* and *Bifidobacterium* and a decrease in the *Escherichia coli*, *Enterococcus*, and Yeast in the fecal contents of experimental sheep and lambs indicated the potentiality of Enzimsporin™ on maintaining good gut health.
Conclusion: Spore-forming bacteria *B. subtilis* B-2998D, B-3057D, and *B. licheniformis* B-2999D can be used in feeding sheep and lambs of 2 months of age to increase body weight gain, improve intestinal microbiota, strengthen the immune system, and maintain normal metabolic processes.

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Introduction

The productive qualities of farm animals are directly dependent on the processes occurring in the body. In this aspect, a unique role is assigned to the use of biologically active substances (BAS), including probiotic additives, which contribute to increasing the resistance and safety of livestock [1,2].

The pharmaceutical industry's intensification, which contributes to the market's saturation with many medicines [3,4], does not always reduce or alleviate diseases [5]. The resulting disorders are often associated with strict regimes of production processes [6], which cause a high functional load on the animal body [7], which is directly related to the full and balanced nutrition of farm animals with nutrients, energy, macro and microelements and vitamins [8,9]. As metabolic processes increase, there is a need for operational support of the digestive system through the use of complex biologically active feed additives [10]. in

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particular probiotics and prebiotics, which mostly correspond to the features of the digestive system of ruminants, increase the biological value of the diet and the efficiency of feed assimilation [11].

Live competitive strains of microorganisms or their metabolites in probiotic supplements are well colonized and take root in the host organism's gastrointestinal tract, optimizing the normal flora [12,13], improving feed digestibility, biological status, and natural resistance of the body [14,15]. It is known that probiotic drugs have pronounced enzymatic and proteolytic properties [16].

Many studies have been devoted to the use of probiotics in agriculture. Extensive scientific and practical material have been accumulated [13,17,18,19] describing their role in improving the microbiocenosis of the gastrointestinal tract, increasing the body's natural resistance, and changing the host's biochemical reactions by optimizing its microbiological status, immunomodulatory, and anti-infective effects [20,21]. Simultaneously, the incidence of newborns is reduced to 20%, safety increased to 95.0%, productivity increased by 8.0%–12.9%, and feed costs are reduced by 6.0%–11.4% [16]. This is especially true for young animals (lambs) in early weaning when the gastrointestinal tract is formed, and animals are susceptible to various diseases [22].

Many spore-forming bacteria's ability to have a probiotic effect has led to the development of drugs related to the generation of so-called self-eliminating antagonists [23,24]. Spores of bacilli are initiated after entering the gastrointestinal tract. In the process of germination, they begin to produce a complex of BAS, which lyzes pathogenic and opportunistic microorganisms sensitive to them, freeing-up adhesion sites for representatives of the normal flora. *Bacilli*, in the process of division, synthesizes amylase, protease, lipase, hemicellulase, and regulatory peptides. The gastric secrete is further enriched with enzymes, other BAS, which contribute to the normalization of digestive processes and strengthen the body as a whole. The reproduction of Lacto- and bifidobacteria is stimulated together with other microflora representatives, which, in turn, synthesize amino acids (including essential) and vitamins that enhance the complex therapeutic and preventive action spore-forming probiotics at the microecological level. Besides, when administered orally, probiotic bacilli significantly increase the non-specific and specific resistance of the macroorganism, i.e., they restore the immune status disturbed by pathology and improve endogenous production interferon, the functionality of macrophages of monocytes, and neutrophils [12,13,16].

Bacteria of the genus *Bacillus licheniformis* produce several proteins, peptides, enzymes, and vitamins. This fact contributes to interferon production in the body, which suppresses pathogenic microbes and viruses, leading to normalization of intestinal microbiota, better food digestion, and eliminating food and chemical toxicity [25,26,27,28]. *Bacillus subtilis* are antagonists of pathogenic and conditionally pathogenic microorganisms such as *Salmonella*, *Proteus*, *Staphylococci*, *Streptococci*, yeast, and fungi [29,30]. They increase the non-specific resistance of the host organism, produce enzymes that remove the products of putrefactive tissue decay, and the number of lactobacilli and other microorganisms that make up the normal flora of the gastrointestinal tract, and ensure its normal functioning is growing [31,32]. They synthesize amino acids, vitamins, and immunoreactive substances, have increased thermal stability, are producers of proteolytic and amylolytic enzymes, and participate in the initial fiber cleavage stages. They also produce antibiotic substances, cellobiase, and endo-beta-gluconase [14,31,33–36].

Probiotic drugs prescribed for preventive and curative purposes should be safe but necessarily effective since these properties affect the enterprise's economic component. The clinical effectiveness of probiotics is determined by the characteristics of the strains and the daily and course dose adequacy, which should not be underestimated or exceeded. Modern research proves the identity of the impact on animal health of probiotic agents and functional foods containing probiotic strains in optimal concentrations. Determining the preventive and therapeutic doses of probiotics in lambs at an early age is an urgent task of animal husbandry. The presented studies reflect the features of growth, biochemical composition, and immunological parameters of sheep and lambs' blood when feeding strains of *B. subtilis* and *B. licheniformis*, which are part of the probiotic Enzimsporin.

Materials and Methods

Ethical approval

The work was carried out following the state order with the Foundation's financial support for basic scientific research of the Russian Academy of SCIENCES, state registration number of R&D AAAA-A18-118021590136-7 in the Department of physiology and biochemistry of agricultural animals and the laboratory of Microbiology of the L.K. Ernst Federal Research Center for Animal Husbandry.

Probiotic Enzimsporin™

The consortium of Bacteria of the genus *B. subtilis* B-2998D, B-3057D, and *B. licheniformis* B-2999D make up the probiotic Enzimsporin™, registered in the Rosselkhoznadzor 77-2-8. 16-6957 no. PVR-2-8.16/03297 from 26.09.2016. From white to light beige color, the fine powder is well soluble in water and milk, and is mixed with the diet's main feed. The content of viable spores in the preparation is not less than 5×10^9 colony-forming units (CFU)/gm, which

causes a wide range of actions of the drug against pathogenic and opportunistic microorganisms. The L.K. Ernst Federal Research Center for Animal Husbandry provides scientific support for industrial probiotic tests [16,30,36].

Keeping and feeding animals

To achieve the study's goals in the conditions of the physiological yard of the L.K. Ernst Federal Science Center for Animal Husbandry, investigations were conducted by using the group-period method on six 18-month-old analog sheep with an average weight of 40 kg. The duration of each period was 30 days. In the first control group period, the sheep received a basic diet consisting of 1.5 kg of mixed-grass hay and 0.3 kg of crushed barley. In the second (group 1) and third (group 2) periods, in addition to the main diet, 1.0 and 3.0 g per head/day (recommended by the developer) of spore-forming bacteria *B. subtilis* and *B. licheniformis* (probiotic Enzimsporin™) was added, respectively, in a mixture with barley milling. The sheep were kept in separate rooms, fed from feeders, and watered from carpoolers.

Lambs used in this study were divided into two groups (10 heads each group, 2 months of age, and an average live weight of 9.4 kg) by the method of analog pairs taking into account body weight and age. The scientific and economic experiment was conducted under production conditions of the breeding reproducer LLC farm "Pokrov" Zubtsovsky district of the Tver region to breed Romanov sheep.

The control group received a basic diet consisting of ground wheat, corn, sunflower meal, and flattened oats. Bean and cereal hay were freely available. Lambs of the experimental group and the primary diet received spore bacteria *B. subtilis* and *B. licheniformis* in the form of probiotic Enzimsporin™ for 30 days at a dose of 1 gm/head (0.08 gm/kg live weight). The dosage of feeding the drug was taken from previously obtained studies on sheep (Ref.). The lambs are kept in groups in two pens. All animals in the experiments were under constant veterinary control for health, appetite, and behavior.

Weighing lambs

The gross and average daily increase in live weight of lambs was estimated by control weighing on electronic floor platform scales TV-M-300.2-A1 in the morning before feeding throughout the experiment.

Blood and fecal sampling

On day 30 (research completion), before morning feeding, two blood samples were taken from five lambs and six sheep from each group by puncturing the jugular vein in disposable vacuum system tubes Vacuette (GreinerBio-One, Austria). One blood sample was taken with an anticoagulant for hematological studies; the other was placed in a non-heparinized test tube and left for 30 min for blood clotting, then centrifuged for 15 min at 3,000 rpm at 4°C. The serum was separated for further analysis and stored at −20°C. Indicators of protein, carbohydrate-fat, and lipid metabolism, hematological indicators, and non-specific resistance indicators were determined in the blood. Fecal samples were collected from the rectum of the animals in sterile containers.

Hematological and biochemical analysis

Serum concentrations of total protein (biuretic method), albumin (colorimetric), urea (enzymatic colorimetric Bertelot), creatinine (kinetic Jaffe method), glucose (GLU) (enzymatic GLU oxidase), and cholesterol (enzymatic colorimetric), phospholipids (enzymatic colorimetric); bilirubin (quantitative determination by Walters and Gerarde's method); calcium (O-cresolphthalein complex), phosphorus, magnesium, and iron (colorimetric); the activity of alanine aminotransferase (UV-kinetic), aspartate aminotransferase (UV-kinetic), alkaline phosphatase (ALP) (kinetic method) were determined using an automatic biochemical analyzer ChemWell (Awareness Technology, Palm City, FL) with reagents from Analyticon Biotechnologies AG (Germany) and Spinreact (Spain). In whole blood, the content of hemoglobin (HGB) was determined using the ABC VET analyzer (Horiba ABZ, France) using uni-Gem reagents (reamed, Russia).

Indicators of non-specific resistance of blood

To assess the cellular defense link's anti-infective state, the absorption activity of neutrophils [phagocytic activity (FA), phagocytic index (PHI), FE phagocytic capacity, and PH, phagocytic number] was determined. The state of the humoral defense link was evaluated by the bactericidal activity of blood serum (BASC) indicators BASC.

FA was calculated as a percentage of active white blood cells involved in phagocytosis to the total number of counted white blood cells with the use of the following:

$$\%FA = \frac{F1}{F2} * 100$$

where F1 is the number of white blood cells involved in phagocytosis; F2 is the total number of counted white blood cells.

To calculate the number of phagocytic leukocytes, 0.5 ml of inoculum of a test strain of daily culture of *Escherichia coli* with an optical density concentration of 4.5 McF (Densi-La-Meter, Czech Republic). Tubes with the prepared mixture were kept in a thermostat at 37°C for 30 min under constant shaking. Thin smears were fixed with 96% ethanol and stained using the Romanovsky–Giemsa method. The smear was viewed under immersion microscopy with a WF 16× eyepiece and a 90° lens. The number

of phagocytic leukocytes was calculated from the total number counted (at least 100).

The following formula determined PHI (FI):

$$FI = \frac{Mf}{La}$$

where Mf is the total number of phagocytic microorganisms and *La* is the number of active white blood cells.

Phagocytic amount (FAM) is an additional indicator that characterizes both the aggressiveness of white blood cells and their activity and is calculated using the following formula:

$$FAM = \frac{Mf}{Lt}$$

where Mf is the total number of phagocytic microorganisms and *Lt* is the total number of counted white blood cells.

The percentage of lysis determined lysozyme activity of blood serum, the amount of lysozyme (lysozyme, mg/ml) in 1 ml of blood serum, the specific unit of activity in terms of 1 mg of protein (AU/TP).

The analysis used a culture of the genus *Micrococcus* growing well on the nutrient agar. The culture of *Micrococcus luteus* (lysodeicticus) obtained from the all-Russian Collection of Industrial Microorganisms of FSUE Gosniigenetika under the registration number RCAM 01016 was seeded on mown meat-peptone agar. Washing the test culture with a sterile phosphate buffer (pH = 7.2), standardized on a photoelectrocolorimeter (FEK-2, Russia) against a phosphate buffer, using a green light filter (wavelength 540 nm) in cuvettes with a working length of 3 mm. The standard culture suspension corresponds to 0.6–0.62 McF (Mcfarland). Test tubes with 0.1 ml of blood serum and a control tube with 0.1 ml of phosphate buffer were heated at 56°C for 30 min. 1.4 ml of traditional culture was added to the cooled test tubes at 37°C for 5 h. After 1 and 3 h, the results were measured.

Calculation of the percentage of lysozyme activity is as follows:

$$\%LA = \frac{(\Delta\, Do) * 100}{Do1} - \frac{(\Delta\, Dk) * 100}{Dk1}$$

where ΔDo is the difference in the optical density of a test sample, ΔDk is the difference of the optical density of control, Do1 is absorbance of test sample immediately, and Dk1 is optical density of control.

The amount of lysozyme in 1 ml of blood serum was determined by the standard lysozyme solution's calibration curve. The lysozyme activity level was converted into activity units per 1 mg of serum protein and expressed in conventional units of activity (activity units per 1 mg of TP or AU/TP). BASC (BA). A daily *E. coli* test culture suspension with an optical density of 1.9 McF was prepared for the study. 4.5 ml of meat-peptone broth, 0.5 ml of blood serum, and 0.005 ml of culture inoculum were poured into sterile cuvettes. The control contained 0.5 ml of a sterile 0.9% saline solution incubated at 37°C for 5 h. Measurements were performed at 3 and 5 h on a photoelectrocolorimeter (FEK-2, Russia) at a wavelength of 540 Nm (green light filter) in cuvettes with a working length of 5 mm.

%BA is calculated using the following formula:

$$\%BA = \frac{(\mathrm{Dk} - \mathrm{Do})}{\mathrm{Dk}} \cdot 100$$

where Dk is the optical density of the control and Do is the optical density of the prototype.

Microbiological studies of feces

Samples of the contents of the large intestine of sheep and lambs were examined by seeding successive 10-fold dilutions on accumulative and differential diagnostic media by deep (1.0 ml) and surface (0.2 ml) methods, followed by counting the number of (CFU/g or ml). Species identification of microorganisms was carried out by evaluating the morphology and microscopy results of colonies grown on microbiological differential diagnostic media such as MRS and Bifidum medium for lactic acid microorganisms ("FBUN SSC of applied Microbiology and biotechnology", Moscow region, HiMedia, India), agar Endo-GRM for *E. coli* ("Central research Institute state scientific center of applied Microbiology and biotechnology", Moscow region), meat-peptone agar for hemolytic *Streptococcus* spp. and *E. coli* (MPA, "FBUN SSC of applied Microbiology and biotechnology", Moscow region), polymyxin agar for enterococci ("FBUN SSC of applied Microbiology and biotechnology", Moscow region), Saburo agar with the addition of chloramphenicol for yeast and yeast-like fungus (HiMedia, India), and panels of API test systems ("BioMerieux", France).

Statistical analysis

To measure the statistical significance of the experimental and control groups' different paired values, the reliability of differences between the two data groups was determined using Student's *t*-test. The readings were considered statistically reliable at $p < 0.05$. During all periods of the experiment, the clinical and physiological states of the animals were determined by daily examinations. Simultaneously, attention was paid to general behavior, appetite, water consumption, and mobility.

Results and Discussion

The main factor that ensures the health, high level of productivity, and duration of animals' economic use is the state

of metabolism. The size and speed of metabolic processes can be indirectly determined by changes in the number of blood metabolites. The functioning of the multicomponent blood system is based on the basic principle of the living system-stability with constant dynamic variability, reflecting all the processes in the body of animals [37].

As a result of the conducted research, it was found out that the concentration of the studied metabolites of metabolism in the blood of animals of all groups was within acceptable physiological norms (Table 1). The use of spore-forming bacterial strains contributed to positive changes in protein and carbohydrate fat metabolism in the body of sheep and lambs, with an increase in the total protein in the blood serum of sheep of the experimental groups by 4.5%–6.4% ($p \leq 0.001$), lambs by 5.3%. In sheep, mainly due to the fraction of albumin (A) by 9.7% in group 1 ($p \leq 0.01$) and by 14.5% ($p \leq 0.001$) in group 2, with a simultaneous increase in the ratio of albumin-globulin (A/G). The growing lambs have a 10.8% increase due to the globulin (G) fraction, which is a carrier of antibodies and performs a protective function. Similar results from the use of probiotics were obtained by other scientists [10,38].

The primary role of albumin, a fine fraction of proteins, is to maintain the colloidal osmotic pressure of plasma and the volume of circulating blood and the transport and deposition of various substances. It binds such non-polar substances as bilirubin and fatty acids, cholesterol, and a carrier of several hormones, such as thyroxine, triiodothyronine, cortisol, and aldosterone [39].

An increase in albumin level may indirectly indicate an increase in the liver's protein-forming function since albumin synthesis occurs in this organ. A decrease in the level of albumin would indicate its pathology [40,41]. Positive changes in the direction of protein metabolism are confirmed by the indicators of transamination enzyme activity, particularly the revealed tendency to increase the level of Alanine transaminase (ALT) in sheep of the experimental groups by 2.3%–4.2% and lambs by 3.9%. Simultaneously, the lower indicators of Aspartate transaminase levels (AST) were found; in experimental group 1, it was 15.2%. In group 2, it was 12.8%, with a General tendency to lower the AST/ALT ratio. Other researchers obtained similar data that associate a decrease in AST levels with the liver's better functional state [38,39,42].

The activation of nitrogen metabolism under spore-forming bacteria's action caused increased creatinine concentration in the sheep by 2.6% and 3.1% and in the lambs by 17.6%. Creatinine, like urea, is a product

Table 1. Biochemical and hematological parameters of blood and its serum.

Indicator	Group of sheep			Group of lambs	
	Control	Experimental-1	Experimental-2	Control	Experimental
Total protein, gm/l	72.01 ± 0.86	75.23 ± 1.50	76.65 ± 0.57***	58.53 ± 4.69	61.61 ± 5.31
Albumin (A), gm/l	24.94 ± 0.61	27.37 ± 0.43**	28.56 ± 0.33***	21.82 ± 1.15	21.74 ± 0.89
Globulin (G), gm/l	47.07 ± 0.35	47.86 ± 1.08	48.09 ± 0.55	36.71 ± 5.11	40.67 ± 5.38
A/G	0.53 ± 0.01	0.57 ± 0.06	0.59 ± 0.01	0.59 ± 0.12	0.53 ± 0.09
ALT, U/l	17.66 ± 1.48	18.06 ± 1.46	18.41 ± 0.94	13.72 ± 0.95	14.25 ± 0.71
AST, U/l	86.61 ± 4.10	75.19 ± 3.19	76.77 ± 4.57	79.28 ± 4.24	79.58 ± 2.20
Urea,mmol/l	5.60 ± 0.38	5.69 ± 0.24	5.70 ± 0.29	3.86 ± 0.24	4.10 ± 0.29
ALT/AST	4.9	4.2	4.2	5.78	5.58
Creatinine, µmol/l	92.74 ± 2.50	95.11 ± 1.98	95.61 ± 4.06	69.65 ± 8.48	81.94 ± 7.90
Bilirubin, µmol/l	6.69 ± 1.13	6.37 ± 0.45	5.88 ± 0.25	7.08 ± 0.68	6.61 ± 0.89
Cholesterol, mmol/l	2.47 ± 0.10	2.36 ± 0.05	2.27 ± 0.03	1.76 ± 0.09	1.8 ± 0.22
ALP, U/l	151.93 ± 39.89	147.52 ± 26.92	145.73 ± 43.77	596.71 ± 147.67	617.14 ± 128.86
GLU	3.66 ± 0.17	3.51 ± 0.26	3.47 ± 0.11	4.70 ± 0.32	4.82 ± 0.29
Ca, mmol/l	2.91 ± 0.16	2.85 ± 0.16	2.99 ± 0.19	2.19 ± 0.08	2.17 ± 0.13
P, mmol/l	2.28 ± 0.32	2.37 ± 0.28	2.39 ± 0.19	2.14 ± 0.25	2.19 ± 0.12
Ca/P	1.40	1.28 ± 0.19	1.29 ± 0.16	1.08 ± 0.15	0.99 ± 0.04
Mg, mmol/l	2.29 ± 0.07	2.28 ± 0.13	2.32 ± 0.16	1.44 ± 0.06	1.47 ± 0.05
Fe, µmol/l	34.21 ± 2.42	34.71 ± 0.76	34.78 ± 1.33	14.74 ± 3.24	18,79 ± 2,15
HGB, gm/l	130.78 ± 4.26	131.73 ± 5.37	135.22 ± 5.71	96.0 ± 5.15	104.04 ± 7.59

ALT = Alanine transaminase; AST = Aspartate transaminase; ALP = Alkaline Phosphatase; GLU = The glucose; HGB = The hemoglobin.
The differences compared with the control group are statistically significant for the *p* value **) – < 0.01, *) – < 0.001.

of the metabolism of protein. Its content depends on the protein level as the intensity of metabolism, the synthesis of which involves amino acids methionine, glycine, and arginine [43,44]. This may indicate that it is possible to activate energy metabolism through creatine phosphate, a reserve energy accumulator for complete protein synthesis. It is known that creatinine phosphate is a donor of the phosphorous residue for ADP, reducing the latter to ATP increases tissue cells' energy potential [44]. Creatinine is formed in the body at a constant rate, and its concentration in the blood serum is usually stable and correlates typically with the volume of muscle tissue. This corresponded to the same live weight of the sheep. The level of creatinine directly depends on age, so it was lower in lambs.

Based on the fact that lambs in the experimental group weighed more, the relatively high creatinine values obtained can be physiologically justified. The indirect sign of this can be a tendency of a slight increase in phosphorus level by 3.9%–4.8% in the blood serum of sheep and by 2.3% in lambs. The other researchers also note the improvement of metabolic processes in the body of ruminants due to the physiological effect of probiotics on the digestive tract's microflora, which increases the digestibility of food and increases the body's exchange fund in nutrients and energy [16,40,43,44].

As ALP catalyzing the hydrolysis of monoamino phosphoric acid is a marker enzyme reflecting the state of energy and mineral metabolism, the lower indicators (2.9% and 4.1%) with a tendency to reduce the level of GLU in the sheep's organism of the experimental groups can indicate activation of energy metabolism, increase in consumption of the enzyme for the energy supply of tissue cells in the form of ATP [42]. The production of ALP mainly occurs in the intestinal mucosa, and a decrease in this indicator can indirectly show its better state, the absence of inflammatory processes. The liver's state since the enormous amount of the enzyme is located in the liver cells and bile ducts [42,43,45].

With the increase in the level of GLU in growing lambs of the experimental group by 2.6%, the main source of energy in all vital processes occurring in the body, which is one of the most critical parameters characterizing carbohydrate metabolism, we can talk about a better energy supply of their body. The increase in ALP levels can be explained by active bone growth, confirmed by better gains in their live weight than control (Table 2). The existing differences may also be due to growing and adult sheep [46–48].

The use of spore-forming bacteria has affected lipid metabolism, with the revealed tendencies to decrease serum levels of total bilirubin in sheep (by 4.8% and 12.1%) and cholesterol (by 4.5% and 8.1%). In the experimental group of lambs, bilirubin levels were 6.6% lower, with a 2.3% increase in cholesterol. A decrease in bilirubin concentration may indicate an increase in the ability to exchange and transfer bilirubin to bile by liver cells, indirectly suggesting an improvement in liver function [43,44,49].

The analysis of hematological studies has allowed us to judge the increase in redox processes in the body of animals of experimental groups. With relatively similar indicators of mineral metabolism, there was a tendency to increase the level of iron in the blood of lambs in the experimental group by 27.5%, indirect relationships with an increase in the level of HGB by 0.7%–3.4% in sheep and 8.4% in lambs. Iron is a vital trace element that is part of the redox processes that regulate the respiratory, metabolic activity of cells and tissues, and oxygen transport. The body's principal amount of iron is part of the red blood cell HGB and muscle myoglobin. Since the mass of red blood cells and the concentration of HGB in them are of particular importance for the transportation of oxygen and carbon dioxide, a higher concentration of iron and HGB is a positive physiological indicator that characterizes a higher level of metabolic processes in the body [50–52]. It was found that with age, the level of iron in the blood decreases; apparently, this factor can explain the difference in indicators in sheep and grow young [53].

The changes in live body weight and absolute growth allow us to judge the growth rate of animals and their development to a certain extent, considering that fast-growing animals spend significantly fewer feed nutrients per unit of production than animals growing slowly [53]. The increased intensity of metabolic processes in the body with the lambs in the experimental group under the influence of spore-forming strains of *B. Subtilis* and *B. licheniformis* had a positive impact on their growth dynamics (Table 2). The individual weighing lambs at statement and removal from the experiment showed that the experimental group's gross increment was 12.1 ± 0.62 kg, 0.43 kg more than the control ($p < 0.05$). The average daily increments were 18.8% higher and amounted to 90.3 ± 0.004 gm compared to 76.0 ± 0.01 g in control ($p < 0.05$). The effect of probiotics on the increase in live weight gain was also noted in similar studies by other authors, who also connected this with an improvement in the flow of carbohydrate-fat and protein metabolism in the body of experimental animals [54,55,56].

Non-specific resistance is the ability to maintain optimal functioning in organs, systems, or throughout the body, both in stereotypical and changed life conditions under various influences [52]. It is the first protective barrier to the introduction of an unfavorable infectious agent. It is associated with anatomical, physiological, and genetic features of the body; its mechanisms, humoral and cellular non-specific protection factors, inflammation, normal antibodies, largely depend on proper feeding and compliance with veterinary rules for keeping animals [53].

Table 2. The dynamics of the growth of lambs.

Indicator	Control group	Experimental group
Of the experiment, day	30	30
Number of animals	10	10
Live weight at the beginning of the experiment, kilo	9.4 ± 0.61	9.4 ± 0.84
Live weight at the end of the experiment, kilo	11.68 ± 0.64	12.11 ± 0.62[a]
The absolute gain in live weight, kilo	2.28 ± 0.15	2.71 ± 0.12[a]
Daily average gain, gram	76.0 ± 0.005	90.3 ± 0.004[a]
Average daily increase in% of control	100.00	118.82

[a]Differences in comparison with the control group are statistically significant at the *p*-value *($p < 0.5$)

Table 3. Indicators of non-specific resistance of the blood.

Indicator	Group of sheep			Group of lambs	
	Control	Experiment-1	Experiment-2	Control	Experimental
Total protein, gm/l	72.01 ± 0.86	75.23 ± 1.50	76.65 ± 0.57	58.53 ± 4.69	61.61 ± 5.31
% lysis	12.16 ± 1.65	23.23 ± 1.24	14.76 ± 0.58	43.22 ± 1.19	40.10 ± 1.59
Lysozyme	0.27 ± 0.03	0.41 ± 0.02	0.31 ± 0.01	0.76 ± 0.02	0.72 ± 0.04
Bactericidal activity %	64.17 ± 2.70	69.37 ± 1.10	71.67 ± 3.63	49.69 ± 1.91	51.71 ± 2.44
Phagocytic activity %	27.23 ± 1.56	28.00 ± 1.18	27.95 ± 1.26	54.83 ± 6.36	69.33 ± 4.68
PHI	1.88 ± 0.08	2.44 ± 0.19	2.81 ± 0.05	2.06 ± 0.19	2.43 ± 0.34
Phagocytic number	0.51 ± 0.01	0.61 ± 0.07	0.69 ± 0.03	1.14 ± 2.05	1.69 ± 0.27

The natural resistance of animals to various adverse effects is provided by non-specific protection factors present in the body from birth and persist throughout life. It is phagocytosis with its protective cellular mechanisms and humoral resistance factors that play a crucial role, the most important of the lysozyme and bactericidal factors. This means that a unique position among the protection factors is occupied by phagocytes (macrophages and polymorphonuclear leukocytes) and a system of blood proteins called complement. They can be attributed to both non-specific and immunoreactive protection factors [54].

The level of lysozyme activity, a thermally stable protein that stimulates phagocytosis of neutrophils and microphages, antibody synthesis, and the degradation of lipopolysaccharide surface layers of most of the cell walls pathogenic bacteria in our studies was stable, which may indicate the health of animals in the experiments [58]. The level of lysozyme activity can change depending on preventive measures. Higher indicators in lambs in the control group can be associated with medications to treat four cases of diarrhea during the experiment (Table 3).

The BASC is a full display of antimicrobial processes caused by humoral factors of natural resistance, both to Gram-positive and Gram-negative microflora part of the blood serum. The degree of manifestation of protective properties of animals to microbial agents when feeding *B. subtilis* and *B. licheniformis* well illustrates a higher rate of BASC in sheep by 8.1%–11.7%,, in lambs by 4.1%, as well as an increase in phagocytic activity in sheep by 2.8% and 2.6%, in lambs by 26.4%, with higher PHI values of 1.3–1.5 times in sheep and 1.2 times in lambs compared to the control group. This may indicate a more stable non-specific cellular immunity system, increasing animals' resistance in experimental groups to possible infection [56–59].

The intestinal microbiota (normal flora), which performs numerous functions to maintain the body's homeostasis, plays a vital role in food digestion. Normal flora's role is to maintain natural resistance mechanisms by competing with pathogens for intestinal mucosal receptors at their primary adhesion and colonization stage. Under the influence of normal microflora, the complement system and phagocytosis are activated, which occupies an important place in the body's disinfecting from pathogens of intestinal infection [60–63].

Intestinal microbiocenosis is the most numerous and diverse in its qualitative composition and the most sensitive to adverse factors. Therefore, intestinal dysbacteriosis should be considered an early signal of an imbalance in the body [64]. In our studies on lambs, we observed four diarrhea cases in the control group, indicating a weakening of their natural resistance mechanisms. Some scientists in their work also show a positive effect of probiotics on diarrhea's nature, improving the fecal microbiota [65–69].

Table 4. Microbiological studies of the contents of the large intestine, log10 CFU/gm.

Indicator	Group of sheep			Group of lambs	
	Control	Experiment-1	Experiment-2	Control	Experimental
Lactobacillus, CFU/gm	5.57 ± 0.30	6.67 ± 0.30	7.17 ± 0.76	7.63 ± 0.12	7.68 ± 0.15
Bifidobacterium pp., CFU/gm	6.62 ± 0.93	7.72 ± 0.21	8.64 ± 0.21	10.0 ± 0.42	10.32 ± 0.24
Lactose- positive *E. coli* bacteria, CFU/gm	5.77 ± 1.38	5.46 ± 0.49	4.77 ± 0.62	0	0
Enterococcus spp., CFU/gm	7.64 ± 0.12	6.26 ± 0.14	6.44 ± 0.14	8.57 ± 15.37	6.89 ± 0,13
Yeast, CFU/gm	4.81 ± 0.43	0	0	4.79 ± 0.61	0

Many microorganisms inhabit the last parts of the large intestine, primarily sticks of the genus *Bifidus*. They are involved in destroying enzymes from the small intestine with chyme, the synthesis of vitamins, the exchange of proteins, phospholipids, fatty acids, and cholesterol. The function of normal flora can be disrupted after prolonged use of antibiotics, which leads to the development of yeast and fungi [69,70]. In our experiments, we noted yeast's presence in the feces of animals of control groups against their complete absence in animals of experimental groups. Feeding spore bacteria affected normal intestinal microflora development, which acts as an antagonist of pathogenic microbes and inhibits their reproduction [13,71,72].

Lactic acid bacteria and bifidobacteria that colonize the intestinal epithelium are of greater importance as eubiotics. They can inhibit the growth and development of *Pseudomonas*, *Escherichia, Salmonella, Shigella, Streptococci, Staphylococci*, anaerobic bacteria, including *Clostridium* [73,74].

Antagonistic activity is caused by the synthesis of organic acids and bacteriocins, which are fixed on specific pathogen receptors, changing the structure and permeability of their cell wall, causing in some cases its lysis, limiting its number. Lactic acid bacteria stimulate the immune system and lysozyme production. Like bifidobacteria, *Lactobacilli* are actively involved in the metabolism, synthesis of vitamins, amines, and biologically active compounds [73,74,75].

The physiological value of normal biocenosis is characterized by a high titer of bifidobacteria and *E. coli*, which have high antagonistic properties that prevent the development of pathogenic and opportunistic microorganisms [68,74,76,77]. The main groups of microorganisms' comparative characteristics in the intestinal contents showed that spore bacteria's probiotic effect did not affect lacto- and bifidobacteria in lambs. There was an increase in the content of lactobacilli in the feces of sheep of group 1 by 19.7% and group 2 by 28.7%, bifidobacteria by 16.6% and 30.5%, degressions of lactose-positive *E. coli* by 5.3% and 17.3%, enterococci by 18.1% and 15.7% (Table 4). *Enterococci* are grouped into the genus Streptococcus, which includes more than 90 species. Enterococci are more active than other representatives of normal microflora. They are fixed on the wall of the mucous membrane of the proximal small intestine [78,79,80].

Feeding spore-forming bacteria in our experiments had a negative effect on the content of enterococci in the feces of animals. However, an increase in the number of lacto- and bifidobacteria in animals of experimental groups generally indicates an improvement in the microbial landscape of the contents of the rectum, the potential for development and functioning of the gastrointestinal tract, as well as an increase in protective properties and immunity in lambs and sheep [81,82].

Spore-forming bacteria are not elements of the normoflora in microbial communities of animals, but they have properties that enable the body to maintain microbocinosis at an environmentally natural level, optimize metabolism and supply the body with biologically active and building substances, and ensure high-quality digestion of food.

Conclusion

Studies have shown that the addition of spore-forming bacteria *B. subtilis* and *B. licheniformis* to the diet of sheep in the amount of 1 and 3 gm and lambs in the amount of 1 gm per head/day (0.08 gm/kg live weight) through the use of probiotic Enzimsporin helps to improve the health of the organism as a whole. This is manifested in an improvement in the direction of protein and carbohydrate-fat metabolism, with a significant increase in the serum concentration of total protein by 4.5%–6.5% ($p \leq 0.001$), albumin by 9.7%–14.5% ($p \leq 0.01$), and globulins. An increase in creatinine and GLU concentration indicates the activation of nitrogen, carbohydrate, and energy metabolism. A decrease in serum levels of total bilirubin and cholesterol in animals of experimental groups may indicate a more favorable course of lipid metabolism. Hematological studies allowed us to judge the increase in redox processes in the experimental groups' bodies of animals. The growth of bactericidal, lysozyme, and phagocytic activity indicates an increase in the state of animals' natural resistance. An increase in the number of lacto-and

bifidobacteria in animals of experimental groups with a simultaneous decrease in enterococci, in General, indicates an improvement in the rectum contents' microbial landscape the potential development and functioning of the digestive tract protective properties and immune status of lambs and sheep.

An increase in the intensity of metabolic processes and natural resistance in the experimental groups' animals positively affected the lambs' growth dynamics. Their average daily increases were 18.8% higher and amounted to 90.3 gm than 76.0 gm in control ($p < 0.05$). The addition of spore bacteria prevents dysbacteriosis development, compared to the control group, where four disease cases were recorded.

Even though in the experiment on sheep, a positive effect was obtained from feeding both 1 and 3 gm per head/day, the conducted research allows us to recommend feeding the spore-forming bacteria *B. subtilis* and *B. licheniformis* in the form of probiotic Enzimsporin with a titer of 5×10^9 CFU/gm. in the amount of 3 gm/head/day, or 0.08 gm per kilogram of live weight, and confirms the prospects for widespread use as a means of optimizing metabolic processes, the state of natural resistance and microbiocenosis of the intestines of sheep and lambs. To study the synergistic and symbiotic relationships of spore-forming bacteria with the host organism, improving health, normalizing metabolism, and increasing productive qualities, more in-depth and thorough research is required.

List of abbreviations

A colony-forming unit (CFU), Adenosine phosphate (ADP), Alanine transaminase (ALT), Alkaline phosphatase (ALP), Aspartate transaminase (AST), Biologically active substances (BAS), Colony-forming units (CFU), Glucose (GLU), Grams (gm), Grams per head (gm/head), Grams per kilogram of live weight (gm/kg), Hemoglobin (HGB), Kilograms (kg), Limited liability company (LLC), Liter (L), Lysozyme activity (LA), Mcfarland (McF), Milliliters (ml), Minute (min), Number of hydrogen ions (PH), Phagocytic activity (FA), Phagocytic amount (FAM), Phagocytic index (PHI), Phagocytic index (PHI), Phagocytic leukocytes (PHI), Revolutions per minute (pm), The bactericidal activity of blood serum (BASC), The specific unit of activity in terms of 1 mg of protein (AU/TP).

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Conflict of interest

The authors responsibly declare that they have no disagreements about the publication of this article.

Authors' contribution

Vladimir Devyatkin took part in developing the research project, summarized the information, and wrote a manuscript draft. Alexey Mishurov conceived the research, participated in its development, reviewed, and edited the manuscript. Yevgenia Kolodina conducted microbiological research and participated in the writing and proofreading of this document. All authors participated in the article's drafting, and writing unanimously approved the final version of the manuscript and agreed to its publication.

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