

Antimicrobial and therapeutic effect of probiotics in cases of experimental purulent wounds

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Probiotics based on bacteria of the genus *Bacillus* with a multifactorial mechanism of action are considered as a possible alternative to antibiotics in the treatment of purulent wounds. The aim of the study was to determine the antimicrobial and therapeutic effect of the Arederma probiotic preparation containing probiotic strains of the genus *Bacillus* in an experimental model of a purulent wound in animals. The antimicrobial efficacy of the probiotic against test strains and clinical isolates of pathogenic and opportunistic microorganisms was studied using the method of delayed antagonism. *Staphylococcus aureus* ATCC 6538 and *Streptococcus pyogenes* K-7 were used to model a purulent wound. From the surface of the wounds, bacteria of *Staphylococcus*, *Streptococcus*, Enterobacteriaceae, *Pseudomonas* genera and Enterobacteriaceae family were sown on appropriate selective media for the cultivation and enumeration of different groups of microorganisms by generally accepted microbiological research methods. The formation of a purulent wound in rabbits caused by mechanical skin damage and subsequent double infection with *Staphylococcus aureus* ATCC 6538 and *Streptococcus pyogenes* K-7 strains was accompanied with a pronounced inflammatory process, necrosis, the formation of purulent exudate and general intoxication. Representatives of the genera *Staphylococcus* and *Streptococcus*, microscopic fungi and, to a lesser extent, members of the family Enterobacteriaceae and *Pseudomonas* were found on the surface of purulent wounds, which confirmed the development of the infectious-inflammatory process. Treatment of purulent wounds with a suspension of probiotic preparation once a day for 4 days led to their faster healing (gradual attenuation of the inflammatory process, reduction of edema and discharge, as well as their disappearance) compared with untreated purulent wounds (control). Representatives of the *Staphylococcus* and *Streptococcus* genera, as well as microscopic fungi, presented in purulent wounds treated with probiotic preparation in much smaller numbers than in the control, and bacteria of the *Pseudomonas* genus and the Enterobacteriaceae family were not detected at all. The effective antimicrobial effect of this probiotic preparation against opportunistic and pathogenic microorganisms was confirmed by *in vitro* studies. Therefore, the Arederma probiotic preparation showed an effective therapeutic and antimicrobial effect in the experimental model of a purulent wound in animals, so it can be recommended for further preclinical and clinical studies.

Keywords: probiotic bacteria; preparation; bacilli; skin; infection; rabbits.

Introduction

In recent years, there has been a rapid increase in antibiotic resistance of microorganisms that cause infectious and inflammatory diseases, as well as the emergence of multidrug-resistant bacterial strains, which leads to a decrease in the effectiveness of antibacterial therapy. Therefore, the problem of search for new highly effective antimicrobial agents, in particular created on the basis of non-pathogenic commensal microorganisms, which have antagonistic properties against pathogenic and opportunistic microorganisms and the ability to influence the development of inflammation, remains relevant (Safronova & Ilyash, 2017; Nolan et al., 2020; Kabanangi et al., 2021; Kumar et al., 2021; Safronova et al., 2021).

Physiological wound healing is a highly organized process that begins with tissue damage and ends with the restoration of its integrity. This process includes several stages: hemostasis, inflammation, proliferation and reconstruction. The hemostasis phase is triggered immediately after injury and accompanied by immediate vascular contraction, platelet aggregation, and the formation of fibrin clots, which initiate the next phase – inflammation (Kuzin & Kostyuchenok, 1990; Lombardi et al., 2019).

During this phase, platelets are activated and the damaged tissue secretes a panel of growth factors, cytokines and chemoattractants, which in

turn attract neutrophils, macrophages and lymphocytes to the site of injury. While physiological wound healing then successfully goes on to subsequent phases, chronic non-healing wounds/ulcers are unable to complete individual stages and the entire healing process. There are many risk factors that can be reversed (stress, alcohol use, smoking, obesity, malnutrition, etc.) as well as those that cannot be reversed (genetic diseases and aging) that contribute to impaired wound healing (Avishai et al., 2017).

Current statistics show that cases of chronic non-healing wounds are becoming more common among the population, which creates a significant socio-economic burden for the health sector and society as a whole. Thus, innovative concepts of prognostic, preventive and personalized medicine are crucial for implementation in this area. First of all, two important tasks are solved in the complex individualized therapy of purulent wounds: inhibition of the growth of pathogenic microorganisms, which are the causative agents of the infectious-inflammatory process; as well as creating conducive conditions for their healing. In the treatment of patients with purulent wounds local and general therapy is used, the type of therapy is determined by the phase of the wound process. Thus, surgical remediation of the source of infection, antibacterial, detoxification and transfusion therapy, as well as nutritional support and other therapeutic measures can be used. Since it is known that many pathogens that are causative

agents of wound infections are resistant to a wide range of antibiotics, other antimicrobial drugs against certain bacteria are additionally used in antibacterial therapy, such as: bacteriophages – streptococcal, staphylococcal, coli-phage, the bacteriophage that affected *Proteus*, as well as complex phages, for example, pyophage, consisting of several species of bacteriophages (Villaruel et al., 2017; McCallin et al., 2018; Chang et al., 2020).

The pandemic caused by the SARS-CoV-2 virus has significantly changed the world, as it has led to a sharp increase in the use of antibiotics and the frequency of the use of antiseptics of various natures, which in turn has accelerated the spread of antibiotic resistance (multidrug resistance) and chemical resistance among pathogenic and opportunistic microorganisms (Cantón et al., 2020; Getahun et al., 2020; Lai et al., 2020).

According to many experts, the era of pure antibiotics is gradually coming to its end, because the rate of resistance development in most microorganisms far exceeds the capabilities of the pharmaceutical industry. All this necessitates the search for new drugs as antiseptics and natural antimicrobial preparations for the treatment of infections including purulent wounds (Francolini et al., 2019; Punjataewakupt et al., 2019) that will meet several criteria: a wide range of antimicrobial action, safety and positive effects on the macroorganism.

Bacillus genus bacteria are among the most promising microorganisms for the development of natural safe probiotic preparations with antimicrobial properties for the treatment of patients with purulent wounds. Firstly, they show high antagonistic activity against a wide range of pathogenic and opportunistic microorganisms; secondly, lipopeptide, biosurfactants (surfactin, pumilacidin and fengycin), produced by them, have antioxidant activity, which promotes wound healing (Ben Ayed et al., 2015; Zouari et al., 2015); thirdly, their proteolytic enzymes with thrombolytic effect prevent scarring and necrotic tissue lysis and therefore accelerate tissue regeneration (Sinclair & Ryan, 1994; Shahzad et al., 2015). At the same time, it is convenient to clone foreign genes of pro- and eukaryotic origin in bacteria of the *Bacillus* genus, so they are promising for the development of recombinant probiotics with multifactorial action (Kordon, 2014).

Antagonism of bacilli is directly related to the ability of different species to synthesize antibiotics. About 800 antibacterial compounds that they synthesize have been identified (Stein, 2005; Pohilenko & Perelygin, 2007; Sang & Blecha, 2008; Sumi et al., 2015; Caulier et al., 2019). Thus, the most well-known among them are bacitracin, pumulin, butirosin, tyrocidine, which are effective against Gram-positive bacteria; colistin and polymyxin, which are active against Gram-negative bacteria, while mycobacillin and zwittermicin have antifungal activity. The use of live bacterial cells that produce a wide range of antibiotics prevents the suppression of local and systemic immunity, the development of side complications such as dysbacteriosis or allergic reactions and the spread of antibiotic-resistant strains of microorganisms in the environment (Sorokina et al., 2013; Safronova & Ilyash, 2017). Therefore, today probiotics based on the *Bacillus* genus bacteria are considered as a possible alternative to antibiotics in the treatment of purulent wounds.

Thus, it has long been proven that probiotics based on bacteria of the *Bacillus* genus, such as Endosporin (*B. amyloliquefaciens* subsp. *plantarum* UCM B-5139 and UCM B-5140), Sporobacterin (*Bacillus subtilis* 534), Bactisubtil (*B. cereus* IP 5832), Bioseptin (*B. amyloliquefaciens* VKPM B-10642 and *B. amyloliquefaciens* strain VKPM B-10643), Bactisporin (*B. subtilis* 3H), etc. have a positive effect on wound healing in the treatment of patients with purulent-necrotic processes, dermatitis and burns, as well as in veterinary medicine (Vinnik et al., 1998; Kalynovskiy et al., 2000; Nikitenko, 2004; Shahsafi, 2017). Such antimicrobial probiotic preparations have accelerated the elimination of pathogens of the infectious-inflammatory process from wounds, stimulated the formation and growth of granulations, promoted faster wound healing. Therefore, further search for strains of *Bacillus* genus bacteria, promising for the development of antimicrobial probiotic preparation of multifactorial action, as well as the study of new biological properties of already known probiotics based on these bacteria is one of the promising areas of modern biotechnology and microbiology.

Such scientific developments can be useful for the military medics working in the Anti-terrorist Operation Zone. Given the specifics of such injuries, probiotic preparations based on probiotic microorganisms and

their metabolites can be used immediately after injury to form a protective biofilm and prevent wound infection, and during treatment as a wound healing, antimicrobial and anti-inflammatory agent. Therefore, one of the promising areas of modern biotechnology and microbiology is the further search for strains of the *Bacillus* genus bacteria, which have wound-healing, antimicrobial, anti-inflammatory and immunomodulatory properties, and nanomaterials that can enhance the biological properties of probiotic strains, in order to develop a multifactorial probiotic preparation for the prevention and personalized treatment of purulent wounds of the skin and mucous membranes. In connection with the above, the aim of the work was to determine the antimicrobial and therapeutic effect of the Arederma probiotic preparation containing probiotic strains of the *Bacillus* genus (*B. subtilis* and *B. megatherium*) in an experimental purulent wound in rabbits.

Materials and methods

The object of the study was the Arederma probiotic preparation (Sirion LLC) in the form of a spray. It consists of *B. subtilis* (5×10^7 CFU/mL) and *B. megatherium* strains (5×10^7 CFU/mL), as well as didecylidimethylammonium chloride (0.1%). The number of live bacterial cells was determined using conventional microbiological methods (Krivoshin, 1986; Krasilnikov, 1995).

The antimicrobial efficacy of the probiotic against test strains and clinical isolates of pathogenic and opportunistic microorganisms was studied using the method of delayed antagonism (Ermolenko et al., 2004). At least two cultivations using a suitable nutrient medium with incubation under standard conditions were performed to restore lyophilized cultures of test strains. To obtain isolated colonies, the second cultivation was performed on a suitable agar medium with subsequent incubation under standard conditions. The morphology of the growing colonies was studied; bacteria were stained by the Gram method for microscopic evaluation. Test strains of microorganisms had typical morphological, tinctorial, cultural, biochemical properties in accordance with the presented collection certificate.

The resulting suspension was plated with a bacteriological loop with a diameter of 3.5 ± 0.5 mm on 6 Petri dishes with nutrient agar medium, conducting two parallel strokes with a length equal to the diameter of the Petri dish. After incubation for 48–96 h at a temperature of 37.0 ± 1.0 °C under adequate conditions, daily broth cultures of test strains or clinical isolates (1×10^9 cells/mL) were plated with a loop with a diameter of 1.75 ± 0.25 mm in perpendicular direction to the growth zone of the probiotic strain and without touching it. The distance between cultures was 1.0–1.5 cm. Petri dishes with cultures were placed in a thermostat, lids down in stacks not more than six units high and incubated at a temperature of 37.0 ± 1.0 °C for 18–24 hours. The growth control of test strains and clinical isolates was their parallel hanging on Petri dishes with the same nutrient medium, but without probiotic bacteria. The results were assigned by measuring the distance of growth absence of test strains and clinical isolates of pathogenic and opportunistic microorganisms. The size of growth inhibition zone of the test strain or clinical isolate, expressed in mm, was taken into account. The growth inhibition zone of test cultures should be more than 10 mm. The antagonistic activity of probiotic bacilli strains was considered high if the growth inhibition zones of test strains or clinical isolates were not less than 20 mm. Growth inhibition zones size 0–10 mm were considered as a manifestation of their weak antagonistic effect, and 10–20 mm – moderate.

Experimental studies were performed using outbred rabbits aged one year, weighing 4.3–4.5 kg. The animals were kept in the vivarium of the Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine in standard conditions in metal cages in a separate room at a constant air temperature (22–25 °C). The studied animals obtained appropriate fed and had free access to water. The animals were quarantined for at least two weeks before the experiment. All experimental studies were conducted taking into account the norms of the European Convention for the Protection of Vertebrate Animals used for Research and other Scientific Purposes, from 18.03.1986 (Strasbourg) and the law of Ukraine No. 3447-IV “On protection of animals from cruel treatment” (Zapadnyuk, 1983; Reznikov, 2001).

Staphylococcus aureus ATCC 6538, deposited in the Depository of Microorganisms of the Zabolotny Institute of Microbiology and Virology of the NAS of Ukraine, as well as clinical strain *Streptococcus pyogenes* K-7 were used to model a purulent wound. The purulent wounds in rabbits were modeled using opportunistic bacteria as follows: skin of rabbits in different sections of the back was exposed by removal of fur (in diameter up to 5 cm), treated with alcohol, and then mechanical damage to the skin was carried out with a sterile scalpel, accompanied by a violation of its integrity. Surrounding tissues were damaged to a small extent. The main clinical sign of cut wounds was capillary bleeding. *S. aureus* ATCC 6538 and *S. pyogenes* K-7 strains each separately were subcutaneously injected around the cut wound in a dose of 100 µL (1 × 10⁹ CFU/mL) immediately after violating of the integrity of the skin. These strains of bacteria were re-injected in the same dose and quantity to damaged skin areas 48 hours after the moment of injury.

The suspension of the probiotic strains was applied to the formed purulent wounds by irrigating them once a day for 4 days. Before treatment and 4 days after treatment of the purulent wounds with the probiotic preparation, swabs were taken from their surfaces. The samples were diluted ten-fold using sterile 0.15 M NaCl to a concentration of 10⁻¹–10⁻³ and plated on Petri dishes with selective solid nutrient media for different groups of microorganisms. Similarly, swabs were taken from unaffected areas of skin and fur of animals. Obtained samples were plated on Petri dishes with the following nutrient media: meat-peptone agar (MPA) – medium for cultivation of aerobic and optionally anaerobic bacteria; KF-*Streptococcus* agar (“Merck”, Germany) – selective medium for *Streptococcus* bacteria; BAIRD-PARKER-Agar (“Merck”, Germany) – selective medium for *Staphylococcus* bacteria; Sabouraud agar (“HiMedia”, India) – selective medium for microscopic fungi; ENDO (“HiMedia”, India) – selective medium for coliform bacteria; *Pseudomonas* agar (“HiMedia”, India) – selective medium for *Pseudomonas* bacteria using conventional microbiological research methods (Lebedev & Ponyakina, 1990; Egorov, 1995; Brown & Perry, 1999).

After 24 hours of incubation, a preliminary record of the results was taken, and after 48 hours – the final. The number of colonies of microorganisms in each of the parallel studies of one dilution was counted. The result was calculated as the arithmetic mean of the number of colonies in different studies of one dilution. The multiplicity of sample dilutions was taken into account during the calculation. The result was expressed in logarithms of colony-forming units (CFU) in 1 mL of the studied sample. The final result was the number of colonies in one Petri dish. If it was impossible to count the colonies on the surface of the Petri dish, then “continuous growth” was noted in the protocol.

Streptococcus bacteria colonies on KF-*Streptococcus* agar medium were small (0.1–1.0 mm), white or light brown. *Staphylococcus* bacteria colonies on BAIRD-PARKER-Agar medium were shiny, small (0.01–0.10 mm) or medium in size (0.6–3.0 mm), from milky-white to yellow-orange, surrounded by pure halos. Coliform bacteria colonies on ENDO medium were different in sizes, the colour of the colonies varied from dark pink to pink-red with a green metallic luster. Colonies of microscopic fungi on Sabouraud agar were medium in size, milky-white, shiny, waxy, usually smooth, 0.1–4.0 mm in size. The criterion for the purity of the culture was the presence in the field of view of the microscope of morphologically homogeneous cells.

All obtained digital data were processed using the computer program Epi Info (version 8.0) using the method of variation statistics. Numerical data was presented in the form of arithmetic average and standard deviations ($\bar{x} \pm SD$). Null hypothesis for the comparison groups was verified using non-parametric Wilcoxon-Mann-Whitney (U) and Kolmogorov-Smirnov criteria. Differences between the groups were considered statistically significant at $P < 0.05$.

Results

The studied probiotic contained living cells of *Bacillus* genus bacteria in the concentration of 1 × 10⁷ CFU/mL. *In vitro* studies showed that *B. subtilis* and *B. megaterium* strains, which are part of the Arederma probiotic preparation, had a strain-dependent antimicrobial activity against the museum strains of opportunistic and pathogenic microorganisms

(Fig. 1). Thus, under their influence, the growth of *Proteus vulgaris* 72 and *Candida albicans* 690 was the most effectively inhibited, to a lesser extent – *S. aureus* 209 and *Salmonella typhimurium* 11. Minor growth zones after cultivation with these strains of bacilli were noted for *Escherichia coli* 028 and *Shigella flexneri* GISK 337.

Therefore, in further studies, we tested the antimicrobial activity of this probiotic preparation on purulent wounds, which were modeled on rabbits (Fig. 2). After removal of the fur, the skin of rabbits was intact (Fig. 2a), cut wounds appeared as a result of mechanical damage to the skin (Fig. 2b). The following typical clinical signs testified to the formation of a purulent wound on the 4–5th day from the moment of injury after double infection of rabbits with strains *S. aureus* ATCC 6538 and *S. pyogenes* K-7: pronounced inflammation, necrosis, formation of purulent exudate and the phenomenon of general intoxication (Fig. 2c). Thick pus of mostly yellowish hue was formed. The data received testify the development of the infectious and inflammatory process during phase I of wound healing – the phase of inflammation, in accordance with the popular classification of the phases of wound healing, which usually lasts for 1–5 days from the moment of injury and consists of several successive periods, in particular vascular changes and cleaning of the wound from necrotized tissues.

Animals were under observation during the phase II of wound healing – the regeneration phase, which continues over the next 6–14 days and is accompanied by collagenization and an increase in the number of blood cells, as well as lymphatic vessels. However, the data obtained indicate that pronounced signs of infectious-inflammatory process in purulent wounds were present in rabbits during this phase.

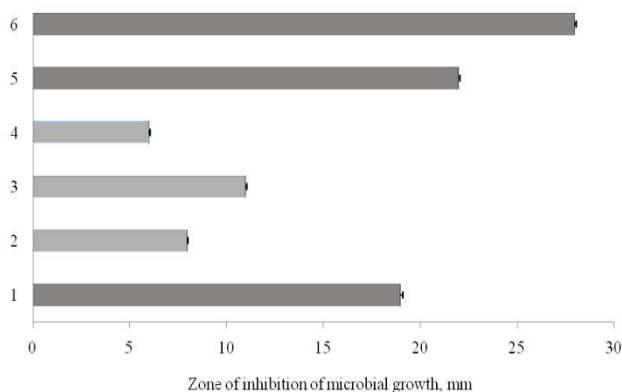


Fig. 1. Antagonistic activity of probiotic strains of bacilli against opportunistic microorganisms (method of delayed antagonism, $n = 3$, $\bar{x} \pm SD$): 1 – *S. aureus* 209; 2 – *E. coli* 028; 3 – *S. typhimurium* 11; 4 – *S. flexneri* GISK 337; 5 – *P. vulgaris* 72; 6 – *C. albicans* 690

The results of microbiological studies, which we conducted on the 8th day from the moment of injury, showed that aerobic and optionally anaerobic microorganisms, including representatives of *Staphylococcus* and *Streptococcus* genera, as well as microscopic fungi were present in purulent wounds in large quantities (Table 1). Representatives of the Enterobacteriaceae family were present only in one purulent wound, and the representatives of the genus *Pseudomonas* were not detected.

It should be noted that staphylococci, streptococci and microscopic fungi were also present on fur and unaffected skin areas adjacent to the purulent wound at the same observation period. A small number of *Pseudomonas* genus bacteria, and representatives of the Enterobacteriaceae family (on the 12th day) were present on fur and unaffected skin (Table 1).

The probiotic preparation was used to treat wounds during the phase of their healing – the phase of regeneration. Thus, 8 days from the moment of injury after a microbiological study, the surface of purulent wounds was treated with this probiotic preparation by irrigation once a day for the next 4 days. Animals were under observation over the next 8 days (before completing the phase II of regeneration). The results of the influence of the probiotic preparation on the healing of purulent wounds are presented in Figure 3.

Thus, a gradual attenuation of the inflammatory process, reduction of edema and the number of secretions was detected. Under the influence of

the probiotic preparation, wound healing processes were more expressive, starting from the 5th day after treatment (Fig. 3b, wound No. 1 and 2) compared with untreated purulent wounds (Fig. 3b, wound No. 3). On the 9th and 12th days after the treatment of purulent wounds with the probio-

tic preparation (Fig. 3c, d, wound No. 1 and 2), a faster healing of wounds compared with control (Fig. 3c, d, wound No. 3) was observed. It should be noted that during this period of observation a recovery of fur cover around the wound was also observed.

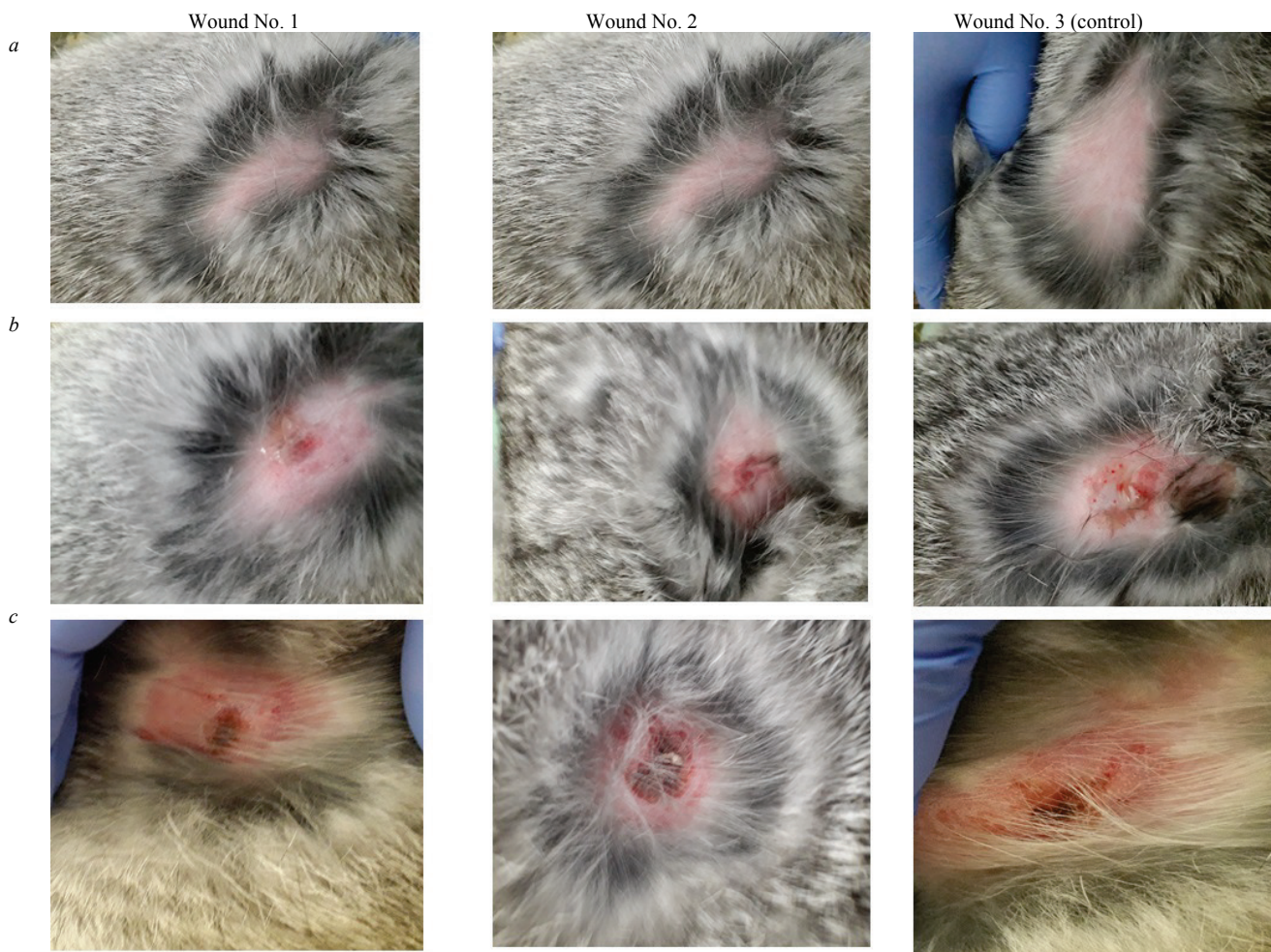


Fig. 2. Purulent wounds modeling in rabbits (I phase of wound healing – 1–5 days from the moment of injury):

a – areas of skin of the back, freed from fur before mechanical damage (1st day of the experiment); *b* – areas of skin of the back, freed from fur, after mechanical damage with a scalpel before the injection of clinical strains of bacteria and treatment with the probiotic preparation; *c* – the formation of a purulent wound after two injections of clinical strains of bacteria before treatment with the probiotic preparation (5th day after injections)

Table 1

The microbiota spectrum of the wounds surface of rabbits ($\bar{x} \pm SD$)

Group of animals	Days from the moment of injury	The number of microorganisms detected using selective nutrient media, Lg CFU/mL					
		MPA	BAIRD-PARKER-Agar	KF-Streptococcus agar	Sabouraud agar	ENDO agar	Pseudomonas agar
Before treatment with the probiotic preparation							
Wound No. 1	8	5.12 ± 0.04	4.67 ± 0.06	3.13 ± 0.06	2.14 ± 0.12	1.14 ± 0.08	0.00
Wound No. 2		5.07 ± 0.07	4.18 ± 0.05	3.86 ± 0.03	2.61 ± 0.04	0.00	0.00
Wound No. 3		4.48 ± 0.28	4.66 ± 0.22	3.82 ± 0.04	2.34 ± 0.04	0.00	0.00
Skin and fur**		2.14 ± 0.11	2.41 ± 0.12	1.10 ± 0.05	1.25 ± 0.15	0.00	1.10 ± 0.10
After treatment of wounds No. 1 and 2 with the probiotic preparation							
Wound No. 1	12	2.44 ± 0.08*●	1.40 ± 0.10*●	2.21 ± 0.09*●	1.14 ± 0.07*●	0.00	0.00
Wound No. 2		3.01 ± 0.11*●	1.20 ± 0.10*●	2.99 ± 0.08*●	1.94 ± 0.08*●	0.00	0.00
Wound No. 3 (control)		4.92 ± 0.07	4.25 ± 0.14	4.27 ± 0.07●	2.78 ± 0.07●	1.25 ± 0.10●	1.50 ± 0.15●
Skin and fur**		2.32 ± 0.12	2.33 ± 0.03	1.66 ± 0.12	1.60 ± 0.10	2.14 ± 0.12●	1.22 ± 0.07

Notes: * – $P < 0.05$ compared to the indicators for the wounds not treated with the probiotic preparation (control); ● – $P < 0.05$ compared to the indicators for the same study group on the 8th day of the study; ** – intact skin and fur of rabbits.

Microbiological study of the surface of purulent wounds was carried out for on the 5th day from the beginning of the treatment with the probiotic preparation (on the 12th day from the moment of injury), when a pronounced attitude of clinical manifestations of the pathological process was revealed. As shown in Table 1, after four treatments of purulent wounds with this probiotic preparation, a significant decrease in the number of

representatives of the genus *Staphylococcus* (by 3 orders of magnitude) was observed compared with the indicators obtained before its use (Table 1, Fig. 4). Instead, on the 12th day from the moment of injury, the number of representatives of the *Staphylococcus* genus bacteria did not decrease in purulent wounds that were not treated with the probiotic preparation (Table 1, Fig. 4).



Fig. 3. Healing of purulent wounds in rabbits treated (Wounds No. 1 and 2) and untreated (Wound No. 3, control) with the probiotic preparation: *a* – the formation of purulent wounds on the 8th day from the moment of injury before treatment with the probiotic preparation; *b* – purulent wounds on the 12th day from the moment of injury (on the 5th day from the beginning of treatment with the probiotic preparation); *c* – purulent wounds on the 16th day from the moment of injury (on the 9th day from the beginning of treatment with the probiotic preparation); *d* – purulent wounds on the 19th day from the moment of injury (on the 12th day from the beginning of treatment with the probiotic preparation)

The number of representatives of the *Streptococcus* genus bacteria in purulent wounds, which were treated with the probiotic preparation, on the 12th day from the moment of injury decreased by 1 order of magnitude, compared with the indicators obtained before its application. At the same time, *Streptococcus* genus bacteria were present in the wounds not treated with this probiotic preparation even in greater quantities than on the 8th day after the injury.

A similar tendency was noted for microscopic fungi: their number from 8th to 12th days from the moment of injury decreased in purulent wounds that were treated with the probiotic preparation, but slightly increased in wounds that were not treated (control) (Table 1).

The representatives of *Pseudomonas* genus and Enterobacteriaceae family were not found in purulent wounds that were treated with the probiotic preparation on the 12th day from the moment of injury (Table 1). However, in the untreated purulent wounds, we found not only streptococci and staphylococci in large quantities, but also the representatives of the *Pseudomonas* genus and Enterobacteriaceae family, which also cau-

sed a delay in their healing. Presumably, the latter got into an infected wound from unaffected skin and fur around it.

Discussion

Probiotics are determined as live microorganisms that are introduced in sufficient quantities to improve host health (Food and Agriculture Organization / World Health Organization, 2006). Probiotics produce antimicrobial substances, including bacteriocins, short-chain fatty acids and organic acids, and they modulate disorders of the gastrointestinal tract using antimicrobial and anti-adhesive action against pathogenic and opportunistic microorganisms, and may also affect the immune system, so they are used as natural prophylactic and therapeutic preparations for many diseases of man and animals. Probiotic potential is mainly set for bacteria of *Lactobacillus* and *Bifidobacterium* genera, but have bacteria of the genus *Bacillus* also have high prophylactic and therapeutic activity. Such commercial probiotic strains of *Bacillus*: *B. cereus*, *B. clausii*,

B. coagulans, *B. licheniformis*, *B. polyfermenticus*, *B. pumilus*, and *B. subtilis* etc., which have antimicrobial, antioxidant and vitamin-producing properties are well known (Lee et al., 2015; Safronova, 2017; Lee et al., 2019). Despite the useful probiotic properties of *Bacillus* strains belonging to the security group 1, a number of their strains can be a significant risk for health, since they can produce toxins and biogenic amines, as well as contain antibiotic resistance genes that are a key factor in their yet limited application. Therefore, useful *Bacillus* probiotic properties and safety must be carefully proven in preclinical and clinical studies (Jeżewska-Frackowiak et al., 2018; Lee et al., 2019).

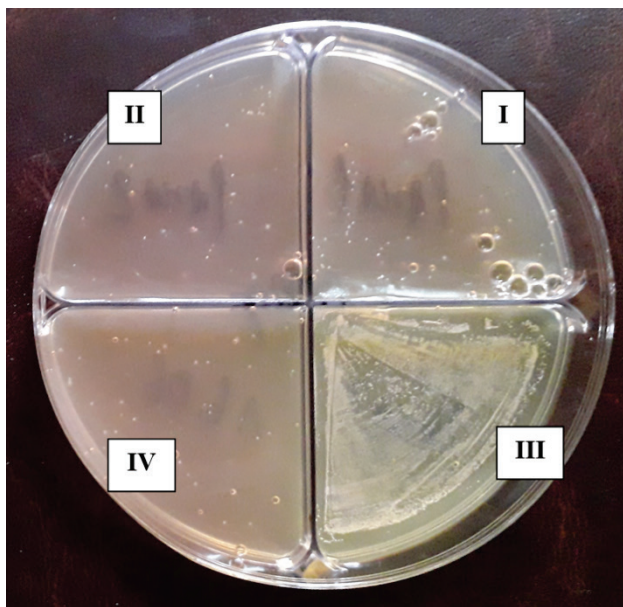


Fig. 4. Microbiological studies for the presence of *Staphylococcus* genus bacteria in purulent wounds on the 12th day from the date of injury (5 days after the start of treatment with the probiotic preparation): I – wound No. 1 treated with the probiotic preparation; II – Wound No. 2 treated with the probiotic preparation; III – Wound No. 3, not treated with the probiotic preparation; IV – unaffected skin and fur of rabbits; as can be seen, *Staphylococcus* was present in much larger quantities in the wound No. 3 (control, III) than in the other wounds and unaffected skin and fur of rabbits

An analysis of PubMed and ClinicalTrials.gov databases has shown that oral and local use of probiotics can be effective for the treatment of certain inflammatory skin diseases, including psoriasis, atopic dermatitis, seborrheic dermatitis, acne vulgaris, cutaneous neoplasms and chronic wounds. However, in order to confirm the therapeutic and prophylactic effectiveness of probiotics for inflammatory diseases of the skin, wounds and skin cancer, more research is required (Yu et al., 2020), possibly focused on determining the mechanisms of their actions for these pathologies. The most effective strategy of wound management is to prevent infections, promote healing, and prevent excess scarring. It is known that probiotics can contribute to the process of healing of wounds by modulating an inflammatory reaction, limiting the colonization of the pathogen of the infectious-inflammatory process and the reduction of the wound. In particular, a number of probiotics based on bacteria of the genus *Lactobacillus* (most often used *Lactobacillus plantarum*, *L. casei*, *L. acidophilus* and *L. rhamnosus*) demonstrated efficiency in the treatment of chronic wounds, diabetic ulcers, burn wounds, as well as in prevention and treatment of skin infections (Tsiouris et al., 2017; Fijan et al., 2019; Yu et al., 2020). Exogenous and oral use of these probiotics showed a decrease in wound infections, especially when used as an adjuvant to antibiotic therapy (Fijan et al., 2019). The potential use of probiotics for wound healing remains a valuable subject for further study.

Some probiotics based on bacteria of the *Bacillus* genus also had a therapeutic effect in the inflammatory skin diseases (Vinnik et al., 1998; Kalynovskyj et al., 2000; Bakulina et al., 2001; Nikitenko, 2004; Zouari et al., 2016; Shahsafi, 2017). These bacteria synthesize not only a variety

of antimicrobial substances, but also produce enzymes, polysaccharides, amino acids and vitamins. It is known that extracellular polysaccharides have the ability to increase the protective potential of macroorganism to bacterial and viral infections, to activate phagocytosis, lysozyme synthesis, exhibit antitumour, anti-inflammatory, wound-healing effect, which indicates the wide possibilities of their use in medicine and veterinary medicine (Enikeev, 2011; Ramanathan et al., 2011; Van Dyk et al., 2012). Aerobic bacilli strains synthesize the enzymes of different classes, which allow them to exist on different substrates. They also participate in the regulation and stimulation of digestion, have an anti-allergy and antitoxic effect. Among the hydrolytic enzymes, proteases that occupy a key position due to their important physiological role in the organism should be highlighted. An important area of use of proteases is medicine and veterinary medicine. For example, the subtilisin which is a proteolytic enzyme produced by *B. licheniformis* acts on some species of gram-negative bacteria – intestinal rods, pathogenic clostridium and has thrombolytic and anticoagulant properties. Antagonistically active bacteria that are included in the drugs Sportserine and Baktosporinplast, produce a wide range of proteolytic enzymes that contribute to the purification of foci of damage from necrotic tissues in cases of local use with purulent infections (Safronova, 2017).

Our studies indicate an effective antimicrobial and therapeutic effect of the probiotic Arederma containing representatives of the *Bacillus* genus bacteria (*B. subtilis* and *B. megaterium*) in the form of a spray in cases of experimental purulent wounds in rabbits. Treatment of purulent wounds with this preparation led to faster healing (attenuation of the inflammatory process, reduction of edema and the number of secretions, as well as their gradual disappearance) compared with untreated purulent wounds. Antimicrobial properties of the probiotic preparation are confirmed in the *in vitro* experiments, as well as *in vivo* by a significant decrease of the number of aerobic and optional anaerobic microorganisms, including representatives of the *Staphylococcus* and *Streptococcus* genera, as well as microscopic fungi in purulent wounds treated with the probiotic preparation compared to the untreated wounds.

Pseudomonas genus bacteria and the representatives of the Enterobacteriaceae family were not detected at all in the purulent wounds treated with this probiotic preparation, as opposed to control. That is, the probiotic preparation had an antimicrobial effect on the representatives of those genera which are often the causative agents of purulent-inflammatory processes in humans and farm animals. It should be noted that the Arederma probiotic is a complex preparation, since it is based on two strains of bacilli – *B. subtilis* and *B. megatherium*, which can significantly expand the range of antimicrobial action and the degree of therapeutic and prophylactic efficiency.

Antimicrobial and therapeutic effect of representatives of the genus *Bacillus* for experimental wounds and veterinary medicine were detected in other studies. The strain *B. subtilis* 534, the suspension of which was directly injected into the wounds, accelerated their healing and prevented the development of purulent infections in farm animals (Bakulina et al., 2001). The probiotic preparation based on two strains *B. subtilis* IMV B-7142 and *B. subtilis* IMV B-7142, which suppressed the development of opportunistic microorganisms – representatives of *Escherichia*, *Salmonella*, *Shigella*, *Proteus*, *Pseudomonas*, *Staphylococcus*, *Streptococcus*, *Acinetobacter* genera, and mutually complemented each other according to some biological properties, while also showing a high therapeutic and prophylactic effect in cases of purulent wounds in farm animals. Under the influence of the suspension of this biological preparation in the form of application on damaged places of wounds, the tightening and rejection of necrotized tissues occurred faster; the exudation stopped, the wounds were covered with a scab. Thus, this biopreparation accelerated the regeneration of damaged tissue (Safronova et al., 2015). Bioseptine ointment with the content of some recombinant strains *B. subtilis* and *B. licheniformis* and their metabolites with antiseptic, anti-inflammatory and proteolytic properties are used to treat aseptic, purulent-necrotic wounds and dermatitis in animals. Under the influence of Bioseptin, the processes of epithelization and scarring of the wounds are accelerated (Bakulina et al., 2001). Faster wound healing in experimental animals is also found under the influence of a gel containing of *B. subtilis* SPB1 lipopeptide biosurfactant. Its therapeutic effect was confirmed by histological studies. As a result of these

studies, the use of a gel based on a lipopeptide from *B. subtilis* SPB1 was substantiated for the treatment of normal and complicated wounds, as well as skin diseases (Zouari et al., 2016).

The probiotic preparation Sporobacterin, which includes *B. subtilis* 534 strain, that produces broad-spectrum antibacterial substances, also has an effective wound healing ability, inhibiting the development of pathogenic and opportunistic microorganisms, as well as accelerating the clearance of postoperative wounds and foci of inflammation from necrotized tissue. Thus, this strain of bacilli had an effective antimicrobial effect against *Staphylococcus* (34 of 37 studied strains), *Streptococcus* (11 of 12 strains), *Proteus* (6 of 6 strains), *P. aeruginosa* (7 of 8 strains), *E. coli* (7 of 8 strains), as well as yeast fungus (1 of 1 strain). This strain also produced proteolytic enzymes with antiallergic properties. It was found that oral administration of Sporobacterin in the complex treatment of patients with burns and granulation wounds resulted in the restoration of normal intestinal biocenosis, acceleration of regenerative processes in the wound, as well as the processes of granulation and epithelialization. After the use of this probiotic preparation, the length of patients' stay in a surgical hospital and the time of their rehabilitation reduced (Nikitenko & Guryanov, 2008; Sorokina et al., 2013).

Another approach to improving the therapeutic and prophylactic efficacy of bacilli-based probiotics in infectious and inflammatory diseases, including purulent wounds, is to create biocomposites that provide their prolonged action. In particular, the biocomposite, which included a gel-film of bacterial cellulose with immobilized living *B. subtilis* cells, had high therapeutic efficacy in experimental wounds in rats. It was found that immobilized *B. subtilis* cells significantly inhibited the growth of causative agents of wound infections, such as *S. aureus*, *S. epidermidis*, *E. coli*, *P. aeruginosa*. The use of this biocomposite for the treatment of excisional wounds in laboratory animals (rats) on the one hand, stimulated reparative processes, and on the other – reduced the time of their healing (Savitskaya et al., 2019). Recently, a new approach to the treatment of patients with open purulent wounds has been described: the use of sticky and soluble microparticles of polyvinyl alcohol containing live culture of *B. subtilis*, which produced antimicrobial molecules and had a significant antagonistic effect against staphylococci including methicillin-resistant *S. aureus*. This probiotic preparation effectively shortened the healing time and did not have such side effects as skin irritation, infection development, etc. in experimental model of open wound in C57BL/37 line mice (Ben David et al., 2021).

In our studies, a suspension of Arederma probiotic preparation containing live cells of *B. subtilis* and *B. megatherium* was used as a spray for the treatment of experimental purulent wounds in rabbits once a day for 4 days from the 8th day after injury. This provided long-term contact on the surface of purulent wounds with live bacterial cells capable of producing antimicrobial and other biologically active substances that contributed to the inhibition of the growth of pathogenic and opportunistic microorganisms, and the decay of necrotized damaged tissue, which ultimately led to faster wound healing.

Bacillus genus bacteria have not only antagonistic properties against causative agents of infectious and inflammatory diseases, but also the ability to penetrate and concentrate in the localization of the pathological process, where they excrete antibacterial compounds, proteolytic enzymes, immunomodulators, which also enhances their effectiveness (Sumi et al., 2015). It should be noted that spores of *Bacillus* genus bacteria which have significant antioxidant and detoxifying properties and are able to persist for a long time in the affected areas can also have a therapeutic effect on purulent wounds (Sumi et al., 2015; Petruk et al., 2018; Mazzoli et al., 2019). The immunomodulatory properties of *Bacillus* genus bacteria, which are or will be part of probiotics with antimicrobial properties, should also be studied in various experimental models and in clinical practice. It is known that bacilli can affect the factors of innate and acquired immunity (Matsuzaki & Chin, 2000; Timoshok et al., 2006; Guo et al., 2017; Lv et al., 2020), which results in changes in the production of a number of immunoregulatory cytokines and other biologically active substances involved in the processes of repairing and healing of tissues.

Therefore, probiotic preparations based on *Bacillus* genus bacteria with proven wound healing, antimicrobial, anti-inflammatory and immunomodulatory effects can be used in health care to increase the effective-

ness of prevention and comprehensive individualized treatment of patients with purulent inflammatory wounds of the skin and mucous membranes. Based on the analysis of these literature sources, we can conclude that their action is aimed at accelerating the healing of purulent wounds, elimination of pathogenic microorganisms and normalization of inflammation and immune response, as well as the restoration, formation and maintenance of normal microbiota. The use of these probiotic preparations, which include live bacterial cells, will also prevent the suppression of local and systemic immunity, the development of side complications such as dysbacteriosis or allergic reactions and the spread of antibiotic-resistant strains of microorganisms in the environment.

Such probiotic preparations based on *Bacillus* genus bacteria may have advantages over antibiotics, which are widely used in the treatment of purulent wounds, resulting in a rapid increase in antibiotic resistance of microorganisms, as well as the emergence of multidrug-resistant bacterial strains. They have a wider range of antimicrobial activity than other drugs with similar effects, such as bacteriophages, because the probiotic bacteria are able to produce a wide range of antibiotics effective against pathogenic and opportunistic microorganisms of various genera and species.

In addition to the above mentioned antibacterial compounds, the antagonism of bacilli is also directly related to the ability of their various species to synthesize lytic enzymes (lysin) – enzyme complexes with different substrate specificity (such as lysozyme and enzyme complexes, including glucanase, mannanase, protease, acetylhexosaminidase, amidase, etc.), which together or separately degrade the cell wall of microorganisms. Under their influence there is an effective lysis of cells of Gram-negative and Gram-positive bacteria, as well as yeast-like fungi (Lee & Lee, 2011). Free amino acids, in particular essential, synthesized by *Bacillus* genus bacteria, also play an important role in the formation of anti-infective defenses, as they participate in protein and carbohydrate metabolism, provide the process of new cells' formation with oxygen, they are building blocks for enzymes, antibodies and some hormones, serve as a source of energy at the cellular level, etc. (Nuttawut et al., 2012). The body's protective potential against bacterial and viral infections is also increased under the influence of extracellular polysaccharides of bacilli, which have immunomodulatory, antitumour, anti-inflammatory and wound-healing effects, indicating the wide possibilities of their use in medicine (van Dyk et al., 2012). Therefore, the use of probiotic bacteria of the genus *Bacillus* opens the prospect of creating fundamentally effective natural wound-healing probiotic preparations with a broad spectrum of action, which can increase the effectiveness of treatment of patients with purulent wounds.

Conclusion

Thus, treatment of purulent wounds in rabbits with Arederma probiotic preparation containing two live strains – *B. subtilis* and *B. megatherium* – led to their faster healing (gradual attenuation of the inflammatory process, reduction of edema and discharge, as well as their disappearance). This probiotic preparation also had an effective antimicrobial effect, which was proven in experiments *in vitro* and *in vivo*. The number of aerobic and optionally anaerobic microorganisms, including representatives of the *Staphylococcus* and *Streptococcus* genera, as well as microscopic fungi decreased in purulent wounds under its influence. Bacteria of the *Pseudomonas* genus and the Enterobacteriaceae family were not detected at all in purulent wounds treated with this preparation. The results of our experimental studies showed that Arederma probiotic preparation had an effective therapeutic and antimicrobial effect in experimental purulent wounds in rabbits. From the data obtained by us, we can conclude that this probiotic preparation can be used for treatment of purulent wounds, so we recommend it for further preclinical and clinical studies.

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