

<https://doi.org/10.15407/microbiolj85.03.048>

L.M. LAZARENKO^{1*}, L.P. BABENKO¹, L.A. SAFRONOVA¹,
O.M. DEMCHENKO², V.V. BILA², G.M. ZAITSEVA³, M. Ya. SPIVAK¹

¹ Zabolotny Institute of Microbiology and Virology, NAS of Ukraine,
154 Akademika Zabolotnoho Str., Kyiv, 03143, Ukraine

² Kyiv Perinatal Center,
9 Preslavinskaya Str., Kyiv, 03150, Ukraine

³ O.O. Bogomolets National Medical University,
13 Shevchenko Blvd., Kyiv, 01601, Ukraine

* Author for correspondence; e-mail: LazarenkoLM@gmail.com

ANTIMICROBIAL AND IMMUNOMODULATORY ACTION OF PROBIOTIC COMPOSITION OF BACILLI ON BACTERIAL VAGINITIS IN MICE

The **purpose** of this study was to investigate the antimicrobial and immunomodulatory action of a probiotic composition of *Bacillus subtilis* and *B. megatherium* strains (UnicaUro, Sirion (Ukraine)) for experimental bacterial vaginitis. **Methods.** Experimental studies were conducted on female BALB/c mice; we used *Staphylococcus aureus* strain B-918 (ATCC 6538) to induce bacterial vaginitis. The strain was vaginally introduced into mice before treatment with probiotic bacteria. In the vagina of mice, aerobic and optionally anaerobic bacteria, including representatives of the genera *Staphylococcus*, *Streptococcus*, *Lactobacillus*, *Bifidobacterium*, *Pseudomonas*, coliform bacteria, and microscopic fungi were identified in different periods of observation using generally accepted microbiological methods. Serum antibody titer to *S. aureus* was determined by the bacterial agglutination reaction. The phagocytic activity and oxygen-dependent bactericidal activity of peritoneal exudate macrophages (PEM) were evaluated using generally accepted immunological methods. **Results.** The formation of bacterial vaginitis in the BALB/c mice line infected with *S. aureus* B-918 (ATCC 6538) was evidenced by the appearance of external clinical manifestations of the infectious and inflammatory process against the background of the increased number of aerobic and optionally anaerobic microorganisms, including representatives of the genus *Staphylococcus* and *Streptococcus*, microscopic fungi, and decreased number of lactobacilli in different observation periods. The probiotic introduction to mice with bacterial vaginitis led to a dynamic change in the vaginal microbiota: the number of aerobic and optionally anaerobic microorganisms decreased, primarily due to the normalization of the number of representatives of *Staphylococcus* genus accompanied by a decrease in the antibody titer to staphylococcus in the blood serum. The effective therapeutic action of the probiotic was confirmed by the gradual disappearance of the external clinical signs of the infectious-inflammatory process in the vagina against the background

Citation: Lazarenko L.M., Babenko L.P., Safronova L.A., Demchenko O.M., Bila V.V., Zaitseva G.M., Spivak M.Ya. Antimicrobial and Immunomodulatory Effect of Probiotic Composition of Bacilli on Bacterial Vaginitis in Mice. *Microbiological journal*. 2023 (3). P. 48—60. <https://doi.org/10.15407/microbiolj85.03.048>

© Publisher PH «Akademperiodyka» of the NAS of Ukraine, 2023. This is an open access article under the CC BY-NC-ND license (<https://creativecommons.org/licenses/by-nc-nd/4.0/>)

of the functional activity of PEM. **Conclusions.** The probiotic composition of *B. subtilis* and *B. megatherium* (UnicaUro, Sirion, Ukraine) is a promising antimicrobial formulation that may be used in the treatment of bacterial vaginitis; however, further studies are required to confirm its therapeutic, antimicrobial, and immunomodulatory efficacy.

Keywords: bacilli, probiotic, vaginitis, immunity, mice.

Infectious-inflammatory diseases of the genitourinary system, induced by pathogenic or conditionally pathogenic microorganisms, are characterized by a high incidence without a clear tendency to decrease. Therefore, they may be a cause of obstetric complications and gynecological disorders. Antibacterial therapy, which is most often used to treat such patients, does not prevent relapse of the disease, because it is one of the reasons for antibiotic resistance and can cause a significant transformation of the genitourinary microbiota against the background of disrupted local immunity. Thus, about 30% of cases of the standard treatment of women with metronidazole or clindamycin face relapse of urogenital infections within a month after the completion of antibiotic therapy [1, 2]. Therefore, alternative methods of treatment are widely used in today's clinical practice, in particular, probiotics based on commensal probiotic cultures (mostly lacto- and bifidobacteria), which can inhibit the growth of pathogens, as well as restore the vaginal microbiota after antimicrobial therapy [2–5].

Normal or abnormal microbiota of the vagina is a complex ecosystem, which may include more than 200 species of bacteria. Their interaction is influenced by genes, various environmental and behavioral factors, the ethnic origin of patients, etc. [4–6]. The commensal microbiota of the vagina, which is mostly represented by lactobacilli, is crucial for protection against the translocation of microbiota from other biotopes, as well as conditionally pathogenic and pathogenic microorganisms that can be transmitted sexually. The change in the qualitative and quantitative composition of the vaginal microbiota is clinically manifested by such nosological forms of the disease as bacterial vaginosis, vaginal candidiasis, as well as vaginitis

with a predominance of aerobic microorganisms that can manifest pathogenic properties [4, 7]. The results of some studies revealed a clear link between the use of antibiotic therapy along with probiotics containing mainly lactobacilli and the restoration of the vaginal microbiota leading to a decrease in the risk of urogenital infection relapse. Probiotics can significantly reduce the risk of preterm birth by preventing the development of bacterial vaginitis [2].

Effective therapeutic and prophylactic probiotics for medical treatment also include aerobic spore-forming safe bacteria of the genus *Bacillus*, mainly species *B. subtilis*, *B. clausii*, *B. coagulans*, *B. licheniformis*, *B. amyloliquefaciens* [8] (except for *B. cereus* and *B. anthracis*). Due to the ability to form spores, bacteria of this genus are resistant to aggressive environmental factors; therefore, they are widespread in nature (in soil, air, and water). They can be found in the food and body of humans and animals [9, 10].

Their specific feature is a strain-related multifactorial biological effect, specifically (i) marked antagonism to a wide range of pathogenic and conditionally pathogenic microorganisms, pathogens of infectious diseases of humans and animals, (ii) the ability to synthesize compounds different in the nature and mechanism of their antimicrobial activity, as well as enzymes, amino acids, polysaccharides, vitamins, etc. [8, 11], and (iii) immunomodulatory properties [10]. Therefore, bacteria of the genus *Bacillus* have been used as probiotics for more than 55 years and remain one of the most promising groups of microorganisms for the development of new therapeutic and prophylactic antimicrobial drugs for clinical and veterinary practice. Interest in bacilli in the composition of probiotics has grown significantly over the past 15 years,

as scientific data demonstrate their therapeutic efficacy in the treatment of various diseases of the gastrointestinal tract, disruption in the immune system, metabolism, etc. [12]. The search for such promising probiotic bacteria strains of the genus *Bacillus* is an urgent task in microbiology. The development of new probiotics based on these bacteria is important since the efficacy of probiotics available in the market may decrease due to mutations of the strains of bacilli which they contain, or mutations of the major pathogenic strains, pathogens of infectious and inflammatory diseases [13].

Noticeably that the therapeutic action of probiotics based on bacteria of the genus *Bacillus* for infectious and inflammatory diseases of the genitourinary system has not been enough studied. It was found that some probiotics containing bacteria of the genus *Bacillus* had an antimicrobial effect on uropathogenic microorganisms, effectively prevented relapse of vaginal infections, and facilitated their clinical course, particularly by reducing heartburn, micturition, itching, soreness, etc. [14–19].

Given the above, **the purpose** of our study was to establish an antimicrobial and immunomodulatory probiotic composition based on strains of *B. subtilis* and *B. megatherium* (UnicaUro, Sirion (Ukraine)) for experimental bacterial vaginitis through studying its effect on the vaginal microbiota spectrum and indicators of the body immunoreactivity.

Materials and methods. The object of the study was a probiotic composition comprising strains of *B. subtilis* and *B. megatherium* bacteria of the genus *Bacillus* (UnicaUro, Sirion (Ukraine)). To simulate bacterial vaginitis, we used *Staphylococcus aureus* strain B-918 (ATCC 6538) deposited in the Ukrainian Collection of Microorganisms of the Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine (IMV).

Experimental studies were conducted on female BALB/c line mice aged 6–8 weeks and

weighing 17–20 g. The animals were kept in the vivarium of the IMV in standard conditions in plastic cages in a separate room at an air temperature of 22–25 °C. They received a full meal and had free access to auto-drinkers. Before the experiment, the animals were kept in quarantine for at least two weeks. All experimental studies were carried out taking into account the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, dated 18.03.1986 (Strasbourg) and the Law of Ukraine No. 3447-IV «On the Protection of Animals from Cruelty» [20, 21].

Before the experiment, the mice were randomly divided into 3 groups, each of 5 individuals. Staphylococcal infection was simulated by injecting a daily culture of *S. aureus* strain B-918 (ATCC 6538) in 0.15 M NaCl into the vagina of mice at a dose of 25 µL (5×10^7 cells per animal). 24 h later, a probiotic composition was injected into the vagina of mice at a dose of 25 µL (1×10^6 cells per animal) once a day for 7 days. Individual comparison groups included infected and intact mice that were injected with 25 µL of 0.15 M NaCl only.

Thus, the following groups of animals were formed:

1. Intact mice, with 0.15 M NaCl injected into the vagina.
2. Mice infected with *S. aureus* strain B-918 (ATCC 6538); 0.15 M NaCl was injected into the vagina (control).
3. Mice infected with *S. aureus* strain B-918 (ATCC 6538); the probiotic composition was injected into the vagina.

On the 3rd, 7th, and 10th days after injection of the probiotic composition, vaginal discharge was obtained from live mice of all comparison groups with the aid of sterile uniform cotton swabs, which then were placed in individual sterile Eppendorf-type tubes with 1 mL of 0.15 M NaCl and suspended. The obtained suspension was adjusted to a concentration of 10^{-5} and

10^{-7} in a series of ten-fold dilutions with 0.15 M NaCl, and 100 μ L was sampled for inoculation of solid selective nutrient media for cultivation of various groups of microorganisms.

Aliquot inoculation was performed in the following selective nutrient media: (i) meat-peptone agar (MPA) for aerobic and facultatively anaerobic bacteria, (ii) BAIRD-PARKER-Agar (Merck, Germany) for *Staphylococcus* spp., (iii) KF-Streptococcus agar (Merck, Germany) for *Streptococcus* spp., (iv) MRSA (MRSA, HiMedia, India) for *Lactobacillus* spp., (v) Bifidum agar (HiMedia, India) for *Bifidobacterium* spp., (vi) Endo (HiMedia, India) for coliform bacteria, (vii) Sabouraud agar (HiMedia, India) for microscopic fungi, and (viii) Pseudomonas agar (HiMedia, India) for *Pseudomonas* spp. [22–24]. After 24 h of incubation, we recorded preliminary data, and after 48 h, — the final ones. The data were expressed as colony-forming units (CFU) in 1 mL of a tested sample. The final result was the number of colonies in a single Petri dish. The criterion for the culture purity was the presence of morphologically homogeneous cells (stained according to Gram) in the microscope field.

On the 14th day of the experiment, peripheral blood was taken from the tail vein of all live mice, from which serum was extracted. Serum antibody titer was determined by the bacterial agglutination reaction. Then on the same day, mice of all comparison groups were killed by decapitation after complete anesthesia with chloroform, and peritoneal exudate was obtained. The phagocytic activity and oxygen-dependent bactericidal activity of peritoneal exudate macrophages (PEM) were evaluated using generally accepted methods [25]. The percentage of cells (phagocytic index (PI)) that phagocytized latex and the average number of latex particles that were absorbed by a single macrophage (phagocytic number (PN)) were determined. Oxygen-dependent bactericidal activity of PEM was assessed by the indicators of spontaneous and stimulated cytochemical tests of nitro-blue tet-

razolium recovery (NBT tests). In the field of view of the microscope, the percentage of cells containing dark blue granules of diformazan per 100 phagocytes was calculated. Functional reserve (FR) was determined by the difference between the indicators of stimulated and spontaneous tests (in%).

All the data were processed by the method of variation statistics using the Epi Info computer software (version 8.0) and Excel of Microsoft Office (versions 2007 and 2010). The data were presented as the arithmetic mean and standard error ($M \pm m$). The null hypothesis for the comparison groups was tested using nonparametric Wilcoxon-Mahn-Whitney (U) and Kolmogorov-Smirnov criteria. Differences between groups were considered statistically significant at $p < 0.05$.

Results. The spectrum of the vaginal microbiota of intact mice was represented by bacteria of genera *Staphylococcus*, *Streptococcus*, *Lactobacillus*, and *Bifidobacterium*, as well as representatives of the *Enterobacteriaceae* family and microscopic fungi (Fig. 1). Representatives of the genus *Pseudomonas* in the vagina of intact mice have not been identified. After infection of mice with *S. aureus* strain B-918 (ATCC 6538), starting from the 1st day and up to 10 days, we found the following clinical manifestations of the infectious-inflammatory process: a significant increase in the number of whitish mucous secretions from the vagina, an increase in body temperature, weakness, a decrease in appetite, etc. From the vagina of infected mice, aerobic and facultative anaerobic microorganisms were seeded in the MPA medium in an amount significantly greater than from the vagina of intact mice throughout the observation period (Fig. 1). At the same time, from the 3rd to 10th day of observation, a significant increase in the number of representatives of the genera *Staphylococcus* and *Streptococcus* was also identified in the vagina of these mice. The change in the number of representatives of the genus *Lactobacillus* was

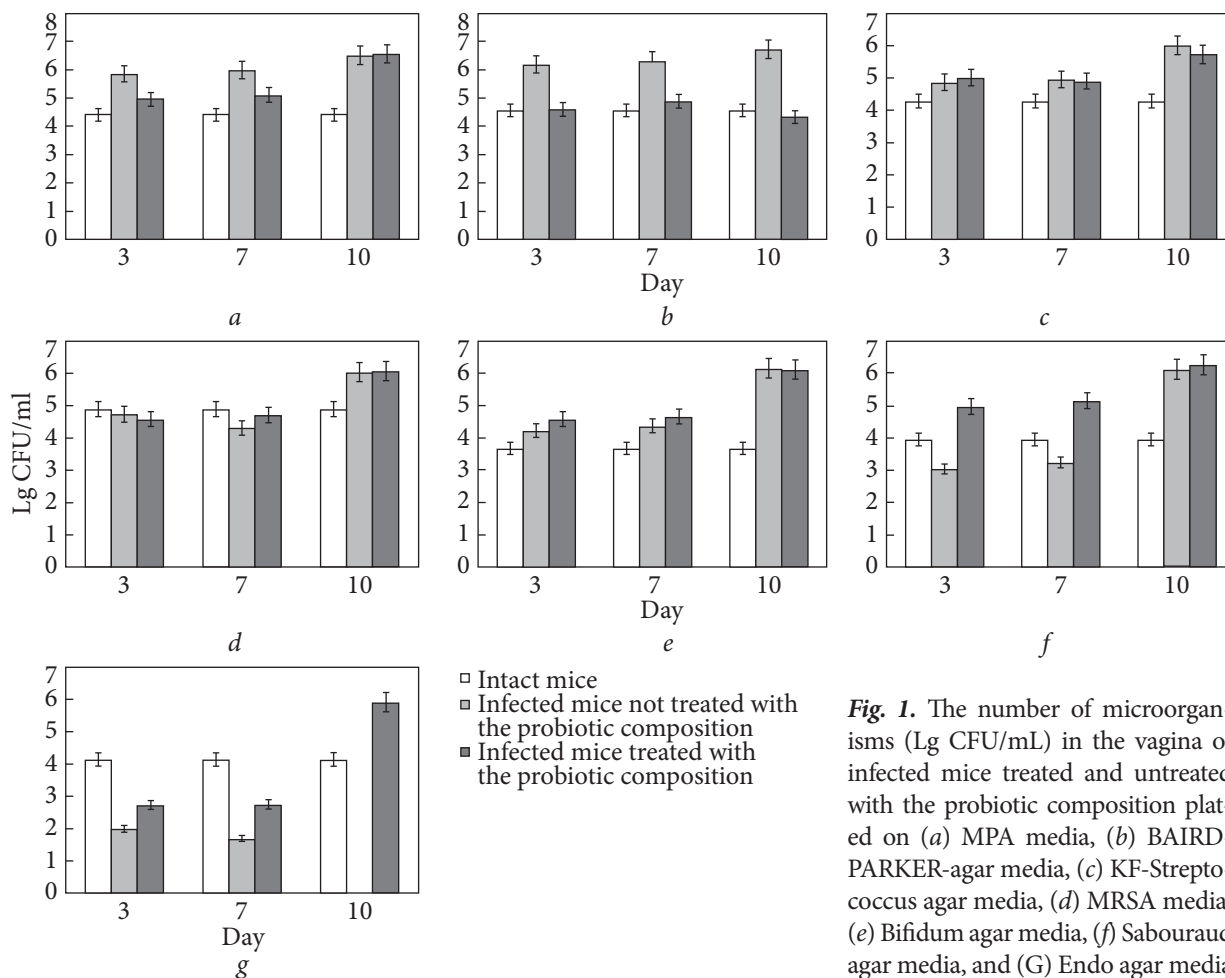


Fig. 1. The number of microorganisms (Lg CFU/mL) in the vagina of infected mice treated and untreated with the probiotic composition plated on (a) MPA media, (b) BAIRD-PARKER-agar media, (c) KF-Streptococcus agar media, (d) MRSA media, (e) Bifidum agar media, (f) Sabouraud agar media, and (g) Endo agar media

dynamic: on the 7th day of the observation, it decreased, while on the 10th day increased compared to intact mice, which presumably reflects the development of the infectious-inflammatory process (Fig. 1). In the vagina of infected mice, the number of representatives of the genus *Bifidobacterium* increased throughout the observation period, especially by the 10th day.

The number of microscopic fungi in the mice vagina decreased by the 3rd and 7th days; however, it significantly increased by the 10th day, compared to the indicators for intact mice (Fig. 1). From the vagina of infected mice, coliform bacteria were seeded in much smaller numbers than from the vagina of intact mice, while representatives of the genus *Pseudomonas* were not

identified at all. That is, the vaginal infection of mice with *S. aureus* B-918 (ATCC 6538) led to the appearance of external clinical signs of bacterial vaginitis and changes in the vaginal microbiota, associated primarily with an increase in the number of representatives of genera *Staphylococcus* and *Streptococcus*, microscopic fungi, and a decrease in the number of representatives of the genus *Lactobacillus* in different observation periods.

After the application of the probiotic composition to infected mice, the external clinical manifestations of bacterial vaginitis gradually disappeared and were not observed at all from the 7th to 10th day. On the 3rd and 7th days, significantly fewer aerobic and optionally anaerobic

microorganisms were seeded from the vagina of mice of this comparison group compared to the control group but more compared to intact mice. We should mention that under the effect of the probiotic composition, on the 10th day of observation, the number of these microorganisms in the vagina of infected mice increased significantly compared to intact mice (Fig. 1). It was shown that the increase in the number of aerobic and facultative anaerobic microorganisms in this group was associated with the appearance of the colony characteristic of bacteria of the genus *Bacillus* in about 50% of cases.

These bacteria were usually large, different in shape, rough, and white-grey, and they grew into agar without forming mucus. Under the microscope, the microorganisms of these colonies looked like gram-positive bacilli, a substantial part of which had an endospore at one end, making the bacterium bulge, which is a characteristic morphological feature of bacilli. As shown in Fig. 1, in the vagina of mice of this group, no representatives of the genus *Pseudomonas* were identified.

Application of the probiotic composition to infected mice resulted in increasing the number of microorganisms seeded in the KF-Streptococcus agar and Sabouraud media compared to intact mice in the whole observation period (Fig. 1). On the 3rd and 7th days, the number of microorganisms that grew in Sabouraud medium was greater even compared to the control mice. However, the morphological features of the colonies formed in the KF-Streptococcus agar and Sabouraud medium were characteristic for representatives of the genus *Bacillus*, which was also confirmed by microscopic studies. Application of the probiotic composition to infected mice resulted in a significant increase in the number of microorganisms from the vagina seeded in the Endo medium compared to both intact mice and control mice throughout the observation period, especially on the 10th day (Fig. 1). However, in 50% of cases, we also found morphological signs

of the colony, characteristic of representatives of the genus *Bacillus*, which was confirmed by the microscopic study. The number of representatives of the genus *Lactobacillus* from the vagina of infected mice of this group did not change compared to intact mice on the 3rd and 7th days of observation but increased significantly on the 10th day. At the same time, the application of the probiotic composition to infected mice led to an increase in the number of representatives of the genus *Bifidobacterium* found throughout the observation period, especially on the 10th day, compared even to the indicators for intact mice (Fig. 1). On the contrary, the number of representatives of the genus *Staphylococcus* seeded from the vagina of infected mice treated with the probiotic composition was significantly smaller than that from the control mice group throughout the observation period, especially on the 10th day (Figs. 1, 2).

A gradual decrease in the staphylococcal load in the vagina of infected mice treated with the probiotic composition was also accompanied by a decrease in the titer of antibodies to staphylococcus in the serum, which confirms the accelerated slowdown of the infectious process under its influence. Thus, on the 7th day, in the blood serum (1:10 dilution) of infected mice of all comparison groups, we found antibodies to *S. aureus* strain B-918 (ATCC 6538).

In the same observation period, when serum was diluted 1:100, antibodies were present in all infected control mice and 66% of mice infected and treated with the probiotic composition. On the 14th day, antibodies to *S. aureus* strain B-918 (ATCC 6538) were detected in 66% of cases in mice of these two groups for the serum dilution of 1:10. For the 1:100 serum dilution, antibodies were present in 66% of the infected control mice but disappeared in the infected mice treated with the probiotic composition.

Immunological studies were conducted to find out if there is a relationship between a decrease in the titer of antibodies to *S. aureus* strain

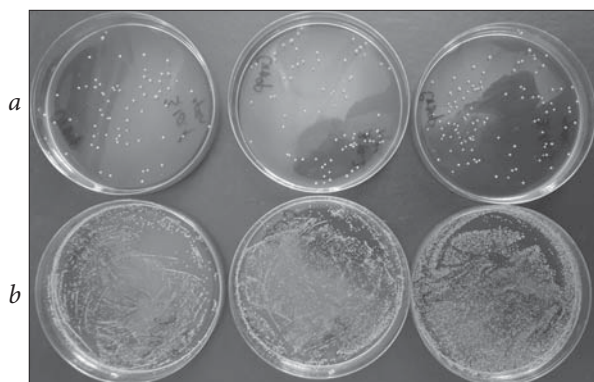


Fig. 2. Microbiological studies of excretions from the vagina of mice of different comparison groups (on the 10th day of observation): *a* — colonies of staphylococcus from the vagina of three infected mice treated with the probiotic composition (concentration 4.34 ± 0.04 lg CFU/mL); *b* — colonies of staphylococcus from the vagina of three infected mice not treated with the probiotic composition (control, concentration 6.73 ± 0.04 Lg CFU/mL)

B-918 (ATCC 6538) in serum and some factors of innate immunity involved in the mechanism of protection against staphylococcus. Given in Table 1 are the data on PEM functional activity in infected mice treated with the probiotic composition.

We found a decrease in the PI of PEM of infected mice that were not treated with the probiotic composition, compared to intact mice. The PN of PEM of infected mice of this group was maintained at the control level. At the same

time, vaginal infection of mice with staphylococcus did not lead to a significant change in the oxygen-dependent bactericidal activity of PEM in terms of the spontaneous NBT test. In response to additional stimulation of PEM in mice of this group, the comparisons did not correspond to an increase in their activity in the NBT test. Therefore, the indicators of the stimulated NBT test were lower than those of the spontaneous NBT test, which indicates the depletion of the reserve capabilities of phagocytes. Application of the probiotic composition to infected mice led to an increase in PI PEM compared to infected mice of the control group but did not exceed the indicators of intact mice. As shown in Table 2, the PN of PEM obtained from mice of this group did not change compared to the control and intact mice. As evidenced by the results of the NBT test, there was observed normalization of oxygen-dependent bactericidal activity of PEM of infected mice treated with the probiotic composition.

Thus, the data obtained by us show that the probiotic composition of *B. subtilis* and *B. megatherium* has a therapeutic action on bacterial vaginitis in mice, which was confirmed by the gradual disappearance of the external clinical signs of the infectious-inflammatory process in the vagina against the background of the normalization of the microbiota composition and activation of innate immunity factors.

Table 1. Indicators of functional activity of macrophages of the peritoneal cavity of infected mice treated with the probiotic composition

Group of mice	Activity indicators				
	PI (%)	PN (standard units)	NBT test spontaneous (%)	NBT test stimulated (%)	FR (%)
Intact	28.2 ± 7.5	2.6 ± 0.7	26.5 ± 6.8	$43.1 \pm 2.6^{**}$	16.0 ± 3.1
Infected and treated with 0.15 M NaCl	$12.1 \pm 4.4^{*\wedge}$	2.7 ± 0.8	21.1 ± 9.3	14.2 ± 2.9	0
Infected and treated with the probiotic composition	$36.2 \pm 13.8^*$	2.8 ± 0.7	38.8 ± 9.8	$48.2 \pm 5.9^{**}$	10.0 ± 6.2

Note: *compared to intact mice; \wedge compared to infected mice treated with the probiotic composition; **compared to the indicators of spontaneous NBT test.

Discussion. It has been found that probiotic therapy can be applied along with various antimicrobials or as an alternative treatment method for patients with urinary infections of various age groups [2–4]. In our view, the use of probiotic strains of bacilli is beneficial for the treatment of patients of this category. First of all, the probiotic strains are more powerful in terms of antibacterial properties and capable of spore formation, as a result of which they can remain longer in a certain epitope. They also have a transient effect, as bacilli are not representatives of the obligate microbiota of many biotopes, including biotopes of the vagina.

In an experimental model of vaginitis in mice induced with *S. aureus* strain B-918 (ATCC 6538), we established the therapeutic effect of the new probiotic composition consisting of two strains of bacilli, namely *B. subtilis* and *B. megatherium* (UnicaUro, Sirion (Ukraine)). Note that we have used this model of staphylococcal vaginitis in mice to effectively select probiotics based on probiotic strains of lacto- and bifidobacteria with a multifactorial mechanism of action [26]. To model bacterial vaginitis in mice, *S. aureus* strain B-918 (ATCC 6538) with proven hemolytic properties and a high ability to form biofilms was chosen [27]. Its genome contains genes for staphylococcal leukotoxin and staphylococcal enterotoxin [28]. It should be noted that in the presence of some antibiotics, the ability of *S. aureus* strain ATCC 6538 to form biofilms even increased [29], so it is a good model for testing the effect of antimicrobial drugs on biofilm formation [30, 31]. The formation of a polymicrobial biofilm in the vagina is known [29] to protect bacteria from lactic acid, H_2O_2 , bacteriocins, and antibiotics, as well as resistance to the immune defense factors (for example, phagocytic system cells); therefore, it can be one of the reasons for the high frequency of relapses of infectious and inflammatory diseases. The formation of bacterial vaginitis in mice infected with *S. aureus* strain B-918 (ATCC 6538) was

evidenced by the appearance of external clinical manifestations of the infectious-inflammatory process against the background of an increase in the number of aerobic and facultatively anaerobic microorganisms, including representatives of the genera *Staphylococcus* and *Streptococcus*, and microscopic fungi and a decrease in the number of lactobacilli in different observation periods. The therapeutic action of the probiotic composition under study was confirmed by the gradual cessation of external clinical signs of the infectious-inflammatory process in the vagina along with the dynamic normalization of its microbiota, primarily due to a decrease in the number of representatives of the genus *Staphylococcus* and an increase in the number of representatives of the genera *Lactobacillus* and *Bifidobacterium*.

It should be noted that scarce scientific literature has been published on experimental studies and clinical observations regarding the effectiveness of spore probiotics in the treatment of urinary tract infections. In particular, it was shown that the elimination of uropathogenic microorganisms from the vagina of patients with nosocomial urinary tract infections significantly increased under the action of Bactisporin formulation of probiotic strain *B. subtilis* [17]. The use of another probiotic strain, *B. coagulans* UniqueIS-2 (Unique Biotech Limited, India), along with antibiotic therapy in the treatment of patients with bacterial vaginosis led to a decrease in the disease relapse and the disappearance of concomitant symptoms such as discharge, burning, urination, itching, and soreness [18]. The probiotic strains *B. subtilis* KATMIRA 1933 and *B. amyloliquefaciens* B-1895 as well as their cell-free supernatants demonstrated antimicrobial efficacy against the biofilm-forming uropathogenic *Proteus mirabilis*, which is one of the most common causative agents of the urinary tract infections [19].

We have not established the mechanisms of therapeutic action, including the antibacterial

action of the strains *B. subtilis* and *B. megatherium*, which compose the probiotic composition under study. However, it has long been known that the antimicrobial efficacy of bacilli against uropathogenic microorganisms is associated with their ability to produce metabolites, primarily bacteriocins [17, 19, 32–35]. For example, Subtilosin A, which is produced by *B. amyloliquefaciens* and *B. subtilis*, effectively inhibited the growth of *Gardnerella vaginalis*, the causative agent of bacterial vaginosis and other human uropathogens through the induction of cells of transitional pores in the cytoplasmic membrane, which led to the leakage of intracellular ions and ATP and cell death [33, 36–38]. The growth of uropathogens such as *Escherichia coli*, *Serratia marcescens*, *Enterobacter cloacae*, *P. mirabilis*, *Citrobacter freundii*, and *Enterococcus faecalis* was effectively suppressed under the effect of Iturin A lipopeptides (LPs) C14 and C15, which are potent biosurfactants synthesized by the *B. subtilis* 11a strain [34]. The cell-free supernatant of the *B. subtilis* strain R-18 also has an antimicrobial effect on *S. marcescens*, which causes various nosocomial infections (mostly urinary tract infections) and shows increased resistance to conventional antibiotics [35]. Metabolites of *B. subtilis* have been shown to reduce the resistance of uropathogenic microflora to antibiotics [17]. Lactosporin *B. coagulans* ATCC 7050 [32] and Subtilosin bacilli in the composition of vaginal hydrogel [39] have been used for the treatment of patients with bacterial vaginosis.

At the same time, in this study, we showed that the therapeutic action of the spore probiotic composition based on *B. subtilis* and *B. megatherium* strains, can be associated not only with their antimicrobial properties but also with the effect on some indicators of innate immunity. In particular, the application of this spore probiotic composition to mice with bacterial vaginitis led to partial activation of the absorbing activity of PEM and elevated indicators of oxygen-dependent bactericidal activity according to the

indicators of the spontaneous NBT test of PEM and the restoration of their FR. A decrease in the number of representatives of the genus *Staphylococcus* spp. in the vagina of mice with vaginitis treated with the probiotic composition was accompanied by a decrease in the titer of antibodies to staphylococcus in the blood serum. However, further studies are required to establish the immunomodulatory properties of *B. subtilis* and *B. megatherium* strains. In particular, the immunomodulatory properties of other bacilli strains have been confirmed by their ability to balance the immune response by affecting both the activity of macrophages and the production of cytokines that determine the development of T helper cells of the Th1 and Th2 types, which prevents the development of chronic inflammation and promotes long-term positive effects on the host's health [10].

Previously, we have established that the strains of bacilli included in this probiotic composition have strain-dependent antimicrobial effectiveness against some museum strains of opportunistic and pathogenic microorganisms (*Proteus vulgaris* 72, *Candida albicans* 690, *S. aureus* 209, and *Salmonella typhimurium* 11). Insignificant zones of growth retardation were also observed for *Escherichia coli* 028 and *Shigella flexneri* GISK 337, which were cultivated with these strains of bacilli [35]. Therefore, the antimicrobial effect of this probiotic composition in bacterial vaginitis can be related both to the antagonistic activity of the strains of bacilli, and to their immunomodulatory effectiveness. In our opinion, the basis of the therapeutic effect of bacilli for bacterial vaginitis lies in their powerful antagonistic activity against staphylococcus and other opportunistic microorganisms, which leads to limiting the growth and number of the latter in the vagina and, thus, has a therapeutic effect, including normalizing the state of the immune system. At the same time, to prove the presence of immunomodulatory efficiency in these strains of bacilli, it is necessary

to conduct additional studies, which will necessarily include *in vitro* experiments.

In addition, bacilli that are not representatives of the normal microbiota of the vagina should gradually be eliminated from this biotope. Therefore, in further studies of the effectiveness of these strains of bacilli in vaginitis, we plan to determine the term of their complete disappearance from the vagina. But it is known that probiotics from bacilli are self-eliminating antagonists; 10–14–30 days after the last injection, they are not registered in the macroorganism [41–43].

Conclusions. Thus, the results of our study suggest that the spore probiotic composition

of two strains of bacilli, namely *B. subtilis* and *B. megatherium* (UnicaUro, Sirion (Ukraine)) may be effective in the treatment of bacterial vaginitis. However, further preclinical and clinical studies are required to confirm its therapeutic, antimicrobial, and immunomodulatory actions. It should be noted that contraindications for the application of probiotics based on bacilli for the treatment of women with urinary tract infections must be clearly defined, since different bacteria and their associations may be involved in the development of other pathological conditions, in particular, pre-tumor diseases of the anogenital region [44].

REFERENCES

1. Polatti F. Bacterial vaginosis, *Atopobium vaginae* and nifuratel. *Curr Clin Pharmacol*. 2012; 7(1):36–40. doi: 10.2174/157488412799218824
2. Li X, Wang Q, Hu X, Liu W. Current status of probiotics as supplements in the prevention and treatment of infectious diseases. *Front Cell Infect Microbiol*. 2022; 12:789063. doi: 10.3389/fcimb.2022.789063
3. Homayouni A, Bastani P, Ziyadi S, Mohammad-Alizadeh-Charandabi S, Ghalibaf M, Mortazavian AM, et al. Effects of probiotics on the recurrence of bacterial vaginosis: a review. *J Low Genit Tract Dis*. 2014; 18(1):79–86. doi: 10.1097/LGT.0b013e31829156ec
4. Mendling W. Vaginal microbiota. *Adv Exp Med Biol*. 2016; 902:83–93. doi: 10.1007/978-3-319-31248-4_6.
5. Roth RS, Liden M, Huttner A. Theurobiome in men and women: a clinical review. *Clin Microbiol Infect*. 2022; S1198-743X(22)00426-8. doi: 10.1016/j.cmi.2022.08.010
6. France M, Alizadeh M, Brown S, Ma B, Ravel J. Towards a deeper understanding of the vaginal microbiota. *Nat Microbiol*. 2022; 7(3):367–78. doi: 10.1038/s41564-022-01083-2
7. Severgnini M, Morselli S, Camboni T, Ceccarani C, Salvo M, Zagonari S, et al. *Gardnerella vaginalis* clades in pregnancy: New insights into the interactions with the vaginal microbiome. *PLoS ONE*. 2022; 17(6): e0269590. <https://doi.org/10.1371/journal.pone.0269590>
8. Cutting S. *Bacillus* probiotics. *Food Microbiology*. 2011; 28(2):214–20. doi: 10.1016/j.fm.2010.03.007
9. Sorokulova I. Modern status and perspectives of *Bacillus* bacteria as probiotics. *J Prob Health*. 2013; 1(4):1000–106.
10. Ghelardi E, Abreu Y, Abreu AT, Marzet CB, Álvarez Calatayud G, Perez M. 3rd Moschione Castro AP. Current progress and Future Perspectives on the Use of *Bacillus* clausii. *Microorganisms*. 2022 Jun 17;10(6):1246. doi: 10.3390/microorganisms10061246
11. Jeżewska-Frąckowiak J, Seroczyńska K, Banaszczyk J, Jedrzejczak G, Żylicz-Stachula A, Skowron PM. The promises and risks of probiotic *Bacillus* species. *Acta Biochim Pol*. 2018; 65(4):509–19. doi: 10.18388/abp.2018_2652
12. Safronova LA, Ilyash VM. [Biosynthetic activity of bacilli, which determines their probiotic effect]. *Mikrobiol Z*. 2017; 79(6):120–36. Russian.
13. Milian VO, Harhota MA, Nechipurenko OO. Study of probiotic properties of strains of *Bacillus* sp. 1.1. and *B. amyloliquefaciens* UKM B-5113. *ScienceRise*. 2014; 5(5(1)):15. doi:10.15587/2313-8416.2014.32023.
14. Tsimaris P, Giannouli A, Tzouma C, Athanasopoulos N, Creatsas G, Deligeoroglou E. Alleviation of vulvovaginitis symptoms: can probiotics lead the treatment plan? *Benef Microbes*. 2019;10(8):867–72. doi: 10.3920/BM2019.0048
15. Ratna Sudha M, Yelikal KA, Deshpande S. Clinical study of *Bacillus coagulans* Unique IS-2 (ATCC PTA-11748) in the treatment of patients with bacterial vaginosis. *Indian J Microbiol*. 2012; 52(3):396–99. doi: 10.1007/s12088-011-0233-z

16. Pushkarev AM, Tuĭgunova VG, Zaĭnullin RR, Kuznetsova TN, Gabidullin Iu Z. Use of antagonistic *Bacillus subtilis* bacteria for treatment of nosocomial urinary tract infections. *Zh Mikrobiol Epidemiol Immunobiol.* 2007; (2):90—3.
17. Ingersoll MA, Albert ML. From infection to immunotherapy: host immune responses to bacteria at the bladder mucosa. *Mucosal Immunol.* 2013; 6(6):1041—53. doi: 10.1038/mi.2013.72.
18. Algburi A, Alazzawi SA, Al-Ezzy AIA, Weeks R, Chistyakov V, Chikindas ML. Potential probiotics *Bacillus subtilis* KATMIRA1933 and *Bacillus amyloliquefaciens* B-1895 co-aggregate with clinical isolates of *Proteus mirabilis* and prevent biofilm formation. *Probiotics Antimicrob Proteins.* 2020; 12(4):1471—83. doi: 10.1007/s12602-020-09631-0
19. Li S, Wang Y, Feng L, Yu Z, Qiu M, Wang Y, et al. *Bacillus subtilis* ameliorates *Escherichia coli*-induced endometritis in mice via maintaining endometrial barrier and inhibiting inflammatory response. *Microb Pathog.* 2022; 166:105487. doi: 10.1016/j.micpath.2022.105487
20. Reznikov O. [Problems of ethics during experimental medical and biological studies on animals]. *Bulletin of NASU.* 2001; 1:5—7. Russian.
21. [Laboratory animals: breeding, maintenance, use in the experiment]. I.P. Zapadnyuk, editor. K.: Vishcha school, 1983. Russian.
22. Egorov NS. [Guide to practical exercises in microbiology]. Moscow: Publishing House of Moscow State University. 1999. Russian.
23. Lebedev KA, Ponyakina ID. [Immunogram in clinical practice]. Nauka. 1990. Russian.
24. Brown DF, Perry SF, Methods used in the United Kingdom for the culture of microorganism. *J Clin Pathol.* 1999; 45:468—74. doi: 10.1136/jcp.45.6.468
25. Spivak NYa, Lazarenko LN, Mikhailenko ON. Interferon and the system of mononuclear phagocytes Kyiv: Phytosociocenter, 2002. Russian.
26. Babenko, L.P., Lazarenko, L.M., Bubnov, R.V., Spivak, M.J. Prophylactic effect of lactobacilli and bifidobacteria probiotic strains on experimental bacterial vaginitis. *Biosystems Diversity.* 2019; 27(2):170—76. doi 10.15421/011923
27. Zhou G, Peng H, Wang YS, Huang XM, Xie XB, Shi QS. Enhanced synergistic effects of xylitol and isothiazolones for inhibition of initial biofilm formation by *Pseudomonas aeruginosa* ATCC 9027 and *Staphylococcus aureus* ATCC 6538. *J Oral Sci.* 2019; 61(2):255—263. doi: 10.2334/josnusd.18-0102
28. Makarova O, Johnston P, Walther B, Rolff J, Roesler U. Complete Genome Sequence of the Disinfectant Susceptibility Testing Reference Strain *Staphylococcus aureus* subsp. *aureus* ATCC 6538 Genome Announc. 2017; 5(19): e00293—17. doi: 10.1128/genomeA.00293-17
29. Berlutti F, Frioni A, Natalizi T, Pantanella F, Valenti P. Influence of sub-inhibitory antibiotics and flow condition on *Staphylococcus aureus* ATCC 6538 biofilm development and biofilm growth rate: BioTimer assay as a study model. *J Antibiot (Tokyo).* 2014; 67(11):763—9. doi: 10.1038/ja.2014.66.
30. Ceresa C, Hutton S, Lajarin-Cuesta M, Heaton R, Hargreaves I, Fracchia L, et al. Production of mannosyl-erythritol lipids (mels) to be used as antimicrobial agents against *S. aureus* ATCC 6538. *Curr Microbiol.* 2020; 77(8):1373—1380. doi: 10.1007/s00284-020-01927-2
31. Mathys J, Redelinguys, Janri Geldenhuys, Hyunsul Jung, Marleen M. Kock Bacterial Vaginosis: Current Diagnostic Avenues and Future Opportunities. *Front Cell Infect Microbiol.* 2020; 10:354. doi: 10.3389/fcimb.2020.00354
32. Riaz S, Dover SE, Chikindas ML. Mode of action and safety of lactosporin, a novel antimicrobial protein produced by *Bacillus coagulans* ATCC 7050. *J Appl Microbiol.* 2012; 113(3):714—22. doi: 10.1111/j.1365-2672.2012.05376.x
33. Noll KS, Prichard MN, Khaykin A, Sinko PJ, Chikindas ML. The natural antimicrobial peptide subtilisin acts synergistically with glycerol monolaurate, lauric arginate, and ϵ -poly-L-lysine against bacterial vaginosis-associated pathogens but not human lactobacilli. *Antimicrob Agents Chemother.* 2012; 56(4):1756—61. doi: 10.1128/AAC.05861-11
34. Bernat P, Paraszkiwicz K, Siewiera P, Moryl M, Płaza G, Chojniak J. Lipid composition in a strain of *Bacillus subtilis*, a producer of iturin A lipopeptides that are active against uropathogenic bacteria. *World J Microbiol Biotechnol.* 2016; 32(10):157. doi: 10.1007/s11274-016-2126-0
35. Devi KR, Srinivasan S, Ravi AV. Inhibition of quorum sensing-mediated virulence in *Serratia marcescens* by *Bacillus subtilis* R-18. *Microb Pathog.* 2018; 120:166—75. doi: 10.1016/j.micpath.2018.04.023

36. Babasaki K, Takao T, Shimonishi Y, Kurahashi K. Subtilosin A. A new antibiotic peptide produced by *Bacillus subtilis* 168: isolation, structural analysis, and biogenesis. *J Biochem.* 1985; 98:585—603. doi: 10.1093/oxford-journals.jbchem.a135315
37. Stein T, Düsterhus S, Stroth A, Entian KD. Subtilosin production by two *Bacillus subtilis* subspecies and variance of the sbo-alb cluster. *Appl Environ Microbiol.* 2004; 70:2349—53. doi: 10.1128/AEM.70.4.2349-2353.2004
38. Sutyak KE, Wirawan RE, Aroutcheva AA, Chikindas ML. Isolation of the *Bacillus subtilis* antimicrobial peptide from the dairy product-derived *Bacillus amyloliquefaciens*. *J Appl Microbiol.* 2007;104:1067—74. doi: 10.1111/j.1365-2672.2007.03626.x
39. Sundara Rajan S, Cavera VL, Zhang X, Singh Y, Chikindas ML, Sinko PJ. Polyethylene glycol-based hydrogels for controlled release of the antimicrobial subtilosin for prophylaxis of bacterial vaginosis. *Antimicrob Agents Chemother.* 2014; 58(5):2747—53. doi: 10.1128/AAC.02446-14
40. Babenko LP, Tymoshok NO, Safronova LA, Demchenko OM, Zaitseva GM, Lazarenko LM, et al. Antimicrobial and therapeutic effect of probiotics in cases of experimental purulent wounds. *Biosyst Divers.* 2022; 30(1):29—37. doi: 10.15421/012204
41. Uyeno Y, Shigemori S, Shimosato T. Effect of Probiotics/Prebiotics on Cattle Health and Productivity. *Microbes Environ.* 2015; 30(2):126—132. doi: 10.1264/jsme2.ME14176
42. Cameron A, McAllister TA. Could probiotics be the panacea alternative to the use of antimicrobials in livestock diets? *Benef Microbes.* 2019; 10(7):773—799. doi: 10.3920/BM2019.0059
43. Devyatkin V, Mishurov A, Kolodina E. Probiotic effect of *Bacillus subtilis* B-2998D, B-3057D, and *Bacillus licheniformis* B-2999D complex on sheep and lambs. *J Adv Vet Anim Res.* 2021; 8(1):146—157. doi: 10.5455/javar.2021.h497
44. Chen Y, Qiu X, Wang W, Li D, Wu A, Hong Z, et al. Human papilloma virus infection and cervical intraepithelial neoplasia progression are associated with increased vaginal microbiome diversity in a Chinese cohort. *BMC Infect Dis.* 2020; 20(1):629. doi: 10.1186/s12879-020-05324-9

Received 13.03.2023

Л.М. Лазаренко¹, Л.П. Бабенко¹, Л.А. Сафронова¹,
О.М. Демченко², В.В. Біла², Г.М. Зайцева³, М.Я. Співак¹

¹ Інститут мікробіології і вірусології ім. Д.К. Заболотного НАН України,
вул. Академіка Заболотного, 154, Київ, 03143, Україна

² Київський перинатальний центр,
вул. Предславинська, 8, Київ, 03150, Україна

³ Національний медичний університет ім. О.О. Богомольця,
бульвар Тараса Шевченка, 13, Київ, 01601, Україна

АНТИМІКРОБНА ТА ІМУНОМОДУЛЮВАЛЬНА ДІЯ ПРОБІОТИЧНОЇ КОМПОЗИЦІЇ НА ОСНОВІ БАЦИЛ ЗА БАКТЕРІАЛЬНОГО ВАГІНІТУ В МИШЕЙ

Бактерії роду *Bacillus* є однією з найперспективніших груп мікроорганізмів для розробки лікувально-профілактичних антимікробних препаратів, які можна використовувати у клінічній практиці та ветеринарії. Пробиотики на основі бацил мають терапевтичну і лікувальну ефективність при різних захворюваннях шлунково-кишкового тракту, а також порушенні імунного статусу, обміну речовин тощо. Пошук перспективних пробіотичних штамів бактерій роду *Bacillus* є актуальною проблемою мікробіології. **Метою** роботи було визначення антимікробної та імуномодулювальної дії пробіотичної композиції на основі штамів *Bacillus subtilis* та *B. megatherium* (UnicaUro, Sirion (Україна)) за експериментального бактеріального вагініту. **Методи.** Експериментальні дослідження проводили на самках мишей лінії BALB/c. Для індукції бактеріального вагініту використовували штам *Staphylococcus aureus* B-918 (ATCC 6538), який вводили мишам у піхву перед лікуванням пробіотичними бактеріями. У піхві мишей в різні терміни спостереження ідентифікували аеробні та факультативно анаеробні бактерії, у тому числі представники *Staphylococcus*, *Streptococcus*, *Lactobacillus*, *Bifidobacterium*, *Pseudomonas*, а також коліморфні бактерії та мікроскопічні гриби за допомогою загальноприйнятих мікробіологічних методів дослідження. Титр сироваткових антитіл до *S. aureus* визначали за допомогою реакції бактеріальної аглютинації. Фагоцитарну та кисневозалежну бактерицидну

активність макрофагів перитонеального ексудату (МФПЕ) оцінювали за їхньою здатністю до поглинання латексу та рівнем кисневозалежної бактерицидної активності у тесті відновлення нітросинього тетразолію (НСТ-тест). **Результати.** Про формування бактеріального вагініту в мишей лінії BALB/c, інфікованих *S. aureus* В-918 (АТСС 6538), свідчила поява зовнішніх клінічних проявів інфекційно-запального процесу на тлі збільшення кількості аеробних і факультативно анаеробних мікроорганізмів, у тому числі представників родів *Staphylococcus* і *Streptococcus*, мікроскопічних грибів, а також зменшення кількості лактобактерій у різні терміни спостереження. Введення пробіотичної композиції мишам із бактеріальним вагінітом приводило до динамічної зміни мікробіоти піхви: зменшувалась кількість аеробних і факультативно анаеробних мікроорганізмів, насамперед за рахунок нормалізації кількості представників роду *Staphylococcus*. Поступове зменшення стафілококового навантаження в піхві інфікованих мишей, які отримували пробіотичну композицію, супроводжувалось зниженням титру антитіл до стафілококу у сироватці крові, що підтверджує пришивиджене затухання інфекційного процесу. У результаті проведених досліджень встановлено, що після введення пробіотичної композиції інфікованим мишам нормалізувалась функціональна активність клітин фагоцитарної системи, які відіграють ключову роль у захисті від стафілококової інфекції. Так, зростала інтенсивність фагоцитозу МФПЕ за показниками фагоцитарного індексу, а також відновлювалась до рівня норми їх кисневозалежна бактерицидна активність в НСТ-тесті. **Висновки.** Пробіотична композиція на основі *B. subtilis* та *B. megatherium* (UnicaUro, Sirion (Україна)) мала ефективну лікувальну дію за бактеріального вагініту у мишей, що підтверджувалось поступовим зникненням зовнішніх клінічних ознак інфекційно-запального процесу у піхві та титру антитіл до стафілококу у сироватці крові на тлі нормалізації функціональної активності МФПЕ. Тому ця пробіотична композиція є перспективним антимікробним засобом, який може бути використаний у терапії бактеріального вагініту, але для підтвердження його лікувальної, антимікробної та імуномодулювальної ефективності потрібні подальші дослідження.

Ключові слова: *бацили, пробіотик, вагініт, імунітет, миші.*