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CORRECTION OF VAGINAL DYSBIOSIS CAUSED BY THE BIOFILM FORMING STRAIN OF *STAPHYLOCOCCUS AUREUS*, USING PROBIOTIC BASED ON SPORE MICROORGANISMS

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Changes of microbiocenosis of different biotopes of the human body are known as dysbiosis. For the renovation of the composition of the microbiota, use the bacteriotherapeutic preparations – probiotics. The aim of the research was to study the effectiveness of the use of the mixed suspension of cells of probiotic strains *Bacillus subtilis* and *Bacillus licheniformis* for the correction of the composition of the microbiota of the vagina of white laboratory mice, induced by the intravaginal introduction of a biofilm forming strain of *Staphylococcus aureus*. It was shown that in cases of dysbiosis of genitourinary tract of mice, induced by the intravaginal introduction of a biofilm-forming strain of *S. aureus,* the use of probiotic microorganisms *B. subtilis* and *B. licheniformis* led to the tendency to normalization of the vaginal microbiota. Renovation of vaginal microbiome characterized by the increase of ratio anaerobic to anaerobic bacteria to 1: 71–1: 87 that in fact corresponded to normal index; the decrease of the frequency of determination and quantity of opportunistic bacteria, such as staphylococci and enterobacteria.

Key words: vaginal dysbiosis, microbiota, correction, probiotic, bacilli.

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КОРЕКЦІЯ ДИСБІОЗУ ПІХВИ, ЗУМОВЛЕНОГО ВВЕДЕННЯМ ПЛІВКОУТВОЮЮЧОГО ШТАМУ ЗОЛОТИСТОГО СТАФІЛОКОКУ ІЗ ЗАСТОСУВАННЯМ ПРОБІОТИКІВ НА ОСНОВІ СПОРОВИХ МІКРООРГАНІЗМІВ

Стан порушення мікробіоти, що характеризується поняттям «дисбактеріоз», є доволі актуальною та поширеною проблемою. Для відновлення складу мікробіоти використовують бактеріотерапевтичні препарати на основі мікроорганізмів – пробіотики. Метою роботи було дослідження ефективності використання змішаної суспензії клітин пробіотичних штамів *Bacillus subtilis* та *Bacillus licheniformis* для корекції складу мікробіоти піхви білих лабораторних мишей, індукованого шляхом інтравагінального введення біоплівкотвірного штаму *Staphylococcus aureus*. Показано, що у випадку дисбіозу урогенітального тракту мишей, викликаного інтравагінальним введенням біоплівкотвірного штаму золотистого стафілокока, використання пробіотичних мікроорганізмів *B. subtilis* та *B. licheniformis* сприяло відновленню мікробіоти піхви. Відновлення вагінального мікробіому виражалося у зростанні індексу відношення аероби: анаероби до 1: 71–1: 87 і практично наближалося до показника норми; зниженні частоти виявлення та кількості умовно-патогенних мікроорганізмів, зокрема, стафілококів та ентеробактерій.

Ключові слова: дисбіоз піхви, мікробіота, корекція, пробіотик, бацили.

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The ecological balance of the microbiocenosis of the reproductive system is an important component of human health. Thus, a disorder of the composition of the microbiocenosis of the reproductive system of a woman can lead to pregnancy loss, and can also adversely affect the child's body when passing through the birth ways of the mother [3], therefore the study of the correction of microbiota with using of preparations that are safe for humans and effective against opportunistic microorganisms. It is known that the composition of the indigenous microbiota, including the female reproductive system, formed over a long evolutionary interaction of macro- and microorganisms and today is represented by a number of such microorganisms, mainly lactobacilli, bifidobacteria and others, and temporarily include a number of transient species [3]. Among the latter, opportunistic microorganisms pose a threat, especially those capable to form biofilms, which is associated with an increase of their colonization activity [2, 15]. Among these bacteria, staphylococci are one of the most well-known. Most clinical isolates of it are able to form biofilms [2]. Their penetration into the vaginal microflora system has many significant negative consequences. In this regard, it should be noted that staphylococci quickly become resistant to antibiotics and spread these genes among cells in the microbiome, which becomes especially relevant for polycomponent microbiocenosis of the open cavities of the human body. As part of the latter, microorganisms are often prone to film formation. In a biofilm, staphylococci become significantly more resistant to antibiotics [14], and this situation requires control and treatment, which can be done with antibiotics or probiotics.

The use of bacteriotherapy drugs – probiotics, is considered to be more optimal for suppressing of opportunistic bacteria and for correcting the ecological balance of microbiota [8, 10]. Although for the normalization of the composition of the microbiota of the reproductive system the preparations based on

lactobacilli are considered optimal [8], nevertheless, probiotic preparations based on spore microorganisms of the genus Bacillus, can also be considered promising.

One else prospect of use belongs to natural components for correction of microbiota. Among them can be listed plant essential oils combined with mexidol that realise the significant antimicrobial effect on opportunistic bacteria including Staphylococcus aureus [4].

It is known that bacilli realize high antagonistic activity against many bacteria and able to regulate the composition of the microbiota of various biotopes of the microorganism. The vast majority of the bacilli species are safe to macroorganism. Among different species of bacilli, some strains with high antagonistic activity were selected. This activity is more pronounced and manifests against a much wider spectrum of pathogenic and opportunistic microorganisms than in other representatives of exogenous and endogenous microbiota; they are stable during storage and environmentally safe [13].

Usually, bacilli probiotics are used for the correction of dysbacteriosis of the gastrointestinal tract, however, the high efficiency of these preparations allows one to anticipate their possible use for the correction of the microbiota of other biotopes, in particular, the vagina. Strains of bacilli – component of probiotics – *B. subtilis* and *B. licheniformis*, have a certain degree of kinship with lactic acid bacteria [7]. This provides the basis for the effective use of it in complex with other bacteria for solution of problems associated with bacterial vaginosis [11]. For the study of this issue, experimental modeling is the optimal way. Modeling of the development of dysbacteriosis provides unique opportunities: clearly measure the effect of a particular factor, study the dynamics of development, the particular composition and metabolic properties of bacteria that make up the vaginal microflora, and investigate the effects of various probiotic and other preparations on the model.

The purpose of the study was to access the effectiveness of the use of the mixed suspension of cells of probiotic strains Bacillus subtilis and Bacillus licheniformis for the correction of the composition of the microbiota of the vagina of white laboratory mice, induced by the intravaginal introduction of a biofilm forming strain of Staphylococcus aureus.

Materials and methods. For the study mice model of dysbacteriosis of the genitourinary tract was used. Studied groups of animals included female outbred white laboratory mice: control group (n=10) – animals injected with saline, experimental group 1 (n=10) – animals injected with the suspension of bacilli from "Biosporin" only, experimental group 2 (n=10) – animals, which at the first stage were injected with cells of the film-forming strain of S. aureus (strain 4v), isolated from the mouse, and in the second stage – the suspension obtained from the lyophilic preparation of the probiotic "Biosporin" (Biopharma, Ukraine) was injected.

Animals for the experiments were selected randomly from the total population of female mice aged 20–25 weeks and weighing 20–24 g, which were kept in standard conditions [1]. All research were carried out in accordance with the standards established by the Law of Ukraine No. 3447-IV "On protecting animals from ill-treatment" and the norms adopted to the "European Convention on the Protection of Vertebrate Animals used for Experimental and Scientific Purposes" (1986).

A strain S. aureus 4v used for modeling of dysbiosis of the reproductive tract was isolated from the mouse vagina. Identification was done by use of the ApiStaph test-system (bioMérieux, France). The production of pathogenicity factors were studied using standard techniques [1].

Ability to form a biofilm determined in the immunological tablet. Suspension of cells of the isolated strain was sown in the wells: 50 μl with a cell content of 10^5 CFU/ml. The culture was incubated at 37 ºC for 72 h. The formation of noticeable surface or near-bottom film growth in the well during this period was considered to be a sign of the film formation of the strain.

For modeling of a dysbacteriosis an intravaginal administration of a suspension of cells of strain S. aureus 4v isolated from the mouse was realized. The microorganisms were injected once with a dispenser into the vagina of the animals of experimental group 2 (n=10): 50 μl of cell suspension, containing 1×10^9 CFU/ml. The control group of animals was carried out with the introduction of sterile saline (mass fraction of sodium chloride 0.9 %) – 50 μl. After the introduction of bacteria, the animals were kept in an isolated room under the same conditions as the control group. The signs of vaginal dysbacteriosis were monitored on the 10^{th} day after the addition of the microbial suspension according to the following features: a decrease of the aerobic/anaerobic ratio index, a decrease of the number of lactobacilli and an increase in the number of opportunistic microorganisms.

The isolation and identification of microorganisms was carried out by standard methods [1] in accordance with the signs given in the Bergey's Manual of determinative bacteriology [9].

For the study of the correction efficacy of the microbiota composition in the vagina by bacterial strains from probiotic "Biosporin", animals with dysbacteriosis (experimental group 2 (n=10)) received

intravaginally 50 µl of cell suspension from the preparation "Biosporin", containing 1×10^9 CFU/ml once a day for 5 days.

In parallel, healthy animals of the experimental group 1 (n=10) received only cell suspension with probiotic strains by the same scheme. The suspension was prepared in accordance with the manufacturer's recommendations. Changes in the composition of microbiocenosis were monitored for 25 days after the completion of the probiotic course. Changes in the qualitative and quantitative composition of the microbiota, including the disappearance of bacteria as components of the probiotic or, conversely, the colonization of the vagina of animals by them were evaluated.

Statistical data processing was performed by the program OriginLab 7.5 ($p<0.05$).

Results of the study and their discussion. A strain of staphylococcus was isolated from a randomly selected female mouse from the general group. It was identified as an *S. aureus*. The strain produced plasmocoagulase, lipase, lecithinase, producted hemolysins on the medium with blood (hemolysis zone diameter 5 mm). After 72 h cultivation in the plate, it produced a biofilm deposited on the walls of the three wells inoculated by cell suspension. A 50 µl suspension of daily culture of the strain, with a cell content of 1×10^9 CFU/ml, was administered intravaginally to the animals of the experimental group 2. All animals were included in the experiments at the same time and control studying of the contents of the vagina was done in the same time periods. The composition of microbiota was studied at the qualitative (table 1) and quantitative (table 2) levels.

Table 1

The frequency of detection of bacteria of different genera from the vagina of animals of different experimental groups before and after the use of the probiotic strains of bacilli (abs/%)

Group / Bacteria	Control (use of saline), n=10	Experimental group 1 (use probiotic only), n=10		Experimental group 2 (modeling dysbiosis and use of probiotic), n=10		
		Time of use and $^{\Delta}$time after completion of use				
		$5+5^{\Delta}$	25^{Δ}	Before the probiotic use	$5+5^{\Delta}$	25^{Δ}
		Day from the start of the experiment			Day from the start of the experiment	
		10	30		10	30
Obligate anaerobic bacteria						
Fusobac-terium	7/70	6/60	6/60	7/70	8/80	6/60
Peptococcus	5/50	4/40	5/50	8/80	6/60	6/60
Peptostrepto-coccus	6/60	5/50	5/50	6/60	5/50	5/50
Bacteroides	9/90	9/90	9/90	10/100	8/80	7/70
Lactobacillus	9/90	9/90	10/100	6/60	7/70	9/90
Facultative anaerobic bacteria						
Staphylo-coccus	8/80	6/60	5/50	10/100	9/90	6/60
Streptococcus	7/70	6/60	7/70	7/70	7/70	6/60
Enterococcus	2/20	0/0	0/0	4/40	2/20	1/10
Micrococcus	3/30	1/10	1/10	4/40	2/20	1/10
Bacillus	2/20	10/100	4/40	3/30	10/100	5/50
Enterobacte-riaceae	2/20	1/10	1/10	8/80	7/70	3/30
Gardnerella	0/0	0/0	0/0	3/30	1/10	1/10
Microaerophile bacteria						
Lactobacillus	9/90	8/80	9/90	5/50	7/70	8/80

After intravaginal administration of the probiotic strains of bacilli (mix of B. subtilis and B. licheniformis) to healthy mice, a slight decrease of the detection rate of all bacterial genera, except the bacilli itself, was noted. It was established that the use of probiotic bacilli had almost no effect on the qualitative and quantitative composition of microaerophilic and anaerobic lactobacilli, which are the basic component of the vaginal microbiome. It was a positive indicator of the probiotic action. At the same time, the frequency of detection of obligate-anaerobic bacteria in the experimental group 1 after use of the probiotic cells' suspension was somewhat reduced compared with the control on the 10^{th} and 30^{th} day, but without significant differences compared to the control.

For the bacilli, a tendency to decrease the frequency of their detection and quantity over the course of the experiment was revealed. So, in the control group, the frequency of their detection was 20 %, and in the experimental group 1 on the 10^{th} day was 100 %, after which their elimination on the 30^{th} day to 40 % was observed. The number of these bacteria in the vaginal microbiome of animals from the experimental group 1 remained significantly higher than that of the control group by 3 and 2 times respectively on the 10^{th} and 30^{th} days, although, as with the detection rate, the dynamics of reducing their number were observed.

Table 2

The quantitative composition of bacteria of different genera from the vagina of animals of different experimental groups before and after the introduction of the probiotic strains of bacilli (M±m lg CFU/ml)

Group / Bacteria	Control (use of saline), n=10	Experimental group 1 (use probiotic only), n=10		Experimental group 2 (modeling dysbiosis and use of probiotic), n=10		
		Time of use and $^{\Delta}$time after completion of use				
		5+5$^{\Delta}$	25$^{\Delta}$	Before the probiotic use	5+5$^{\Delta}$	25$^{\Delta}$
		Day from the start of the experiment			Day from the start of the experiment	
		10	30		10	30
Obligate anaerobic bacteria						
Fusobac-terium	4.3±3.1	4.3±3.3	4.4±3.3	4.4±3.1	4.7±3.7×	4.0±3.0×
Peptococcus	4.3±3.2	4.4±3.5	4.8±3.6×	4.3±3.0	4.5±3.7×	4.1±2.7×
Peptostrepto-coccus	4.1±3.6	4.1±3.3	4.0±2.9	4.3±3.3×	4.5±3.4×	4.0±3.5
Bacteroides	4.0±3.6	4.2±3.9×	4.4±3.2×	4.5±3.7	4.6±3.2×	4.1±3.2
Lactobacillus	4.1±3.4	3.9±2.1×	4.2±3.2	4.0±3.0	3.9±2.3×	4.2±3.3
Facultative anaerobic bacteria						
Staphylo-coccus	2.1±0.8	2.5±1.6×	2.3±1.3×	2.6±1.4×	2.4±1.2×	2.2±1.3
Streptococcus	2.6±1.1	2.7±1.2	2.6±1.2	2.7±1.3	2.6±1.3	2.5±1.3
Enterococcus	1.6*	-	-	1.8±0.8	1.3*	1.3*
Micrococcus	1.6±1.0	1.3*	1.6*	1.8±0.7	1.3*	1.8*
Bacillus	1.3*	1.8±0.8	1.6±1.1×	1.8±1.1×	1.7±0.8×	1.6±0.8
Enterobacte-riaceae	1.6*	1.6*	1.3*	2.5±1.6×	1.8±1.1×	1.3±1.0
Gardnerella	–	–	–	1.6±0.8	1.6*	1.3*
Microaerophile bacteria						
Lactobacillus	2.4±1.3	2.3±1.3	2.3±1.6	1.7±0.6×	1.7±0.7×	1.8±0.8×

Notes: * data obtained for 1–2 animals; × identified statistically significant changes compare to control (P<0.05)

It was found that the introduction of the film-forming strain of S. aureus led to changes in the composition of the microbiota on the 10th day in animals of the experimental group 2. It could be assessed as dysbacteriosis, based on the following signs: the ratio of aerobic to anaerobic bacteria was 1: 169 compared to control – 1: 82, the number of anaerobic lactobacilli decreased by 1.6 times and microaerophilic – in 5.0 times. In addition, the number of opportunistic microorganisms, especially enterobacteria, increased by 7.9 times and staphylococci – by 2.0 times.

The use of suspension of probiotic strains of bacilli for animals with experimental dysbiosis led to changes in the composition of the vaginal microbiota. Thus, the active antagonistic action of suspension of B. subtilis and B. licheniformis against opportunistic bacteria leading to a decrease in their number was recorded. Changes in the composition of the microflora showed a tendency to gradually renovation of it to criteria of normal state.

Therefore, on the 10th day, the ratio of aerobes to anaerobes was 1: 71 compared to the stage before the use of probiotic strains of bacilli – 1: 169, and on the 30th day – 1: 87, which almost corresponded to the state of the control group. A decrease in the frequency of detection and the number of opportunistic microorganisms, especially representatives of the family Enterobacteriaceae, was also noted. So, on the 10th day, the number of enterobacteria was slightly higher than the norm – 1.6 times, and on the 30th day, it was 2.0 times lower than the control value. At the same time, there was a steady downward trend in the number of these microorganisms compared with dysbiosis.

The tendency to reduce of the frequency of detection was also noted for staphylococci, however, their number decreased less significantly and even on the 30th day remained higher than the control group by 1.3 times. In addition to these microorganisms, there is also a tendency to decrease in the number of transient microorganisms such as micrococci and enterococci.

For the bacilli – the components of the probiotic preparation, a gradual decrease in the frequency of their detection and quantity was also observed, approaching the normocenosis indices. Therefore, on the 10th day the number of bacilli was higher than the control group by 3.2 times, and on the 30th – by 2.0 times that indicates their gradual elimination.

The composition of the microbiome of the vagina of mice has a composition that is similar to the microbiome of the human reproductive tract, as was shown in one of our previous research [12], thus there is a possibility of extrapolating the data obtained on the effect of probiotics on the possibility of correction of microbiocenosis in humans.

Bacilli are also present among the components of the microbiocenosis, although their number is minimal. However, this allows us to consider the use of probiotics based on spore microorganisms to

correction of the microflora of the reproductive tract. The presence of bacilli also takes place in the microbiocenosis of healthy people, where their number can reach 10^4–10^6 CFU/g for healthy pregnant women and newborns [6]. At the same time, it is noted that these bacteria are transient representatives of microflora. They are not capable of adhesion to the epithelial cells of the body cavities and, over time they are eliminated spontaneously from the macroorganism [7], which is a prerequisite for the selection of such microorganisms for probiotic.

The decrease in the frequency of detection of staphylococci was significant, but even on the 30th day, the indicator remained higher than the control. In comparison with our previous experimental studies [12], the tendency to a decrease in the number of staphylococci was less pronounced, which probably could be due to the fact that the introduced strain of S. aureus has the ability to film formation, and therefore may have enhanced adhesive properties, which enhances its colonization potential [5].

The obtained results confirm the data of different clinical research with the use of mix of probiotic strains B subtilis and B. licheniformis, that this preparation is harmless, well tolerated and has a high clinical efficacy, and also leads to a significant reduction in the number or complete elimination of opportunistic microorganisms such as enterobacteria and staphylococci, as well as *Candida* fungi with the renovation of microbiome [7, 8].

Conclusion

It was shown that dysbiosis modeled by intravaginal administration of the film-forming strain of *S. aureus* 4v was characterized by a decrease of the aerobic to anaerobic ratio to the level of 1: 169 compared to the control – 1: 82, by a decrease of the number of anaerobic and microaerophilic lactobacilli by 1.6 and 5 times respectively, increase of the number of opportunistic microorganisms, especially enterobacteria and staphylococci.

The tendency to normalization of the microflora of the vagina of mice by the use of mixed suspension of probiotic strains B. subtilis and B. licheniformis was shown, which was expressed as an increase of the ratio of aerobes to anaerobes to 1: 71 on the 10th day and 1: 87 on the 30th day of the experiments and was characterized by a decrease of the frequency of detection and number of enterobacteria and staphylococci more than 3 times with the establishment of compliance indicators with the control.

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