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## **IMUNOBIOTICS ARE THE NOVEL BIOTECH DRUGS WITH ANTIBACTERIAL AND IMMUNOMODULATORY PROPERTIES**

*This contribution deals with recently developed technology to obtain the novel biotech probiotic drugs on the basis of pre-selected and characterized strains of lactobacilli and bifidobacteria, which have antibacterial action and high level of ability to balance the immune response in infectious and inflammatory diseases of bacterial, viral and fungal origin by induction of various cytokines. We created an experimental model for the study of immunomodulatory activity of probiotic strains of lactobacilli and bifidobacteria in vitro and in vivo. We established that the probiotic strains Lactobacillus acidophilus IMV B-7279, L. casei IMV B-7280, Bifidobacterium animalis VKL and B. animalis VKB, as well as different compositions based on probiotic strains might be promising for creating high-effective immune biotics with antibacterial and immune modulatory effects for treatments of infectious-and inflammatory diseases, caused by pathogenic and opportunistic microorganisms.*

*K e y w o r d s: lactobacilli, bifidobacteria, immunity, infection, diseases, biotech drugs.*

Rapid increase in number of infectious diseases caused by pathogen and opportunistic bacteria, viruses and fungi as well as emergence of new pathogens of mainly viral nature has been observed in the world in recent years. These phenomena in their turn are caused by general decrease in organisms protection abilities, spread of immune systems imbalances (depressions or overexcitations) which are caused by environmental changes, uncontrolled use of chemical preparations of different origin including modern antibiotic preparations and formation of resistance to them, and negative changes to the normal gut microbiota [6, 10, 16, 28]. Dysfunction of the immune system is directly related to increased aggressiveness of opportunistic microorganisms and can cause emergence of oncological diseases, chronic inflammatory and allergic states etc. [7, 9, 12, 26].

Taking into account a constant negative influence of environmental factors that significantly adversely affect the functioning of major systems and organs of the macroorganism, primarily the immune system, probiotics, prebiotics and probiotic products use in the treatment of patients with the most common infections is becoming increasingly important. The term “probiotics” mean pharmaceutical drugs, special foods and dietary supplements that are based on

living cells of normal microbiota that putting a positive effect on physiological, biochemical and immune response of the host organism under natural way by optimizing and stabilizing functions of its normal microbiota through adhesive immunomodulatory and antagonistic properties [7, 8, 9, 11, 14, 15, 25, 27, 31].

The most promising strains of lactobacilli (LAB) and bifidobacteria for development of probiotics are those which easily accustom to organisms, show high antagonistic activity towards pathogenic and opportunistic microorganisms and have immunomodulation properties related to activation of innate immunity and balancing cytokine, that affecting development of specific immune response [23, 27]

Today few hundreds of probiotic preparations with declared high level of immunomodulation properties able to activate immune response mainly related to induction of anti-inflammatory cytokines are presented on the pharmaceutical market of developed countries. However only few probiotic strains stored in research laboratories and only a number of ready forms of probiotic preparations are able to balance immune response mainly through synthesis of regulating anti-inflammatory cytokines such as interleukin-10 (IL-10), transforming growth factor- $\alpha$ , and induction of TLR-NF- $\kappa$ B or some CD-antigens expression which regulate of the immune response [23]. But the actual problem is the selection of probiotic strains and probiotic compositions with a maximum level of induction both pro- and anti-inflammatory cytokines (IL-4, IL-10, IL-12, interferon- $\gamma$ , (IFN- $\gamma$ )) able to align the imbalance of Th1 / Th2 immune response in case of particular level of its disorder being a result of an infectious disease. This is the most efficient way of influencing immune response under infectious, inflammatory and other diseases. Therefore research and development of preparations based on human mucous tunics commensal microbiota able to regulate immune response of an organism without side effects which are difficult to foresee and compensate in every particular case still is one the most important tasks of modern molecular immunology and immunotherapy.

Over the last 10 years our efforts were aimed at the creating of a new research-based approaches to development of technologies for production of advanced biotechnological preparations – immunobiotics on the basis of the LAB and bifidobacteria which have antibacterial properties and high level ability to balance immune response by inducing cytokines of various nature under infectious and inflammatory diseases of bacterial, viral and fungal genesis. The objects of our study were *Lactobacillus acidophilus* IMV B-7279, *L. casei* IMV B-7280, deposited in the Depository of microorganisms of the D.K. Zabolotny Institute of Microbiology and Virology, NAS of Ukraine and also *Bifidobacterium animalis* VKL and *B. animalis* VKB.

It was established that non-pathogenic, non-toxic, genetically homogeneous *L. acidophilus* IMV B-7279, *L. casei* IMV B-7280, *B. animalis* VKL and *B. animalis* VKB strains, which were not subjected to mutagenic influences and genetic transformation, are promising to create immunobiotics with antibacterial and immunomodulatory action. It was shown that the main probiotic properties of these strains of LAB and bifidobacteria, that allow to use them as the main component of immunobiotics, are: the ability to survive in the harsh conditions of the gastrointestinal tract; significant biosynthetic activity (ability to substantial synthesis of lactic acid, enzymes, which are associated

with their cholesterol-lowering activity); middle and high adhesiveness to mucosal epithelium; antagonistic activity against a wide range of pathogenic and opportunistic microbiota; immunomodulatory properties. So, *L. acidophilus* IMV B-7279, *L. casei* IMV B-7280, *B. animalis* VKL and *B. animalis* VKB *in vitro* effectively inhibit the growth of museum and clinical pathogenic and opportunistic bacteria strains. Most sensitive to LAB and bifidobacteria antagonistic activity appeared bacteria of *Staphylococcus* genus. The probiotic strains *L. acidophilus* IMV B-7279, *L. casei* IMV B-7280, *B. animalis* VKL and *B. animalis* VKB have different sensitivity to various types of antibiotics – cephalosporins, lincosamides, fluoroquinolones, nitrofurans, penicillins, aminoglycosides, macrolides, tetracyclines, glycopeptides, sulfonamides, carbapenems and sulfonamides that should be taken into account for developing of individual schemes of complex treatment, which involve combined use of antibiotics and probiotics [17–19, 21–24, 29].

Determination of biocompatibility of *L. acidophilus* IMV B-7279, *L. casei* IMV B-7280, *B. animalis* VKL and *B. animalis* VKB has shown that they are not antagonists, so not inhibit the growth of each other [5]. The obtained data showed that it is possible to combine these strains of LAB and bifidobacteria in different compositions with different volume ratios to obtain preparation with various probiotic properties. Immunomodulatory activity of *L. acidophilus* IMV B-7279, *L. casei* IMV B-7280, *B. animalis* VKL and *B. animalis* VKB *in vitro* and *in vivo* is proved: in case of physiological norm they promoted effector functions of phagocytic system cells, induced production of varied immunoregulatory cytokines, but *in vivo* left unchanged phenotypic composition of spleen lymphocytes of the intact BALB/c mice. After use of these probiotic strains for treatment intact mice (at the dose of 25 ml in the amount of  $4 \times 10^8$  CFU/ml to the 7-day schedule) the parameters of the peritoneal macrophages activity such as phagocytosis and respiratory burst were increased in different periods of observation. It was found that probiotic strains *L. acidophilus* IMV B-7279, *L. casei* IMV B-7280, *B. animalis* VKL or *B. animalis* VKB after administration to intact mice did not cause a change in subpopulation of the spleen cells: the number of CD3<sup>+</sup>-, CD4<sup>+</sup>-, CD8<sup>+</sup>-, CD19<sup>+</sup>-cells and NKC were kept on the level of control. Increase of the immunoregulatory index in different periods of observation under the influence of *L. acidophilus* IMV B-7279 and *B. animalis* VKB indirectly indicate their potential ability to guide the development of the immune response by cell type [5, 13, 21–23].

In case of physiological norm probiotic strains did not change the pro-inflammatory cytokine production – tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), however production of other Th1-type cytokines – interferon- $\gamma$  (IFN- $\gamma$ ) and / or IL-12 production was increased and the decreasing of Th2-type cytokines IL-4 and/or IL-10 were observed in different periods of observation [19]. We have also observed the significant increasing of IgA production in all the mucosal sites including the popliteal, mesenteric lymph nodes, Peyer's patches, as well as in mucosal sites of vagina and uterus induced by using of intravaginal implementation LAB strains into intact mice. A fundamentally new approach for preliminary selection of LAB strains with immunomodulatory properties to create immunobiotics that takes into account correlation between the degrees of rigidity of the cell wall of bacteria and their influence on the activity of phagocytic cells (ability to accumulate NO/NO<sub>2</sub> and reactogenic oxygen

metabolites) and production of immunoregulatory cytokines were established. The rigidity of cell wall of LAB was distributed as follows: *L. acidophilus* IMV B-7279 > *L. casei* IMV B-7280. Among the strains of bifidobacteria *B. animalis* VKB had tougher cell wall than *B. animalis* VKL. It was found that effector functions of phagocytic system cells increased *in vitro* intensively under the influence of LAB strains with less rigid cell wall, which were better digested in these cells than the strains with more rigid cell wall. However, more effective activator of cytokine production (IFN- $\gamma$  and IL-12) *in vitro* among LAB strains appeared strains with more rigid cell wall. It is proved that *L. acidophilus* IMV B-7279, *L. casei* IMV B-7280, *B. animalis* VKL and *B. animalis* VKB is biocompatible, which allows to combine these strains of LAB and bifidobacteria with different degrees of the cell wall rigidity in different composition, thereby getting probiotic preparation with specified properties [20].

Therefore, we detected *in vitro* and *in vivo* the potential of *L. casei* IMV B-7280, *L. acidophilus* IMV B-7279, *B. animalis* VKB or *B. animalis* VKL to guide the development of the immune response mainly by Th1-type, at least in case of physiological norm. Thus, these probiotic strains were promising to create immunobiotics with antibacterial and immunomodulatory action for treatment of infections disease, accompanied by the development of immune deficiency. In our further investigations the experimental model of the vaginal staphylococcal infection in mice BALB/c for determination of the antibacterial and immunomodulatory effect of probiotic strains of LAB and bifidobacteria was created. *S. aureus* 8325-4 was used for creation of an experimental intravaginal staphylococcosis model because it contain the stability plasmid to gentamicin so it could be separated from other strains of staphylococci by way of cultivation on elective nutrient medium (BAIRD-PARKER-Agar, Merck, Germany) with this antibiotic. The mice received a one-time intravaginal injection of *S. aureus* 8325-4 of 25  $\mu$ l ( $2 \times 10^8$  CFU/ml) [5].

After a single injection of *S. aureus* 8325-4 into the vagina the occurrence of external clinical signs of infectious-inflammatory process such as discharge of yellowish-greenish color, edema and hyperemia of the vulva was observed [5, 13]. Morphological features of infectious-inflammatory process in the vagina were: significant swelling of the epithelial layer, it's magnaview infiltration of polymorphonuclear leukocytes and lymphoid cells, clusters of which were found also in subepithelial areas; and moderate infiltration of inflammatory cells in the submucosa, muscular and even adventitia shell. The development of the inflammatory process at the local level was evidenced by the increase in the number TL2- and TL4-positive macrophages in the epithelium of the vaginal mucosa.

The vaginal microbiota of the infected mice was significant changed. The *S. aureus* 8325-4 strain was seeded out from the vagina of these mice throughout the observation period. The number of aerobic and facultative anaerobic microorganisms, staphylococci, streptococci, coliform bacteria and fungi flora after injection in vagina of the Staphylococcus infected mice were increased. These mice demonstrated the following changes in the species spectrum of the mucous layers of vagina: the number of LAB fell to its lowest level; the number of *P. aeruginosa* and *S. aureus* was increased for both of these pathogens; the number of *S. agalactiae* also was increased compared to the intact mice (data non published).

We first found that infection of mice with *S. aureus* 8325-4 into vagina contributed to the development of ascending staphylococcal infections and led to changes in gut because this strain of staphylococcus was seeded out from their kidneys and intestinal contents. Unlike control group indicators, in the kidneys of Staphylococcus infected mice the number of staphylococci was increased; in a small number appeared streptococci, coliform bacteria and fungal flora in the different period of the observation. It should be noted, that from kidneys of intact mice these microorganisms were not seeded out at all. In the intestinal contents of Staphylococcus infected mice the number of staphylococci also increased, but the number of microorganisms that were plated on the elective medium for streptococci and fungal flora did not change; but the number of coliform microorganisms decreased [5].

In the case of vaginal staphylococcal infection in mice an impairments of immune parametrs such as decreased activity of macrophages, reduce the number of natural killer cells (NKC), CD3+ and CD4+ T-lymphocytes in the spleen and dropped the immunoregulatory index CD4/CD8 in different periods of observation was observed. In the same time after injection of *S. aureus* 8325-4 into the vagina the imbalance of Th1/Th2 type cytokine production at local and systemic levels with a predominance of the IL-4 production and decreased production of the IL-12 were found. Established imbalance in Th2/Th1-type cytokines production can be the basis of a violation in the cellular immune response that was observed in cases of vaginal staphylococcal infection [5, 13].

The probiotic strains *L. acidophilus* IMV B-7279, *L. casei* IMV B-7280, *B. animalis* VKL and *B. animalis* VKB (individually) and in various compositions after the administration to Staphylococcus infected Balb/c line mice (once per day for 7 days at a dose of  $5 \times 10^6$  cells/animal) had both antibacterial and immunomodulatory properties. It should be noted, that *L. acidophilus* IMV B-7279, *L. casei* IMV B-7280, *B. animalis* VKL, *B. animalis* VKB and their different compositions in the case of the vaginal staphylococcal infection caused the disappearance of the external visual clinical signs of infectious-inflammatory process in the vagina, as well as the gradual decrease or even the disappearance of its morphological changes in the tissue of the vagina, starting from 1<sup>st</sup> day and during the next period of observation. Our data indicate that the injection of *L. casei* IMV B-7280 into vagina and per os is more effective in the treatment of mice with vaginal staphylococcal infection, because in this scheme of LAB probiotic strain administration full normalization of the morphological structure of the vaginal wall was observed. However, morphological signs of inflammation of the vagina still remained after the injection of infected mice with other probiotic starins (data non published).

After use for treatment of the probiotic starins the accelerate elimination of *S. aureus* 8325-4 from the vagina, kidney and intestinal contents was observed. The strains *L. casei* IMV B-7280, *B. animalis* VKL and *B. animalis* VKB had more efficient antagonistic activity against *S. aureus* 8325-4 than *L. acidophilus* IMV B-7279. The most effective antagonistic activity against *S. aureus* 8325-4 was in probiotic compositions such as *L. casei* IMV B-7280 - *B. animalis* VKL - *B. animalis* VKB and *L. casei* IMV B-7280 - *B. animalis* VKL - *L. acidophilus* IMV B-7279.



It was established that microbiota of the vagina of infected mice normalized under the influence only of the strain *L. casei* IMV B-7280, and such probiotic compositions like *L. acidophilus* IMV B-7279 - *B. animalis* VKB, *L. acidophilus* IMV B-7279 - *L. casei* IMV B-7280, *L. casei* IMV B-7280 - *B. animalis* VKB, *L. casei* IMV B-7280 - *B. animalis* VKB - *B. animalis* VKL, *L. casei* IMV B-7280 - *B. animalis* VKL - *L. acidophilus* IMV B-7279, *L. casei* IMV B-7280 - *B. animalis* VKB - *L. acidophilus* IMV B-7279. In generally, the number of aerobic and facultative anaerobic microorganisms, staphylococci, streptococci, coliform bacteria and microscopic fungi in the vagina of the Staphylococcus infected mice after use for treatment of the *L. casei* IMV B-7280 and these probiotic compositions was decreased in certain periods of the observations.

Vaginal microbiota of mice infected with *S. aureus* 8325-4 that were administered for therapeutic purposes the intravaginal *L. casei* IMV B-7280 saw the following changes: the number of *L. acidophilus* and *L. casei* by the end of the experiment was increased, the number of *P. aeruginosa* and *S. aureus* was decreased; the number of *S. agalactiae* was dropped. So, it was detected a normalization of the microbiota spectrum in the vagina of *Staphylococcus* infected mice treated with probiotic strains [1–5, 13].

*L. acidophilus* IMV B-7279, *L. casei* IMV B-7280, *B. animalis* VKL, *B. animalis* VKB strains and their different compositions *in vivo* in the case of the vaginal staphylococcal infection increased functional activity of phagocytic system cells, normalized the amount of NKC, CD4+ T-lymphocytes in the spleen and immunoregulatory index CD4/CD8 [5, 13]. All of these probiotic strains and the composition *L. casei* IMV B-7280 - *B. animalis* VKB - *B. animalis* VKL directed the development of the immune response by Th1-type, which was confirmed by increased production of IL-12 and IFN- $\gamma$  due to impaired production of IL-4 in the system level. Under the influence of *L. casei* IMV B-7280 the number of TLR2- and TLR4- positive macrophages in the epithelium of the vaginal mucosa of infected mice was decreased, and the local production of Th1-type cytokine – IL-12 was increased, and the synthesis of anti-inflammatory cytokines – IL-10, IL-17A was suppressed at the local levels (data non published).

In further studies probiotic strain *L. casei* IMV B-7280 was used in limited clinical trials for the correction of intermediate type of biocenosis of the vagina in women and to treat women with vaginal dysbiosis. Our data indicate a high clinical efficacy of *L. casei* IMV B-7280 in the treatment of female patients with vaginal dysbiosis and for the correction of the vaginal microbiota of women with an intermediate type, as evidenced by normalization of the number of LAB, bifidobacteria, staphylococci, streptococci, as well as the disappearance of microscopic fungi and coliform flora on the background of the normalization of the cytological picture.

As a result of our studies it was developed and approved Technical specification of Ukrain TSU 21.1-2960512097-004:2015 “Freeze-dried concentrate of probiotic microorganisms” about the use of *L. acidophilus* IMV B-7279, *L. casei* IMV B-7280, *B. animalis* VKL and *B. animalis* VKB for production of biologically active drugs, including immunobiotics [30]. The test sample of the preparation based on *L. casei* IMV B-7280 was received. Probiotic strain *L. casei* IMV B-7280 in limited clinical studies have

demonstrated a high capacity for normalization the species composition of biocenosis of the vagina in cases of dysbiosis and vaginosis. The laboratory regulations to obtain of the “Lactokas” (PreVaLac) preparation on the basis of *L. casei* IMV B-7280 had been developed.

Our results confirm the validity of the requirements of the European regulatory legislation in the field of probiotics regarding the need for comprehensive studies of biological activity of both separate cultures and their combinations, which would allow for the creation of effective probiotic drugs based on monocultures of LAB and/or bifidobacteria or their various combinations. We suggest that LAB and bifidobacteria and novel strains might be an additional or supplementary therapy in the cases of differenty pathological process and may have potential for preventing wide scope of immunity-related diseases due anti-inflammatory effect. The next generation probiotics strains should be properly studied and suggested to clinical application. Translation of the obtained data on animal model to human organism may allow considering diet correction with probiotics for balancing immunity, in particular in for promotion of health based on patient profiling with relevant gut microbiome data and immune response patterns guided physiologic diet and lifestyle in the integrated vision of an interactome.

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## **ІМУНОБІОТИКИ – НОВІТНІ БІОТЕХНОЛОГІЧНІ ПРЕПАРАТИ З АНТИБАКТЕРІАЛЬНИМИ ТА ІМУНОМОДУЛЮВАЛЬНИМИ ВЛАСТИВОСТЯМИ**

### **Резюме**

Розроблено технологію отримання новітніх біотехнологічних пробіотичних препаратів на основі попередньо відібраних та охарактеризованих штамів лактобацил і біфідобактерій, які мають антибактеріальну дію і високий рівень здатності збалансувати імунну відповідь при інфекційних і запальних захворюваннях бактеріального, вірусного та грибового генезу шляхом індукції цитокінів різної природи. Створено експериментальні моделі для вивчення імуномодулювальної активності пробіотичних штамів лактобацил і біфідобактерій *in vitro* та *in vivo*. Встановлено, що пробіотичні штами *Lactobacillus acidophilus* IMB B-7279, *L. casei* IMB B-7280, *Bifidobacterium animalis* VKL та *B. animalis* VKB, а також різні композиції на основі цих пробіотичних штамів є перспективними для створення вискоєфективних імунобіотиків з антибактеріальною та імуномодулювальною дією для лікування інфекційно-запальних захворювань, викликаних патогенними та умовно-патогенними мікроорганізмами.

**Ключові слова:** лактобацили, біфідобактерії, імунітет, інфекції, хвороби, біотехнологічні препарати.

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## **ИММУНОБИОТИКИ – НОВЕЙШИЕ БИОТЕХНОЛОГИЧЕСКИЕ ПРЕПАРАТЫ С АНТИБАКТЕРИАЛЬНЫМИ И ИММУНОМОДУЛИРУЮЩИМИ СВОЙСТВАМИ**

### **Резюме**

Разработана технология получения новейших биотехнологических пробиотических препаратов на основе предварительно отобранных и охарактеризованных штаммов лактобацилл и бифидобактерий, которые обладают антибактериальным действием и высоким уровнем способности балансировать иммунный ответ при инфекционных и воспалительных заболеваниях бактериального, вирусного и грибкового генеза путем индукции цитокинов различной природы. Созданы экспериментальные модели для изучения иммуномодулирующей активности пробиотических штаммов лактобацилл и бифидобактерий *in vitro* и *in vivo*. Установлено, что пробиотические штаммы *Lactobacillus acidophilus* IMB B-7279, *L. casei* IMB B-7280, *Bifidobacterium animalis* VKL и *B. animalis* VKB, а также различные композиции на основе этих пробиотических штаммов являются перспективными для создания высокоэффективных иммунобиотиков с антибактериальным и иммуномодулирующим действием для лечения инфекционно-воспалительных заболеваний, вызванных патогенными и условно-патогенными микроорганизмами.

**Ключевые слова:** лактобациллы, бифидобактерии, иммунитет, инфекции, болезни, биотехнологические препараты.

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