Annals of Mechnikov Institute, N 2, 2014
www.imiamn.org.ua /journal.htm

UDC 615.372 : 57.083.3

IMMUNOBIOLOGICAL PROPERTIES OF MEDICINAL PRODUCT OBTAINED ON THE BASIS OF STRAINS OF BIFIDOBACTERIA AND LACTOBACILLUS

Khizhnyak O. S.

National Technical University "Kharkov Polytechnic Institute"

Deteriorating environment condition, quick pace of life, improper and unbalanced diet, great number of stresses and pernicious habits lead to microflora imbalance. The maximum sensitivity to these effects is characteristic of microflora of intestine which reacts to unfavorable factors long before appearance of clinical symptoms by reduction of indigenous microflora level and increase of quantity of opportunistic pathogens, that is, by condition of micloflora imbalance called dysbiosis [1,2].

Lately dysbacterioses of various etiologies and degree of severity are rather frequent phenomena, in spite of great choice of medicinal products and biologically active additives offered for their prevention. Among all products used for prevention and treatment of dysbiotic disorders, the medicinal products on the basis of bacteria typical for normal microflora of healthy people, or probiotics, provide the widest range [3].

For rational development of new probiotics, it is necessary to take into consideration a quantitative ratio of different strains in intestine, their physiological features and ability to survive in aggressive conditions (acid environment in stomach, bile acids), to provide the maximum stay of bacteria in the appropriate loci in case of taking *per os* [4]. For increase of effectiveness of medicinal products, it is also rather important to choose correctly a prebiotic component for certain consortium. It is necessary to indicate that the prebiotic component comprises not only an important supplement to probiotic medicinal products but also provides a source of feed for weakened representatives of patient's intestine micloflora.

We proposed a medicinal product based on consortium of bifidobacteria belonging to *Bifidobacterium bifidum LVA-3* strain and lactobacilli belonging to *Lactobacillus Plantarum* strain with addition of lactitol as a prebiotic component [5].

Lactitol used as a prebiotic component is chosen for a number of reasons: lactitol is actively metabolized both by the pure cultures indicated above and in case of mutual cultivation of strains of the said bacteria. Besides, fatty acids formed during lactitol fermentation by large intestine microflora have no influence on the level of glucose and insulin in blood, so composition of the probiotic medicinal product proposed by us may be recommended for patient suffering from diabetes mellitus [6]. In addition to this, in case of treatment of hepatic encephalopathy, cirrhosis and hepatitis, lactitol is able to change ratio of proteolytic and saccharolytic bacteria in favor of the latter, which leads to a considerable reduction of endotoxins level in patients [7,8]; lactitol has no influence on bone tissue metabolism, including concentration of parathormone, osteocalcin and alkaline phosphatase, has no influence on concentration of inorganic phosphate and calcium in blood plasma [9]. An important criterion that determined choice of lactitol as prebiotic used for achievement of symbiotic effect consists in that comparative study of influence of lactitol and lactulose on opportunistic pathogens and pathogens showed possibility of its selective action [10,11,12,13]. In particular, it is not split by coliform bacterium (*E. coli*) and is split by much lesser quantity of strains of staphylococcus (*S. aureus*) and clostridium (*Cl. Perfringens*) [9,14].

Work objective was aimed at examination and evaluation of safety of the proposed combined probiotic medicinal product on the basis of consortium of *Bifidobacterium bifidum LVA-3* strain and *Lactobacillus Plantarum* strain with addition of lactitol as a prebiotic component.

Materials and methods

To assess safety of the developed probiotic medicinal product, its integral action on the body was determined according to the requirements of Guiding document РД 42-28-8-89 "Preclinical testing of new immunobiological medicinal products. Fundamental principles" by the following indices: 1) general toxicity; 2) embryotoxicity; 3) general pharmacology [15].

The preclinical studies were conducted on laboratory mice from ICR closed colony, males and females, with mass of 18 to 20 g, age of 60 days, as well as on newborn mice. Husbandry: conventional, controlled by bacterial load parameters. The studies were conducted with introduction of the studied medicinal product to animals *per os* in quantity corresponding to the dose of commercial medicinal products intended for man. The medicinal product was given to experimental animals once per day before feeding for 14 days.

For obtaining the medicinal product, lyophilized *Bifidobacterium bifidum LVA-3* strain was dissolved with Blaurock culture medium (pH 6.5±0.1) and cultivated at a temperature of (38±0.5)°C for 96 hours, by means of two successive inoculations having 48 hours of cultivation each. Then this culture, in quantity of 2.5%, was introduced into modified casein-yeast KD-5 culture medium [16] prepared for cultivation.

Lyophilized *Lactobacillus Plantarum* strain was dissolved with MRS-1 culture medium (pH 6.7±0.1) and cultivated at a temperature of (37±0.5)°C for 24 hours, then subsequent generations were obtained on media MRS-2 (pH 7.3±0.1) and MRS-4 (pH 7.9±0.1). After taking the required quantity of stock culture of lactobacilli of the generation V, for further experiment, the culture was cultivated on MRS-1 medium (pH 6.7±0.1) for 24 hours, to obtain the generation VI stock culture. Then the obtained cultures (7.5% v/v) were introduced into modified casein-yeast KD-5 culture medium prepared for cultivation.

Mutual cultivation of bifidobacteria and lactobacillus (generations V and VI) was carried out on

Annals of Mechnikov Institute, N 2, 2014
www.imiamn.org.ua /journal.htm

modified casein-yeast KD-5 culture medium (pH 7.0±0.1), with addition of lactitol in quantity of 1%, for 24 hours at a temperature of (38±0.5)°C.

To determine immunobiological properties of culture fluid, we examined sterile filtrate of culture fluid (SFCF) of *Bifidobacterium bifidum LVA-3* strain bifidobacteria and *Lactobacillus Plantarum* strain lactobacilli in ratio of 1:3. The filtrate acidity is (228±5)°T.

The immunopotentiating activity [17] was studied on mice (12 in each group) by means of single subcutaneous introduction of adsorbed tetanus toxoid in a dose of 0.2 ml (2 binding units) manufactured by JSC "Biolek". The sterile filtrate of culture fluid was introduced to animals intraperitoneally or *per os* in a dose of 0.5 ml for 21 days. 0.9% solution of sodium chloride was introduced to control group of mice. After 21 days, we intraperitoneally introduced tetanus toxin in a dose of 50 dlm (dosis letalis minima) and observed survival capability of the animals for 7 days.

We studied antibacterial activity of the sterile filtrate of culture fluid. To determine antibacterial activity, we used *Sh. Flexneri* culture as a test strain in a dose of 200 mln. The studies were conducted along two lines: we introduced to mice mixture of strains culture and sterile filtrate of culture fluid in ratio of 1:1 incubated at (37±0.5)°C, or infected the animals through intraperitoneal injection of 0.1 ml dose, with subsequent intraperitoneal introduction of sterile filtrate of culture fluid in a dose of 0.5 ml for 7 days. Only *Sh. Flexneri* culture was introduced to the control group.

The safety of developed probiotic medicinal product was studied in parallel with that of commercially available medicinal products "Bifidumbacterin" and "Lactobacterin" manufactured by PrJSC "Biofarma".

Results and discussion

While studying chronic toxicity of the medicinal product, we observed its stimulating action on growth, development and survival ability of the animals. The blood hematological parameters were within normal physiological limits (Table 1).

Table 1-Main hematological parameter characterizing blood of animals

Hematological parameter characterizing blood of animals	Results	
	Intact animals	Experimental animals
Red blood cells, mln/mm^3	5,80 ± 0,15	5,95 ± 0,26
Leucocytes, thou./mm^3	5,24 ± 0,12	6,05 ± 0,12
Hemoglobin, %	13,20 ± 0,13	14,6 ± 0,14

Absence of changes in blood hematological parameters and death of animals also testifies that strains included in composition of the proposed medicinal product are nonpathogenic.

Histological examination of target organs (thymus, spleen, liver and duodenum) showed that there is no toxic action on these organs. The medicinal product showed itself as immunologically safe not having suppressive action on quantitative parameters of circulating lymphocytes and hematogenesis function [18]. For example, absolute values of immune competent cells in experimental animals approached physiological normal values: quantity of lymphocytes was $2{,}70\cdot10^6$ cells·cm^{-3}, T- and B cells – $1{,}60\cdot10^6$ cells·cm^{-3} and $1{,}18\cdot10^6$ cells·cm^{-3}, respectively [19,20,21].

No immunotoxic action of the medicinal product on lymphoid system organs when they were examined histologically was found: studies conducted on pregnant mouse females showed total absence of any embryotoxicity of the medicinal product. Besides, when it is used, a considerable improvement of organism condition of pregnant female animals, reduction of embryonic mortality, great live weight gain and better survival of newborn mice are observed; the conducted studies showed that there is no penetration bifidobacteria and lactobacilli into epithelial cells, no damaging action on structure of intestinal epithelium; check of effectiveness of physiological action of the medicinal product on mice showed a stabilizing influence on functioning gastrointestinal tract, fast establishment of microbiocenosis in mice; the conducted study on action of the medicinal product on condition of adult animals when they are kept at elevated temperatures testify of sustaining physiological activity of experimental mice, which provides their survival in extreme conditions.

The obtained results testify that the medicinal product passed the tests successfully. As a whole, the presented data give evidence of good prospects of further clinical study of the medicinal product and expediency of its use in combined therapy for the purpose of treatment and prophylaxis. Comparison with standard medicinal products shows that the proposed consortium of bifidobacteria and lactobacilli does not differ by its safety from commercially available medicinal products "Bifidumbacterin" and "Lactobacterin" manufactured by PrJSC "Biofarma" but simultaneously provides higher parameters of biological activity.

Comparative analysis of the proposed medicinal product and commercially available standard medicinal products is presented in Tables 2 and 3 [22].

Table 2 -Main growth characteristics

	Acid formation, °T		Survival capability	
	On Blaurock medium after 48 hours	On MRS-1 medium after 24 hours	On Blaurock medium, GFU/ml	On MRS-1 medium, billion microbial cells
Dry Bifidumbacterin	230,0 ± 5,0	-	10^9	-
Dry Lactobacterin	-	220,0 ± 5,0	-	4,1 ± 0,2
Complex preparation	392,0 ± 5,0	290,0 ± 5,0	10^{12}	6,4 ± 0,2

Table 3 -Antagonistic activity of medicinal products (M ± m)

Tested medicinal product	Growth inhibition zone of test strains, mm						
	Sh. Flexneri 170	*Sh. Sonnei 5063*	*Sh. Flexneri 337*	*E.coli 157*	*P. Vulgaris 170*	*P. Mirabilis*	*St. Aureus 209*
Dry Bifidum-bacterin	18,80 ± 0,27	19,30 ± 0,34	18,60 ± 0,44	21,30 ± 0,49	19,8 ± 0,7	17,60 ± 0,53	-
Dry Lacto-bacterin	25,30 ± 0,41	20,80 ± 0,28	22,7 ± 0,42	23,80 ± 0,49	21,60 ± 0,27	20,60 ± 0,37	21,80 ± 0,29
Complex preparation	27,40 ± 0,32	22,5 ± 0,18	23,8 ± 0,45	23,90 ± 0,35	22,40 ± 0,37	21,90 ± 0,43	22,10 ± 0,31

Lately a great interest is taken in creation of medicinal products on the basis of acellular components of probiotic cultures. The culture fluid of these bacteria has a high concentration of biologically active components including bacteriocins, proteases and products of bacterial activity, which makes it possible to consider culture fluid as a promising material for development on its basis of medicinal products and biologically active additives.

In the further group of experiments, we studied biological activity of culture fluids not containing microorganisms.

While studying immunopotentiating activity of components of the culture fluid obtained during cultivation of tested strains, we found that survival capability of control group of mice was 58%. In the group of animals which obtained the sterile filtrate of culture fluid, survival capability reached 75% for introduction *per os* and 83% for intraperitoneal injection.

Study of immunopotentiating activity of sterile filtrate of culture fluid showed that the products of bacterial activity in case of introduction to animals immunized with vaccine enhance the immune response to antigen of sterile filtrate of culture fluid.

Study of antibacterial activity showed that the products accumulated in the cultivation media (bacteriocins, acids, adhesions, enzymes) may inactivate pathogenic microflora, protecting organisms of infected animals from death both in vitro and in vivo. It was found that use of sterile filtrate of culture fluid leads to reduction of death rate of mice both in case of incubation with pathogenic bacteria and introduction to animals. In comparison with control group, the death rate was reduced by 17-25% in case of preincubation and by 8-17% in case of direct introduction to animals.

At present, some authors propose to obtain concentrated and purified probiotic medicinal products. In our opinion, proceeding from obtained data, it may be concluded that reduction of quantity of bacterial metabolism products and all the more their removal from medicinal product is unjustified since it reduces immunobiological activity of probiotic medicinal products.

Conclusions

There is provided an optimized process of cultivation and obtaining an effective bacterial culture in laboratory conditions and in conditions of industrial production and developed a technology of mutual cultivation of bifidobacteria of *Bifidobacterium bifidum LVA-3* strain and lactobacilli of *Lactobacillus Plantarum* strain. The conducted studies made it possible to choose the optimum cultivation media and optimum balance of cultivated strains, as well as to study the main technological parameters.

The conducted studies demonstrated a high biological activity (acid formation, antagonistic activity, survival ability) of life functioning products of bacterial strains making part of the developed probiotic medicinal product.

It is confirmed that it is possible to create a medicinal product on the basis of biologically active compounds secreted by microorganisms and having immunopotentiating and antibacterial action.

References

1. The modern state of development and application of probiotics, prebiotics and synbiotics [Text] / S.V. Kalinichenko [et al.] // Annals of Mechnikov Institute. – 2013. - № 3. – P. 5-12.

2. The normal microbiota of the human gastrointestinal tract: history of analysis, succession, and dietary

influences [Text] / A.L. McCartney, G.R. Gibson // Gastrointestinal Microbiology. – 2006. - №2. – P. 51–73.
3. Probiotics and gastrointestinal disease: Clinical evidence and basic science [Text] / E.O. Petrof // Antiinflammatory, antiallergy agents medical chemistry. – 2009. - №3. – P.260 – 269.
4. Probitics in gastrointestinal and liver diseases [Text] / D. Jonkers // Aliment. Pharmacol. Ther. – 2007. - №26. – P. 133 – 148.
5. Khizhnyak, O.S. Prebiotics – required component of combined probiotic medicinal product [Text] / O.S. Khizhnyak// Problems and Prospects of Science Development: Materials of VIII International Research-to-Practice Conference. – Chernivtsi, 2013. – P. 180 – 181.
6. Lactitol prebiotic – an effective product for maintenance of health of modern man [Text] / S.I. Artyukhova, Yu.A. Gavrilova // International Magazine of Applied and Fundamental Research. – 2013. – № 4. - P. 95-96.
7. Artyukhova, S.I. Use of probiotics and prebiotics in biotechnology designed for production of bioproducts [Text] / S.I. Artyukhova, Yu. A. Gavrilova. – O. : PHofOSTU, 2010. – 112 p.
8. "Eksportal" (Lactitol) - safe and effective osmotic laxative [Text] / O.P. Minushkin [et.al] // RJGGK. - 2010. - № 5. - P.118.
9. Effects of lactitol on intestinal microflora and plasma endotoxin in patients with chronic viral hepatitis [Text] / C.Chen [et al] // Infection. - 2007. - № 54(1). – P. 98-102.
10. Metabolic effects and their role in functioning of gastrointestinal tract of patients suffering from celiacia [Text] / L.S. Oreshko [et al] // Gastroenterology. – 2012. – № 1. - P. 27-28.
11. New opportunities for prevention and treatment of chronic constipation complex prebiotic agents: choice of optimal combination [Text] / L.I.Butorova // Gastroenterology. - 2012. - № 4. - P. 2-10.
12. Prebiotic properties of lactitol and lactulose for creating symbiotic bacteria [Text] / M. A. Harhota [et al] // Mikrobiol. Journal. – 2012. - №1. – P. 52 – 58.
13. Prebiotic lactitol as a component of a biological product of aerobic bacilli [Text] / L.A.Safronova [et al] // Mikrobiol. Journal. - 2008. - № 6. - P. 34-41.
14. Lactitol or lactulose in the treatment of chronic constipation: result of a systematic review [Text] / A. Maydeo // Indian Med. Assoc. – 2010. - №11. – P. 789 – 792.
15. Bondarenko, V. M. Molecular and cellular mechanisms of therapeutic action of probiotics [Text] / V. M. Bondarenko // Farmateka. - 2010. -№ 2. – P. 26-32.
16. Khizhnyak, O.S. Joint cultivation of probiotic strains of lactobacilli and bifidobacteria [Text] / O.S Khiznyak // Scientific Discussion: Issues of Mathematics, Physics, Chemistry, Biology: Proceedings of II International Conference. – Moscow, 2013. – P. 87 – 91.
17. Commensal gut bacteria: mechanism of immune modulation [Text] / D.Kelly [et al] // Trends Immunology. - 2005. - №26. – P 326 - 333.
18. Experimental evaluation limfotsitotoksic action of bifidobacteria and lactobacillus [Text] / A.S. Erdyakova [et al] // Practical Medicine. - 2012. - №3. – P.194-196.
19.Examination of some immunological parameters of blood serum of experimental animals during their pregnancy period [Text] / L.I. Drozdova, N.V. Arkhipenko // Agrarian Messenger of the Ural. - 2011.- №9. - P. 8-12.
20. Immunomodulatory activity of lacto- and bifidobacteria probiotic strains in vitro and in vivo [Text] / L. M. Lazarenko [et al.] // Theses of XIIIth congress of Vynograds'kyj Society of Microbiologists of Ukraine. – Yalta, 2013. – P. 278.
21. Chicherin, I.Yu. Investigation of the influence of large doses of probiotic microorganisms and Bifidumbacterin autoflora intestinal organism of white mice and indicators of cellular immunity [Electronic resource] / I. Yu. Chicherin [et al] // Intestinal microflora: Collection of scientific articles. – 2013. - №2. – P. 61 – 66. Access mode: http://gastroportal.ru/files/mikroflora.pdf
22. Comparative evaluation of microbial survival of probiotics in selling drugs in in vitro [Text] / I.V. Darmov // Experimental and clinical gastroenterology. - 2011. - № 9. - P. 96-101.

UDC 615.372 57.083.3

IMMUNOBIOLOGICAL PROPERTIES OF MEDICINAL PRODUCT OBTAINED ON THE BASIS OF STRAINS OF BIFIDOBACTERIA AND LACTOBACILLI

Hizhnyak O.S.

The article treats issues of examination of safety of combined bacterial medicinal product on the basis of probiotic strains of bacteria belonging to two genera obtained by submerged mutual cultivation. During conducting experiments, it was found that the medicinal product causes no changes in blood hematological parameters of experimental animals, does not lead to their death, has no immunotoxic action, histological examination shows no deviations from the norm. In comparison with standard medicinal products, the proposed consortium of the said strains of bifidobacteria and lactobacilli does not differ by its safety from commercially available medicinal products "Bifidumbacterin" and "Lactobacterin" produced by PrJSC "Biofarma" but provides higher parameters of biological activity.
There also was conducted examination of culture fluid activity after removal of proposed bacterial consortium. A high antibacterial activity of the sterile filtrate of culture fluid was found.

Key words: prebiotic component, microflora, probiotics, sterile filtrate of culture fluid, immunopotentiating activity, antibacterial activity, safety.

УДК 615.372 : 57.083.3

ИММУНОБИОЛОГИЧЕСКИЕ СВОЙСТВА ПРЕПАРАТА, ПОЛУЧЕННОГО НА ОСНОВЕ ШТАММОВ БИФИДОБАКТЕРИЙ И ЛАКТОБАЦИЛЛ

Хижняк О.С.

В статье рассмотрены вопросы изучения безопасности комбинированного бактериального препарата на основе пробиотических штаммов бактерий двух родов, выращенных совместным глубинным культивированием. В ходе проведенных экспериментов было установлено, что препарат не вызывает изменений в гематологических показаниях крови подопытных животных, не приводит к гибели животных, не имеет иммунотоксического воздействия, при гистологических исследованиях не обнаруживается отклонений от нормы. При сравнении со стандартными препаратами, предложенный консорциум указанных штаммов бифидобактерий и лактобацилл по своей безопасности не отличается от коммерческих препаратов «Бифидумбактерин» и «Лактобакерин» производства ЧАО «Биофарма», но дает более высокие показатели биологической активности. Также было проведено исследование активности культуральной жидкости, после удаления предлагаемого консорциума бактерий. Была установлена высокая иммуностимулирующая и антибактериальная активность стерильного фильтрата культуральной жидкости.
Ключевые слова: пребиотический компонент, микрофлора, пробиотики, стерильный фильтрат культуральной жидкости, иммуностимулирующая активность, антибактериальная активность, безопасность.

УДК 615.372 : 57.083.3

ІММУНОБІОЛОГІЧНІ ВЛАСТИВОСТІ ПРЕПАРАТУ, ОТРИМАНОГО НА ОСНОВІ ШТАМІВ БІФІДОБАКТРІЙ ТА ЛАКТОБАЦИЛ

Хижняк О.С.

У статті розглянуто питання вивчення безпеки комбінованого бактеріального препарату на основі пробіотичних штамів бактерій двох родів, отриманих за допомогою сумісного глибинного культивування. В результаті проведених експериментів було встановлено, що препарат не викликає зміни гематологічних показників крові дослідних тварин, не приводить до загибелі тварин, не має імунотоксичного впливу, при гістологічних дослідах не спостерігається відхилень від норми.
При порівнянні зі стандартними препаратами, запропонований консорціум вказаних штамів біфідобактерій і лактобацил за рівнем безпеки не відрізняється від комерційних препаратів «Біфідумбактерин» та «Лактобактерин» виробництва ПАТ «Біофарма», але дає значно вищі показники біологічної активності.
Також було проведено дослідження активності культуральної рідини, після відділення запропонованого консорціуму бактерій. Була встановлена висока імуностимулююча та антибактеріальна активність стерильного фільтрату культуральної рідини.
Ключові слова: пребіотичний компонент, мікрофлора, пробіотики, стерильний фільтрат культуральної рідини, імуностимулююча активність, антибактеріальна активність, безпечність.