THE EFFICIENCY OF NEW ASSOCIATIONS OF BACTERIA AS PROBIOTICS FOR BIRDS

BOGDAN Victoria, VRABIE Valeria

Abstract. The identification of new probiotics as antimicrobial agents is important in connection with the evolution of the antimicrobial resistance of pathogenic bacteria as a result of the intensive use of antibiotics. In experiments on gnotobiotic chickens, the ability of monocultures and associations of bifidobacteria, lactobacteria and bacteroides to inhibit and replace pathogenic bacteria – *Escherichia* and *Salmonella* – was tested. It has been established that monocultures and combinations of beneficial bacteria in a single dose (1 million bacterial cells for each animal) do not significantly influence the inhibition of pathogenic microbial agents. In order to eliminate them from the intestines, a new scheme was applied, which provided the additional administration of beneficial bacteria in a dose of 100 billion microbial cells for 15 days, over a day. The administration of beneficial bacteria according to the proposed scheme resulted in the obvious suppression of the pathogens of colibacteriosis and salmonellosis in experimental chickens. Also, the tested microorganisms positively influenced the growth and development of experimental animals and contributed to the general resistance of the organism and protection against gastrointestinal diseases. The obtained results can be applied not only to poultry industry, but also to the captive-breeding of pet and wild birds.

Keywords: probiotics, bifidobacteria, lactobacteria, bacteroids, pathogenic microbes, birds.

Rezumat. Eficiența unor noi asociații de microorganisme utile în calitate de probiotice pentru păsări. Identificarea de noi probiotice în calitate de agenți antimicrobieni are importanță în legătură cu evoluția rezistenței antimicrobiene a bacteriilor patogene în rezultatul utilizării intensive a antibioticilor. În experimente pe pui gnotobionți a fost testată capacitatea monoculturilor și asociațiilor de bifidobacterii, lactobacterii și bacteroizi de a inhiba și substitui bacteriile patogene – *Escherichia* și *Salmonella*. A fost stabilit că monoculturile și asociațiile de bacterii folositoare într-o singură doză (1 mln de celule bacteriene pentru fiecare animal în parte) nu influențează semnificativ inhibarea agenților microbieni patogeni. În scopul eliminării acestora din intestine a fost aplicată o schema, ce prevedea administrarea suplimentară a bacteriilor utile în doză de 100 mlrd de celulele microbiene timp de 15 zile, peste o zi. Administrarea bacteriilor benefice conform schemei propuse a avut ca rezultat suprimarea evidentă a agenților patogeni ai colibacteriozei și salmonelozei. De asemenea, microorganismele testate au influențat pozitiv creșterea și dezvoltarea animalelor experimentale și au contribuit la rezistența generală a organismului și protecția față de bolile gastrointestinale. Rezultatele obținute pot fi aplicate nu numai la fermele de păsări, dar și pentru creșterea și menținerea în captivitate a păsărilor de companie și a păsărilor sălbatice.

Cuvinte cheie: probiotice, bifidobacterii, lactobacterii, bacteroizi, microbi patogeni, păsări.

INTRODUCTION

The gut microbiota is an integral part of the host organism, participating in the protection against pathogenic microorganisms and in maintaining homeostasis. The disturbance of the intestinal microbiocenosis or intestinal dysbiosis leads to the destabilization of the functioning of the entire organism, primarily of the immune system.

The main means/ways for the correction or prevention of dysbiosis are probiotics – food products, dietary supplements or medical preparations containing live microorganisms that have a positive effect on the physiological, biochemical and immune reactions of the body by normalizing the microbiota. Recently the FAO/WHO adopted the definition for probiotics: "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). Thus, probiotics are live non-pathogenic microorganisms, which are administered through the digestive route and are beneficial for the host's health (GUILLOT, 1998). Despite the successful use of probiotics in medicine and veterinary medicine, their application in some cases may not give the expected effects and even lead to serious complications. With the development of gnotobiology, the process of colonization by certain types of microorganisms in the various cavities of a macroorganism, as well as the interaction between these microorganisms, can be studied (BÄUMLER & SPERANDIO, 2016).

It was established that the microflora of the digestive tract of humans and animals has a strict specificity, depending on which types of bacterial cultures can be used for the correction of intestinal biocenosis. Biological bacterial preparations are used for the treatment of intestinal diseases of humans and some farm animals, in particular bifidobacteria. In the poultry industry, however, the application of such preparations is limited, despite the fact that, according to a number of authors (TIMOSHCO, 1979; GROND et al., 2018) probiotics, including bifidobacteria, are also representative for the normal microflora of the digestive tract of chickens.

The study of the intestinal microflora and ways of its correcting is essential not only for the poultry industry or for pet birds, but also for the breeding and keeping of wild birds in captivity (in zoo). This is especially important in cases of restoring the population of rare birds for nature reserves. Knowledge in the field of avian microbiology is extremely diffused (uneven) and research on gut microbiota of birds has lagged behind mammalian research. Most data are obtained in microbiological investigations on agricultural birds such as chickens and turkeys, as well as birds of evolutionary or conservation interest (WAITE & TAYLOR, 2014). The wild bird core gut microbiota is more similar to domesticated chickens than to non-human mammals, and most different from the core gut microbiota of humans. The

significance of studying the intestinal microflora of wild birds also derives from the fact that they can be a source of a number of human and animal diseases through direct transmission, or by acting as vectors for zoonotic pathogens (GROND et al., 2018). It is known that gut avian microbiota is dominated by members of the *Firmicutes*, with *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria.* The role or function of *Firmicutes* in wild birds is not yet established, but in domestic chickens, several studies found a positive relationship between *Firmicutes* abundance and mass gain and immune function (LIAO et al., 2012; ZHANG et al., 2015). A positive correlation was established between the supplementing of chicken diets with *Bacillus subtilis*, *Enterococcus faecium*, and other *Firmicutes* (as probiotics) and nutrient uptake and metabolic efficiency (ZHENG et al., 2016). A higher proportion of *Proteobacteria* was found in the gastrointestinal tract of wild birds than in mammals or domestic chickens. A wide range of these microorganisms are opportunistic pathogens (*Campylobacter*, *Escherichia*, *Helicobacter*, *Rickettsia*, *Salmonella* and *Vibrio*) (GROND et al., 2018). *Actinobacteria* are the fourth most abundant phylum of microbes in the wild bird gastrointestinal tract, but no studies have investigated the function of *Actinobacteria* in wild or domestic birds. The *Actinobacteria* include some pathogens (*Corynebacterium*, *Mycobacterium* and *Nocardia* species) and commensal bacteria (*Bifidobacterium*) that can be used as a probiotic (GROND et al., 2018). In birds compared to mammals, a relatively low abundance of *Bacteroidetes* was established, that may be attributed to dietary differences (GROND et al., 2018). The avian gut microbiota is broadly involved in the digestion of food products, and specifically microbiota associated with the crop and ceca may be involved in the detoxification of plant materials and other food compounds.

Generally, the knowledge in the field of avian microbiology and application of probiotics for the amelioration of intestinal microbiocenosis in birds is extremely uneven. Most of the results are related to microbiological investigations of agriculturally important birds, such as chickens and turkeys and pet birds. Thus, the investigation of a new microbial association as probiotics is of interest not only for poultry industry, but also for the breeding of birds of evolutionary or conservation interest.

MATERIAL AND METHODS

The new strains of beneficial microorganisms (bifidobacteria, lactobacteria and bacteroides) were selected based on their capacity to inhibit the growth and development of pathogenic microbes (test-microbes) in *in vitro conditions*. As a result, four types of bifidobacteria, five types of lactobacteria and one type of bacteroides with high antagonistic properties against pathogenic microbes were identified (data are not presented in the article). The numbering of the types of microorganisms was performed during the selection and preventive testing of the strains.

The efficiency of the new associations of microorganisms (cultures of bifidobacteria, lactobacteria and bacteroides) was verified in experiments on chickens (gnotobionts), in farm conditions. For this purpose, 330 of chickens with sterile microflora were divided into 11 groups of 30 chickens each. The associations of useful bacteria were administered according to the following scheme: group I - received bifidobacteria 1 and lactobacteria 1; group II - received bifidobacteria 1, lactobacteria 1 and enzymes; group III - received bifidobacteria 545 and lactobacteria 404; group IV - received bifidobacteria 457 and lactobacteria 578; group V - received bifidobacteria 443 and lactobacteria 597; group VI - received a complex of 3 cultures of bifidobacteria (545, 457, 443) and 3 cultures of lactobacteria (404, 578, 597); group VII - received bacteriodes 18 and lactobacteria 597; group VIII - received bacteriodes 18 and lactobacteria 457; group IX - received bacteroides 18; group X - received *Escherichia* (control group); group XI - received *Salmonella* (control group). The number of microorganisms in the administered dose was 10^6 (1 mln) of microbial cells.

In order to test the efficiency of the selected microorganisms, the experimental groups were infected with test-microbes – *Salmonella* and *Escherichia coli.* Experimental groups I-VI and IX were infected with *Escherichia*, and groups VII and VII – with *Salmonella*. Experimental groups X and XII received only pathogenic microbes and served as control. In order to amplify the effect of the new associations of microorganisms and the displacement of pathogenic microbes from the intestine, an additional administration scheme has been developed. After testing, the most effective scheme (data are not presented in the article) was selected: administration of useful microorganism in the amount of 100 billion microbial cells for 15 days, over a day. This scheme was applied to all experimental groups.

The quantitative index of the microbial species was estimated in dynamics: before and after infection with test-microbes (*Salmonella* and *Escherichia coli*) and after the application of the additional scheme. Microbiological and immunological investigations were performed 3 days after the introduction of useful microorganisms; after 3 days of infection with *Escherichia* and *Salmonella*, and in the 3rd day after the end of additional scheme application. The content of microorganisms was determined using classical microbiological methods (GARMASHEVA & KOVALENKO, 2010). Their inoculation of microorganisms was performed on agar culture medium, (produced and marketed by the company "Himedia"). Over 72 hours after incubation of the inoculated samples on Petri dishes at 37±1 °C, quantitative indices of microorganisms were calculated at 1 g of intestinal contents (by multiplying the number of colonies by diluting the sample). The final results are expressed in decimal logarithms (log) (GOST 30518-97, 2000). The biochemical analysis of blood in farm animals was performed according to the methods described by VASILIEVA (1982) and KONDRAHINA (2004).

The experiments were carried out in accordance with Directive 86/609 /EEC of 24 November 1986 on the Protection of Animals Used for Experimental and Other Scientific Purposes and were approved by the Methodical Committee and the Ethics Committee of the Institute of Physiology and Sanocreatology.

RESULTS

The investigation of new associations of beneficial microorganisms on gnotobiotic chickens was performed in order to highlight their probiotic action with their subsequent use in poultry industry. At the same time, these investigations are important for the captive breeding of wild birds, especially in the case of rare and endangered bird species.

The effect of new associations of microorganisms was established in the dynamics. The data obtained are presented in Table 1.

Table 1. The influence of beneficial bacteria on the control of *Escherichia* and *Salmonella* in the intestines of gnotobiotic chickens.

The experimental groups of animals	Bacterial species	The average number of microbial cells in 1 g of intestinal contents (in decimal logarithms)		
		Before the infection with test-microbes	After 3 days of infection with test-microbes	After administration of beneficial bacteria according to the elaborated scheme
1	Bifidobacteria	10,57	10,71	10,90
	Lactobacteria	9,36	9,52	9,72
	Escherichia	-	8,43	0,30
2	Bifidobacteria	10,49	10,62	10,81
	Lactobacteria	9,68	9,67	9,65
	Enzymes	9,44	9,09	8,32
	Escherichia	-	7,90	0,40
3	Bifidobacteria 545	10,20	10,17	10,29
	Lactobacteria 404	9,13	8,80	8,90
	Escherichia	-	7,90	1,30
4	Bifidobacteria 457	9,89	10,17	10,29
	Lactobacteria 578	8,47	8,80	8,90
	Escherichia	-	7,90	1,30
5	Bifidobacteria 443	10,37	10,51	10,65
	Lactobacteria 597	9,39	9,42	9,51
	Escherichia	-	7,65	0,80
6	Complex of bifidobacteria 545, 457, 443	10,90	11,52	11,60
	Lactobacteria 404, 578, 597	9,76	9,80	9,87
	Escherichia	-	7,16	0,00
7	Bacteroides 18	9,78	10,09	10,15
	Lactobacteria 597	9,15	9,27	9,36
	Salmonella	-	7,51	0,00
8	Bacteroides 18	10,09	10,27	10,16
	Bifidobacteria 457	9,75	9,87	9,90
	Salmonella	-	7,95	0,30
9	Bacteroides 18	10,54	10,65	10,70
	Escherichia	-	8,05	1,70
10	*Escherichia*	-	9,75	9,80
11	*Salmonella*	-	8,87	8,90

Note: Groups 1-9 – experimental; groups 10-11 – control

According to the results presented in the table, a high generative capacity of associations of microorganisms (bifidobacteria, lactobacteria and bacteroides) was revealed even in the first days of their administration. Thus, after three days of administration of the tested microorganisms, their intestinal content increased (from 8.47 to 10.9 lg microbial cells / gram) and was maintained at a high level throughout the experimental period (Table 1).

The infection of experimental chickens with test microbes (*Salmonella* and *Escherichia*) did not negatively influence the amount of bifidobacteria and lactobacteria in the intestine. It should be mentioned that chickens from the experimental groups, which received the associations of microorganisms, did not manifest cases of gastrointestinal dysfunctions, and are thus maintaining their vitality. In the chickens from the control group (groups 10 and 11) after three days from the infection with test-microbes, diarrheal symptoms appeared, and during the experimental period, 50% and respectively 80% of tested animals died. Therefore, bifidobacteria and lactobacteria have positively influenced the growth and development of experimental chickens and have contributed to their protection against gastrointestinal diseases. At the same time, monocultures and associations of beneficial bacteria administered in the dose of 10^6 (1 mln) of microbial cells per animal in a single dose do not inhibit the growth and multiplication of *Escherichia* and *Salmonella* bacteria. Therefore, in order to exclude test-microbes from the animal intestine, additional bacteria had to be used according to the scheme: in the amount of 100 billion microbial cells for 15 days, over 1 day.

According to the obtained results, the administration of new associations of microorganisms (bifidobacteria and lactobacteria and bacteroides) according to the proposed scheme resulted in an obvious suppression of the pathogens of colibacteriosis and salmonellosis in the animal intestine (Table 1).

The cultures of tested microorganisms also positively influenced the resistance of organisms (Table 2).

Table 2. Nonspecific immunity of gnotobiotic chickens experimentally infected with *Escherichia* and *Salmonella*, and under the influence of beneficial bacteria.

The experimental groups of animals	Sampling time	Indices of nonspecific immunity					
		Phagocytic activity, %	Lysozyme, µg/ml	Ascorbic acid, mg%	Haemoglobin, g%	Erythrocytes, mln (M)	Leukocytes, thousand (K)
1	1	8,4	1,0	5,6	10,4	2,0	12,0
	2	10,9	1,4	16,0	10,7	2,1	21,3
	3	10,7	2,4	12,5	10,0	2,4	20,4
2	1	10,6	1,1	6,6	10,1	2,0	12,7
	2	10,0	1,6	16,8	10,1	2,2	21,9
	3	12,6	2,8	14,6	11,1	2,6	22,1
3	1	12,1	0,9	23,3	10,0	2,0	15,0
	2	10,8	1,1	20,6	10,2	2,1	22,1
	3	10,8	1,3	26,5	11,4	2,6	22,5
4	1	13,7	1,0	21,4	9,0	2,0	14,3
	2	13,1	1,3	19,3	10,0	2,2	18,2
	3	11,3	1,7	22,6	10,0	2,6	23,5
5	1	10,7	1,0	21,1	10,0	2,0	13,3
	2	8,5	1,2	16,4	10,6	2,4	21,0
	3	9,6	1,1	21,8	10,3	2,4	20,7
6	1	10,7	1,2	23,3	10,3	2,2	15,7
	2	15,1	1,5	20,8	11,0	2,2	21,0
	3	14,1	1,8	29,9	10,2	2,3	21,2
7	1	13,8	-	16,4	10,6	2,0	13,1
	2	12,5	-	19,2	11,4	2,1	19,8
	3	17,1	-	19,2	10,4	2,2	22,1
8	1	13,8	-	16,4	10,6	2,0	13,1
	2	13,8	-	20,4	10,8	2,2	21,2
	3	24,0	-	21,0	11,2	2,5	23,0
9	1	13,8	-	16,4	10,6	2,0	13,1
	2	12,5	-	17,0	10,4	2,1	20,1
	3	11,5	-	19,2	11,1	2,4	22,6
10	1	6,7	0,7	4,4	10,4	2,1	11,9
	2	10,3	1,2	9,4	10,6	2,1	18,3
	3	9,3	2,1	15,2	10,4	2,2	18,8
11	1	6,7	-	12,0	10,2	2,1	11,9
	2	13,1	-	17,0	10,1	2,3	19,5
	3	13,5	-	16,2	10,2	2,7	20,8

Note: 1 - investigation after the initial administration of beneficial microorganisms; 2 - after the infection with test-microbes; 3 - after the subsequent administration of beneficial bacteria according to the proposed scheme.

Groups 10 and 11: 1 - gnotobiotic chickens, which did not receive microorganism cultures; 2 - 3 days after the introduction of *Escherichia* and *Salmonella*; 3 - control data for groups 1-9 after the additional introduction of beneficial bacteria according to the proposed scheme.

As we can see from the obtained data, the phagocytic activity in the blood of chickens from the nine experimental groups (after 3 days of oral administration of cultures of useful bacteria, in a single dose, 1 mln (M) microbial cells per chicken head) varied from 8.4% to 13.8%. In the control group (10 and 11), these values were 6.7%, i.e., 1.7-7.1% lower compared to the groups that received beneficial bacteria. At the same time, the ascorbic acid content in the liver of chickens from groups 1-9 was in the range of 5.6 to 23.3 mg %, and in the control group it was 4.4 mg %, i.e. lower than in the experimental groups with 1.6-18.9 mg %. The indices of lysozyme activity of the blood serum of chickens, which received pure cultures of beneficial bacteria, were higher compared to those in the control group, by 0.2-0.5 mg/ml. Also, no difference was found between the control and experimental groups in terms of the amount of haemoglobin and erythrocytes in the blood. The quantitative index of leukocytes in the animals from the experimental groups is higher compared to the control groups (by 0.1-3.8 K). So, this index is lower in gnotobiotic chickens than in contaminated experimental groups. This confirms that the immunocompetent system of gnotobionts is reactive to antigenic stimulation.

The introduction of *Escherichia* and *Salmonella* monocultures in experimental chickens revealed an increase of phagocytic activity of leukocytes in groups 10 and 11, by 3.6 and 6.4% respectively, and in group 1 and 6 – by 2.5 and 4.4%, compared to the primary data. Without changes, this index remained in group 8. In the other chicks, the phagocytic activity of the blood serum decreased by 0.6-2.2%. The infection with *Escherichia* of animals in groups 2-4 had an inhibitory action on the phagocytosis. The same influence was on the chickens from groups 7 and 9, after three days with salmonella infection. In the liver of chickens from group 1 and 2, an increase in the vitamin C content, by 10.2-10.4 mg %, was accordingly found. At the same time, in groups 3-6 of experimental animals, the negative action of the infection is obvious. The increase in the haemoglobin level (by 0.1-0.8 mg %) was observed in groups 1-8, and the decrease (by 0.1-0.2 mg %) – in groups 9 and 10. In all experimental chickens, the number of leukocytes increased by 3.9- 9.3 K.

As a result, the additional administration of beneficial bacteria according to the proposed scheme (i.e., 100 billion microbial cells for 15 days over a day) showed a high phagocytic activity of leukocytes in the blood of

experimental animals in groups 2,5,7 and 8, respectively with 2.6, 1.1, 4.6 and 10.2%. At the same time, the decreasing tendency of this index in chickens from groups 4, 6 and 10, respectively by 1.8, 1.0 and 1.0%, was revealed. In the control group infected by *Escherichia* (group 10) we observed the inhibition of the phagocytic process in the blood. In other control group (11), infected with salmonella such influence in monoculture was not revealed, while in association with bacteroides they contributed to a decrease in the phagocytic activity (group 9). The administration of useful bacteria had a positive influence on the content of vitamin C in the liver of chickens from groups 3-6, which increased respectively by 5.9, 3.3, 5.4 and 9.1 mg% compared to data obtained after 3 days of the infection with *Escherichia*. The increase of this level was also observed in other groups (except for groups 1 and 2, where it was found a decrease by 3.5 and 2.2 mg %). The tested microorganisms positively influenced the haemoglobin level in groups 2, 3, 8 and 9, as well as the lysozyme activity of the blood serum in all experimental groups (except for group 5).

Thus, the obtained data demonstrated the efficiency of new associations of beneficial microorganisms (bifidobacteria, lactobacteria and bacteroides) for the health of the gastrointestinal tract of birds and for improving the general condition of the avian organism.

DISCUSSIONS

The wide use of antibiotics in the livestock industry has led to the evolution of antimicrobial resistance to pathogens, thus contributing to the increase of intestinal dysfunctions cases, causing enormous damage to the animal husbandry sector. At the same time, recently, in veterinary medicine, in particular in avian medicine, studies that reveal the efficacy of probiotics in the elimination of pathogenic microbes from the gastrointestinal tract have been documented. This principle or concept of probiotics mechanism of action is called competitive exclusion.

In this context, probiotics are used, as an alternative to antibiotic treatment, in the control of intestinal pathogens in farm animals. The process of pathogen elimination by probiotic administration has also been found in poultry. It has been shown that hatched chickens can be protected from intestinal infections (salmonella colonization) by dosing a suspension with intestinal content from healthy adult chickens (LUFTUL KABIR, 2009). Probiotics and their consumption, either as direct "fed microbials", "and nutritional supplements" (SMITH, 2014) have a broad spectrum of beneficial impact, such as promoting growth and production, immune enhancement and health protection (ABD EL-HACK et al., 2020). The prompt use of probiotics immediately after birth is more important and useful for bird species than for other animals, because, the chickens, unlike other animals, lack contact with their mother or other adults from which they can receive beneficial intestinal bacteria. Thus, the only way to colonize the intestine is supplementation with microbial preparations designed to restore the protective intestinal microflora (FULLER, 2001).

The species of microorganisms currently used in probiotic preparations are varied, the most widespread being the *Lactobacillus* and *Bifidobacterium* bacteria. Other bacteria can also be used as probiotics, such as: *Streptococcus thermophilus*, *Enterococcus faecium*, *Enterococcus faecalis* (LUFTUL KABIR, 2009; FIJAN, 2014). Some probiotics are microscopic fungi, such as yeast strains belonging to the species *Saccharomyces cerevisiae* (FIJAN, 2014). However, different types of probiotics can have different effects.

For these reasons, associations of beneficial microorganisms (bifidobacteria, lactobacteria, bacteroides) were used in the performed experiments. It has been shown that the associations of different species of microorganisms, as well as of monoculture strains of these bacteria, are the most effective in inhibition of pathogenic intestinal microbes (*Escherichia* and *Salmonella*). Thus, the greatest effect of inhibition of pathogenic microbes was found in experimental groups 5-8, especially in group 6 where an association of bifidobacteria and lactobacteria strains was used (Table 1). A stronger action of pathogen suppression was also revealed in group 7, where the combination of lactobacteria and bacteroides was tested (Table 1). The effect of competitive exclusion of pathogenic microorganisms is greater when useful bacteria are administered additionally, in a higher dose and for a longer period of time.

It has been shown that the beneficial effect of probiotics is achieved not only by the competitive exclusion of pathogens, but also by other mechanisms that influence metabolism, digestion and immunity (APATA, 2008). Data on non-specific immunity parameters confirm the advantage of using different types of microorganisms as probiotics in order to improve the health of the digestive tract and of the whole organism of birds (Table 2).

It has been established that a combination of useful microorganisms must be used in order to eliminate or inhibit pathogenic microbes from the intestinal microbiota of birds, according to a scheme that provides the administration of a higher number of microbial cells in one dose, over a longer period of time.

CONCLUSIONS

The administration of useful bacteria has a more obvious effect when they are additionally applied according to the scheme: in the amount of 100 billion microbial cells for 15 days, over 1 day.

The selected microorganisms, in the used associations and scheme, contributed to the inhibition of the gut pathogenic microbes, to the increase of the general resistance of the organism and, respectively, of the vitality of experimental animals.

The new associations of useful microorganisms (bifidobacteria, lactobacteria and bacteroides) can be used as probiotics to colonize the gastrointestinal tract of farm birds (chickens), pet birds and other captive-bred birds, in order to exclude pathogens.

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Bogdan Victoria, Vrabie Valeria
The Institute of Physiology and Sanocreatology,
1 Academiei str., Chișinău, Republic of Moldova.
E-mails: valvrabie@yahoo.com: victoriabogdan@gmail.com

Received: April 02, 2021
Accepted: August 17, 2021