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Use of Carbon Materials In Medicine

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Abstract: Immobilization of *Lactobacilli* on sorbents made by carbonizing husk at high temperature lead to increase in physiological activity and production of antibacterial metabolites resulting in higher antagonistic activity of *Lactobacilli*. It is implied that the use of nanosorbents for immobilization of probiotic microorganisms may be very promising for solution of some important problems such as probiotics delivery and adsorption to intestinal mucosa with subsequent normalization of its microecology.Besides, carbonized sorbents with nanostructural surface are capable of adsorbing and in this way detoxifying toxic shock - LPS.

Key words: Lipopolysaccharides · Endotoxin · Carbonized rice husk · Immobilization

INTRODUCTION

Normal human microflora maintains biochemical, metabolical and immunological equilibrium, which is essential for health [1]. Intestinal microflora plays the key role in colonial resistance of the host's intestines with respect to pathogenic and opportunistic microorganisms. Gut microflora biological equilibrium is disturbed by various endo- and exogenic factors. As a result a clinical laboratory syndrome may arise which is characterized by gastrointestinal upset defined as disbacterioses [2].

Over the past years one can observe an increase in the number of people with severe diseases caused by gram-negative bacteria endotoxins which represent non-secreted heat-stable lipopolysaccharides (LPS) that are the main components of the cell's outer membrane. The endotoxin is released when pathogenic microorganisms die which results in general infections with the endotoxic shock in the most severe cases. Gram-negative gut microflora is the constant source of LPS in human organism. Normally in healthy persons small amounts of LPS entering the bloodstream are considered to be necessary to support immunity. When human gastrointestinal microecology is disturbed one can often observe an increase in the number of gram-negative opportunistic enterobacteria thatare transferred to other organs resulting in endotoxinemiya [3].

Probiotics are the main therapeutically for correction of intestinal microbiocenose. Probiotics represent preparations of living microorganisms and their metabolites which have positive physiological, biochemical and immunological effects on the host organism via optimization of the host's microbial ecosystem when introduced in a natural way [1]. Since lactobacilli are the essential component of the normal microflora which is usually the first to decrease in number when disbacteriosis develops it is obvious why lactobacilli-containing probiotics are used to re-establish intestinal microbiocenose [2].

Probiotics are primarily designed to improve microecological condition of the large intestine. However the precalculated activity of such preparations is usually significantly reduced on passage through upper parts of the gastrointestinal tract. That is why it is necessary to develop completely new approaches not only to the production but also to the delivery of probiotics to target organs. The solution to this problem may be found in new techniques in biotechnology based on the use of

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immobilized preparations in which bacterial cells are adsorbed on the surface of a carrier. Among these of special interest are the carbonized sorbents withnanostructural surface which manifest not only high affinity to bacterial cells but also detoxifying activity [3]. It means that such sorbent will act as carrier for probiotical microorganisms and at the same time neutralizes various toxins which enter the gastrointestinal tract.

It can expect that an increase in probiotical activity as a result of a more effective delivery of health-giving exogenic bacteria to the large intestine will allow re-establishing intestinal microbiocenose relatively quickly and efficiently by means of elimination of pathogenic and opportunistic microflora. On one hand such technique will bring to the decrease in the number of gram-negative bacteria and on the other hand will reduce the amount of endotoxins due to the sorption on the carrier.

Since the range of probiotical preparations with immobilized lactobacilli cells is extremely limited and nanostructured sorbents have not been used for these purposes the main goal of this study was to investigate adhesive and detoxifying properties of new carbonized sorbents with nanostructured surface with the aim of developing probiotical preparations and detoxifying agents capable of binding toxic shock lipopoly saccharides.

MATERIALS AND METHODS

Lactobacillus fermentum AK-2 strain with good probiotical parameters i. e. high antagonistic and adhesive activity [5] was used in this work. A nanostrucured sorbent produced by high temperature rice husk (RH) carbonization in the Combustion Research Institute of KazNU named after al-Faraby was used to immobilize the test strain [6].

Immobilization was performed during 24 hours. Then the carrier was flushed with isotonic solution to remove loosely adsorbed cells and used in the subsequent experiments. Sorption efficiency was derived from the difference between the cell counts in the culture medium before and after sorption [7]. The JCXA-7334 scanning electronic probe microanalyzer was used for visual evaluation of modified sorbents surfaces and their interaction with bacterial cells [8].

Experiments to assess the immobilized cells probiotical activity were performed using 6-8 weeks old not purebred rats with disbacteriosis induced by the antibiotic ciprofloxacin [5]. For this purpose the test animals had been divided into 3 groups after a bacteriological examination. Ciprofloxacin was administered to all rats but in the first group only the antibiotic was used whereas in the second group free lactobacilli cells of the above-said strain were introduced along with ciprofloxacin. For this purpose an aqueous suspension of lactobacilli cells at the concentration of 10^8 cells/ml was introduced intergastrally during 10 days after the etiotrops therapy. At the same time lactobacilli cells of the strain in question immobilized on the carbonized rice husk (CRH) were introduced in the third group of test rats. Analysis was done on the expiration of 24 hours after ciprofloxacin introduction was cancelled and after 15 days from the start of the experiment. In accord with the aim of the experiment enterobacteria were quantified in different parts of the gastrointestinal tract.

Antimicrobial activity of the culture medium used for growing free and immobilized lactobacilli was estimated by agar diffusion test. The following enterobacteria from the KazNU chair of microbiology museum were used as test strains: *Escherichia coli K-12*, *Klebsiellapneumoniae AK-2, Proteus vulgaris Z-53, Proteus mirabilis 54*, *EnterobacteraerogenesE-12*, *Salmonella typhimurium P-1*, *Shigellazonnei A-22*, *Shigellaflexneri A-2*. Antimicrobial activity level was assessed by the inhibitory zones size. One more method was applied when enterobacteria strains were grown along with free and immobilized *Lactobacillus fermentum AK-2* cells. In this case antimicrobial activity was estimated by viable test strains cells counts.

Lipopolysaccharide concentration in the culture medium was determined photometrically in microtablets with the help of QCL-1000 Chromogenic LAL Endpoint Assay kit (Lonza Group Ltd, Switzerland) and photometric scanner (Bio-Rad Co., USA).

RESULTS AND DISCUSSION

The carrier material pore size is very important for efficient adhesive immobilization. Sorbents with pores 2-3 times larger than microbial cells are considered the most suitable for these purposes [9]. One can see on Fig. 1 (A) that RH natural surface is very solid and doesn't have any pores.

The sorbent surface undergoes changes in the process of high temperature carbonization. A more complex and solid structure with a large specific surface is formed. Pores increase in number and size, new ones appear, two or more pores can merge into one, pores surface and volume change (Fig. 1- B, C).

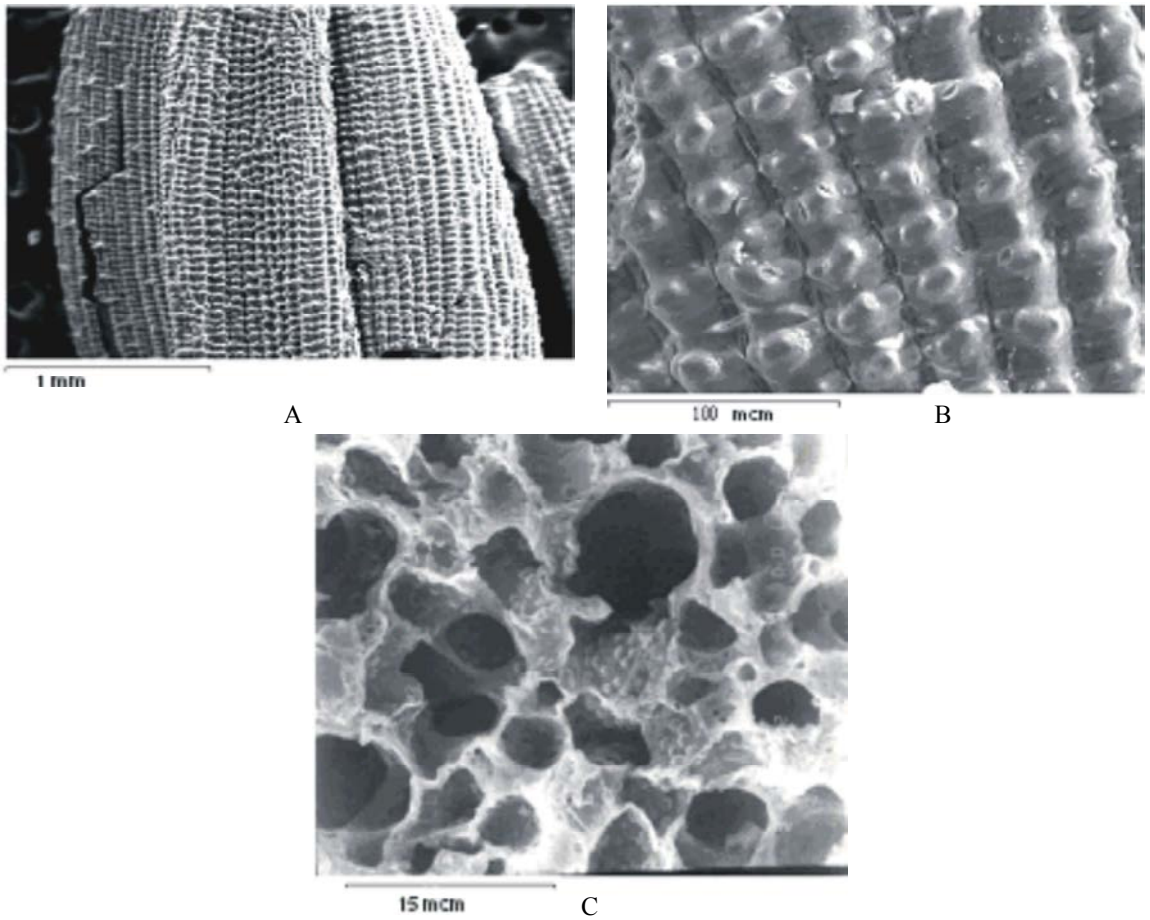

Fig. 1: Rice husk surface, natural (A) and carbonized at 650°C (B, C). Electron microscope image

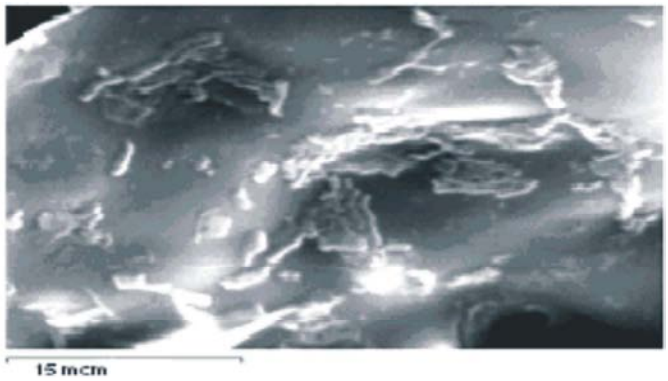

Fig. 2: *Lactobacillus fermentum* AK-2R microcolonies adsorbed on the CRH surface

It can see on rice husk surface electron microscope images that a regular structure which consists of rows of cells appears after carbonization. Lactobacilli cells fit well into them (Fig. 2).

Sorptional interaction between microbial cells and carbonized material has been revealed by electron microscopy. One can see that the cells are not scattered one by one over the CRH surface but form microcolonies.

The fact may be of essential importance because intercellular aggregation in microcolonies is the initial stage in the formation of biofilms in which bacteria are much better protected against various adverse factors. Besides their metabolic activity is higher as it is controlled in mcirocolonies and biofilms by "Quorum sensing". This system is called so because it coordinates the genes that are expressed only at a certain density of a microbial

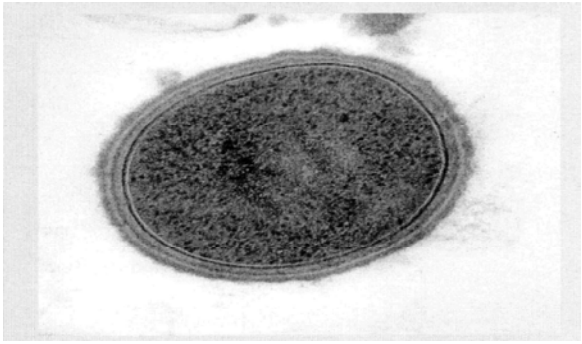

Fig. 3: Ultrathin S-layer *Lactobacillus acidophilus* D75 (foto: O.V.Rybalchenko) [2]

population (not less than 10^7 cells/ml). The system also controls the genes which code for enzymes involved in the production of some antimicrobial factors such as bacteriocines and microcines [2].

Cellular loading on the sorbent is pretty high and reaches 62 % with 10^8 adsorbed cells/g at an optimal cells/carrier ratio. The ability of microorganisms to fix themselves on a carrier is determined by their adhesive properties. Adhesion includes a number of stages in which microbes become fixed on or stick to the surface. At the first stage non-specific mechanisms are involved based on a number of physical, chemical and biological factors of which hydrophobic interaction is the most important one [10]. Hydrophobic is one of the most important features of the cell surface. It underlies such biological processes as biofilms formation, microbial cells adsorption on solid surfaces andhost organism tissues [11, 12]. However these long-range disperse interactions as they are do not result in a firm sorption. They just provide optimal thermodynamic conditions. Sorption becomes stronger due to specific covalent, electrostatic bonds and other interactions within the direct contact zone in which various reactive clusters on the surface of the carrier take part. Thus, phenolic, carboxylic, carboxylic, esteric, enolic and various types of lactonic clusters have been found on the surface of carbonized nonmaterials [13]. At the same time phosphoric, amines, sulphohydrilic, phenolic, hydroxyl, carboxylic, clusters are present on the surface of lactobacilli cells [14]. Special of importance for the adhesion processes is the so-called S-layer which is found in many lactobacilli (Fig. 3).

The S-layer consists of non-glycolized proteins which contain primarily asparagus and glutamic acids [2, 14]. This means that peptide bonds may be formed between the cells and the carrier surfaces (Fig. 4).

Lactobacilli have a number of other surface biopolymers. Glycoprotein's, polysaccharides, teyhoice acids also have various functional clusters [15]. Carbohydrate composition of lactobacilli glycopolymers is pretty varied but D-mannose, D-galactose, L-phucose, D-glucose, N-acetyl-D-glucoseamine, N-acetyl-D-galactoseamine, sialic acid can always be found [16]. These glycocalix components can stand out of the fragmented S-layer [17]. This may cause the cell surface charge to change which often enhances interaction between oppositely charged clusters on the surface of disperse particles (Fig. 5).

When the cells interact with these particles various types of bonds and their combinations can arise, namely: electrostatic, hydrogen, covalent, hydrophobic [18]. Besides, new binding siteswhich may exhibit a higher specificity towards lactobacilli cells emerge on the surface of carbonized sorbents during the high temperature treatment.

Immobilized bacterial cells are known to be more resistant to various negative environmental factors. In our experiments high stomach acidity was one of such factors. Most microbial cells die on passage through this part of the digestive tract. Gastric juice obtained by gastroscopy was used to study free and immobilized cells sensitivity. The juice was added to a lactobacilli culture in MRS-1 medium with 10^9 cells/ml. The culture was then incubated for one hour and viability measured afterwards.

Lactobacilli biotitre (viable cells concentration) in the liquid suspension is reduced by a factor of 10^4 after exposure to gastric juice. This means that only a small portion of viable lactobacilli cells in a suspension taken orally reaches the large intestine. For this reason such a suspension should be considered a valuable source of enzymes and vitamins but not a medicine designed to colonize intestines with lactobacilli.

In the experiments with the "stomach model" and immobilized lactobacilli cells it has been revealed that microbial cells adsorbed on the CRH are to a certain extent protected against the action of gastric juice. Most probably this phenomenon is ascribable to a membrane which covers every microcolony as a whole and provides additional protection against bactericides and adverse environmental factors [2]. Therefore immobilized probiotics surpass liquid cell suspension in resistance to gastric juice significantly and are capable to easily overcome the "stomach" barrier when taken orally.

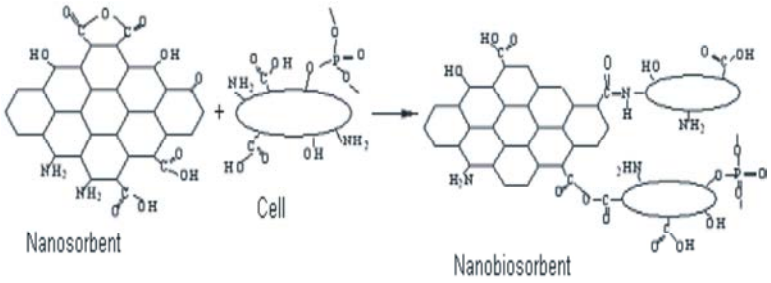

Fig. 4: Interaction of microbial cells with a carrier surface

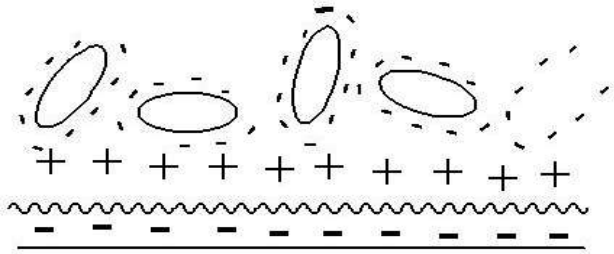

Fig. 5: Interaction of microbial cells with a carrier surface

Table 1: Enterobacteria number in laboratory animals with ciprofloxacin-induced disbacteriosis

	Bacteria number in 1 g (*M*±*m*)			
	Largeintestine		Smallintestine	
Group of animals	Wall	Cavity	Wall	Cavity
	Beforeantibioiticstherapy			
Control	$(2,8\pm0,4)\times10^4$	$(7,2\pm0,6)\times 10^6$	$(0,9\pm0,2)\times10^2$	$(1,5\pm0,2)\times10^3$
	24 hours after the end of therapy			
Withoutprobioitic	$(3,1\pm0,5)\times10^5$	$(8,1\pm0,7)\times10^7$	$(3,5\pm0,3)\times10^4$	$(1,2\pm0,4)\times10^5$
LLS	$(0,7\pm0,6)\times10^5$	$(1,2\pm0,3)\times10^7$	$(8,4\pm0,4)\times10^4$	$(9,1\pm0,5)\times10^5$
ProbioticonCRH	$(7,1\pm0,5)\times10^5$	$(1,0\pm0,2)\times10^7$	$(7,9\pm0,3)\times10^4$	$(8,2\pm0,6)\times10^5$
	15 days after the end of therapy			
Withoutprobioitic	$(2,1\pm0,4)\times10^5$	$(1,2\pm0,3)\times10^8$	$(8,4\pm0,6)\times10^4$	$(9,5\pm1,1)\times10^5$
LLS	$(6,1\pm0,7)\times10^4$	$(9,6\pm0,8)\times10^7$	$(3,1\pm0,4)\times10^3$	$(2,1\pm0,9)10^4$
ProbioticonCRH	$(4,2\pm0,6)\times10^4$	$(8,5\pm0,7)\times10^6$	$(2,2\pm0,3)\times10^2$	$(1,2\pm0,7)\times10^3$

Note: LLS - liquid lactobacilli suspension; CRH - carbonized rice husk

Table 2: *L. fermentum* AK-2 free and CRH-immobilized cells antagonistic activity in joint cultivation with enterobacteria

	Viable test strain cells number, %		
Variants	*SalmonellatyphimuriumP-1*	*ShigellazonneiA-22*	*Proteusvulgaris 3-53*
ProbioticonCRH	5,8	4,6	2,3
Probiotic	17,5	26,5	9,6
CRH	67,5	70,3	72,5
Withoutprobioitic	100	100	100

Another advantage of immobilized lactobacilli is that their microcolonies on carbon sorbents possess an ability to adhere relatively quickly to the intestinal mucous membrane [2]. As a result therapeutical effect of such probioticsin the treatment of disbacteriosis shows faster. Disbacterioses with an increased number of gram-negative bacteria particularly lactose-negative *E. coli* and other opportunistic enterobacteria attract special attention [3]. In order to test therapeutical effect of lactobacilli immobilized on CRH an experimental disbacteriosis was induced in laboratory animals with the help of a of phtorhinolones antibiotic ciprofloxacin. The use of this

medicine brings to an excessive number of enterobacteria in the large intestine of experimental animals. Enterobacteria are transferred to the small intestine resulting in bacterial overgrowth syndrome [5] accompanied by endotoxinemia which in its turn leads to the cytokine chain activation and toxic shock [3].

Enterobacteria number was determined by plating homogenized small and large intestines walls on Endo agar before the antibiotic administration, 24 hours and 15 days after the end of therapy. This data as well as data on enterobacteria quantification in rats which CRH-immobilized probiotical cells were fed to following antibiotic therapy are summarized in Table 1.

It was established that opportunistic enterobacteria number on the wall and in the cavity of the small intestine increased significantly (by a factor of 10^2) after 5 days of antibiotic therapy. At the same time bifidobacteria and lactobacteria number fell drastically.

Situation was different in rats which got liquid lactobacilli suspension. *Enterobacteria* number on the wall and in the cavity of their small and large intestines was much smaller after the end of antibiotic therapy and lactobacillifeeding than in rats which didn't get probiotics.

The use of lactobacilli immobilized on CRH was even more effective in suppressing antibiotic-induced disbacterosis. Opportunistic enterobacteria number in this group decreased by a factor of 10^2 whereasbifidobacteria and lactobacteria number returned to normal.

It's been established that a colony of at least 20 bifidobacteria cells is needed to colonize 1 mm^2 of the intestinal mucous membrane. Under the electron microscope one can see lactobacilli colonies of 20-200 cells on the surface of carbonized rice husk sorbent particles. Such number provides conditions for cells to remain viable and multiply in the community. The results obtained testify that indigenous bacteria colonize intestinal mucous membrane more efficiently when introduce into the host organism in the form of microcolonies adsorbed on the sorbent.

Decreasing in enterobacteria number in animals of this group can be related with CRH-immobilized lactobacilli antimicrobial activity and with a more effective enterobacterial LPS sorption by the sorbent. Antimicrobial activity is the most important characteristic of probiotic effectiveness. The fact was a good reason to investigate the influence of immobilization on this parameter. Two methods were applied to compare antimicrobial activity of *L. fermentum* AK-2 free and immobilized cells. First an inhibitory zone was measured on an agarmedium surrounding holes with liquid culture medium used to grow free and immobilized antagonist strain cells. 8 species of *Enterobacteriacea* family were used as target (test) strains (Fig. 6). LPS sorption by the sorbent. Antimicrobial activity is the most important characteristic of probiotic effectiveness. The fact was a good reason to investigate the influence of immobilization on this parameter. Two methods were applied to compare antimicrobial activity of *L. fermentum* AK-2 free and immobilized cells. First an inhibitory zone was measured on an agar medium surrounding holes with liquid culture medium used to grow free and immobilized antagonist strain cells. 8 species of *Enterobacteriacea* family were used as target (test) strains (Fig. 7). It was found that CRH-immobilized cells antimicrobial activity increased by 25-60 % depending on a test strain. The result was confirmed with the use of another technique. In this case the number of viable enterobacteria test strain cells was determined following combined cultivation of the test strain with free or immobilized lactobacilli at the concentration of 10^8 CFU/ml. The sorbent without any cells was used for control. *Lactobacilli* were added to 10 ml suspension of test strains (10^8 CFU/ml) i. e. the cultures ratio was 1:10.

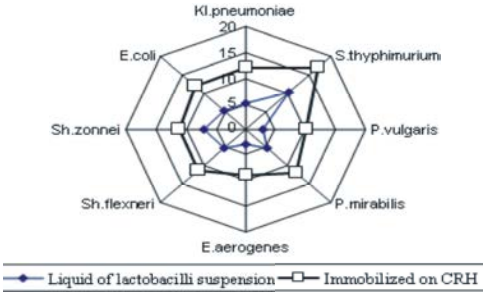

Fig. 6: Antimicrobial activity of *L. fermentum* AK-2 free and CRH-immobilized cells with respect to enterobacteria

It is seen from the table that free lactobacilli cells obviously suppress the growth of all 3 test strains. At the same time the sorbent itself can bind up to 28-33 % of enterobacteria cells. However probiotic immobilized on CRH is more effective in suppressing test strains. Test strains growth and viability tends to zero within 48 hours of combined cultivation.

As it was mentioned earlier carbonized nanostructured sorbents have a high specific surface (per unit of mass) resulting in a high adsorption capacity. We measured CRH adsorption capacity for LPS (Fig. 7). Since LPS causes acute toxic shock its binding to the sorbent means that the latter is capable of detoxifying LPS.

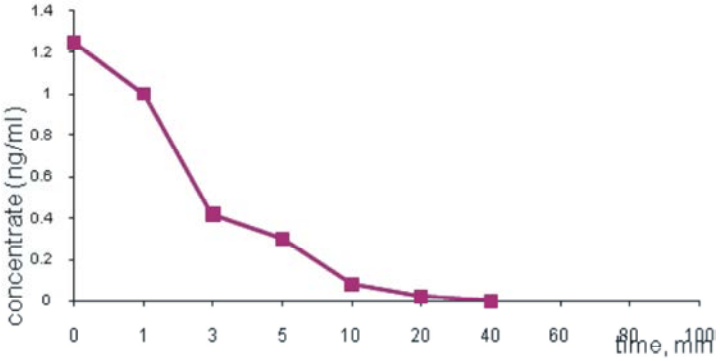

Fig. 7: Adsorption of LPS on CRH

It can see on the graph on figure 8 that LPS concentration is reduced due to adsorption on CRH by 90 % within the first 10 min. After 40 min the test substance in not detected in the solution at all. This means that CRH is a promising material for detoxification of the toxic shock LPS.

Thus it's been found upon investigation that the use of *Lactobacillus fermentum* AK-2R strain cells immobilized on CRH returned experimental animal's microbiocenose to normal. The enhancement of the positive probiotical effect by immobilization may be ascribable firstly to lactobacilli cells fixed on the surface of a sorbent surviving in the upper parts of gastrointestinal tract. This means that CRH takes part in the delivery of a probiotic to target organs. Secondly antimicrobials are produced in larger amounts when lactobacilli exist on the surface of a sorbent in the form of microcolonies and "quorum sensing" is induced. In the third line the sorbent itself exerts sanogenic effect. Finally, lactobacilli immobilization provides a dense local epithelium colonization which in its turn promotes faster normoflora rehabilitation and speeds up reparation processes in the intestinal mucous membrane.

Carbonized sorbents with nanostructured surface are promising materials for immobilization of probiotical microorganisms since they provide an opportunity to solve a complex problem of delivering a probiotic to its destination, colonizing intestinal wall with the following gastrointestinal tract detoxification and microecology normalization.

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