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A technology of experimental studies on the xenobiotic element sorption characteristics of representatives of the intestinal normal flora

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ABSTRACT

Modern views on the general level of intoxication of an organism by heavy metals are based solely on the extent of effects on organs and organ systems of macroorganisms while the impacts on representatives of the normal flora and their role in the organism's mechanisms of detoxification as a whole are not taken into account. We proposed technology of experimental studies based on a complex variety of methods. The technology allows estimating the degree of effects of xenobiotic elements on representatives of the intestinal normal flora and their sorption characteristics, which is an important factor in the assessment of mechanisms of detoxification in oral intoxication. The obtained experimental data indicate high sorption characteristics of lead cations in the studied microorganisms, which, in our view, is primarily associated with microorganisms' mechanisms of detoxification. The study found that the most pronounced bioaccumulative characteristics for lead were registered in *Escherichia coli* (65% of the total volume of the elements introduced in the substrate) and, in the case of cadmium, – *Enterococcus faecium* (33.2%). The minimal accumulation scores were revealed for *Lactobacillus acidophilus* (42.3% and 5.3%, respectively).

Keywords: *technology of sorption characteristic assessment, normal flora, microbiota, xenobiotics, heavy metals.*

1. INTRODUCTION

Nowadays, only cadmium, lead, mercury, and antimony are unequivocally considered as toxic elements. Activities of other numerous heavy metals in biological systems vary. It is true that there are no harmful substances – there are harmful concentrations [1].

The role of lead ions as a biogenic element is not great; it is known that lead participates in the metabolic processes of the bone tissue. In the environment, lead is widespread as a result of natural processes, including erosion, forest fires, the decay of radon, and as a result of human activities – waste from ore extraction, burning leaded fuel [2, 3, 4].

But there is a group of metals which vital necessity has not been revealed. They include cadmium [5, 6]. It is known that cadmium is an active antagonist of several essential elements such as copper and zinc. Mutual antagonism between these elements is a consequence of their isomorphic substitution in biological systems. Namely, increased consumption of one of these elements can cause deficiency of another owing to the substitution of it in some functional sites of binding [7, 8, 9].

Some metal ions are essential for microorganisms. When viewing the patterns of interaction of heavy metals with microorganisms, one should also take into account the toxic effects of heavy metal ions on microorganisms [10]. Metal accumulation by microorganism cells is of two-phase nature. The initial phase is not dependent on the energy status of the cell and is caused by sorption of metal by components of the cell wall [11].

Summarizing the above, we set the aim to develop a technology of xenobiotic sorption characteristic evaluation of normal flora representatives in the intestine.

2. MATERIALS AND METHODS

As a xenobiotic factor, we used salts of lead and cadmium with high levels of dissociation in aqueous solutions. Applying these salts enables to create maximal concentrations of cations of the elements in a nutritious substrate. Selected and identified representatives of the normal flora of the intestine served as the research objects.

To achieve our aim, we implemented the following methods of research:

1. The method of microorganism selection and identification. The necessity of applying this method lies in the fact that the intestinal microbiota is represented by a variety of microorganisms. Toward this end, we extracted a fragment of intact rat intestines in compliance with the rules of asepsis for selection of representatives of the optionally anaerobic normal flora. One gram of that content was introduced in 10 ml of the physiological solution, and then it was thoroughly pipetted with subsequent transferring one ml into the next test tube with the same amount of the isotonic solution. Only eight shifts were made (this was necessary for the maximal dilution with a view to determining the number of colony-forming units (CFU)). The contents of the tubes in the volume of 50 µl were transferred into sterile Petri dishes filled with 1.5% beef-extract agar followed by incubation at the

temperature of 37°C for 24 hours. As the period of cultivation finished, cell cultures were counted and identified on cultural characters and tinctorial properties.

The next phase of the research was to obtain isolated colonies (strains) of the studied microorganisms – the pour plate method was implemented for this. To solve the task, we did passaging of grown colonies on 1.5% beef-extract agar (MSR) with an inoculation loop by the method of thinning dash on four sectors with subsequent processing at the thermostat at 37°C for 24 hours. The passaging was held up to obtaining morphologically identical isolated cultures.

The final step of this task was to identify microorganisms up to the species level. For this, we utilized conventional techniques based on cultural, morphological, tinctorial, and biochemical properties. In order to assess the biochemical characteristics of the isolated strains, we used commercial test-systems by the company BioMerieux. The final criterion of relating selected strains to certain species was based on the summation of the sign totality on Bergey's Manual.

2. Method of consecutive (serial) dilutions. We applied two variants of it: in dilution 1 to 10, which was mentioned in the previous technique, and in dilution 1 to 1. In the second case, the technique was used to determine inhibiting and sub-inhibiting concentrations of heavy metal salts.

To complete this phase of the study, we used a liquid nutrient broth (MRS agar), 13 sterile test tubes for each series of studies, aqueous solutions of heavy metals and a suspension of the studied microorganisms in the isotonic solution of NaCl in the concentration of 0.5 by McFarland (McFarland standard). For the preparation of aqueous solutions of salts of the metals, weighted amounts got dissolved in 100 ml of distilled water at a rate of 0.02 mmol/ml with subsequent autoclaving at the temperature of 121°C for 15 minutes and brought to pH values equal 7. The experiment was carried out in 10 replicates for the purpose to obtain significantly meaningful results.

3 ml of MSR was injected in tubes except the first one (where 5 ml of the medium and 1 ml of a solution of a metal salt at a rate of 0.02 mmol/ml were brought to). The content of the first tube was thoroughly pipetted and then 3 ml was transferred into the second tube, from the second to the third, and so on until the tenth which 3 ml was removed from. This manipulation was carried out with the aim of reducing the concentration of each tube by two times. To evaluate the quality of the research, each series had three controls: the control of the medium, microbial growth, and the metal; in the case of the growth in the first and the third controls, experimental data were not taken into account. The first ten tubes received a suspension of microorganisms in the volume of 30 µl with subsequent incubation of the content for 24 hours at the temperature of 37 °C. The evaluation of the inhibitory action of different concentrations of heavy metals was carried out by two methods: a visual assessment of growth and seeding from each tube on firm nutritive media.

3. The colorimetric method. This method was used to assess the effects of xenobiotic elements on the growth of the studied microorganisms in batch culture. To implement this technique of research, we had previously prepared sterile 100 ml bottles, a sterile liquid nutritional medium (MSR), daily culture of the studied microorganisms and sterile solutions of metals (in accordance with the technique described above). Experimental studies were conducted in ten replications.

Isolated strains were incubated in the liquid nutrient medium using the batch method of cultivation. Four bottles were filled with MSR agar, a suspension of microorganisms in the concentration of 0.5 by McFarland (McFarland standard), and the working concentration of xenobiotic elements composed of salts with a high level of dissociation in aqueous solutions (the first concentration with no sub-inhibiting effect). Like in the serial dilutions method, we utilized three control samples allowing the qualitative assessment of the research; in case of deviations in the control samples, the results were not taken into account.

The colorimeter CFC-2 was used for the analysis. The mean values of the series of measurements were considered as the result of the analysis. The measurement of the optical density of the bacterial suspension was carried out with an interval of three hours starting with a background measurement. The duration of the study was determined by the time of the beginning of the M-phase of growth concentration in the studied strains as evidenced by the presence of three relatively close values of the relative optical density of the suspension in different time intervals. During the period of incubation, the microorganisms were at the thermostat at 37 °C set on the shaker for constant stirring.

4. Atomic absorption spectrophotometry. The use of this technique was the final stage of our study. The method is based on the property of atoms formed when ash solutions are sprayed in acetylene-air flame to absorb light of a specific wavelength. As atomic absorption spectrophotometer (AASP), there was used the device AAS-1 (Germany) with a set of spectral lamps.

For the preparation of the samples, the sterile bottles were filled with 400 ml of sterile MSR, working concentrations of heavy metals and 4 ml of a suspension of the studied microorganism followed by incubation at 37°C till the stationary phase of growth in batch culture. After the cultivation, the content of bottles was poured into sterile test tubes and centrifuged for 30 minutes at 3000 rpm. The supernatant was separated from the biomass with an automatic pipette, the biomass of the studied microorganisms was lysed with 5% solution of KOH and further held in a water bath at 96 °C for 20 minutes (for the complete destruction of cells). Both the biomass and the supernatant were exposed to the analytical study for the purposes of determining concentrations of metals in both samples. The presence of metals in the biomass of cells indicates the sorption characteristics of the studied microorganisms. The mean values of ten series of experiments were converted into percentages in the form of concentrations. A loss in the biomass and the supernatant was determined by calculating the difference between the values of the injected concentration of the metal and the amount of its content in the biomass and the supernatant.

5. All the data were subjected to statistical processing by calculating the means and the errors of the mean, one-way

ANOVA. The significance of the results was proved using Student's t-test between the experimental and the reference values.

3. RESULTS

During the preliminary study aimed at selecting and identifying representatives of the intestinal microbiota of lab rats, we selected four microorganisms that accounted maximal concentrations of CFU: *Lactobacillus acidophilus*, *Escherichia coli*, *Enterobacter cloacae*, and *Enterococcus faecium.*

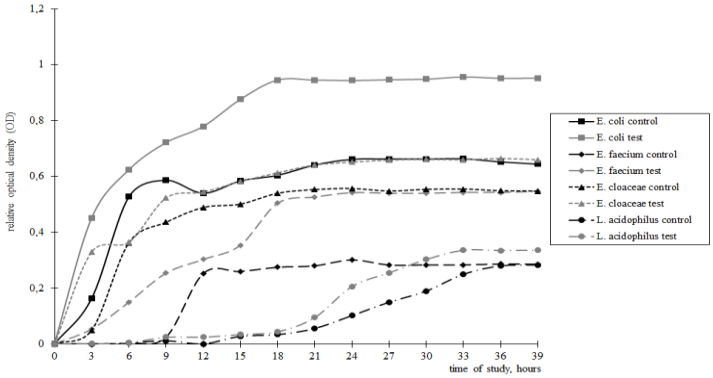

Figure 1. Influence of $Pb(NO_3)_2$ on representatives of the optionally anaerobic microbiota of rats' intestines.

Studies of evaluating the resistance of the studied strains of microorganisms to xenobiotic factors (table 1) revealed a general trend of relative resistance to cations of lead (from 0.005 to 0.00125 mmol/ml) and pronounced sensitivity to cadmium (from 0.0003 to 0.0001 mmol/ml).

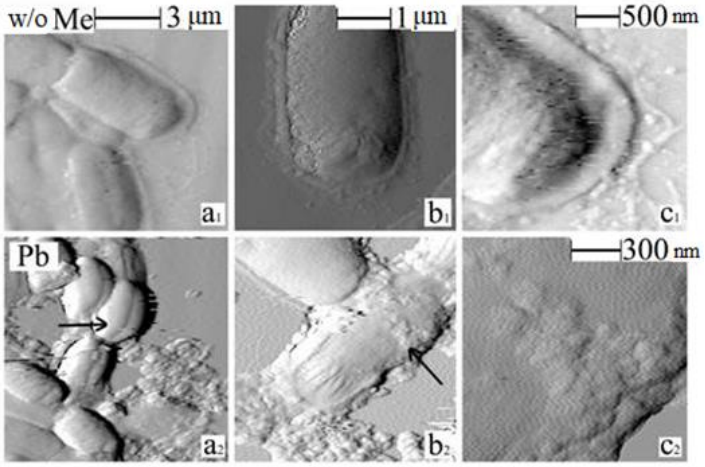

Figure 2. Photos of microorganisms without metals and with lead. Scanning atomic force microscopy. a1, b1, c1, — without metal; a2, b2, c2 — in the presence of lead salts (Peshkov et al., 2015).

From the presented in the table data, it should be concluded that the most resistant to lead strains are *Escherichia* coli and *Enterococcus faecium* (sub-inhibiting concentration of 0.005 mmol/ml), while the level of sustainability to cadmium had values 0.0003 and 0.0001 mmol/ml, respectively. *Lactobacillus acidophilus* and *Enterobacter cloacae* have lower values of resistance to lead (0.0025 and 0.00125 mmol/ml) and similar to the other two strains' values of sensitivity to cadmium (0.0003 and 0.0001 mmol/ml).

The studies allowed us to define the working concentrations of salts of xenobiotic elements for further research. So they amounted to 0.0025 mmol/ml of $Pb(NO_3)_2$ and 0.0001 mmol/ml of $CdSO_4$ for *Escherichia coli*, for *Enterococcus faecium* – 0.0025 and 0.00005 mmol/ml, for *Lactobacillus acidophilus* – 0.00125 and 0.0001 mmol/ml, for *Enterobacter cloacae* – 0.00063 and 0.00005 mmol/ml, respectively.

Table 1. Evaluation of the level of resistance of studied microorganisms to the effects of various concentrations of lead and cadmium.

Strain	Salts	Concentration, M/l							
		0.02	0.01	0.005	0,0025	0,00125	0,00063	0,0003	0.0001
Escherichia coli	$Pb(NO_3)_2$	-	-	±	+	+	+	+	+
	$CdSO_4*8H_2O$	-	-	-	-	-	-	±	+
Enterococcus faecium	$Pb(NO_3)_2$		-	±	+	+	+	+	+
	$CdSO_4*8H_2O$	-	-	-	-	-	-	-	±
Lactobacillus acidophilus	$Pb(NO_3)_2$	-	-	-	±	+	+	+	+
	$CdSO_4*8H_2O$	-	-	-	-	-	-	±	+
Enterobacter cloacae	$Pb(NO_3)_2$	-	-	-	-	±	+	+	+
	$CdSO_4*8H_2O$	-	-	-	-	-	-	-	±

«–» - inhibiting concentration
«±» - sub-inhibiting concentration
«+» - lack of inhibiting action

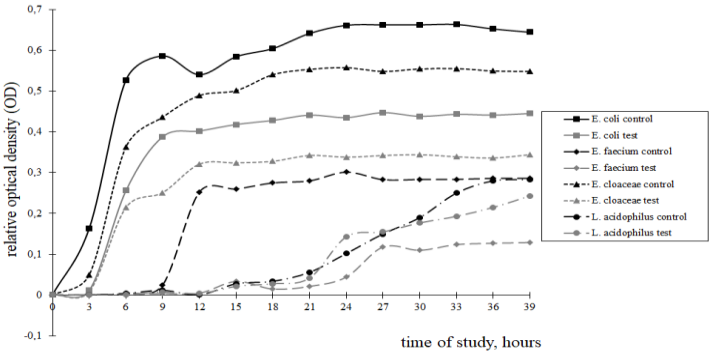

Figure 3. Influence of $CdSO_4$ on representatives of the optionally anaerobic microbiota of rats' intestines.

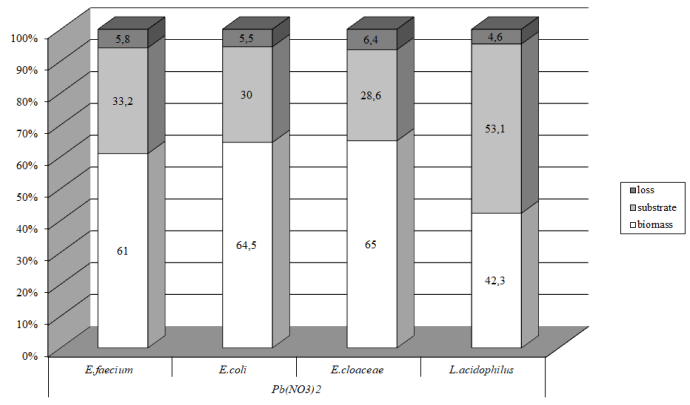

Figure 4. Accumulation of lead ions from the nutrient substrate in representatives of the rats' optionally anaerobic intestinal microbiota.

The next phase of our research aims to assess the extent of influence of the studied heavy metals on growth of the studied microorganisms and the time of the beginning of the M-phase of growth concentrations (Figures 1, 3). The process of metal accumulation occurs in the stationary growth phase, this is due to the fact that this phase includes substrate depletion and accumulation of toxic products forcing bacteria to search for alternative sources of energy and detoxification of the environment.

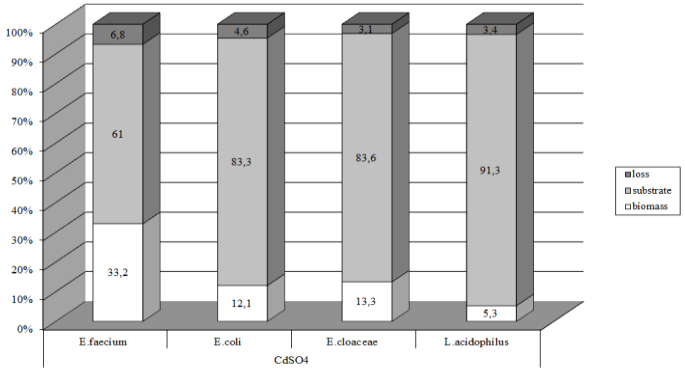

Figure 5. Accumulation of cadmium ions from the nutrient substrate in representatives of the rats' optionally anaerobic intestinal microbiota.

The data from Figure 1 show a pronounced degree of influence of lead on growth of microorganisms while the values of the relative optical density of the experimental groups were significantly higher. This may be associated with detoxification mechanisms of bacterial cells. According to S.A. Peshkov (2015) [12], atomic force microscopy found that bacteria are capable to actively accumulate metal particles on its surface (Figure 2) and, as a consequence, it will distort data on the influence of lead on growth of the studied microorganisms.

Visually on the graphs, lead stimulates growth, but when seeding the studied microorganisms, the CFU value is lower than the control ones, which, we believe, is associated with a high concentration of introducing a lead salt with a high level of dissociation in aqueous solutions and cation accumulation on the surface, which, in turn, affects the values of relative optical density. It was recorded that the presence of lead in a nutritious substrate had no significant effect on the time of the beginning of the stationary phase.

The analysis of the data presented in Figure 3 shows a pronounced inhibiting action of cadmium cations on the growth of the studied microorganisms.

The final phase of the study was to assess the sorption characteristics of representatives of the optionally anaerobic normal flora in rats' intestines (Figures 4, 5).

Presented in the figures data showed the expressed sorption characteristics of the studied microorganisms with respect to cations of the studied metals. Lead is the most actively extracted from the substrate at the surface of a bacterium from 65% (*Escherichia coli*) to 42.3% (*Lactobacillus acidophilus*), which correlates with data obtained in previous experiments. The sorption level of cadmium cations (Figure 5) had lower values from 33.2% (*Enterococcus faecium*) to 5.3% (*Lactobacillus acidophilus*).

4. CONCLUSIONS

The conducted experimental studies indicate high sorption characteristics of the studied microorganisms. It should be noted that the level of sorption characteristics is of individual (species) nature and depends on biochemical features of the species, in our view.

The technology of evaluation of biotoxicity and accumulative characteristics of microorganisms can be used not only for studying the mechanisms of detoxification, but also for designing and synthesis of new antibacterial compounds based on essential elements of pathogenic microorganisms, which currently has great practical significance at seeking for alternative methods of chemotherapy of infectious diseases taking into account the minimization of negative effects on the normal flora of the organism of humans and animals, as well as for elemental status biocorrection.

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