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PERSPECTIVES OF GENUS BACILLUS-BASED PROBIOTIC STRAIN APPLICATION IN THE CORRECTION OF COPPER-DEFICIENT STATES

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ABSTRACT

Mineral-deficient states largely occur in humans and animals because of the nutrition imbalance. One of the criteria of low copper content in food is a feature of the geological province which produces agricultural products [4]. One of the criteria of the cell elemental status regulation of an organism can be associated with the use of probiotic microorganisms that possess not only high sorption characteristics but also are able to deposit excessive content of essential elements in the biologically active form. To assess the perspectives of the application of probiotic microorganisms of the genus Bacillus as micronutrients we used such methods as the agar basin method relating to diffusion

methods, it allows us to estimate the biotoxicity of copper chemical compounds; the colorimetric method allows to study the impact of copper cations on the microorganisms based on optical density changes; the method of atomic absorption spectrometry let us rate bioaccumulative characteristics of studied bacteria, as well as determine the metal content in the tissues of the investigated animals [5]. Obtained in the study data indicate a higher level of resistance to the copper in the probiotic strain B. amyloliquefaciens 10642, which showed resistance to $Cu(CH_3COO)_2$ concentration in 0.063 mol/l, for B. subtilis 10641 the concentration of 0.03 mol/l is non-toxic. Assessing the impact on the growth of this microorganism we found that excessive concentration does not make a distinct influence, as we see from the close values to the control group. High storage characteristics were also shown by this microorganism, which allows supposing the direct correlation between sustainability and accumulation. In vitro studies were confirmed by in vivo results which revealed effective application of micronutrients on the basis of probiotic bacterial strains for correction of mineral-deficient copper states.

Keywords: copper, probiotic, micronutrients, mineral-deficient state correction

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1. INTRODUCTION

Mineral substances entering the body of animals during feeding participate in the processes of digestion, metabolism, blood circulation, as well as they affect the protective reactions of an organism. Copper plays a great role as one of the obligatory and normable trace elements [4].

Copper deficiency cannot always be overcome by the introduction of high doses of micronutrients, since entering the digestive tract in forms of salts and oxides, it reduces the absorption of other elements. As a structural component of hormones, copper provides growth, increase cellular immunity, and also participates in metabolic processes [5, 6].

Other researchers showed that copper deficiency can lead to the impaired synthesis of elastin and collagen. Copper belongs to strong cytoplasmic poisons, but a small quantity of it is required for the production of hemoglobin (despite the fact that copper is not included in its composition). Copper deficiency in organism violates iron absorption and life duration of red blood cells, it increases the speed of iron metabolism in plasma and embedding of it in erythrocytes, it develops microcytic anemia [7, 8].

Copper gets out of body mainly with bile, the excretion is intense, and so copper is needed to constantly arrive with feed. However, copper surplus is harmful because it violates hematopoiesis.

Mineral-deficient states largely occur in humans and animals because of the nutrition imbalance. One of the criteria of low copper content in food is a feature of the geological province which produces agricultural products. One of the criteria of the cell elemental status regulation of an organism can be associated with the use of probiotic microorganisms that possess not only high sorption characteristics but also are able to deposit excessive content of essential elements in the biologically active form [4].

In this work, we propose the use of probiotic strains for delivering copper into organisms of lab animals. It is known that bacteria of the genus Bacillus have the ability to accumulate metals; they are able to extract and concentrate metals [12, 16].

On the basis of the above data, we set the goal to study the possibilities of alternative application of micro-organisms' probiotic strains in the system of copper delivery. Earlier we obtained data on the application of probiotic preparations "Sporobacterin" and "Bactisubtil" for the iron and zinc delivery into organisms of lab animals. We discovered that the inactivated probiotic strains deliver a deficient element into the tissues of body.

2. MATERIALS AND METHODS

For the experimental part of the study we chose the following probiotic strains: *B. subtilis 10641* ("Vetom 1.1", manufacturer – LLC SPC "Issledovatelsky Tcentr" (Russia)), *B. amyloliquefaciens 10642* ("Vetom 3", manufacturer – LLC SPC "Issledovatelsky Tcentr" (Russia)). As the source of the essential element in the first phase of the study we used copper chloride, copper sulfate and copper acetate; a common criterion for selection of these salts was their high level of salt dissociation in aqueous solutions and, as a consequence, the creation of copper cation peak concentrations in substrates. As objects of study *in vivo* model, we used the Wistar line rats under conditions of the vivarium of the Institute of bioelementology of Orenburg State University. Treatment to animals and performing procedures during the studies we carried out in accordance with the requirements and recommendations of Russian rules (order of Ministry of the health of the USSR № 755 from 12.08.1977) and "The Guide for the Care and Use of Laboratory Animals (National Academy Press Washington, D.C. 1996)". To perform experimental studies we selected 96 rats, which formed 4 groups of identical counterparts by sex, age, and weight. We caused mineral-deficient states in animals to assess the effectiveness of the high copper bioavailability drug application (that level of bioavailability we got from cultivation the studied microorganisms in the presence of redundant copper cation content in the substrate ($Cu(CH_3COO)_2$). Three of the studied groups were on diet consisting of distilled water and boiled-off within 15 minutes polished rice (followed by distilled water backwashing) for 20 days before the start of the experiment and throughout the period of the study [11]. In order to avoid the avitaminosis development, we injected complex multivitamin preparations into the diet of animals. The group of intact animals was on diet in accordance with the requirements and recommendations of Russian rules (order of Ministry of the health of the USSR № 163 from 10.03.1966).

The first block of experimental research aimed to assess the bioaccumulative ability of probiotic strains of microorganisms and identification of working copper acetate concentrations to create an experimental preparation. For the implementation of this phase of the research, we used the diffusion method of agar basins. This method combines two methods: the method of basins in the agar layer and the method of serial dilution. As the regulating factors in the work, we used various copper salts (chloride, sulfate, and acetate). The advantage of this method is a visual estimate of the toxicity of chemicals in different concentrations under identical conditions, in addition, this technique is not only of quality but also quantification of biotoxicity of the studied chemical compounds [10].

To determine the optimal time for metal sorption from nutrient substrate we observed the influence of copper salts with a various anionic component on the growth of the studied microorganisms. The necessity for this study can be explained by that in the process of growth in periodic culture bacteria are forced not only to search for alternative sources of energy and substrate due to the depletion of the nutrient substrate, and also to turn on the mechanisms of detoxification from secondary metabolites and other toxigenic factors. Based on this, one of the stages of our investigation was to determine the start of the stationary phase of growth in the

presence of copper salts with various anionic components. We used the colorimetric method to solve this task [9].

To evaluate the effect of copper on growth of the studied microorganisms and the time of the stationary phase of growth we carried out measuring the optical density of the bacterial suspension with an interval of three hours starting from the background research, and continued till we received three identical values, which indicated the beginning of the stationary phase of growth. We conducted Gram's stain at all points of the study to assess the purity of the experiment. Aiming to visualize the obtained results on assessing of the copper impact on the growth of probiotic strains and the time of the beginning of the stationary phase we drew growth curves of the studied microorganisms using Microsoft Excel. Hereat each point of the curves represented an average of three measurements in parallel observations.

The final phase of the first block of the experiments was to evaluate copper ion sorption characteristics of probiotic strains. To implement this task we used atomic absorption method based on a property of atoms of chemical elements, formed when sprayed ash solutions into acetylene-air flame, absorb light of a specific wavelength. Studies were performed using atomic-absorption spectrophotometer AAS-1 (GDR) with a set of spectral lamps [20]. For the preparation of the samples we conducted a preliminary cultivation of the studied microorganisms in liquid nutrient medium with the addition of working copper concentration (absence of bactericidal and bacteriostatic effect), we did periodic cultivation before the beginning of the stationary phase of growth followed by division of the vial content into biomass and supernatant using centrifugation within 10 minutes at 3000 rpm. Next, the supernatant was separated from biomass by automatic pipettes. Biomass of bacteria was lysed by 5% solution of KOH and further boiling in the water bath within 20 minutes. We analyzed both the supernatant and the biomass. Mean value of a series of measurements was supposed to be the result of the analysis.

Findings that we received in the first block of experiments allowed us to embark upon the second (basic) block of research aimed at investigating the prospective use of probiotic preparations with accumulating ability to create products with a high level of copper bioavailability.

We conducted preparation of test samples by means of cultivation of the studied probiotic strains of microorganisms in a liquid substrate in the presence of working metal concentrations before the beginning of the stationary phase of growth. However, at this stage, there arose the need to inactivate microorganisms for maximum release of the essential element and in order to avoid competition with macroorganisms. The technique of making the preparation was adding the slurry of daily culture of studied probiotic strains of micro-organisms to liquid nutritional medium of the working copper acetate concentrations of 0.063 M/l for *B. amyloliquefaciens* 10642 and 0.03 M/l for *B. subtilis* 10641 with the subsequent cultivation for 24 hours at a temperature of 37° C. Biomass of bacteria was derived in a similar way as in the preparation technique for atomic adsorption studies with subsequent inactivation of microorganisms by autoclaving for 40 minutes. The biomass was used for individual introduction into laboratory animals per os.

We formed four groups of animals counterparts – two control ones and two test groups with 24 animals in each for the realization of tasks on the assessment of the prospective use of probiotic strains of microorganisms as a regulatory factor of elemental status with the development of copper-deficient states. The first control group was on a mineral-deficient diet (K_1). The second control group of intact animals was on a normal diet and served as a criterion score of the physiological norm (K_2). The test groups, like the group K_1, were on the mineral diet resulting in the development of copper-deficient states for 20 days. In the 1st test group (O_1) we injected (within 10 days from the start of the experiment) into the diet the biomass of inactivated *B. subtilis* 10641 (Vetom 1.1) after their cultivation in liquid nutrient medium in the presence of excessive

amounts of $Cu(CH_3COO)_2$ in dosages: O_1 – 1 ml per a head. In the second test group (O_2) we used *B. amyloliquefaciens* 10642 (Vetom-3) as the source of the copper with analogical injection and dosage. The study was conducted by means of the comparative method of biological research, i.e., animals were in identical conditions and at the same time.

The impact of the experienced preparation application was carried out using atomic absorption spectrophotometry. For this purpose throughout the experiment, we were selecting biomaterial with a periodicity of 5 days (a background study, the fifth, the tenth, and the fifteenth days). We used bone and muscular tissue, as well as cutaneous covering as the biological material of the studies.

All the obtained results were subjected to statistical processing by using Student's t-test to assess the reliability of the data.

3. RESULTS

We conducted the series of experiments aimed at assessing the biological toxicity of copper salts with a various anionic component on the growth of the studied microorganisms to reach our objectives to study the use of probiotic preparations as a source of copper with high bioavailability.

The study found that all investigated compounds have expressed bactericidal effect (Figure 1, 2) in respect of probiotic strains. This mechanism of action in our view is that copper belongs to the group of essential elements and, as a result, microorganisms have no mechanisms of detoxification for this element. As it is known [8], there are two mechanisms of interaction with heavy metals in prokaryotes: detoxifying and depositing.

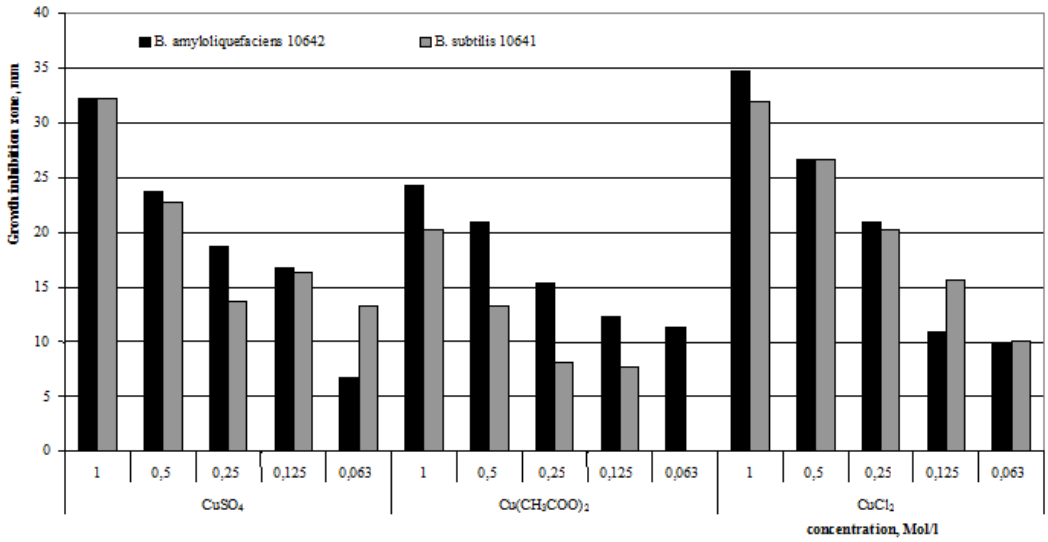
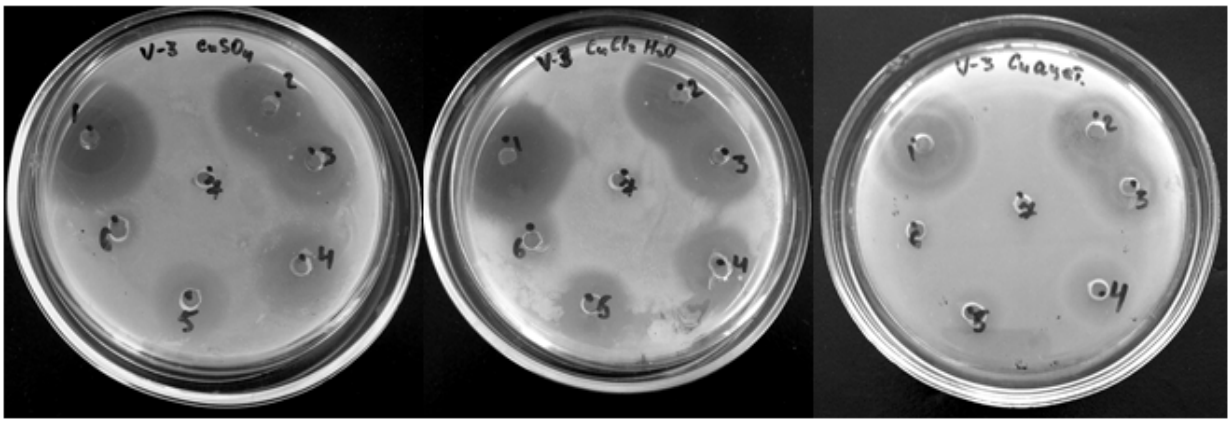


Figure 1 Studying biological toxicity of copper in salts with the various anionic component to the probiotic strains of microorganisms

Storage of the elements takes place mainly on surface structures of cells as non-toxic compounds, metal adsorption process on the surface of microorganisms includes linking it with the cell wall, cytoplasmic membrane, and also with substances of capsules and extracellular excretions. The primary interaction is mainly connected with the negative charge of these surface structures.



1 **2** **3**

1 – $CuSO_4$, 2– $CuCl_2$, 3 – $Cu(CH_3COO)_2$

Figure 2 Assessment of the impact of copper salts composed of cations with a different anionic component on the growth of *B. amyloliquefaciens* 10642

Received data (Figure 1) indicate pronounced toxic effects of the investigated salts. It should be noted that the bacterial strain of *B. amyloliquefaciens* 10642 has distinct resistance to copper acetate and this microorganism faces no toxic effect compared to *B. subtilis* 10641 in the concentration of this compound of 0.063 mol/l. Sulfate and chloride of copper in all the studied concentrations provided pronounced bactericidal effect, their values of growth suppression zones exceeded the same value of copper acetate for *B. subtilis* 10641 in 37.1% and 36.5%, respectively, *B. amyloliquefaciens* 10642 in 24.7% and 29.9%, respectively. The exception was *B. amyloliquefaciens* strain 10642, for which copper chloride turned out to be more toxic.

One of the defining parameters of heavy metals accumulation from substrates with periodic cultivation is the determination of the time when the stationary growth phase starts (Figure 3).

The obtained experimental data (Figure 3) show the pronounced activity of all observed salts on the growth of bacterial strains of microorganisms. It should be noted that the presence of redundant copper concentrations in the substrate has a significant impact on both the growth of the population as a whole and the start time of M concentration of growth. Copper chloride discovers the most pronounced sub-inhibiting effect on the growth of the studied microorganisms, its presence gives the minimum values of the population density of the bacterial strain of *B. subtilis* 10641. The closest values to the control of growth of the studied microorganisms were obtained in samples cultured in the presence of $Cu(CH_3COO)_2$. The obtained data confirm preliminary studies evaluating the biotoxicity of the observed salts on the growth of probiotic strains.

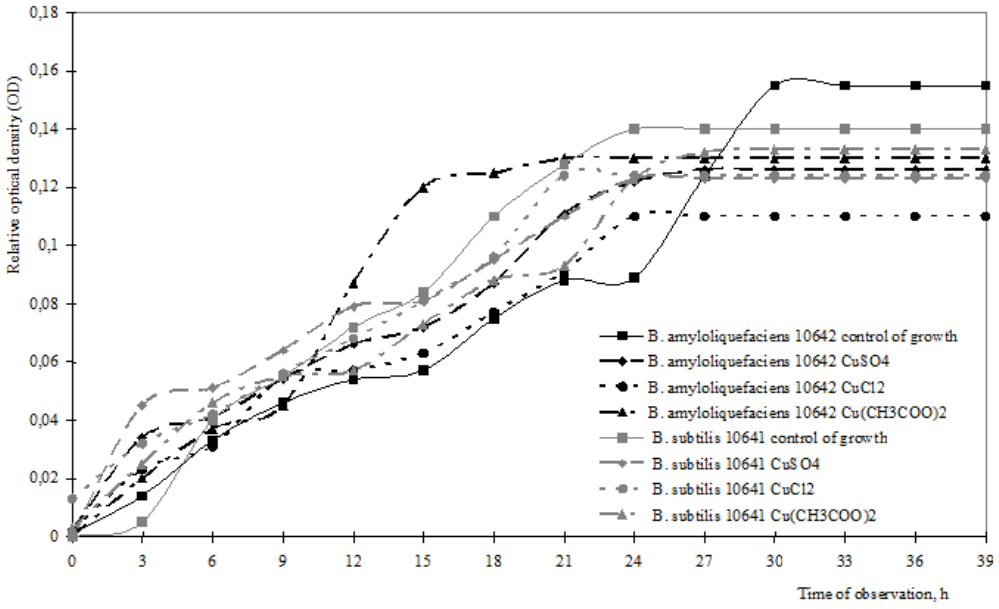


Figure 3 Assessment of the impact of copper salts composed of cations with a different anionic component on the growth dynamics of the studied microorganisms in the periodical culture

The obtained data on the influence of copper on the growth of the studied microorganisms enabled us to determine the time of cultivation of microorganisms for the maximum accumulation of deposited copper. For both of the studied strains, we selected 39th hour of incubation in the presence of copper salts as the basic time interval for sampling.

The next stage of our research was to study the bioaccumulative capacity of bacterial strains (Figure 4) in the presence of excessive copper cation contents in the liquid substrate. We carried out our studies of the content of copper in the composition of the liquid substrate to exclude the possible impact on any of other factors. The minimum concentration of the observed element determined by atomic absorption spectrophotometer is 0.00006 m/l, copper content was not revealed in the liquid substrate. The concentration of copper injected in the nutritious medium as salts ranged from 0.03 to 0.063 m/l.

The experimental data show that both of the investigated strains extract copper cations from the substrate with sorption levels of 10.29%, 10.10%, and 9.55% out of the nutrient mediums containing $CuSO_4$, $CuCl_2$, и $Cu(CH3COO)_2$ for *B. subtilis* 10641. For *B. amyloliquefaciens* 10642, these values amounted to 4.60%, 4.50%, and 13.97 percent, respectively.

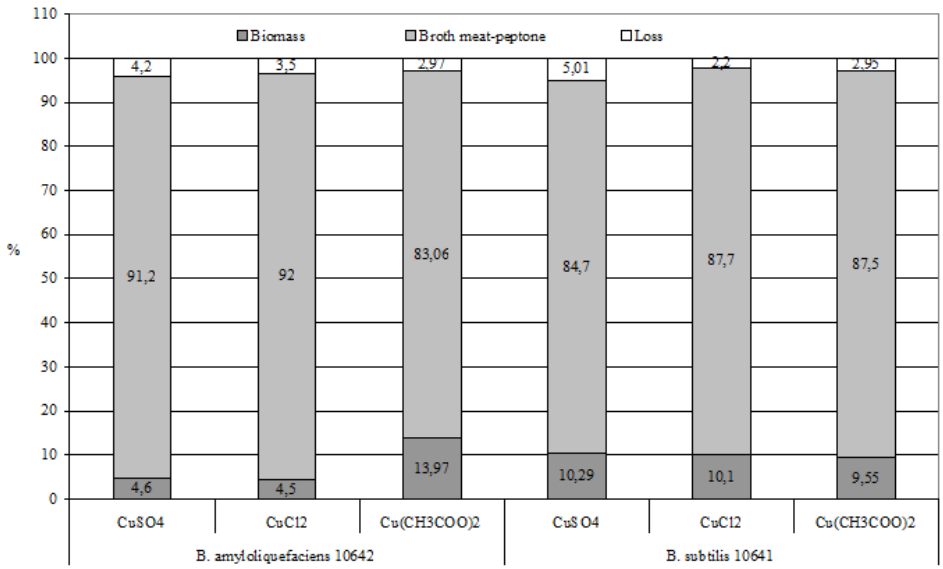


Figure 4 Studying ion copper accumulating characteristics of probiotic strains from salts with a various anionic component

Generalizing the obtained data, we should note that in the course of our research we discovered the consistent pattern between the toxicity of copper ions in salts with a various anionic component, their impact on the population growth in the periodic culture and the sorption characteristics.

In vitro experiments let us identify the most promising source of copper for a further study aimed at evaluating perspectives of probiotic strain application to create preparations that contain copper in a biologically available form. We chose $Cu(CH_3COO)_2$ as a source of copper for both strains.

At the final stage of our studies, we assessed the effectiveness of probiotic transient strain application in creating products with high levels of biologically available copper (table 1).

Analysis of the obtained experimental data allows us to postulate the effectiveness of the application of the resultant prototypes used as micronutrients.

Obtained in experiments data indicate the development of copper-deficient states in groups of experimental animals (K_0, O_1, and O_2). The total content of the analyzed element in the biological samples of these groups was 32.14% lower than that of the intact animal group (K_1).

Using test preparations as a regulatory factor of copper-deficient state correction for 10 days allowed us to bring close the values in the experimental groups to those of the intact animals.

Table 1 Evaluation of the copper concentration in the tissues of laboratory animals at different times of study, µg/kg

Group	Background study	In 5 days	In 10 days	In 15 days
Copper concentration in the cutaneous covering				
K_0	0,24±0,01	0,24±0,013	0,23±0,01	0,24±0,012
K_1	0,41±0,02	0,40±0,023	0,39±0,022	0,40±0,021
O_1	0,24±0,012	0,31±0,018	0,36±0,011*	0,34±0,024
O_2	0,24±0,01	0,32±0,012	0,37±0,013*	0,32±0,015
Copper concentration in muscular tissue				
K_0	0,37±0,08	0,36±0,02	0,36±0,022	0,36±0,012
K_1	0,52±0,04	0,51±0,03	0,49±0,02	0,50±0,014

O_1	0,37±0,02	0,39±0,024	0,46±0,024*	0,42±0,021
O_2	0,37±0,01	0,43±0,02	0,49±0,024*	0,42±0,023
Copper concentration in bone tissue				
K_0	0,34±0,02	0,34±0,012	0,33±0,01	0,33±0,012
K_1	0,47±0,01	0,46±0,03	0,48±0,02	0,47±0,023
O_1	0,34±0,012	0,39±0,02	0,45±0,03**	0,43±0,024*
O_2	0,33±0,014	0,40±0,01*	0,46±0,024**	0,43±0,024*
*p < 0,5; **p < 0,05; ***p < 0,005 Comparison of group K_1 to the experimental groups (O_1 and O_2)				

The data we found indicate a significant increase in the concentration of copper ions in biological samples by the 5th day of the experiment. So, at this stage of the study, the copper content in the experimental groups exceeded that value of the copper deficiency state group (K_0) to 14.74% and 21.05% for groups O_1 and O_2. On the 10th day of the experiment, there was the difference between these groups estimating as 33.68% and 38.95% for O_1 and O_2, respectively. At the final stage of this experiment (from the 10th to 15th day), the animals of the test groups were stopped getting the experimental preparations with maintaining them on a mineral-deficient diet. The data received at the final point of research in the experimental groups indicate a 6.30% (O_1) and 11.36% (O_2) decrease in the concentration of the analyzed metal compared to the 10th day of the experiment.

4. CONCLUSION

Summarizing and interpreting the results, we should note that in the course of the experiments *in vitro*, we revealed the consistent pattern between the toxicity of copper ions in the structure of salts with a various anionic component, their impact on the population growth in the periodical culture, and sorption characteristics. From the literature review, it is known that bacterial cells have the two-phase nature of the bioaccumulation of essential trace elements, including metals. The first phase is characterized by biosorption of elements by components of the cell wall and is not related to the energy status of the cell. The second phase is due to the energy-dependent intracellular accumulation involving ion-carrier enzymes [2]. Our studies suggest the relationship between the toxicity of the element, dissociation level of compounds whose structure the element is in, and, as a result, the sorption characteristics.

Biochemical mechanisms of microorganism persistence to ions and compounds of heavy metals can be associated with the mechanism of decrease in accumulation due to competition and exchange with protons on the cell surface. Decontamination of heavy metals is a result of their binding, sedimentation, and transformation into low-toxic and non-toxic forms associated with changing of their valency, demethylation or formation of organometallic compounds [1].

One of the possible versions for the elemental status regulation in organisms of animals and humans is the application of biotic drugs based on waste products of transient microflora with high sorption characteristics [3].

Summarizing the results we obtained *in vivo* experiments, we should note that the probiotic strains of microorganisms as a candidate for correction of mineral-deficient states, in particular, the copper-deficient states. The criterion score for selection of microorganism which is a producer of the essential bioavailable element is a preliminary assessment of its sorption characteristics be the method of agar basins.

Being aimed at developing a micronutrient preparation, our studies allow us to assess perspectives of researches on probiotic preparation application for correction of elemental status in a microorganism and, depending on the research direction, can be used both in the case of deficient states and as detoxification agents.

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