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Influence of Biologically Active Substances On the Basis of Medusomyces Gysevii (The Tea Mushroom) On Bacteria of the Genus Lactobacillus.

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abstract

Symbiont *Medusomyces gysevii* (a tea mushroom) at the expense of a wide range of biochemical components of a microbic origin can meet nutritious requirements of bacteria of the genus Lactobacillus. The significant role of these microorganisms defines requirement of their growing both *in vitro*, and *in vivo*. At an inactivation of microorganisms forming this simbiont and at bringing of substances on the basis of *Medusomyces gysevii* to a form, digestible for lactobacilli, it is important to stop on those methods of technological processing which will provide the maximum growth-stimulating effect. The obtained results demonstrate that the growth-stimulating effect on bacteria of the genus Lactobacillus in vitro is rendered by the nanostructured substance of a tea mushroom added at concentration of 10% of MRS medium volume that it is possible to explain with the better comprehensibility of the nutrients contained in it, and also with existence of the low-molecular stimulating factors.

Keywords: *Lactobacillus*, *Medusomyces gysevii*, tea mushroom, microbic simbiont, zooglea, growth, growth stimulator, prebiotic.

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INTRODUCTION

Bacteria of genus *Lactobacillus* are one of the dominating components of obligate microflora of a human body which takes an active part in regulation of the physiological processes directed to maintenance of a homeostasis of a macroorganism [14]. Lactic bacteria are widespread practically in all biotopes of a human body. They are normal residents of an integument; a mouth, a stomach, a small intestine, a female reproductive path are also populated with them, but the greatest number of these microorganisms is concentrated in a large intestine. The lactobacilli which are a part of normal intestinal microflora participate in formation of such structure of its mucous membrane as a biofilm (bacterial glycocalyx) and cause its high adsorbtive ability [2].

In the process of vital activity lactic bacteria form a number of biologically active agents and enter into multiple interrelations with other microorganisms. They can act inhibitory on causative agents of acute intestinal and vaginal infections, exert impact on various factors of immunity, take part in digestion process [16,19;24].

Their basic significance in microflora of a digestive tract is caused by ability to production of growth components for other eubiotic microorganisms, in particular the bifidobacteria, promoting a stimulation of their growth [17,24].

Thus, bacteria of genus *Lactobacillus*, carrying out various physiological functions, exert positive impact on a macroorganism in general, and stability of course of many vital processes depends on their quantity and quality. Therefore maintenance of a homeostasis of their qualitative and quantitative composition is very important [9,23,25]. The significant role of these microorganisms defines requirement of their growing both *in vitro*, and *in vivo*, with preservation of quality of all properties of the last ones.

The quantity of lactobacilli and their qualitative characteristics directly depend on degree of satisfaction of their nutritional requirements. Lactobacilli need a full range of amino acids, B vitamins, nitrogenous bases, mineral elements, carbohydrates, etc. [5,21].

Now there is an actual problem of search of new substances which can meet nutritious needs of lactobacilli and stimulate their growth, and also to be available and cheap. As a source of such substance the tea mushroom (*Medusomyces gysevii*) can be used.

Tea mushroom (*Medusomyces gysevii*) is a natural symbiont of acetic bacteria and yeasts. As a result of a metabolism these microorganisms form a zooglea in the form of the thick film consisting of cellulose threads (4-6%) and the culture liquid(94-96%) having a rich chemical composition. It includes such amino acids as glycine, valine, histidine, phenylalanine, threonine; carbohydrates of various degree of complexity; a complex of vitamins: A, B1, B2, B15, PP, C, P, K, E; and also compounds of a calcium, potassium, zinc, manganese and phosphorus. Active accumulation of the listed substances, according to many authors, happens on 7-10 days of cultivation [3,13,27,28].

It is very important at the choice of methods of inactivation of microorganisms forming *Medusomyces gysevii* and technological processing of substances on the basis of this symbiont to stop on those which will allow to ensure safety of possibly bigger amount of biologically active agents, and to bring them into the form, most available, i.e. digestible for lactobacilli.

Due to the above, influence of biologically active substances on the basis of *Medusomyces gysevii* developed with application of various methods of technological processing on a bacterium of genus *Lactobacillus* was investigated.

MATERIALS AND METHODS

In experiment have been used the 10-days-old cultural liquid and *Medusomyces gysevii* zooglea located therein, exposed to various technological processing. As a result 6 samples have been received:

- Hydrolyzate of *Medusomyces gysevii* zooglea prepared by enzymatic hydrolysis (the process providing production of the substances, widely applied in microbiology for preparation of traditional bases of nutrient mediums and growth factors for microorganisms);
- The nanostructured cultural liquid of *Medusomyces gysevii* representing a homogeneous product which has been received by triple homogenization of cultural liquid under pressure of 1000 bars that provides destruction of cells and other components to midget particles in the nano range. Production of this sample was performed with use of high-pressure homogenizer APV Lab Series Homogenizers 2000;
- Autoclaved cultural liquid of *Medusomyces gysevii* produced by use of a steam sterilizer of SPVA-75-1NN (Trans-signal) within 15 minutes at a temperature of 121 °C and pH 3,39;
- Autoclaved cultural liquid of *Medusomyces gysevii*, brought to pH 7,0 with use of 20% NaOH;
- Cultural liquid of *Medusomyces gysevii*, exposed to tyndallization on a water bath LAB-TZH-TB-01/26 (MEDLAB, Russia) at a temperature 60°C for 60 minutes quintuply at pH 3,39. Tyndallization is the gentle way of sterilization at which the bigger quantity of biologically active agents persist;

Inasmuch as activity and composition of biologically active agents under the influence of physical and chemical factors depends on pH of environment in which they are, part of samples of cultural liquid before application of various ways of sterilization (autoclaving, tyndallization) were brought to neutral reaction.

The preliminary estimate of sterility has shown that all used methods of technological processing have provided a complete inactivation of microorganisms constituting this symbiont.

As a basis of a nutrient medium the classical MRS-Agar(Base) medium (Germany) was used; besides, this nutrient medium without addition of substances of tea mushroom was used as control. The studied forms of tea mushroom was added in the concentrations recommended in microbiological practice (0,1%; 1,0%; 10% of the volume of a nutrient medium), with the subsequent bringing of pH of environment to 6,2±0,2.

In the experiment was used commercial preparation "Lactobacterin dry", made by Federal State Unitary Enterprise "SPA "Mikrogen" of the Russian Ministry of Health, containing *L. plantarum* 8P-A3, *L. fermentum* 90T-C4.

The taken live cultures at volume of 0,1 ml from cultivation 102 were inoculated on Petri dishes. For each chosen concentration of a certain sample of substance on the basis of *Medusomyces gysevii* three Petri dishes in three iterations were used.

Incubation of lactobacilli was carried out in the thermostat at 37°C within 48 hours.

Calculation of colonies it was made by use of the counter of colonies of microorganisms Scan 100 (Interscience, France).

Microbiological smears prepared from the grown colonies were subjected to Gram's staining.

Visualization of the image of micropreparations was made with use of the specialized photocamera AxioCam MRc5 and the software Zena 2012 Pro (Carl Zeiss Microscopy, Germany).

The microscopy of lactobacilli was carried out with use of Axio Imager 2 (A2) (Carl Zeiss Microscopy, Germany), Axio Observer A1 (Carl Zeiss Microscopy, Germany) and the scanning probe atomic-force microscope (AFM) NTegra Life (Russia).

For atomic-force microscopy bacterial cells after reaching of an exponential phase were collected by washing off from a surface of a nutrient medium, then the bacteria-containing sediment was washed out with use of 0,9% saline to remove surplus of components of a nutrient medium. Process of washing was repeated twice [8,18,]. After that a drop (~ 4 µl) of freshly prepared bacterial suspension was applied on a surface of a fresh chip of a mica sheet. The sample was maintained within 5-7 minutes and then, after small drying, was washed out with use of the deionized water for removal of salt crystals and at once dried by a big stream of compressed air at the room temperature.

Then the samples were carried out for a natural final drying within 1-2 hours. The atomic-force microscopy was carried out in air by means of AFM NTegra Life (NT-MDT, Russia) in the tapping mode with use of cantilevers HA_NC Etalon, resonant frequency 154 kHz, force constant 3,5±20%N/m, curvature radius <10nm. The scanning speed didn't exceed 0,5 Hz. The analysis of the produced images was carried out by means of an application program package Nova Px.

Use of atomic-force microscopy allowed presenting the studied samples in the form of two- and the three-dimensional scans containing information on length, width and height of bacterial cells [1,6,7]. Height was measured by construction of profile of a surface along the line focused in the direction of scanning. As the lower point the level of a mica plate, as top – the highest point of a bacterium were chosen. Width of a bacterium was measured by construction of profile of a surface along the line directed perpendicular to a long axis of a cell. The measure planes were disposed on a cell half-height for leveling of effect of convolution. Length of a cell was measured by creation of a profile of a surface along the line directed on the longest axis of a cage. The measure planes were disposed on a cell half-height. The direction of measurement was chosen randomly [20,22].

In addition the parameter of a roughness (RMS) of a surface of bacterial cell was evaluated with high resolution. Measurements of dimensional characteristics and also of the value of root mean square roughness (RMS) were carried out both on different sites of a mica plate, and on various sites of a surface of a bacterial cell [15].

Statistical analysis

All analyses were performed using the Statistical Package for the Social Sciences (SPSS) for Windows, version 11.0 packed program. Data were presented as mean ± standard. Difference between the control and experimental groups was analyzed using Mann-Whitney U test. $P < 0.05$ was considered statistically significant. Statistical processing of results of research was carried out with use of the program Primer of Biostatistics (Version 4.03).

RESULTS

Subsequent to time of incubation the counting of the grown colonies (Table 1) was carried out. It is established that at addition of an enzymatic hydrolyzate a zooglea of tea mushroom to the extent of 0,1%, 1,0% and 10% of the volume of a nutrient medium, the quantity of colonies of lactobacilli has decreased in comparison with control by 32%, 11% and 64% respectively.

Increase in number of the grown colonies of lactobacilli for 14% in comparison with control is noted on a nutrient medium with addition of the nanostructured cultural liquid of a tea mushroom to the extent of 10% of the total amount of the medium.

Table 1: Quantity of colonies of the lactobacilli grown under the influence of biologically active substances on the basis of a tea mushroom (*Medusomyces gysevii*).

Quantity of substance in a nutrient medium, %	Hydrolyzate of zooglea of Medusomyces gysevii	Nanostructured cultural liquid of Medusomyces gysevii	Autoclaved cultural liquid of Medusomyces gysevii	MRS medium without additions (control)
0.1	25.0±0.5*	36.0±1.2	23.3±0.7*	36.7±0.5
1.0	32.5±0.9*	35.0±0.8	23.3±0.6*	
10.0	13.3±0.3*	41.7±0.8*	28.0±0.8*	

Quantity of substance in a nutrient medium, %	Autoclaved cultural liquid of Medusomyces gysevii, (pH 7.0)	Tyndallized cultural liquid of Medusomyces gysevii	Tyndallized cultural liquid of Medusomyces gysevii, (pH 7.0)
0.1	36.7±0.6	28.3±0.5*	26.7±0.7*
1.0	32.0±0.4*	26.7±0.6*	25.0±0.6*
10.0	31.4±0.7*	34.0±0.9*	23.3±0.5*

*P≤0.05 (in comparison with parameters of control)

On a nutrient medium with addition of autoclaved cultural liquid of a tea mushroom in concentration of 0,1%, 1,0%, and 10% of the volume of a nutrient medium reduction of quantity of the grown colonies of lactobacilli in comparison with control for 37%, 37% and 24% respectively was observed.

At addition of the autoclaved cultural liquid of a tea mushroom brought to pH 7,0 in number of 1,0% and 10% the quantity of colonies of lactobacilli, smaller in comparison with control, for 13% and 14% respectively is established.

The number of the grown colonies of lactobacilli on a nutrient medium with addition of cultural liquid of a tea mushroom exposed to tyndallization in concentration of 0,1%, 1,0%, and 10% of volume of medium, also decreased in comparison with control by 23%, 27%, 7% respectively.

Decrease in quantity of colonies of lactobacilli in comparison with control is noted also at addition of cultural liquid of a tea mushroom exposed to tyndallization with pH 7,0 in concentration of 0,1%, 1,0%, 10% of the volume of a nutrient medium for 27%, 32%, 37% respectively.

The nanostructured cultural liquid brought in number of 0,1% and 1,0% and also autoclaved cultural liquid of a tea mushroom in concentration of 0,1% of the volume of a nutrient medium doesn't render authentically significant, in comparison with control, influence on quantity of colonies of lactobacilli.

Thus, it is established that only the nanostructured cultural liquid of a tea mushroom brought in a nutrient medium in quantity of 10% has the stimulating effect on growth of lactobacilli. For the purpose of deeper studying of influence of the specified *Medusomyces gysevii* substance cultural-morphological properties of lactobacilli have been investigated.

At research of cultural-morphological properties of lactobacilli their invariance in comparison with control is proved: colonies with a diameter of 2-4 mm, convex, with unbroken edge, opaque, not pigmented, lustrous, white colored. At microscopy of smear non-sporing gram-positive bacteria with rhabdoid regular shape of cells, located as single cells and short chains, are observed.

On images made by means of atomic-force microscopy, at fields of vision from 4,0x4,0 µm to 55,0x55,0 µm were visualized both single cells, and groups containing from 12 to 42 cells. At a atomic-force morphometry of cells of Lactobacillus (fig. 1, a) the last ones were found as objects of a rhabdoid form with the roundish ends which dimensional characteristics made 2,46±0,84 µm on length, 0,84±0,22 µm on diameter and 0,28±0,08 µm on height. The three-dimensional image of group of cells (fig. 1, b) and the enlarged image of single cells (fig. 1, c), also confirm compliance of morphological parameters to normative data.

The measured profile of the bacterial surface area (Fig. 1) allowed to fix the value of surface roughness (RMS) of Lactobacillus cells at the level of 4,96±0,89 nm.

The results received by means of AFM, allow characterizing morphometric parameters of the studied cells in general as appropriate to physiological norm.

DISCUSSION AND CONCLUSION

As a result of the conducted research it has been established that the growth-stimulating effect on bacteria of the genus *Lactobacillus* in vitro is rendered by the nanostructured substance of a tea mushroom (*Medusomyces gysevii*) added at concentration of 10% of MRS medium volume that is possible to explain with the best assimilability of the nutrients contained in it, and also with existence of the low-molecular stimulating factors. The data obtained as a result of cultural-morphological researches confirm lack of disorganization of cellular structures of the microorganisms which have grown on the medium with addition of the specified substance. These facts allow to consider nanostructured substance of a tea mushroom (*Medusomyces gysevii*) as potential prebiotic, that is a stimulator of own eubiotic strains of lactobacilli, and as a component of nutrient mediums in case of production of probiotics.

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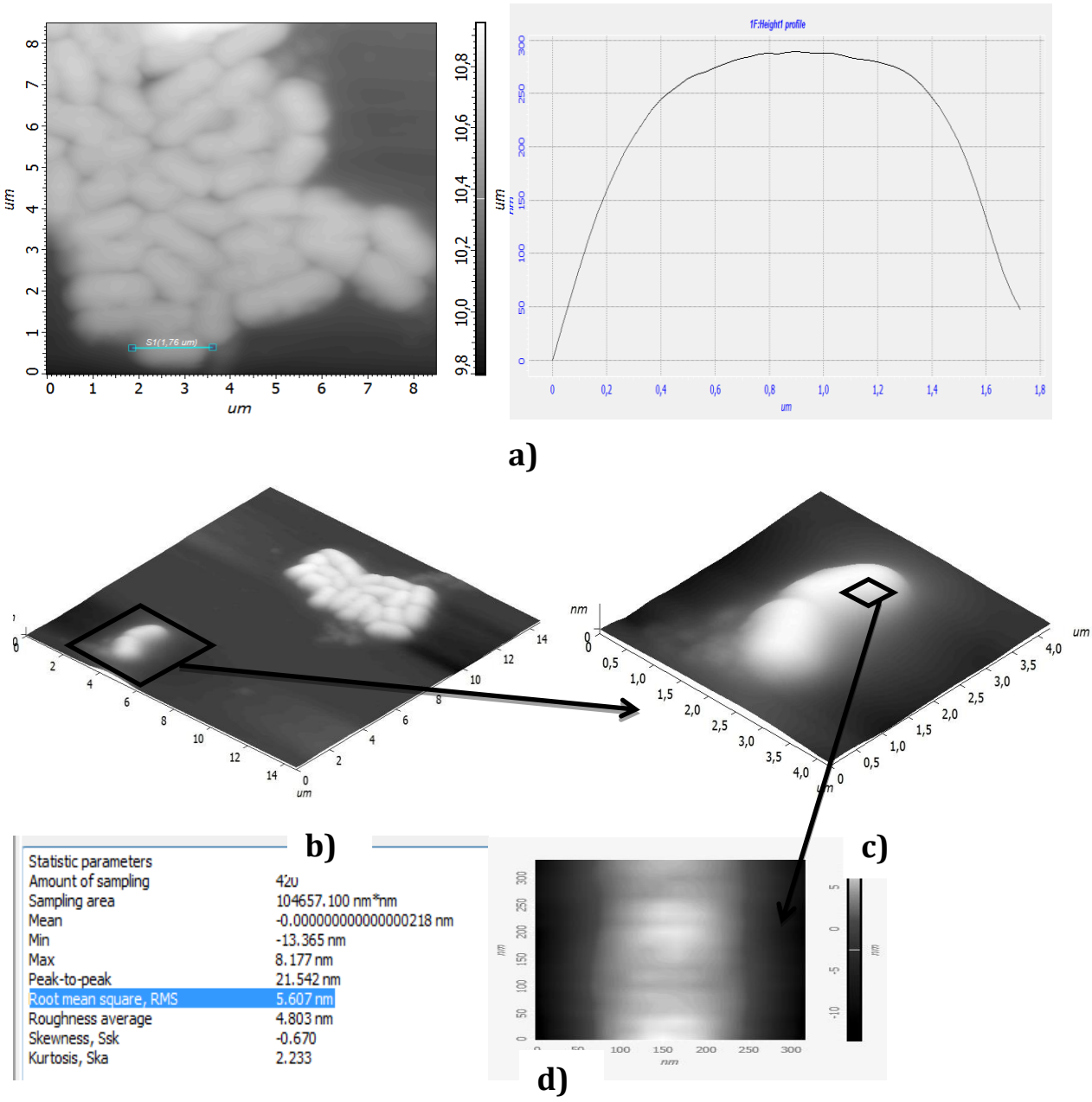

Figure 1. AFM of cells of bacteria of genus *Lactobacillus*. a) 2-dimensional image of group of cells and lateral profile of section; b) 3-dimensional image of group of cells and single cell; c) the enlarged 3-dimensional image of single cells; d) parameters of level of a roughness (RMS) of a site of a single cell.

All other biologically active substances on the basis of *Medusomyces gysevii* produced with application of various methods of technological processing have the inhibiting effect on growth of lactobacilli. The received results correspond with data of other researchers [11] which describe bacteriostatic and bactericidal action of this natural simbiont against of large number of microorganisms.

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