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Influence of biologically active substances from Kombucha (*Medusomyces gisevii)* on rat gut microbiota with experimental antibiotic-associated dysbiosis

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ABSTRACT

The present study was conducted to evaluate the effect of biologically active substances from *Medusomyces gisevii* zoogloea (MG zoogloeas) on intestine microbiocenosis of white rats during the experimental antibiotic-associated dysbiosis. The intestinal dysbiosis of rats was induced by gentamicin sulfate in dose of 10 mg/ rat twice a day for 10 days for all the rats. Animals in the control group received a standard diet recommended by the Institute of Nutrition, the animals of the experimental group received 120 mg of active substance of MG zoogloea/ rat every day during the experiment, in addition to the usual diet. After the termination of the oral administration of gentamicin the animals of the second sub-group (experimental 2) received 120 mg of active substance of MG zoogloea every day during the experiment, in addition to the basic diet.

The animals in the control group showed a dysbiosis symptoms, observed a significant decrease in the total bacterial counts, as well as the decrease of *Bifidobacterium* spp*, Lactobacillus* spp*, Escherichia coli* level starting from the fourth until 10th day of the experiment, the number of *Candida* spp. increased. Animals of the experimental group avoided the symptoms of dysbiosis, reduce of the test groups of bacteria occurred only on the eighth day, and *Candida* level did not rise. After discontinuation of gentamicin administration rats treated with MG showed rapid disappearance of dysbiosis symptoms, the number of microflora started to improve significantly already in the fourth day, while the control sub-group animals showed small increase occurred only on the eighth day. Results indicated that using biologically active substances from *Medusomyces gisevii* zoogloea has positive effects on intestine microbiocenosis of rats during the experimental antibiotic-associated dysbiosis.

Key words: Antibiotic, Dysbiosis, Experimental antibiotic-associated dysbiosis, Gentamicin, Kombucha, *Medusomyces gysevii*, Microbic simbiont, Microflora, Prebiotic, Rat, Tea mushroom, Zooglea

Nowadays it is well known that intestinal microbiota plays a very important role in the general condition and health of the whole organism (Bohnhoff *et al.* 1954, Freter 1954, 1955, 1956, Collins *et al.* 1978, Schrezenmeir *et al.* 2007). It is the collection of microbes that reside in the gastrointestinal tract (GIT) and comprised over 1,000 different species, including bacteria which live in a symbiotic relationship with their host and some microbes which have potentially pathogenic characteristics (Brown *et al.* 2012). The normal work of this intricate microbial system provides resistance to diseases (Bohnhoff *et al.*1954, Collins *et al.* 1978, Freter 1954, 1955, 1956, Schrezenmeir *et al.* 2007), crucially influences the normal structural and functional development of the immune system (O'Hara *et al.* 2003). However, these health-promoting aspects of the gut microbiota are not infallible and can be overcome by pathogens, psychological and physical stress, altered GIT peristalsis, dietary changes, radiation, chemotherapy or chronic disease. This realization has lead to the concept of modulating gut health through the diet, development of foods and supplements specifically designed to fortify the gut microbiota (Tuohy *et al.* 2003). Among the diversity of functional foods the most effective for these purposes are prebiotics.

Any dietary component that reaches the colon intact is a potential prebiotic; however, 3 criteria are required for success, in that the ingredient should resist host digestion, and absorption processes; be fermented by the microflora colonizing the gastrointestinal system; and selectively stimulate the growth and/or the activity of 1 or a limited number of bacteria within the gastrointestinal system. The latest prebiotic definition is "a selectively fermented

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ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora, that confer benefits on the host's well-being and health" (Kolida *et al.* 2007). For the gut microflora, non-digestible carbohydrates of plant origin are the main prebiotic substrates and include resistant starch as well as non-starch polysaccharides such as cellulose, hemicellulose, pectin and inulin which are referred to as dietary fiber. However, the extent of microbial breakdown of dietary fibers differs–it depends on the matrix and the type of polysaccharides. For example, purified wood cellulose is not broken down by the intestinal microflora, while a considerable proportion of the cellulose in cabbage is degraded (Blaut 2002).

One of the most promising raw materials for the dietary fiber production is a natural symbiont *Medusomyces gisevii* (MG) Lindau also known as Kombucha or Tea Fungus which represents a complex microbial community of yeasts and acetic acid bacteria, that produce a cocktail of diverse metabolites, such as organic and amino acids, ethanol, polyphenols such as catechin, lysine, water-soluble vitamins such as vitamin C and B, antioxidants, catalase, carbon dioxide, enzymes, essential elements (Na, K, Ca, Cu, Fe, Mn, Ni, and Zn) (Ramana *et al*. 2000, Muthukumarasamy *et al.* 2002, Chau *et al*. 2008, Rathore *et al.* 2009, Ren *et al.* 2009, Ramyadevi *et al*. 2012, Neera *et al*. 2015). All of these micronutrients are referred to as protective agent against colo-rectal cancer (Blaut 2002). The composition of *Medusomyces gisevii* is also rich in cellulose fiber, which are produced by bacteria forming a jellyfish-like zoogleal mat where microbial cells are attached (Kozyrovska *et al.* 2012). This hydrogel has already found several applications in food, cosmetics, and pharmaceutical industries (Ramana *et al*. 2000, Muthukumarasamy *et al.* 2002, Chau *et al*. 2008, Rathore *et al.* 2009, Ren *et al.* 2009, Ramyadevi *et al*. 2012, Neera *et al.* 2015). Being one hundred times thinner than cellulose of fibrils obtained from plants this type of microcellulose was confirmed to be free from contaminants such as lignin. X-ray diffraction studies showed that the overall degree of crystallinity index of dried tea fungal biomass was slightly lower than that of microbial cellulose (Gayathry *et al.* 2014).

The prebiotic effect of a substrate can be measured as a selective effect upon the growth of major bacterial groups commonly found in the gut, in particular a selection for increased numbers of *bifidobacteria* and *lactobacilli* in comparison with 'undesirable' micro-organisms (Gomez *et al.* 2009). *In vivo* experiments are the most representative approach for evaluating the success of any administration, since physiological parameters and interactions with the host organism are taken into account (Hawrelak *et al.* 2004). This is why the aim of this study was to evaluate the potential prebiotic properties of *Medusomyces gisevii* zooglea *in vivo*. As especially the usage of antibiotics is the most common and significant cause of major alterations in normal GIT microbiota we decided to check this affect during the antibiotic-associated dysbiosis.

MATERIALS AND METHODS

Medusomyces gisevii zooglea preparation: Zooglea of *Medusomyces gisevii* (MG) was obtained from the liquid medium, cultivated under standard conditions: a gauze bag containing 10 g of black tea purchased from the local market was placed for in 1000 mL of hot boiled water 20 min, the leaves were removed by filtration, 100 g of sugar was added until it dissolved, then the solution cooled down to room temperature (24–26°C). After that 10% of the culture (of the previous fermentation brew from MG with the same origin, corresponding to the aforementioned starter culture) was added to the solution. The container was covered with a 100% cotton towel and fixed with an elastic band. The culture was fermented at the room temperature (23–24° C). After 20 days of cultivation the formed cellulose pellicle of MG zooglea was removed from the medium, continuously washed with water within 10–15 min to remove soluble medium components. The pellicle was cut into small pieces with sterilized scissors and then put into a low temperature refrigeration chamber (Tefcold se10–45, Denmark) at the temperature of –40° C for 48 h, it was dried by freeze-drying (P.I.T., LC-500, Russia) to a moisture level 8–10% at the operating pressure of sublimator 70–80 Pa, the condenser temperature was –45° C, and the total drying cycle of 35 h. After the drying, the substance was ground to a particle size of 20–150 microns with a laboratory grinder, placed in a glass container, tightly stoppered and sterilized by autoclaving at 110° C for 15 min. The powder prepared according to the proposed technology was used for the further experiment.

Animals: Experiments were performed on 40 male Wistar rats, about 9 months old, the average weight 250 g. All animals were maintained in conventional conditions in an environmentally controlled room (20–22°C, 12 h light:dark cycle), with food and water *ad lib*. All procedures were performed according to EC Directive 86/609/EEC and in accordance with the Russian legislation governing the conduct of experiments on animals.

Treatment design: At the first stage of experimental influence rats were divided into 2 groups (n=20/group). Intestinal dysbiosis was induced by gentamicin sulfate in dose of 10 mg/rat twice a day through a gastric tube for 10 days for all the rats. The dose of antibiotic was selected according to the method of dysbiosis simulations in laboratory animals (Chicherin *et al.* 2013), considering the conversion factor / unit body surface for rats (Habriev 2005). Animals in the control group received a standard diet recommended by the Institute of Nutrition, and the animals of the experimental group received 120 mg of active substance of MG zoogloea/rat considering conversion factor/unit body surface every day during the experiment, in addition to the usual diet.

After the termination of the oral administration of gentamicin, the control group of animals with significant dysbiotic changes in fecal microflora were randomly divided into 2 sub-groups of 10 rats each (second stage of the experiment). Animals of the first sub-group (control 2)

received a standard diet recommended by the Institute of Nutrition for the next 10 days, and the animals of the second sub-group (experimental 2) received 120 mg of active substance of MG zoogloea / rat considering conversion factor / unit body surface every day during the experiment, in addition to the basic diet. During the experiment the monitoring of general state of the animals, their activity, body weight, water absorption, the frequency and consistency of stool was also conducted.

Bacteriological examination: At the initial stage of the experiment, before antibiotic treatment, the background bacteriological examinations of the large intestinal microflora of all animals were conducted. Then fecal examinations on 2, 4, 6, 8, 10 day of treatment was performed by selection of faeces individually from each animal in sterile sealable glass containers with the aim of counting the number of *Bifidobacterium* spp*, Lactobacillus* spp*, Escherichia coli, Candida* spp. The total number of microbial cells/1 g of feces of animals was determined by counting in a Goryaev chamber (hemocytometer). The number of viable bacteria was determined by serial dilutions method. Serial tenfold dilution of cecal contents were prepared with sterile phosphate buffer saline (pH 7.2) solution. Planting on selective solid culture media (Lactobacillus Selection Agar Base (HiMedia, India), Agar for bifidobacteria (HiMedia, India), Hottinger agar (Medgamal, Russia), Endo (HiMedia, India), Candida Medium (HiMedia, India)) in Petri plates and counting colonies of bacteria after the incubation time of 48 h at 37 °C was carried out. Cultivation of *bifidobacteria* and *lactobacilli* was performed on solid nutrient media under microaerophilic conditions using the anaerobic system for the cultivation (INFORS MT Multitron, Switzerland). Colony counting of *Bifidobacteria, Lactobacilli, Escherichia coli* and *Candida* spp. was carried out using microorganisms counter Scan 100 (Interscience, France). The identification was performed by the characteristic cultural properties on the corresponding nutrient media, as well as by the presence of specific cells in the smears stained by Gram. Microscopy was carried out using a Carl Zeiss Axio Imager 2 (A2) (Jena, Germany) with Zeiss AxioCam MRm for obtaining digital images (Zeiss AxioVision Release 4.8.1). Morphologically distinct organisms were counted in appropriate aliquots and an estimate of the abundance of distinct organisms was obtained.

Statistical method: To facilitate the statistical presentation of data on the number of colony forming units of bacteria logarithms were used (base 10). All analyses were performed using the Statistical Package for the Social Sciences (SPSS) for Windows, version 11.0 packed program. Data are presented as mean ± standard deviation. The difference between the control and experimental groups was analyzed using Mann-Whitney U test. $P < 0.05$ was considered statistically significant. Statistical processing of the results of the research was carried out using the program Primer of Biostatistics (Version 4.03).

RESULTS AND DISCUSSION

During the antibiotic treatment, animals in both groups showed no significant change (increase or decrease) of body weight. Water intake was similar across the experimental groups. However, starting from the 4th till the 10th day of the experiment the animals in the control group who did not receive supplements from MG showed a decline of the total activity, passive state, poor appetite, that was probably associated with flatulence, abdominal pain, or bloating – symptoms provoked by strong dysbiosis, significant number of animals (14 rats) had frequent defecation with the release of liquid faeces. On the contrary the experimental group of animals willingly accepted feed, actively moved in the cell, frequency and stool consistency was normal.

After the termination of the oral administration of gentamicin animals of the control sub-group showed improvement in general condition only on the seventh day, the animals resumed their activity, improved feed intake, stool returned to normal consistency, some animals (2 rats) did not restore their state until the 10th day of the experiment. Animals that received a substance of MG zoogloea showed improvement in general condition already on the third day.

It should be noted that pathogens and microorganisms of the genus *Proteus*, *Klebsiella*, *Sitrobacter*, hemolytic flora were not detected in any of the animals before and after the experiment. Total bacterial counts and individual bacterial groups after the gentamicin induced disbiosis are shown in Table 1, there were no differences in the numbers of bacteria in any of the bacterial groups at the beginning of the experiment.

In the control group of animals, in a state of antibiotic-associated dysbiosis on a standard diet, on the second day after the application of gentamicin observed a significant decrease in the total bacterial counts, as well as the decrease of *Bifidobacterium* spp*, Lactobacillus* spp*, Escherichia coli* level, which regularly continues until 10th day of the experiment. On the background of the general decline in the number of microorganisms a significant increase in the number of *Candida* spp begins from the sixth day which was caused by the general decline in the body's immune system. At the same time the animals of the experimental group exposed by gentamicin but receiving MG substance showed a significant reduction in the total bacterial counts and *Bifidobacterium* spp*, Lactobacillus* spp*, Escherichia coli* only on the eighth day of the experiment (it is possible due to the cumulative effect of the antibiotic). Comparing the corresponding values in the control and experimental group it can be noted that, on the 10th day of the experiment the level of microorganisms of the experimental group animals was significantly higher than that of the control group. In the group of experimental animals the level of *Candida* spp did not change significantly.

The results obtained after the termination of gentamicin exposure are shown in Table 2. It can be noted that in the animals of the first subgroup (control), that are on a standard diet, reduction of the number of *Bifidobacterium* spp*,*

Table 1. Dynamics of the intestinal microflora of rats with experimental antibiotic-associated dysbiosis in applying them a biologically active substance from *Medusomyces gysevii* zoogloea

Bacterial group	Numbers of the monitored bacterial groups in 1 g of faecal microflora of experimental animals Log_{10} CFU/g											
	Before gentamicin influence		Day 2		Day 4		Day 6		Day 8		Day 10	
	I	II	I	II	I	II	I	II	I	II	I	II
Total	9.98± 0.25	10.11± 0.12	8.95± 0.32*	10.02± 0.45	7.25± 0.22*	9.14± 0.11	6.12± 0.32*	8.61± 0.22	5.55± 0.25*	8.02± 0.21*	4.42± 0.25*	7.25± 0.24*
Bifidobacterium spp	8.87± 0.58	8.92± 0.22	7.01± 0.25*	8.22± 0.27	5.58± 0.44*	7.25± 0.17	5.05± 0.48*	7.15± 0.51	4.24± 0.45*	6.65± 0.17*	4.25± 0.11*	6.61± 0.21*
Lactobacillus spp	8.25± 0.13	8.64± 0.11	6.88± 0.67*	8.02± 0.14	5.02± 0.18*	7.14± 0.26	5.95± 0.21*	6.52± 0.68	4.03± 0.48*	6.11± 0.22*	4.01± 0.18*	6.10± 0.14*
Escherichia coli	7.21± 0.13	7.45± 0.28	6.15± 0.15*	7.04± 0.45	4.15± 0.18*	6.1± 0.21	3.25± 0.15*	5.85± 0.41	2.28± 0.25*	5.82± 0.17*	2.14± 0.54*	5.25± 0.22*
Candida spp	2.67± 0.4	2.04± 0.15	2.85± 0.22	2.06± 0.22	2.95± 0.16	2.09± 0.28	3.01± 0.24*	2.75± 0.28	3.01± 0.27*	2.21± 0.45	4.12± 0.24*	2.81± 0.31

I, Control group; n,20, animals received a usual diet II, experimental group; n,20, animals received 120 mg of active substance of MG zoogloea with the usual diet.* Indicates difference from beginning of treatment ($P < 0.05$).

Table 2. Dynamics of the recovery of intestinal microflora of rats after antibiotic-associated dysbiosis in applying them a biologically active substance from *Medusomyces gysevii* zoogloea

Bacterial group	Numbers of the monitored bacterial groups in 1 g of faecal microflora of experimental animals Log_{10} CFU/g										
	The end of the gentamicin influence	Day 2		Day 4		Day 6		Day 8		Day 10	
	Unified group	I	II	I	II	I	II	I	II	I	II
Total	4.42± 0.25	4.01± 0.14	4.95± 0.22	4.98± 0.4	5.59± 0.22*	5.51± 0.14*	6.45± 0.36*	5.67± 0.33*	7.02± 0.12*	6.01± 0.36*	7.89± 0.31*
Bifidobacterium spp	4.25± 0.11	3.62± 0.25*	4.58± 0.28	4.22± 0.15	5.98± 0.12*	4.86± 0.21	6.21± 0.14*	5.26± 0.22*	6.75± 0.16*	6.02± 0.35*	7.76± 0.25*
Lactobacillus spp	4.01± 0.18	3.25± 0.24*	4.04± 0.18	4.02± 0.12	5.35± 0.15*	4.74± 0.28	5.94± 0.26*	5.27± 0.19*	6.95± 0.28*	5.81± 0.34*	7.82± 0.18*
Escherichia coli	2.14± 0.54	2.00± 0.17	2.88± 0.22	2.22± 0.18	3.68± 0.14*	3.25± 0.24	5.01± 0.19*	3.68± 0.14	5.55± 0.19*	4.22± 0.22*	7.02± 0.26*
Candida spp	4.12± 0.24	4.85± 0.28	4.21± 0.22	4.88± 0.28	4.21± 0.22	4.22± 0.16	3.98± 0.21	4.01± 0.17	3.31± 0.24*	3.79± 0.28	3.03± 0.12*

I, Control sub-group; n,10, animals received a usual diet; II, experimental sub-group; n,10, animals received 120 mg of active substance of MG zoogloea with the usual diet. * Indicates difference from beginning of treatment ($P < 0.05$).

Lactobacillus spp, continued until the second day after the cessation of gentamicin exposure. Self-healing microflora is very slow, significant changes in its number are marked only on the eighth day. The number of *Candida* spp during all 10 days were not significantly changed and remained quite high.

The animals received the additive of the MG did not show reduction of *Bifidobacteria* and *Lactobacilli*. On the contrary, already on the fourth day after the initiation of the substance animals we observed a significant increase in the total bacterial counts and *Bifidobacterium* spp, *Lactobacillus* spp, *Escherichia coli*, which continues until the termination of the experiment. On the 10th day of the experiment the number of these microorganisms was almost close to the original values obtained before the effects of antibiotics on animals. Also on the eighth day after having taken substances level of *Candida* spp a significant decrease begins.

The present study is the first one to have been undertaken to investigate the potential prebiotic efficacy of a biologically active substance made from zooglea of *Medusomyces gisevii*. The literary sources does not contain specific data on the prebiotic properties of MG, however Kozyrovska *et al*. (2012) mentioned about its possible effect, because of its ability to produce a microcellulose. Zooglea MG represents an insoluble cellulose fiber, and it

was doubtful whether this type of cellulose can have a positive effect or at least any effect on the host's own microflora, because the data of various authors differ. For example, Barry *et al*. (2010) tested a diet containing 4% of cellulose on cats, and did not note any influence on the level of *Bifidobacterium* spp., *Lactobacillus* spp. and *Escherichia coli* in the feces of the test animals, against to the results obtained after addition to their diet of pectin and fructooligosaccharide, which he recommended as useful fibers. Paturi *et al.* (2010) notes, that cellulose is minimally fermented in the large bowel of rats. According to his research using of a low-fat diet in the combination with cellulose during a long period of time resulted to a decrease of *Bifidobacterium* spp. in rats feces. In this case our results showed the effect of MG substance on members of *Bifidobacterium* spp. *Lactobacillus* spp. and *Escherichia coli* in the significant positive direction of their dynamics, are in contrast with the findings of the above authors.

During the study, there were no cases of toxicity of MG substance applied at the selected dose that is in agreement with the results obtained by Kotkoskie *et al.* (1996). The general condition of the rats treated with the substance of the MG was significantly better compared with the control animals, which in our opinion is due to the leveling of the brightest signs of intestinal dysbiosis as flatulence, abdominal pain or bloating. These observations are in agreement with the results obtained by Bianchi *et al.* (2002), who reported that the use of microcrystalline cellulose results in less gas production and reducing abdominal distension. Our common results, showing a positive effect of biologically active substances from MG during the experimental antibiotic-associated dysbiosis in the total amount of the important representatives of the microflora of rats, their general condition, normal consistency of their stools, are in agreement with Bamba *et al*. (2002) who applied the new prebiotic from germinated barley containing glutamine-rich protein and hemicellulose rich fiber on patients with ulcerative colitis, and showed that fiber fraction modulates stool water content because of its high water-holding capacity, supported maintenance of epithelial cell populations, increased short-chain fatty acid (especially butyrate) production by luminal microflora, which includes *Bifidobacterium.*

Thus, summarizing the above, we believe that the use of biologically active substances from MG may play an important role in treatment of different kinds of dysbiosis as well as diseases of the gastrointestinal tract.

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