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Application of probiotics in complex treatment of tuberculosis

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Abstract
The probiotic bacteria possessing ability to suppress growth of Mycobacterium B_5 are revealed. Antagonistic activity in selected strains studied during the growth on various nutrient media. Strains adapted to the low pH exposure. They are steady against a number of the antibiotics, used at tuberculosis treatment. This testifies to the prospects of further studies on the use of probiotics in the complex therapy of tuberculosis.
Kaywords: probiotic bacteria, antagonistic activity, antibiotics, steady, tuberculosis.

Tuberculosis (TB) is an infectious disease of humans and animals. The most common cause of human disease is the bacteria called Mycobacterium tuberculosis. In the past, another major cause of human tuberculosis was Mycobacterium bovis, the causative agent of tuberculosis in cattle. But tuberculosis can also be found in other animals; and these animals can also pass TB to humans [1].

Besides social, environmental and economic reasons, prevalence of tuberculosis is associated with emergence of forms of the disease that are resistant to the used medicines. Today tuberculosis has been recognized as an important international concern due to increasing incidence and share of acutely progressing forms and forms resistant to treatment, late detection of diseases, spread of strains resistant and multiresistant to specific chemotherapeutic drugs.

According to the World Health Organization (WHO), there was a total number of 9,270,000 new cases of tuberculosis in 2007. About 14 million people worldwide suffer from HIV/AIDS and tuberculosis simultaneously. In 2007, there were 1,370,000 new cases of tuberculosis among people suffering from HIV. In the same year, 456 thousand people who suffered from both infectious diseases died. A particular concern of WHO experts is a form of drug-resistant tuberculosis. In 2007, approximately 500 thousand people suffered from this form of tuberculosis. Less than 1% of them received necessary treatment [2].

According to the latest WHO estimates, about 9 million new cases of TB and approximately 2 million deaths occurred in 2011, though the statistics from developing countries could be underestimated [3].

The development of drug resistance in the population has increased concern that TB may once again become an incurable disease, thereby establishing an urgent need to develop new, effective agents.

One of the ways to enhance the efficacy of struggle against multi-drug resistant tuberculosis can be application of probiotics in the complex treatment based on lactic acid bacteria and their metabolites.

Lactic acid bacteria are symbionts of the gastrointestinal tract and are harmless to humans and animals. They have therapeutic effect due to antimicrobial activity, immune system activation and normalization of intestinal microbial population. It is important that in relation to probiotics, resistant forms of microorganisms are not formed. At the moment, lactic acid bacteria are mainly used in the treatment of intestinal infections. Probiotics for the treatment of extraintestinal infections are not produced. However, according to published data, lactic acid bacteria can be used for the treatment of suppurative wounds and inflammatory diseases of the urinogenital tract. Our research in 2000-2005 also confirmed high efficiency of the association of lactic acid and propionic acid bacteria for treatment of inflammatory diseases caused by purulent infection [4]. In animal experiments, we have found that the strain of *Lactobacillus salivarius* 8g has high prophylactic and therapeutic efficacy in experimental brucellosis. Moreover, therapeutic effectiveness of lactic acid bacteria equals that of gentamicin, doxycycline, rifamcycline antibiotics. An antibiotic has been identified to which the culture is slightly sensitive, and the use of which in the treatment of brucellosis with lactic acid bacteria had higher therapeutic efficacy as compared with monotherapy [5].

I. Materials and methods

The strains of bacteria grow in liquid nutrient mediums MRS , combinate on base of the corn extract and skimmed milk. As test culture used strain of Mycobacterium B_5, which is not the causative

agent of tuberculosis. Nonetheless, this strain is suitable for researches as Mycobacterium tuberculosis and it have significant structural similarity , and their most widespread clinical manifestation - a disease of lungs. Being in many respects similar to extremely infectious M. tuberculosis, the strain of Mycobacterium B_5 has low infectivity and therefore doesn't demand exclusively safe conditions for experimenting. This fact does very valuable pretenders of Mycobacterium B_5 for tuberculosis laboratory research.

Antagonistic activity was determined by agar diffusion method in the zones of assay culture inhibition using a sampling device of 7 mm in diameter. To determine resistance of selected lactic acid bacteria strains to antibiotics, standard disks saturated with antibiotics were used. Disks were put on a dense culture medium MRS which surface was inoculated with strains. Antibiotic sensitivity was determined according to zones of growth inhibition of assayed cultures. Resistance of selected bacterial strains to the following anti-tuberculosis therapeutic antibiotics at a concentration of 100 mkg/ml was determined - ethambutol, pyrazinamide, ofloxacin, ciprofloxacin, streptomycin, kanamycin, isoniazid.

At least 4 replications were to be performed.

II. Rhesults and Discussion

For the prevention and treatment of tuberculosis we made selection of lactic acid bacteria strains which had bactericidal activity against *Micobacterium* B_5. Lactic acid bacteria isolated from healthy people and animals and having antagonistic activity against enteropathogenic bacteria. From testing 60 strains, more than 30 strains inhibited *Mycobacterium* B_5 growth. From these 30 strains of lactic acid bacteria, the most prominent cultures were selected, and a limited study was conducted to identify the factors influencing anti-TB efficacy. Resistance of probiotic strains to conventional anti-TB antibiotics was examined as an important factor for combinatorial application of probiotics along with antibiotics. For this purpose, the antibiotic susceptibility of selected strains was assessed by the Kirby-Bauer method by placing small wafers containing antibiotics onto a plate upon which bacteria were growing (Table 1). One can see that all examined strains were sensitive (susceptible) to rifampicin, which is a component of the first-line antibiotics cocktail conventionally applied to treat susceptible and MDR *Mycobacteria.* This is itself an undeniable evidence of the negative effect of rifampicin upon GI essential bacteria. However, no sensitivity to the other anti-TB conventional antibiotics was observed.

Table 1. Effect of Antibiotics on the Growth of Selected Bacteria Possessing Anti-*Mycobacterium* B_5Activity

Probiotic cultures	Growth inhibition zone, ∅mm							
	Rifampicin	Ethambutol	Ppyrazinamide	Ofloxacin	Ciprofloxacin	Streptomycin	Kanamycin	Isoniazid
L. brevis B-3	21	0	0	0	0	0	0	0
L. plantarum 22	24	0	0	0	0	0	0	0
L. plantarum 2b	25	0	0	11	0	0	0	0
L. plantarum 14d	20	0	0	0	0	0	0	0
L. cellobiosis 20	29	0	0	12	0	15	0	0
L. fermentum 127	25	0	9	0	0	0	0	0
L. plantarum 2b&14d, *L. brevis* B-3&P. *shermanii*-15	9	0	0	0	0	0	0	0
L. plantarum 22&*L. fermentum* 127	21	0	0	0	0	0	0	0

Spot-on-lawn tests performed with cell-free culture broths revealed that the components of the medium used for probiotic cultures growth influenced its anti-TB efficacy (Table 2).

Table 2. Inhibition of *Mycobacterium* B_5 Growth by Cell-free Culture Broth of Selected Bacteria as the Function of Growth Medium

Strains	Ggrowth medium			Strains	Growth medium		
	MRS	Corn syrup	Skimmed Milk		MRS	Corn syrup	Skimmed milk
	Mycobacteria B_5 growth inhibition zone, ∅mm				Mycobacteria B_5 growth inhibition zone, ∅mm		
1	2	3	4	5	6	7	8
L. plantarum 1n	15±0.7	17±0.3	0	*L. brevis* 27_2	9±0.5	12±0.3	0
L.plantarum 53n	15±0.7	15±0.0	10±0.6	*L.cellobiosus* 7n	19±0.6	19±1.0	10±0.5
L.plantarum 25m	10±0.6	15±0.7	0	*L. cellobiosus* 28	9±0.7	14±0.3	0
L.plantarum 14d	9±0.7	12±0.5	0	*L. cellobiosus* 58n	10±0.5	15±0.6	9±0.3
L. plantarum 2b	19±0.6	22±0.6	10±0.3	*L. cellobiosus* 20	18±0.0	18±0.3	9±0.3
L.plantarum 17d	11±0.6	14±0.5	9±0.5	*L. casei* 7	9±0.7	12±0.3	0
L. plantarum 22	18±0.8	20±1,0	10±0.3	*L. casei* 27_1	9±0.5	12±0.3	0
L .fermentum 15	9±0.8	12±0.6	0	*L. casei* 173a	13±0.3	15±0.0	9±0.8
L. fermentum 16	13±0.3	15±0.5	9±0.0	*L. casei* 26L	15±0.5	17±0.6	9±0.5
L. fermentum 17_{5-2}	15±0.4	17±0.6	0	*L. acidophilus* 15	14±0.0	16±0.3	0
L. fermentum 27	15±0.3	18±0,0	9±0.3	*L. brevis* B-3	15±0.3	18±0.6	9±0.5
L. fermentum 17	11±0.5	14±0.9	0	*L. plantarum* 2b&14d *L. brevis* B-3&P *Propionibacterium shermanii*-15	19±1.0	19±0.8	10±0.6
L. fermentum 7n	14±0.9	14±0.0	9±0.5				
L.fermentum 127	16±0.5	18±0.0	10±0.5				

table continued 2

1	2	3	4	5	6	7	8
				L. plantarum 22& *L. fermentum* 127	17±0.0	18±0.3	9±0.0

The average of probiotic cultures grown in the medium containing corn syrup demonstrated higher anti-TB activity. When cultured in milk, bacterial antagonism is exhibited to a lesser extent.

Limited oligosaccharides that are non-digestible in the human GI tract but stimulate growth of probiotic bacteria, so-called prebiotics, were examined. It was demonstrated that a significantly higher yield of selected bacteria was achieved using a synthetic, non-digestible sugar lactulose as compared to raffinose, which is also a non-digestible sugar in the human GI tract. The probiotic bacteria yield on raffinose was similar to that on sucrose and glucose, which are sugars that are perfectly digestible in the human GI tract (Table 3).

Table 3 - Titers of lactic and propionic acid bacteria on the media containing fructo-oligosaccharides

Bacterial strains	Titers, CFU/ml in various variants			
	glucose	sucrose	raffinose	lactulose
L. plantarum 2b	$2.2x10^{8}$	$4.1x10^{8}$	$2.0x10^{8}$	$2.0x10^{10}$
L. salivarius 8d	$1.0x10^{9}$	$1.0x10^{9}$	$5.4x10^{8}$	$3.6x10^{13}$
L.fermentum 127	$6.6x10^{8}$	$4.5x10^{8}$	$3.3x10^{7}$	$1.2x10^{13}$
L. cellobiosus 20	$1.4x10^{9}$	$2.3x10^{9}$	$1.1x10^{7}$	$9.7x10^{13}$
L. plantarum 22	$3.8x10^{11}$	$4.8x10^{11}$	$2.9x10^{9}$	$2.6x10^{13}$
L. fermentum 27	$3.8x10^{11}$	$3.7x10^{11}$	$1.5x10^{10}$	$1.5x10^{13}$
Propionibacterium shermanii-15	$1.4x10^{10}$	$1.2x10^{10}$	$3.4x10^{6}$	$1.6x10^{13}$

It was determined that lactic and propionic acid bacteria cultures grow on all assayed oligosaccharides. The largest amount of bacterial cells was found on MRS medium with lactulose compared to glucose (Table 3). For example, while the amount of lactic acid bacteria cells was n x 10^{8} - n x 10^{11} on the glucose medium, it was n x 10^{10} - n x 10^{13} on the lactulose medium. The number of propionic acid bacteria was 1.4 x 10^{10} in 1 ml of the glucose medium, and 1.6 x 10^{13} on the lactulose medium. The strain of *L. plantarum* 2b grows both on glucose, sucrose, and raffinose (2.0 x 10^{8}); however, accumulation is much higher (2.0 x 10^{10}) on the lactulose medium. A number of strains (15, 127, 20, 22, 27) showed a decrease in growth on raffinose compared to other carbohydrates.

Antagonistic activity of the assayed monocultures and associations was determined essentially on the glucose, lactulose, sucrose and raffinose media (Figure).

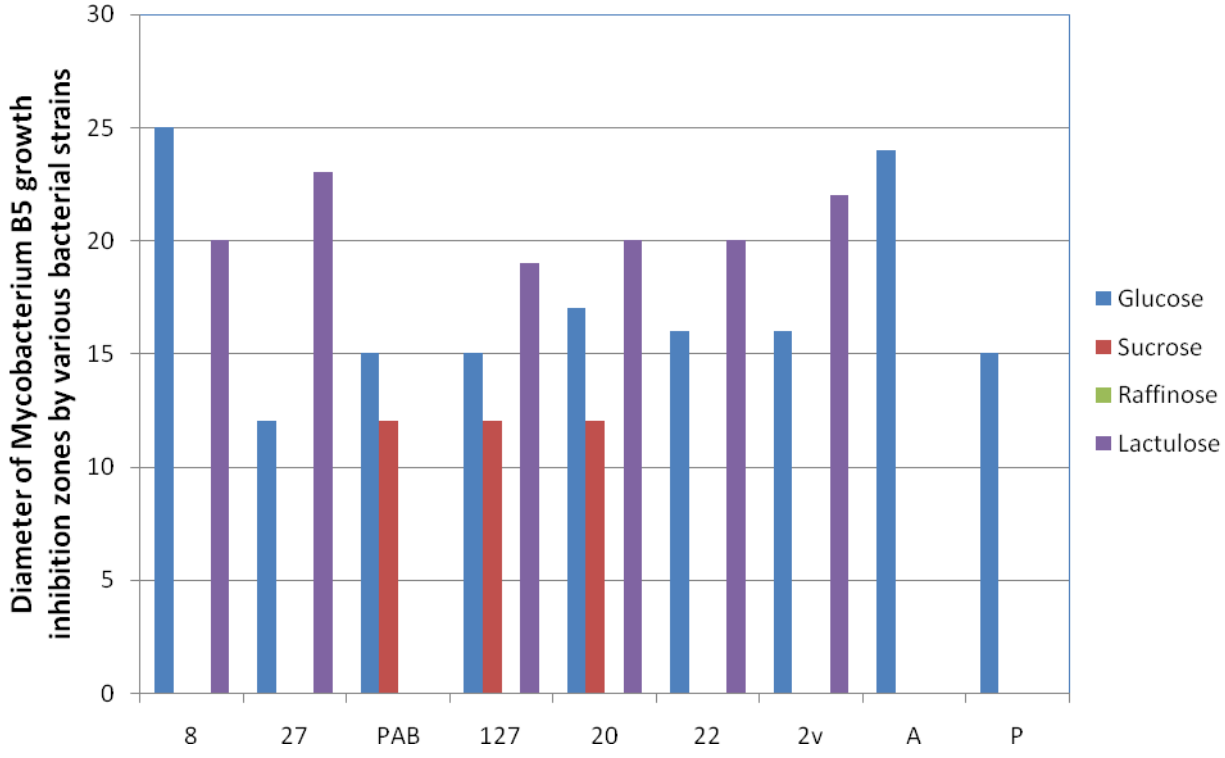

Figure - The antagonistic activity of lactic and propionic acid bacteria when grown on the media containing fructo-oligosaccharides

The strains of *Lactobacillus plantarum* 22 and 2b, *Lactobacillus fermentum* 27 and *Lactobacillus salivarius* 8d revealed the antagonistic activity against *Mycobacterium* B_5 on the glucose (16, 16, 12 and 25 mm respectively) and lactulose (20, 22, 23 and 20 mm respectively) media, and *P. shermanii* 15 (PAB) showed its antagonistic activity on the glucose (15 mm) and sucrose (12 mm) media.

The cultures of *Lactobacillus fermentum* 127 and *Lactobacillus cellobiosus* 20 were active to this assayed culture on the glucose (15 and 17 mm respectively), sucrose (12 mm each) and lactulose (19 and 20 mm respectively) media.

The best carbohydrate for the association A (*L. plantarum* 2b+ *L.brevis B-3*+ *P.shermanii*-15) is glucose where it shows antagonistic activity against *Mycobacterium* B_5 (24 mm). Association P (*L.plantarum* 22+ *L.fermentum* 27) exhibits antagonistic activity against *Mycobacterium* B_5 on the glucose (15 mm), lactulose (14 mm) and sucrose (12 mm) media. None of the individual cultures and associations showed antagonistic activity against mycobacteria when grown on raffinose.

Adaptation of probiotic strains to low pH was investigated in the light of naturally low pH in the stomach and upper intestine, areas which are proposed as the habitat for probiotics in the human organism. For this purpose, freshly grown cells were sequentially passed through solutions of the pH 3.0 and incubated at 37˚C. The experiment was carried out until the variants resistant to low pH values were obtained without loss of productively essential properties.

Nine strains that tolerated low pH better than the others have been selected (Table 4).

Table 4. Effect of Sequential Passes through Low pH to Selected Microorganisms

Microorganisms	Titer, CFU/ml		
	Incubation time at pH 3.0, min		
	0	40	60
L. plantarum 22	$3.2x10^9$	$3.2x10^7$	$2.5x10^7$
L. plantarum 14d	$2.7x10^9$	$2.2x10^8$	2.2x107
L. plantarum 2b	$6.0x10^9$	$3.0x10^8$	$2.7x10^7$
L. cellobiosis 20	$1.3x10^8$	$5.2x10^6$	$2.0x10^6$
L. fermentum 27	$8.0 x10^9$	$1.0x10^8$	$1.3x10^7$
L. fermentum 127	$8.8 x10^9$	$7.9x10^7$	$7.5x10^7$
L. salivarius 8d	$1.0 x10^9$	$8.5x10^8$	$2.0x10^7$
L. brevis B-3	$1.2 x10^8$	$2.0x10^7$	$4.0x10^7$
P. shermanii-15	$3.0x10^8$	$1.2x10^7$	$1.5x10^7$

The growth-inhibiting activity of probiotic strains adapted to low pH was compared to those not adapted to low pH. It was shown that the strains adapted to low pH had 1.5-3 times higher *Mycobacterium* growth-inhibiting activity than that of original strains (Table 5).

Table 5. Growth-inhibiting Activity of the Original LAB Strains and the Low pH-adapted Strains

Microorganism	*Mycobacterium* B5 Growth inhibition zone, ∅ mm	
	Original	pH 3- adapted
L. plantarum 2b	16.0	30.0
L. plantarum 14d	17.0	38.0
L. plantarum 22	16.0	22.0
L. salivarius 8d	11.5	30.0

The long-term stability (loss of viability) of selected probiotic strains in liquid medium was studied at room temperature (25˚C - 28˚C) and at +4˚C in the presence of various compounds such as pectin, microcrystalline cellulose, zeolite, dietary fiber, bentonite, starch, sucrose, vitamin C, among others. It was shown that in variants with addition of dietary fiber, probiotic strains insignificantly reduced the number of viable cells and retained anti - *Mycobacterium* B_5 activity during 4 months at +4˚C, and during 4 weeks at room temperature.

Thus, the probiotic bacteria possessing ability to suppress growth of *Mycobacterium* B_5 are revealed. Antagonistic activity in selected strains studied during the growth on various nutrient media. Strains adapted to the low pH exposure. They are steady against a number of the antibiotics, used at tuberculosis treatment. It was shown that in variants with addition of dietary fiber, probiotic strains in liquid culture insignificantly reduced the number of viable cells and retained anti-*Mycobacterium* B_5 activity during 4 months at +4˚C, and during 4 weeks at room temperature. This testifies to the prospects of further

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