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Effectiveness of combined use of antibiotics, essential metals and probiotic bacterial strain complexes against multidrug resistant pathogens

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ABSTRACT

Large-scale uncontrolled use of antibiotics in various spheres of human activities (agriculture, veterinary medicine, and food industry) has led to the emergence of multi-resistant pathogen isolates. The aim of this study was to assess the complex potential effects of antibiotics and metals on *P. aeruginosa* clinical isolate. In this study, clinical isolates of *P. aeruginosa* were used as test organisms in respect of which combinations based on antibiotics, salts of essential metals with a high level of dissociation in aqueous solutions and the probiotic bacterial strain of *B. subtilis 534* were examined. The specific selection criterion for antibiotics and metals was their resistance to specific concentrations of *B. subtilis 534* strain and their inhibitory or subinhibitory effect against *P. aeruginosa.* In the course of the research it was found that the most promising is the use of CuSO4, since it has a more pronounced bactericidal effect on *P. aeruginosa* at a concentration of 40 mg/ml, in contrast to *B. subtilis 534* for which this concentration is not toxic. Of all the studied antibacterial drugs, we selected fosfomycin for further research as it meets the requirements of the studies performed, with more pronounced resistance of *B. subtilis 534* (0.25 mg/ml) compared to *P. aeruginosa* (0.03125 mg/ml). Generalized experimental data indicate that studies of the antibacterial complex based on fosfomycin, CuSO4, and the probiotic strain *B. subtilis 534* are the most promising.

Keywords: *P. aeruginosa; B. subtilis*; antibiotics; essential elements; high potency preparations; multidrug resistance.

1. INTRODUCTION

Over the past decade, the proportion of pathogenic bacteria highly resistant to antimicrobials and multi-resistant to various antibiotics has increased dramatically. The World Health Organization considers this phenomenon as a global problem. The causes of antibiotic resistance against pathogens include the abuse of antimicrobial drugs in medicine and agriculture. The accumulation of antimicrobial agents in the environment also results in mutations and R-genes spreading in microbial populations at high rates. Due to this fact, antibiotics used for therapeutic purposes are becoming less effective in combating infectious diseases. An increase in multi-drug resistant microorganisms in health facilities, which provide ideal conditions for horizontal R-gene transfer and spread of resistance, poses a significant threat. It is facilitated by an increased number of patients, a constant influx of pathogenic micro flora and frequent use of antibiotics [1-2].

The development of microbial resistance to antibacterial drugs is a process whereby bacteria acquire mechanisms to protect cells from antimicrobial medicinal products effect used for therapeutic purposes. However, antibiotics are secondary metabolites (molecules of different chemical nature) produced by microorganisms in traces. Such molecules can perform a variety of functions, ranging from effectors of different types and toxins to pigments. Perhaps, the synthesis of these molecules in natural habitats gives an advantage to some types of microorganisms and makes them more competitive. Commercially produced antimicrobial compounds are mainly isolated from actinomycetes, which actively compete with other types of microorganisms in natural conditions. In this regard, bacteria have acquired several mechanisms to protect them from antibiotic effect. These mechanisms include efflux pumps aimed at reducing the content of antibacterial compounds in the cell, and enzymes, which action is associated with changes in the structure and activity, as well as with the destruction of antimicrobial molecules. Thus, there are several ways of developing resistance to antimicrobial compounds in nature. The first one is antibiotic producers which should have mechanisms to protect their cellular structures from the action of antibacterial molecules. The second one is selective environmental pressure, which can contribute to the development of antimicrobial resistance mechanisms. There is evidence from the genome and functional analyses of deep-frozen soils indicating that antibiotic resistance is found in bacteria dating back thousands of years. Phylogenic analyses of enzymes that destroy β-lactam antibiotics show a high probability of the joint R-gene evolution with antibiotic biosynthesis genes for millions of years. Although antibiotic resistance appeared naturally in ancient times, modern methods in medicine and agriculture show a sharp spike and a full spread of resistance among micro populations [3-4].

The problem of the emergence of highly-resistant pathogenic bacteria is particularly relevant in surgery treatment, due to the high frequency of post-surgery complications and the

development of infectious processes in the cavity of the wound [5].

There is evidence that the primary agents of the infection process in wounds are *P. aeruginosa, S. aureus,* and *E. faecalis.* Less markedly, there are representatives of *Enterobacteriaceae* family (*E. coli, Proteus vulgaris, S. marcescens, and K. pneumoniae*) and different cocci [6].

P. aeruginosa refers to rod-shaped gram-negative motile bacteria. *P. aeruginosa* is ubiquitous, being a pathogenic microorganism and causing various diseases in humans. It is often isolated from the micro flora of suppurative wounds and is found in abscesses. Due to the increased resistance to various antibiotics, *P. aeruginosa* is a frequent cause of death in patients with mucoviscidosis and is one of the leading nosocomial pathogens. Also, in most cases, *P. aeruginosa* causes a variety of infection processes, being in association with other pathogenic microorganisms. Infections caused by *P. aeruginosa* are hardly treatable. This is due to resistance to many antimicrobials, including antibiotics commonly used in medical practice: aminoglycosides, cephalosporins, fluoroquinolones and carbapenem antibiotics. The tendency to increase in antibiotic and multi-drug resistance for these bacteria worldwide is reported [7-8].

With regard to the above, to date, the most promising is the research into inhibitors of antibiotic resistance in microorganisms, the identification of mechanisms to enhance the effectiveness of existing antibiotics and the development of new antibacterial agents [16].

Research of combined use of antibiotics and probiotic strains of bacteria together with various metals is particularly relevant. There are many studies aimed at studying antibiotic-resistant bacteria strains that are part of various probiotic drugs. Examples of such studies include the assessment of antimicrobial resistance of the *L. casei, E. faecium, L. gasseri, B. longum, E. coli, Nissle 1917* and other probiotic bacterial strains. Of particular interest is the evaluation of antibiotic resistance of genus Bacillus bacteria, especially for the co-use of probiotic preparations based on these microorganisms with different antibiotics. *B. subtilis 534* bacteria included in the «Sporobacterin» probiotic preparation, were isolated from microbial populations in septic wounds. These Gram-positive bacteria are pervasive in nature, having a pronounced antagonistic activity, which is manifested in the synthesis of antimicrobial compounds. This ability of *B. subtilis 534* underlies their use in medical practice. At the same time, data indicating the resistance of *B. subtilis 534* to some antibiotics are accumulated [9].

A lot of metals are known to have a bactericidal effect against various microorganisms. Silver, zinc and copper have the most significant antimicrobial activity. There is evidence that cations of these metals inhibit the growth of pathogenic bacteria, which are the causes of infectious processes in wounds. Relevant research is aimed at studying the use of antibiotics in combination with metals. For example, zinc compounds have a positive effect on the antimicrobial properties of nitroimidazole and nitrofuran [10].

Thus, this research aims to determine the antibiotic resistance of *B. subtilis 534* and *P. aeruginosa* and the bactericidal action of zinc and copper salts against these microorganisms, to explore the possibility of co-use of antibiotics in combination with probiotics and essential elements.

2. MATERIALS AND METHODS

The probiotic strain *B. subtilis 534* isolated from per primam wound healing and the clinical isolate *P. aeruginosa* were used as study objects.

To determine the antibiotic resistance of the target microorganisms, «Bio Merieux» test systems were used. Further evaluation of resistance to some antimicrobial agents in selected bacteria was performed using agar well diffusion method. This method combines two methods in its structure: agar well diffusion assay and serial dilutions. Salts (sulphates, nitrates, chlorides, and acetates) of copper and zinc with a high level of dissociation in aqueous solutions and antibacterial preparations were used as a source of excessive cation concentrations of essential elements.

The advantage of this method is a visual assessment of the toxicity of chemical compounds in different concentrations under identical conditions; besides, this technique is not only a qualitative but also a quantitative assessment of the biotoxicity of the target chemical compounds [11-15].

3. RESULTS

The first stage of sampling evaluated the resistance to antibiotics of different groups against *B. subtilis 534* and *P. aeruginosa* bacteria, using the «Bio Merieux» test systems, the results of which are presented in Table 1.

Table 1. The target microorganisms' antibiotic resistance using «Bio Merieux» test systems.

Antibiotics	Target Microorganisms	
	B. subtilis 534	*P. aeruginosa*
Penicillins		
Penicilline G	R	R
Amoxicilline	S	S
Ticarcilline	R	R
Cephalosporins		
Cefamandole	S	S
Cefotaxime	R	R

Antibiotics	Target Microorganisms	
	B. subtilis 534	*P. aeruginosa*
Ceftazidime	R	R
Carbapenems		
Imipeneme	R	R
Monobactams		
Aztreonam	R	R
Aminoglycosides		
Amikacine	S	S
Gentamicine	S	S
Netilmicine	S	S
Kanamycine	S	S
Tetracyclines		
Tetracycline	S	S
Minocycline	S	I
Lincosamides		
Lincomycine	R	S
Nitroimidazoles		
Metronidazole	R	R
Macrolides		
Erytromycine	S	I
Pristinamycine	S	S
Nitrofurans		
Nitrofurantoine	S	S
Quinolones / Fluoroquinolones		
Pefloxacine	S	R
Ciprofloxacine	S	I
Other		
Fosfomycine	R	R
Spectinomycine	S	S

Clinical susceptibility breakpoints: R – resistant; S – susceptible; I – intermediate

As can be seen from the table, probiotic strain *B. subtilis 534* is resistant to most β-lactam antibiotics. Exceptions are amoxicillin and cefamandole, to which these bacteria are sensitive. Also, it is noted that bacilli do not grow in the presence of antibiotics belonging to the groups of aminoglycosides, tetracyclines, macrolides, quinolones, and fluoroquinolones; spectinomycinshows an antibacterial effect against *B. subtilis 534* as well. Probiotic bacteria are also resistant to lincomycin, metronidazole and fosfomycin.

Similarly to *B. subtilis 534*, *P. aeruginosa* is resistant against most β-lactams, and is sensitive to aminoglycosides, tetracyclines, macrolides, nitrofurans and spectinomycine. These bacteria are resistant to metronidazole and fosfomycin. However, it is noted that the growth of *P. aeruginosa* is suppressed in the presence of lincomycin.

For further studies, Bitsillin-3 (penicillin), Imipenem (carbapenem), Lincomycin (lincosamide), Cefotaxime and Ceftazidime (cephalosporins), and Monural (fosfomycin) were selected.

At this stage of the study, we evaluated the bactericidal action of the selected antibacterial drugs against the target microorganisms using agar well diffusion method, which involves taking into account the halo of bacterial growth inhibition in millimetres.

Table 2. The evaluation of antibiotic resistance for *B. subtilis 534* and *P. aeruginosa* against Bicillin-3.

Target Microorganisms Concentration	*B. subtilis* 534	*P. aeruginosa*
300000 U/ml	≥35	—
150000 U/ml	28,25 ± 0,6292	—
75000 U/ml	27,75 ± 0,75	—
37500 U/ml	22,5 ± 0,6455***	—
18750 U/ml	19,5 ± 0,5**	—
9375 U/ml	17,5 ± 0,2887**	—
4687,5 U/ml	15,00 ± 0,7071*	—
2344 U/ml	18,75 ± 0,4787**	—
1172 U/ml	18,25 ± 0,25	—
586 U/ml	23,75 ± 1,25**	—
293 U/ml	20,00 ± 0,4083*	—

*p≤0,050, **p≤0,010, ***p≤0,001
* Related to the previous concentration

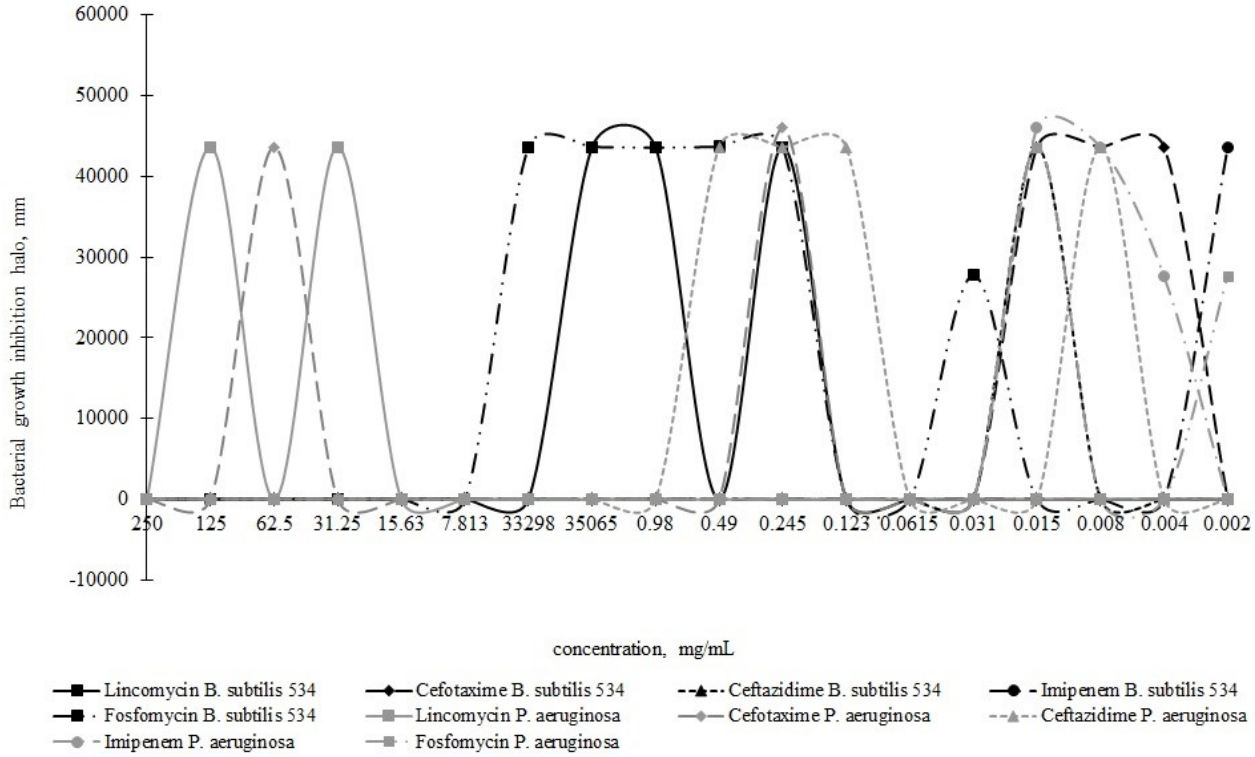

Figure 1. Biotoxicity of antibacterial drugs against *B.subtilis 534* and *P.aeruginosa*.

According to the determination of the bactericidal action of Bicillin-3, it is evident that *P. aeruginosa* has a pronounced resistance to this antibiotic, in contrast to *B. subtilis 534* (Table 2). This phenomenon is explained by the fact that the selected antibiotic is active mainly against gram-positive microflora. Besides, it is known that *P. aeruginosa* microorganisms have developed defense mechanisms against β-lactam antibiotics.

The results of determining the target microorganisms resistance to lincomycin similarly indicate a pronounced resistance of *P. aeruginosa* as compared with probiotic microorganisms (Figure 1).

P. aeruginosa growth is inhibited at concentrations of lincomycin from 300 mg/ml to 37.5 mg/ml. *B. subtilis 534* is resistant to this antibiotic at concentrations below 0.0733 mg/ml.
Figure 1 presents data on the study of the bactericidal action of Cefotaxime and Ceftazidim against target microorganisms.

P. aeruginosa is mostly resistant to cefotaxime and ceftazidim, while probiotic microorganisms of the genus Bacillus are sensitive to these antibiotics. However, *P. aeruginosa* is more resistant to cefotaxime. The growth stops when the concentration of cefotaxime reaches the equivalent of 0.123 mg/ml. At the same time, resistance to ceftazidim concentrations below 0.0039 mg/ml is noted. It can be observed that the selected antibiotics from the group of cephalosporins are not susceptible to the action of β-lactamases, therefore having a more pronounced antimicrobial effect against *P. aeruginosa* bacteria than Bicillin-3.

Imipenem has the highest antimicrobial effect in comparison with other selected antibacterial drugs (Figure 1). This is because imipenem has a wide range of action and is resistant to β-lactamases, penicillinases and cephalosporinases. The most pronounced antimicrobial effect of this drug is noted for *B. subtilis 534* microorganisms, while *P. aeruginosa* bacteria can grow in the presence of imipenem at concentrations below 0.1906×10-2 mg/ml.

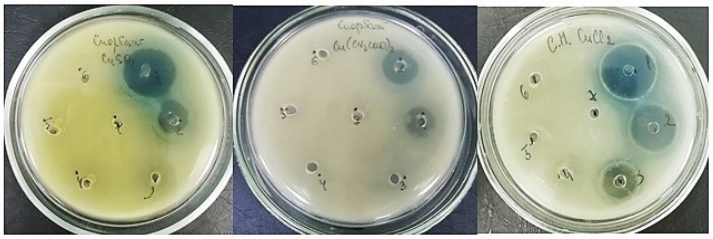

Figure 2. Effect of copper salts on *B. subtilis* 534 growth.

Figure 3. Effect of copper salts on *P. aeruginosa* growth.

B. subtilis 534 has a pronounced resistance to fosfomycin in comparison with *P. aeruginosa* (Figure 1).
Growth inhibition of probiotic microorganisms is observed at concentrations from 0.25 mg/ml to 0.03125 mg/ml. There is evidence of increased activity of this antibiotic against *P. aeruginosa*, which explains the results.

The next stage of our study was to determine the bactericidal action of copper and zinc salts against *B. subtilis 534* and *P. aeruginosa*.

As can be seen from Figure 2 and Figure 3, copper salts, namely CuCl2, CuSO4 and Cu (CH3COO) 2 have a more significant bactericidal effect on *P. aeruginosa* than *B. subtilis 534*.

Table 3. The evaluation of bactericidal effect of copper salts against *B. subtilis 534* and *P. aeruginosa.*

Target Microorganisms Concentration	*B. subtilis 534*	*P. aeruginosa*
$CuSO_4$		
160 mg/ml	20,5 ± 0,8238	15,625 ± 0,9808
80 mg/ml	16,25 ± 0,4119**	12,00 ± 0,7792*
40 mg/ml	—	10,25 ± 0,3660
20 mg/ml	—	—
$Cu(CH_3COO)_2$		
200 mg/ml	16,375 ± 0,375	14,125 ± 0,7181
100 mg/ml	10,375 ± 1,625**	12,625 ± 0,4978
50 mg/ml	—	10,875 ± 0,6928
25 mg/ml	—	7,375 ± 1,0680*
12,5 mg/ml	—	5,375 ± 1,1943
6,25 mg/ml	—	—
$CuCl_2$		
134,5 mg/ml	23,5 ± 0,6268	19,25 ± 0,8183
67,25 mg/ml	19,00 ± 0,3273***	15,875 ± 0,6665*
33,625 mg/ml	15,00 ± 0,5***	11,5 ± 0,4629***
16,8125 mg/ml	—	9,375 ± 0,3239**
8,4063 mg/ml	—	6,75 ± 0,25***
4,2031 mg/ml	—	—

*p≤0,050, **p≤0,010, ***p≤0,001
* Related to the previous concentration

Table 4. The evaluation of bactericidal effect of zinc salts against *B. subtilis 534* and *P. aeruginosa.*

Target Microorganisms Concentration	*B. subtilis 534*	*P. aeruginosa*
$ZnSO_4$		
287 mg/ml	28,375 ± 0,7304	23,5 ± 0,7792
143,5 mg/ml	22,875 ± 0,5154***	19,375 ± 0,5957**
71,75 mg/ml	20,75 ± 0,4532*	13,75 ± 0,5261***
35,875 mg/ml	17,00 ± 0,8018**	10,75 ± 0,4119**
17,9375 mg/ml	14,00 ± 0,3780**	9,375 ± 0,4199*
8,96875 mg/ml	9,125 ± 0,7425***	—
4,4844 mg/ml	—	—
$Zn(NO_3)_2$		
297 mg/ml	27,5 ± 1,1339	21,375 ± 0,4199
148,5 mg/ml	24,75 ± 0,9402	15,875 ± 0,4407***
74,25 mg/ml	22,375 ± 0,7778	12,375 ± 0,2631***
37,125 mg/ml	17,875 ± 0,5154**	10,75 ± 0,3134**
18,563 mg/ml	12,875 ± 0,6105***	8,875 ± 0,2266**
9,2813 mg/ml	9,75 ± 1,4237	—
4,641 mg/ml	—	—
$Zn(CH_3COO)_2$		
203 mg/ml	26,125 ± 0,8952	18,00 ± 0,9636
101,5 mg/ml	23,875 ± 0,8332	14,125 ± 0,7425*
50,75 mg/ml	21,625 ± 0,7055	11,375 ± 0,4978*
25,375 mg/ml	17,625 ± 0,5957**	8,625 ± 1,2669
12,688 mg/ml	12,5 ± 0,4226***	—
6,3438 mg/ml	8,5 ± 2,1876	—
3,172 mg/ml	—	—

*p≤0,050, **p≤0,010, ***p≤0,001
* Related to the previous concentration

Table 3 presents the data of the bactericidal effect of copper salts on target microorganisms.

Probiotic microorganisms of *B. subtilis 534* are more resistant to copper salts, as compared with *P. aeruginosa*. Growth of *P. aeruginosa* is inhibited at a concentration of CuSO4 from 160 mg/ml to 40 mg/ml, Cu (CH3COO)2 – from 200 mg/ml to 12.5 mg/ml, CuCl2 – from 134.5 mg/ml to 8.4063 mg/ml. At the same time, the minimum inhibitory concentrations of CuSO4, Cu (CH3COO)2 and CuCl2 for probiotic microorganisms of *B. subtilis 534* are 80 mg/ml, 100 mg/ml and 33.625 mg/ml, respectively. Zinc salts have the most significant bactericidal effect on the target microorganisms, unlike copper salts (Figure 4 and Figure 5).

Zinc salts have the most pronounced bactericidal effect on *B. subtilis* 534 probiotic microorganisms. The lowest inhibitory concentration of ZnSO4 for bacilli is 8.96875 mg/ml, Zn(NO3)2 – 9.2813 mg/ml, Zn(CH3COO)2 – 6.3438 mg/mL, while *P. aeruginosa* is able to grow under these conditions (Table 4).

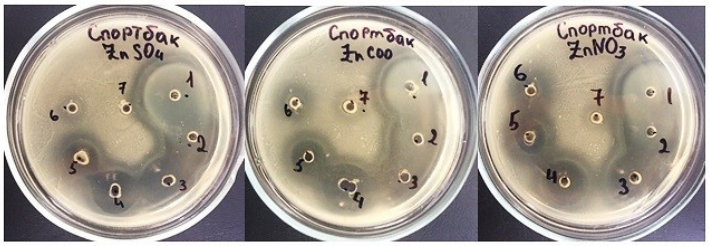

Figure 4. Effect of zinc salts on *B. subtilis* 534 growth.

Figure 5. Effect of zinc salts on *P. aeruginosa* growth.

4. CONCLUSIONS

Probiotic preparations based on Bacillus bacteria are widely used in medicine and veterinary practices for the prevention of postoperative complications. These transient forms of microorganisms have a pronounced antagonistic effect against various pathogens. However, their use in the treatment of complicated wounds is ineffective because of interspecies competition. This fact was the basis for studies to assess the complex potential effects of antibiotics and metals on *P. aeruginosa* clinical isolate, taking into account the resistance of *B. subtilis 534* against concentrations of chemical and chemotherapeutic substances used.

Summarizing all the above mentioned, it should be noted that the target *P. aeruginosa* clinical isolate is highly resistant to most antibacterial drugs under study. The exception to this was fosfomycin in relation to which the probiotic strain of *B. subtilis 534* has a specific resistance, which, in its turn, makes it a drug candidate for the development of a complex preparation for the treatment of complicated wound infections.

As a source of essential elements with inhibitory characteristics, we used copper and zinc salts with a high level of dissociation in aqueous solutions. The most promising for further research are copper compounds, as for these compounds, the probiotic strain showed more pronounced resistance in comparison with the clinical isolate.

Hypothetically, each of the studied elements in the composition of the complex drug candidate will perform the functions of inhibitors of different levels on the pathogenic flora, allowing the growth and reproduction of the probiotic strain and activation of its antagonistic characteristics.

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