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Original research

Lactobacillus reuteri cell-free extracts against antibiotic-resistant bacteria

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

The aim of the research was to evaluate the antimicrobial potential of cell-free extracts obtained in various ways from the probiotic strain *Lactobacillus reuteri* DSM 17938 with respect to their ability to influence the proliferation of antibiotic-resistant bacteria.

Materials and methods. Cell-free extracts were obtained: 1) from *L. reuteri* cell suspension, subjected to disintegration by repeated freezing-thawing, L; 2) from *L. reuteri* culture, cultivated in its own disintegrate (ML); 3) from *L. reuteri* culture, cultivated in its own disintegrate supplemented with glycerol (73.7 mg/ml) and glucose (72.1 mg/ml) (MLG); 4) from *L. reuteri* culture, cultivated in its own disintegrate supplemented with ascorbic acid (20 mg/ml) (MLA). Multidrug-resistant (MDR) and extensively drug-resistant (XDR) clinical isolates: *Escherichia coli, Klebsiella pneumoniae, Lelliottia amnigena* and *Corynebacterium xerosis* were used as a test cultures. The investigation of the inhibitory activity of cell-free extracts was carried out by spectrophotometric method using a microplate analyzer "Lisa Scan EM" ("Erba Lachema s.r.o.", Czech Republic).

Results. Cell-free extract L exerted predominantly stimulatory effect on the proliferation of all studied test cultures. Cell-free extract ML had significant inhibitory effect on the proliferation of *E. coli* and *C. xerosis* (growth inhibition indices were 24.8 % and 96.1 %, respectively) and did not have significant effect on the proliferation of *K. pneumoniae* and *L. amnigena.* Cell-free extracts MLG and MLA caused pronounced inhibition of the proliferative activity of all tested microorganisms. Growth inhibition indices were: 75.0 % and 90.7 % *(E. coli),* 77.9 % and 88.9 % *(K. pneumoniae),* 40.9 % and 77.9 % *(L. amnigena),* 99 % and 100 % (*C. xerosis),* respectively.

Conclusions. The cell-free extracts obtained by cultivation of *L. reuteri* DSM 17938 in its own disintegrate supplemented with glycerol and glucose or ascorbic acid have shown a pronounced antimicrobial activity against antibiotic-resistant bacteria *in vitro*. After confirming safety and efficacy *in vivo*, they can be used to increase the efficiency of the therapy of diseases caused by antibiotic-resistant microorganisms. The results of the study indicate the prospects of obtaining probiotic derivatives with high antimicrobial activity by applying a combinatorial (precursor directed) biosynthesis strategy.

Key words: *Lactobacillus reuteri* derivatives, inhibitory activity, combinatorial (precursor-directed) biosynthesis.

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Безклітинні екстракти *Lactobacillus reuteri* проти антибіотикорезистентних бактерій

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Мета роботи – оцінити протимікробний потенціал безклітинних екстрактів, що отримані різними способами з пробіотичного штаму *Lactobacillus reuteri* DSM 17938, за їхньою здатністю впливати на проліферацію бактерій, стійких до антибіотиків.

Матеріали та методи. Безклітинні екстракти отримували: 1) із суспензії клітин *L. reuteri,* підданих дезінтеграції шляхом багаторазового заморожування-відтавання (L); 2) із культури *L. reuteri*, яку культивували в дезінтеграті власних клітин (ML); 3) із культури *L. reuteri*, яку культивували в дезінтеграті власних клітин, доповненому гліцерином (73,7 мг/мл) і глюкозою (72,1 мг/мл) (MLG); 4) із культури *L. reuteri*, яку культивували в дезінтеграті власних клітин, доповненому аскорбіновою кислотою (20 мг/мл) (MLA). Клінічні ізоляти з множинною лікарською стійкістю (MDR) і з широкою лікарською стійкістю (XDR): *Escherichia coli, Klebsiella pneumoniae, Lelliottia amnigena* та *Corynebacterium xerosis* були використані як тестові культури. Дослідження інгібіторної активності безклітинних екстрактів проводили спектрофотометричним методом із використанням мікропланшетного аналізатора «Lisa Scan EM» («Erba Lachema s.r.o.», Чеська Республіка).

Результати. Безклітинний екстракт L виявив переважно стимуляторну дію на проліферативну активність усіх досліджених культур. Безклітинний екстракт ML спричиняв суттєве пригнічення проліферації *E. coli* та *C. xerosis* (індекси пригнічення росту становили 24,8 % та 96,1 % відповідно) та не мав істотного впливу на проліферацію *K. pneumoniae* та *L. amnigena*. Безклітинні екстракти MLG і MLA викликали виражене пригнічення проліферативної активності всіх досліджених мікроорганізмів. Індекси пригнічення росту становили 75,0 % та 90,7 % (*E. coli*), 77,9 % та 88,9 % (*K. pneumoniae*), 40,9 % та 77,9 % (*L. amnigena*), 99 % та 100 % (*C. xerosis*) відповідно.

Висновки. Безклітинні екстракти, що отримані шляхом культивування *L. reuteri* DSM 17938 у дезінтеграті власних клітин, доповненому гліцерином із глюкозою або аскорбіновою кислотою, продемонстрували виражену протимікробну активність щодо антибіотикорезистентних бактерій *in vitro*. Після підтвердження безпечності та протимікробної активності *in vivo* вони можуть бути використані для підвищення ефективності терапії захворювань, котрі спричинені стійкими до антибіотиків мікроорганізмами. Результати дослідження свідчать про перспективність отримання пробіотичних похідних із високою протимікробною активністю шляхом застосування стратегії комбінаторного (спрямованого прекурсором) біосинтезу.

Ключові слова: похідні *Lactobacillus reuteri*, інгібіторна активність; комбінаторний (спрямований прекурсором) біосинтез.

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Ключевые слова: производные *Lactobacillus reuteri*, ингибиторная активность, комбинаторный (направленный прекурсором) биосинтез.

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Бесклеточные экстракты *Lactobacillus reuteri* против антибиотикорезистентных бактерий

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Цель работы – оценить противомикробный потенциал бесклеточных экстрактов, полученных разными способами из пробиотического штамма *Lactobacillus reuteri* DSM 17938, по их влиянию на пролиферацию бактерий, устойчивых к антибиотикам.

Материалы и методы. Бесклеточные экстракты получали: 1) из суспензии клеток *L. reuteri*, подвергнутых дезинтеграции путем многократного замораживания-оттаивания (L); 2) из культуры *L. reuteri*, культивированной в дезинтеграте собственных клеток (ML); 3) из культуры *L. reuteri*, культивированной в дезинтеграте собственных клеток, дополненном глицерином (73,7 мг/мл) и глюкозой (72,1 мг/мл) (MLG); 4) из культуры *L. reuteri*, культивированной в дезинтеграте собственных клеток, дополненном аскорбиновой кислотой (20 мг/мл) (MLA). Клинические изоляты с множественной лекарственной устойчивостью (MDR) и с широкой лекарственной устойчивостью (XDR): *Escherichia coli*, *Klebsiella pneumoniae*, *Lelliottia amnigena* и *Corynebacterium xerosis* были использованы как тестовые культуры. Исследование ингибиторной активности бесклеточных экстрактов проводили спектрофотометрическим методом с использованием микропланшетного анализатора «Lisa Scan EM» («Erba Lachema s.r.o.», Чешская Республика).

Результаты. Бесклеточный экстракт L проявил преимущественно стимуляторное действие на пролиферативную активность всех исследованных культур. Бесклеточный экстракт ML вызывал значительное подавление пролиферации *E. coli* и *C. xerosis* (индексы угнетения роста составляли 24,8 % и 96,1 % соответственно) и не оказывал существенного влияния на пролиферацию *K. pneumoniae* и *L. amnigena*. Бесклеточные экстракты MLG и MLA вызывали выраженное угнетение пролиферативной активности всех исследованных микроорганизмов. Индексы угнетения роста составляли 75,0 % и 90,7 % (*E. coli*), 77,9 % и 88,9 % (*K. pneumoniae*), 40,9 % и 77,9 % (*L. amnigena*), 99 % и 100 % (*C. xerosis*) соответственно.

Выводы. Бесклеточные экстракты, полученные путем культивирования *L. reuteri* DSM 17938 в собственном дезинтеграте, дополненном глицерином и глюкозой или аскорбиновой кислотой, продемонстрировали выраженную противомикробную активность в отношении антибиотикорезистентных бактерий *in vitro*. После подтверждения безопасности и противомикробной активности *in vivo* их можно использовать для повышения эффективности терапии заболеваний, вызванных устойчивыми к антибиотикам микроорганизмами. Результаты исследования указывают на перспективность получения производных пробиотиков с высокой антимикробной активностью путем применения стратегии комбинаторного (направленного прекурсором) биосинтеза.

Uncontrolled, inappropriate and massive imprudent use of antibiotics has led to spread of antibiotic resistance among microorganisms. In recent years, antibiotic resistance has become a serious problem for modern medicine. Its spread entails the increasing morbidity and mortality due to infectious diseases [1,2]. In February 2017, the World Health Organization (WHO) published so-called "Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics" [3]. It lists the most important at the global level antibiotic-resistant bacteria, which constitute the greatest threat to human health. The priority pathogens list (PPL) is divided into three categories according to the level of priority for the development of new treatments. The Priority 1 category (critical) includes gram-negative bacteria: *Acinetobacter baumanii, Pseudomonas aeruginosa* and *Enterobacteriaceae (Klebsiella pneumoniae, Escherichia coli, Enterobacter spp., Serratia spp., Proteus spp., Providencia spp., Morganella spp.)*. These bacteria are resistant to the wide range of antibiotics, including the most effective of existing antibiotics for the treatment of bacterial infections with multiple drug resistance: carbapenems and third-generation of cephalosporins. The second and third categories are high and medium priority pathogens (vancomycin-resistant *Enterococcus faecium;* methicillin-resistant, vancomycin intermediate and resistant *Staphylococcus aureus* and others). They can cause severe and often fatal infections. It is assumed that the PPL will be constantly updated in accordance with the new data.

The highest levels of antibiotic resistance are found among less pathogenic but more common bacteria that are prevalent in healthcare institutions [4]. Any opportunistic microorganism that becomes resistant to antibiotics, poses a threat to human health. During the last decade, some reports have described various cases of infections caused by opportunistic species of the genus *Corynebacterium* [5,6]. *Corynebacterium xerosis,* a representative of the normal flora of the human body, can cause serious and life-threatening diseases, such as septicaemia, endocarditis, pleuropneumonia, peritonitis, osteomyelitis, septic arthritis, mediastinitis, meningitis, ventriculitis, wound infections in immunocompromised or post-operative patients [7]. Some researchers report that among *C. xerosis* strains isolated from patients with urogenital pathology and healthy people, the number of antibiotic resistant pathogens reached 96.0 %. More than 58 % of these strains showed resistance to three or more antibiotics [8]. The hazard to patient health and life dramatically increases when a pathogen exhibits resistance to antibiotics, which are usually effective. The emergence of drug resistance among coryneform isolates is of most concern and requires constant monitoring for the correct and timely treatment of patients with such infections [5].

The basis for solving the problem of antibiotic resistance is the WHO global plan of action to combat the resistance of microorganisms to antimicrobial drugs, which involves strengthening the groundbreaking work, research and development of new antimicrobials. It is hoped that there is an enormous potential of alternative unconventional approaches to antimicrobial therapy: the use of antibodies, probiotics, bacteriophages and lysins, vaccines, antimicrobial peptides (AMPs), host/innate defense peptides (HDPs/IDRs), antibiofilm peptides, immune stimulation and others [9]. There are two promising microbial strategies for restricting antibiotic resistance spread: bacteriophage- and bacteria-based. Commensal

("health-promoting", "beneficial" or probiotic) bacteria can inhibit growth and transmission of antibiotic-resistant pathogens by direct and indirect ways: by their own production of antimicrobial substances (bacteriocins, antimicrobial peptides and organic acids) and by causing the activation of host defense mechanisms (the production of antimicrobial peptides and IgA, an increase in the mucus layer thickness). They not only contribute to elimination of antibiotic-resistant pathogens but there is also evidence that they may be able to inhibit the horizontal transfer of antibiotic resistance genes [10].

It is known that the beneficial effect of probiotic bacteria on the host organism is realized due to the action of their structural components and metabolites [11]. Therefore, using the biological activity of the probiotic derivatives can also be considered as an alternative way to combat the spread of antibiotic-resistant bacteria [10,11]. Recent studies have confirmed the validity of this approach to the fight against antibiotic-resistant strains. Although the results were encouraging, they showed the variability and selectivity of the antimicrobial activity of the probiotic cell-free supernatants against various antibiotic-resistant microorganisms [12,13].

L. reuteri is one of the heterofermentative lactobacilli with a powerful probiotic potential [14,15]. However, the functional properties of different strains of *L. reuteri* are not the same. For example, the different strains of *L. reuteri* are capable of producing specific antimicrobial substances (reutericin, reutericycin and reuterin), but there is no evidence that single strain is capable of producing two or more of them simultaneously [16]. The spectrum of the produced antimicrobial metabolites varies a lot according to the culture conditions of the producer [17]. *In vitro* and *in vivo* studies (in animal models and in clinical trials), which proved the anti-inflammatory, antimicrobial and immunotropic activity of lactobacillus species *L. reuteri*, were carried out using various strains. *L. reuteri* ATCC 55730 and ATCC 17938 are the most commonly used strains in human trials. Research interest in this probiotic is due not only to its well-known immunotropic, anti-inflammatory and antimicrobial activity. Recent attention of researchers has been focused on the *L. reuteri* as a producer of reuterin, which is a precursor-induced antimicrobial agent [18,19].

Precursor-directed biosynthesis strategy is a promising way to develop new antimicrobials, in particular against antibiotic-resistant pathogens. Obviously, this approach involves the study of various substances as precursors. In our opinion, one of the relevant candidates for the role of a precursor is ascorbic acid. There is convincing data on the pronounced intrinsic antimicrobial activity of ascorbic acid [20,21]. However, our preliminary studies have shown its weak antimicrobial activity against antibiotic-resistant strains at concentrations of 0.15–0.60 %.

Aim

The aim of this research was to evaluate the antimicrobial potential of cell-free extracts obtained in various ways from the probiotic strain *Lactobacillus reuteri* DSM 17938 with respect to their ability to influence the proliferation of antibiotic-resistant bacteria.

Materials and methods

Probiotic strain *L. reuteri* DSM 17938 (from dietary supplement "BioGaia ORS", BioGaia AB, Sweden) was used as source of biologically active structural components and as producer of metabolites. The additional components included in the dietary supplement were removed as follows: the lyophilisate from the sachet was rehydrated with distilled water and centrifuged; the supernatant with the dissolved salts was decanted. Precipitated bacterial cells were inoculated into a liquid nutrient medium: thioglycollate medium or Man, Rogosa and Sharpe broth (Biolife, Italy) and cultivated for 20–24 hours. After checking the purity, the culture was washed three times with sterile saline solution to remove the culture medium. Bacterial suspensions with an optical density of 10 units on the MacFarland scale were prepared from the precipitated cells. Cell-free extracts were obtained from disintegrates (disintegrated cell suspensions) and cultures of lactobacilli.

Disintegrates were prepared by a repeated freezing-thawing of the bacterial suspensions in physiological saline with optical density of 10.0 units according to the McFarland scale (measured with Densi-La-Meter, Lachema, Czech Republic). 10 cycles of freeze-thawing were carried out according to the following regimen: passive cooling and freezing in the freezing chamber of Samsung RB29FSRNDSA refrigerator down to –23 °C, thawing in water bath at 37 °C up to complete thawing.

Probiotic cultures were obtained by cultivating of lactobacilli in their own disintegrates without supplementation and supplemented with glycerol & glucose or ascorbic acid. To this end, the probiotic suspension in physiological saline of turbidity 10.0 units on the McFarland scale was added into disintegrate with or without supplementation in 1:9 ratio and cultured at 37 °C for 72 hours in the microaerobic conditions.

Disintegrates and cultures of lactobacilli were centrifuged at 3000g for 10 minutes in order to remove remained cells and cellular debris. Supernatant was passed through sterile membrane filters with pore diameter of 0.2 micron (Vladipor, RF).

Four cell-free extracts have been studied:

1) L – filtrate of *L. reuteri* disintegrate;

2) ML – filtrate of *L. reuteri* culture, cultivated in its own disintegrate;

3) MLG – filtrate of *L. reuteri* culture, cultivated in its own disintegrate supplemented with glycerol (73.7 mg/ml) and glucose (72.1 mg/ml);

4) MLA – filtrate of *L. reuteri* culture, cultivated in its own disintegrate supplemented with ascorbic acid (20 mg/ml).

Clinical isolates: *E. coli, K. pneumoniae, L. amnigena* and *C. xerosis* were used as a test cultures. They were isolated from patients with urinary tract infection or purulent-inflammatory surgical diseases. The cultures were stored in the Collection of Microorganisms of the Laboratory of Respiratory Infections Prevention of IMI NAMS, Kharkiv, Ukraine. All isolates were identified based on the phenotypic traits: morphological, tinctorial, cultural, physiological and biochemical properties. Susceptibility of bacteria toward antimicrobial agents of different classes was determined on Mueller Hinton agar (Merck, Germany) by the Kirby-Bauer disc diffusion test. Antibiotic-containing disks were produced by JSC Aspect (Ukraine) and company HiMedia (India). The lists of tested antimicrobial agents corresponded

to those previously published [22]. Clinical isolates were considered as resistant, intermediate or susceptible according to inhibition zone diameters. Quality control was performed using *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. Susceptibility interpretations were conducted considering CLSI (Clinical and Laboratory Standards Institute) document M100-S26 (Performance Standards for Antimicrobial Susceptibility Testing, 2016) and/or EUCAST breakpoints (European Committee on Antimicrobial Susceptibility Testing, 2019. Breakpoint tables for interpretation of MICs and zone diameters. Version 9.0). Based on the antimicrobial resistance profiles, all isolates were categorized according to the criteria previously proposed [22].

Preparation of the inoculum: the test cultures were cultivated overnight aerobically at 37 °C on Mueller-Hinton agar (Merck, Germany). After verifying the purity of the culture, some colonies from the overnight incubated agar medium were emulsified in a physiological saline for obtaining a suspension of turbidity 0.5 on the McFarland scale. The turbidity of the suspension was measured using the Densi-La-Meter II device (PLIVA-Lachema Diagnostika, Czech Republic).

Effect of cell-free extracts on proliferative activity of clinical isolates was studied using spectrophotometric microplate method previously described in detail [23]. Briefly, an increase in the optical density of test cultures was compared in the presence (test samples) or absence (control samples) of the studied cell-free extracts. Test cultures were exposed to whole cell-free extracts for one hour and after adding of Meat-Peptone Broth (HiMedia, India), the concentration of the extracts in the incubation medium was 30 % vol. The concentration of test cultures in the incubation medium was ~10^5 CFU/ml. The optical density (OD) of the wells was measured at 578 nm using a microtiter-plate reader "Lisa Scan EM" ("Erba Lachema s.r.o.", Czech Republic) immediately before and after incubation of plates aerobically for 24 hours at 35–37 °C in static conditions. Growth inhibition (or stimulation) indices were calculated by the formula: GII (GSI) = $(\Delta OD-\Delta OD_{PC}) \div \Delta OD_{PC} \times 100$ %, where ΔOD and ΔOD_{PC} were the changes in optical density of the control and test samples within 24 hours, PC – positive control sample containing test-culture without cell-free extract.

All experiments were performed three times. Each sample was tested in triplicate. Average values of obtained indices (ΔOD) with standard deviations (SD) were determined. Obtained data were statistically processed with Excel 2010 software (Microsoft, USA). One-way analysis of variance (ANOVA) followed by post hoc Bonferroni's multiple comparison test was performed. Differences were considered significant at $P < 0.05$.

The study was conducted in the Laboratory of Respiratory Infections Prevention of IMI NAMS.

Results

In accordance with the results of the antibiotic susceptibility study by disc diffusion, test clinical isolates were defined as MDR (multidrug-resistant) and XDR (extensively drug-resistant). It is important to note that *E. coli, K. pneumonia* and *L. amnigena* were resistant to carbapenems and third-generation of cephalosporins. This allowed them to be subsumed under the first category according to the level of priority for the development of new treatments [3]. *C. xerosis* was resistant to penicillins, fluoroquinolones, aminoglycosides, macrolides, tetracyclines, moderately resistant to rifampicin and susceptible only to vancomycin and linezolid.

Incubation of two test-cultures, *L. amnigena* and *C. xerosis,* in the cultivation medium containing cell-free extract L was accompanied by pronounced increase in their optical density (*Fig.1*). The GSIs calculated by the formula for these cultures were 56.0 % and 71.7 %, respectively. *E. coli* and *K. pneumoniae* were cultures, the optical density increase of which in the presence of this extract was not significantly higher than in case of its absence (GSIs were 17.3 and 4.3 %, respectively). Thus, extract L did not significantly affect the proliferative activity of some cultures and significantly stimulated the growth of others. Notably, the differences in the extract L effect on cultures did not depend on their Gram stain.

As shown in *Fig. 2*, the presence of ML extract in the cultivation medium did not significantly affect the optical density increase of two test-cultures: *K. pneumoniae* and *L. amnigena*. GSIs, calculated for them, were 1.8 % and 18.3 %, respectively. The optical density increase of other two cultures (*E. coli* and *C. xerosis*) in the presence of this extract was significantly inhibited: GIIs were 24.8 % and 96.1 %, respectively. There was no association between the nature of the ML extract effect and Gram stain of the tested cultures.

MLG cell-free extract showed remarkable antibacterial activity against all studied antibiotic resistant test-cultures regardless of their Gram stain (*Fig. 3*). This extract presented excellent inhibitory activity against *C. xerosis*. The optical density increase of this test-culture in the presence of MLG extract in the cultivation medium was negligible or absent (GII was 99 %). This extract also possesses moderate antibacterial activities against *E. coli, K. pneumoniae* and *L. amnigena* as evidenced by a significant inhibition of the optical density increase of these test cultures in the presence of MLG extract in the incubation medium compared to controls (GIIs were 75 %, 77.9 % and 40.9 %, respectively).

As can be seen from the data presented in *Fig. 4*, MLA extract showed high inhibitory activity against all studied test-cultures. Growth of *C. xerosis* was ceased under the influence of this extract. The optical density increase of the other three test cultures (*E. coli, K. pneumoniae* and *L. amnigena*) in the presence of this extract was small. GIIs, calculated for them, were 90.7 %, 88.9 % and 77.9 %, respectively.

Discussion

The cell-free extract L contains the disintegration *L. reuteri* products obtained by repeated cyclic freeze-thawing. At the freeze-thawing stages, cells receive sublethal and lethal damage through undergoing the thermal, osmotic, thermomechanical, dehydration, rehydration shock and mechanical disruption by ice crystals. Because of cellular integrity violation, not only superficial, but also intracellular structural components and metabolites of

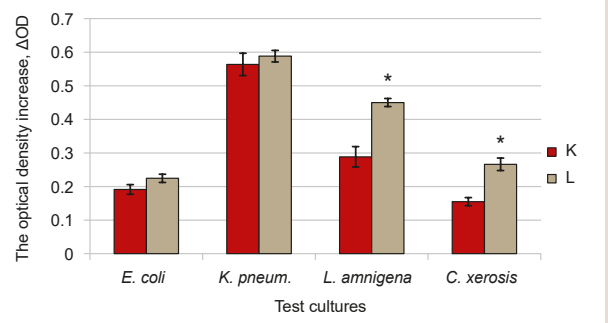

Fig. 1. The effect of cell-free extract L on the proliferation of antibiotic-resistant test-cultures (average ΔOD ± SD at 578 nm, n = 3): **K:** positive control; **L:** filtrate of *L. reuteri* disintegrate; ***:** the differences are significant compared to the K; P < 0.05.

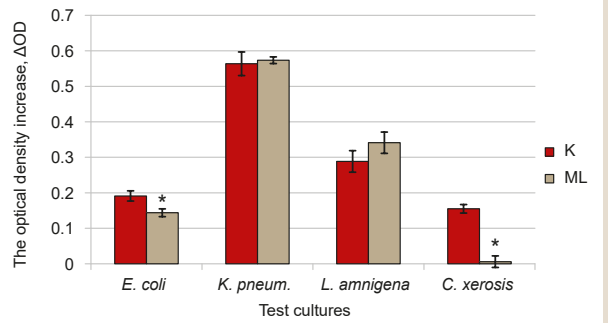

Fig. 2. The effect of cell-free extract ML on the proliferation of antibiotic-resistant test-cultures (average ΔOD ± SD at 578 nm, n = 3): **K:** positive control; **ML:** filtrate of *L. reuteri* culture, cultivated in its own disintegrate; *: the differences are significant compared to the K; P < 0.05.

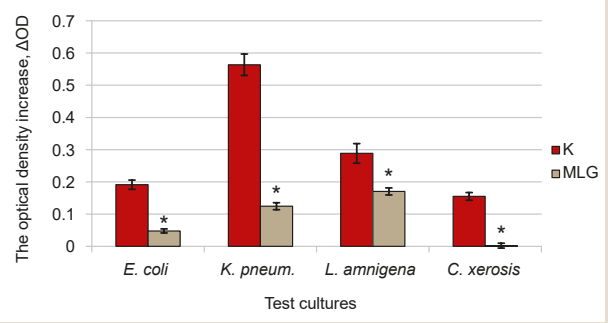

Fig. 3. The effect of cell-free extract MLG on the proliferation of antibiotic-resistant test-cultures (average ΔOD ± SD at 578 nm, n = 3): **K:** positive control; **MLG:** filtrate of *L. reuteri* culture, cultivated in its own disintegrate supplemented with glycerol and glucose; ***:** the differences are significant compared to the K; P < 0.05.

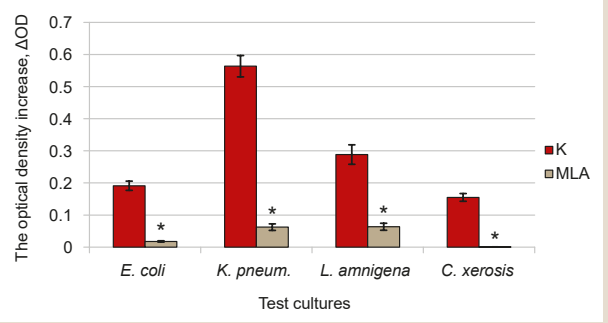

Fig. 4. The effect of cell-free extract MLA on the proliferation of antibiotic-resistant test-cultures (average ΔOD ± SD at 578 nm, n = 3): **K:** positive control; **MLA:** filtrate of *L. reuteri* culture, cultivated in its own disintegrate supplemented with ascorbic acid; ***:** the differences are significant compared to the K; P < 0.05.

stressed cells enter the extracellular space. Obviously, the extract from *L. reuteri* disintegrate contains both MAMPs (microbe-associated molecular patterns) and DAMPs (damage-associated molecular patterns). It is known that these bacterial structures possess powerful bioregulatory potential. For example, cold shock proteins have the ability to orchestrate multiple cellular processes, including proliferation and differentiation by regulation of transcription, translation, and splicing [24]. The results of this research stage indicate that the cell-free extract obtained from *L. reuteri* disintegrate contains derivatives of lactobacilli that cause a predominantly stimulating effect on proliferation of antibiotic resistant test-cultures. Since the extract has a complex composition, only the fractionation and studying the effects of its individual fractions will help to elucidate the mechanism of its action.

In contrast to the L extract, which contains the disintegration products of bacteria, the cell-free extract ML contains lactobacillus metabolites obtained by cultivation them in their own disintegrates. It is known that *L. reuteri* are capable of producing a number of metabolites with antimicrobial activity: organic acids (lactic, phenyllactic and acetic), ethanol, hydrogen peroxide, bacteriocin-like high-molecular-mass compounds and others [14,15,17]. Production of antimicrobial compounds strictly depends not only on the lactobacillus strain, but also on the cultivation conditions (pH, temperature and the adequate concentration of specific precursors, glucose, salts, and enzyme cofactors) [17]. Clearly, the ability of ML extracts to inhibit the proliferation of *E. coli* and *C. xerosis* is associated with the presence of the above-mentioned antimicrobial substances. At the same time, the antimicrobial potential of this extract is insufficient to suppress the proliferative activity of antibiotic resistant isolates of *K. pneumoniae* and *L. amnigena*. To date, a number of studies have been published, indicating the antimicrobial activity of *L. reuteri* metabolites obtained by cultivation of producer on conventional nutrient media [13,25–27]. The results of these studies allow us to draw some conclusions: the main extracellular antibacterial agents of lactobacilli are organic acids (pH-neutralization elicited the most significant impact on the antimicrobial activity of cell-free supernatants, while proteinase and heat treatment had little or no impact); longer cultivation of the producer in microaerobic conditions results in stronger inhibition of test bacteria. Unfortunately, among the published works there are few studies on the effect of *L. reuteri* metabolites on antibiotic resistant bacteria. Some authors have shown that supernatant of *L. reuteri* strain isolated from a commercial oral product exhibits relatively weak inhibitory activity (20 %) against MDR clinical isolates of *P. aeruginosa* [27]. Despite the differences in the method of obtaining the metabolite-containing product and the method of studying its antimicrobial activity, the results obtained by Jamalifar et al. and the results of this study on the effect of the ML extract on Gram-negative bacteria are similar. In contrast to the data obtained in this study, other authors found out a significant antagonistic activity of *L. reuteri* against multiple antibiotic-resistant *K. pneumoniae*

strains [13]. The suppression degree of the *K. pneumoniae* by *L. reuteri* varied in the range of 56–73 %. However, such pronounced inhibition was obtained by the co-cultivation of microorganisms and may indicate the existence of a mechanism for the induction of *L. reuteri* antimicrobial activity by co-cultured microorganism. Cell-free supernatants of other *Lactobacillus* species (*L. acidophilus*, *L. paracasei*, *L. delbrueckii*, *L. casei*, *L. helveticus*, *L. brevis*, *L. salivarius*, *L. fermentum*, *L. rhamnosus*, *L. animalis*, and *L. plantarum*) showed different antimicrobial activity against a number of MDR uropathogens: *Candida albicans*, *K. pneumoniae*, *P.aeruginosa*, *E. coli*, and *Enterococcus fecalis*. Only one indicator strain (*Staphylococcus aureus* DPC 6867) showed resistance to all the lactobacillus supernatants [12]. Among fifty-seven *Lactobacillus* spp. strains, only five exerted high anti-carbapenem-resistant *Enterobacteriaceae* activity. The minimum inhibitory percentage of supernatants of these five strains ranged from 10 % to 30 % [28]. These data indicate the variability and selectivity of the antimicrobial activity of cell-free supernatants of probiotic origin.

It is known that *L. reuteri* can produce and excrete reuterin [14,15]. This antimicrobial compound is able to inhibit or inactivate a wide range of microorganisms: bacteria, fungi, protozoa and viruses. At the same time, *L. reuteri* strains are resistant to it. Reuterin is a mixture of different forms of 3-hydroxypropionaldehyde (3-HPA). Production of reuterin is the result of glycerol fermentation. In the gastrointestinal tract, where *L. reuteri* usually inhabited, small amounts of glycerol are available. However, for complete growth inhibition, for example, enterobacteria *L. reuteri* requires at least 0.2 % of glycerol. These circumstances should encourage researchers to reconsider existing methods of using probiotic supplements for more effective clinical use of their antimicrobial potential [18]. Precursor-directed antimicrobial biosynthesis is considered as a promising strategy for next-generation probiotics development [19]. The results of this study have confirmed its promise. The cell-free extracts, obtained by cultivating *L. reuteri* in its own disintegrate supplemented with glycerol & glucose, have shown high and moderate antimicrobial activity against antibiotic-resistant bacterial strains. However, it is worth remembering that *in vitro* activity cannot be equated with an *in vivo* effect. In addition, there are circumstances that must be taken into account before *in vivo* use of the extracts thus obtained; it is known that reuterin can be reversibly converted into the toxic substance acrolein. Moreover, some authors have suggested that acrolein is an active compound responsible for the antimicrobial activity attributed to reuterin [29]. Therefore, thorough toxicological studies are necessary before the therapeutic use of these extracts.

MLA cell-free extract exerted the most pronounced antimicrobial activity against antibiotic-resistant bacteria among all the studied extracts. The results are consistent with the data from other authors who previously reported inhibitory activity of ascorbic acid against Gram-positive and Gram-negative bacteria [20,21,30]. Some authors have suggested that the antibacterial activity of ascorbic acid was not due to acidification or a decrease in pH, but owing to specific action of ascorbic acid on the cell membrane or cell enzymes [21]. Other authors experimentally confirmed the existence of another mechanism of bacterial growth inhibition (another than acidification) [30]. The discovered in this study high inhibitory effect of MLA extract cannot be explained only by the intrinsic antimicrobial activity of ascorbic acid. Firstly, ascorbic acid at a similar concentration had a significantly weaker inhibitory effect on the studied microorganisms. Secondly, a chromatographic study showed that the substance found in the greatest quantity in MLA cell-free extract was a modified ascorbic acid (data not shown). It is possible that just the modified ascorbic acid is responsible for the high antimicrobial activity of MLA extract. Thirdly, the high antimicrobial potential of the MLA extract can be partially explained by the presence of others organic acids, in particular lactic acid. The synergistic inhibitory effect caused by combination of ascorbic and lactic acid was discovered earlier [30]. Further study is necessary to clarify the mechanism of antimicrobial activity of MLA extract.

Conclusions

1. The cell-free extracts obtained by cultivation of *L. reuteri* DSM 17938 in its own disintegrate supplemented with glycerol and glucose or ascorbic acid have shown a pronounced antimicrobial activity against antibiotic-resistant bacteria *in vitro*.

2. After confirming safety and efficacy *in vivo*, they can be used to increase the efficiency of the therapy of diseases caused by antibiotic-resistant microorganisms.

3. The results of the study indicate the prospects of obtaining probiotic derivatives with high antimicrobial activity by applying a combinatorial (precursor directed) biosynthesis strategy.

Prospects for further research: obtained results will be used to develop new antimicrobial agents of probiotic origin, effective against antibiotic-resistant bacteria.

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