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SENSITIVITY OF ANTIBIOTIC-RESISTANT MICROORGANISMS TO METABOLITES OF LACTOBACTERIA AND COMBINATION OF LACTOBACILLUS RHAMNOSUS GG AND SACCHAROMYCES BOULARDII METABOLITES

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The study investigates the antibacterial activity of metabolites (cultured separately Lactobacillus rhamnosus GG or in combined with Saccharomyces boulardii in disintegrates of L. rhamnosus GG) against multidrug-resistant microorganisms. Metabolites of lactobacillus and L. rhamnosus with S. boulardii demonstrated a 100 % bactericidal effect on the Corynebacterium xerosis regardless of the initial test culture seeding dose. All the experimental samples were bactericidal on the strains Lelliottia amnigena, Acinetobacter baumannii, Pseudomonas aeruginosa with a lower initial seeding dose (0.5 units by the McFarland scale, diluted 10 times). Increasing the dose of test cultures to 1.0 unit by the McFarland scale was accompanied by the presence of the pathogens viable cells (48.0±8.32 – 68.72±7.16) % after exposure to cell-free substances. The metabolites obtained by means of our own technology, due to its high antibacterial activity against antibiotic-resistant pathogens, open the prospect for further designing a new class of antimicrobial agents for broad spectrum.

Key words: metabolites, disintegrates, antibiotic-resistant microorganisms, Lactobacillus rhamnosus GG, Saccharomyces boulardii.

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ЧУТЛИВІСТЬ АНТИБІОТИКОРЕЗИСТЕНТНИХ МІКРООРГАНІЗМІВ ДО МЕТАБОЛІТІВ ЛАКТОБАКТЕРІЙ ТА КОМБІНАЦІЇ МЕТАБОЛІТІВ LACTOBACILLUS RHAMNOSUS GG І SACCHAROMYCES BOULARDII

В дослідженні вивчається антибактеріальна активність метаболітів (культивованих окремо Lactobacillus rhamnosus GG або спільно з Saccharomyces boulardii в дезінтеграгах лактобактерій) щодо полірезистентних мікроорганізмів. Метаболіти лактобактерій і L. rhamnosus з S. boulardii проявляли 100 % бактерицидний ефект щодо Corynebacterium xerosis незалежно від початкової посівної дози тест-культури. Всі дослідні зразки бактерицидно впливали на штами Lelliottia amnigena, Acinetobacter baumannii, Pseudomonas aeruginosa за умови застосування нижчої вихідної посівної дози (0,5 одиниць за шкалою МакФарланд, розведеною в 10 разів). Збільшення дози тест-культур до 1,0 одиниці за шкалою МакФарланд супроводжувалося наявністю життєздатних клітин збудників (48,0±8,32 – 68,72±7,16) % після витримки з речовинами. Отримані за власною технологією метаболіти завдяки високій антибактеріальній активності відкривають перспективу майбутнього конструювання нового класу протимікробних препаратів з широким спектром дії.

Ключові слова: метаболіти, дезінтеграти, антибіотикорезистентні мікроорганізми, Lactobacillus rhamnosus GG, Saccharomyces boulardii.

The work is a fragment of the research project "Study of biological and physico-chemical preconditions for the development of anti-diphtheria agents based on probiotic strains metabolites", of the laboratory for respiratory infections prevention at the SI "IMI NAMS", state registration No. 0116U000864.

Wrestling to antibiotic-resistant pathogens of various diseases is a modern world problem. The rapid spread of multidrug-resistant cultures is caused by the free movement of people: migration, travelling and economically low living standard. Also, self-medication and inappropriate use of antibiotics promotes the development of resistant microorganisms, which traditional drugs do not effect on. The lack of bacterial sensitivity to antibiotics makes the treatment process using low-active drugs ineffective, which is a common problem for medical workers, patients, pharmaceutical products manufacturers and researchers. At the same time, more and more antibiotic resistance is observed in those pathogens that were previously susceptible to antimicrobial agents.

Resistance of microorganisms also draws attention due to the widespread development of multiple drug resistance simultaneously to several drugs of different groups with wide spectrum of action. Multidrug-resistant agents more frequently cause intestinal diseases, upper respiratory tract infections, wound and surgical infections caused mainly by methicillin-resistant staphylococci, enterococci, pseudomonads and other enterobacteria [1].

The priority of this problem is confirmed by constant appeals of the WHO with proposals for solving this difficult situation concerning the permanent increase of pathogens strains with multiple drug resistance. In 2017, the WHO has published resistant microorganisms, divided according to the risk groups, among which the threat is primarily presented by: Acinetobacter, Pseudomonas, various types of Enterobacteriaceae (Klebsiella, E. coli, Serratia, Proteus) [10].

Particular attention deserve resistant to antimicrobial drugs strains, such as Pseudomonas aeruginosa (P. aeruginosa), whose number is constantly growing worldwide [5, 10]. According to the WHO, additional fatal cases caused by resistance to P. aeruginosa carbapenems amount up to 7%. It is known that multidrug resistance to P. aeruginosa is due to several mechanisms: production of enzymes, loss of the outer membrane protein, mutations, etc..

Alarming is the multidrug-resistant of A. baumannii to traditional antibacterial agents [1]. In studying the sensitivity of 90 hospital strains of Acinetobacter baumanii to 8 antimicrobials, a high level of resistance to most of the antibiotics under study was established, which permitted the authors to prove the epidemic spread of the extreme-resistant A. baumanii strains.

According to the WHO, the number of deaths caused by Klebsiella pneumoniae and Escherichia coli, resistant only to the third generation cephalosporins, has grown by 52%, the same for each pathogen [5]. The difficulty is that even among the highly sensitive types of microorganisms to a certain antibacterial agent, there will always be individual cells in the isolates that spontaneously have become resistant to this or that antimicrobial agent.

A promising alternative to ineffective antibacterial agents is the non-cellular metabolic probiotics due to their pronounced antimicrobial activity and broad spectrum of action. Their significant advantage is also a low rate of the bacterial resistance induction, i.e. the difficulty of developing bacteria resistance to substances of the probiotic origin. Metabolic products (exometabolites) of microorganisms, or, as they are called, metabolic-type drugs are the substitution therapy means, which virtually do not produce side effects due to the absence of toxic properties.

The purpose of the study was to substantiate the promising use of the original metabolites in combination with the cell disintegrates of Lactobacillus rhamnosus GG against multidrug resistance microorganisms for designing antimicrobial drugs with a wide spectrum of action.

Materials and methods. When receiving the disintegrate (structural components – L), the lactobacilli cells Lactobacillus rhamnosus GG (L. rhamnosus) (PREEMA® symbiotic, Schonen, Switzerland) with an optical density of 10.0 units by the McFarland scale (Densi-La-Meter device, Czech Republic) were irradiated by means of the G3-109 ultrasonic generator.

To obtain the lactobacilli metabolites, L. rhamnosus microbial suspension was cultivated in its own ultrasonic disintegrate (ML). lactobacilli suspension was introduced into the disintegrate at a ratio of 1: 9, cultivated at 37 ° C for three days, centrifuged at 1000 g for 30 minutes, and filtered using 0.2 μm pore diameter membrane filters [8].

Biologically active metabolic compounds of the L. rhamnosus and Saccharomyces boulardii (S. boulardii) probiotic strains (BULARDI®, Schonen, Switzerland), which are a combination of bacterial and fungal metabolites, were obtained by cultivating producer cells in disintegrates of lactobacilli (MLS).

The study of disintegrate filtrates, L. rhamnosus metabolites and a mixture of bacteria and fungi antimicrobial activity was carried out on multidrug resistance to antibiotics test cultures: Corynebacterium xerosis PR, Klebsiella pneumonia PR, Pseudomonas aeruginosa PR, Acinetobacter baumannii PR, Lelliottia amnigena (Enterobacter amnigenus) PR (to levofloxacin, ceftriaxone, ciprofloxacin, doxycycline, ampicillin, cefepime).

The optical density of the probiotic strains and pathogens suspensions was determined using McFarland's turbidity standard on the Densi-La-Meter device (PLIVA-Lachema Diagnostika, (Czech Republic)). The cultures were synchronized using a single-dose low-temperature effect.

For antimicrobial effect determination of disintegrates and lactobacilli metabolites, experimental suspensions of test strains were prepared with an optical density of 0.5 units by the McFarland scale (McF) and diluted by 10 times (conditionally designated as 0.05), as prescribed by the guidance [3]. This is the first concentration of bacteria cultures suspensions taken for the experiment. The second suspension exceeded the recommended by the guidelines concentration by 20 times and amounted 1.0 units by the McFarland scale. Suspensions of microorganism cultures, in a ratio of 1:9, were introduced into a filtrate with metabolites: experimental samples, and into a physiological solution of sodium chloride: control samples. Exposure of the samples under study was carried out at 37^{o} C for 2 hours. Seeding from the control and experimental samples was carried out on a solid nutrient medium, depending on the test culture species, with determination of the colony-forming units (CFU) number in the test strains. The living microorganisms' number in the

experimental suspension was determined by the presence of colonies. The results of the CFU definition were expressed in decimal logarithms (lg).

All experiments were performed in three or four repetitions. The statistical processing of the study results was carried out using the Microsoft Excel 2010 software package. The average values of the obtained indices (M) and their standard errors (m) were determined. The reliability of the difference between the obtained indices was determined using the Student's t-criterion. Differences in the studied samples indices were considered statistically reliable in relation to the control samples at $p<0.05$.

Results of the study and their discussion. Taking into account the preliminary data on a wide spectrum of antimicrobial activity concerning the reference test strains of the obtained L. rhamnosus substances concerning, the next stage of our work was the study of the lactobacilli metabolic by-products influence on resistant microorganisms strains separately or in combination with saccharomycetes. In the previous studies, a high sensitivity to probiotic microorganisms disintegrates and metabolites was manifested by the biofilm forms of polyresistant gram-negative bacteria. The antibacterial activity study of L. rhamnosus (ML) metabolite filtrates and mixtures of saccharomyces and lactobacillus (MLS) metabolites with regard to the non-diphtheric corynebacterium strain multidrug resistance to antibiotics demonstrated a 100% bactericidal effect irrespective of the test culture seeding dose (fig. 1.a). Quantitative indices of the selected pathogen viability in lactobacilli disintegrates (L) samples, when applying the seeding culture test dose of 1.0 unit by the McFarland scale, were reliably lower compared to the control values. Thus, a reduction of C. xerosis PR cells CFU under the influence of structural components (L) occurred by 2.3 times less, amounting 56.76±8.18% compared to the control values. The two-hours exposure of a non-diphtheria corynebacteria strain with a seeding dose of microbial cells amounting 0.5 units by the McFarland scale (diluted 10 times) in the L. rhamnosus (L) disintegrate filtrates resulted in the viability loss of the selected test culture.

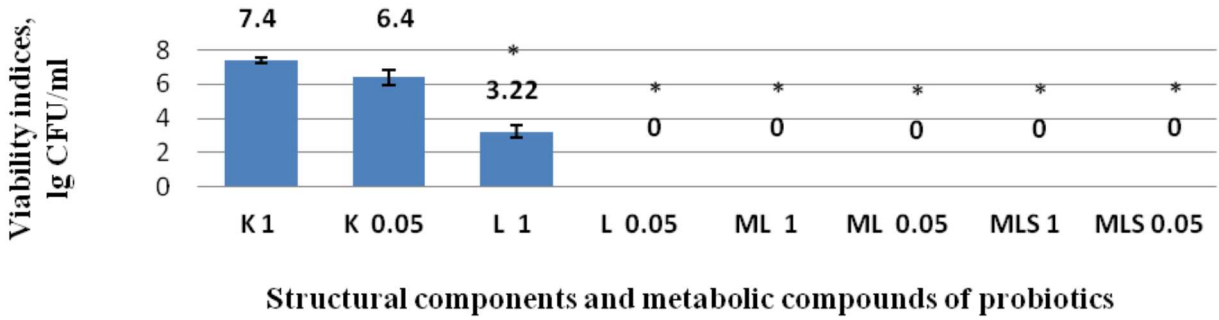

Fig. 1.a. Viability indices of multiple drug resistance Corynebacterium xerosis microorganism after exposure to ultrasonic disintegrates and metabolites of lactobacilli

Note: C – control, L – disintegrate (structural components) of lactobacilli, ML – metabolites (metabolic compounds) of lactobacilli, MLS – combination of saccharomycetes and lactobacilli metabolites, S – disintegrate (structural components) of saccharomycetes, MS – metabolites (metabolic compounds) of saccharomycetes grown on saccharomycetes, LS – metabolites (metabolic compounds) of saccharomycetes grown on lactobacilli;

*the differences are statistically significant compared to the control indices: – ($p<0.05$).

The results of studies on the antimicrobial properties of lactobacilli (L) disintegrates L. rhamnosus (ML) metabolites, mixture of fungal and bacterial metabolites (MLS), with respect to the multiple drug resistance Lelliottia amnigena (Enterobacter amnigenus) strain, display the data that reliably differ from the control ones (fig. 1.b).

Thus, the lower CFU test cultures with a seeding dose of 1.0 unit by the McFarland scale after the two-hours exposure were observed in all the trial samples compared to the control values: L – by 1.45, ML – by 1.5, MLS – by 1, 49 times ($P<0.05$). The two-hours exposure of L. amnigena (E. amnigenus) PR in the presented samples provided using seeding doses of 0.5 units by the McFarland scale (diluted 10 times) causes a complete (100%) loss of of the test culture's viability.

The reduction in the A. baumannii living microbial cells number under the influence of bacteria (L) disintegrates occurred by 1.78 times, after exposure to lactobacilli metabolites (ML) – by 2.08 times, and when incubated with a combination of fungi and bacteria (MLS) – by 2.05 times (fig. 2). Provided a lower seeding dose of pathogenic agents, 0.5 units by the McFarland scale (diluted 10 times), a lack of growth (100%) of the selected test culture ($P<0.05$) was established.

A comparative study using a quantitative method to assess the antimicrobial properties of lactobacilli filtrates against the multiple drug resistance strain of K. pneumonia showed a bacteriostatic action of bacterial disintegrates (fig. 1.c). Thus, the number of viable cells reduced by 1.53 and 2.25 times, depending on the initial seeding dose of the pathogen test cultures taken for the experiment.

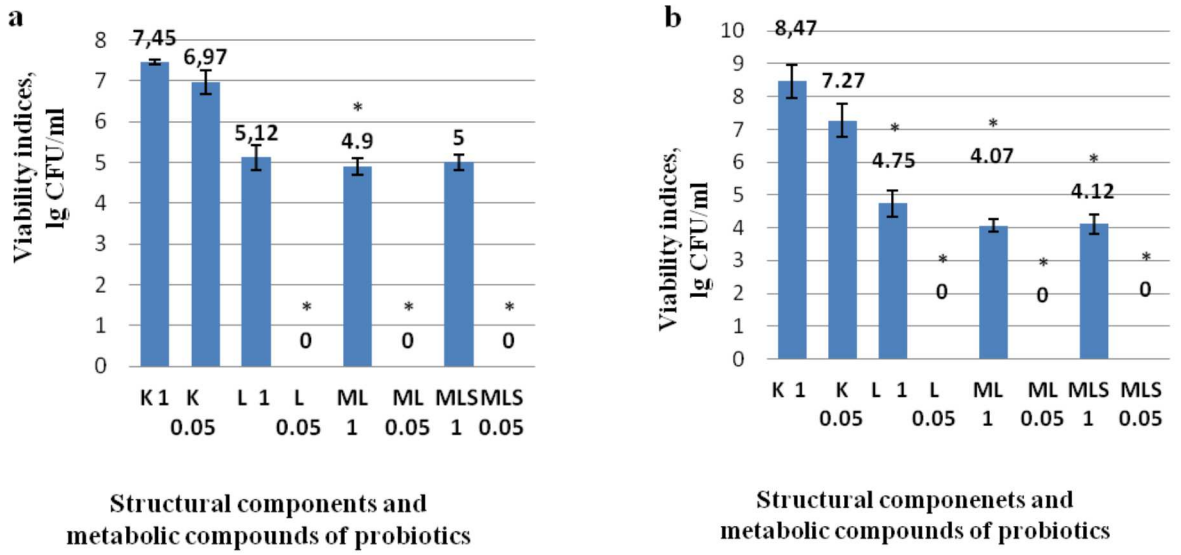

Fig. 1.b. Viability indices of multiple drug resistance *Lelliottia amnigena* (*Enterobacter amnigenus*) (a) and *Acinetobacter baumannii* (b) microorganisms after exposure to ultrasonic disintegrates and metabolites of lactobacilli

Note: C – control, L – disintegrate (structural components) of lactobacilli, ML – metabolites (metabolic compounds) of lactobacilli, MLS – combination of saccharomycetes and lactobacilli metabolites, S – disintegrate (structural components) of saccharomycetes, MS – metabolites (metabolic compounds) of saccharomycetes grown on saccharomycetes, LS – metabolites (metabolic compounds) of saccharomycetes grown on lactobacilli;

*the differences are statistically significant compared to the control indices: – ($p<0.05$).

The CFU indices of multiple drug resistance strain K. pneumonia after exposure to the lactobacilli disintegrate amounted 44.37±8.22% (with the initial concentration of 0.5 units by the McFarland scale, diluted 10 times) and 65.33±7.54% (with the initial concentration of 1.0 units by the McFarland scale).

Consequently, according to its antibacterial activity, the bacteria (L) structural components are inferior to other samples. The presence of bactericidal effect produced by lactobacilli (ML) metabolic compounds as well as bacteria metabolites and fungi (MLS) combination to the selected pathogen was observed after two hours exposure under the condition of applying a lower test culture seeding dose (fig. 1.c).

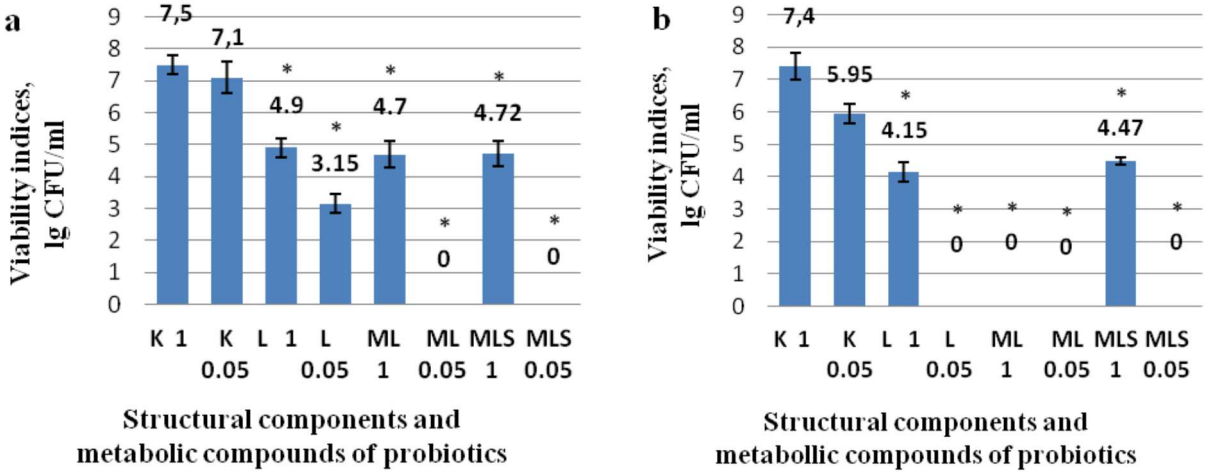

Fig. 1.c. Viability indices of multiple drug resistance Klebsiella pneumonia (a) and Pseudomonas aeruginosa (b) microorganisms after exposure to ultrasonic disintegrates and metabolites of lactobacilli

Note: C – control, L – disintegrate (structural components) of lactobacilli, ML – metabolites (metabolic compounds) of lactobacilli, MLS – combination of saccharomycetes and lactobacilli metabolites, S – disintegrate (structural components) of saccharomycetes, MS – metabolites (metabolic compounds) of saccharomycetes grown on saccharomycetes, LS – metabolites (metabolic compounds) of saccharomycetes grown on lactobacilli;

*the differences are statistically significant compared to the control indices: – ($p<0.05$).

The pronounced antimicrobial activity of the experimental samples against the P. aeruginosa multiple drug resistance pathogen was established (fig. 1.c). The complete loss of viability of the specified strain was observed with the test culture exposure at the initial seeding dose of 0.5 units by the McFarland scale, diluted 10 times in the disintegrate and both types metabolites samples (L, ML, MLS). A similar effect was obtained with the use of L. rhamnosus (ML) metabolites in relation to P. aeruginosa with the initial seeding dose of 1.0 units by the McFarland scale, indicating a greater sensitivity of the selected pseudomonas strain to the lactobacilli products. The antimicrobial properties of other probiotic origin samples with respect to P. aeruginosa with a higher seeding dose were manifested by bacteriostatic action, which was accompanied by

the pathogen viable cells number reduction by 1.78 times after interaction with the bacteria disintegrate (L) and by 1.65 times with the use of lactobacilli metabolites (ML).

The results of the studies performed demonstrate a rather high level of antimicrobial activity of the disintegrate filtrates and the probiotic metabolic compounds against gram-negative and gram-positive multidrug-resistant strains of microorganisms.

In the domestic pharmaceutical market, a representative of metabolic drug dominates, containing the metabolic products of Escherichia coli DSM 4087, Streptococcus faecalis DSM 4086, Lactobacillus acidophilus DSM 4149, Lactobacillus helveticus DSM 4183. In research studies, the expressed antimicrobial properties of probiotic strains metabolites against gram-negative and gram-positive microorganisms have been established. Thus, LHG2 Lactobacillus casei bacteriocin has high antibacterial properties relative to gram-positive pathogens, especially to Staphylococcus aureus. In future, it is planned to be used for improvement of the existing antimicrobial peptides aimed at combating antibiotic-resistant pathogenic microorganisms [7]. The AMPs LR14 Lactobacillus plantarum bacteriocin has a pronounced antifungal effect up to four fungi: Aspergillus niger, Rhizopus stolonifer, Mucor racemosus and Penicillium chrysogenum [6]. The nukacin lantibiotic (nukacin ISK-1) demonstrates high antibacterial activity against planktonic forms of pathogens [1]. The Lactobacillus acidophilus and L. fermentum metabolites are potent antimicrobial agents against K. pneumoniae biofilms [4].

We have suggested a new technology for obtaining probiotic candidates: drugs containing no traditional nutrient media, designed for cultivation of probiotic microorganisms: separately lactobacilli or in combined with saccharomycetes in disintegrates of L. rhamnosus GG. The most pronounced antimicrobial properties with respect to antibiotic-resistant agents were found in metabolic compounds of L. rhamnosus.

In the chemist's (pharmacist's) protocol, the list of over-the-counter medications for the symptomatic treatment of diarrhea includes probiotics and products of their vital functions, namely lactobacilli. Regarding the particulars of the drugs application, it is known that "probiotics may be used against the background of antimicrobial therapy, since they include antibiotic-resistant strains of microorganisms" [2]. The above enhances the possibilities of probiotic drugs and their metabolic products practical application in medicine for scientific research purposes.

The metabolites obtained by means of our own technology, due to its high antibacterial activity against antibiotic-resistant pathogens, open the prospect for further designing a new class of antimicrobial agents with a wide spectrum of action.

Conclusions

1. The antibacterial effect of experimental filtrates samples, which manifested itself in a bacteriostatic or bactericidal action against multiple drug resistance strains of Corynebacterium xerosis, Klebsiella pneumonia, Pseudomonas aeruginosa, Acinetobacter baumannii, Lelliottia amnigena (Enterobacter amnigenus), depends on the initial concentration of pathogenic microorganisms in the experiments.

2. Lactobacilli metabolites and the mixture of L. rhamnosus and S. boulardii in relation to the multiple drug resistance strain of non-diphtheria C.xerosis corynebacteria display the 100% bactericidal effect irrespective of the test culture seeding dose.

3. All experimental samples were bactericidal to L. amnigena (E. amnigens), A. baumannii, P. aeruginosa multiple drug resistance strains provided that a lower seed yield was applied (0.5 units by the McFarland scale, diluted 10 times). An increase in the initial seeding dose of test cultures to 1.0 unit by the McFarland scale was accompanied by the presence of the selected pathogens viable cells (48.0±8.32 – 68.72±7.16) % after exposure to cell-free substances.

4. The antimicrobial activity of the both types metabolites (lactobacilli and a combination of bacteria and fungi) was manifested by the complete loss of the K. pneumonia test culture viability at a low seeding dose, and after the influence of the disintegrate a bacteriostatic effect was observed, which was accompanied by the presence of living microbial pathogen cells amounting 44.37±8.22%.

5. The highest antimicrobial effect among all the L. rhamnosus samples of the selected multiple resistances to antibacterial drugs pathogens was achieved with the use of metabolic lactobacilli compounds.

Prospects of further research: L. rhamnosus disintegrates and metabolites presented in the paper, as well as a combination of lactobacilli and saccharomycetes metabolites possess high antibacterial properties in relation to gram-positive and gram-negative pathogens with multiple resistance to antibacterial drugs, which prompts a further comprehensive study of the authors designed cell-free probiotic substances to permit further designing a new class of antimicrobial agents with a wide spectrum of action.

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