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ANTIBIOFILM ACTIVITY OF LACTOBACILLUS RHAMNOSUS GG AND SACCHAROMYCES BOULARDII METABOLITES IN RELATION TO POLYRESISTANT GRAM-NEGATIVE MICROORGANISMS

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In order to avoid the chronic forms of diseases development and to effectively treat patients with acute manifestations of infections it is necessary not only to fight pathogenic microorganisms with antibacterial drugs, but also to learn how to influence the pathogens biofilm formation. In this work, the study of the antibiofilm activity of the original metabolites of *Lactobacillus rhamnosus GG* and *Saccharomyces boulardii*, obtained by cultivating probiotics in disintegrated microorganisms, was carried out. The antibiofilm effect of probiotic substances concerning strains polyresistant to antibacterial drugs (*Pseudomonas aeruginosa PR, Klebsiella pneumoniae PR, Lelliottia amnigena (Enterobacter amnigens) PR*) is dependent on the activity of *L. rhamnosus GG* and *S. boulardii* filtrates and on the individual sensitivity of the pathogens test culture. Statistically significant reduction of the biofilms formation by pathogens' microbial cells occurred with the use of metabolites of bacteria and of fungi ($p<0.03$). High antibiofilm properties inherent in the combination of saccharomyces and lactobacilli metabolites, with the exception of the polyresistant *K. pneumoniae* culture, which tended to reduce biofilm formation. The disintegrated *L. rhamnosus GG* and *S. boulardii* were inferior to the activity of these pathogens as to their antibiofilm properties.

Key words: biofilms, products of lactobacilli and saccharomyces metabolism, gram-negative microorganisms polyresistant to antibacterial drugs.

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In recent decades, the ability to effectively treat patients with bacterial diseases is rapidly faltering: intractable diseases are replenished due to the reduced activity of antibiotics and, as a result, emergence of pathogens resistant to antimicrobial drugs. The problem of fighting against resistant microorganisms continues aggravating and is acquiring the global significance. According to the World Health Organization (WHO), due to the misuse and excessive use of antibiotics, the time may come when widespread infections and minor injuries are likely to become fatal again [3, 8, 13]. WHO published a list of antibiotic-resistant "priority pathogens" ("supermicrobs"): Acinetobacter, Pseudomonas, various species of Enterobacteriaceae (Klebsiella, E. coli, Serratia, Proteus).

Pathogenic microorganisms contained in biofilms are almost not sensitive to antibiotics [2]. It was established that the formation of biofilms is going on in stages: bacteria join each other in a few minutes, within 2-4 hours they form dense colonies of microscopic size, within 6-12 hours they become tolerant to biocides , after 2-4 days, depending on the microorganism's species and the conditions of its cultivation, lose planktonic forms of bacteria and create complete biofilm colonies, which are characterized by high resistance to biocides, and, with mechanical destruction applied, have the ability to quickly recover and within 24 hours to re-create a mature biofilm [1, 7]. The presented dynamics of the mature microorganism biofilm formation, testifies to the complexity of its formation process and to the difficulty of affecting the bacteria being inside. According to the literature, 60% of all chronic and recurrent infections occurred with the participation of biofilms [4, 6, 7, 11].

Consequently, in order to avoid the development of chronic disease forms and to effectively treat patients with infections caused by bacteria and fungi, it is necessary to fight against pathogenic and polyresistant opportunistic microorganisms, as well as to develop means affecting the pathogens biofilm formation [3]. Studies of recent years show the promising use of substances that can suppress the biofilm formation of Staphylococcus aureus and Staphylococcus epidermidis opportunistic bacteria, based on of lactobacilli metabolites (Lactobacillus plantarum L3 and Lactobacillus fermentum 97) [2].

The purpose of the study was to substantiate the promising application of Lactobacillus rhamnosus GG and Saccharomyces boulardii metabolism products against the biofilm forming activity of polyresistant gram-negative microorganisms to design the new generation antimicrobials.

Materials and methods. Probiotic strains were used to obtain metabolic products: Lactobacillus rhamnosus (LGG®) ATCC 53103 derived from the PREEMA® symbiotic (Schonen, Switzerland) and Saccharomyces boulardii CNCM I-745, isolated from BULARDI® drug (Schonen, Switzerland).

Ultrasonic disintegrates of lactobacilli (L) and saccharomycetes (S) were derived from suspensions of microorganisms with an optical density of 10.0 units by McFarland (Densi-La-Meter, PLIVA-Lachema Diagnostika, Czech Republic). The metabolic products deriving from lactobacillus (ML) and saccharomycetes (MS) was carried out by cultivating a probiotic in its own disintegrate according to the procedure [9]. The original products of microorganism metabolism were obtained by introducing saccharomycetes suspension into the ultrasonic lactobacilli (LS) disintegrate, or by adding a mixture of lactobacilli and saccharomycetes cells to the lactobacilli disintegrate (MLS) [9].

Were used as test-strains polyresistant to antibacterial drugs: Pseudomonas aeruginosa PR (P. aeruginosa PR), Klebsiella pneumoniae PR (K. pneumoniae PR), Lelliottia amnigena (Enterobacter amnigenus) (L.amnigena (E.amnigenus) PR) and Pseudomonas aeruginosa (P. aeruginosa) and the reference strain of Pseudomonas aeruginosa ATSC 27853 (P. aeruginosa ATSS 27853). Concentration microbial cells complied $1{,}5 \times 10^7$ CFU / ml [8, 12].

The study of the metabolic complexes impact on the process of biofilm formation was performed by means of spectrophotometric method in polystyrene flat-bottom 96-well plates (TOV Eximcargotrade, Ukraine) [12]. To each well was added 110 μl of TSB (trypticase-soy broth) with 1% glucose. To the experimental wells, 30 μl of metabolites were added, and to the control ones - a physiological solution of sodium chloride. Bacterial cultures were introduced in the amount of 10 μl into the experimental and positive control wells (control of culture), and into the negative control wells (control of medium), test cultures suspension was replaced with sodium chloride physiological saline solution. Incubation was carried at a temperature of 37 ° C. for 24 hours. The contents of the wells being removed, the plates were washed three times with 0.1 M phosphate-saline buffer (FSB, pH 7.2) and dried at 60° C for 60 minutes. After fixation, the biofilms were stained with 1% solution of crystal violet (150 μl / well) followed by thorough washing and addition of 150 μl of 96 ° ethanol (30 minutes at room temperature). Changes in the optical densities were measured using Erba LisaScantem EM. The degree of biofilm formed was calculated according to the formula:

$$OD_{exp} = OD_{av} - OD_{cut}, \text{ where}$$

$$OD_{cut} = OD_{av.neg/c} + (3\times SD_{neg/c}), \text{ where}$$

OD_{exp} - optical density of the experimental strain, OD_{av} - average test strain, OD_{cut} - cut off value, $OD_{av.neg/c}$ - average negative control, $SD_{neg/c}$ - standard deviation of negative control [12]. The study was carried out three times in three replicates. Statistical processing of the study results was carried out with a personal computer using Excel 2010 software package (Microsoft, USA). The mean values of the obtained indices (M) with standard deviations (m) were calculated. The significance of the difference between the indices obtained was determined using the Student's criterion (t). The presented differences of the results processed were reliable and amounted $p<0.03$ and $p<0.05$.

Results of the study and their discussion. Probiogenic metabolic products developed by the authors have a broad spectrum of action and possess high antimicrobial activity compared to many obligate pathogenic and opportunistic gram-positive and gram-negative microorganisms. Studying the antibiofilm properties of the original biologically active Lactobacillus rhamnosus GG and Saccharomyces boulardii complexes' effects on the biofilm formation by gram - negative microorganisms polyresistant to antimicrobial agents is carried out for the first time.

In determining the degree of biofilms formation by pathogens, according to the literature, the researchers measured the optical density of the wells content according to different wavelengths of 492 nm, 570 nm, 620 nm, etc. Our preliminary analysis of the experiments on studying the ability of the Pseudomonas aeruginosa (P. aeruginosa) circulating strain to form a biofilm in 96-well microplates was carried out with measurements of optical densities available at three wavelengths: 578 nm, 630 nm, and using the double reading option of 578 nm + 630 nm (Table 1). Depending on the light filter's wavelength, the excellent indices of absolute values for the optical density of the P. aeruginosa eluates were observed, both in the control and in the experimental samples. Despite the different values of the optical density, the initial intensity of the pathogen biofilm formation and the change in the biofilm formation degree under the influence of the Lactobacillus rhamnosus GG and Saccharomyces boulardii samples, measured at the same wavelength, answered to the changes between similar indices fixed with other wavelengths applied.

Using the obtained results on the Pseudomonas aeruginosa strain's ability to biofilm formation by means of three-wavelengths light filters, it was concluded that any wave may be used to determine the optical density of the experimental microorganisms eluates. Consequently, further experiments on the effects of the Lactobacillus rhamnosus GG and Saccharomyces boulardii test substances on the biofilms formation by bacterial test cultures were carried out using filters with the wavelength of 630 nm.

Table 1

Optical density of biofilm formation by Pseudomonas aeruginosa strain after exposure to lactobacilli and saccharomycetes metabolism products when using light filters with different wavelengths (OD_{exp})

Wavelength, nm	C	Structural components and metabolite compounds, (M±m)					
		L. rhamnosus			S. boulardii		
		L	ML	MLS	S	MS	LS
578	1.903± 0.004*	1.49± 0.006*	0.916± 0.012**	1.305± 0.013**	1.323± 0.0123**	1.614± 0.007*	1.377± 0.012**
630	0.736± 0.011**	0.219± 0.013**	0.161± 0.009**	0.343± 0.013**	0.435± 0.014**	0.533± 0.006*	0.703± 0.011**
578+630	2.095± 0.003*	1.577± 0.013**	0.915± 0.01**	1.881± 0.009**	1.362± 0.005**	1.859± 0.006*	2.067± 0.006*

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: * - $p<0.02$; ** - $p<0.05$

The results of studying the work on determining the effect of lactobacilli filtrates and saccharomycetes metabolic products on the biofilm formation in two representatives of Pseudomonas aeruginosa showed a different degree of biofilms formation inhibition by pathogens (fig. 1). Thus, one of the selected representatives, P. aeruginosa ATCC, was sensitive to all the studied samples of L. rhamnosus GG and S. boulardii. The maximum statistically reliable inhibition of biofilms formation by the reference strain among the studied probiotic substances was observed with the use of lactobacilli (ML) metabolites: the reduction was more than by 100 times ($p = 0.03$).

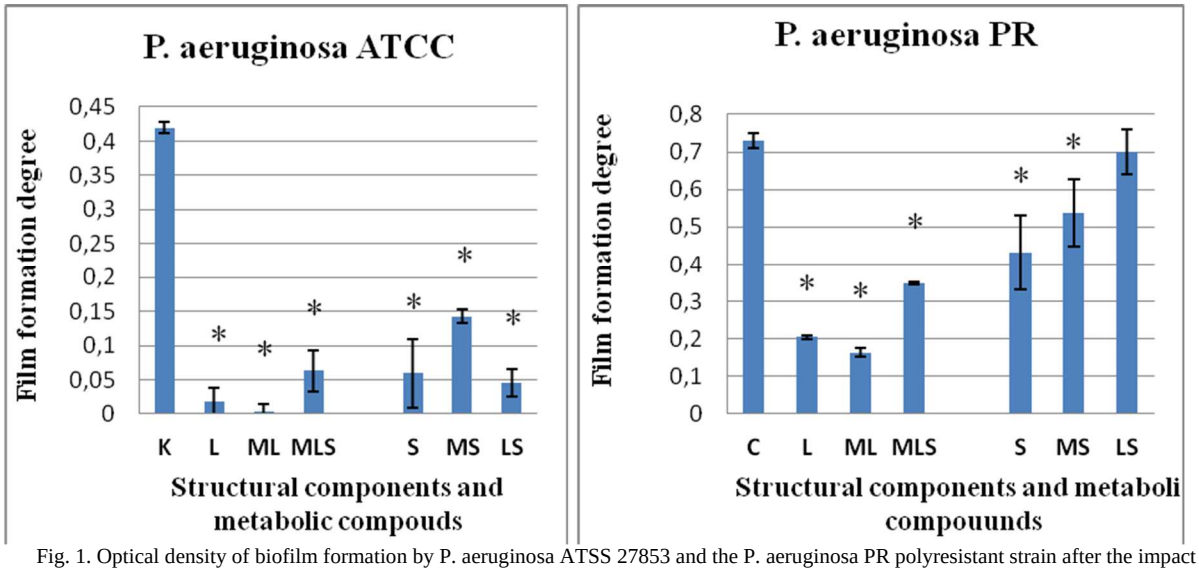

Fig. 1. Optical density of biofilm formation by P. aeruginosa ATSS 27853 and the P. aeruginosa PR polyresistant strain after the impact of lactobacilli and saccharomycetes metabolites. Note: C- control, L - disintegrate (structural components) of lactobacilli, ML - metabolites (metabolic compounds) of 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: - $p<0.03$.

Biofilm formation of the second microorganism, P. aeruginosa PR, which is polyresistant to antibacterial drugs, was reduced under the impact of all experimental samples except for the metabolites of saccharomycetes grown on lactobacilli (LS): the pseudomonade culture was insensitive to the said samples. The greatest inhibition of the biofilms formation is performed by opportunistic pathogens, among the presented L. rhamnosus GG and S.boulardii filtrates, has been established with the use of bacterial metabolites (ML).

The performed experiments permitted to establish that, regardless of the pseudomonade strain, the more pronounced antibiotic effect was observed after incubation of microbial cells with metabolites of lactobacilli (ML).

The results of studying the antibiofilm activity of Lactobacillus rhamnosus GG and Saccharomyces boulardii compared to the K. pneumoniae PR polyresistant strain differed from the previous data (figure 2). The use of both types of disintegrators (saccharomycetes (S) and lactobacilli (L)) did not cause statistically significant changes in the biofilm formation of the selected opportunistic microorganism. Under the influence of lactobacilli and saccharomycetes (MLS) metabolites combination, there was observed a tendency to reduce the biofilms formation by a polyresistant strain, however, no significant

differences between the experimental and control samples were observed. The statistically significant reduction in the biofilm formation by K. pneumoniae PR was established after application of metabolites samples: ML - by 1.7 times (p = 0.01), MS - by 1.3 times (p = 0.01), and LS - by 1.6 times (p = 0.02). Determination of the most active filtrate among L. rhamnosus GG, S. boulardii disintegrates, metabolites of lactobacilli and saccharomycetes, mixture of bacteria and fungi probiotic strains metabolites, which reduces the formation of biofilms by the K. pneumoniae polyresistant strain, confirmed the results of the performed pseudomonades studies. A more pronounced inhibition of the biofilm formation by opportunistic cultures was observed after the preliminary microbial cells incubation with L. rhamnosus GG (ML) metabolites samples.

Polyresistant strain of L.amnigena (E.amnigenus) PR was more sensitive to the lactobacilli and saccharomycetes metabolites (fig. 2). As the study results showed, all experimental filtrates produced an adverse effect on the pathogen's biofilm formation. The complete suppression of the above culture biofilm formation was observed after the use of bacteria and fungi (L, S) disintegrates, Lactobacillus rhamnosus GG and Saccharomyces boulardii (ML, MS) metabolites and combination of saccharomycetes and lactobacilli (MLS). Antibiofilm action of the saccharomycetes metabolites grown on lactobacilli (LS) on the selected test culture was manifested by the biofilm formation reduced by 2.2 times (p = 0.006).

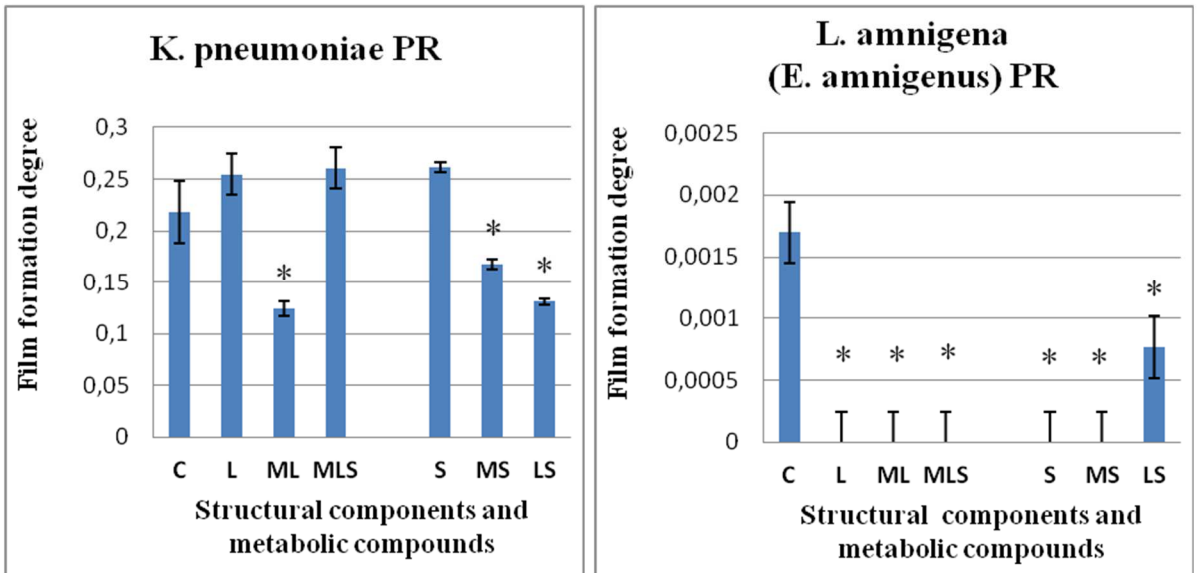

Fig. 2. Optical density of biofilm formation by polyresistant strains of K. pneumoniae PR and L.amnigena (E.amnigenus) PR impacted with metabolic products of lactobacilli and saccharomycetes. Note: C- control, L - disintegrate (structural components) of lactobacilli, ML - metabolites (metabolic compounds) 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: - $p<0.03$.

The results of the high antibiofilm activity of metabolites obtained by the author's method coincide with the data of other researchers [5, 8, 12, 13]. The degradation by peptide substances of biofilms *Pseudomonas aeruginosa* is known after 4 hours, but the concentration of the pathogen was less than own in 10-fold (2×10^6) [8]. In the following experiment, the effect on an even smaller number of pathogen microbial cells (0.5×10^5 CFU / ml) was studied in contrast to our own study (1.5×10^7 CFU / ml) [5]. The installed lack of effectiveness of the 1-hour effect of biologically active substances Lactobacillus jensenii and Lactobacillus rhamnosus on the pre-formed biofilms of antibiotic resistant strains of Acinetobacter baumannii, Escherichia coli and Staphylococcus aureus [13]. The increase in incubation time to ~ 18 hours was accompanied by degradation of 75 - 99.9% of the biofilms of all three pathogens. The data are consistent their own work pronounced antibiotic effect on antibiotic resistant strains of microorganisms and incubation time. A similar duration of exposure was tested by Osama et al. [12]. They also established the anti-film activity of metabolites of Lactobacillus rhamnosus EMCC 1105, but at a lower concentration of the microorganism (2×10^5 CFU / ml). In the previous own work the expressed anti-biofilm properties of metabolic complexes of lactobacilli and saccharomycetes were obtained in relation to pre-formed biofilms Corynebacterium ulcerans tox + 112, Corynebacterium diphtheriae gravis tox + 108; Pseudomonas aeruginosa ATCC 27853 with a high concentration of microbial cells (1.5×10^7 CFU / ml). The decrease in optical density of the formed biofilm of all tested strains occurred in 1.3 - 5.3 times depending on the strain and the test substances [11].

Conclusions

1. All the selected bacterial test cultures to various extents are sensitive to samples of L. rhamnosus and S. boulardii. The antibiotic effect of metabolic products on polyresistant to anti-bacterial drugs strains depends on the activity of lactobacilli and saccharomycetes metabolic products and on the individual sensitivity of the pathogens test culture.

2. Comparative characteristics of the L. rhamnosus GG and S. boulardii filtrates impact on the biofilm formation by the presented gram-negative strains showed that, under the same conditions of the experiment performed, the statistically significant reduction in the biofilms formation by microbial pathogens cells occurred with the use of bacteria and fungi metabolites obtained by means of cultivation on their own disintegrates ($p<0.03$). The combination of saccharomycetes and lactobacilli metabolites possesses high antibiofilm properties, which is established due to a significant reduction of the biofilms formation by selected test strains, except for the polyresistant K. pneumoniae culture, which tended to reduce its biofilm formation. Other metabolic products of Lactobacillus rhamnosus GG and Saccharomyces boulardii, in terms of their antibiofilm properties against the said patogens, were inferior to the above mentioned ones.

Prospects for further research lie in the comprehensive study of disintegrates and original metabolic products of L. rhamnosus GG and S. boulardii in order to apply them in development of new generation antimicrobials against pathogenic and polyresistant to antibacterial drugs opportunistic pathogens.

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Реферати

АНТИБІОПЛІВОЧНА АКТИВНІСТЬ ПРОДУКТІВ МЕТАБОЛІЗМУ LACTOBACILLUS RHAMNOSUS GG І SACCHAROMYCES BOULARDII ПО ВІДНОШЕННЮ ДО ПОЛІРЕЗИСТЕНТНИХ ГРАМНЕГАТИВНИХ МІКРООРГАНІЗМІВ

Ісаєнко О.Ю., Книш О.В., Рижкова Т.М., Коцар О.В., Дюкарева Г. І.

Щоб уникнути розвитку хронічних форм захворювань та ефективно проводити лікування хворих з гострими проявами інфекцій необхідно не лише боротися з патогенними мікроорганізмами за допомогою антибактеріальних препаратів, а й навчитися впливати на біоплівкоутворення збудників. В роботі проведено вивчення антибіоплівочної активності оригінальних продуктів метаболізму Lactobacillus rhamnosus GG і Saccharomyces boulardii, отриманих шляхом

АНТИБИОПЛЕНОЧНАЯ АКТИВНОСТЬ ПРОДУКТОВ МЕТАБОЛИЗМА LACTOBACILLUS RHAMNOSUS GG И SACCHAROMYCES BOULARDII ПО ОТНОШЕНИЮ К ПОЛИРЕЗИСТЕНТНЫМ ГРАМОТРИЦАТЕЛЬНЫХ МИКРООРГАНИЗМОВ

Исаенко Е.Ю., Кныш О.В., Рыжкова Т.Н., Коцарь Е.В., Дюкарева Г.И.

Чтобы избежать развития хронических форм заболеваний и эффективно проводить лечение больных с острыми проявлениями инфекций необходимо не только бороться с патогенными микроорганизмами с помощью антибактериальных препаратов, но и научиться влиять на биопленкообразование возбудителей. В работе проведено изучение антибиопленочной активности оригинальных продуктов метаболизма Lactobacillus rhamnosus GG и Saccharomyces boulardii, полученных путем культивирования

культивування пробіотиків у дезінтеграті мікроорганізмів. Антибіоплівочний ефект пробіотичних речовин відносно полірезистентних до антибактеріальних препаратів штамів (Pseudomonas aeruginosa PR, Klebsiella pneumoniae PR, Lelliottia amnigena (Enterobacter amnigenus) PR) має залежність від активності фільтратів L. rhamnosus GG та S. boulardii та від індивідуальної чутливості тест-культури збудників. Статистично значуще зменшення утворення біоплівок мікробними клітинами патогенів відбувалося при застосуванні метаболітів бактерій і грибів ($p<0,03$). Високими антибіоплівочними властивостями володіє комбінація метаболітів сахароміцетів та лактобактерій, за винятком полірезистентної культури K. pneumoniae, у якої спостерігалась тенденція до зменшення біоплівкоутворення. Дезінтеграти L. rhamnosus GG і S. boulardii за своїми антибіоплівочними властивостями відносно даних збудників поступалися активністю.

Ключові слова: біоплівки, продукти метаболізму лактобактерій і сахароміцетів, полірезистентні до антибактеріальних препаратів грамнегативні мікроорганізми.

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пробиотиков в дезинтеграте микроорганизмов. Антибиопленочный эффект пробиотических веществ относительно полирезистентных к антибактериальным препаратам штаммов (Pseudomonas aeruginosa PR, Klebsiella pneumoniae PR, Lelliottia amnigena (Enterobacter amnigenus) PR) зависит от активности фильтрата L. rhamnosus GG и S. boulardii и от индивидуальной чувствительности тест-культуры возбудителей. Статистически значимое уменьшение образования биопленок микробными клетками патогенов происходило при применении метаболитов бактерий и грибов ($p<0,03$). Высокими антибиопленочными свойствами обладает комбинация метаболитов сахаромицетов и лактобактерий, за исключением полирезистентной культуры K. pneumoniae, у которой наблюдалась тенденция к уменьшению биопленкообразования. Дезинтеграты L. rhamnosus GG и S. boulardii по своим антибиопленочным свойствам в отношении данных возбудителей уступали активностью.

Ключевые слова: биопленки, продукты метаболизма лактобактерий и сахаромицетов, полирезистентные к антибактериальным препаратам грамотрицательные микроорганизмы.

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ENERGY VALUE OF DIETARY INTAKE AND ITS CONFORMITY TO DAILY NEEDS IN YOUNG PEOPLE

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The paper presents the energy value of dietary intake of people with different body weight and its compliance with daily energy needs. 96 people of both gender aged 18-25 have been involved into the study. In the groups with normal body weight, overweight (body mass index of 25.00-29.99 kg/m^2) and Class I obesity (body mass index of 30.00-34.99 kg/m^2) nutritional status has been studied by the method of 24-hour nutrition reproduction, as well as basal metabolism and recommended daily energy dietary intake. The energy value of the dietary intake of the subjects of both gender with overweight and Class I obesity significantly exceeded the energy value of the dietary intake of controls. In the group with overweight, the level of positive energy balance in male subjects on a weekday was 16.02%, in male subjects and female subjects on a weekend day was 26.47% and 24.46%, respectively. In male subjects with obesity, the level of positive energy balance on a weekday was 20.09%, on a weekend day was 33.92%, whereas in female subjects with obesity it was 10.48% and 37.28% on a weekday and a weekend day, respectively. Strong to moderate correlation has been established between anthropometric and energy metabolism values.

Keywords: overweight, Class I obesity, energy value of dietary intake, energy metabolism, positive energy balance.

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The rapid increase in the number of overweight and obese people is attracting the attention of health professionals, since the above conditions are the precursors for the formation of a large number of diseases, namely, diabetes mellitus, metabolic syndrome, cardiovascular disease, diseases of the musculoskeletal system [8].

Recently, the nutrition pattern of the population worldwide has changed in the direction of the predominance of products high in fat and carbohydrates but poor in microelements and low quality nutrients. The current diet contains predominantly highly refined foods, long-term storage products, fast food, which contributes to the development of metabolic disorders, leading to overweight and obesity [9]. Young people may be one of the most vulnerable groups in terms of weight gain and obesity, given the numerous lifestyle factors that can potentially affect health [15]. These are energy imbalance, hypodynamia, significant emotional stress, chronic insomnia, irregular nutrition, inadequate distribution of the daily dietary intake, the presence of bad habits, etc. [5, 6].

Energy imbalance, caused by constant increased energy consumption with food and low levels of its expenditure is one of the main factors in the formation of overweight and obesity [10]. Information on studying the compliance of the energy value of the dietary intake with the energy needs of young people is quite limited.