The effects of cell-free extracts derived from probiotic strains
*Bifidobacterium bifidum* and Lactobacillus reuteri
on the proliferation and biofilm formation by Lactobacillus reuteri in vitro

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The aim of the research was to investigate the ability of cell-free extracts, containing derivatives of probiotic strains *Bifidobacterium bifidum* and *Lactobacillus reuteri*, to influence the proliferation and biofilm formation by *Lactobacillus reuteri* in vitro; to evaluate the prospects for the creation of new metabolics and means of increasing the overall productivity of probiotic cell biomass based on them.

Materials and methods. Cell-free extracts were obtained from probiotic strains *B. bifidum* 1 and *L. reuteri DSM 17938* by the authors’ method. Probiotic strain *L. reuteri* DSM 17938 was used as a test culture. The investigation of the proliferation and biofilm formation by *L. reuteri* was carried out by spectrophotometric method using a microtiter-plate reader "Lisa Scan EM" (Erba Lachema s.r.o., Czech Republic).

Results. It has been established that the cell-free extract obtained from *L. reuteri* culture grown in its own disintegrate, supplemented with glycerol and glucose, is the only one of all studied, which stimulates both proliferation and biofilm formation by *L. reuteri*. The cell-free extracts, obtained from *L. reuteri* and *B. bifidum* disintegrate and from *L. reuteri* culture, grown in its own disintegrate, stimulate proliferation of test-culture to varying degrees (depending on the type of extract and its content in the culture medium), but have a significant inhibitory effect on the biofilm formation by *L. reuteri*. The extract, obtained from *B. bifidum* culture, grown in its own disintegrate, does not have a significant effect on proliferation and greatly suppresses the biofilm formation by *L. reuteri*.

Conclusions. The results of the study allow us to positively evaluate the prospects for the creation of new metabolics based on probiotic derivatives. Cell-free extract, obtained from *L. reuteri* culture, grown in its own disintegrate supplemented with glycerol and glucose can contribute to the survival and facilitate inoculation of the introduced probiotic in the gastrointestinal tract when used together.

Derivative-containing extracts with a pronounced growth-stimulating effect can be the basis for creating the means of increasing the overall productivity of the probiotic cell biomass. The use of such means will increase the economic efficiency of probiotic cultures production. Obtained data induce further study of the biochemical composition, elucidation of the mechanism of cell-free extracts action and confirmation of their efficacy in vivo.

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Вплив безклітинних екстрактів, що отримані з пробіотичних штамів
*Bifidobacterium bifidum* та *Lactobacillus reuteri*, на проліферацію та біоплівкоутворення *Lactobacillus reuteri* in vitro

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Мета роботи – дослідити здатність безклітинних екстрактів, що містять деривати пробіотичних штамів *Bifidobacterium bifidum* та *Lactobacillus reuteri*, впливати на проліферацію та біоплівкоутворення *Lactobacillus reuteri* in vitro; оцінити перспективи створення на їхній основі нових метабіотиків, засобів підвищення загальної продуктивності клітинної біомаси пробіотика.

Матеріали та методи. Безклітинні екстракти отримували з пробіотичних штамів *B. bifidum* 1 і *L. reuteri DSM 17938* за методом, що розроблені авторами. Пробіотичний штам *L. reuteri DSM 17938* використовували як тест-культуру. Проліферацію та біоплівкоутворення *L. reuteri* досліджували спектрофотометричним методом за допомогою мікропланшетного аналізатора «Lisa Scan EM» («Erba Lachema s.r.o.», Чеська Республіка).

Результати. Безклітинний екстракт, який отримали з культури *L. reuteri*, що вирощена у власному дезінтеграті з додаванням гліцерину та глукози, є одним з усіх досліджених, котрий стимулює і проліферацію, і біоплівкоутворення *L. reuteri*. Безклітинні екстракти, що отримані з дезінтегратів *L. reuteri* і *B. bifidum* і з культури *L. reuteri*, вирощеної у власному дезінтеграті, стимулюють проліферацію тест-культури різною мірою (залежно від типу екстракту та його вмісту в культурі), але мають значущий інгібіторний вплив на біоплівкоутворення *L. reuteri*. Екстракт, що отриманий з культури *B. bifidum*, яка вирощена у власному дезінтеграті, не має істотного впливу на проліферацію та суттєво пригнічує біоплівкоутворення *L. reuteri*.

Висновки. Результати досліджень дають можливість позитивно оцінити перспективи створення нових метабіотиків на основі пробіотичних похідних. Безклітинні екстракти, що отримані з культури *L. reuteri*, яка вирощена у власному дезінтеграті з додаванням гліцерину та глукози, може сприяти вживанню та полегшувати інокуляцію введеного пробіотика у шлунково-кишковому тракті при поєднаному використанні.

Дериват-вмісні екстракти з вираженим ріст-стимулюючим ефектом можуть стати основою для створення засобів підвищення загальної продуктивності клітинної біомаси пробіотика. Використання таких засобів підвищить економічну ефективність виробництва пробіотичних культур. Результати спонукають до продовження вивчення біохімічного складу, з’ясування механізму дії безклітинних екстрактів і підтвердження їхньої ефективності in vivo.
Влияние бесклеточных экстрактов, полученных из пробиотических штаммов Bifidobacterium bifidum и Lactobacillus reuteri, на пролиферацию и биопленкообразование Lactobacillus reuteri in vitro

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Цель работы – исследовать способность бесклеточных экстрактов, содержащих дериваты пробиотических штаммов Bifidobacterium bifidum и Lactobacillus reuteri, влиять на пролиферацию и биопленкообразование Lactobacillus reuteri in vitro; оценить перспективы создания на их основе новых метабиотиков и средств повышения общей производительности клеточной биомассы пробиотика.

Материалы и методы. Бесклеточные экстракты получали из пробиотических штаммов B. bifidum 1 и L. reuteri DSM 17938 методом, разработанным авторами. Пробиотический штамм L. reuteri DSM 17938 использовали в качестве тест-культуры. Пролиферацию и биопленкообразование L. reuteri исследовали спектрофотометрическим методом с помощью микро-планшетного анализатора «Lisa Scan EM» («Erba Lachema s.r.o.», Чешская Республика).

Результаты. Бесклеточный экстракт, полученный из культуры L. reuteri, выращенной в собственном дезинтеграте с добавлением лигнинов и глюкозы, – единственный из всех исследованных, который стимулирует пролиферацию и биопленкообразование L. reuteri. Бесклеточные экстракты, полученные из дезинтегратов L. reuteri и B. bifidum и культуры L. reuteri, выращенной в собственном дезинтеграте, стимулируют пролиферацию тест-культуры в разной степени (в зависимости от типа экстракта и его содержания в среде культивирования), но имеют значительное ингибирующее влияние на биопленкообразование L. reuteri. Экстракт, полученный из культуры B. bifidum, выращенной в собственном дезинтеграте, не имеет существенного влияния на пролиферацию и значительно подавляет биопленкообразование L. reuteri.

Выводы. Результаты исследования позволяют положительно оценить перспективы создания новых метабиотиков на основе пробиотических производных. Бесклеточный экстракт, полученный из культуры L. reuteri, выращенной в собственном дезинтеграте с добавлением лигнинов и глюкозы, может способствовать выживанию и облегчать инокуляцию введенного пробиотика в желудочно-кишечном тракте при совместном использовании.

Дериват-содержащие экстракты с выраженным рост-стимулирующим эффектом могут стать основой для создания новых метабиотиков на основе пробиотических производных. Бесклеточный экстракт, полученный из культуры L. reuteri, выращенной в собственном дезинтеграте с добавлением лигнинов и глюкозы, может способствовать выживанию и облегчать инокуляцию введенного пробиотика в желудочно-кишечном тракте при совместном использовании.

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the gastrointestinal tract by beneficial microorganisms and enhancing the protective properties of the immune system is using the biological activity of the probiotics’ derivatives [11–13].

The aim

The aim of the research was to investigate the ability of cell-free extracts, containing derivatives of probiotic strains Bifidobacterium bifidum and Lactobacillus reuteri, to influence the proliferation and biofilm formation by Lactobacillus reuteri in vitro; to evaluate the prospects for the creation of new metabolites and means of increasing the overall productivity of probiotic cell biomass based on them.

Materials and methods

Five cell-free extracts containing probiotics’ derivatives were investigated:
- L – filtrate of L. reuteri disintegrate;
- ML – filtrate of L. reuteri culture, grown in L. reuteri disintegrate;
- MLG – filtrate of L. reuteri culture, grown in L. reuteri disintegrate supplemented with 0.8 M (73.7 g/l) glycerol and 0.4 M (72.1 g/l) glucose;
- B – filtrate of B. bifidum disintegrate;
- MB – filtrate of B. bifidum culture, grown in B. bifidum disintegrate.

Disintegrates and probiotics’ cultures were obtained from probiotic strains B. bifidum 1 (from medical product “Bifidumbacterin-Biopharma”, JSC Biopharma, Ukraine) and L. reuteri DSM 17938 (from medical product “BioGaia”, BioGaia Production AB, Sweden) using the method developed by the authors [14]. Disintegrates were prepared by tenfold cyclic freezing-thawing of the bacterial suspensions with an optical density of 10 units on the McFarland scale. Cultures were obtained by growing probiotic microorganisms in their own disintegrates for 72 hours. Disintegrates and cultures were centrifuged and passed through membrane filters (Vladipor, RF) with a pore diameter of 0.2 μm to remove microbial cells and cellular debris. As a result, cell-free extracts were received.

The probiotic strain L. reuteri DSM 17938 was used as a proliferating and biofilm-forming test culture. The lyophilized microbial mass was rehydrated and recovered by cultivation in tryptone soya broth (TSB; HiMedia, India) in microaerophilic conditions for 24 hours at 37 °C. After checking the purity of the culture, the cells were washed three times to remove the components of the nutrient medium. For this, the cell suspension was centrifuged for 10 min at 1500 g and the pellet was resuspended in a sterile isotonic saline solution (0.9 % NaCl). The inoculum was prepared from the suspension of washed cells. It was a microbial suspension in a physiological saline solution with an optical density of 10 units on the McFarland scale (~10⁶ CFU/ml). The turbidity of the suspension was measured using the Densi-La-Meter II device (PLIVA-Lachema Diagnostika, Czech Republic).

The study of the effect of cell-free extracts on the L. reuteri proliferation was performed in sterile 96-well polystyrene microtiter plates (JSC “Exincargotrade”, Ukraine). TSB supplemented with 1 % glucose, cell-free extracts and inoculums were added into the test wells in the ratio of 8:1:1; 6:3:1 and 4:5:1. Thus, the final concentration of each extract in the cultivation medium was 10 % vol, 30 % vol or 50 % vol. TSB supplemented with 1 % glucose, physiological saline (PhS) and inoculums were added into the positive control wells (PC) in the same ratio: PC₈₃₀ (10 % vol of the PhS in the cultivation medium); PC₅₀ (30 % vol of the PhS in the cultivation medium); PC₃₀ (50 % vol of the PhS in the cultivation medium). The final concentration of microbial cells in the test and positive control wells was ~10⁷ CFU/ml. The negative control wells (NC) contained only TSB. The plates were covered with lids and incubated in microaerophilic static conditions for 24 hours at 35–37 °C. 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). The inhibition (or stimulation) indices were calculated by the formula: Inh (S/I) = (ΔOD – ΔOD₃₀) + ΔOD₃₀×100%, where ΔOD and ΔOD₃₀ are the optical density gain in the test and positive control wells within 24 hours, respectively.

The study of the effect of cell-free extracts on the biofilm formation by L. reuteri. Cultivation of biofilms in the wells and subsequent detection by stain for biofilm recognition were performed according to the method of Stepanovic’ et al. [15]. After measuring the optical density of the wells the plates were continued to incubate in microaerophilic static conditions for 24 hours at 35–37 °C. Than the contents of the wells were decanted into discard container. Each well was carefully washed three times with sterile 0.1 M phosphate-buffered saline (PBS; pH 7.2) using an appropriate micropipette. Plates were drained in an inverted position. Attached biofilms were fixed by exposing them to hot air at 60 °C for 60 min. Then they were stained by 1 % crystal violet for 15 min. After that, wells were washed ten times with purified water using a microspette. Ethanol as an eluent was gently added into the wells for resolubilization of the dye. Microtiter plate covered with the lid and was left at room temperature for 30 min. The optical density (OD) of the eluate in the test and control wells was measured at 630 nm by using a microtiter-plate reader “Lisa Scan EM” (“Erba Lachema s.r.o.”, Czech Republic). Based on the OD values obtained for NC and test samples, biofilm formation was defined as:
- weak (OD₃₀ < OD ≤ 2xOD₃₀);
- moderate (2xOD₃₀ < OD ≤ 4xOD₃₀);
- strong (4xOD₃₀ < OD);
- no biofilm formation (OD ≤ OD₃₀), according to the method for quantification of biofilm formation proposed by In Lee S. et al. [16].

The inhibition (or stimulation) indices were calculated by the formula: Inh (S/I) = (OD – OD₃₀) + OD₃₀×100%, where OD and OD₃₀ are optical density of the test and control samples, respectively.

All experiments were performed three times. Each sample was tested in triplicate. Obtained data were statistically processed with Excel 2010 software (Microsoft, USA). Average values of obtained indices (OD or ΔOD) with standard deviations (SD) were determined. The significance of the difference between the obtained indices of the test and control groups was determined by Student’s t-test. Differences were considered significant at P < 0.05.
Results

At the first stage of research, the effect of cell-free extracts on *L. reuteri* proliferation was studied. The data presented in Fig. 1 suggests that an increase in the volume of the PhS replacing the nutrient medium in the control sample from 10 % to 30 % and from 10 % to 50 %, is accompanied by a decrease in the optical density gain of the culture by an average of 13.8 % and 26.0 %, respectively. This is a dilution effect due to a decrease in the amount of nutrients in the cultivation medium. Introduction into the cultivation medium of the L extract at a concentration of 10 % leads to a significant increase in the optical density gain of the culture compared to PC<sub>10</sub> (StIs are 63.6 % and 51.3 %, respectively). The InhIs after the introduction of MLG: filtrate of *L. reuteri* disintegrate supplemented with glycerol and glucose; *: the differences are significant compared to the PC<sub>10</sub>; #: compared to the PC<sub>50</sub>; †: compared to the PC<sub>10</sub>, P < 0.05.

As can be seen from the data presented in Fig. 2, cell-free extract B in the composition of the cultivation medium at a concentration of 10 % does not have a significant effect on the optical density gain of the culture compared to PC<sub>10</sub>. An increase in the content of this extract in the composition of the cultivation medium to 30 % and 50 % leads to a significant increase of the culture optical density gain compared to the PC<sub>10</sub> and PC<sub>50</sub> (StIs are 19.5 % and 16.3 %, respectively). The presence of B extract in the cultivation medium does not affect the culture optical density gain.

At the second stage of research, the effect of cell-free extracts on biofilm formation by *L. reuteri* was studied. Optical density of the eluate enables to indirectly judge the biofilm formation by bacteria. An increase in the volume of the PhS replacing the nutrient medium in the control sample from 10 % to 30 % and from 10 % to 50 %, is accompanied by a decrease in the optical density of the eluate by an average of 8.9 % and 29.3 %, respectively (Fig. 3). The biofilm formation by *L. reuteri* culture at 10 %, 30 % and 50 % content of the PhS in the cultivation medium is defined as strong. The introduction into the cultivation medium of extract L at a concentration of 10 % is accompanied by a decrease in the optical density of the eluate compared with PC<sub>10</sub> (InhIs is 16.3 %). Extract ML at a concentration of 10 % does not affect the optical density of the eluate. An increase in the concentration of extracts of L and ML to 30 % in the cultivation medium is accompanied by a significant decrease of the biofilm formation by test-culture compared to PC<sub>10</sub> (InhIs are 63.6 % and 51.3 %, respectively).

But, despite this, the biofilm formation of the test culture is defined as strong. An increase in the content of L and ML extracts in cultivation medium up to 50 % leads to pronounced inhibition of biofilm formation by *L. reuteri* compared to PC<sub>10</sub> (InhIs are 78.0 % and 48.3 %, respectively). It should be noted that in the presence of L extract, biofilm formation by *L. reuteri* culture becomes moderate. MLG extract at 10 % and 30 % concentration in the cultivation medium significantly stimulates the biofilm formation of the test culture (StIs are 32.9 % and 25.5 %, respectively). The degree of biofilm formation by *L. reuteri* in a cultivation medium containing 50 % MLG extract does not differ from biofilm formation by test culture in the PC<sub>10</sub>.

As can be seen from the data presented in Fig. 4, B and MB extracts significantly inhibit biofilm formation of *L. reuteri* in a dose dependent manner. Calculated InhIs of biofilm formation under the influence of introducing B extract into the cultivation medium at concentrations of 10 %, 30 % and 50 % are: 50.0 %; 82.9 % and 83.7 %, respectively. The InhIs after the introduction of MB extract into the cultivation medium at the same concentrations are: 31.0 %; 73.8 % and 73.1 %, respectively. Biofilm formation by *L. reuteri* culture in the presence of extracts B and MB in the cultivation medium at concentrations of 30 % and 50 % is defined as moderate.
Discussion

Summarizing the data obtained at the first stage of research, it should be noted that cell-free extracts obtained from L. reuteri disintegrate and culture, grown in its own disintegrate, stimulate the proliferative activity of L. reuteri to a much greater degree than the extract obtained from B. bifidum disintegrate. According to the degree of stimulatory effect on the L. reuteri proliferation, cell-free extracts arranged in ascending order: B < MLG < ML < L. The stimulation abilities of cell-free extracts enhance with increasing concentration in the cultivation medium from 10% to 30% and weaken with increasing concentration to 50% due to the dilution effect. The most pronounced stimulatory effect on L. reuteri proliferation is exerted by extract L, containing disintegration products of L. reuteri. The less pronounced effect of ML and MLG extracts on the proliferative activity of L. reuteri is obviously associated with the inhibition effect of some metabolites contained in these extracts. One of these metabolites can be reuterin, produced by L. reuteri when cultivated in a glycerin-containing medium. It is known that reuterin exhibits pronounced antimicrobial activity toward to representatives of many genera of bacteria, fungi and protozoa in concentrations 4–5 times lower than those that can inhibit the growth of lactic acid bacteria. It is noteworthy that among all species of the genus Lactobacillus L. reuteri produces the highest concentrations of reuterin and is most resistant to its antimicrobial activity [1]. In addition to reuterin, proliferative activity can be suppressed by lactic acid produced by lactobacilli in the process of cultivation. Lactic acid due its chelating properties can capture essential for growth elements, such as iron [17,18].

Data obtained at the second stage of research, allow us to conclude that all cell-free extracts, with the exception of MLG, inhibit biofilm formation by L. reuteri. According to the degree of inhibitory effect on the L. reuteri proliferation, cell-free extracts arranged in ascending order: ML < L < MB < B. The inhibitory and stimulatory abilities of cell-free extracts are in a concentration-dependent way. The chemical stimuli that influence the development of biofilms include nutrients and metabolites, quorum sensing molecules, and antimicrobials. According to recent studies, bacterial exposure to subinhibitory antimicrobials of many chemically different classes increases biofilm formation. This phenomenon can be considered as a quick and nonspecific way to protect the population from chemical threats. It can be explained by relying on the fundamental biomedical paradigm of hormesis, according to which small doses stimulate, and large inhibit biological parameters [19]. We assume that stimulatory effect of MLG extract on biofilm formation by L. reuteri is related to the content in the extract of reuterin, which has antimicrobial activity. To date, we have not established which mechanisms underline the inhibitory effect of other extracts on the biofilm formation by L. reuteri. This issue is to be studied further. However, we assume the existence of at least two mechanisms of inhibition. The first mechanism is based on the ability of disintegration products, which are signal molecules, to suppress the expression of genes responsible for biofilm formation. The second mechanism is associated with the involvement of some structural components of bacteria (EPSs, proteins, for example) in non-specific interactions of cells with abiotic and biotic surfaces [18]. Supposedly, the disintegration products, which are structural components of lactic acid bacteria, can disrupt the biofilm formation by shielding cell surface adhesins and changing its physicochemical properties.

Analysis of the data suggests that the different directionality and degree of influence of the studied cell-free extracts on the basic physiological functions of L. reuteri are due to differences in their composition, which is predetermined by the method of preparation and by the type of derivatives’ producer. It is obvious that extracts from disintegrates, obtained by repeated freezing-thawing of a bacterial suspension, contain the structural components of bacterial cells (MAMPs, microbe-associated molecular patterns) and bacterial cold shock proteins (DAMPs, damage-associated molecular patterns). These structures possess powerful bioregulatory potential. For example, cold induced proteins have the ability to orchestrate multiple cellular processes, including proliferation and differentiation by regulation of transcription, translation, and splicing.
Conclusions

1. The results of the study allow us to positively evaluate the prospects for the creation of new metabolites based on probiotic derivatives.

2. Cell-free extract, obtained from *L. reuteri* culture, grown in its own disintegrate supplemented with glycerol and glucose can contribute to the survival and facilitate inoculation of the introduced probiotic in the gastrointestinal tract when used together.

3. Derivative-containing extracts with a pronounced growth-stimulating effect can be the basis for creating of means for increasing the overall productivity of probiotic cell biomass. The use of such means will increase the economic efficiency of the production of probiotic cultures.

4. Obtained data induce further study of the biochemical composition, elucidation of the mechanism of action of cell-free extracts and confirmation of their efficacy in vivo.

Prospects for further research: obtained results are of practical importance and will be used in the development of new metabolites and means for increasing the overall productivity of cell probiotic biomass based on *B. bifidum* and *L. reuteri* derivatives.

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Conflicts of interest: author has no conflict of interest to declare.

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