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BIOLOGY

DOI 10.26724/2079-8334-2018-4-66-235-240
УДК 579.871.1:579.262:615.372

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IMPACT OF BIFIDOBACTERIUM BIFIDUM AND LACTOBACILLUS REUTERI DERIVATIVES ON BIOFILM FORMATION BY CORYNEBACTERIA

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The work is devoted to the study of corynebacteria biofilms formation process under the influence of Bifidobacterium bifidum and Lactobacillus reuteri probiotic strains bacterial derivatives, obtained by the authors-developed method. Five samples of cell-free extracts containing structural components and probiotics metabolites have been studied: filtrates of disintegrates and bifido- and lactobacilli cultures, grown in their own disintegrates. Corynebacteria biofilms were obtained in polystyrene microtiter plates. It has been shown that cell-free derivative-containing extracts have a multidirectional effect on the biofilm formation by corynebacteria at the initial stage, stimulating the biofilm formation by one species of corynebacteria and preventing or inhibiting the biofilm formation by others. Irrespective of the species' belonging of the corynebacteria, most cell-free extracts inhibit the further growth of the biofilms formed during 24 hours and can cause their degradation. The most pronounced inhibitory effect on biofilm formation is exerted by cell-free extracts obtained from cultures of probiotics grown in their own disintegrates. Anti-biofilm formation activity of the studied cell-free extracts indicates the promising of creating on the basis of probiotics' derivatives new therapeutic and prophylactic agents for increasing the efficacy of the toxigenic corynebacteria carrier state and diseases caused by opportunistic and pathogenic corynebacteria control.

Key words: corynebacteria, biofilm formation, cell-free extracts, probiotics' derivatives, Bifidobacterium bifidum, Lactobacillus reuteri.

The study is a fragment of the research project "Studying the biological and physico-chemical preconditions for the development of anti-diphtheria agents based on probiotic strains metabolites", (state registration No. 0116U000864).

Corynebacteria play an important role both in preserving health and in the development of human infectious pathology. Most of them are representatives of normal microbiocenosis of various human body biotopes and are involved in the colonization resistance formation. Non-diphtheritic corynebacteria, producing diphtheria-like exotoxins and pathogenicity enzymes, constitute an ultimate danger to human health. Other non-diphtheritic corynebacteria, being commensals, are potentially dangerous due to their ability to cause infectious diseases of skin, mucosa, wounds, and internal organs in immunocompromised individuals or patients with multiple-organ pathology. Non-diphtheritic corynebacteria can cause nosocomial infections, in particular, associated with the use of medical equipment and devices [1].

Particular attention should be drawn to diphtheritic corynebacteria, the circulation of which in the population remains, despite vaccine prophylaxis with the use of a highly immunogenic diphtheria toxoid. The reservoirs of the pathogen are bacteria-carriers with a high level of antitoxic antibodies [11]. One of the main pathogenetic mechanisms of long-term persistence on bacteria-carriers' mucous membranes is the ability of corynebacteria to biofilm formation. This form of existence protects bacteria not only from unfavorable factors in the host's organism, but also allows them to survive on abiotic surfaces for a long time [3, 6].

The intensity of biofilm formation depends on the strains' belonging of corynebacteria and surrounding conditions of existence [4]. In the human body, biofilms represent communities of mainly different microorganisms' species, aggregated in microcolonies and surrounded with a protective matrix. Community members permanently interact with each other through signaling molecules and each of them performs specialized metabolic functions [8]. It is known that in polymicrobial biofilms exometabolites of some species microorganisms can promote the growth and development of another microorganism species [5]. Based on the natural antagonism in bacteria, the assumption about the possibility of inhibition the biofilm formation of some microorganisms by others was proved by the results of studies on the bifidobacteria exombetabolites effect on the biofilm formation by Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae, Staphylococcus aureus, Candidia albicans and Rhodotorula rubra [10]. Therefore, studies aimed at searching ways to regulate the composition of microbial biofilms in mucous membranes by means of metabolites and structural components of bacteria are promising. The inhibiting effect on biofilm formation of pathogenic and opportunistic microorganisms can be realized by blocking mechanisms of bacterial

adhesion to the surface and synthesis of polymer matrix, destruction of the formed matrix, disturbance in the intercellular information exchange and enhanced action of bactericidal agents [7, 9].

The purpose of the present work was to study the influence of Bifidobacterium bifidum and Lactobacillus reuteri derivatives on corynebacteria biofilm formation.

Materials and methods. Cell-free extracts were obtained using the method developed by the authors [2]. The disintegration of the microbial mass was performed by the repeated freezing-thawing. Probiotic bacteria cultivated in their own disintegrate. Obtained disintegrates and cultures were filtered. The studied extract samples contained structural components and metabolites of the commercial strains Bifidobacterium bifidum No. 1 (from medical product "Bifidumbacterin-Biopharma", JSC Biopharma, Ukraine) and Lactobacillus reuteri DSM 17938 (from medical product "BioGaia", BioGaia Production AB, Sweden).

The impact of five types of cell-free extracts (derivative-containing filtrates) on the corynebacteria biofilm formation processes was studied: 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.

As test cultures forming biofilms, two strains of toxigenic diphtheritic corynebacteria were used: Corynebacterium diphtheriae gravis tox^+ No. 149, Corynebacterium diphtheriae mitis tox^+ No. 97; two strains of non-diphtheritic corynebacteria: toxigenic – Corynebacterium ulcerans tox^+ No. 112 and non-toxigenic – Corynebacterium xerosis No. 41 (stored in the microorganisms collection at the Laboratory for Prevention of Droplet Infections at SI "IMI NAMS", Kharkiv).

Corynebacteria were cultured for 48 hours under aerobic conditions at the temperature of 37° C on the blood agar. After checking the cultures purity, inoculums were prepared of them. Inoculum was a microbial suspension in a physiological saline solution with an optical density of 0.5 units on the McFarland scale (~10^8 CFU/ml).

Biofilms of corynebacteria were obtained in sterile polystyrene 96-well microtiter plates (JSC "Eximcargotrade", Ukraine) by modified Stepanovic S. method [12].

Study of the cell-free extracts' effect on the biofilm formation initial stages. The tryptone soya broth (TSB; HiMedia, India) supplemented with 1% glucose was added into all wells of the microtiter plate. Cell-free extracts were introduced into the experimental wells achieving a final concentration of 20 % vol. Physiological saline solution was introduced into the control wells in the same volume. Inoculums were injected into experimental and positive control wells (PC) containing growth medium in the ratio of 1:9. Negative control wells (NC) contained physiological saline solution instead of inoculums. Hermetically sealed plates were incubated in a thermostat for 24 hours at 37° C.

Then the contents of the wells were removed. The wells were gently washed three times with sterile 0.1 M phosphate-buffered saline (PBS; pH 7.2). After that, the plates were dried. Fixed for 60 minutes at 60° C, the biofilms were stained with 1% solution of crystal violet. After ten-times flushing with distilled water 96° ethanol was gently poured into the wells and was held for 30 minutes at room temperature. The optical density of the experimental and control samples was measured using a microtiter-plate reader "Erba LisaScan™ EM" (Germany) at a wavelength of 630 nm.

The difference between the optical density of the experimental and control samples was the basis to judge about the presence or absence of the studied cell-free extracts effect on biofilm formation of corynebacteria. Each test culture, depending on the difference between the optical density of the test specimen (OD) and the negative control (OD_{NC}), was attributed to one of the following groups by their intensity of biofilm formation:

- weak ($OD_{NC} < OD \leq 2xOD_{NC}$);
- moderate ($2xOD_{NC} < OD \leq 4xOD_{NC}$);
- strong ($4xOD_{NC} < OD$).

If the $OD<OD_{NC}$ or the difference between the indicated indices was not significant ($p\geq0.05$), then biofilm formation was considered absent.

Study of the cell-free extracts' effect on the 24-hour corynebacteria biofilm. At the first stage, 24-hour corynebacteria biofilms were obtained by inoculating the test culture suspension in TSB supplemented with 1% glucose and by incubating the hermetically sealed microtiter plates in the thermostat for 24 hours at 37° C. At the second stage, TSB supplemented with 1% glucose and the cell-free extracts at a final concentration of 20 % vol were added into the wells with the formed biofilms. The hermetically sealed microtiter plates were re-incubated in a thermostat for 24 hours at 37 ° C.

Control positive samples (PC) instead of cell-free extracts, and control negative samples (NC) instead of extracts and inoculums contained a physiological saline solution. Removing the contents and

washing the wells, fixation, staining, washing, stain extraction, measuring the optical density and evaluating the results were carried out as described above.

The study was performed three times in triplicate. The statistical processing of the results was carried out using the software package Excel 2010 ("Microsoft", USA). The arithmetic mean (M) with standard deviations (SD) was calculated. The significance of the difference between the obtained values was determined by the Student's criterion. The value of $p \leq 0.05$ was considered statistically significant.

Results of the study and their discussion. The C. diphtheriae gravis tox$^+$ No. 149 test culture was characterized by weak biofilm formation during the first 24 hours of incubation (fig. 1). Introduction of extracts at a final concentration of 20 % vol into the incubation medium resulted in insignificant (L extract), significant inhibition or loss (B, MB, ML and MLG extracts) of the test culture's ability to biofilm formation. The 24-hour biofilm produced by corynebacteria after the removal of planktonic bacteria and the addition of a nutrient medium continued to grow within the next 24 hours. OD_{PC} value permitted to attribute this culture to a group with moderate biofilm formation. At the same time, in the presence of ML, MLG and MB cell-free extracts, the biofilm formation was significantly inhibited. The mean OD value of samples containing extracts L and B were also lower than the OD_{PC} value, but the difference was not reliable. Thus, the filtrates of B. bifidum and L. reuteri cultures grown in their own disintegrates revealed a more pronounced inhibitory effect on the biofilm formation of C. diphtheriae gravis tox + No. 149 test culture than the filtrate of the disintegrates of probiotics.

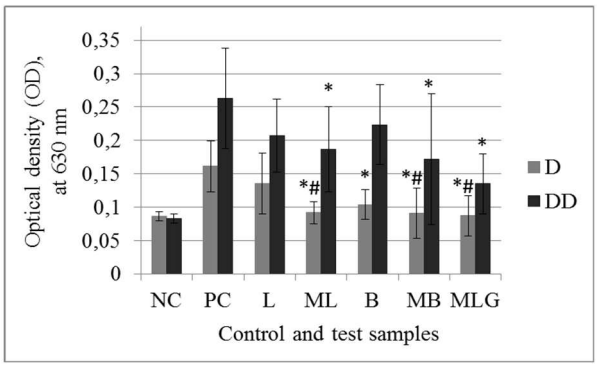

Fig. 1. - Influence of probiotic derivatives on biofilm formation by C. diphtheriae gravis tox$^+$ No. 149 test culture.

Note: D – influence on the initial stages of biofilm formation; DD – effect on 24-hour biofilms; NC – negative control; PC - positive control; 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 glycerol and glucose; B – filtrate of B. bifidum disintegrate; MB – filtrate of B. bifidum culture, grown in B. bifidum disintegrate; * – differences reliable compared to positive control (PC) ($p < 0.05$), # - differences unreliable compared to negative control (NC) ($p \geq 0.05$).

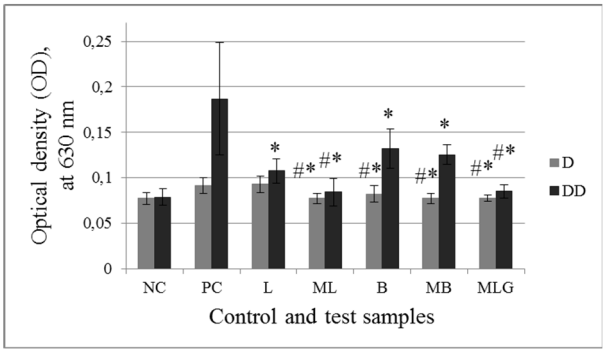

Fig. 2. - Influence of probiotic derivatives on biofilm formation by the C. diphtheriae mitis tox$^+$ No. 97 test culture.

Note: D - influence on the initial stages of biofilm formation; DD - effect on 24-hour biofilms; NC - negative control; PC - positive control; 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 glycerol and glucose; B – filtrate of B. bifidum disintegrate; MB – filtrate of B. bifidum culture, grown in B. bifidum disintegrate; * - differences reliable compared to positive control (PC) ($p < 0.05$), # - differences unreliable compared to negative control (NC) ($p \geq 0.05$).

The C. diphtheriae mitis tox$^+$ No.97 test culture, according to OD_{PC} value, after 24 hours of incubation was referred to the group with weak biofilm formation (fig. 2). During the next 24 hours, the biofilm continued to grow and, according to the OD_{PC} value, the test culture referred to the group with moderate biofilm formation intensity. The addition of ML, MLG, B and MB extracts into the incubation medium at the first stage led to loss of test culture ability to biofilm formation. The introduction into the incubation medium of these extracts at the second stage caused significant inhibition (B and MB extracts) of the test culture's ability to biofilm formation and even degradation of the formed biofilm (ML and MLG extracts). The exception was the L extract, which did not have a significant effect on the biofilm formation of this test culture during the first day, but significantly inhibited further growth of the 24-hour biofilm. Thus, the C. diphtheriae mitis tox + No. 97 test culture was also more susceptible to cell-free extracts contained disintegration products and probiotics' metabolites.

The C. ulcerans tox$^+$ No. 112 test culture after 24-hour incubation was characterized as a strong biofilm producer (fig. 3). Due to the presence of L and ML cell-free extracts in the incubation medium, the biofilm formation was greatly enhanced. With the presence of B, MB and MLG extracts, the mean OD indices of the experimental samples were higher than the control ones, but the difference between them was unreliable. During the next 24 hours, the culture's biofilm formation has grown significantly, as evidenced by the mean OD_{PC} growth by 2.2 times. OD values of the test samples in the presence of L and B extracts did not differ significantly from OD_{PC} value. The presence in the incubation medium of ML, MLG and MB extracts caused the inhibition of the further growth of the 24-hour corynebacteria biofilm.

This is evidenced by significantly lower OD values of the corresponding test samples compared to the OD_{PC} value.

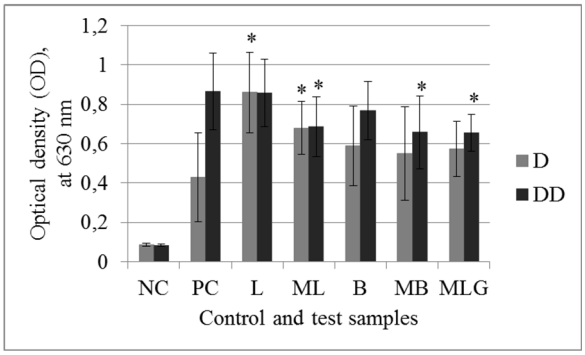

Fig.3. - Influence of probiotic derivatives on biofilm formation by C. ulcerans tox+ No. 112 test culture.

Note: D - influence on the initial stages of biofilm formation; DD - effect on 24-hour biofilms; NC - negative control; PC - positive control; 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 glycerol and glucose; B – filtrate of B. bifidum disintegrate; MB – filtrate of B. bifidum culture, grown in B. bifidum disintegrate; * - differences reliable compared to positive control (PC) ($p < 0.05$).

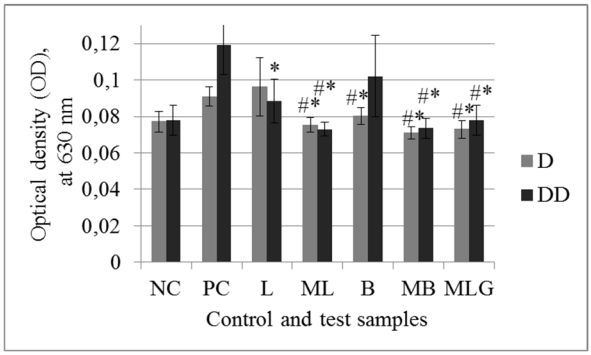

Fig. 4. - Influence of probiotic derivatives on biofilm formation by C. xerosis No. 41 test culture.

Note: D - influence on the initial stages of biofilm formation; DD - effect on 24-hour biofilms; NC - negative control; PC - positive control; 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 glycerol and glucose; B – filtrate of B. bifidum disintegrate; MB – filtrate of B. bifidum culture, grown in B. bifidum disintegrate; * - differences reliable compared to positive control (PC) ($p < 0.05$), # - differences unreliable compared to negative control (NC) ($p \geq 0.05$).

The C. xerosis No. 41 test culture, according to OD_{PC} value after the 24 hours incubation, was determined as weak biofilm producer. The presence of ML, MLG, B and MB extracts in the growth medium prevented the formation of biofilm. The extract L did not have a significant effect on this process. Over the next 24 hours, OD_{PC} has grown significantly, but the culture remained in the group with weak biofilm formation ability. Adding of B extract to the formed 24-hour biofilm of C. xerosis No. 41 test culture resulted in an insignificant suppression of its further growth. The L extract caused a significant decrease in the mean OD value of the experimental samples compared to OD_{PC} value. The introduction of ML, MLG and MB extracts into the growth medium resulted in a decrease in the average OD value of the experimental samples to the level of OD_{NC}, indicating the degradation processes of the formed biofilm in the presence of these extracts. Consequently, all cell-free extracts inhibit the formation of biofilm by C. xerosis No. 41 culture to some extent. However, this culture was more sensitive to extracts containing both disintegration products and probiotic metabolites.

To quantify the ability of cell-free extracts to influence the biofilm formation and the degree of sensitivity of test cultures, we used a scoring system.

Table 1

Influence of probiotic derivatives on biofilm formation by corynebacteria

No.	Test-culture	Biofilm formation:		Arrangement extracts on the ability to influence the biofilm formation (according to OD)				
		Stage	Intensity	1	2	3	4	5
1	C. xerosis	D	weak	L	**B**	**ML**	**MLG**	**MB**
		DD	weak	B	**L**	**MLG**	**MB**	**ML**
2	C.diphtheriae mitis tox+	D	weak	L	**B**	**MLG**	**MB**	**ML**
		DD	moderate	**B**	**MB**	**L**	**MLG**	**ML**
3	C.diphtheriae gravis tox+	D	weak	L	**B**	**ML**	**MB**	**MLG**
		DD	moderate	B	L	**ML**	**MB**	**MLG**
4	C.ulcerans tox+	D	strong	L	ML	B	MLG	MB
		DD	strong	L	B	**ML**	**MB**	**MLG**

Note: D - influence on the initial stages of biofilm formation; DD - effect on 24-hour biofilms; A – stimulates (-1 points); A- does not affect (0 points); **A** – inhibits (+1 points); **A** - prevents the biofilm formation or destroys the formed biofilm (+2 points).

According to the degree of inhibitory effect on test cultures, cell-free extracts were arranged in the ascending order: L (1 point) → B (6 points) → ML (11 points); MB (11 points) → MLG (12 points).

Analysis of the study results showed: cell-free extracts containing derivatives of B. bifidum No. 1 and L. reuteri DSM 17938 have a multidirectional effect on the biofilm formation by corynebacteria at the initial stage: stimulating the biofilm formation by one species of corynebacteria and preventing or

inhibiting the biofilm formation by others. Irrespective of the species' belonging of the corynebacteria, most cell-free extracts inhibit the further growth of the biofilms formed during 24 hours and can cause their degradation. The most pronounced inhibitory effect on biofilm formation is exerted by cell-free extracts containing disintegration products and probiotic metabolites.

The corynebacteria sensitivity to the influence of derivative-containing extracts was different. C. xerosis and toxigenic diphtheritic corynebacteria, characterized as weak and moderate biofilm producers, were more susceptible. Less sensitive was C. ulcerans, which was defined as a strong biofilm producer. According to the degree of sensitivity to the inhibitory effect of cell-free extracts of the culture were arranged in ascending order: C. ulcerans tox+ (1 point) → C. diphtheriae gravis tox+ (10 points) → C. diphtheriae mitis tox+ (15 points); C. xerosis (15 points).

The study motivates further research of the influence of probiotics' derivatives on the corynebacteria in microbial associations in vitro and in vivo.

Conclusion

Anti-biofilm formation activity of the studied cell-free extracts indicates the promising of creating on the basis of probiotics' derivatives new therapeutic and prophylactic agents for increasing the efficacy of the toxigenic corynebacteria carrier state and diseases caused by opportunistic and pathogenic corynebacteria control.

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Реферати

ВПЛИВ ДЕРИВАТІВ BIFIDOBACTERIUM BIFIDUM ТА LACTOBACILLUS REUTERI НА БІОПЛІВКОУТВОРЕННЯ КОРИНЕБАКТЕРІЙ

Книш О.В., Ісаєнко О.Ю., Перетятко О.Г., Бабич Є.М.

Робота присвячена дослідженню процесу утворення біоплівок коринебактеріями за впливу дериватів бактерій пробіотичних штамів Bifidobacterium bifidum і Lactobacillus reuteri, отриманих розробленим авторами методом. Досліджено п'ять зразків безклітинних екстрактів, що містять структурні компоненти та

ВЛИЯНИЕ ДЕРИВАТОВ BIFIDOBACTERIUM BIFIDUM И LACTOBACILLUS REUTERI НА БИОПЛЕНКООБРАЗОВАНИЕ КОРИНЕБАКТЕРИЙ

Кныш А.В., Исаенко А.Ю., Перетятко О.Г., Бабич Е.М.

Работа посвящена исследованию процесса образования биопленок коринебактериями под влиянием дериватов бактерий пробиотических штаммов Bifidobacterium bifidum и Lactobacillus reuteri, полученных разработанным авторами методом. Исследовано пять образцов бесклеточных экстрактов, содержащих структурные компоненты и

метаболіти пробіотиків: фільтрати дезінтегратів та культур біфідо-і лактобактерій, вирощених у власних дезінтегратах. Біоплівки коринебактерій отримували в полістиролових мікротитрувальних планшетах. Показано, що безклітинні дериват-вмісні екстракти мають різноспрямований вплив на утворення біоплівок коринебактеріями на початковій стадії: стимулюють біоплівкоутворення одним видом коринебактерій і запобігають або інгібують біоплівкоутворення іншими. Незалежно від видової належності коринебактерій, більшість безклітинних екстрактів гальмують подальший ріст утворених протягом 24 годин біоплівок і можуть викликати їх деградацію. Найбільш виражене пригнічення біоплівкоутворення викликають безклітинні екстракти, отримані з культур пробіотиків, вирощених у власних дезінтегратах. Протибіоплівкова активність досліджених безклітинних екстрактів свідчить про перспективність створення на основі дериватів пробіотиків нових профілактичних і лікувальних засобів підвищення ефективності боротьби з носійством токсигенних коринебактерій і захворюваннями, викликаними умовно-патогенними і облігатно патогенними коринебактеріями.

Ключові слова: коринебактерії, біоплівко-утворення, безклітинні екстракти, деривати пробіотиків, Bifidobacterium bifidum, Lactobacillus reuteri.

Стаття надійшла 1.06.18 р.

метаболиты пробиотиков: фильтраты дезинтегратов и культур бифидо- и лактобактерий, выращенных в собственных дезинтегратах. Биопленки коринебактерий получали в полистироловых микротитровальных планшетах. Показано, что бесклеточные дериват-содержащие экстракты оказывают разнонаправленное воздействие на биопленкообразование коринебактериями на начальной стадии: стимулируют биопленкообразование одним видом коринебактерий и предотвращают или ингибируют биопленкообразование другими. Независимо от видовой принадлежности коринебактерий, большинство бесклеточных экстрактов препятствуют дальнейшему росту биопленок, образованных в течение 24 часов, и могут вызвать их деградацию. Наиболее выраженное ингибирующее действие на биопленкообразование оказывают бесклеточные экстракты, полученные из культур пробиотиков, выращенных в собственных дезинтегратах. Антибиопленочная активность исследованных бесклеточных экстрактов свидетельствует о перспективности создания на основе дериватов пробиотиков новых профилактических и лечебных средств повышения эффективности борьбы с носительством токсигенных коринебактерий и заболеваниями, вызванными условно-патогенными и облигатно патогенными коринебактериями.

Ключевые слова: коринебактерии, биопленко-образование, бесклеточные экстракты, дериваты пробиотиков, Bifidobacterium bifidum, Lactobacillus reuteri.

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