Staphylococcal proliferation and biofilm formation in vitro under the influence of cell-free extracts of probiotic origin

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

The paper presents the results of the study on proliferation and biofilm formation by Staphylococcus aureus and Staphylococcus epidermidis under the influence of cell-free extracts obtained by the author’s method and containing derivatives of probiotic strains Bifidobacterium bifidum and Lactobacillus reuteri.

The aim of this work was to study the ability of cell-free extracts containing derivatives of probiotics Bifidobacterium bifidum and Lactobacillus reuteri to influence proliferation and biofilm formation by staphylococci in vitro, to evaluate the prospects of their use for the correction of medical microbiological disorders and adjuvant therapy of staphylococcal infection.

Materials and methods. Cell-free extracts were obtained from commercial strains B. bifidum and L. reuteri by the authors’ method. Reference strain of S. aureus ATCC 25923 and clinical isolate of S. epidermidis were used as a test cultures. The investigation of the proliferation and biofilm formation by staphylococci 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 effect of cell-free extract on proliferation and biofilm formation depends on the type of extract and on the species of staphylococci. Among the five studied extracts, only one significantly inhibits the proliferation and biofilm formation of both staphylococci species. It is the cell-free extract, obtained from L. reuteri culture, grown in its own disintegrate supplemented with glycerol and glucose. The proliferative activity of S. aureus is sensitive to the L. reuteri derivatives while the proliferative activity of S. epidermidis is sensitive to the B. bifidum derivatives. The filtrates of disintegrates have stimulatory effect, while the filtrates of cultures have inhibitory effect on the staphylococcal proliferation. The biofilm formation by S. aureus is significantly inhibited by B. bifidum derivatives and is stimulated by L. reuteri derivatives. The biofilm formation by S. epidermidis is stimulated by derivatives of bifidobacteria and does not change in the presence of derivatives of lactobacteria in the growth medium.

Conclusions. Obtained results indicate a high bioregulatory potential of cell-free extracts of probiotic origin and the possibility of drugs development for medical microbiological disorders correction on their basis. They also confirm that the method of obtaining probiotic derivatives with bacteriotropic activity through precursor-directed biosynthesis is promising. Cell-free extract, obtained from L. reuteri culture, grown in its own disintegrate supplemented with glycerol and glucose, exhibits pronounced anti-staphylococcal activity in vitro. After confirming efficacy in vivo, it can be recommended for the adjuvant therapy of staphylococcal infections.
Among the more than 70 species of the genus *Staphylococcus*, the most pathogenic for humans is *Staphylococcus aureus*. It persistently colonizes approximately 20 % of the human adult population. The main biotopes that *S. aureus* colonizes: the anterior nares and skin. It is considered the main causative agent of nosocomial and community-acquired infections [1,2]. There are many known factors of *S. aureus* virulence and pathogenicity: degradative enzymes, pore-forming toxins, superantigens, immuno-stimulatory exo-proteins and others [3]. *S. aureus* can cause both acute and chronic infectious processes. Acute infections are generally caused by planktonic cells. In this case, the production of secreted toxins and exo-enzymes is the main pathogenicity factor. In contrast, chronic infections are associated with the ability of *S. aureus* to attach to host tissues or implanted medical devices, to form a biofilm and persist in this state for a long time [2–4].

Traditionally considered, that *Staphylococcus epidermidis* is a harmless commensal that lives on human skin. By preventing the attachment of pathogenic staphylococci, it provides colonization resistance of mucous membranes and skin. But *S. epidermidis* can cause an infectious process in newborns, immuno-compromised patients or patients undergoing surgery, with multiorgan pathology. The widespread use of medical devices has made *S. epidermidis* the most clinically important opportunistic agent that often causes nosocomial infections [4,5]. The main pathogenicity factor of *S. epidermidis* is the ability to biofilm formation [5,6].

Today, quite a lot of work has been devoted to the study of biofilm formation by bacteria of *S. aureus* and *S. epidermidis* species [1–3,7]. Biofilm formation protects microbial cells from antimicrobial agents, the host’s immune system and makes them more difficult to eliminate [2]. The resistance of bacteria in the composition of a biofilm can be in tens of thousands of times greater than the resistance of the same microorganisms in planktonic form [8]. In recent years, an active search for substances and compounds with anti-biofilm activity has been conducted. It was found out that cationic antimicrobial peptides varnerin and hominin can not only prevent biofilm formation by coagulase-negative staphylococci, but also actively destroy the formed biofilm [9]. Bacteriocins also had an inhibitory effect on biofilm formation *in vitro* [10]. It has been shown that enzymes can have different effects on biofilm formation: while DNase can inhibit, α-galactosidase can stimulate this process [11]. The enzyme oligoribonuclease effectively suppresses the exchange of chemical signals between individual bacterial cells. It can be used to stop or prevent the biofilm formation by bacteria [8].

The development of new effective drugs with antimicrobial and anti-biofilm activity is one of the priorities of the modern pharmaceutical industry [9,17]. The ability of some species of staphylococci to inhibit biofilm formation and destroy the biofilms formed by other species of staphylococci suggests the promise of finding effective regulators of this process among natural microbial antagonists [7]. Recently, *in vitro* studies and clinical trials have accumulated much evidence of the effect of probiotics on infectious agents through a competition and counteraction. However, data on the use of probiotics as tools to prevent the formation or destruction of preformed biofilms is not enough [12,13,20,21]. Considering the natural antagonism of probiotics in relation to conditionally pathogenic and pathogenic microorganisms, it seems logical to search for antimicrobial and anti-biofilm substances among probiotic derivatives.
The aim
The aim of this work was to study the ability of cell-free extracts containing derivatives of probiotics *Bifidobacterium bifidum* and *Lactobacillus reuteri* to influence proliferation and biofilm formation by *staphylococci in vitro*, to evaluate the prospects of their use for the correction of microbiological disorders and adjuvant therapy of staphylococcal infection.

Materials and methods
Two commercial probiotic strains were used as sources of biologically active structural components and as producers of metabolites. These were *B. bifidum* No 1 (from medical product “Bifidumbacterin-Biopharma”, JSC “Biopharma”, Ukraine) and *L. reuteri* DSM 17938 (from medical product “BioGaia”, BioGaia AB, Sweden). Five types of cell-free extracts have been 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.

Test samples were obtained by the authors’ method [14]. Disintegrates were obtained by physical method of disintegration. It was a multiple freezing-thawing of the microbial mass. To obtain the disintegrate, the microbial mass was exposed to 10 cycles of freeze-thawing according to the following regimen: freezing – passive cooling in freezing chamber of Samsung RB29FSRNDSA refrigerator down to -23 ± 1 °C, heating – in water bath at 37 ± 1 °C up to complete thawing.

Cultures were obtained by cultivating of probiotic microorganisms in their own disintegrates. To this end the probiotic suspension in physiological saline of turbidity 10.0 units according to the McFarland scale measured using the Densi-La-Meter (PLIVA-Lachema, Czech Republic) was exposed to 10 cycles of freeze-thawing according to the following regimen: freezing – passive cooling in freezing chamber of Samsung RB29FSRNDSA refrigerator down to -23 ± 1 °C, heating – in water bath at 37 ± 1 °C up to complete thawing.

Cultures were obtained by cultivating of probiotic microorganisms in their own disintegrates. To this end the probiotic suspension in physiological saline of turbidity 10.0 units on the McFarland scale was added into disintegrate in 1:9 ratio and cultured at 37 ± 1 °C for 72 hours in anaerobic conditions.

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

Coagulase-positive reference strain *S. aureus* ATCC 25923 (from the Museum of Microorganisms of IMI NAMS, Kharkiv) and coagulase-negative clinical isolate *S. epidermidis* № 558 (from the Collection of Microorganisms of the Laboratory of Respiratory Infections Prevention of IMI NAMS, Kharkiv) were used as test cultures. The test cultures were cultivated overnight aerobically at 37°C on appropriate solid nutrient medium. It was blood agar.

Preparation of the inoculum: after verifying the purity of the culture, few colonies from the overnight incubated agar medium were emulsified in a physiological saline for obtaining a suspension of turbidity 0.5 on the McFarland scale.

Assessment of biofilm formation by *staphylococci*. Staphylococcal biofilms were obtained in sterile 96-well polystyrene microtiter plates (JSC “Eximcargotrade”, Ukraine) by modified S. Stepovanovic’s microtiter-plate technique [15]. The tryptone soya broth (TSB; HiMedia, India) supplemented with 1% glucose aseptically was added into all wells of the microtiter plate. Cell-free derivative-containing extracts were added into the test wells to achieve a final concentration 20 % vol. Physiological saline was added to the positive control wells (PC) in the same volume. The inoculum was vortexed and then inoculated into a test and positive control wells of the microtiter plate. The final concentration of cells in the incubation medium was ~10^7 CFU/ml. The negative control wells (NC) contained TSB supplemented with 1 % glucose and physiological saline instead of cell-free extracts and inoculum. The plates were covered with lids and incubated aerobically for 24 hours at 35–37 °C in static conditions. After incubation, the contents of the wells have been removed. Each well was washed three times with sterile 0.1 M phosphate-buffered saline (PBS; pH 7.2). Remaining attached biofilms were fixed by exposing them to hot air at 60 °C for 60 min; they were stained by 1% crystal violet for 15 min. After that, wells were washed ten times with distilled water. Ethanol was gently added and thereafter the microtiter plate covered with the lid. It was left at room temperature for 30 min. The optical density (OD) of 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 (OD _nc_ < OD ≤ 2xOD _nc_);
- moderate (2xOD _nc_ < OD ≤ 4xOD _nc_);
- strong (4xOD _nc_ < OD);
- no biofilm formation (OD ≤ OD _nc_), according to In Lee S. et al. [16].

The inhibition (or stimulation) index was calculated by the formula: Inh (StI) = (OD – OD _pc_)/OD _pc_×100%, where OD _pc_ and OD were optical density of the control and test samples, respectively.

Assessment of staphylococcal proliferation. The study of the effect of cell-free extracts on the proliferation of staphylococci was performed by modified Gladisheva’s method [17]. Cell-free extracts and diluted 10 times inoculums were added into the test wells in the ratio of 9:1. Physiological saline and inoculums were added into the positive control wells (PC) in the same ratio. The negative control wells (NC) contained only physiological saline. The plates were covered with lids and incubated aerobically for 1 hour at 35–37 °C in static conditions. After incubation, pepted meat broth (PMB; HiMedia, India) supplemented with 1 % glucose aseptically was added into all wells of the microtiter plate in volume exceeding the total volume of cell-free extract and inoculum by 2 times. The final concentration of microbial cells in the incubation medium was ~10^8 CFU/ml. The optical density (OD) of the wells was measured at 578 nm using a microtiter-plate reader ‘Lisa Scan EM’ (Erba Lachema s.r.o., Czech Republic) immediately after adding PMB and after incubation of plates aerobically for 24 hours at 35–37 °C in static conditions. The inhibition (or stimulation) index was calculated by the formula: Inh (StI) = (ΔOD_ΔOD _pc_)/ΔOD _pc_×100%, where ΔOD and...
ΔOD

were the changes in the optical density of the control and test samples within 24 hours.

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 with standard deviations 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

As shown in Fig. 1, extract L had a significant stimulatory effect on proliferation of S. aureus (StI = 27.6 %, P < 0.04). Pronounced inhibition of staphylococcal proliferation was observed under the influence of extracts ML (InhI = 53.2 %, P < 0.009) and MLG (InhI = 76.2 %, P < 0.003). Taking into account InhI, the filtrate of L. reuteri culture, grown in L. reuteri disintegrate supplemented with glycerol and glucose (MLG), causes more pronounced inhibition of S. aureus proliferation, than filtrate of L. reuteri culture grown in its own disintegrate without additives (ML). Extracts B and MB had no significant effect on the proliferation of this test culture.

Fig. 2 shows, that extract B exerted a significant stimulatory effect on the proliferation of S. epidermidis (StI = 42.5 %, P < 0.03). Introduction into the growth medium of MLG and MB extracts resulted in inhibition of staphylococcal proliferation by 95.2 % (P < 0.0002) and 27.7 % (P < 0.05), respectively. It should be noted that MLG filtrate, obtained from L. reuteri culture, grown in L. reuteri disintegrate supplemented with glycerol and glucose, has a significantly more pronounced inhibitory effect on the proliferation of S. epidermidis than MB extract, obtained from B. bifidum culture, grown in B. bifidum disintegrate. Extracts L and ML did not affect proliferative activity of S. epidermidis.

Fig. 3 shows the ability of the S. aureus to produce biofilm in the polystyrene microplate. Based on a comparison of the mean value OD

with the mean value of OD

the culture was classified as moderate biofilm-producer. A significant stimulation of biofilm formation by this culture occurred under the influence of extracts L and ML. StI were 44.8 % (P < 0.00001) and 77.2 % (P < 0.009), respectively. Extracts MLG, B, MB in the growth medium, on the contrary, inhibited biofilm formation by S. aureus. InhI were 23.7 % (P ≤ 0.003); 15 % (P < 0.03) and 20.3 % (P < 0.02), respectively.

S. epidermidis showed weak ability to produce biofilms on polystyrene microplates within 24 hours (Fig. 4). Introduction into the growth medium of L and ML extracts did not have a significant effect on the biofilm formation by this test-culture. The presence in the growth medium of extract MLG resulted in a significant inhibition of biofilm formation by staphylococcus, the InhI was 21.8 % (P < 0.0006). Cell-free extracts, containing derivatives of B. bifidum (B and MB), had a powerful stimulating effect on biofilm formation by S. epidermidis. StI were 43.7 % (P < 0.0002) and 77.2 % (P < 0.000002), respectively.
Discussion

In the work, the slow disintegration by the freezing-warming method was used. This method does not provide for the rapid and complete destruction of bacterial cells to obtain a set of structural components. A distinctive feature of this method is the creation of shock conditions for microbial cells. They are exposed to thermal, osmotic, thermomechanical, dehydration and rehydration shock. In created conditions under the influence of a complex of damaging factors there are sublethal, lethal injuries and diversity of metabolic changes in response to stress occur in cells. Bacteria specifically respond to an abrupt decrease in temperature. The cold shock response (CSR) is a cascade of cellular reactions, accompanied by a high level of temporarily expression of cold-induced proteins (CIPs). CIPs have pleiotropic functions, such as the regulation of transcription, translation, and splicing. They have the ability to orchestrate multiple cellular processes, including proliferation and differentiation [18].

Thus, disintegrate obtained by repeated freezing-thawing of a bacterial suspension contains not only the structural components of bacterial cells (microbe-associated molecular patterns, MAMPs), but also bacterial cold shock proteins (damage-associated molecular patterns, DAMPs), which possess powerful bioregulatory potential. In addition, disintegrate also contains a set of substances suitable for nutrition of microorganisms. We used this property of disintegrate, applying it as a nutrient medium in the cultivation of probiotics.

It is known that a change in cultivation conditions has a significant effect on metabolic processes in microbial cells, and hence on the composition of metabolites. Live, growing, proliferating lactic acid bacteria release into the environment a large number of metabolites – organic acids, hydrogen peroxide, ethyl alcohol, bacteriocins, and other biologically active compounds. They have antimicrobial, antiviral, immunomodulatory, anti-inflammatory, antitumor and other types of activity. Cell-free filtrates have a complex composition. The directionality and intensity of the filtrates’ effects on the biological processes are the result of the summation of its components effects. The determination of each component’s effect is possible after fractionation of the cell-free filtrate. At present, we are studying the biochemical composition of disintegrates and cultures, grown in their own disintegrate. The content of some important biologically active components (protein, peptides, amino acids, nucleic acids, teichoic acids, etc.) has been determined. A separate article will be devoted to the coverage of the results.

The results of the experimental study demonstrate the different directionality of the effects of cell-free extracts, introduced into the growth medium, on proliferation and biofilm formation by staphylococci and revealed some regularity. The direction of the effect depends on the species of staphylococci and the type of cell-free extract. Cell-free extracts have a different composition depending on the source and method of their obtaining. Extract L, containing products of disintegration of L. reuteri, stimulate proliferation and biofilm formation by S. aureus, but does not have a significant effect on proliferation and biofilm formation by S. epidermidis. Extract B, containing products of disintegration of B. bifidum, has a stimulatory effect on proliferation and biofilm formation by S. epidermidis. It does not have a significant effect on proliferation, but inhibits biofilm formation by S. aureus. Obtained results confirm the existence of a mechanism of influence on the ability to form biofilms, which does not involve growth inhibition [7,19,20]. Recent studies in this area demonstrate that inhibition of biofilm formation without affecting the growth is possible due to the inhibition of ica operon expression by metabolites or lipoteichoic acid (LTA) of probiotics [19,20]. Ica operon responsible for the production of poly-N-acetyl-glucosamine (a key molecule required for S. aureus biofilm development) [19,20]. Remarkably, whereas metabolites of L. fermentum inhibit the biofilm formation by S. aureus, metabolites of L. plantarum do not appear such ability [19]. LTA without D-alanine moieties do not inhibit biofilm formation by S. aureus [20].

The introduction into the growth medium of ML extract, which contains products of disintegration and metabolic products of L. reuteri, leads to the inhibition of proliferation and stimulation of biofilm formation by S. aureus, but does not affect the indicated abilities of S. epidermidis. Inhibitory effect of ML extract on proliferation can be explained by the antimicrobial activity of L. reuteri metabolites against S. aureus. The introduction into the growth medium of MB extract, containing the products of disintegration and metabolic products of B. bifidum, causes inhibition of proliferation and stimulation of biofilm formation by S. epidermidis. It does not affect proliferation and inhibits biofilm formation by S. aureus. Inhibitory effect of MB extract on proliferation can be explained by the antimicrobial activity of B. bifidum metabolites against S. epidermidis. Our data is only partially consistent with the results of studies by other authors, who showed that exometabolites of B. bifidum had a pronounced inhibitory effect on the proliferative activity and biofilm formation of staphylococci [13]. The difference in the results indicates the importance of the method for obtaining probiotic derivatives, which predetermines the composition of the final product. Extract MLG demonstrated a significant inhibitory effect on the proliferation and biofilm formation by both species of staphylococci. It contains products of disintegration and metabolic products of L. reuteri obtained by cultivation in its own disintegrate supplemented with glycerol and glucose. It is known that L. reuteri can convert glycerol into the antimicrobial compound reuterin [21]. We suggest that inhibitory effect of MLG extract on the proliferation and biofilm formation by staphylococci is due to reuterin.

Conclusions

1. Obtained results have a practical importance. They indicate a high bioregulatory potential of cell-free extracts of probiotic origin and the possibility of drugs development for microecological disorders correction on their basis.

2. The results also confirm that the method of obtaining probiotics’ derivatives with bacteriostatic activity through precursor-directed biosynthesis is promising.

3. Cell-free extract obtained from L. reuteri culture grown in its own disintegrate supplemented with glycerol and glucose, exhibits pronounced anti-staphylococcal activity in vitro. After confirming efficacy in vivo, it can be recommended for the adjuvant therapy of staphylococcal infections.
Prospects for further research. Obtained results will be used in the planning and conducting further experimental studies towards the development of new metabolics based on *B. bifidum* and *L. reuteri* derivatives.

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**Conflicts of interest:** authors have no conflict of interest to declare.

**Conflіkt інтересів:** відсутній.

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