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POTENTIATION OF THE ANTIMICROBIAL EFFECT OF *LACTOBACILLUS REUTERI* DSM 17938 CELL-FREE EXTRACTS BY ASCORBIC ACID

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Ключові слова: *комбінаторний (спрямований прекурсором) біосинтез, похідні L. reuteri DSM 17938, модифікована аскорбінова кислота, ВЕРХ-аналіз.*

Ключевые слова: *комбінаторный (направленный прекурсором) биосинтез, производные L. reuteri DSM 17938, модифицированная аскорбиновая кислота, ВЭЖХ-анализ*

Abstract. **Potential of the antimicrobial effect of *Lactobacillus reuteri* DSM 17938 cell-free extracts by ascorbic acid.** Knysh O.V., Martynov A.V. *The purpose of this study was to evaluate the effect of supplementing the culture medium with ascorbic acid on the antimicrobial properties of Lactobacillus reuteri cell-free extracts (CFEs). CFEs were prepared using commercial strain L. reuteri DSM 17938 by culturing lactobacilli in its own disintegrated cell suspension (DCS) supplemented with ascorbic acid in sub-inhibitory (5 mg/ml, CFE₅) or minimal inhibitory concentration (20 mg/ml, CFE₂₀) and without supplementation (CFE₀). Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 25922 reference strains and Pseudomonas aeruginosa extensively drug resistant (XDR) clinical isolate were used as indicator cultures. Screening of the inhibitory properties of the studied CFEs and elucidation of the nature of inhibitory products were done using modified Micro scale Optical Density Assay (MODA). The inhibition indices (InhI) were calculated for the studied CFEs and ascorbic acid of appropriate concentrations. CFEs were subjected to HPLC-analysis. CFE₅ and CFE₂₀ showed significantly higher antimicrobial activity toward to indicator cultures than CFE₀. InhI calculated for extracts CFE₅ and CFE₂₀ mainly exceeded the sum of the corresponding indicators calculated for CFE₀ and ascorbic acid (AA) of appropriate concentrations: InhI CFE₅ ≥ InhI CFE₀ + InhI AA₅; InhI CFE₂₀ > InhI CFE₀ + InhI AA₂₀. Acidic metabolic products have made the greatest contribution to the antimicrobial effect of the studied CFEs. HPLC-nalysis showed that the modified ascorbic acid was the substance found in CFE₂₀ in the greatest quantity. The revealed effect of potentiation of antimicrobial activity of CFEs by ascorbic acid should be taken into account when developing new biotechnological products based on derivatives of L. reuteri DSM 17938.*

Реферат. Потенціювання протимікробного ефекту безклітинних екстрактів *Lactobacillus reuteri* DSM 17938 аскорбіною кислотою. Книш О.В., Мартинов А.В. Метою цього дослідження було оцінити вплив аскорбінової кислоти в культуральному середовищі на протимікробні властивості безклітинних екстрактів (БКЕ) *Lactobacillus reuteri*. БКЕ були отримані з використанням штаму *L. reuteri* DSM 17938 шляхом культивування лактобактерій у дезінтегрованій суспензії власних клітин без додавання (БКЕ₀) та з додаванням аскорбінової кислоти в субінгібіторній (5 мг/мл, БКЕ₅) або мінімальній інгібіторній концентрації (20 мг/мл, БКЕ₂₀). Референтні штами *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 та клінічний ізолят *Pseudomonas aeruginosa* з широкою лікарською стійкістю були використані як індикаторні культури. Скринінгове дослідження інгібіторних властивостей досліджуваних БКЕ та з'ясування природи інгібіторних продуктів проводили з використанням модифікованого спектрофотометричного мікроплатиетного методу. Інгібіторні індекси (ІнгІ) розраховували для дослідних БКЕ та аскорбінової кислоти відповідних концентрацій. БКЕ піддавали ВЕРХ-аналізу. БКЕ₅ та БКЕ₂₀ виявили значно вищу протимікробну активність по відношенню до індикаторних культур, ніж БКЕ₀. ІнгІ, розраховані для БКЕ₅ та БКЕ₂₀, переважно перевищували суми аналогічних показників, розрахованих для БКЕ₀ та аскорбінової кислоти (АК) відповідних концентрацій: $\text{ІнгІ БКЕ}_5 \geq \text{ІнгІ БКЕ}_0 + \text{ІнгІ АК}_5$; $\text{ІнгІ БКЕ}_{20} > \text{ІнгІ БКЕ}_0 + \text{ІнгІ АК}_{20}$. Найбільший внесок у протимікробний ефект дослідних БКЕ належав кислим продуктам метаболізму. ВЕРХ-аналіз показав, що в БКЕ₂₀ у найбільшій кількості містилася модифікована аскорбінова кислота. Виявлений ефект посилення аскорбіною кислотою протимікробної активності БКЕ слід урахувувати при розробці нових біотехнологічних продуктів на основі похідних *L. reuteri* DSM 17938.

The wide spread of antibiotic resistance among bacteria dictates the need to change the strategy and tactics of combating diseases of an infectious nature. Promising direction is the development of etiopathogenetic agents with a wide spectrum of action (antimicrobial, immunotropic and anti-inflammatory). Currently, probiotics are considered as an alternative to classic antibacterial agents [1]. In addition, there are evidences of their anti-inflammatory and immunomodulatory effects [9, 12]. Most of the beneficial effects of probiotics are due to the biological activity of cell structural components and metabolic products [12, 14]. For the implementation of the probiotic action, the colonization of a certain biotope by probiotic cells and the time for their activation are required. However, during transit through the gastrointestinal tract, significant cell loss occurs. This is the main reason for the lack of effectiveness of cell probiotics [4].

Probiotic bacteria can be considered as a valuable source of postbiotic products (metabiotics). They are ready-made biologically active structural components and/or metabolites, the effects of which can be realized immediately after introduction. In addition, they make it possible to avoid undesirable effects associated with the introduction of whole cells [14].

Lactobacillus reuteri is one of the important probiotic bacteria capable of exerting antimicrobial, immunomodulatory and anti-inflammatory effects in the host organism. It can produce a number of antimicrobial substances [9]. There are conflicting data regarding the antimicrobial activity of cell-free supernatants obtained from the *L. reuteri* culture. Some authors have shown that the supernatant of *L. reuteri* exhibits the lowest antimicrobial activity compared to the supernatants of other probiotic lactobacilli [3]. Other authors have stated the high

antibacterial potential of the supernatants of four *L. reuteri* strains. They argue that the antimicrobial potential is determined by the profile of the produced metabolites in specific cultivation conditions [2]. Certain probiotic *L. reuteri* strains have the ability to convert of glycerol into a broad-spectrum antimicrobial compound reuterin. Moreover, *L. reuteri* has the most pronounced ability to produce this aldehyde and the least sensitivity to it compared to other microorganisms. The production of reuterin is an example of a precursor-directed antimicrobial biosynthesis [10]. Nowadays, the precursor-directed biosynthesis is considered as one of the three major strategies for combinatorial biosynthesis and a promising way to obtain antimicrobial and anticancer agents [10, 13]. Enzyme-level modification and pathway-level recombination are no less promising classical approaches for combinatorial biosynthesis of multiple natural product analogs [13].

Ascorbic acid is an organic compound known for its high reducing potential and ability to regulate oxidation-recovery processes. Recent studies indicate the ability of ascorbic acid to inhibit the growth and biofilm formation by opportunistic and pathogenic bacteria [11, 16]. Limited evidence exists regarding the role of ascorbic acid as a precursor to biologically active metabolites of living organisms.

The purpose of this study was to evaluate the effect of supplementing the culture medium with ascorbic acid on the antimicrobial properties of *L. reuteri* cell-free extracts.

MATERIALS AND METHODS OF RESEARCH

Cell-free extracts (CFEs) were prepared using commercial strain *L. reuteri* DSM 17938 (from medical product "BioGaia", BioGaia Production AB, Sweden).

Obtaining of CFEs was performed as described previously with slight modifications [7].

Preparation of disintegrated cell suspension (DCS). The suspension of *L. reuteri* in isotonic saline solution (0.9 % NaCl) corresponding optical density of 10 units on the McFarland scale ($\sim 10^8$ CFU/ml) was subjected to 10 cycles of slow freezing-thawing. Freezing of 50 mL samples was carried out to -23°C . Then samples were heated in a water bath at a temperature of 37°C until completely thawed. The obtained DCSs were divided into three portions. The first portion of them was retained without additives. The second and third portions were supplemented with ascorbic acid (PA, Sigma) in a final concentration of 5 mg/ml and 20 mg/ml.

Cultivation of *L. reuteri* in DCS. The suspension of *L. reuteri* in isotonic saline solution ($\sim 10^8$ CFU/ml) was inoculated into DCS (supplemented and not supplemented with ascorbic acid) at a ratio of 1:9 and was cultured at 37°C for 72 hours.

Preparation of CFEs. The cultures obtained by cultivating of *L. reuteri* in DCS were centrifuged at 3000 g for 10 min. Cell free supernatants were passed through membrane filters (Vladipor, Russian Federation) with a pore diameter of $0.2\ \mu\text{m}$ to remove microbial cells and cellular debris. Thus, three types of extracts were obtained from *L. reuteri* cultured in DCS: 1) without supplementation (CFE₀); 2) supplemented with ascorbic acid, 5 mg/ml (CFE₅) and 3) supplemented with ascorbic acid, 20mg/ml (CFE₂₀).

Indicator strains. Reference strains: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* extensively drug resistant (XDR) clinical isolate were used as indicator cultures.

Preparation of the inoculum. After verifying the purity of the indicator culture, few colonies from the overnight incubated agar medium were emulsified in a physiological saline for obtaining a suspension corresponding optical density of 0.5 units on the McFarland scale. Then the suspension was diluted ten times.

Inhibitory properties. Screening of the inhibitory properties of the studied CFEs and elucidation of the nature of inhibitory products were carried out using Micro scale Optical Density Assay (MODA) described by Lash et al. with some modifications [8].

Screening of the inhibitory properties. Briefly, the optical density gain of indicator bacteria in the liquid nutrient broth for 24 h at 37°C was compared in the presence (test sample) or absence (control sample) of the studied CFEs and ascorbic acid in appropriate concentrations. The ratio of the nutrient broth, inoculum and CFE (or ascorbic acid) in the

incubation medium was 6:1:3, respectively. The optical density was measured using a microtiter-plate reader «Lisa Scan EM» («Erba Lachema s.r.o.», Czech Republic) at 578 nm. The growth inhibition indices (InhI) were calculated by the formula: $\text{InhI} = (\Delta\text{OD} - \Delta\text{OD}_{\text{PC}}) \div \Delta\text{OD}_{\text{PC}} \times 100\%$, where ΔOD and $\Delta\text{OD}_{\text{PC}}$ were the changes in the optical density of the control and test samples within 24 hours [7].

Elucidation of the nature of inhibitory products. Two types of CFEs from *L. reuteri* cultured in DCS: 1) without supplementation and 2) supplemented with ascorbic acid (20 mg/ml) were divided into equal portions for different assays. One portion was retained as untreated. The second portion was treated with proteolytic enzyme pepsin (1 mg/ml, Sigma) for elucidate the contribution of the proteinaceous component to inhibitory activity of CFEs. For determination of a contribution of organic acids the third portion was adjusted to pH 6.8-7 using 1 N NaOH. The fourth portion after neutralization was treated with catalase (0.5 mg/ml, Sigma) at 25°C for 1h to determine the contribution of hydrogen peroxide to the inhibitory activity. The fifth portion after neutralization was treated with proteolytic enzyme trypsin (1 mg/ml, Sigma) at 37°C for 2 h to find out the possible inhibitory effect of substances of a proteinaceous nature. The optical density gain of indicator bacteria in the presence of untreated and treated CFEs was determined as described above.

Chromatographic study was performed according to the procedure described previously [15]. Acetonitrile (Sigma, USA) and lithium perchlorate, perchloric acid in a kit for chromatograph BD2003 (Econova, RF) were used for HPLC-analysis. For analysis of experimental substrates was used HPLC-system Milichrome A-02 (Econova, RF). In addition, we used a set of chromatography conditions for analysis of riboflavin derivatives: gradient separation of acetonitrile (from 0% to 100%) / 0.05 M lithium perchlorate buffer + 0.01 M perchloric acid at 40°C , and fraction detection in UV region. As a comparison, we used ascorbic acid.

Statistical analysis. All experiments were performed three times in triplicate. Obtained data were statistically processed using Windows® XP Professional OEM Software, Excel 2003 (license No 74017-640-0000106-57973). Average values of obtained indices with standard deviations were determined ($\bar{x} \pm \text{SD}$). One-way analysis of variance (ANOVA) was performed followed post hoc multiple comparison using Bonferroni adjustment and critical value of p equaled to $0.05 / (\text{number of possible comparisons})$ [6].

RESULTS AND DISCUSSION

Screening of the inhibitory activity of the studied CFEs. The growth inhibition indices presented in the Table indicate that the strongest decrease of the optical density gain of indicator cultures occurred under the influence of CFEs obtained by culturing *L. reuteri* in DCS supplemented with ascorbic acid (CFE₅ and CFE₂₀). More pronounced inhibition was observed with the addition of ascorbic acid at a higher concentration (InhI CFE₂₀ > InhI CFE₅). It should be noted that the Inhs

calculated for extracts CFE₅ and CFE₂₀ mainly exceeded the sum of the corresponding indicators calculated for CFE₀ and ascorbic acid (AA): InhI CFE₅ ≥ InhI CFE₀ + InhI AA₅; InhI CFE₂₀ > InhI CFE₀ + InhI AA₂₀. Among the studied indicator cultures, the least inhibitory effect of CFE₀ and ascorbic acid was observed toward to *P. aeruginosa* (XDR). CFE₂₀ inhibited the growth of all studied indicator cultures to the same extent.

Inhibitory activity of cell-free extracts (CFEs) and ascorbic acid (AA) against indicator cultures

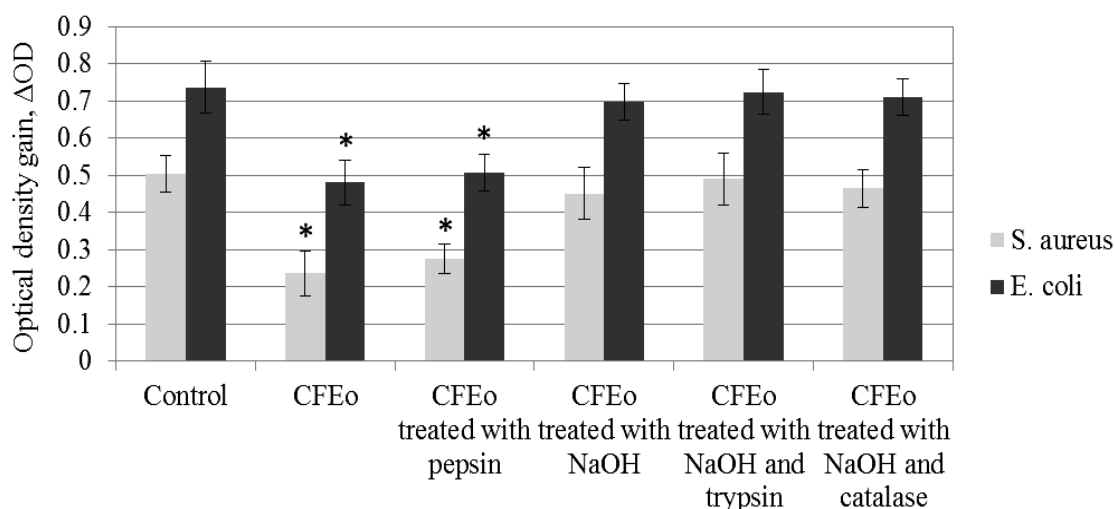
Indicator strains	Inhs, % (x ± SD)				
	CFE ₀	CFE ₅	CFE ₂₀	AA ₅	AA ₂₀
<i>S. aureus</i> ATCC 25923	53.2 ± 5	80.4 ± 7*	90.9 ± 8*	22.9 ± 2	38.5 ± 5
<i>E. coli</i> ATCC 25922	34.7 ± 6	63.3 ± 4*	96.0 ± 4*	17.5 ± 5	30.0 ± 6
<i>P. aeruginosa</i> (XDR)	26.5 ± 5	85.2 ± 5*	93.0 ± 7*	0.1 ± 3	2.4 ± 2

Notes. * — the differences are statistically significant with respect to the inhibition indices calculated for CFE₀ and AA of appropriate concentration, p < 0.05.

Elucidation of the nature of inhibitory products.

The data presented in Fig. 1 show that CFE₀ treated with pepsin significantly inhibited the growth of both indicator cultures: *S. aureus* and *E. coli*. InhI were 45.2% and 31.2%, respectively. CFE₀ after neutralization and treatment with trypsin and catalase did not significantly affect the optical

density gain of both indicator cultures. An exception was *S. aureus*: the InhI of culture under the influence of CFE₀ treated only with NaOH was 10.6%. Thus, the study revealed that the contribution of the proteinaceous component to the inhibitory activity of the CFE₀ against *S. aureus* was small, and against *E. coli* it was negligible.



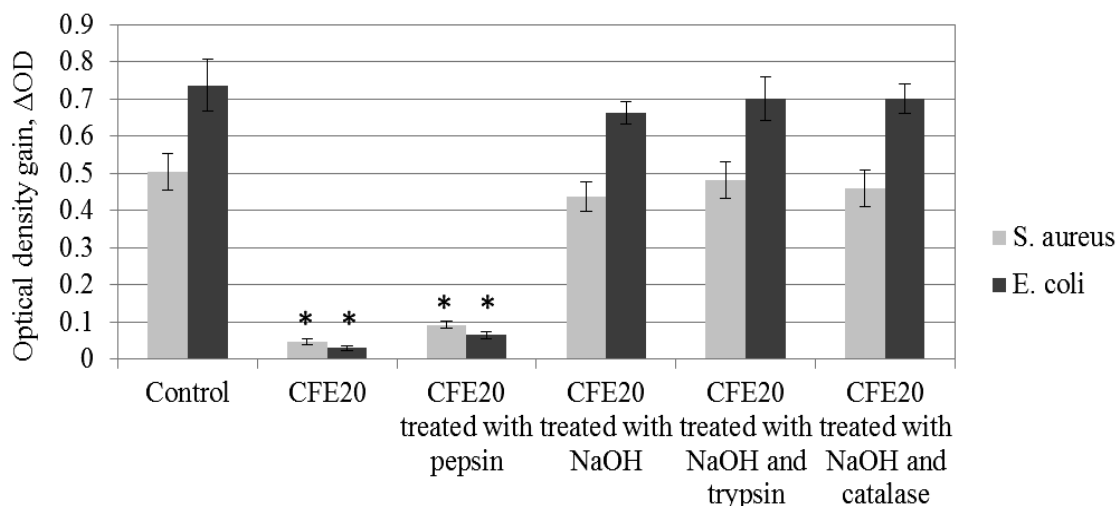
Notes. * — the differences are statistically significant with respect to the control indices, p < 0.05

Fig. 1. The effect of treated and untreated CFE₀ on the growth of indicator cultures



As can be seen from the data presented in Fig. 2, pepsin-treated CFE₂₀ reduced the optical density gain of staphylococcal culture by 81.8%. Inhl calculated for pepsin-treated CFE₂₀ toward *E. coli* was 91.2%. Neutralization led to a significant decrease in antimicrobial activity of CFE₂₀. Inhl calculated for CFE₂₀ treated with NaOH toward *S.*

aureus was 13.6%, and toward *E. coli* it was 10.2%. Optical density gain of both cultures under the influence of neutralized CFE₂₀ treated with trypsin or catalase did not significantly differ from the benchmarks. This indicated an almost complete loss of antimicrobial activity of the CFE₂₀ after neutralization and treatment with enzymes.



Notes. * – the differences are statistically significant with respect to the control indices, $p < 0.05$

Fig. 2. The effect of treated and untreated CFE₂₀ on the growth of indicator cultures

Chromatographic study. Fig. 3 shows the HPLC-chromatogram of degradation products / ascorbic acid as result of ascorbic acid modification by *L. reuteri*. The greatest quantity of the substance in the CFE₂₀ is modified ascorbic acid. Although UV-Spectrum (peak 1) is like ascorbic acid, but retention time of (peak 1) is shifted in a hydrophobic region. This indicates that the substance (peak 1) is a modified product derived as a result of the ascorbic acid conversion by *L. reuteri*, alike as this bacterium converts glycerol to reuterin.

There are a number of mechanisms for the antimicrobial action of probiotics. Different strains of *L. reuteri* can produce and excrete a variety of antimicrobial molecules: lactic acid, acetic acid, ethanol, hydrogen peroxide, carbon dioxide, diacetyl, bacteriocin (reuterin 6), reutericyclin and reuterin [2, 9, 10]. However, there is no evidence that single strain can produce reuterin, reutericyclin, reuterin, or two of these antimicrobial compounds simultaneously. Bacteriocin has a peptide nature; therefore, it loses its activity after treatment with proteolytic enzymes or after prolonged heat treatment. Its production is highly dependent on the cultivation conditions. Reute-

ricyclin is a tetramic acid molecule that functions as a proton ionophore and dissipates the transmembrane ΔpH . It exhibits a powerful antimicrobial effect against Gram-positive microorganisms in low concentrations. The toxicity of reutericyclin to humans and animals remains unstudied.

As mentioned earlier, *L. reuteri* has the ability to convert glycerol into a broad-spectrum antimicrobial compound reuterin. For adequate antimicrobial activity of *L. reuteri*, glycerol is required in amounts significantly exceeding those usually available in the gastrointestinal tract. Therefore, the intake of probiotic bacteria *L. reuteri* requires additional administration of glycerol [5]. A lot of work has been devoted to confirming the antimicrobial activity of reuterin *in vitro*, but there are no works demonstrating its antimicrobial activity *in vivo*. This aldehyde is resistant to proteolytic and lipolytic cleavage. In aqueous solution, reuterin is a three-component dynamic system consisting of various (monomeric, hydrated monomeric, and cyclic dimeric) forms of 3-hydroxypropionaldehyde (3-HPA) and can be reversibly dehydrated into the toxic acrolein. Therefore, the toxicity and stability of 3-HPA should be fully studied before its use *in vivo*.

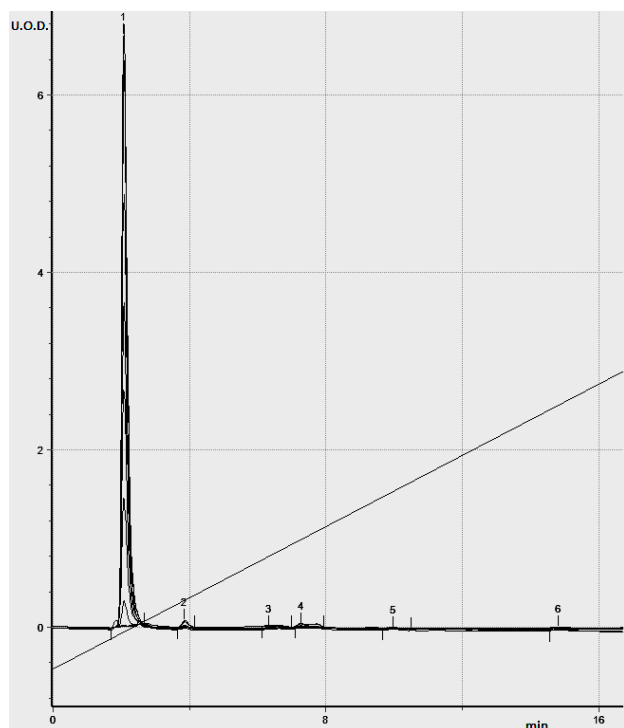


Fig. 3. HPLC-analysis of CFE₂₀.

Analysis of the available data suggests the need to study the role of other important metabolic substances of *L. reuteri*. Precursor-directed antimicrobial biosynthesis strategy is considered one of the most promising in the development of next-generation probiotics [10]. This strategy served as the basis for the search for new precursors.

Well-known biological functions of ascorbic acid are related to its chemical properties as a reducing agent. Recently, there has been an interest in ascorbic acid as an antimicrobial agent. Some of studies have demonstrated the antimicrobial activity of ascorbic acid against Gram-positive, Gram-negative bacteria and fungi [11, 16]. Different sensitivity of bacteria to ascorbic acid was revealed: non-acid resistant bacteria were sensitive to the dissociated form; the lactobacilli were more sensitive to the non-dissociated form. Important conclusion was made that the sensitivity of microorganisms did not depend on sensitivity to antibiotics. Based on their own observations, the authors expressed doubt that the antibacterial activity of ascorbic acid is associated only with acidification or a decrease in pH. They suggested

that ascorbic acid has a specific effect on the cell membrane or cell enzymes [11].

The present study showed that the supplementation of culture medium with ascorbic acid leads to potentiation of the antimicrobial effect of CFEs obtained from the *L. reuteri* culture. The potentiating effect of ascorbic acid was depend on the concentration and did not depend on the indicator culture. It should be noted that ascorbic acid was added to the culture medium in sub-inhibitory and minimal inhibitory concentrations. The choice of these concentrations was based on data previously obtained by other authors in determining the minimal inhibitory concentrations of ascorbic acid for pathogenic, opportunistic bacteria and lactobacilli (*L. plantarum*) [11, 16].

The results of this study confirmed that the greatest contribution to the antimicrobial effect of CFEs was made by organic acids provided a low pH level. Neutralization of CFSs led to complete loss of antimicrobial activity or their inhibitory activity toward test bacteria became negligible. The contribution of the proteinaceous component to the antimicrobial activity of CFE₂₀ was little or insignificant.

Chromatographic studies showed that during *L. reuteri* cultivation in presence ascorbic acid in sub-inhibitory and minimal inhibitory concentrations observed chemical changes in molecular structure of ascorbic acid. This modification leads to potentiation of the inhibitory effects of CFEs toward to indicator cultures.

CONCLUSIONS

1. Ascorbic acid in sub-inhibitory and minimal inhibitory concentrations potentiates the antimicrobial activity of *L. reuteri* DSM 17938 CFEs against reference strains: *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *P. aeruginosa* XDR clinical isolate in vitro.

2. The results of this study show that acidic metabolic products make the largest contribution to the antimicrobial effect of the studied CFEs.

3. The revealed effect of potentiation of the antimicrobial activity of CFEs by ascorbic acid should be taken into account when developing new biotechnological products based on derivatives of *L. reuteri* DSM 17938.

Conflict of interest. The author declares no conflict of interest

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