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EFFECT OF Ca AND Mg IONS ON THE GROWTH AND ANTIBACTERIAL ACTIVITY OF *LACTOBACILLUS DELBRUECKII* SUBSP. *BULGARICUS* RIN-2003-LS

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The effect of Ca and Mg ions on the growth and antibacterial activity of *Lactobacillus delbrueckii* subsp. *bulgaricus* RIN-2003-LS was studied. Stimulation of RIN-2003-LS growth and antibacterial activity was confirmed. The strongest antibacterial activity of RIN-2003-LS was revealed after addition to the medium 15 mM Ca^{2+} or 5 mM Mg^{2+}. However, simultaneous addition of Ca^{2+} and Mg^{2+} to growth medium didn't cause any change of antibacterial activity. So, the strong antibacterial activity of studied strain can serve as a basis for its application as a starter in production of dairy products and as a probiotic in production of antimicrobial drugs.

Lactic acid bacteria – Ca and Mg ions – antibacterial activity – probiotic

Ուսումնասիրվել է Ca և Mg իոնների ազդեցությունը *Lactobacillus delbrueckii* subsp. *bulgaricus* RIN-2003-LS շտամի աճի և հակաբակտերիական ակտիվության վրա: Հաստատվել է RIN-2003-LS-ի աճի և հակաբակտերիական ակտիվության խթանումը: RIN-2003-LS-ի ամենաբարձր հակաբակտերիական ակտիվությունը դրսևորվել է սննդամիջավայրին 15 մՄ Ca կամ 5 մՄ Mg իոններ ավելացնելուց հետո: Սակայն Ca և Mg իոնների համատեղ ավելացումը աճման սննդամիջավայրին հակաբակտերիական ակտիվության էական փոփոխություն չի առաջացրել: Այսպիսով, ուսումնասիրված շտամի դրսևորած բարձր հակաբակտերիական ակտիվությունը կարող է հիմք հանդիսանալ այս շտամի օգտագործման համար կաթնամթերքի արտադրության մեջ որպես մերան և հակամանրէային դեղամիջոցների արտադրության մեջ որպես պրոբիոտիկ:

Կաթնաթթվային բակտերիաներ – Ca և Mg իոններ – հակաբակտերիական ակտիվություն – պրոբիոտիկ

Изучалось влияние ионов Ca и Mg на рост и антибактериальную активность *Lactobacillus delbrueckii* subsp. *bulgaricus* RIN-2003-LS. Подтверждена стимуляция роста и антибактериальной активности RIN-2003-LS. Самая высокая антибактериальная активность RIN-2003-LS была выявлена после добавления в питательную среду ионов Ca (15 mM) или Mg (5 mM). Однако совместное добавление в среду ионов Ca и Mg не выявило никаких изменений антибактериальной активности. Таким образом, высокая антибактериальная активность изученного штамма может стать основанием для его применения в качестве стартовой культуры в производстве молочных продуктов, а также как пробиотик для возможного применения в производстве антимикробных препаратов.

Молочнокислые бактерии – ионы Ca и Mg – антибактериальная активность – пробиотик

Antibiotic resistance of pathogenic bacteria is the most important reason for search and screening of new bacteria with high biological activity. The best candidates fulfilling these requirements are lactic acid bacteria (LAB).

The great diversity of ecological-geographic conditions of Armenia with its exactly determined vertical zoning contributes the development of unique associations of LAB in traditional dairy products. During centuries Armenians have prepared traditional protein-enriched dairy products such as matsoun, sour cream and different types of cheeses, having substantial physiological, antagonistic, antioxidant and antiallergenic activity [2, 7]. Antibacterial activity is connected to synthesis of special substances-bacteriocins [9]. Bacteriocin producing LAB can be applied as a starter cultures in food fermentation or added to fresh food as bio-preservatives.

Environmental pollution by different metal ions is due to technical development. By accumulating in soil, they cause poisoning of plants, animals and people organism. That is why study of possible ways to reduce the amount of metals is actual and prospective. Due to ability to synthesize lactic acid, LAB acidify environment by causing accumulation of different metal salts in soil and their chelation. Chelated metals don't penetrate into the cells and don't cause toxic effect. But it is significant to differentiate the metals that are essential for cell activity and included in composition of enzymes by ensuring their functionality [10]. Such metals are calcium (Ca^{2+}) and magnesium (Mg^{2+}).

Therefore, the aim of this work was to investigate the effect of Ca^{2+} and Mg^{2+} on LAB growth, acid formation and antimicrobial activity which will allow regulating bacteria growth conditions for maximal production of antimicrobial substances and defining the mechanisms of LAB metallotolerance.

Materials and methods*. *Objects of investigation

The object of investigation was *Lactobacillus delbrueckii* subsp. *bulgaricus* RIN-2003-LS strain isolated from salinized Armenian cheese "Lori", village Gladzor, Vayots Dzor province, Armenia, and stored in the Department of Biochemistry, Microbiology and Biotechnology.

Investigation of effects of different metal ions on biological properties of lactic acid bacteria

To determine the stimulating effect of different metal ions (Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Ba^{2+} and Zn^{2+}) on LAB antibacterial activity the primary screening was carried out. As a source of ions the following salts were used – $CaCl_2$, $CoSO_4$, $MgCl_2$, $MnSO_4$, $Fe_2(SO_4)_3$, $FeSO_4$, $BaCl_2$, ZnS. For study of metal ions effect on LAB biological properties MRS medium was prepared containing 5, 10, 15 mM Ca and Mg ions. The optimal concentration of metal ions was determined. Simultaneous effect of two ions on LAB growth, acid formation and antibacterial activity was studied, too.

Growth curve construction and pH measurement

For construction of growth curve, optical density was measured by spectrophotometric method, the wavelength was 595 nm (КФК-3 -"ЗОМЗ", Russia). Each measurement was carried out every 3 h. pH measurement was carried out by potentiometric method using pH-meter (Knick K-766, Germany).

Determination of antibacterial activity

Determination of LAB antibacterial activity was carried out by agar well-diffusion method [8]. The following test-organisms were used: *Pseudomonas aeruginosa* 272786 (isolated from clinical material and provided by "Prom-test" diagnostic center), *Salmonella enterica* K3 (isolated from pig feces and provided by Institute of Molecular Biology, NAS RA), *Staphylococcus aureus* MDC 5233, *Escherichia coli* VKPM M17, *Bacillus subtilis* WT-A1 (isolated from a soil sample). 0.1 mL of overnight culture was added to wells and tested for antibacterial activity. Then Petri dishes were placed in fridge for 2 h for diffusion of antibacterial substances. After 24 h of incubation at the optimum temperature required for the test-culture growth, diameters of growth inhibition zones were measured. A clear zone of inhibition of at least 2 mm in diameter was recorded as positive.

Results and Discussion. *L. delbrueckii* subsp. *bulgaricus* RIN-2003-LS strain was revealed high antibacterial activity against Gram-positive and Gram-negative microorganisms, as shown before [3]. To study the effect of different metal ions on antibacterial activity of this strain 5 mM metal salts were used. The results are shown in fig. 1.

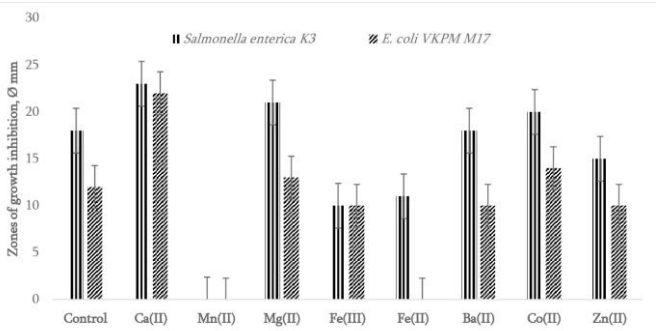

Fig. 1. Dependence of antibacterial activity of *Lactobacillus delbrueckii* subsp. *bulgaricus* RIN-2003-LS on presence of metal ions.

Ca^{2+} and Mg^{2+} induced antibacterial activity of *L. delbrueckii* subsp. *bulgaricus* RIN-2003-LS, vice-versa Mn^{2+}, Fe^{3+}, Fe^{2+} and Zn^{2+} inhibited its activity, and Ba ions didn't show any effect. That is why for further investigation Ca^{2+} and Mg^{2+} were selected (5, 10 and 15 mM). The results are shown in fig. 2.

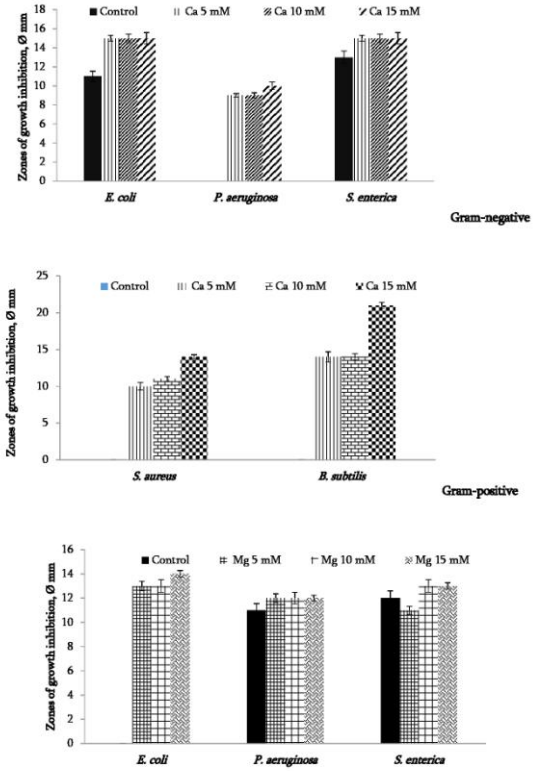

Fig. 2. The effect of different concentrations of Ca and Mg ions on antibacterial activity of *L. delbrueckii* subsp. *bulgaricus* RIN-2003-LS

It was shown before that in case of incubation in milk antibacterial activity of some LAB strains against *S. aureus* MDC 5233 was lower than in case of incubation in MRS [3]. As a rule, concentration of calcium in milk is approximately 15 mM [4]. The results showed that high concentrations of Ca^{2+} induced the antibacterial activity of RIN-2003-LS, especially against Gram-positive test-organisms. It is interesting to note that residual concentration of Ca^{2+} in intestine is 5 mM [1]. Consequently, this allows concluding for strong antibacterial activity of RIN-2003-LS in intestine additional source of calcium is required. The kinetics of *L. delbrueckii* subsp. *bulgaricus* RIN-2003-LS growth, acid formation and antibacterial activity in different Ca^{2+} and Mg^{2+} concentrations had been studied, too. The results are presented in figs. 3 and 4.

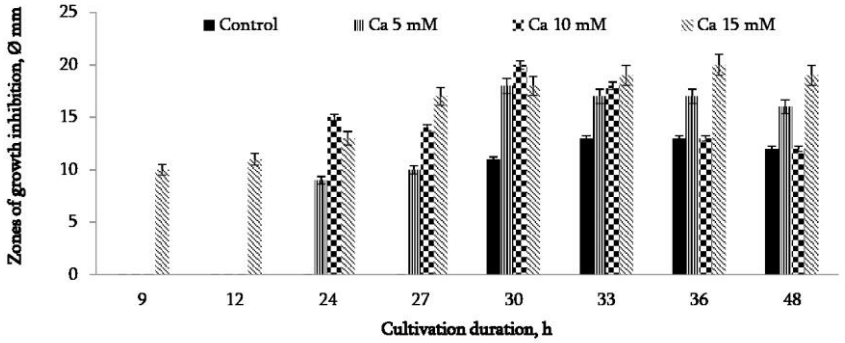

Fig. 3. The effect of different concentrations of Ca^{2+} on kinetics of growth, acid formation and antibacterial activity of *L. delbrueckii* subsp. *bulgaricus* RIN-2003-LS

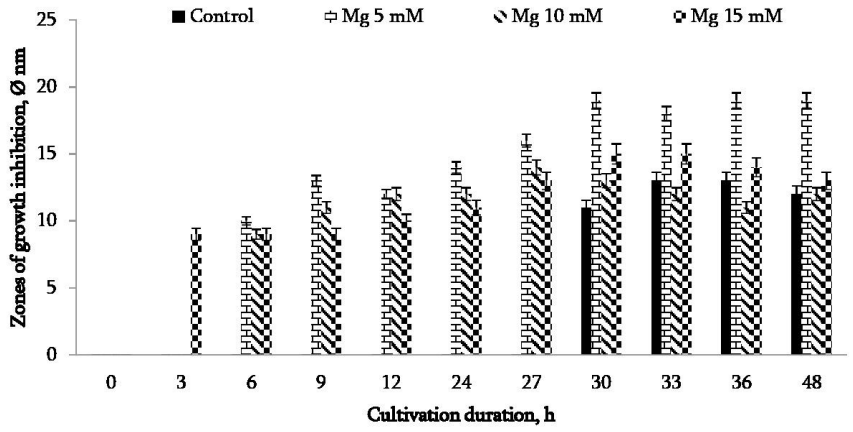

Fig. 4. The effect of different concentrations of Mg^{2+} on kinetics of growth, acid formation and antibacterial activity of *L. delbrueckii* subsp. *bulgaricus* RIN-2003-LS

It is shown from fig. 3 that Ca^{2+} 15 mM in MRS has the strongest effect on these 3 properties. Interestingly, such concentration increases the synthesis of antibacterial substances since 9th hour. However, the stimulating effect of Mg^{2+} was observed in 5 mM concentration (fig. 4). Keryan and coauthors studied the effect of Ca and Co ions on growth, acid formation and antibacterial activity of *L. delbrueckii* subsp. *lactis* INRA-2010-4.2 and *L. crispatus* INRA-2010-5.2 strains and showed that for both strains even 5 mM concentration of Ca^{2+} caused inhibition of growth [6].

For determination of combined effect of Ca and Mg ions 15 mM concentration of Ca^{2+} and 5 mM concentration of Mg^{2+} were added to MRS medium but no significant effect was revealed on antibacterial activity. The similar results were shown by Keryan and coauthors in case of simultaneous addition of Ca and Co ions [6].

Interesting results about antibacterial properties of magnesium were obtained by Duane and coauthors [11]. Particularly, they investigated antibacterial properties of magnesium against Gram-positive (*S. aureus*) and Gram-negative (*E. coli* and *P. aeruginosa*) bacteria. As a source of Mg ions $MgCl_2$ salt was used which was added to the growth medium of test-organisms. The pH of cultures growth medium was increased by 1.0 N NaOH. Authors indicated that Mg metal has a significant effect on CFUs of both Gram-positive and Gram-negative bacteria. Added to the growth media Mg corrosion products would inhibit the growth of *E. coli*, *P. aeruginosa* and *S. aureus* but this is only a hypothesis which needs confirmation.

Ca and Mg ions have other amazing properties. Particularly, it was shown that Ca^{2+} and Mg^{2+} can influence on bacterial biofilm formation [5, 13]. In other work was observed the role of Mg ions in the growth of *Salmonella* Phage Anti-R [14]. Interestingly, Ca^{2+} and Mg^{2+} can also interact with antibiotics and protect the bacterial outer membrane from damage [12].

However, these are not all properties of Ca^{2+} and Mg^{2+} investigated by researchers. Undoubtedly, these metals have many other properties which are not detected yet, and the further works will be devoted to discovery of them.

The obtained results showed that Ca and Mg ions stimulated the antibacterial activity of *L. delbrueckii* subsp. *bulgaricus* RIN-2003-LS. The stimulating effects of Ca^{2+} and Mg^{2+} were depended on their concentrations. Particularly, the best concentration for Ca^{2+} was 15 mM and for Mg^{2+} – 5 mM. However, the simultaneous addition of these ions to growth medium didn't show any crucial effect on antibacterial activity of studied strain. So, it can be considered that in case of presence of two ions competition for entrance to cell or chelation of metal ions occurs which can influence on metal access.

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