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Inhibitation of Growth and Antimicrobial Activity of New Lactobacilli Strains Isolated From Armenian Dairy Product Matsoun with EDTA in Low Concentrations: Comparative Study with Gram-Positive and Gram-Negative Bacteria

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Keywords:*EDTA, bacterial cultivation, antibacterial activity, lactobacilli.*

Introduction. Lactic Acid Bacteria (LAB) are a heterogeneous group of gram-positive microorganisms that synthesize large variety of antimicrobial substances such as organic acids (lactic acid, acetic acid, etc.), hydrogen peroxide, diacetyl, carbon dioxide, bacteriocins and other low molecular weight compounds [1]. Bacteriocins are ribosomally synthesized antibacterial peptides that are usually active against closely related strains. Bacteriocins are found at many gram-positive and gram-negative bacteria but those synthesized by LAB are of particular interest [2]. They may be used as preservatives to inhibit the growth of pathogens and spoilage microorganisms in food technology, thus replacing chemical preservatives [3] because LABhave generally recognized as safe (GRAS) status [4] and their bacteriocins do not change the smell or taste of a product [5]. However, vast majority of bacteriocins produced by LAB are active only against LAB and other gram-positive microorganisms. There are only a small number of bacteriocins reported to be active against gram-negative microorganisms [6]. This difference is due to a protective outer layer of gram-negative bacteria that covers plasma membrane and peptidoglycan layer. This structure acts as a penetration barrier and consists of glycerophospholipids and lipopolysaccharide molecules.Ethylenediaminetetraacetic acid (EDTA) is metal chelating agent that removes Ca^{2+} and Mg^{2+} from outer layer of gram-negative bacteria destabilizing its structure and affecting permeability, thus making cells sensitive to hydrophobic substances such as bacteriocins [7, 8]. EDTA is used in food production to prevent oxidation and other harmful reactions catalyzed by metal ions. It also has antimicrobial activity and is known to enhance the activity of antimicrobials and antibiotics especially against gram-negative microorganisms. Thus, EDTA in combination with other antimicrobials may be effective for inhibition of gram-negative bacteria in food products [1]. However, there is little information on effects of EDTA on gram-positive bacteria.Hansen et al. [9] demonstrated inhibitory effect of 0.9 mM EDTA against various gram-positive microorganisms. Other researchers showed that EDTA prevented the growth of *Staphylococcus epidermidis* [10]. It was reported that EDTA at 10 mM did not inhibit the growth of *Streptococcus agalactiae*[11]. In another work EDTA expressed antimicrobial properties against streptococcal bovine mastitis isolates but only in high concentrations (30-100 mM) [12]. But there are no available data on lactobacilli.

The aim of this study was to investigate the growth of two lactobacilli strains as well as test strains – gram-positive *Micrococcus luteus*and gram-negative *Salmonella typhimurium*inthe presence of low concentrations of EDTA. In order to evaluate the optimal conditions for maximal antagonistic activity the effect of EDTA on lactobacilli antibacterial activity was also determined. The findings might be a good input in development of new probiotics or food preservation technology.

Materials and methods. The objects of the study were *Lactobacillus delbrueckii* subsp. *lactis* INRA-2010-4.2 and *Lactobacillus crispatus* INRA-2010-5.2 that were originally isolated from Armenian traditional dairy product matsoun. They were maintained by subculturing once a month in 10% skim-milk. For long storage MRS broth (Hi Media, India) was used with addition of 20% glycerol. Prior to experiments they were pre-incubated in MRS broth.

Micrococcus luteus WT and *Salmonella typhimurium* WDCM 1474 were used as test strains to determine the antibacterial activity of lactobacilli.They were kept in a viable condition by sub-culturing once a month on slant agar. For experiments LB broth was used (10 g/l peptone, 5 g/l yeast extract, 10 g/l NaCl, 5 g/l sucrose, 0.5 g/l $MgSO_4$).When needed, agar (9 g/l) was added.

To investigate the growth of bacterial strains appropriate media were supplemented with EDTA in concentrations 0.5, 1, 3, 5 and 10 mM. Media were inoculated with overnight cultures of bacteria and incubated at 37 °C. To monitor the growth every hour probes were taken and optical density of bacterial cultural liquids was measured at 595 nm using a spectrophotometer (Thermo Scientific, GENESYS 10S UV-VIS, USA). For lactobacilli pH of a medium was also measured using a pH-meter (Knick 766, Germany).

To determine the antibacterial activity of lactobacilli agar-well diffusion assay was used [13] with some modifications. Briefly, test strains were grown overnight in LB broth. 100 µl of their cultural liquid was put into sterile Petri dishes, melted LB agar was poured on top and shaken. After solidification of a medium wells (6mm) were cut aseptically. Lactobacilli were pre-cultivated in MRS broth with supplementation of different concentrations of EDTA. Sterile MRS containing EDTA was used to study the antibacterial effect of EDTA. 100 µl of these probes was added in wells. Petri dishes were kept at room temperature for 1 h for diffusion of antibacterial substances and then incubated at 37°C for 24h. Then zones of growth inhibition were measured. A clear zone of inhibition of at least 2 mm was recorded as positive.

Results and Discussion. Investigations of EDTA effects on growth of two test microorganisms showed that it inhibited the growth of both strains (Fig. 1). EDTA was inhibitory for *M. luteus* growth in the concentrations >1 mM. *S. typhimurium* was able to grow at presence of EDTA in the concentrations of 0.5 to 3 mM. Obtained results are in accordance with literature data. Boziaris and Adams [14] showed that EDTA had inhibitory effect against gram-negative *Escherichia coli*. In other work the other action of EDTA against *E. coli* different strains was reported which could suggest that the EDTA effect is strain dependent [8]. EDTA showed inhibitory effect to *E. coli* O157:H7 in tryptic soy broth [15] and ground beef [16].

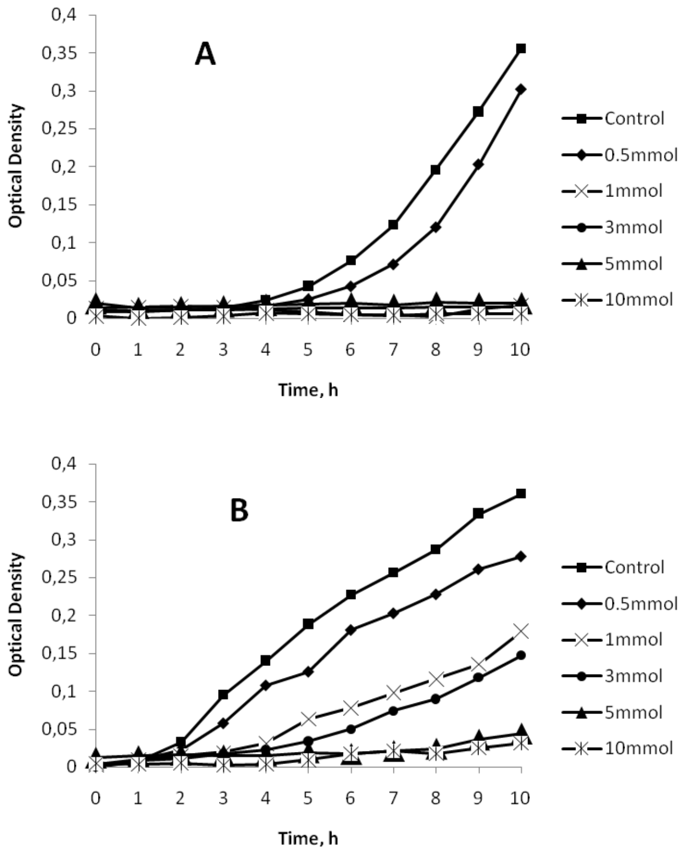

Fig. 1. The growth kinetics of *M. luteus* WT (A) and *S. typhimurium* WDCM 1474 (B) in the presence of EDTA in different concentrations.

It is well established that EDTA enhances the action of antimicrobials, antibiotics and bacteriocins against gram-negative bacteria by permeabilization of their outer membrane. The enhanced effects of EDTA and bacteriocins against Gram-negative bacteria have been demonstrated bothunder laboratory conditions and in some foods [14, 16]. *Salmonella* has been reported tobe more resistant to antimicrobial combinations of bacteriocinsand sensitizing agents than other gram-negative bacteria [14, 17]. Enterocin AS-48 which is a cyclic peptide produced by *Enterococcus faecalis* S-48 actssynergistically with EDTA against *Salmonella Choleraesuis*, as well as against gram-positive *Bacillus cereus* and *Staphylococcus aureus* [7].

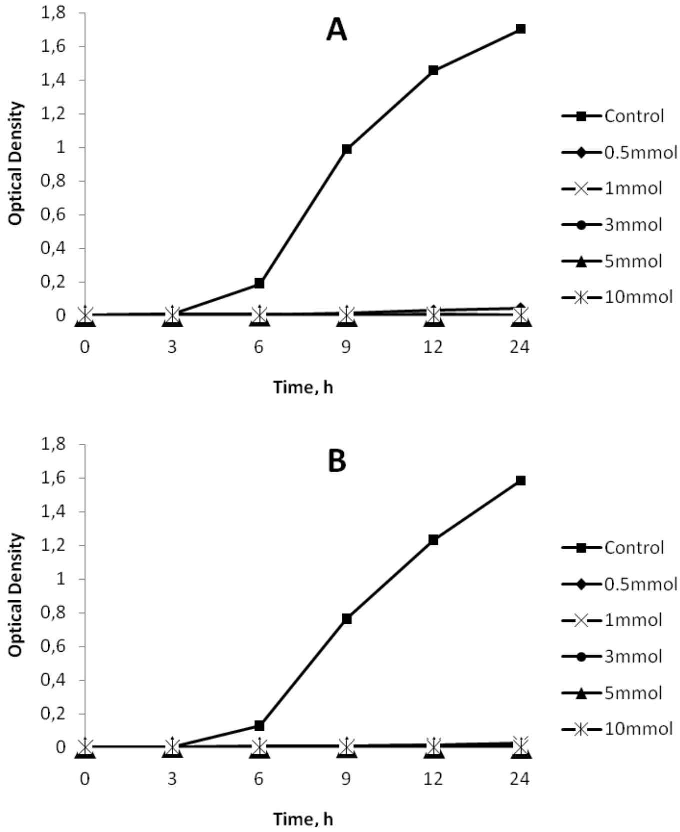

Fig. 2.The growth kinetics of *L. delbrueckii* subsp. *lactis* INRA-2010-4.2 (A) and *L. crispatus* INRA-2010-5.2 (B) in the presence of different concentrations of EDTA.

Taking into account available data about synergistic effect of EDTA with bacteriocins it was interesting to study the effect of EDTA on antimicrobial activity of lactobacilli strains. EDTA expressed inhibitory effect on growth of two lactobacilli strains in low concentrations tested (Fig. 2).Similar results but with *Lactobacillus casei*werereported by Tamura *et al.* [18]. This effect may be connected with chelation of manganese, iron and magnesium ions by EDTA. The growth ofthis strain was completely restored after addition of manganese or iron into assay mixture. It may be hypothesized that similar effect may take place for *L. delbrueckii* subsp. *lactis* and *L. crispatus* but this suggestion needs to be clarified in further experiments. It is interesting to state that in previous works it was suggested that antimicrobial substances synthesized by *L. delbrueckii* subsp. *Lactis* and *L. Crispatus* are connected with cell wall [19]. Also it was demonstrated that the activity of both strains can be induced by addition of Ca^{2+} and Co^{2+} as well as Mg^{2+} (only for *L. crispatus*) [20]. This means that further research is needed in order to elucidate the role of EDTA.

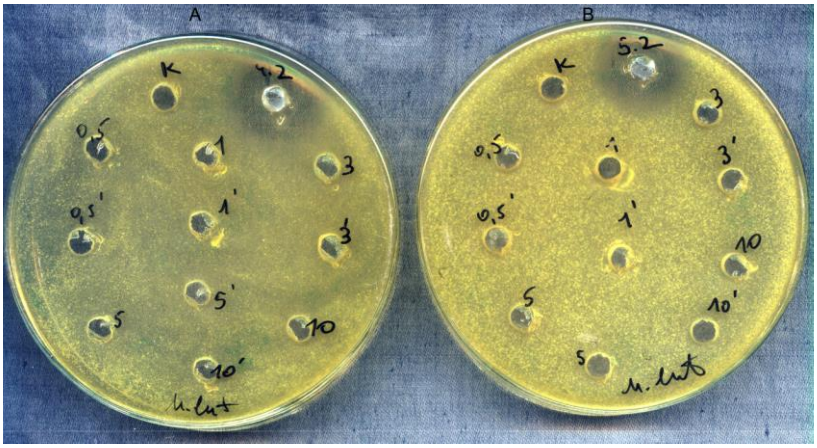

Fig. 3.The effects of EDTA on antibacterial activity of *L. delbrueckii*subsp. *Lactis* INRA-2010-4.2 (A) and *L. crispatus* INRA-2010-5.2 (B) against *M. luteus* WT. 0.5, 1, 3, 5, 10 – concentrations of EDTA; ´- only EDTA, without ´ - EDTA + lactobacilli.

As addition of EDTA into cultivation medium inhibited lactobacilli strains they consequently showed no antibacterial activity (Fig. 3). Interestingly, EDTA alone also had no inhibitory effect on both test strains when applied into Petri dishes during agar well diffusion assay as controls.

Concluding remark. EDTA is able to inhibit the growth of lactobacilli and test strains, and hence has a high antibacterial activity. This might be useful in development of new probiotics and food preservation technology.

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Inhibitation of Growth and Antimicrobial Activity of New Lactobacilli Strains Isolated from Armenian Dairy Product Matsoun with EDTA in Low Concentrations: Comparative Study with Gram-Positive and Gram-Negative Bacteria

The effect of EDTA on growth and antibacterial activity of two lactobacilli strains isolated from Armenian traditional dairy product matsoun, as well as on growth of test strains in medium supplemented with EDTA was studied. The obtained results demonstrate that EDTA totally inhibits the growth of lactobacilli strains and their antibacterial activity in low concentrations of 0.5 mM to 10 mM. It also inhibits the growth of *Micrococcus luteus* and *Salmonella typhimurium*.

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Подавление роста и антибактериальной активности новых штаммов лактобацилл, изолированных из армянского молочного продукта мацун ЭДТА в низких концентрациях: сравнительное исследование с грам-положительными и грам-отрицательными бактериями

Изучено воздействие ЭДТА на рост и антибактериальную активность двух штаммов лактобацилл, изолированных из армянского молочного продукта мацун, а также на рост тест организмов в среде с добавлением ЭДТА. Полученные результаты показали, что ЭДТА ингибирует рост лактобацилл и их антибактериальную активность в низких концентрациях 0.5-10 мМ. Оно также ингибирует рост *Micrococcus luteus* и *Salmonella typhimurium*.

Ա. Գ. Քերյան, Ե. Ա. Սիմոնյան, Ի. Լ. Բազուկյան, ՀՀ ԳԱԱ թղթակից անդամ Ա. Հ. Թռչունյան

Հայկական կաթնամթերք մածունից անջատված լակտոբացիլների նոր շտամների աճի և հակաբակտերիական ակտիվության ճնշումը ԷԴՏԱ-ի ցածր կոնցենտրացիաներով. համեմատական հետազոտություն գրամ-դրական և գրամ-բացասական բակտերիաների նկատմամբ

Ուսումնասիրվել է ԷԴՏԱ-ի ազդեցությունը հայկական կաթնամթերք մածունից անջատված լակտոբացիլների նոր շտամների աճի և հակաբակտերիական ակտիվության, ինչպես նաև թեստ-օրգանիզմների աճի վրա ԷԴՏԱ պարունակող միջավայրում: Ստացված արդյունքները ցույց են տվել, որ ԷԴՏԱ-ի ամբողջովին ճնշում է լակտոբացիլների աճը և նրանց հակաբակտերիական ակտիվությունը ցածր՝ 0.5-10 մՄ կոնցենտրացիաներով: Այն նաև ճնշում է *Micrococcus luteus* և *Salmonella typhimurium* աճը:

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