

COMPARATIVE INHIBITION OF *E. COLI* GROWTH BY PROBIOTIC LACTIC ACID BACTERIA AND THEIR METABIOTICS

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Abstract

Studies have shown, that the inhibition of growth of *E. coli* serotypes, isolated from different sources by probiotic lactic acid bacteria (LAB), their metabiotics (protein-like substances and exopolysaccharides) depends on *E. coli* isolation sources, the concentration and nature of metabiotics. It was shown, that probiotic LAB strains of the *Lactobacillus* genus possessed higher inhibitory activity then probiotic LAB strains of *Enterococcus* genus. The *Lactobacillus helveticus* KG5, *L. acidophilus* 1991 and *L. rhamnosus* 20-12 strains were selected due to their wide spectrum of growth inhibition of different serotypes of *E. coli*, isolated both from sick patients and from food products. The use of these LAB in the process of curd production as bioprotective culture has shown positive results.

Key words: LAB, antimicrobial activity, *E. coli*, protein-like substances and polysaccharides

Introduction. The scientific literature of recent years provides a lot of data that probiotics can balance intestinal microbiota, and thereby regulate proper intestinal function and be effective in the prevention or treatment of several gastrointestinal disorders such as diarrhea (infectious, antibiotic-related, infantile,

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traveller's diarrhea), irritable bowel syndrome or Crohn's disease [1]. Probiotic bacteria also provide health benefits via protection against endogenous/exogenous pathogenic bacteria such as *Salmonella typhimurium*, *Helicobacter pylori* and *Escherichia coli* [2]. Bacteriocin-producing lactic acid bacteria (LAB) strains may offer potential as an alternative to antibiotics, and can prevent pathogen growth, therefore being highly suitable as microbial food additives. During many food fermentation processes *Lactobacillus* strains can be added as a bioprotective culture to contribute in situ bacteriocin production and food preservation [3].

To assess the intestinal microbiome, indicators of a number of properties can be identified, including changes in the total amount of *Escherichia coli*. Clinical trial data show that a decrease in the total number of *E. coli* with normal enzymatic activity to 10^6 or their absence leads to diarrhea, constipation, and increased symptoms of gastrointestinal dyspepsia [4]. Most *E. coli* are harmless, commensal isolates that form part of the normal flora of humans and various animals. But several of them are identified as pathogenic bacteria, which cause infections of the gastrointestinal system (intestinal pathogenic *E. coli*), while others cause infections outside the gastrointestinal system (extraintestinal pathogenic *E. coli* or ExPEC) such as pneumonia, meningitis and bloodstream, urinary tract, abdominal and wound infections [5].

Resistance in *E. coli* serotypes to antibiotics is a serious problem, especially for diabetic patients [6].

Bacteria of *E. coli* serotypes are also known to be a source of food contamination. The food supply, including poultry and dairy products, may transmit antimicrobial antibiotic resistant *E. coli* to humans. Although more than 200 *E. coli* serotypes produce shiga toxins, only a limited but increasing number is considered pathogenic to humans. Serotype O157:H7 is still the predominant serotype among enterohaemorrhagic and most frequently associated with foodborne outbreaks [7].

The growing problem of the prevalence of pathogenic bacteria resistant to antibiotics, requires the search for alternative natural microbial preparations, including those created on the basis of probiotic lactic acid bacteria.

Therefore, it was of interest to study the ability of selected probiotic LAB strains to inhibit the growth of *E. coli* strains isolated from different sources.

Materials and methods. Objectives and growth media. The LAB strains (*Lactobacillus helveticus* KG5, *L. plantarium* 66, *L. acidophilus* 1991, *L. rhamnosus* 20-12, *Ent. faecium* KE-5, *Ent. sp.* KE-13 and *Ent. sp.* KE-18) which have probiotic properties [8], *B. subtilis* G 17-89, *S. typhimurium* G 38 test cultures and conditionally pathogenic multidrug resistant bacteria *E. coli* K12, *E. coli* 5001, *E. coli* 5002, used in the presented study, were taken from the culture collection of Microbial Depository Centre (MDC) of SPC "Armbiotechnology" NAS RA which included in WDCM (World Data Centre for Microorganisms) database (armbiotech.am, www.wfcc.info). The LAB strains were grown in skimmed milk (1.5% of fat), standard MRS agar and broth (Merck (Germany), ISO (Italy), Hi-

Media (India)) and in the nutrient media prepared on the basis of curd whey with addition of the following salts ((%) ± 0.2 , $(\text{NH}_4)_2\text{SO}_4$ -0.8; KH_2PO_4 -0.1; MgSO_4 -0.2; yeast extract-0.3; pepton-0.3; MnSO_4 -0.05; $\text{CH}_3\text{COONa}\cdot 3\text{H}_2\text{O}$ -0.2, pH 6.5–7.0)) [9].

Test cultures. Antibiotic resistant conditionally pathogenic bacteria were isolated from blood, feces, urine and wounds of patients by infectious disease doctors and microbiologists in the Stepanakert Center for Hygiene and Epidemiology and in the “Nork” Infectious Clinical Hospital (Yerevan, RA), during 2010–2022 [10]. Food contaminating *E. coli* serotypes were isolated by us from different industrial food products (preserved meat and vegetables, red caviar, dairy). For growing and determination of *E. coli* strains the selective solid medium Endo (Himedia, India; Merck, Germany) was used. All experiments were performed according to the sanitary-epidemiological protocols (National Committee for Clinical Laboratory Standards) [11, 12].

Obtaining and isolating biologically active substances. LAB strains were grown in 5 ml of MRS broth (37 °C, 24 h), then were transferred into 100 ml-Erlenmeyer’s flask containing 50 ml of MRS broth or milk whey-based medium and were incubated at 37 °C for 24 hours in thermostat. At the end of culture growth, cell count reached $1.0 \pm 5 \times 10^9$ CFU ml⁻¹ and pH was reduced to 3.5–4.2. The culture broth was centrifuged at 6.000 g for 20 min and cell free culture liquid (CFC) was obtained. CFC liquids were concentrated 5 ± 0.5 times (CFC $\times 5$) on a rotary evaporator at 50 °C and residual pressure 0.01 MPa (DM = 8–12%). CFC $\times 5$ were treated with cooled ethanol (96%) to the extent of 30–50% saturation with constant stirring using a magnetic stirrer. Samples, treated with ethanol, were kept for 24 h at +40 °C for precipitation and then centrifuged at 10 000 g at +4 °C for 10 min. Ethanol was removed by evaporation until the initial volume of CFC liquid. The obtained partially purified CFC $\times 5$ liquids were fractioned by gel filtration method on the column with Sephadex G-25(1.5 $\times$ 57 cm, resin vol: 100 mL). Each fraction ($n = 45$) eluted from the column, was tested for antibacterial activity on *B. subtilis* G 17-89 and *S. typhimurium* G 38 test cultures. Protein concentration of fractions was detected on 2800 Scanning UV/visible Spectrophotometer (Cole Parmer USA) and was calculated according to absorbance difference (260–230–320 nm). Fractions displaying bactericidal properties were pooled and vacuum evaporated at a temperature of 55 °C, residual pressure 0.01 MPa. The obtained partially purified antimicrobial preparation (AMP) contains protein-like substances and has antimicrobial activity 2000–5000 AU/ml, pH = 4.2–5.0 depending on strain. The content of dry matter (DM) of the obtained AMP reached 30% (IFT 10HP Reichert Refractometer) [13, 14].

Isolation of the exopolysaccharides (EPS). For isolation of EPS, cell-free culture broth was treated with 10% trichloroacetic acid and kept in the refrigerator at +4 °C for 24 h. Then the sediments were removed by centrifugation at 6000 g for 20 min. The cooled ethanol (96%) in a ratio of 1:3 was added to supernatants and

kept in refrigerator at +4 °C for 24 h. Then the sediments (exopolysaccharides) were removed from supernatants by centrifugation at 6000 g for 20 min. The obtained EPS was kept in thermostat at 50 °C until it reached a constant mass [15]. Dry powder of EPS was resolved in sterile water and 20% EPS liquids (5 mL) were passed through a column filled with Sephadex G50 resin (Sigma Aldrich) (1.5 × 57 cm, volume: 100 mL). The elution was carried out by sterile distilled water. Each fraction ($n = 45$) eluted from the column, was tested for antibacterial activity on *B. subtilis* G 17-89 and *S. typhimurium* G 38 test cultures. EPS concentration of fractions was detected on 2800 Scanning UV/visible Spectrophotometer (Cole Parmer USA) at 200–600 nm). Fractions displaying bactericidal properties were pooled and vacuum evaporated at a temperature of 55 °C, residual pressure 0.01 MPa. The obtained EPS preparation has antimicrobial activity 500–700 AU/ml, pH = 4.2–5.0 depending on strain.

Determination of antimicrobial activity. Antimicrobial activity of metabolites of LAB (AMP and EPS) strains against test cultures was assessed by tube serial dilution method [16] and spot-on-lawn methods, measuring the size of the inhibition zone (diameter) of test culture growth ($\varnothing$, mm) after 24 h incubation in thermostat at 30 °C. The antimicrobial activity was calculated according to PARENTE et al. [14] and expressed in arbitrary units (AU/ml).

Determination of resistance to antibiotics. To determine the resistance of test cultures to antibiotics, the method with antibiotic standard disks was applied [17]. The antibiotic discs of aminoglycosides (Amikacin 30 µg, Azithromycin 15 µg, Gentamicin 120 µg), beta-lactams (Augmentin 30 EG 10 B, Cefalotin 30 µg, Cefazolin 30 µg, Ceftazidime 30 µg, Cefuroxime 30 µg), tetracyclines (Doxycyclin 10 µg, Tetracyclin 30 µg), fluoroquinolone (Ofloxacin 5 µg, Ciprofloxacin 5 µg) were used (Oxoid, UK).

Statistical analysis. The experimental data were analyzed with one-way ANOVA. Each p -value can be considered statistically significant at $p < 0.05$.

Results and discussion. Previously we have conducted studies to evaluate the antimicrobial activity of LAB strains of the *Enterococcus* and *Lactobacillus* genus. The research results showed that strains of the genus *Enterococcus* with probiotic properties had higher antimicrobial activity against different *Salmonella* serotypes than strains of the genus *Lactobacillus* [18].

The comparative investigation of resistance of *E. coli* serotypes, isolated from sick patients and food contaminating *E. coli* strains to antibiotics were carried out. The results of the study of the resistance to *E. coli* are summarized in Fig. 1.

A comparison of the obtained data shows that *E. coli* bacteria, isolated from different sources of patients are especially resistant to various antibiotics. The highest resistance was observed to antibiotics of the tetracycline group for *E. coli* bacteria, isolated both from different sources of sick patients and from different food products.

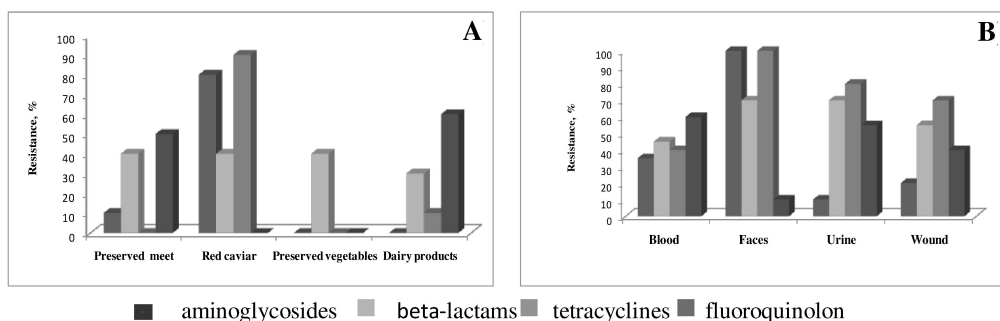


Fig. 1. Resistance of *E. coli* strains to antibiotics
A) Isolated from food products; B) Isolated from patients

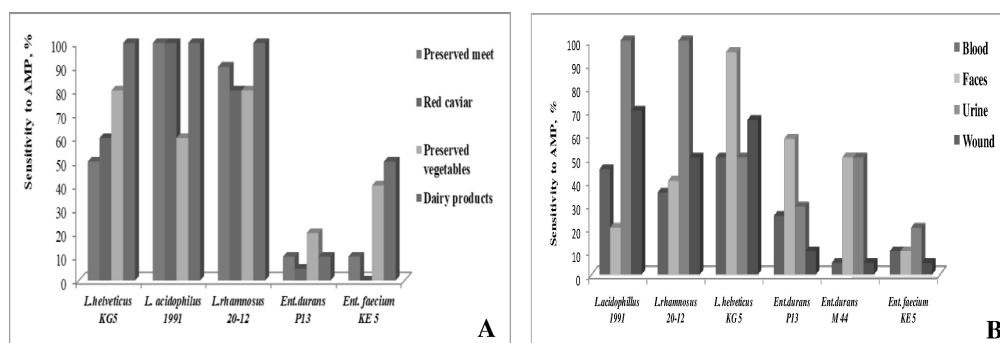


Fig. 2. Sensitivity of *E. coli* serotypes to AMP
A) Isolated from food products; B) Isolated from patients

Figure 2 presents the results of inhibition of the growth of *E. coli* serotypes, isolated from sick patients and food products by antimicrobial preparations (AMP), obtained after purification of culture liquid (CL) of various lactic acid bacteria.

The presented results show that the inhibition of *E. coli* growth by different AMP (100 AU/ml) depends on the isolation source of *E. coli* serotypes and on the species of the lactic acid bacteria strain. Suppression of the *E. coli* growth isolated from patient and food products by AMP of bacteria of the *Lactobacillus* and *Enterococcus* genus was practically different. The LAB strains of *Enterococcus* genus weakly inhibited the growth of *E. coli* serotypes, isolated both from food products and from patients in comparison with AMP, isolated from LAB strains of *Lactobacillus* genus. The LAB of strains *L. helveticus* KG5, *L. acidophilus* 1991 and *L. rhamnosus* 20-12 showing better inhibitory activity against different serotypes of *E. coli*, isolated both from patients and food products were selected.

For the evaluation of bioprotective potential of selected LAB strains, the lyophilized cultures of *L. rhamnosus* 20-12 and *L. helveticus* KG5 were used as

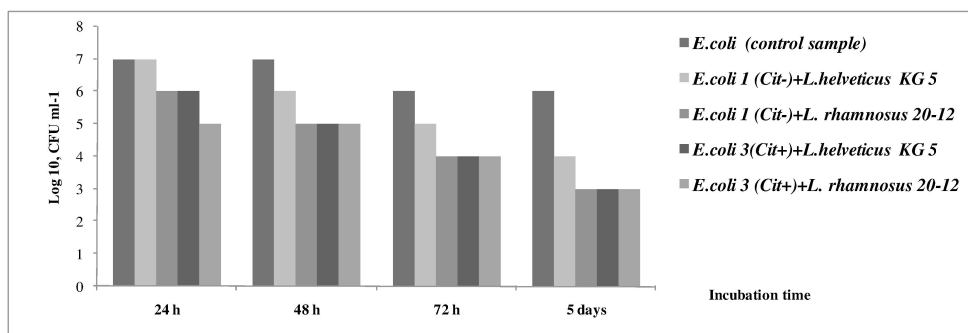


Fig. 3. The influence of lyophilized cultures of LAB on the growth of *E. coli*

adjunct cultures during the production of curd, pre-contaminated with target Citrate-positive and Citrate-negative *E. coli* strains. The results of the study are summarized in Fig. 3.

The results of the study show that *L. rhamnosus* 20-12 and *L. helveticus* KG 5 strains equally suppress the growth of Citrate-positive and Citrate-negative *E. coli* bacteria during curd production and storage. After three days of storage decrease of both *E. coli* strains growth was observed. The strains *L. helveticus* KG 5 and *L. rhamnosus* 20-12 cause a decrease of *E. coli* cell count for 3Log₁₀ degree for 72 h of storage. After five days of storage the use of both strains causes a decrease of cell count for 4Lg₁₀ degree.

A number of authors have shown the ability of exopolysaccharides to suppress the growth of many pathogenic strains [19, 20]. It was shown that out of the 8 exopolysaccharides possessing antimicrobial activity against a number of pathogenic bacteria, only 3 exopolysaccharides obtained from CL of *Ent. faecium* KE-5, *Ent. sp.* KE-13, *Ent. sp.* KE-18, strains (isolated from fermented donkey milk) inhibited the growth of pathogenic *E. coli* serotypes with equal effectiveness. The effect of polysaccharides, obtained by us from CL of *L. rhamnosus* 20-12 and *L. helveticus* KG 5 LAB strains on the growth of conditionally pathogenic *E. coli* K 12 serotype was studied. The same result was obtained when the exopolysaccharide of the *Ent. faecium* KE-5 strain was used. It was shown, that different concentrations (2, 4, 8%) of EPS inhibits the growth of *E. coli* K12 bacteria with same efficiency. Within 1–5 h of influence of *Ent. faecium* KE-5 EPS strain on the growth of *E. coli*, a decrease of cell count for 4Lg₁₀ degree was observed.

Thus, investigation results showed that the inhibition of the *E. coli* growth did not depend on species belonging of LAB strains from which the EPS was obtained.

Conclusion. Based on the obtained data it is obvious, that the strains *L. acidophilus* 1991, *L. helveticus* KG 5, *L. rhamnosus* 20-12, and *Ent. faecium* KE-5 have great potential for use as probiotic preparations due to the wide spectrum

of inhibition activity against different serotypes of *E. coli*, isolated from various sources. They can also be used as bioprotective cultures for biopreservation of some food products.

REFERENCES

- [1] FITTON N., J. S. THOMAS (2009) Gastrointestinal dysfunction, Surgery, **27**(11), 492–495.
- [2] BEGUM P. S., G. MADHAVI, S. RAJAGOPAL et al. (2017) Probiotics as Functional Foods: Potential Effects on Human Health and its Impact on Neurological Diseases, Int. J. Nutr. Pharmacol. Neurol. Dis., **7**(2), 23–33.
- [3] REDA F. M., B. M. HUSSEIN, G. ENAN (2018) Selection and Characterization of Two Probiotic Lactic Acid Bacteria Strains to be used as Starter and Protective Cultures for Food Fermentations, J. Pure Appl. Microbiol., **12**(3), 1499–1513.
- [4] IVANOVA E., L. V. RYCHKOVA, U. M. NEMCHENKO et al. (2017) The structure of the intestinal microbiota and the frequency of detection of pathogenicity genes (stx1, stx2, bfp) in *Escherichia coli* with normal enzymatic activity isolated from children during the first year of life, Mol. Genet. Microbiol. Virol., **32**, 42–48.
- [5] PITOUT J. D. D. (2012) Extraintestinal pathogenic *Escherichia coli*: a combination of virulence with antibiotic resistance, Front. Microbiol., **3**, 9, <https://doi.org/10.3389/fmicb.2012.00009>.
- [6] LAMBOU J. C. S., M. NOUBOM, B. E. D. GOMSEU et al. (2022) Multidrug-resistant *Escherichia coli* causing urinary tract infections among controlled and uncontrolled type 2 diabetic patients at Laquintinie Hospital in Douala, Cameroon, Can. J. Infect. Dis. Med. Microbiol., <https://doi.org/10.1155/2022/1250264>.
- [7] YOHANNES G. (2018) Isolation, identification and antimicrobial susceptibility testing of *Escherichia coli* isolated from selected dairy farms in and around Mekelle, Ethiopia, J. Dairy Vet. Anim. Res., **7**(6), 287–291.
- [8] KHACHATRYAN T. V., F. N. TKHRUNI, T. R. BALABEKYAN et al. (2023) A Study of Lactic Acid Bacteria and Prospects for their Application, Biol. J. of Armenia, **2–3**(75), 75–83.
- [9] TKHRUNI F., A. AGHAJANYAN, T. BALABEKYAN et al. (2020) Characteristic of bacteriocins of *Lactobacillus rhamnosus* BTK 20-12 potential probiotic strain, Probiotics Antimicrob. Proteins, **12**(2), 716–724.
- [10] ISRAYELIAN A., T. BALABEKYAN, Z. SARGSYAN, F. TKHRUNI (2024) Effect of Some Metabiotics from Strains of the Genus *Enterococcus* on the Growth of Pathogenic Bacteria that Cause Pneumonia, Biol. Bull., **51**(1), 91–97.
- [11] MUK 4.2.2495-09 (2010) Determination of the sensitivity of pathogens of dangerous bacterial infections (plague, anthrax, cholera, tularemia, brucellosis, glanders, melioidosis) to antibacterial drugs, MUK Guidelines, 4.2.2495-09 RF.
- [12] MU 2.3.2.2789-10 (2011) Sanitary epidemiological safety assessment and functional potential of probiotics microorganisms used for food production.
- [13] ISRAYELIAN A. (2018) Investigation of lactic acid bacteria and their metabiotics isolated from milk of different domestic animals of Artsakh, PhD thesis, Yerevan, Armenia, Armbiotechnology NAS RA, 116, (in Armenian).

- [14] PARENTE E., C. BRIENZA, M. MOLES, A. RICCIARDI (1995) A comparison of methods for the measurement of bacteriocin activity, *J. Microbiol. Methods*, **22**(1), 95–108.
- [15] SØRENSEN H. M., K. D. ROCHFORD, S. MAYE et al. (2022) Exopolysaccharides of Lactic Acid Bacteria: Production, Purification and Health Benefits towards Functional Food, *Nutrients*, **14**, 2938.
- [16] BEN-DAVID A., C. E. DAVIDSON (2014) Estimation method for serial dilution experiments, *Journal of Microbiological Methods*, **107**, 214–221.
- [17] EUCAST Disk Diffusion Method for Antimicrobial Susceptibility Testing, Version 9.0 January (2021), www.eucast.org.
- [18] ISRAYELYAN A., K. J. KARAPETYAN, F. TKHRUNI et al. (2015) Sensitivity of different pathogens to biological antimicrobial agents, *European Journal of Biomedical and Life Sciences*, **3**, 61–66.
- [19] ZHOU K., Y. ZENG, M. YANG et al. (2016) Production, purification and structural study of an exopolysaccharide from *Lactobacillus plantarum* BC-25, *Carbohydr. Polym.*, **144**, 205–214.
- [20] LIU Z., Z. ZHANG, L. QIU et al. (2017) Characterization and bioactivities of the exopolysaccharide from a probiotic strain of *Lactobacillus plantarum* WLPL04, *J. Dairy Sci.*, **100**(9), 6895–6905.

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