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Review Article

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Bacteriocin-Producing Probiotic Lactic Acid Bacteria against Hospital Strains of *Staphylococcus aureus* and *Escherichia coli*

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Introduction

It is now accepted that the human microbiome plays a key role in human health, and disturbances in the microbiome can cause or aggravate a range of diseases, from metabolic to immune disorders and mood disorders [1]. In addition, microbiota imbalance or dysbiosis may arise due to careless use of antibiotics in humans and animals, leading to the emergence of antibiotic-resistant bacteria that may compromise the effectiveness of antibiotics, so there is an urgent need to identify and develop new strategies to combat antibiotics resistance [2,3]. So far, probiotics are considered as perspective alternatives to antibiotics [4]. Probiotics are defined by the FAO/WHO as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" and they are successfully used for preservation of the microbial community [5].Moreover, bacteriocin-producing commensal microbes have the capacity to eliminate specific colonizing pathogens from various human body sites, and, as many bacteriocins have only narrow activity ranges they can be used for precision therapy and infections prevention [6]. Lactic acid bacteria, particularly lactobacilli, are the best known probiotics that modulate the immune system, and produce significant amounts of bioactive compounds, including bacteriocins, to eliminate pathogens and limit the clinical effects of antibiotic use [7, 8].

Earlier, at the Research Institute of Health and Physical Medicine of the Ministry of Health of the Republic of Armenia, clinical study showed the antimicrobial properties of the probiotic mixture of selected strains *L. rhamnosus* and *L. acidophilus,* which significantly reduce the manifestations of intestinal dysbiosis in various diseases, especially in pathologies of the gastrointestinal tract [9]. After a course of treatment with probiotics, microbiological analysis of the patients' stool showed that the number of lactobacilli, bifidum bacteria and lactose-positive *E. coli* was within normal limits, and accompanied by a significant decrease in the number of staphylococci, streptococci, clostridia and yeast-like fungi. The probiotic mixture also had an antagonistic activity against a wide range of pathogenic and opportunistic microorganisms, including *Shigella, Salmonella, Proteus, Candida albicans,* and corrected disturbed microbiocenosis, restoring the intestinal microbiota and stimulating local reparative processes that improved digestion, metabolic processes, and stimulated the immune response [10]. The results obtained suggest that these probiotics may be effective in treating dysbiosis and relieving various pathological conditions. In addition, malignant neoplasms of the colon in most cases are accompanied by the development of dysbiosis of the intestinal microbiota with a decrease in the number of asporogenous anaerobic bacteria, in particular microbes of lactic acid fermentation, lactobacilli up to their absence, and an increase in the number of hemolytic and lactose-negative *E. coli*.So far, probiotics are concidered as perspective alternatives to antibiotics, spore-forming anaerobes and conditionally pathogenic Enterobacteriaceae that may cause endogenous infections. These changes can also contribute to the side effects of radiation and chemotherapy (mutagenicity, carcinogenicity, toxicity). Surprisingly, the use of the mentioned probiotics during chemotherapy prevents the toxic effects of chemotherapy and the manifestation of enteropathy, as well as reduces dyspeptic disorders and have a lasting clinical effect regarding the healing of mucous membranes.

The present study characterize the optimal culture conditions (pH, temperature, culture time, culture media) for *Lactobacillus rhamnosus V 300, L. acidophillus ИНМИА 9602* and *L. casei ВКПМВ-7657* strains to increase the synthesis and efficiency of AMPs/bacteriocins. Thereafter, isolate them from non-living cell-free supernatants that exhibit antimicrobial activity against the most common multidrug-resistant pathogens such as hospital strains of *Escherichia coli* and *Staphylococcus aureus.*

Materials and Methods

Bacterial Strains and Growth Conditions

Homofermentative lactobacilli *Lactobacillus rhamnosus V 300, L. casei BKΠMB-7657* and *L. acidophilus ИНМИА 9602* were used as a producer of AMPs / bacteriocins. Each *Lactobacillus* strain was grown on MRS broth (de Man Rogosa and Sharpe) from HiMedia India, skimmed milk and whey (milk serum), incubated for 48 h at 37°C under aerobic conditions, centrifuged at 8000 rpm for 30 min. The resulting supernatants were filtered through a Millex GV 0.22 µm filter to remove bacteria.

After incubation in MRS broth, the amount of probiotics (CFU per ml of culture medium) was determined on MRS agar.

Antimicrobial Activity

The hospital strains of *Staphilococcus aureus* and *Escherichia coli* were obtained at the Department of Epidemiology of Yerevan Medical University named after M. Heratsiconfirmed to be multidrug resistant were used in this study as indicator microorganisms to determine the antagonistic activity of cell-free supernatants of the studied lactobacillus strains. The antimicrobial activity was measured using the standard agar well diffusion technique on Nutrient agar plates. Cell-free supernatants were adjusted to pH 6.0 with 6% NaOH to prevent the inhibitory effect of lactic acid and cultured on agar at 37°C for 24 h under aerobic conditions, after which zones of inhibition were measured in mm.

Separation of Peptides/Bacteriocins was performed using reversed-phase high-performance liquid chromatography (RP-HPLC), which is preferred for peptide separation (Molt). We used a gradient-mode high pressure pump (Shimadzu LC-20AD) fitted with aPerkin Elmer Quasar SPP 100 mm x 4.6 mm (i.d.) C18 RP-HPLC column with particle size 2.7 µm, phase A: 0.1% aqueuos trifluoroacetic acid (TFA) and phase B: acetonitrile (ACN, CH3CN). starting with 100% phase A and increasing phase B concentration with time from 0 up to 60% (0-30min) and kept at 60% for 10 min with flow rate 0.6 ml/min, Absorbance at 220 nm was evaluated with Shimadzu SPD-20A PDA detector.

Statistics Data were expressed as the mean (M) ± standard deviation (SD) based on three independent experiments conducted in triplicate. The differences among groups were assessed using analysis of variance (one-way ANOVA), followed by post hoc Holm-Sidak test. Data are considered significant at $P<0.05$.

Results

The cultivation condition of the studied strains of Lactobacillus were evaluated to establish the optimal ones for their successful growth and antimicrobial properties. For all of them, the highest growth rate was observed at a temperature of 37°C, similar to human body temperature and pH 6.0 ($p<0.05$). Each Lactobacillus strain capable of growing in various culture media, whey, skim milk and MRS broth at 37°C was tested at culture times of 24, 36 and 48 hours (Tables 1-3).

Lactobacillus Strains	Cultivation time (h) in whey		
	24	36	48
	lgCFU/ml	lgCFU/ml	lgCFU/ml
L. acidophillus ИНМИА 9602	8.7 ± 0.25	9.5 ± 0.123	9.8 ± 0.125
L. rhamnosus V 300	7.3 ± 0.213	8.3 ± 0.2	8.7 ± 0.26
L. casei ВКПМ B-7657	8.7 ± 0.24	9.4 ± 0.122	9.7 ± 0.115

Table 1: Effect of cultivation time on the growth of probiotic Lactobacilli in whey.

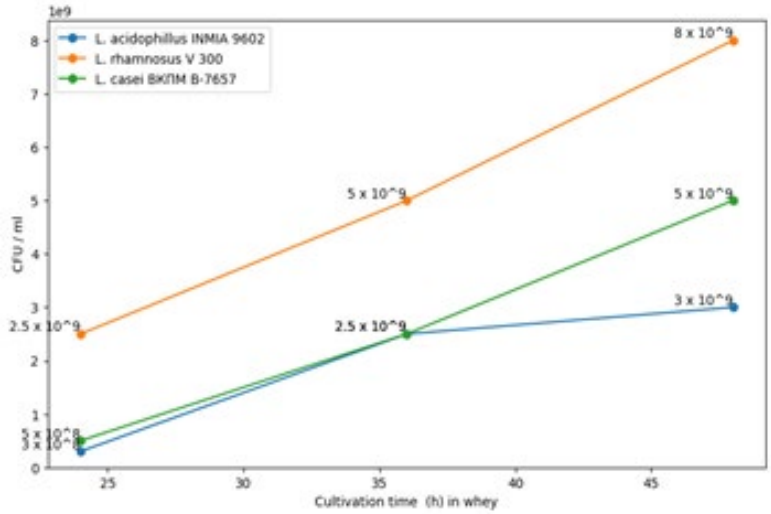
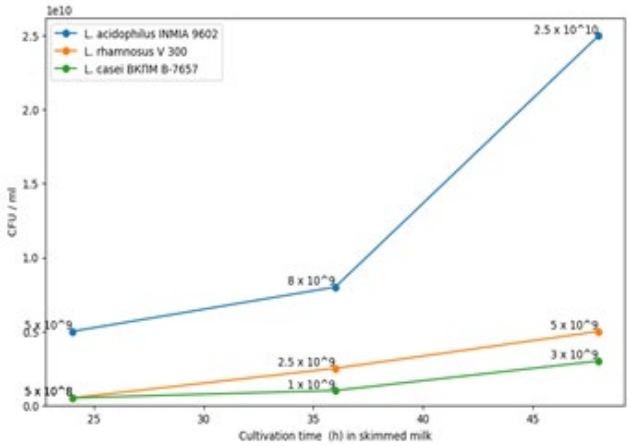


Table 1: Effect of cultivation time on the growth of probiotic Lactobacilli in whey.

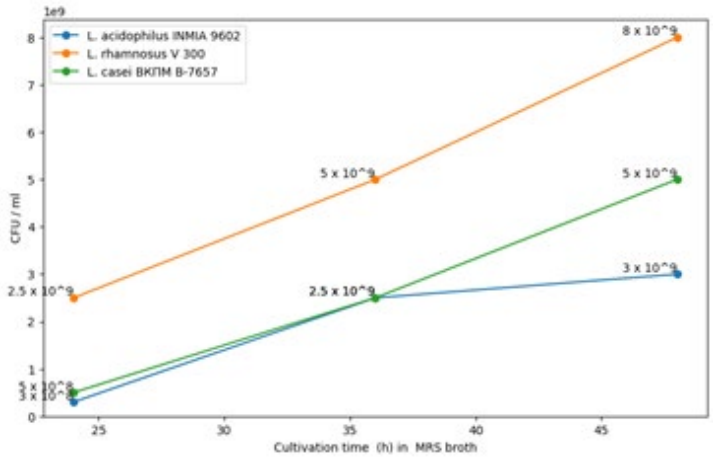
Lactobacillus Strains	Cultivation time (h) in skimmed milk		
	24	36	48
	lgCFU/ml	lgCFU/ml	lgCFU/ml
L. acidophillus ИНМИА 9602	9.7 ± 0.21	9.9 ± 0.215	10.4 ± 0.25
L. rhamnosus V 300	8.7 ± 0.2	9.4 ± 0.2	9.7 ± 0.22
L. casei ВКПМ B-7657	8.7 ± 0.23	9.0 ± 0.122	9.5 ± 0.15

Table 2: Effect of cultivation time on the growth of probiotic Lactobacilli in skimmed milk.



Lactobacillus Strains	Cultivation time (h) in MRS broth		
	24	36	48
	lgCFU/ml	lgCFU/ml	lgCFU/ml
L. acidophillus ИНМИА 9602	8.5 ± 0.23	9.4 ± 0.124	9.5 ± 0.23
L. rhamnosus V 300	9.4 ± 0.126	9.7 ± 0.16	9.9 ± 0.19
L. casei ВКПМ B-7657	9.7 ± 0.14	9.4 ± 0.2	9.7 ± 0.13

Table 3: Effect of cultivation time on the growth of probiotic Lactobacilli in MRS broth.



For all Lactobacillus strains, the pH of the culture medium at the end of cultivation was approximately 3.5. Optimal culture conditions were 37°C, pH 6.0 and 48 h in MRS broth for each strain studied, which were then used to measure the zone of growth inhibition of indicator strains in double-layer agar. Probiotic-containing fractions were detected during the early stationary phase of growth (24, 36 and 48 hours) and remain active up to 48 hours after the start of fermentation. The maximum amount of bacteriocins was detected after 48 h of fermentation.

The cell-free supernatants of L. acidophilus ИНМИА 9602, exhibited antimicrobial activity with zone of inhibition measurements of 25 mm forS. aureus and 23 mm for E. coli. Lactobacillus rhamnosus V 300 showed a diameter of inhibition zoneson nutrient agar for both S. aureus and E. coli of 23 mm, while L. casei ВКПМВ-7657 showed the inhibition zone of 23 mm for S. aureus and 18 mm for E. coli (Figure 1).

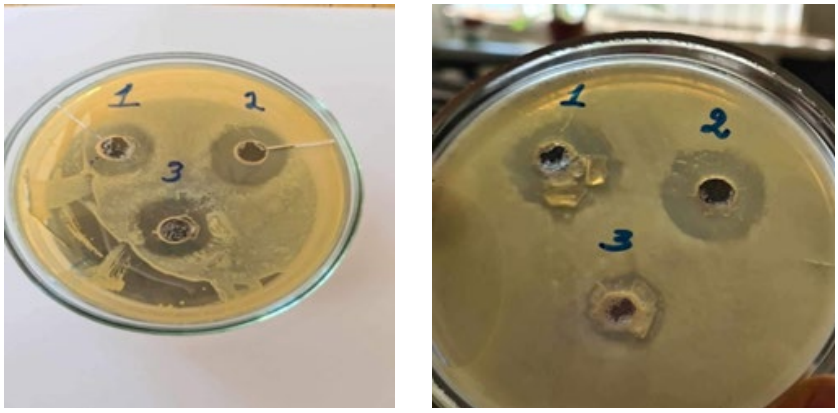


Figure 1: Zones of growth inhibition of a multidrug-resistant hospital strains: *Staphylococcus aureus* (I) and *Escherichia coli* (II) demonstrating the antimicrobial activity of cell-free supernatants of selected probiotic strains.

Note: (1) L. rhamnosus; (2) L. acidophilus; (3) L. casei

Separation of AMPs/Bacteriocins

Cell-free culture supernatants obtained from *Lactobacilli* strains were applied to RP-HPLC (see Materials and methods). The chromatographic conditions were the same for *L. rhamnosus* and *L. acidophilus* (Figures 2 and 3). Fractions were collected at 1.5 min intervals, starting at 1.5 min for the first fraction. The chromatograms show the results of a 22-minute analysis as no spikes were observed after that.

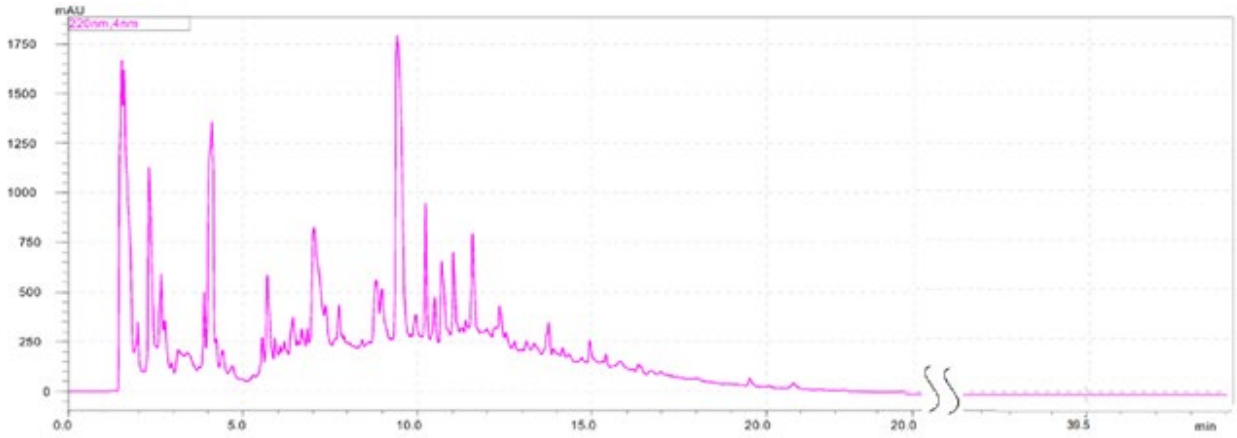


Figure 2: Isolation of peptides from the *L.rhamnosus V 300* by reversed-phase chromatography. Column: Perkin-Elmer Quasar SPPC18, 2.7 µm, 4.6 x 100 mmEluent: 0–60 % ACN in 0.1% TFA over 22 minutes at 1.5 ml/minute, Sample: cell-free culture supernatant, 12 fractions.

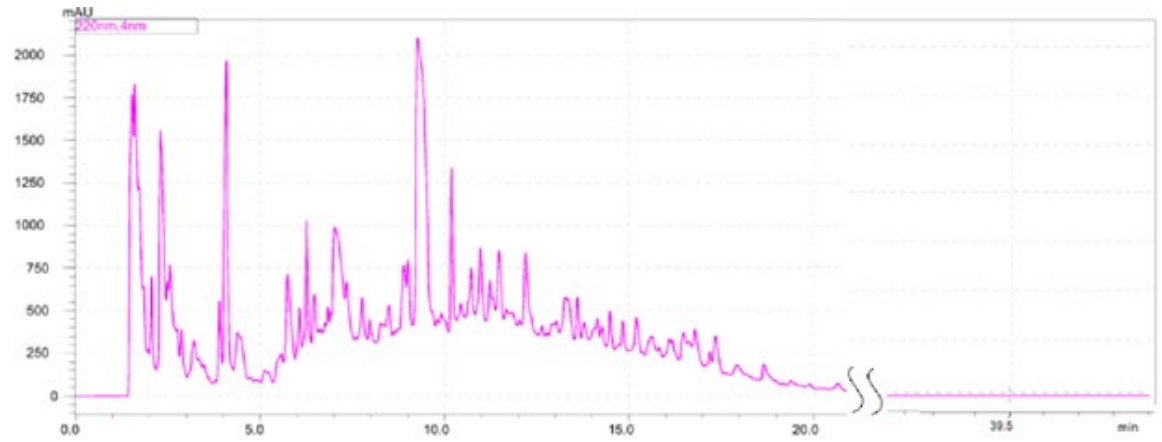


Figure 3: Isolation of peptides from the *L. acidophilus ИНМИА 9602* cell-freeculture supernatant by reversed-phase chromatography Column: Perkin-Elmer Quasar SPP C18, 2.7 µm, 4.6 x 100 mmEluent: 0–60 % ACN in 0.1% TFA over 22 minutes at 1.5 ml/min. 12 fractions.

Fractions of the resulting peptides were tested for antimicrobial activity against the target strains by measuring the zone of inhibition. The effects of the most active fractions (collected at 1.5-3 min and 9-10.5 min for *L. rhamnosus*/1.5-3 min and 10.5-12 min for *L. acidophilus*) are shown in Figure 4.

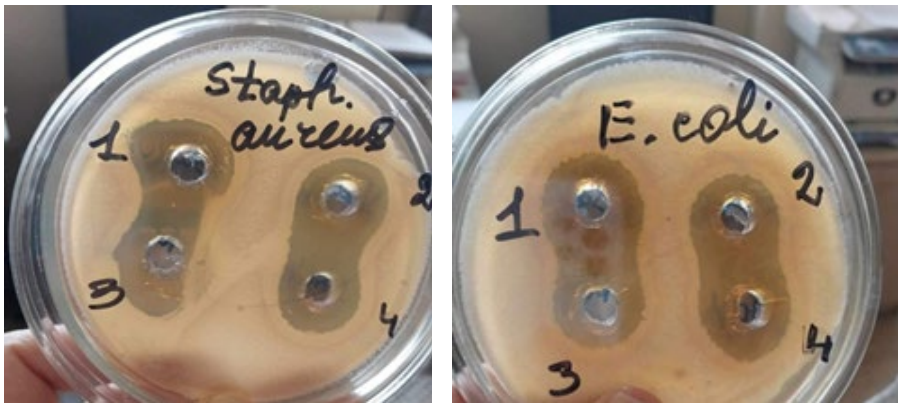


Figure 4: Zones of growth inhibition of Staphilococcus aureus (I) and Escherichia coli (II) by AMP /bacteriocins isolated from the supernatants of probiotic cell-free cultures of Lactobacilli.

Note: AMPs /bacteriocins from *L. rhamnosus* (1) and (3); *AMPs / bacteriocins* from *L. acidophilus* (2) and (4)

As shown in the fig. 4, the separated fractions of AMPs /bacteriocins from *L. acidophilus ИНМИА* 9602 exhibited antimicrobial activity with zone of inhibition of 23 mm for *S. aureus* and 23 mm for *E. coli.* Those from *Lactobacillus rhamnosus* V 300 showed a diameter of inhibition zoneson nutrient agar for both *S. aureus* and *E. coli* of 23 mm.

Thus, the productions of antimicrobial compounds, such as our identified AMPs/bacteriocins, which have been shown to inhibit the multidrug-resistant pathogens studied, are responsible for the inhibitory properties of Lactobacilli strains and are therefore selected for further study for their practical application.

Discussion

Today, instead of confronting pathogens with vaccines and antibiotics, the new field of medical microbiology is emerging, which seeks to translate research into the human microbiome into new probiotic strategies to promote human health and prevent disease [11]. Despite the widespread use of probiotics, safety issues continue to be debated, especially in vulnerable subjects. Moreover, given the existence of opportunistic microbes in the microbiome itself. Approximately 30% of the population is colonized with *Staphylococcus aureus*, which is both a commensal bacterium and a human pathogen, and can be the leading cause of bacteremia and infective endocarditis (IE), as well as osteoarticular, skin and soft tissue, pleuropulmonary and device-related infections reviewed elsewhere [12] (1). *Candida albicans* is a commensal fungus that colonizes the genital and gastrointestinal mucosa without causing disease, but in immunocompromised individuals can *C. albicans* become pathogenic, causing diseases ranging from

superficial infections of the mucous membranes (oral and vaginal) to invasive candidiasis accompanied by bloodstream infection (candidemia) [13]. Misuse of *L. rhamnosus* has been associated with development of septicemia, septic shock, or endocarditis in people with symptoms of inflammatory bowel disease [14]. To overcome these problems, there is growing interest in the use of non-living microorganisms, such as heat-killed probiotics, microbial extracts and cell-free supernatants, as they can provide corresponding beneficial effects [15]. In this regard, the high antibacterial activity of bacteriocins produced under optimal conditions by studied Lactobacillus strains can be used in food preservation and as antimicrobial agents in the treatment of human infections, like other lactic acid bacteria, including lactobacilli, as rich source of bacteriocins [16]. Moreover, studied Lactobacilli strains are also capable of successfully suppressing the growth of Clostridia, pathogens of food intoxication, as well as Salmonella, gram-negative bacteria that contaminate food, and both are infectious agents [17,18]. It should be noted that AMPs are able to recruit and activate various subpopulations of leukocytes, exhibiting immunomodulatory and anti-inflammatory properties, and therefore can also be used to treat various inflammatory diseases [19]. Of note, *L. acidophilus* NCFM showed significant effects on the *H. pylori* eradication and reduction of inflammation *in vitro* (human gastric adenocarcinoma cells) and *in vivo* (mouse model C57BL/6 inbred mice), as well as on the infiltration of inflammatory cells in lamina propria of the gastric mucosa [20-31].

Conclusion

Thus, we have developed optimal cultivation conditions for selected strains of *Lactobacillus rhamnosus* V 300, *L. acidophillus* INMIA 9602 and *L. casei BKIIMB-7657.* These conditions significantly increase the antimicrobial activity in their cell-free supernatants, in particular the effectiveness of their production the AMPs/bacteriocins against the most common multidrug-resistant pathogens, namely, hospital strains of *Escherichia coli* and *Staphylococcus aureus.* This could form the basis for further study of probiotics and the bacteriocins they produce instead of antibiotics for use in the clinic, as well as preservatives in the food industry for the development of functional foods and beverages. However, further research is needed to confirm their effectiveness and safety in improving human health and treatment.

Author Contributions

Conceptualization, A.S., R. M and S. Ch.; methodology, R.M., V. A. and S. Ch; software, V.A., A.S.; validation, R.M., A.S., H.H. and V.A.; investigation, A.S., R. M., H.H. and S. Ch; data curation, S.Ch. and N.A.; writing-original draft preparation, A.S. and N.A.; writing-review and editing, A.S. and N.A.; visualization, R.M., A.S. and V.A; supervision, S.Ch.; project administration, S.Ch. and A.S. All authors have read and agreed to the published version of the manuscript.

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