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Research Paper

The *Lactobacillus acidophilus* Supernatant: An Effective and Safe Alternative to Antibiotics

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ABSTRACT

Background: Given the global problem of antibiotic resistance among pathogens, researchers are looking for appropriate treatment alternatives to eliminate infections. Application of probiotics and their products can be a practical solution. This study aimed to investigate the inhibitory effect of cell-free supernatant (CFS) of probiotics against *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*).

Methods: The effect of CFS of eight strains of probiotics against E. coli and *S. aureus* was evaluated by well diffusion method. The agent with the highest inhibition diameter was selected to investigate other antibacterial properties. They included minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and timed kill effect. Surface electron micrographs were taken to compare the treated versus untreated bacteria with CFS of *Lactobacillus acidophilus* (SLA) LAFTI-L10 DSL. Finally, the percent viability of the Hu02 cells was investigated after 24, 48 or 72 hours of incubation with SLA at various concentrations.

Results: Among the tested strains, SLA showed the highest inhibitory diameter against E. coli and *S. aureus* ($P \leq 0.005$). Also, the MIC of SLA was equal to those of *E. coli* and *S. aureus* (12.5 µL/mL) but was different in their MBC. Almost 100% of bacteria removed after exposure to SLA (20 min.). The results of log CFU/mL demonstrated that SLA had bactericidal effect against *S. aureus* and E. coli. The toxicity assays showed that the percent viability of the Hu02 cells was 31.71 to 81.09 after 24, 48 or 72 hours exposure.

Conclusion: Our results suggest that SLA can be a suitable, effective and safe alternative to antibiotics.

Keywords: Cell-free supernatant; *Escherichia coli*; *Lactobacillus acidophilus*; Probiotics; *Staphylococcus aureus*

Introduction

The use of antibiotics is a standard treatment for numerous bacterial infections; however, the overuse can lead to antibiotic resistance [1-4]. The global emergence of multi-drug resistance (MDR) among bacteria is a major challenge facing infectious disease management. Therefore, preventing or minimizing this phenomenon is one of the main approaches to control infections or the fatal outcomes [5, 6]. In fact, the use of alternative agents to replace conventional and synthetic antibiotics is a logical approach [6, 7]. Currently, bacterial therapy, i.e., application of beneficial and safe bacteria or their products against pathogens, is one of the alternative approaches to infection management [8, 9].

Probiotics are live and useful microorganisms that offer antibacterial properties. These agents can serve roles in the body to fight against infections, while reducing the need for standard antibiotics [9-11]. The cell-free supernatant (CFS) of probiotics are known to be effective antimicrobial agents [12, 13]. Currently, *Lactobacillus spp*. and their supernatants are being investigated in many probiotic research projects. Indeed, probiotics and their bactericidal capacity can be effectively applied in the clinical management of infections as an important treatment strategy [14].

Bacteriotherapy with *lactobacilli spp.* has emerged as a practical alternative to the treatment or prevention of various nosocomial infections [9, 13]. Some strains of *Lactobacillus* and their products have significant inhibitory activity against bacteria. *Lactobacillus spp*. secretes antibacterial substances, such as hydrogen peroxide, lactic acid, bacteriocins and short-chain fatty acids (SCFA) [15, 16]. These products regulate the normal flora population and minimize or inhibit bacterial infection in the human body. The positive role of SCFA, bactriocins and hydrogen peroxide in the cell free supernatant of probiotics has been established in infection control studies. *Lactobacilli* strains have inhibitory effects on the growth of resistant pathogens, such as *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Acinetobacter baumannii* (*A. baumannii*). Thus, they should be considered as effective alternatives in the management of various infectious diseases [16, 17]. A critical point facing such a strategy is that we should search for factors that have antibacterial effects while being safe to human cells.

This study aimed to investigate the antimicrobial and cytotoxic properties of the CFS of *Lactobacillus acidophilus* (SLA) against gram-positive and gram-negative bacteria. The current study was conducted to evaluate one strain of probiotics with antibacterial and cytotoxic properties that has not been studied to date.

Methods

Preparation of Probiotics

We used a total of eight probiotic strains in this study. The five commercial strains included *L. Plantarum 299 V* (DSM 9843), *L. ruteri* (DSM 17938), *L. acidophilus* (LAFTI-L10 DSL), *B. bifidum B94* (DSM 20456) and *Bacillus coagulans* (DSM 1). They were purchased from the Iranian Biological Resource Center, and the Industrial Enzymes Company, which represents the Dutch company DSM in Iran. We also used the following three local strains: *L. ruteri* EF4, *L. salivarius* EF6 and EF7. These strains were purchased from the Iranian Institute of Agricultural Biotechnology and the Probiotic Research Center of Alborz University of Medical Sciences. The probiotics were cultured on de Man Rogosa and Sharpe (MRS) agar media (Merck, Germany), under microaerophilic conditions at 37°C for 48 hours [5].

Preparation of Probiotics from Cell-free Supernatant

The probiotic strains were transferred separately from MRS agar medium to tubes containing MRS broth. These tubes were incubated under micro-aerophilic conditions at 37°C for 24 hours. The tubes were then centrifuged in a refrigerated unit for 10 minutes at 4000 rpm. Next, the supernatant was separated and passed through a syringe filter with a 0.45 pm pore size [5].

Preparation of Bacteria

Strains of *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) were obtained from the Iranian Biological Resource Center (Tehran, Iran). These strains were cultured in Muller-Hinton agar medium (Merck, Germany).

Antibiotic Susceptibility

We performed qualitative evaluations of susceptibility of bacterial strains to all 8 probiotic strains in order to select the appropriate and effective ones. For antibiogram, we used the CFS from probiotics at a concentration of 100 µL/mL. For quality assays, well diffusion method was used on Muller-Hinton agar medium. The bacterial strains with 0.5 McFarland dilutions were cultured separately on multi-well plates containing Muller-Hinton medium. The wells were individually filled with CFS from the probiotics. Next, the plates were incubated at 4°C for two hours. They were then incubated at 37°C for 16 to 18 hours and the inhibition zone was measured (Figure 1) [18].

Selection of Probiotics

To continue the laboratory steps, after performing well diffusion test, the appropriate and effective probiotic was selected based on the largest difference in the inhibitory diameters of CFS against *E. coli* and *S. aureus* strains, and based on Friedman test. Accordingly, the SLA was selected to continue with the experiments.

Minimum Inhibitory Concentration

To determine the MIC of the SLA, we used Müller-Hinton culture medium based on the instructions of the Institute of Laboratory and Clinical Standards (CLSI). The dilution series consisted of 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39 µL/mL of the CFS. For each test, 100 µL/mL of Muller-Hinton Broth medium was added to each of the 12 wells in a 96-well microtiter culture plate. Next, 100 µL/mL of CFS was added to the first well, and the dilution series was performed. Then, 100 µL/mL of the bacterial suspension (10^6 CFU/mL) was prepared from the 24-hour culture, and added to all wells except for the negative control. The last two wells received positive control or microbial growth control (medium + bacterial suspension) and negative control or sterility control (medium alone). Finally, the microplates were incubated at 37°C for 24 hours in duplicates. The minimum concentration of the substance that did not have visible growth was considered as the MIC. In fact, the concentration that completely inhibited the bacterial growth (first clear well) was reported as being the standard MIC.

Minimum Bactericidal Concentration

The MBC is reported as the minimum concentration of the substance that kills 99.9% of the inoculated bacteria on the plate after 18-24 hours of incubation at 37°C. To perform the test, 100 µL/mL of the well contents of the MIC and wells at higher concentrations were transferred to a plate containing Muller-Hinton agar medium [18]. The concentration

of samples with less than 10 colonies on the plate was considered as the MBC. Finally, we also verified whether each antibacterial agent was bactericidal or bacteriostatic. This was based on the MBC/MIC ratio of a given antibacterial agent. Our criteria for being bactericidal was if the MBC/MIC ratio was ≤4 while an agent was considered bacteriostatic if the ratio was greater than 4 [19].

Microscopic Examinations

For the surface electron microscopic (SEM) analysis, we treated the microorganism suspensions with SLA for 10 minutes. To further examine alterations in the bacterial cell membranes, we scanned the treated and untreated bacteria using SEM of the SLA (Figure 4).

Timed Kill Evaluation

We only considered the MBC concentrations of SLA to study the time it took to kill the bacteria. For this purpose, 1000 µL/mL of CFS was poured into a Falcon tube. Then, 1000 µL/mL from the 24-hour culture of bacterial suspension (10^6 CFU/mL) was added to the tube series. The tubes were incubated at 37°C for 5, 10, 15 or 20 minutes. Next, 500 µL/mL was removed from each tube at the predetermined time and used for counting the bacterial colonies. For this purpose, we prepared dilution series up to 10^{-6} in tubes containing normal saline. Then, we removed a 100 µL/mL aliquot from each tube and cultured the bacterial samples on Muller Hinton agar. These plates were incubated at 37°C for 18-24 hours and the colony counting was performed based on CFU/mL, based on the following formula [20, 21]:

$$Number\ of\ Bacteria \times Dilution / Volume = CFU$$

We used MRS broth for the negative control. The log CFA/mL of *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922) were considered at 0, 5, 10, 15 or 20 minutes after exposure to SLA. A control test was performed with the microorganisms but without the agent (Figure 5). The bactericidal or bacteriostatic property of the agent was determined by the assessment of the initial (log CFU/mL) and the final values (log CFU/mL) [22].

Determination of the Reduction Percentage

The cell counts from the plates in previous step were added to the following formula: $Reduction\ Percentage = CFU_0 - CFU_1 / CFU_0 \times 100$. The reduction percentages of *E. coli* and *S. aureus* were derived after exposure to the MBC concentration of SLA.

Cell Culture

The specific cell line samples were purchased from the Iranian Biological Resource Center. Samples of human normal fibroblasts (Hu02, IBRC C10309) were cultured in a T25 flask, containing DMEM (Dulbecco's modified eagle medium) with high glucose (Gibco, USA), 10% fetal bovine serum (FBS), powder penicillin and streptomycin (100 µg/mL; Sigma, USA) in an incubator (37°C, 5% CO_2).

MTT Assay

The cytotoxic effect of the SLA was evaluated on Hu02 cells, using MTT assay after passaging and trypsinizing the cells. For this purpose, the cells were seeded on 96-well micro-titer plates with 10000 cells per well and incubated overnight. Freshly prepared SLA at the concentrations of 1/2 x MIC, MIC or 2 x MIC were added to the wells and incubated for 24, 48 or 72 hours (37°C, 5% CO_2). Wells without SLA were considered as the negative control. Next, 20µL of 5 mg/mL MTT solution (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) was added to each well and the microplate was incubated in the dark at 37°C for 4-hrs. Thereafter, 150µL of DMSO (dimehtylsulfoxide) was added to each well. The microtitre plate was shaken for 5-10 minutes. After the formazan crystals had been dissolved, the supernatant's absorbance was read on a microplate reader at 570 nm (Biotech, elx800, USA) [23]. The percent viability in each group was determined based on the following formula:

Percent Viability: (sample absorbance / average absorbance negative control) × 100 [24].

Statistical Analyses

To analyze the data, descriptive statistical processing, including central tendency indices (means and standard deviations) were used in the form of graphs on Excel and SPSS software, version 26. The mean rank difference in the inhibitions of the SLA was surveyed by Friedman test. The SLA toxicity was examined at varying concentrations and time points by one-way ANOVA and Tukey *post-hoc* tests.

Results

Qualitative Assessment - Agar Well Diffusion

The antimicrobial activity of the strains were evaluated in triplicates against the *E. coli* and *S. aureus* batches. The inhibitory diameter of the probiotics CFS against the *E. coli* and *S. aureus* strains was measured (Figure 2) and the mean was calculated (Figure 1). The results indicated that SLA had the highest inhibition diameter against both *E. coli* (P=0.005) *and S. aureus* (P=0.019) strains based on the Friedman test.

Quantitative Assessment

In this step, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined. Table 1 shows the MIC and MBC of SLA against *E. coli* and *S. aureus*. Based on the data shown in Table 1, the agent was considered bactericidal if the following relation applied: MBC/MIC ratio ≤4.

Table 1. Minimum Inhibitory Concentration (MIC), the Minimum Bactericidal Concentration (MBC) and MBC/MIC ratio of SLA against E. coli (ATCC 25922) and *S. aureus* (ATCC 25923).

	SLA (µL/mL)		
	MIC, mean + SD	**MBC, mean + SD**	**MBC/MIC Ratio**
***Escherichia coli* (ATCC 25922)**	12.5 ±0.00	12.5 ±0.00	1
***Staphylococcus aureus* (ATCC 25923)**	12.5 ±0.00	25 ±0.00	2

Table 2. The reduction percentage of *S. aureus* ATCC 25923 and E. coli ATCC 25922 after exposure to SLA at different culture times.

	Percent Reduction Vs Culture Time (min)			
	5	**10**	**15**	**20**
***S. aureus* (ATCC 25923)**	15	53	77	99.98
***E. coli* (ATCC 25922)**	25	96	97	99.99

Table 3. percentage viability of the Hu02 cells following 24, 48 and 72 hours of incubation at different concentrations (1/2 MIC, MIC, 2 MIC) of SLA, analyzed by one-way ANOVA and Tukey post-hoc test.

Viability Vs Time	**6.25**	**12.5**	**25**	**P-value**
Viability after 24hr	80.02 ± 5.03	50.45 ± 5.25	62.42 ± 7.15	0.002
Viability after 48hr	81.09 ± 5.05	60.94 ± 4.37	64.20 ± 5.01	0.005
Viability after 72hr	48.56 ± 1.87	31.71 ± 5.94	61.12 ± 15.31	0.026

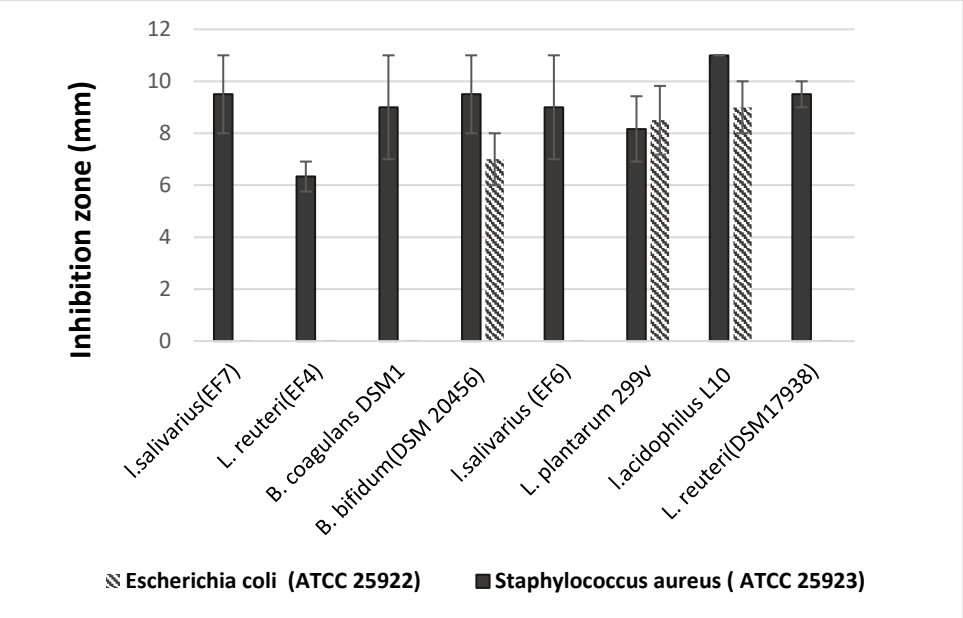

Figure 1. Mean inhibitory diameter of CFS of probiotic strains at a concentration of 100 µL/mL, against *E. coli* and *S. aureus* (means ±SD's).

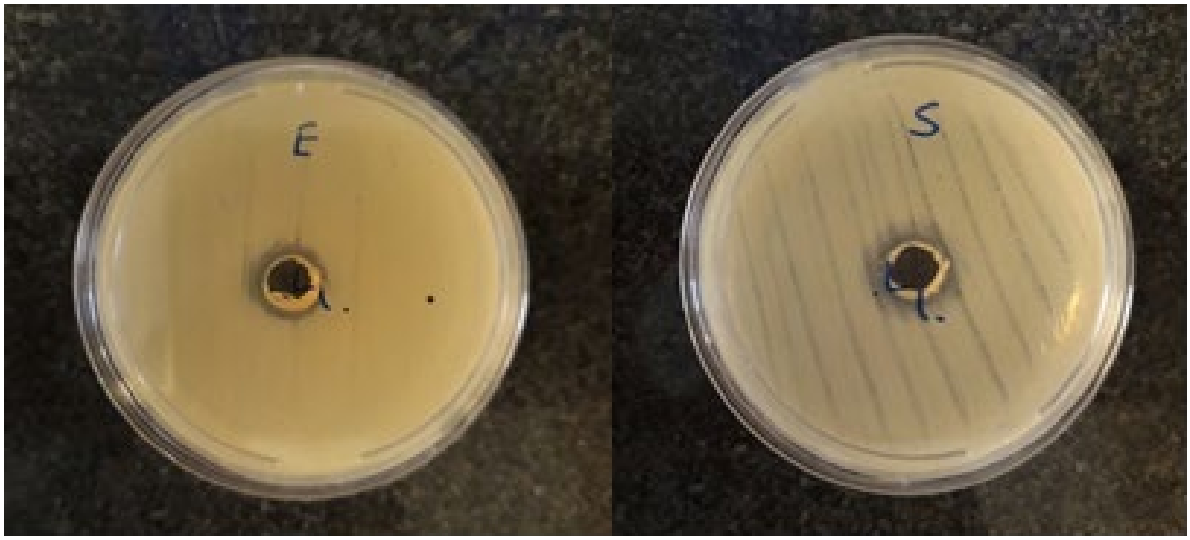

Figure 2. The inhibition zone of SLA (100 µL/mL) against A: E. coli (9 mm) and, B: *S. aureus* (11 mm) using well-diffused method.

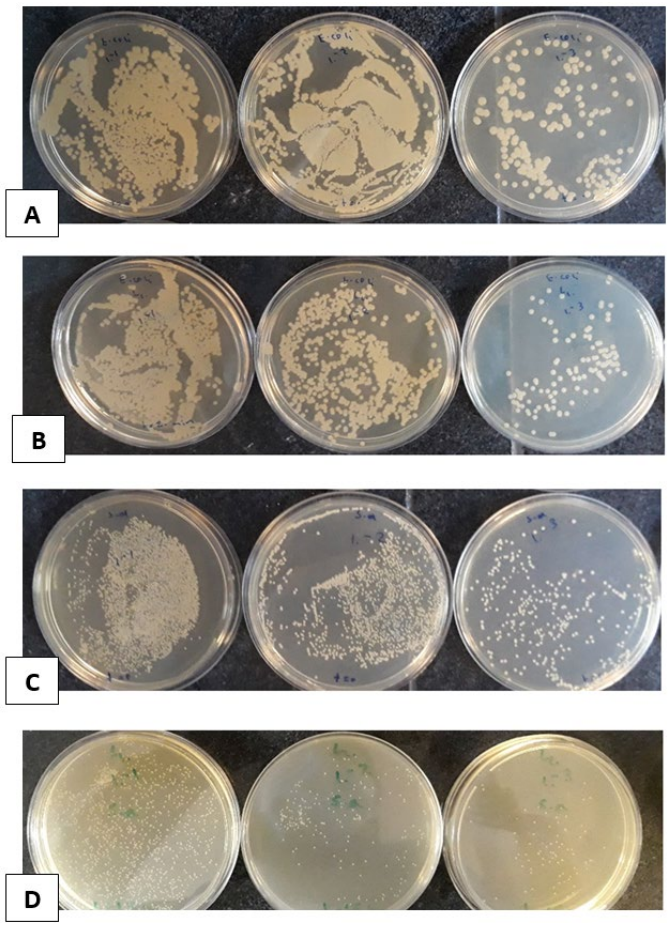

Figure 3. Microbial plates (10-1-10-3), evaluating the killing time effects of *S. aureus* (ATCC 25923) and E. coli (ATCC 25922) treated with SLA.
A: *E. coli* without exposure to SLA at t = 0 min. B: *E. coli* after exposure to SLA at t = 10 min. C: *S. aureus* without exposure to SLA at t = 0 min. D: *S. aureus* after exposure to SLA at t = 10 min.

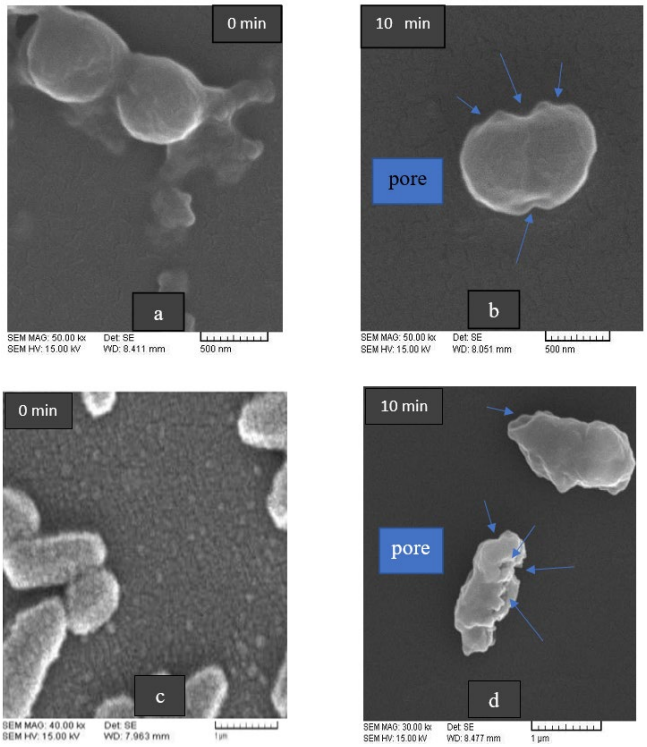

Figure 4. The surface electron micrographs of bacteria, *S. aureus* and *E. coli*, treated versus untreated with SLA. (a): *S. aureus* without wall damage; (b): *S. aureus* with wall damages and numerous pores present in the treated cells; (c): *E. coli* without wall damage; (d) *E. coli* with numerous pores present in the treated cells.

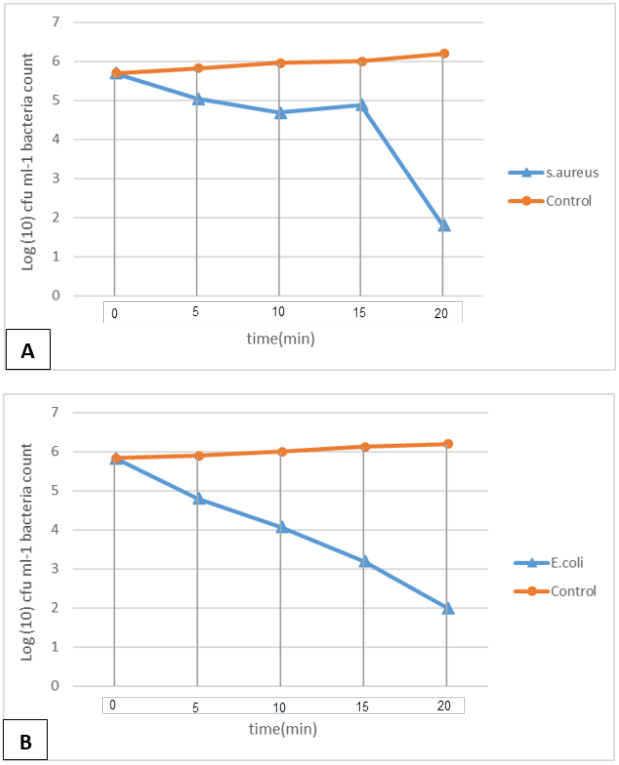

Figure 5. The log counts of (A): *S. aureus* (ATCC 25923) and, (B): *E. coli* (ATCC 25922) at: 0, 5, 10, 15 and 20 minutes.

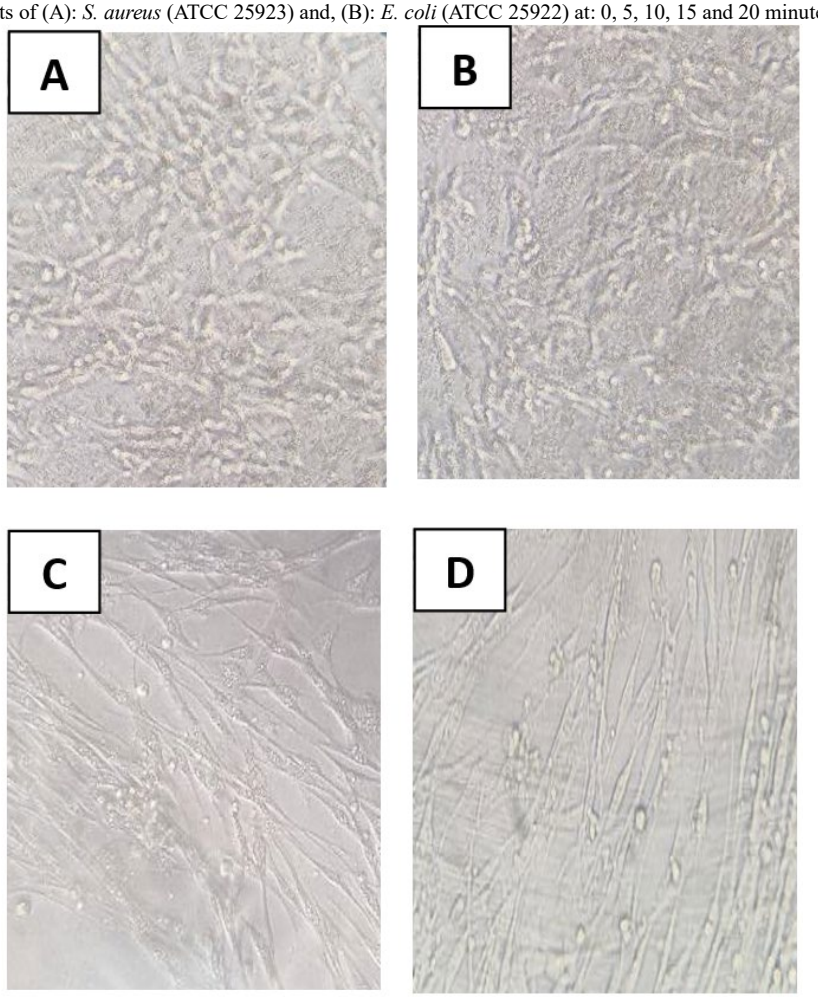

Figure 6. Normal skin fibroblasts (Hu02) before and after treatment with SLA (24 or 72 hrs).
A: Cells before treatment (control; 24 hrs). B: Cells before treatment (control; 72 hrs).
C: Cells treated with SLA at 12.5 µL/mL (24 hrs). D: Cells treated with SLA at 12.5 µL/mL (72 hrs).

Timed Killing

To evaluate the killing time of *S. aureus* and *E. coli*, we considered the MIC concentrations of SLA. Plates were counted based on CFU/mL (Figure 3a-3d), and the SEM micrographs as shown in Figure 4a-4d. The log counts of *S. aureus* and *E. coli* was considered at (t=0, 5, 10, 15 or 20 min) after exposure to the SLA (Figure 5).

According to Figure 3, microbial plates (10^{-1}-10^{-3}) treated and untreated with SLA were used to evaluate of the killing time of *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922). Figure 3 shows cells with and without cell wall damages.

According to Figure 5, SLA had bactericidal effect against *S. aureus* and *E. coli*, reducing the starting log CFU/mL by greater than 3 logs.

The Percent Reduction of Bacterial Cells

The percent reduction of *E. coli* and *S. aureus* after exposure to the MBC concentration of SLA was evaluated and the results are shown in Table 2. These findings demonstrated that 99.98% of *S. aureus* and 99.99% of *E. coli* were eliminated after exposure to SLA following a 20-min incubation.

MTT Assay

For MTT assay, the cytotoxic effect of SLA was evaluated on Hu02 cells (Figure 6 & Table 3), based on one-way ANOVA and Tukey *post hoc* test. Table 3 shows that there was a significant difference in percent viability of the Hu02 cells from the various SLA concentrations for the three exposure times (24, 48 or 72 hours). Increasing the concentration from 6.25 µL/mL to 12.5 µL/mL (P=0.002) and 25 µL/mL (P=0.025) significantly decreased the viability of the Hu02 cells after a 24-hr incubation. However, increasing the concentration from 12.5 to 25 µL/mL decreased the viability of the cells insignificantly after 24 hours of exposure. After the cells were exposed to SLA, increasing the concentration from 6.25 to 12.5 µL/mL (P=0.005) and 25 µL/mL (P=0.012) significantly decreased the percent viability of the cells over a 48-hr exposure. However, changing the concentration from 12.5 to 25 µL/mL decreased the viability of the cells insignificantly (48-hr exposure). Increasing the concentration from 12.5 to 25 µL/mL significantly increased the viability of the Hu02 cells after 72 hours (P=0.022).

Discussion

The current study explored the antibacterial and cytotoxic properties of the cell-free supernatant from *Lactobacillus acidophilus* (SLA) strain against gram-positive and gram-negative bacteria. This subject has not been investigated in previous studies.

This study showed that the strongest inhibition was produced by SLA. Earlier, Piatek, *et al*. have reported that a mixture of *lactobacilli* had a similar inhibition against *E. coli* [25]. The study conducted by Soleymanzadeh, *et al.* in 2020 showed that *Lactobacillus* strain and the product from its supernatant could be potential alternatives to existing antibiotics in controlling resistant infections [16]. Other studies have shown that another probiotic extract has also anti-pathogenic activity against *S. aureus*, *E. coli*, and *P. aeruginosa* [26, 27].

An earlier study conducted by Zahradnik in 2009 has shown that *lactobacillus* spp has a notable role in inhibiting microbial populations and their side effects [28], although the consumption of probiotic cells may cause few complications for patients with severe immune deficiencies [29]. In this context, we used the CFS of a probiotic species in this research. Other researchers have reported that the cell wall components in the broth of living or dead probiotics, and the bacteriocins component produced by probiotics, also contribute to the beneficial effects [30].

In the current study, the MIC and MBC of the SLA against *E. coli* were equal (12.5 µL/mL). Likewise, the MIC and MBC of *L. acidophilus* supernatant against *Proteus* strain was 25 mg/mL as reported earlier by Goodarzi, *et al.* [31]. In another study conducted by Sadatzadeh, *et al.* in 2018, the MIC of *L. casei* against *Streptococcus* spp was reported to be 12.5 mg/mL [26]. The results of these studies were fairly consistent with those of our study, in which SLA reduced 53% and 96% of *S. aureus* and *E. coli* colonies, respectively, after a 10-min exposure. In the current study, the inhibition rate approached essentially 100% against both *S. aureus* and *E. coli* after only a 20-min treatment. In another study, it was reported that *L. casei* has an inhibitory effect against *E. coli* and *P. aeruginosa* about 71% and 80%, respectively, while this rate approached about 75% for *L. plantarum* against *S. aureus* [32].

Anas, *et al.* have reported that the whole culture of *L. plantarum* has antimicrobial effects against *S. aureus* and *E. coli* [33]. In this regard, there have been reports about factors, such as organic acids, hydrogen peroxide, or bacteriocins produced by probiotics, inhibiting the growth of pathogenic bacteria [32, 34]. Another study has reported that *Lactobacillus* spp demonstrates strong inhibitory effects against *S. aureus*, which was consistent with our well diffusion study [32]. In recent years, researchers have shown that *L. acidophilus* has inhibitory effects against the growth of *Enterobacteriaceae* family [30, 35]. Indeed, most of the inhibitory effects have been related to the bacteriocin, the secondary metabolite from probiotics. The highest amount of bacteriocins is generated in the initial stage of bacterial growth. All *Lactobacilli* tend to reduce the pH in the culture environment by secondary metabolites, such as organic acids after a 24-hr exposure. These organic acids are considered to be antimicrobials [31].

In the current study, the viability of Hu02 cells was 81.09% following a 48-hr incubation at a concentration of 6.25 µL/mL. In this context, the study by Dolati, *et al*. conducted in 2021 showed

that the IC_{50} of *Bacillus coagulans* supernatant against cancer cells had low cytotoxic effect (only 23%) against a cell line from human foreskin fibroblasts (HFF) after a 48-hr exposure. But, the viability of HFF cells at 6 mg/mL was 60%, which later reached 100% at a concentration of 1-2 mg/mL [36].

We demonstrated that the viability of the Hu02 cells increased when the concentration was raised from 12.5 to 25 µL/mL following a 72-hr incubation (31.71% vs 61.12%). Metabolites produced by probiotic bacteria, such as organic acids and exopolysaccharides can affect cell proliferation. They can induce apoptosis by up-regulating the genes while down-regulating the anti-apoptotic genes [37, 38]. Nami, *et al.* have shown the effect of *E. lactis* metabolites on FHs-74 normal cells had not toxic effect and 95% of them grew well [39]. It seems that, probiotic supernatant at certain concentrations can be ineffective against growth inhibition or they may even enhance the growth.

Limitation of the Study

We did not use whole cultures of probiotic bacteria, although we used a wide range of supernatants from different probiotic strains. In future studies, we plan to use whole probiotic cultures, and examine the effect of the supernatants with or without microbial cells.

Conclusions

Considering the inhibitory effects of SLA and its low toxicity, we suggest that it is an effective and safe candidate for the inhibition of bacteria, such as *S. aureus* and *E. coli*. In addition, since the examined agent has low toxicity, its application is likely to reduce the side effects caused by the use of numerous antibiotics.

Conflict of Interests

The authors declare no conflict of interests with any entities.

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Compliance with Ethical Guideline

The concept and protocol of this study were reviewed and approved by the Institutional Review Board of Iran University of Medical Sciences, Tehran, Iran, prior to its conduction (Ethics review & approval No.: IR .IUMS.REC1399.863).

Authors' Contributions

Project administration and supervision: Reza Hosseini Doust and Sara Minaean, Investigation and data curation: Ali majidpour, Mahdi Adabi, Microbial assey and writing, editing and approval of final manuscript: All authors.

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