Original Article

Anti-Biofilm Potential of *Lactobacillus casei* and *Lactobacillus rhamnosus* Cell-Free Supernatant Extracts against *Staphylococcus aureus*

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Abstract

Background: Biofilm production is an important virulence factor in *Staphylococcus aureus*. Most of the infections associated with biofilms of this bacterium are very difficult to treat using antibiotics. The present research studied the effects of the two probiotic *Lactobacillus* species *L. casei* and *L. rhamnosus* on *S. aureus* biofilm.

Materials and Methods: Cell-free supernatant (CFS) extracts of *L. casei* ATCC 39392 and *L. rhamnosus* ATCC 7469 culture were prepared. The effects of sub-minimum inhibitory concentrations of the CFS extracts on cell surface hydrophobicity (CSH), initial attachment, biofilm formation, and their ability in eradicating *S. aureus* ATCC 33591 biofilms were assessed. In addition, the effects of CFS extracts on expression of the genes involved in formation of *S. aureus* biofilms (*cidA, hld, sarA*, *icaA,* and *icaR*) were also evaluated through real-time polymerase chain reaction.

Results: CFSs of both *Lactobacillus* spp. significantly reduced CSH, initial attachment, and biofilm formation and eradicated the biofilms. The above findings were supported by scanning electron microscopy results. These two *Lactobacillus* CFSs significantly changed the expression of all studied biofilm-related genes. Expression levels of *cidA*, *hld*, and *icaR* genes significantly increased by 4.4, 2.3, and 4.76 fold, respectively, but *sarA* and *icaA* genes were significantly downregulated by 3.12 and 2.3 fold.

Conclusion: The results indicated that CFS extracts of *L. casei* and *L. rhamnosus* had desirable antagonistic and anti-biofilm effects against *S. aureus*. Consequently, carrying out further research enables us to prepare pharmaceuticals from these CFSs in order to prevent and treat infections caused by *S. aureus* biofilms.

Keywords: Biofilm, *Lactobacillus casei, Lactobacillus rhamnosus,* probiotic, *Staphylococcus aureus*

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Introduction

Staphylococcus aureus is a Gram-positive bacterium and the causal agent of a broad spectrum of infections, including acute infections mostly caused by secretion of exoenzymes and chronic infections such as osteomyelitis, chronic wound infections, eye infections, chronic rhinosinusitis, and endocarditis that develop due to biofilms formed by this bacterium.[1] Biofilm is a collection of adhered microorganisms that is attached to a surface and covered by a matrix of polymeric extracellular matrix.[2] In *S. aureus*, two types of biofilms are produced: polysaccharide intercellular adhesin (PIA)-dependent biofilms and PIA-independent biofilms. In PIA-dependent biofilms, products of the *ica* gene locus that

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include an N-acetylglucosamine transferase gene (*icaA* and *icaD*), a PIA deacetylase gene (*icaB*), a PIA exporter gene (*icaC*), and a regulatory gene (*icaR*) are involved in biofilm biosynthesis. Expression of the *ica* locus can be suppressed by production of *tcar* and *icaR*, which leads to PIA downregulation and hence prevents biofilm formation. In PIA-independent biofilm formation, adhesive proteins such as surface-associated proteins including protein A, fibronectin-binding proteins (FnBPB and FnBPA), *S. aureus* surface proteins (SasG), biofilm-associated protein, and clumping factor B play parts as important components in attachment and development of biofilm matrix.[3,4] These adhesive proteins are controlled by several regulatory systems. Activation of the accessory gene regulator (agr) system reduces expression of adhesive proteins and induces biofilm dispersal by increasing expression of detergent-like peptides and nucleases. On the other hand, the staphylococcal accessory regulator (sarA) system induces attachment and allows initial biofilm formation by suppressing extracellular proteolytic and nucleolytic enzymes.[5]

S. aureus can form biofilms on host tissues and medical implants. Biofilms increase bacterial resistance to host defense mechanisms, and treatment of their resulting infections with antibiotics is difficult. Moreover, *S. aureus* biofilms are considered a source for the spread of infections to the other parts of the body. This increased resistance plays a special role in the development of nosocomial infections.[6,7]

Biofilm formation together with high prevalence of *S. aureus*-induced infections and emergence of antibiotic-resistant strains make necessary to conduct research for finding of new medications for eradicating strains that are resistant to common treatments and/or for eradicating biofilms or preventing their formation. In general, anti-biofilm drugs are based on three main strategies: preventing bacterial adhesion to biotic or abiotic surfaces to reduce the probability of their establishment and biofilm formation, disrupting biofilm structure during its maturation, and disturbing signaling pathways.[8]

One of the treatment methods proposed for drug-resistant or biofilm-forming *S. aureus* infections is to use probiotics such as probiotic *Lactobacillus* strains. Several studies have investigated the effects of some *Lactobacillus* strains in preventing *S. aureus* growth and eradicating its biofilms. Probiotics can exhibit their therapeutic effects in different ways. For example, suppression of *S. aureus* growth by lactobacilli has been attributed to acidification of the culture media by fermentation of lactic acid and production of H_2O_2 and bacteriocins.[9] The present study was carried out to investigate the effects of two probiotic *Lactobacillus* spp. (*L. casei* and *L. rhamnosus*) on *S. aureus* biofilms.

Materials and Methods

The bacterial strains

The standard *S. aureus* strain ATCC 33591 which is a strong biofilm former was used in the present research. This bacterium was kept in Tryptic soy broth (TSB) with 20% glycerol at −70°C. The probiotic *lactobacillus* strains *L. casei* ATCC 39392 and *L. rhamnosus* ATCC 7469 were kept in Man, Rogosa, and Sharpe (MRS) broth with 30% glycerol at −70°C. MRS agar was used to culture these strains. The colonies were regularly evaluated to make sure they were pure in order to reduce the probability of contamination and error in the experiments.

Preparation of cell-free supernatant extracts

Several colonies of the freshly cultured *Lactobacillus* strains were transferred to 10-mL MRS broth and incubated under microaerophilic conditions for 24 h at 37°C. This culture medium was used to inoculate 1000-mL MRS broth. The inoculated culture was incubated for 48 h at 37°C and then centrifuged at 12,000 rpm for 20 min to separate the supernatant. The supernatant was passed through a 0.45-µm filter to completely remove the cells and obtain cell-free supernatant (CFS). Some of the CFSs were used for studying its antimicrobial activity and the rest to prepare CFS extract. The method previously explained by Brosnan *et al.* was employed with some modifications to prepare the extract. The extraction process, in which ethyl acetate was used, lasted for 6 h. The CFS was mixed with ethyl acetate at a ratio of 5:1, and the ethyl acetate was replaced every hour. Finally, the ethyl acetate was removed using a rotary evaporator to obtain the dry matter.[10]

Antimicrobial activity

The agar well diffusion method was used to assess CFS antimicrobial activity of the *Lactobacillus* strains against *S. aureus* and to see whether this antimicrobial effect was dependent on organic acids or on bacteriocin production. This experiment was performed in two ways: once after neutralizing the pH of the CFS with NaOH and once after boiling the CFS for 5 min at 100°C. Briefly, the overnight culture of the *S. aureus* standard strain ATCC 33591 was used to prepare a half McFarland suspension that was cultured on Mueller–Hinton agar plates. A well of 3-mm diameter was punched in the center of each culture plate. Fifty microliters of the prepared CFSs was poured into the wells, and the plates were incubated at 37°C for 24 h. Finally, the diameters of the clearing zones in the plates in the two experiments were compared.[11]

Minimum inhibitory concentration and minimum bactericidal concentration

Broth microdilution was used to determine minimum inhibitory concentration (MIC).[12] Serial two-fold dilutions (0.25–16 mg/mL) of the CFS extracts were made and poured into the wells of the microplate. The wells were inoculated with a suspension prepared from a 24-h culture of *S. aureus* ATCC 33591. The final bacterial concentration of each well was 10^5 cfu/mL. The wells without the extract and without the bacteria were the positive and negative controls, respectively. After 24 h, the wells were examined with respect to growth and the MIC was determined. The number of colonies in the wells that showed no visible signs of turbidity was counted to determine the minimum bactericidal concentration (MBC).

Cell surface hydrophobicity

Microbial adhesion to solvent (MATS) assay was employed to study the effect of sub-MICs of the CFS extracts of the *Lactobacillus* strains on cell surface hydrophobicity (CSH) of *S. aureus*.[13] The bacteria were cultured on TSB at ½, ¼, and ⅛ MICs of CFS extracts for 24 h. The bacterial cells were then centrifuged at 7000 rpm for 5 min. The bacterial culture without the CFS extract was used as the control. The cells were washed once to completely remove the culture medium, and a suspension with OD_{600} = 0.3 was prepared (OD1) by adding the necessary volume of PBS. Hexadecane (0.8 mL) was then added to 5.2 mL of the microbial suspension and vortexed to form an emulsion. This mixture was let stand for 20 min, and two phases were separated; then, the OD of the aqueous phase was measured at 600 nm (OD2). CSH was calculated using the following equation:

$$CSH\% = 1 - (OD2/OD1) \times 100$$

Anti-adhesion assay

In each well of a 96-well microplate, 200 µL of each CFS extract at ½, ¼, and ⅛ MICs was poured. The well containing PBS was the control. The microplate was incubated at 4°C for 18 h, and the wells were then emptied. A suspension with OD_{600} = 0.3 was prepared from a 24-h culture of *S. aureus*, 200 µL of which was poured into each inoculated well, and the plate was incubated at 37°C for 4 h. The wells were then emptied, washed with PBS, and stained with crystal violet. The crystal violet was dissolved by adding 33% acetic acid. Finally, the OD was measured at 570 nm.[14]

Anti-biofilm formation assay

The microtiter plate assay was used for this purpose. The bacteria were first cultured on TSB for 24 h. Suspensions (10^8 cfu/mL) from this culture were inoculated in TSBGlc (TSB + 1% glucose) containing ½, ¼, and ⅛ MICs of the CFS extracts. This suspension (200 µL) was transferred to the wells of a 96-well microplate. The well without the extract was the positive control, and the well without the bacteria was the negative control. The microplate was incubated at 37°C for 24 h. The wells were then emptied, washed with PBS, and stained with crystal violet. The crystal violet was dissolved by adding 33% acetic acid, and the OD of the solution was measured at 570 nm. The OD of negative control well was recorded as ODc, and the OD of tested wells as ODt. Table 1 is used to determine the degree of biofilm formation.[15]

Biofilm dispersal assay

The microtiter plate assay was also used in this experiment with the difference that the biofilms were formed first and then the effect of CFS extracts on them was studied. The 24-h culture of *S. aureus* was diluted with TSBGlc medium at a ratio of 1:100, and 200 µL of this suspension was poured in the wells of the microplate. The microplate was incubated at 37°C for 24 h for biofilm formation. The wells were then emptied and washed with PBS, and each one was filled with 200 µL of TSBG1c medium containing ½, ¼, and ⅛ MICs of the *Lactobacillus* strains' CFS extracts, and the microplate was incubated at 37°C for 24 h. The well containing only TSBGlc was the control. As in the previous experiment, staining was carried out with crystal violet and OD was measured. The same method was employed to determine minimum biofilm eradication concentration (MBEC) with the difference that the extracts were assessed at concentrations of 1–4 times higher than the MIC.[16]

Table 1: Interpretation of the formed biofilm grade[15]

OD	Result
$OD_t \leq OD_c$	Nonbiofilm
$OD_c < OD_t < 2 \times OD_c$	Weak biofilm
$2 \times OD_c < OD_t < 4 \times OD_c$	Moderate biofilm
$OD_t \geq 4 \times OD_c$	Strong biofilm

Scanning electron microscopy of biofilm formation

Overnight culture of *S. aureus* was inoculated in TSBGlc containing ½ MIC of CFS extracts so that the final bacterial concentration was 10^8 cfu/mL. This suspension was transferred to a 6-well microplate. A glass coverslip was placed in each well. The plate was incubated at 37°C for 24 h for biofilm formation. The glass coverslips were then removed and washed three times with PBS and placed in glutaraldehyde for 2 h for the samples to be fixed. In the next stage, serial dilutions of ethanol (50%–100%) were used to dehydrate the samples. The surface of each plate was coated with a thin layer of gold to impart electrical conductivity. Finally, the samples were studied using a scanning electron microscope (MIRA3, TESCAN, Czech Republic).[17]

Effects of cell-free supernatants on expression of genes involved in biofilm formation

Overnight culture of *S. aureus* was inoculated in TSBGlc medium containing ½ MIC of the *Lactobacillus* strains' CFSs so that the final concentration of the bacteria was 10^8 cfu/mL. This suspension was transferred to a 6-well microplate that was incubated at 37°C for 24 h. The cells were then collected and their total RNA was extracted. To this end, TE buffer containing 40 mg/mL lysozyme and 100 µg/mL lysostaphin was added to the pellet to lyse the cells. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. RNA concentration and purity were assessed with NanoDrop (NanoDrop One, Thermo Fisher, USA), and the QuantiTect Reverse Transcription Kit (Qiagen, Germany) was employed to remove genomic DNA contamination and synthesize cDNA. Real-time polymerase chain reaction (PCR) was used to compare the expression levels of the genes involved in biofilm formation in the samples treated with CFSs and the control sample. Table 2 lists the primers that were used (the sequences of the genes of interest were extracted from the GenBank sequence database and the primers were designed using AlleleID 6). Real-time PCR was run in triplicates using QuantiTect SYBR Green PCR Kits. Expression of the genes was normalized using the 16S rRNA gene. The relative expression levels of the genes were determined using the ΔΔCT method.[18,19]

Statistical analysis

All experiments were performed in triplicate. The results of the treatments were compared with those of the control using one-way ANOVA and the Tukey's multiple comparison test. Significant differences were evaluated at $P < 0.05$. GraphPad Prism 8 (GraphPad Software, San Diego, California, United States) was used for these analyses.

Results

Antimicrobial activity

To study the antimicrobial activity of the *Lactobacillus* strains' CFSs using the agar well diffusion method, their pH was neutralized and they were heated. The results showed that the CFS of neither of the *Lactobacillus* strains was able to form clearing zones when its pH was neutralized, whereas the CFSs of both strains formed clearing zones when their pH was not neutralized. The clearing zone diameter for *L. casei* ATCC 39392 was 9 mm and that of *L. rhamnosus* ATCC 7469 was 9.4 mm. Moreover, heating the CFSs to 100°C did not change the clearing zone diameters. Consequently, none of the *Lactobacillus* species produced active bacteriocin against *S. aureus*, and their antimicrobial activity was probably due to production of organic acids.

Minimum inhibitory concentrations and minimum bactericidal concentrations of cell-free supernatants

Using broth microdilution method, it was shown that the MICs and MBCs of CFS extracts for both *Lactobacillus* strains against *S. aureus* ATCC 33591 were 4 mg/mL and 8 mg/mL, respectively. Therefore, concentrations 0.5, 1, and 2 mg/mL of CFSs were used as sub-MICs in the rest of the experiments.

Results of the cell surface hydrophobicity assay

The results of the MATS assay revealed that CFS extracts of both *Lactobacillus* strains at ½ and ¼ MICs significantly reduced surface hydrophobicity of *S. aureus* cells ($P < 0.0001$). At ⅛ MIC, the CFS of *L. rhamnosus* significantly decreased surface hydrophobicity ($P = 0.003$), whereas that of *L. casei* did not cause any significant differences ($P = 0.7$). This reducing effect was dose dependent; i.e., increases in CFS concentrations further reduced surface hydrophobicity of the cells. Figure 1 demonstrates CSH changes in *S. aureus* ATCC 33591 under the influence of various concentrations of the *L. casei* and *L. rhamnosus* CFS extracts.

Effects of CSFs on initial attachment

The results of the experiment on the effects of *L. casei* ATCC 39392 and *L. rhamnosus* ATCC 7469 CFSs on the initial attachment ability of *S. aureus* ATCC 33591 to surfaces indicated that ½, ¼, and ⅛ MICs of CFS extracts significantly reduced attachment of *S. aureus* cells ($P < 0.001$). This attachment inhibitory effect was considerable, so that the *L. rhamnosus* and *L. casei* CFSs at ½ MIC (2 mg/mL) reduced the initial attachment of *S. aureus* by 100% and 98.9%, respectively. Figure 2 presents these results.

Effects of the cell-free supernatants on biofilm formation

Microtiter plate assay was employed to evaluate the effects of *Lactobacillus* strains' CFSs on *S. aureus* biofilm formation. The ODs of the control and the samples treated with ½, ¼, and ⅛ MICs of CFS extracts showed that all concentrations of both extracts significantly reduced biofilm formation ($P < 0.0001$). *S. aureus* ATCC 33591 produced strong biofilms. These biofilms changed to weak ones under the influence of ½ MIC of the CFS extract of *L. casei*. The other concentrations of this CFS extract and all concentrations of the *L. rhamnosus* CFS extract led to the formation of moderate type biofilms. The complete results are listed in Figure 3.

Ability of cell-free supernatants to eradicate biofilms

The ability of *L. casei* ATCC 39392 and *L. rhamnosus* ATCC 7469 CFS extracts in eradicating *S. aureus* ATCC 33591 biofilms was investigated. The results revealed that the CFSs of both *Lactobacillus* strains facilitated eradication of *S. aureus* biofilms. All concentrations of these CFSs significantly decreased the strength of the biofilms ($P < 0.0001$). At ½

Table 2: Sequences and characteristics of the primers used in the experiments

Gene	Primer sequence (5'→3')	Product size (bp)	GenBank
cidA	F: AACTACTACTACAACTAGGAATC	200	AY581892.1
	R: CTACAACTGACGGTATGAAG		
hld	F: CGATAATCCATTTTACTAAGTC	74	X52543.1
	R: AATTAAGGAAGGAGTGATTTC		
sarA	F: CTTTGTTTTCGCTGATGTATGTC	110	U20782.1
	R: GTTGTTATCAATGGTCACTTATGC		
icaA	F: TTATGTAATGTGCTTGGATGC	197	AF086783.1
	R: GTGTCTGACTTCGCTTAATAC		
icaR	F: GATGCTTTCAAATACCAACTTTC	179	AF086783.1
	R: CAATTATCTAATACGCCTGAGG		
16s rRNA	F: CAGAGATATGGAGGAACAC	134	L37597.1
	R: AACACTTAGCACTCATCG		

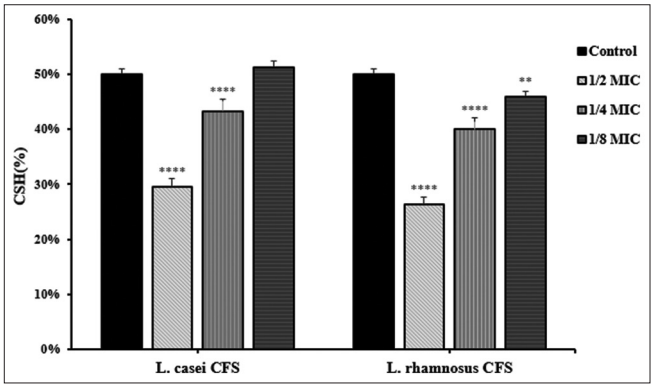

Figure 1: Changes in the cell surface hydrophobicity of *Staphylococcus aureus* ACTT 33591 in ½, ¼, and ⅛ minimum inhibitory concentrations of the *Lactobacillus casei* and *Lactobacillus rhamnosus* cell-free supernatants. Significant differences with the control (**$P < 0.01$, ****$P < 0.0001$)

MIC (2 mg/mL), both CFSs were able to turn strong biofilms into weak ones. Moreover, the strong biofilms were changed into moderate ones at the other MICs. Figure 4 presents these results. At the 4 MIC (16 mg/mL), CFSs of both *Lactobacillus* species were able to completely eradicate *S. aureus* biofilms. Therefore, this concentration was determined as the MBEC.

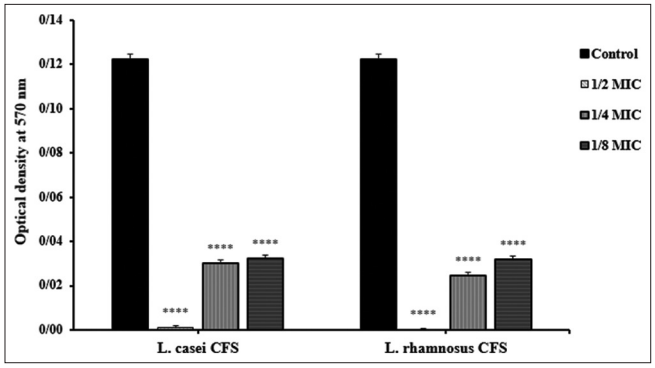

Figure 2: Initial attachment of *Staphylococcus aureus* ATCC 33591 cells in ½, ¼, and ⅛ minimum inhibitory concentrations of the *Lactobacillus casei* and *Lactobacillus rhamnosus* cell-free supernatants. Significant differences with the control (****$P < 0.0001$)

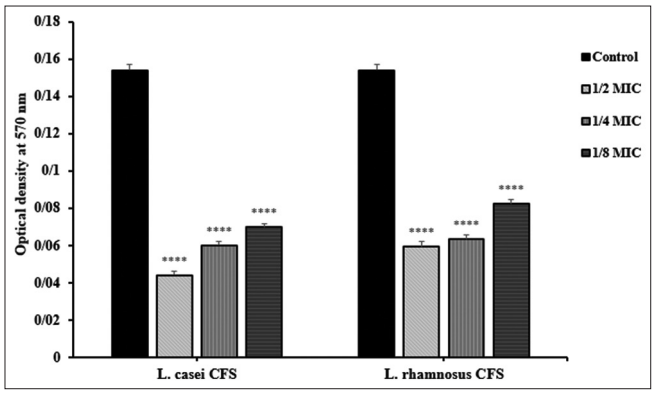

Figure 3: Formation of *Staphylococcus aureus* ATCC 33591 biofilm in ½, ¼, and ⅛ MICs of the *Lactobacillus casei* and *Lactobacillus rhamnosus* CFSs. Significant differences with the control (****$P < 0.0001$)

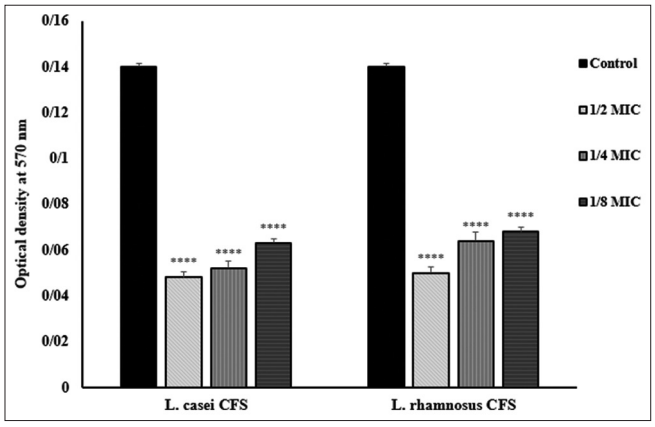

Figure 4: Eradication of *Staphylococcus aureus* ATCC 33591 biofilm in ½, ¼, and ⅛ MICs of the *Lactobacillus casei* and *Lactobacillus rhamnosus* CFSs. Significant differences with the control (****$P < 0.0001$)

Scanning electron microscopy of the biofilms

The effects of the *L. casei* ATCC 39392 and *L. rhamnosus* ATCC 7469 CFS extracts on *S. aureus* biofilm formation were studied using scanning electron microscopy (SEM). As shown in Figure 5, thick biofilms at high cell density were formed in the control sample, whereas the number of biofilm cells considerably decreased in the samples treated with the *Lactobacillus* strains' CFSs.

Results of real-time polymerase chain reaction

Using real-time PCR, the effects of *L. casei* and *L. rhamnosus* CFS extracts at 2 mg/mL on expression of genes involved in *S. aureus* biofilm formation were studied. The studied genes were *cidA*, *hld*, *sarA*, *icaA*, and *icaR*. Some of them are necessary for biofilm formation; i.e., increases in their expression enhance biofilm formation. However, the other genes are expressed under planktonic conditions and their expression decreases formation of biofilms or eradicates them. The CFSs of both *Lactobacillus* species changed the expression levels of all five studied genes: they increased expression of the *cidA*, *hld*, and *icaR* genes but decreased the expression of the *sarA* and *icaA* genes. Figure 6 shows variations in the expression of the studied genes.

Discussion

Although *S. aureus* is may exist as a nonpathogenic microorganism in some parts of the body, especially in the nose and on skin, it can be the causal agent of a broad spectrum of mild to life-threatening infections.[1,2] Considering the high prevalence of *S. aureus* and the difficult treatment of infections caused by this bacterium, especially by its biofilm-forming isolates, it is necessary to conduct research in order to find new treatment methods,[20] which can be either as biofilm formation prevention or as biofilm eradication. Although antibiotics play an important role in modern treatment methods, emergence of antibiotic-resistant strains necessitates constant search for more potent and efficient antibiotics. Among the suggested treatments that have attracted interest is the use of probiotics that have antagonistic properties against other microorganisms. Many studies have demonstrated that probiotics have considerable therapeutic potential for localized and systemic bacterial infections.[21] Several reports have been published on the effects of probiotics on *Staphylococcus*, but few studies have investigated their effects on the steps in biofilm formation and the CSH in *S. aureus*. Consequently, the present research investigated the antimicrobial and anti-biofilm effects of two standard strains (*L. casei* ATCC 39392 and *L. rhamnosus* ATCC 7469) on *S. aureus* ATCC 33591.

The results showed that CFSs of both *Lactobacillus* spp. were able to inhibit *S. aureus* growth. In the broth microdilution method, the MIC of both *Lactobacillus* spp. (*L. casei* and *L. rhamnosus*) for *S. aureus* ATCC 33591 was 4 mg/mL, their MBC was 8 mg/mL, and their MBC/MIC ratio was 2. Since MBC/MIC of ≤4 and >4 indicates that the antimicrobial effect is bactericidal and bacteriostatic, respectively.[22] Therefore,

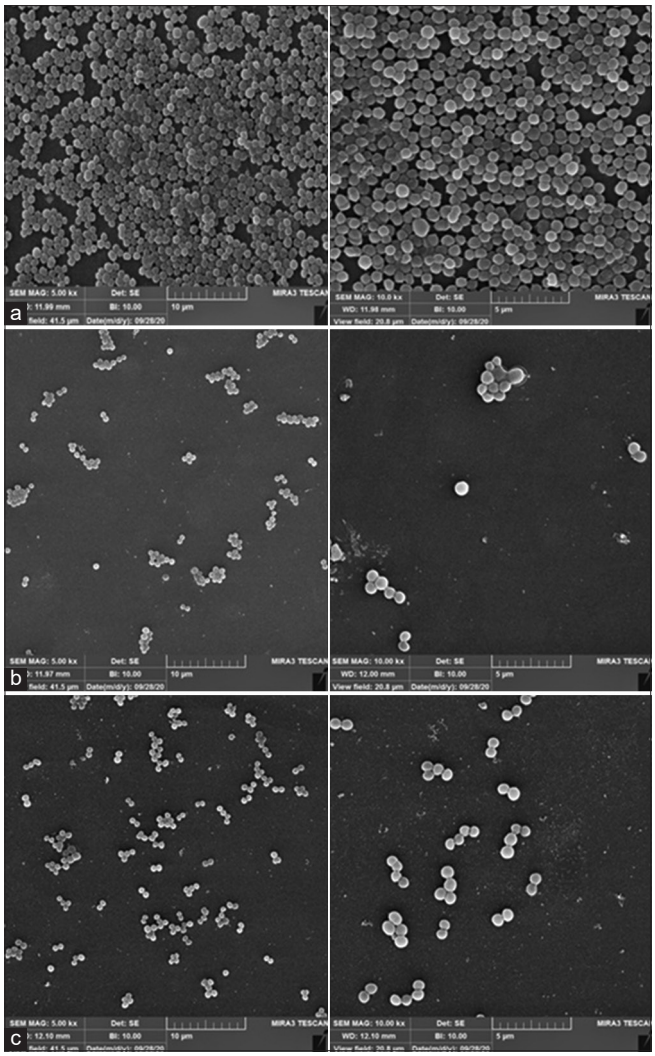

Figure 5: Scanning electron microscopy images of *Staphylococcus aureus* biofilms: (a) The control sample, (b) The sample treated with cell-free supernatant (CFSs) from the cultures of *Lactobacillus casei*, (c) The sample treated with CFSs from the cultures of *Lactobacillus rhamnosus*. Left: ×5000; Right: ×10,000

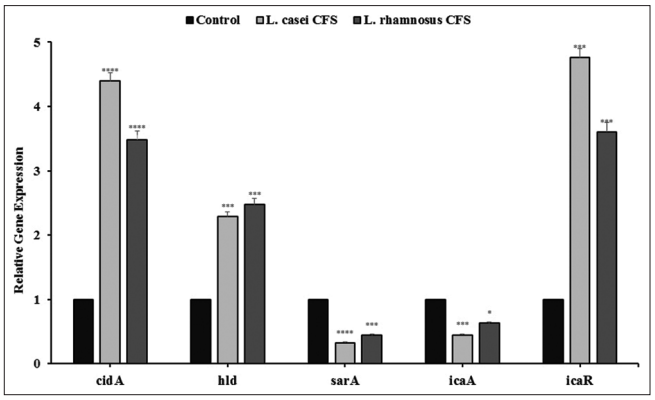

Figure 6: Changes in the expression of genes involved in the formation of *Staphylococcus aureus* ATCC 33591 biofilms in ½ MIC of *Lactobacillus casei* and *Lactobacillus rhamnosus* cell-free supernatant (CFSs). Significant differences with the control (*$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$)

CFSs of both studied *Lactobacillus* spp. had bactericidal effect on *S. aureus*. These results conform to those of previous studies conducted on other lactobacilli (Koohestani *et al.* and Melo *et al.*). In the research by Koohestani *et al.* in which the agar spot method and the broth microdilution method were used, *L. acidophilus* LA5 and *L. casei* 431 formed clearing zones in *S. aureus* ATCC 25923 (diameters of clearing zones of 50 mm and 37 mm for *L. acidophilus* and *L. casei*, respectively) and MIC of 40 mg/mL for both supernatants.[23] In the study carried out by Melo *et al.*, in which the broth microdilution method was employed, the MICs of *L. plantarum* TCUESC02 and *L. fermentum* TCUESC01 CFSs for *S. aureus* CCMB262 were 2.5 and 20 mg/mL, respectively.[17]

In the present research, comparison of antimicrobial activity of the CFSs prior to and following pH neutralization and heating indicated that neither of the studied *Lactobacillus* spp. produced active bacteriocin against *S. aureus* and their antimicrobial activity was probably due to production of organic acids. *L. casei* and *L. rhamnosus* are among heterofermentative lactic acid bacteria and can produce various metabolites including different organic acids in the hydrocarbon fermentation process.[24] Hu *et al.* studied the CFSs of *L. plantarum* strains and identified five different organic acids (lactic, acetic, tartaric, citric, and malic acids). They also showed that CFSs of these strains had antimicrobial activity against different pathogens including *S. aureus*.[25] It seems that, at identical pH values, organic acids exhibit greater antimicrobial activity compared to mineral acids. Minor *et al.* reported that acidification of the culture (to pH 4.6) using lactic acid decreased biomass of *S. aureus* by 99% compared to the control (the culture medium without lactic acid). They also observed that acidification of the culture medium to pH 5 using acetic acid and to pH 4.5 employing citric acid had similar effects and the antimicrobial effects of these organic acids were stronger than the mineral acids (phosphoric and hydrochloric acids), because these two mineral acids were able to exert similar effects at pH values of 4.1 and 4, respectively.[26]

Staphylococci are known as the most prevalent infectious agents associated with biofilms. *S. aureus* can form biofilms in host tissues and on medical catheters and implants.[2,7] The performance of antimicrobial agents in treating infections does not only depend on their bactericidal and bacteriostatic effects but also on their ability to suppress production of virulence factors.[20] The present research investigated the effects of the *L. casei* and *L. rhamnosus* CFSs on factors influencing *S. aureus* biofilms. For this purpose, CSH, initial attachment, biofilm production and eradication, and also expression of genes involved in biofilm formation were assessed. Bacterial adhesion is the first step in biofilm formation, which depends on environmental conditions, characteristics of the surface on which the biofilm is formed, and the extracellular polymers produced by the bacteria. However, biofilm formation is mainly managed by physicochemical properties such as electrostatic

and van der Waals interactions and CSH. In fact, CSH plays the main role in bacterial colonization on biotic and abiotic surfaces. In addition, medical equipment such as catheters, mechanical heart valves, and artificial pacemakers are all made of hydrophobic materials and hydrophobic microorganisms have a propensity to adhere to such surfaces.[27] In this study, sub-MICs of the CFSs of both studied *Lactobacillus* spp. significantly decreased CSH in *S. aureus* ($P < 0.0001$). This result conforms to those of previous studies. Walencka *et al.* reported in their research that the biosurfactant extracted from *L. acidophilus* reduced CSH in *S. aureus,* thereby decreasing its biofilm formation.[28] Melo *et al.* used the microtiter plate assay and SEM in their study and observed that sub-MICs of *L. fermentum* were able to significantly reduce biofilm formation by *S. aureus*. Moreover, they noticed that expression of the biofilm formation of the *icaA* gene declined and that of the *icaR* gene involved in regulation of biofilm formation increased.[17] The gene products of the icaADBC locus are responsible for PIA biosynthesis. PIA is the main molecule responsible for intercellular adhesion in *S. aureus*. Expression of the *icaR* gene suppresses the expression of this gene locus.[29] In the present research also, similar results were obtained. CFSs of both studied *Lactobacillus* spp. significantly reduced biofilm formation. This reducing effect was confirmed by the scanning electron microscope and the microtiter plate assay. Moreover, both CFSs increased expression of the *icaR* suppressor gene and decreased expression of the *icaA* gene.

No studies concerning the effects of supernatants and/or organic acids produced by lactobacilli on expression of the other genes involved in *S. aureus* biofilms were found in a search within the reliable websites. However, some studies had investigated the effects of other metabolites produced by lactobacilli. Yan *et al.* employed the microtiter plate assay and SEM to study the effects of the biosurfactant produced by *L. plantarum* on initial attachment and biofilm formation in *S. aureus*. They also assessed the effects of a number of the genes (*icaA*, *sarA*, *srtA*, and *cidA*) involved in biofilm formation through real-time PCR. The *L. plantarum* biosurfactant significantly reduced initial attachment and biofilm formation and decreased expression of some of the genes involved in biofilm formation (*sarA* and *cidA*).[14] In this research, the *L. casei* and *L. rhamnosus* CFSs reduced the expression of the *sarA* gene in *S. aureus*. It should be mentioned that *sarA* induces attachment and allows initial biofilm formation by suppressing the extracellular proteolytic and nucleolytic enzymes. Consequently, its reduced expression prevents initial attachment of *S. aureus* cells.[30] In the present study also, the *L. casei* and *L. rhamnosus* CFSs increased the expression of the *hld* and *cidA* genes. The *hld* gene is expressed when the agr quorum-sensing system is activated. Therefore, increased expression of the *hld* gene is a sign of improved activity of the agr quorum-sensing system. Researchers have shown that the agr system downregulates the genes encoding adhesion factors and biofilm formation. This can reduce adhesion and hence indirectly decrease initial biofilm formation. In addition, we know that agr upregulates expression of detergent-like peptides and nucleotides that seem to increase biofilm separation.[30] Activity of the cid operon induces autolysis in *S. aureus* cells. Programmed cell death involves *cidA*, and its expression is necessary for biofilm formation because autolysis of a number of bacterial cells provides the eDNA required in biofilm formation.[31] In the present research, expression of the *cidA* gene in the samples treated with the *L. casei* and *L. rhamnosus* CFSs increased several folds (3.48 and 4.4 fold) compared to the control sample. This indicated extensive autolysis and death of *S. aureus* cells.

Conclusion

In summary, the results of this study indicated that *L. casei* ATCC 39392 and *L. rhamnosus* ATCC 7469 had desirable antagonistic effects against the standard strain of *S. aureus* ATCC 33591. CFSs of these two *Lactobacillus* spp. in sub-MIC concentration significantly reduced surface hydrophobicity, initial attachment, and biofilm formation and eradicated biofilms. Moreover, significant changes were observed in expression of all studied genes involved in biofilm formation. Consequently, it is hoped that conducting further research, especially on effective compounds in the CFSs of the studied lactobacilli, will make it possible to produce drugs for preventing/treating infections caused by *S. aureus* biofilms.

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Conflicts of interest

There are no conflicts of interest.

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