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Effects of cell-free supernatant of *Lactobacillus acidophilus* LA5 and *Lactobacillus casei* 431 against planktonic form and biofilm of *Staphylococcus aureus*

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Abstract

This study was carried out to investigate the stability, antibacterial properties and biofilm removal potential of cell-free supernatant (CFS) of *Lactobacillus acidophilus* LA5 and *Lactobacillus casei* 431 against *Staphylococcus aureus* ATCC 25923. Antibacterial activity of both *Lactobacillus* strains was measured according to the agar spot method. The CFS was prepared by centrifugation of bacterial suspension at 4000 *g* for 10 min and the antimicrobial activity was measured using agar-well diffusion. The stability of CFSs during storage at 4.00 ± 2.00 ˚C and 25.00 ± 2.00 ˚C for a period of 4 weeks was measured based on the method of broth micro-dilution assay. Moreover, biofilm removal potential of CFS on 2-days-old biofilm of *S. aureus* developed on polystyrene and glass surfaces was also determined. The efficacy of CFS on bacterial biofilm established on the glass surface was also observed using fluorescence microscope. Results showed that inhibition zones of *L. acidophilus* (50.26 mm) were greater than *L. casei* (37.06 mm). The minimum inhibitory concentration of both CFSs remained stable (40 mg mL^{-1}) during the storage for 28 days at 4.00 and 25.00 ˚C and storage temperature did not affect the antibacterial effectiveness of CFS. The addition of both CFSs significantly removed biofilm developed on both tested surfaces in a concentration-dependent manner. Biofilm removal property of *L. acidophilus* CFS was generally better than *L. casei* CFS which was confirmed by fluorescence microscope. The application of CFS of probiotic strains (i.e. *Lactobacillus*) as antibacterial and biofilm removal compounds could be very suitable to control the growth of food-borne pathogens.

اثرات مایع رویی فاقد سلول *لاکتوباسیلوس اسیدوفیلوس* LA5 و *لاکتوباسیلوس کازئی* ۴۳۱ علیه شکل آزاد و بیوفیلم *استافیلوکوکوس اورئوس*

چکیده

این مطالعه جهت ارزیابی پایداری، خصوصیات ضدباکتریایی و قابلیت حذف بیوفیلمی مایع رویی فاقد سلول (CFS) *لاکتوباسیلوس اسیدوفیلوس* LA5 و *لاکتوباسیلوس کازئی* ۴۳۱ علیه *استافیلوکوکوس اورئوس* انجام گرفت. فعالیت ضدباکتریایی هر دو گونه *لاکتوباسیلوس* بر اساس روش نقطه‌ای تعیین گردید. CFS به روش سانتریفوژ سوسپانسیون باکتری در ۴۰۰۰ شتاب گرانشی به مدت ۱۰ دقیقه تهیه و فعالیت ضدمیکروبی به روش انتشار در آگار تعیین گردید. پایداری CFSs در دمای نگهداری ۲/۰۰ ± ۴/۰۰ و ۲/۰۰ ± ۲۵/۰۰ درجه سانتی‌گراد به مدت چهار هفته به روش میکرودایلوشن بررسی گردید. همچنین، قابلیت CFS در حذف بیوفیلم دو روزه *استافیلوکوکوس اورئوس* تشکیل شده روی سطح پلی استیرن و شیشه نیز مورد بررسی قرار گرفت. اثر بخشی CFS بر بیوفیلم تشکیل شده باکتری روی سطح شیشه نیز با استفاده از میکروسکوپ فلورسنس ارزیابی گردید. نتایج نشان داد ناحیه مهاری *لاکتوباسیلوس اسیدوفیلوس* (۵۰/۲۶ میلی‌متر) بیشتر از *لاکتوباسیلوس کازئی* (۳۷/۰۶ میلی‌متر) بود. حداقل غلظت مهاری هر دو CFS در طول نگهداری ۲۸ روزه در دمای ۴/۰۰ و ۲۵/۰۰ درجه سانتی‌گراد ثابت باقی ماند (۴۰ میلی‌گرم در میلی‌لیتر) و دمای نگهداری اثری بر کارایی ضدباکتریایی CFS نگذاشت. اضافه کردن هر دو CFS به طور معنی‌داری در یک روند وابسته به غلظت باعث حذف بیوفیلم تشکیل شده بر روی هر دو سطح آزمایشی گردید. خاصیت حذف بیوفیلم CFS *لاکتوباسیلوس اسیدوفیلوس* عموماً بیشتر از CFS *لاکتوباسیلوس کازئی* بود که این یافته توسط میکروسکوپ فلورسنس تأیید گردید. استفاده از CFS گونه‌های پروبیوتیک (از جمله *لاکتوباسیلوس*) به‌عنوان ترکیبات ضدباکتریایی و ضدبیوفیلم برای کنترل رشد باکتری‌های بیماری‌زای با منشاء غذایی می‌تواند بسیار مناسب باشد.

واژه های کلیدی: باکتری های اسید لاکتیک، پروبیوتیک، حذف بیوفیلم، ضدمیکروبی

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Introduction

Staphylococcus is a genus of opportunistic Gram-positive bacteria causing a wide spectrum of disease in human and different animals. *Staphylococcus aureus* is well known as a common food poisoning pathogen because the bacteria produce different endotoxins during growth on various food commodity.[1] The bacteria are also parts of the normal skin and mucosa flora of different mammalians, then during food transport and packaging, *S. aureus* may transfer from workers contaminated hands into food matrix and acts as a potential source of contamination in a food plant and processing units.[2]

Biofilm is a community of microorganisms in which the cells adhere to various food and non-food contact surfaces and produce extracellular polymeric substances, which mainly consist of polysaccharides, but also include proteins, lipids and nucleic acids. This type of microbial association is highly resistant to environmental stress and antimicrobial compounds. Biofilm has been known as a permanent source for the propagation of foodborne pathogens and spoilage microorganisms from surfaces into food matrix.[3-5] It is worth pointing out that contamination reduction, control and prevention of biofilm development and proposing new and novel biofilm removal compounds are essential to combat with the biofilm on different surfaces.[6]

Lactic acid bacteria (LAB) and their produced compounds were proposed as a potential biofilm biocontrol agent. The LAB are diverse groups of bacteria with a long history of use in medicine and food. They are Gram-positive bacteria include *Lactococcus, Lactobacillus, Pediococcus, Leuconostoc* and *Streptococcus*.[7] *Lactobacillus* is the most diverse genus of LAB group. Species of the genus produce some compounds such as bacteriocins, organic acids and hydrogen peroxide with special applications in the health and nutrition due to their effective antimicrobial activity.[8] Generally, these compounds are secreted into broth medium, also known as supernatant, during bacterial propagation. Along with LAB which interact with the development of bacterial biofilm on different surfaces,[9,10] the supernatant of LAB also exhibits biofilm removal activity against food-borne pathogens.[4,8,11-13] In line with that, the purpose of this study was to evaluate antibacterial and biofilm removal activity of cell-free supernatant (CFS) of *L. casei* 431 and *L. acidophilus* LA-5 against *S. aureus*.

Materials and Methods

Bacterial preparation. The *L. casei* 431, *L. acidophilus* LA-5 and *S. aureus* ATCC 25923 were kindly obtained from Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, Urmia University. Fresh microbial suspensions of *S. aureus* and *Lactobacillus* strains were prepared by transferring 50.00 µL of lyophilized culture respectively into 5.00 mL tryptic soy broth (TSB; Merck, Darmstadt, Germany) and de Mann, Rogosa and Sharpe (MRS; Scharlau, Barcelona, Spain) broth and standardized approximately by visible-ultraviolet spectrophotometer (Amersham Pharmacia Biotech Inc., Buckinghamshire, UK) at 600 nm.[11]

Antibacterial activity of *Lactobacillus* against *S. aureus.* Antibacterial activity of both *Lactobacillus* strains was measured according to the agar spot method as described by Awaisheh and Ibrahim.[14] An aliquot of 10.00 µL of a 48 hr culture (our preliminary investigation revealed that the 48 hr culture is better than 24 hr culture in the case of antibacterial activity) of *Lactobacillus* was spotted in the middle part of a plate containing MRS agar and the plate was incubated at 37.00 ± 1.00 ˚C for 24 hr in the CO_2 incubator (Sina Lab, Tehran, Iran). The inoculated plates were overlaid with 7.00 mL of soft TSB agar (Merck; 0.80% agar) containing ~6 $\log_{10}$ CFU mL^{-1} of *S. aureus*. Plates were incubated at 37.00 ± 1.00 ˚C for 24 hr and diameter of the inhibition zone was measured using a digital caliper in triplicate.

Preparation of *Lactobacillus* CFS. Each LAB isolate was inoculated in MRS broth and incubated at 37.00 ˚C for 48 hr in the CO_2 incubator. Then, the bacterial suspension was centrifuged (Farzaneh Arman Co, Isfahan, Iran); at 4000 *g* for 10 min and CFS was decanted aseptically, sterilized using a 0.20 µm pore size filter (Millipore Inc., Billerica, USA) and used freshly.[15]

Antibacterial activity of *Lactobacillus* CFS against *S. aureus.* Antibacterial activity of *L. acidophilus* CFS (CFSa) and *L. casei* CFS (CFSc) was determined according to agar-well diffusion method.[16] *S. aureus* lawns (~6 $\log_{10}$ CFU mL^{-1}) on Mueller Hinton agar (Merck) were prepared and 5 mm circular wells were cut using a cork borer. Each well was poured with 100 µL prepared CFS, the plate was incubated at 37.00 ± 1.00 ˚C for 24 hr and diameter of the inhibition zone was measured using a digital caliper in triplicate. The storage stability of CFSa and CFSc during storage at 4.00 ± 2.00 ˚C and 25.00 ± 2.00 ˚C for a period of four weeks was determined according to the method of broth micro-dilution assay in 96 wells polystyrene flat-bottomed microtitre plates based on Clinical and Laboratory Standards Institute (CLSI) guidelines[17] and expressed as minimum inhibitory concentration (MIC).

Biofilm removal activity of *Lactobacillus* CFS. Biofilm removal assay was carried out using 24-well flat-bottomed polystyrene microtiter plate.[18] Each well was poured by 1.80 mL of TSB broth and 0.20 mL bacterial suspension with OD_{600} = 0.10 to obtain a suspension with 10^7 CFU mL^{-1} per well and the plate was incubated at 37.00 ± 1.00 ˚C for 24 hr. Additionally, wells containing only TSB were also prepared as controls. Then, the bacterial content of each well was carefully decanted and the plates were washed using phosphate buffered saline(PBS; Sigma-

Aldrich, St. Louis, USA) and allowed to dry at ambient condition. In next step, aliquots of 2.00 mL of 40, 60, 80 and 100 mg mL^{-1} of CFS were gently added to the corresponding wells and incubated for 30 min at ambient temperature. The CFSs were immediately decanted and plates were washed again using PBS, allowed to dry, then stained with 2.00 mL of 1.00% crystal violet (CV; Sigma-Aldrich) for 30 min, washed gently using tap water to remove the excess color and dried. Biofilm was quantified by eluting CV with 2 mL of acetic acid 33.00% (Sigma-Aldrich) and the optical absorbance determination of the eluted dye at 540 nm. Wells containing TSB and bacteria without any treatment were designed as negative controls. To estimate the reduction percent of biofilms exposed to different concentration of CFS, the below formula was applied:

$$Reduction\ percent = \frac{(C-B)-(T-B)}{(C-B)} \times 100$$

where, C is OD_{540nm} of control wells, B is OD_{540nm} of negative controls and T is OD_{540nm} of treated wells.

Biofilm establishment on glass coupons. Glass coupons (10 × 10 × 1 mm) were cut and sanitized as surfaces to develop biofilm.[19] The procedure was conducted by adding 1.80 mL of TSB broth and 0.20 mL bacterial suspension with OD_{600} = 0.10 to obtain a suspension with 10^7 CFU mL^{-1} per well. Before incubating the plate at 37.00 ±1.00 ˚C for 24 hr, a glass coupon was placed in each well and allowed to develop a biofilm. Subsequently, the procedure was continued in a similar way as described in microtiterplate assay.

Epifluorescence microscopy. The efficacy of CFSa and CFSc on developed biofilm on glass coupons was assessed using fluorescence microscope (BX51TRF; Olympus, Tokyo, Japan). Coupons were stained with acridine orange solution (20 µg mL^{-1}) for 2 min. The dried surface was then visualized by fluorescence microscope.[20]

Statistical analysis. All analyses were performed in triplicates. Data were analyzed using the general linear model of Statistical Analysis Systems (version 9.4; SAS Inst., Inc., Cary, USA). Statistically significant differences were determined using Fisher's least significant difference tests.

Results

Antibacterial activity of *Lactobacillus* spp. The LAB secrete compounds including bacteriocin, lactic acid and hydrogen peroxide exhibiting antibacterial activity against pathogens. Antibacterial activity of *L. acidophilus* and *L. casei* against *S. aureus* was evaluated according to the agar spot method and the results are reported in Figure 1. The diameters of the inhibition zones of *L. acidophilus* were significantly ($p < 0.05$) greater (50.26 mm) than those of *L. casei* (37.06 mm).

Antibacterial activity of *Lactobacillus* CFS. Antagonistic activity of *Lactobacillus* CFS is illustrated in Figure 1. The higher diameter of inhibition zone (16 mm) against *S. aureus* was recorded with CFSa, whereas the inhibitory diameter for CFSc was 13 mm.

Biofilm removal activity of *Lactobacillus* CFS. The addition of CFSa and CFSc significantly ($p < 0.05$) removed biofilm developed on both tested surfaces in a concentration-dependent manner. Biofilm removal property of CFSa was generally better than that of CFSc. On polystyrene surface (Fig. 2), CFSa and CFSc at 100% concentration removed 70.60 and 65.30% of *S. aureus* biofilm, respectively. The biofilm removal activity was dropped significantly as the CFS concentration decreased from 100 to 40.00%. A similar trend was also demonstrated on glass surface (Fig. 3). It should be noted that CFS revealed potent activity on two-day-old biofilm of *S. aureus* on the glass surface, so that, CFSa removed 87.00% of developed biofilm.

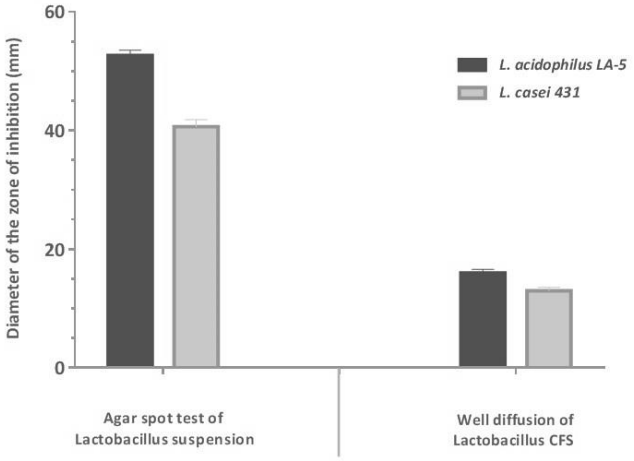

Fig. 1. Antibacterial activity of *L. acidophilus* and *L. casei* suspensions and CFSs against *S. aureus*. CSF: Cell-free supernatant.

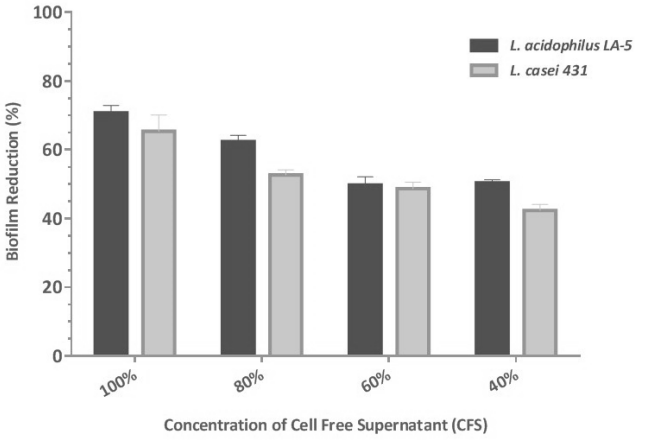

Fig. 2. Biofilm removal activity of different concentrations of *L. acidophilus* and *L. casei* CFSs against 2-day-old biofilm of *S. aureus* established on polystyrene surface. CSF: Cell-free supernatant.

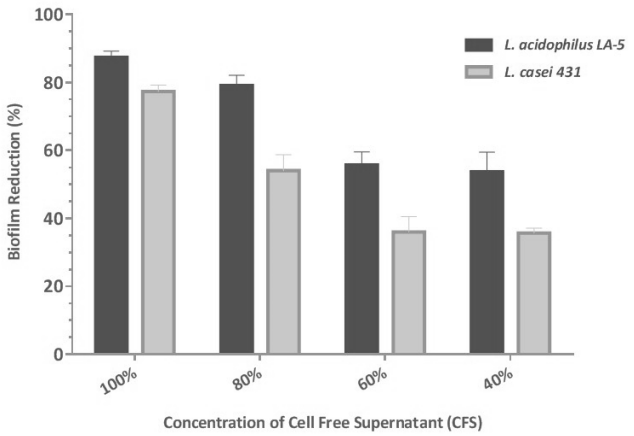

Fig. 3. Biofilm removal activity of different concentrations of *L. acidophilus* and *L. casei* CFSs against 2-day-old biofilm of *S. aureus* established on glass surface. CSF: Cell-free supernatant.

The graphs of biofilm developed on glass coupons and biofilm treated with *lactobacillus* CFS are shown in Figure 4. The photomicrographs revealed the homogeneous development of *S. aureus* biofilm on the glass surface and the removal potential of CFS on developed biofilm after an exposure time of 30 min.

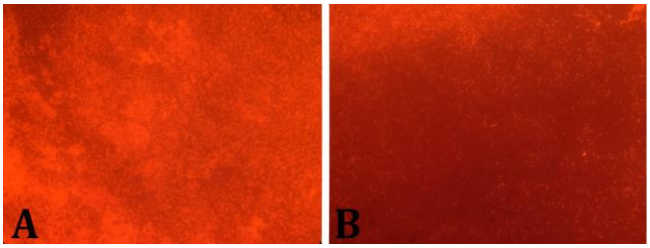

Fig. 4. The surface of glass coupon established with biofilm of *S. aureus* (A) and treated with CFS (B) stained with acridine orange and viewed using fluorescence microscope at 400× magnification. CSF: Cell-free supernatant.

Discussion

The *L. casei* 431 and *L. acidophilus* LA-5 are well-known probiotic LAB widely used in liquid fermented dairy products.[21] Probiotic strains reveal antibacterial activity against pathogenic bacteria through antimicrobial compounds production and competition with harmful pathogens for adhesion to selected surfaces.

Mirnejad *et al.*[22] studied the efficacy of CFSc on multiple drug resistant isolates of *Shigella Sonnei* and *Sh. Flexneri*. In this study, CFSc showed potent antibacterial activity against both pathogens. After adjusting pH to 7, the antibacterial activity was disappeared revealing the importance of organic acid of CFSc in the antibacterial mechanism of action and no involvement of bacteriocins. Similar results were also observed in our study. In contrast, Sharma *et al.*[23] demonstrated that CFS of *Lactobacillus* isolated from curd and human milk does not show antibacterial activity against *S. aureus*, *L. monocytogenes*, *E. coli*, and *Klebsiella pneumonia* revealing that *Lactobacillus* strains exert a varying level of antagonistic activity against indicator pathogens. The moderate-to-good antibacterial activity of *Lactobacillus* CFS was reported against *B. cereus*.[24] In this study, it was shown that subsequent neutralizing of CFS of both *Lactobacillus* strains to pH ~6.50 with 1 N NaOH, eliminated the antibacterial property of CFS. The antibacterial activity of *L. acidophilus* LA-5 was not related to bacteriocin production by the strain. It has been demonstrated that the *L. acidophilus* LA-5 only produces a specific bacteriocin known as lactacin B, when the bacteria grow in co-culture with starter cultures (*S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*).[25] Production of organic acids is the mechanism of action of both probiotic bacteria against *S. aureus*. The CFS of *L. delbrueckii*, a starter culture used for yogurt production, also revealed a suitable antibacterial activity against *E. coli* which was mainly related to lactic acid produced by *L. delbrueckii*.[26]

The stability of CFS during storage at refrigerator and room temperature has a vital importance for the commercial use of this antimicrobial solution. Antibacterial activity of CFS obtained from *L. acidophilus* and *L. casei* during storage at 4.00 ± 2.00 ˚C and 25.00 ± 2.00 ˚C for a period of four weeks showed that MIC of both CFSs at both storage temperatures was 40 mg mL^{-1}. The MICs remained stable during the storage and temperature of incubation did not affect the antibacterial effectiveness of CFS. It was reported that inhibitory compounds of *L. acidophilus* are not influenced by environmental factors.[22,27] The only factor influencing the durability of CFS is an increment in pH. The CFS activity was decreased with an increment in pH. Maximal activity was obtained at pH = 3.20.[28]

A dose-dependent biofilm reduction was also reported when different dilutions of *L. brevis* CD2 CFS (1/10 and 1/100) were evaluated and 1/100 concentration was less effective in *Prevotella melaninogenica* biofilm reduction (31.00%).[29] Twenty-four hr CFS of *L. brevis* dropped an 8.00% decrease in optical density value, whereas 96 hr CFS declined a 56.00%, revealing the importance of incubation time of LAB on the activity of prepared CFS on developed biofilm of bacterial species.

The high biofilm removal activity of CFSa would be related to the certain anti-biofilm compounds including exopolysaccharide and bio-surfactant releasing into CFS in different extents depending on the bacterial species. According to Kim *et al.*,[30] exopolysaccharide of *L. acidophilus* at 1 mg mL^{-1} concentration removed 87.00% and 94.00% of *Escherichia coli* biofilm formed on polystyrene and polyvinyl chloride surfaces, respectively. The next potential biofilm removal agent of CFS is bio-surfactant. The anti-biofilm activity of LAB is a species-dependent phenomenon. *L. reuteri*, *L. acidophilus*, *L. rhamnosus* and *L. paracasei* revealed anti-biofilm activity

only on *Streptococcus mutans* and *S. oralis*.[31] Our conclusion demonstrated that exopolysaccharide is the potential material of CFS in biofilm removal from both hydrophobic and hydrophilic surfaces.

Moreover, our observation showed that greater amounts of biofilm mass were formed on glass coupon compared to polystyrene surface. These results suggested that *S. aureus* had a tendency toward hydrophilic surface rather than hydrophobic surface due to the probable hydrophilic property of the bacterial outer surface. According to Auger *et al*.,[32] hydrophobicity of different bacteria is strongly strain-dependent.

In this work, CFSs of *Lactobacillus acidophilus* LA5 and *Lactobacillus casei* 431 were prepared and their antibacterial and biofilm removal activity were assessed against *S. aureus*. The results revealed a suitable antibacterial activity (> 12 mm diameter of the inhibition zone) and potent biofilm removal potential against biofilm of pathogen established on polystyrene and glass surfaces. Generally, CFS of *L. acidophilus* showed greater antibacterial and biofilm elimination compared to *L. casei*. The current research underlines that the antibacterial activity of both probiotic bacteria was related to the lactic acid production and bacteriocins were not involved.

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Conflict of interest

There is no conflict of intereset to delcare.

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