The antibacterial and antibiofilm activity of *Lactobacillus acidophilus* isolated from vagina against methicillin-resistant *Staphylococcus aureus*

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Background
Bacterial infections caused by the genus *Staphylococcus* represent a grave threat to both humans and animals, and they are a major concern to health authorities. Over the past few decades, methicillin-resistant *Staphylococcus aureus* (MRSA) has been recognized as the principal nosocomial pathogen worldwide.
Objective
To investigate the antibacterial and antibiofilm activity of *Lactobacillus acidophilus* against locally isolated MRSA at different concentrations.
Materials and methods
MRSA isolates were tested against some antibiotics for testing antibiotic sensitivity. The antibacterial activity of *L. acidophilus* based on the minimal inhibitory concentration (MIC) of *L. acidophilus* that inhibits the visible growth of MRSA isolates was assessed using the microdilution method. Estimation of MIC of *L. acidophilus* was done. Biofilm quantification assay was then used for the determination of the antibiofilm activity of *L. acidophilus.* The MIC concentration was assessed by a microtiter reader.
Results and conclusion
The antibacterial activity was tested by agar diffusion method and broth microdilution method. The microdilution method was used to determine the MIC of *L. acidophilus*, whereas the antibiofilm activity was determined by using 96-well polystyrene microtiter plates. The results revealed that *L. acidophilus* has antibacterial activity in a concentration-dependent manner. The average diameter zone of inhibition observed against MRSA isolates ranged from 11±0.5 to 18±0.5 mm Moreover, at subinhibitory concentration, this extract developed an isolate-specific antibiofilm effect and presented highly significant ($P<0.05$) variability in biofilm formation before and after addition of *L. acidophilus*. AA3 and AA12 isolates gave the lowest and highest antibiofilm activity, respectively. In conclusion, the supernatant of *L. acidophilus* is a promising alternative medication that can be used to treat the infection caused by MRSA.

Keywords:
antibacterial, antibiofilm formation, methicillin-resistant Staphylococcus aureus

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Introduction

The widespread therapeutic use of antimicrobials in humans and the administration of antimicrobials as growth promoters in food for animals have been associated with the development of resistant bacteria. Most antibiotics are administered to patients empirically before any diagnosis has been made based on results from cultures [1,2]. In one study, only 17% of methicillin-resistant *Staphylococcus aureus* (MRSA)-infected patients were initially given an effective antibiotic [3]. Such MRSA colonies may be present in an individual's global microbial population as part of the natural balance of his/her own microbial flora [4]. MRSA colonization generally precedes MRSA infection, and it plays a major role in the spread of this organism within human communities and health care facilities [5]. MRSA is a biofilm-forming pathogen that adheres to numerous surfaces. In humans and animals, its main habitats are the nasal membranes and skin. Such colonies cause life-threatening infections such as pneumonia, sepsis, osteomyelitis, and endocarditis. Patients with MRSA colonization are often colonized for long periods of time. Approximately 50% of patients with MRSA infection are still colonized after 1 year [6].

Treatment with selected probiotic strains may be the ultimate answer to decolonization of MRSA because they do not increase the risk of multidrug resistance of

this pathogen [7]. The alarming increase in inappropriate antibiotic use along with bacterial resistance has led to renewed interest in ecological methods to prevent infections, which makes probiotics a very interesting field for further research. For example, a patient in Japan with a decubitus ulcer colonized by MRSA was successfully treated with a probiotic *Lactobacillus* preparation [8]. One nonantibiotic strategy to combat the bacterial infections involves the selection and promotion of endogenous bacterial flora to interfere with pathogenic bacterial adhesion [9]. Lactic acid bacteria (LAB) strains are potentially promising because they generate bactericidal bioactive peptides (bacteriocins) and enzymes that are able to control biofilm formation and the growth of the pathogens. Nisin is the best defined bacteriocin [10] produced by species *Lactococcus* that has been approved for use in food products [11]. Certain LAB strains have been reported to be highly antagonistic to biofilm-forming *Staphylococcus aureus* [12].

The genus *Lactobacillus* has a long history of safe use, especially in the dairy industry, and it plays a major role in the transformation of fermented milk and other food products. Over the past few decades, there has been increased impetus to introduce new *Lactobacillus* strains into foodstuffs with the goal of exerting a beneficial health effect when ingested by humans or animals [13]. Four types of LAB strains have been studied as competitive inhibitors of pathogenic organisms [11]. These strains are *Lactobacillus casei* 99p rhamnosus GG, *L. casei* shirota, *Bifido bacterium breve* yacult, and *Lactobacillus acidophilus* johnsonii [14]. Beneficial effects conferred by *Lactobacilli*, including inhibition of gram-negative and gram-positive pathogenic bacteria, were described by Charlier and colleagues, who reported that *Lactococcus lactis* had a specific antagonistic effect against *S. aureus*. Antimicrobial activity produced by LAB strains appears to be unrelated to the acidification of the medium. LAB strains were reported to exert a strong inhibitory effect on *S. aureus* growth in milk. Several suggestions have been proposed for inhibition of *S. aureus* by LAB. These include production of bacteriocins, hydrogen peroxide, and organic acids such as lactic and acetic acid [15].

Materials and methods

Microorganisms (isolation and identification)

L. acidophilus was obtained from the microbiology laboratory at the Department of Biotechnology, College of Science, University of Baghdad. This isolate was collected from vagina samples. Moreover, 15 MRSA isolates were obtained from the microbiology laboratory at Department of Biology, College of Science, University of Baghdad. These isolates were collected from different clinical infection site (nose, vagina, tongue, and wound pus). Identification was previously performed using 16SrRNA [16], whereas methicillin resistance was tested phenotypically via cefoxitin disk method [17] and molecularly via PCR technique [18].

Antibiotics sensitivity test

MRSA isolates were tested against the following antibiotics: oxacillin (5 µg/disk), vancomycin (20 µg/disk), imipenem (10 µg/disk), gentamicin (10 µg/disk), and erythromycin (15 µg/disk).

Preparation of bacterial suspension

The inoculum was prepared as follows: a few colonies were picked from overnight bacterial cultures and were transferred into 5 ml of normal saline. Subsequently, it was adjusted to be balanced with a 0.5 McFarland tube to give 1.5×10^8 CFU/ml. Then, the bacteria were washed twice with phosphate buffer saline (PBS) after centrifugation for 20 min at 2000 rpm and resuspended in PBS again.

Antibacterial activity of *Lactobacillus acidophilus*

Agar diffusion method: the antibacterial activity of *L. acidophilus* was investigated by the agar well diffusion method as described by Al-Gbouri and Hamzah [17]. Five serial dilutions (80, 40, 20, 10, and 5 mg/ml) were achieved using PBS as a diluent. With the aid of a sterile cotton swab, the inoculum of bacteria (prepared as described previously) was uniformly spread on the surface of a Mueller-Hinton plate. Overall, 50 µl of each dilution was added to each of the five wells (6 mm in diameter holes were cut in the agar). PBS alone was used as control. Finally, all plates were incubated anaerobically by using candle jar with presence of 5% of CO_2 overnight at 37°C. The resultant inhibition zones were measured in mm. Assays were carried out in triplicate.

Microdilution method: for determining the minimal inhibitory concentration (MIC) of *L. acidophilus* that inhibits the visible growth of MRSA isolates, the protocol mentioned by Andrews [18] was followed. The double serial dilutions of *L. acidophilus* were started from 0.087 to 80 mg/ml, which was prepared from a stock solution (100 mg/ml) using the Mueller-Hinton broth as a diluent. Further, an aliquot of 100 µl of each dilution was distributed in microtiter plates. Control wells contained bacteria-free growth media

plus PBS. All wells were inoculated with 10 µl of a bacterial suspension (1.5×10^8 CFU/ml). All trials were repeated in triplicate. Afterward, the microtiter plates were incubated aerobically for 24 h at 37°C.

Biofilm quantification assay: quantification of biofilm formation was assessed as described by Atshan *et al.* [19]. In brief, each isolate was propagated in tryptic soy broth containing 1% glucose at 37°C for 24 h; thereafter, bacterial culture was adjusted to McFarland standard no. 0.5. A volume of 200 µl of an isolated culture was added to three wells of sterile 96-well polystyrene microplates. All plates were covered with their lids to avoid evaporation and incubated under aerobic conditions at 37°C for 24 h. Three wells filled with bacteria-free tryptic soy broth were considered as a negative control. After incubation, growth medium was carefully removed from the biofilm plate, gently washed thrice with distilled water, dried, and fixed at 60°C for 1 h. Afterward, an aliquot of methanol (200 µl) was added to each well for 15 min at room temperature. Subsequently, the plates were washed thrice and covered with 0.1% crystal violet for 15 min at room temperature. Subsequently, plates were washed thrice with tap water and dried at 37°C for ~30 min. The adherent cells were resolubilized by the addition of 96% ethanol for 10 min. The absorbance of stained wells was determined at 630 nm with a microtiter reader (BioTek, USA).

Antibiofilm activity of *L. acidophilus* at MIC concentration: the same protocol was used for the biofilm formation assay that was previously mentioned. However, tryptic soy broth containing bacterial suspension (*L. acidophilus*) at MIC was added after biofilm formation. The microtiter plates were incubated at 37°C for 24 h. After that, all wells were washed, stained, and read at 630 nm. Positive controls were performed as well by adding 200 µl of *L. acidophilus*-free fresh bacterial suspension (compatible to 0.5 McFarland standard). The antibiofilm activity of samples was given as the percentage of inhibition and was expressed as follows:

%inhibition=[OD_{630} of control (without bacterial suspension)-(OD_{630} value in the presence of bacterial suspension)/OD_{630} control×100] [20].

Statistical analysis

Data were presented as mean±SD. *t* test was employed for the evaluation of the efficacy of bacterial suspension (*L. acidophilus*). *P* value of less than 0.05 was considered significant.

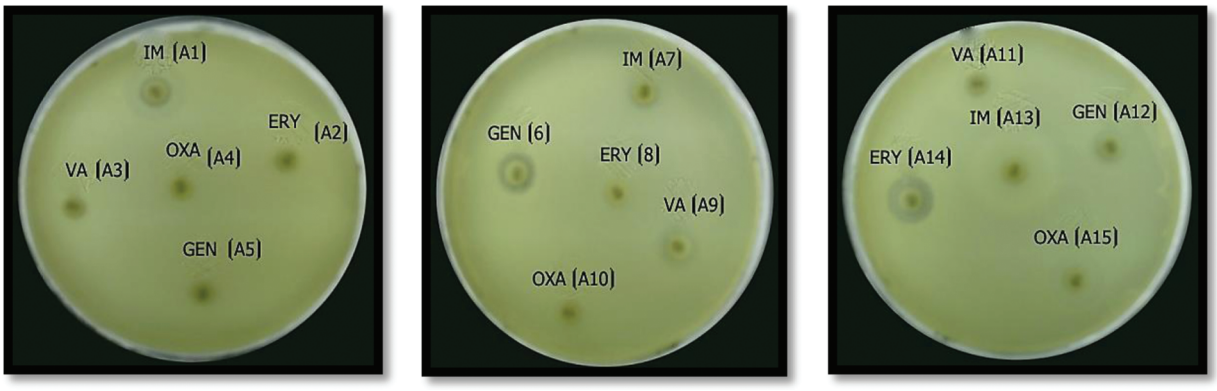
Results and discussion

Regarding the susceptibility of bacteria to antibiotics, the results showed all tested isolates were resistant to all types of antibiotics (imipenem, erythromycin, oxacillin, gentamicin, and vancomycin), as shown in Fig. 1.

Determination of the effect of *Lactobacillus acidophilus* on methicillin-resistant *Staphylococcus aureus*

The results have shown that *L. acidophilus* has good antimicrobial efficacy against MRSA isolates in a concentration-dependent manner. The different concentrations of *L. acidophilus* showed inhibitory properties against all tested isolates. Relatively large zones of inhibition were seen: 18±0.5, 15±0.5, 13±0.5, and 11±0.5 and 9±0.5 mm at 80, 40, 20, 10, and 5 mg/ml, respectively, as shown in Fig. 2. Antimicrobial activity of supernatant from *L*actobacillus *plantarum* (ADK2) isolate had a high inhibitory effect against *S.*

Figure 1



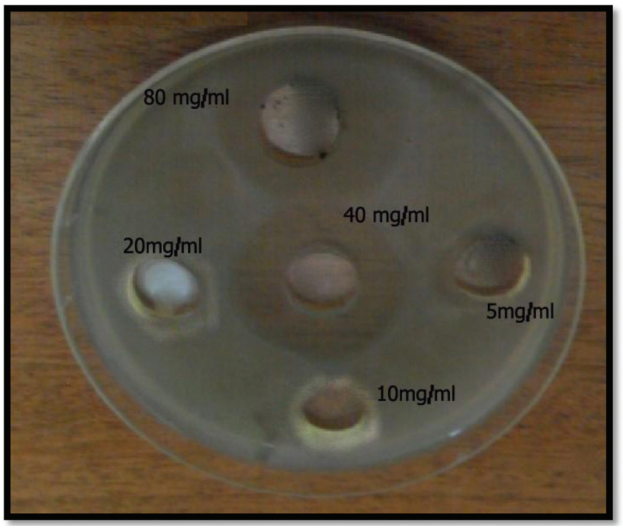
Antibiotic sensitivity of *Lactobacillus acidophilus* against some antibiotics (ERY, erythromycin; GEN, gentamicin; IM, imipenem; OXA, oxacillin; VA, vancomycin).

aureus and Pseudomonas aeruginosa with 34.18 and 38.43 mm, respectively [21]. The antimicrobial activity of purified biosurfactant of *L. plantarum* was examined against some microorganisms. The results showed that the biosurfactant had different antibacterial effect on the bacterial growth [22].

Estimation of minimal inhibitory concentration of *Lactobacillus acidophilus*

The antibacterial efficacy of the *L. acidophilus* calyces was evaluated using MIC assays. The results revealed that the MIC value was 8 mg/ml for all 15 MRSA isolates. The LAB strains were assayed for production of antimicrobial substances such as bacteriocin, hydrogen peroxide, and organic acids using the agar well-diffusion technique described by Touré and colleagues.

Figure 2



Inhibition zone of the *Lactobacillus acidophilus* isolated from vagina against methicillin-resistant *Staphylococcus aureus* at different concentration.

Antibiofilm activity of *Lactobacillus acidophilus*

Biofilm production is considered as a marker of virulence. Numerous new approaches have been suggested for studying biofilm in terms of biofilm physiology and structure. In this study, the ability of biofilm-producing MRSA isolates was evaluated using presterilized 96-well polystyrene microtiter plates, which considered as a standard test for the detection of biofilm biomass [6,23,24]. Due to the crystal violet stained only the cells, not the slime materials and the cell, which is not in the biofilm structure is rinsed off by washing steps [23,24]. To estimate biofilm intensity, absorbance was determined at 630 nm by a microplate reader. Given that, absorbance values represented the intensity of the biofilm thickness that formed by the studied isolates on the surface of the microtiter well. The results of the current study summarized in Table 1 showed that all bacterial isolates were biofilm producer, but the biofilm intensity varies from one isolate to another. Approximately, 70% of isolates formed strong biofilm, whereas only 50% developed moderate biofilms according to OD630 limits (0.110–0.244 represented moderate biofilm, whereas OD630>0.244 represented the strong biofilm). To determine the effect of MIC of *L. acidophilus* on biofilm formation, 15 isolates were incubated with 10 mg/ml of the bacterial suspension; the results clarified that the MIC level was effective against all bacterial isolates. Yet, the effectiveness differs from one

Table 1 Biofilm formation by methicillin-resistant *Staphylococcus aureus* isolates before and after treatment with *Lactobacillus acidophilus* and percentage of inhibition

Isolates	Before treatment		After treatment		*t* test
	OD630±SD	Biofilm intensity	OD630±SD	Percentage of inhibition	
AA1	0.181	Moderate	0.159	10	7.72E-03
AA2	0.129	Moderate	0.101	22	2.57E-06
AA3	0.184	Moderate	0.161	9	6.62E-05
AA4	0.201	Moderate	0.104	45	2.21E-07
AA5	0.224	Moderate	0.103	51	1.11E-08
AA6	0.320	Strong	0.019	61	2.04E-10
AA7	0.380	Strong	0.099	70	8.65E-09
AA8	0.281	Strong	0.088	67	2.00E-09
AA9	0.291	Strong	0.082	69	2.71E-08
AA10	0.317	Strong	0.110	63	1.10E-09
AA11	0.453	Strong	0.241	44	6.62E-05
AA12	0.388	Strong	0.100	71	8.74E-10
AA13	0.221	Moderate	0.091	49	0.90E-08
AA14	0.118	Moderate	0.098	20	2.46E-05
AA15	0.281	Strong	0.079	67	2.51E-07

isolate to another and presented highly significant ($P<0.05$) variability in biofilm formation either with or without the addition of *L. acidophilus*. The lowest antibiofilm activity was exhibited in AA3 isolate with 9% inhibition, whereas the AA12 gave the strongest antibiofilm activity. The percentage of inhibition was 71%. These results indicated that the *L. acidophilus* at MIC level inhibits the biofilm formation of MRSA isolates. Antibiofilm activity of the *L. plantarum* cell-free supernatants (CFS) showed good stability in the presence of different chemicals including EDTA, SDS, and tween 80. High stability of the CFS in the presence of the mentioned chemicals indicates its stable antibiofilm potential to be used as biopreservatives and pharmaceutical compounds. Antibiofilm potential of *L. plantarum* CFS was completely inhibited by proteinase K. This finding has been reported previously for bacteriocins produced by *L. plantarum*, indicating the role of bacteriocins in biofilm inhibition of the CFS from *L. plantarum* spp. [25,26]. In addition, antibiofilm activity of the CFS was not strongly affected by the lipase, which shows the antibiofilm activity is not dependent on the lipid compounds of the CFS.

Conclusion

The supernatant of *L. acidophilus* is a promising alternative medication that can be used to treat the infection caused by MRSA.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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