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ORIGINAL ARTICLE

Investigation of Some *Lactobacillus* SPP. against Methicillin-Resistant *Staphylococcus aureus* Isolated from Iraqi Patients

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ABSTRACT

Evaluate the antimicrobial activity of some *Lactobacillus (L.)* species against *Staphylococcus aureus* isolates with varying biofilm-forming capacities is the aim of this study. *L. acidophilus*, *L. rhamnosus*, and *L. johnsonii* were assessed using the microtiter plate method based on minimum inhibitory concentration (MIC) across serial dilutions. The result demonstrated a significant reduction in bacterial growth at lowered dilutions, with a gradual decrease in inhibition at higher dilutions. *L. rhamnosus* exhibited the highest inhibitory activity, particularly against strong biofilm-production isolates, reaching up to 94.8% inhibition at low dilutions. In contrast, *L. johnsonii* showed the lowest activity, especially at higher dilutions. Moderate biofilm-formation isolates were more susceptible to inhibition compared to strong biofilm. The analysis of supernatants indicated that antimicrobial activity was mainly associated with organic acids, as NaOH treatment reduced inhibitory effects, while trypsin and catalase treatments showed minimal impact. *L. rhamnosus* could be a good alternative to the use of antimicrobial agents in the treatment of *S. aureus* biofilm associated infections.

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1- INTRODUCTION

Opportunistic pathogen *Staphylococcus aureus*, which causes superficial skin infections to life-threatening conditions like bacteremia, endocarditis and sepsis, is a major cause of both community and hospital-acquired infections. The clinical impact of *S. aureus* has also been enhanced by the widespread dissemination of multi-drug resistant strains, especially methicillin-resistant *S. aureus* (MRSA), that restrict the therapeutic options and elevate the morbidity and mortality rates [1, 2]. One of the most important virulence factors that plays a role in the persistence and chronicity of *S. aureus* infections is the ability to form biofilms organized communities of microorganisms within an extracellular polymeric matrix that they self-produce and that allows them to survive harsh conditions, such as antibiotic exposure and host immune responses [3, 4]. Biofilm-associated infections are a serious problem in the clinical environment, particularly in infections associated with indwelling medical devices such as indwelling urinary or vascular catheters, prosthetic joints, and implants. In biofilms, *S. aureus* cells switch to alternative metabolic states and gene expression patterns, which contribute to their resistance to antimicrobial

agents, and enhanced resistance to host defense mechanisms. This has led to the need for other and complementary antimicrobial methods that could also be effective against planktonic and biofilm associated bacterial cells [5, 6].

Over the past few years, the genus *Lactobacillus* has become a big focus of research as a potential therapeutic agent. *Lactobacillus* spp. are well known for their probiotic properties and their ability to regulate the microbial communities at different sites of the human body, such as gastrointestinal, urogenital, and skin niches. These bacteria have antimicrobial properties that can be attributed to several mechanisms such as: organic acids (e.g., lactic acid, bacteriocins, hydrogen peroxide and other bioactive metabolites). Also, they can inhibit pathogens competitively by binding to epithelial surfaces and by changing the microenvironment [7, 8]. There is increasing evidence that the inhibitory effect of *Lactobacillus* spp. is not limited to inhibiting the growth of *S. aureus*, but also disrupts the ability of *S. aureus* to form biofilms and their structural integrity. These antibiofilm activities include down regulation of key genes that are associated with the development of biofilms like Intercellular Adhesion (*icaA*,and *icaD*) and Accessory Gene Regulator (*agr*) [9], and inhibition of adhesion factors and quorum sensing systems. Moreover, some research studies have shown that, in addition to inhibiting the growth of bacteria, cell-free supernatants of *Lactobacillus* cultures can effectively inhibit established biofilms as well, thus presenting them as potential therapeutic alternatives other than antibiotics [10].

While the results were promising, several questions still remain regarding the strain-specific effects, optimal activity conditions and specific molecular mechanisms of the antimicrobial and antibiofilm activity of *Lactobacillus* against *S. aureus*. Furthermore, in vitro results need to be translated to the clinical setting, which further requires elucidation on safety and dosage levels as well as delivery systems. Among the *Lactobacillus* species used in this study, *L. acidophilus* is well known for its strong production of lactic acid and bacteriocins, which contribute to inhibition of pathogenic bacteria including *Staphylococcus aureus.* In addition, it plays an important role in maintaining microbial balance and reducing pathogen adhesion to epithelial surfaces [11]. *Lactobacillus rhamnosus* has demonstrated potent antimicrobial and antibiofilm activity against several multidrug-resistant pathogens, including MRSA, through the production of bioactive metabolites and interference with biofilm formation pathways. It is also characterized by high adherence ability and competitive exclusion of pathogens [12]. Meanwhile, *L johnsonii* exhibits antimicrobial and immunomodulatory properties and has been associated with inhibition of pathogen colonization through acidification of the surrounding environment and secretion of antimicrobial compounds. Recent studies also suggest its role in reducing inflammatory responses and supporting mucosal barrier integrity [13]. Thus, the current study was designed to assess the antimicrobial and antibiofilm properties of the selected strains of *Lactobacillus* and to understand their possible mechanisms of action against *S. aureus*.

2- MATERIALS AND METHODS

2.1 Isolation and identification of *S. aureus*

A total of 961 clinical samples was collected from patients with different infections, including wounds, burns, urine, ear, blood, high vaginal swabs, conjunctiva, synovial fluid, semen, and plural fluid. The samples were collected between May 2025 and February 2026 from Fallujah General Hospital, Ramadi General Hospital, and private laboratories in Anbar Governorate. From these samples, 104 isolates of *S. aureus* were obtained and included in this study. The collected samples were cultured on nutrient agar, blood agar, and mannitol salt agar, followed by incubation at 37°C for 24-48 hours. Identification of isolates was performed using conventional microbiological methods, including Gram staining, catalase test, coagulase test, and DNase test. Final confirmation of isolates was carried out using the VITEK-2 Compact system. Then the identification confirmed by 16S rRNA by Real-time qPCR [14].

2.2 Antibiotic Susceptibility Testing and MRSA Detection

Antimicrobial susceptibility testing was performed using the VITEK-2 Compact System with AST-GP580 cards according to CLSI (2025) [15] guidelines. The system automatically determined the minimum inhibitory concentration (MIC) values and classified isolates as susceptible, intermediate, or resistant. Methicillin-resistant *S. aureus* (MRSA) was identified based on cefoxitin and oxacillin resistance profiles.

2.3 Molecular Detection of *S. aureus*

Genomic DNA was extracted using EasuPure® Bacteria Genomic DNA kit following the manufacturer's instructions. DNA concentration and purity was measured using NanoDrop spectrophotometer. Detection of *S. aureus* was confirmed by real-time PCR targeting the *16S rRNA* gene [16] using specific primers were received

from Macrogen in lyophilized form. Amplification was performed using PerfectStart® Green qPCR SuperMix under optimized thermal cycling conditions.

2.4 Biofilm Formation Assay

2.4.1 Qualitative Method (Congo Red Agar)

Biofilm formation was initially assessed using Congo Red Agar. Black crystalline colonies indicated biofilm production, while pink colonies indicated non-biofilm producers [17, 18].

2.4.2 Quantitative Method (Microtiter Plate Assay)

Microtiter plate assay was used for the measurement of biofilm formation. Brain heart infusion broth with glucose was used to adjust the suspensions of bacteria to 0.5 McFarland and then incubated at 37°C for 24 hours. The wells were washed, fixed, stained with crystal violet and the absorbance was read at 630 nm on an ELISA reader after incubation. The ability of the isolates to form biofilms was determined as weak, moderate, or strong using optical density values [19, 20].

2.5 Antimicrobial Activity of *Lactobacillus spp*.

Three species (*L. rhamnosus, L. acidophilus, and L. johnsonii*) were cultured in de Man, Rogosa and Sharp medium (MRS) broth under anaerobic conditions at 37°C for 48 hours. Several colonies of the freshly cultured *Lactobacillus spp*. were transferred to 10-mL MRS broth and incubated under microaerophilic conditions for 24 h at 37°C. This culture medium was used to inculate 1000-mL MRS broth. The inoculated culture was incubated for 48 h at 37°C and then centrifuged at 6,000 rpm for 15 min to separate the supernatant. The supernatant was passed through a 0.22-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 Jaleel S (2020) was employed with some modifications to prepare the extract. The sterility of the supernatants was confirmed before use. The antimicrobial activity of *Lactobacillus spp*. (CFS) was characterized using the agar well diffusion method according to [21]. With slight modifications, the CFS was divided into four treatments: Trypsin (1 mg/mL) for bacteriocin detection, catalase (0.5 mg/mL) for H_2O_2 identification, pH adjustment to 6.5 for organic acid neutralization, and a non-treated positive control. One handered microliters of the prepared CFSs were introduced into 6mm wells on Mueller-Hinton agar previously seeded with *S. aureus* (1.5×10^8 CFU/mL), and the plates were incubated at 37°C for 24 h. After incubation, the inhibition zones were measured to identify the specific inhibitory agents.

2.6 Determination of the minimal inhibitory concentration (MIC) for *Lactobacillus* supernatant against *S. aureus*

Serial two-fold dilutions (1/1 to 1/512) of the CFS were prepared in 96-well micro plates. Each well was inoculated with a suspension prepared from a 24-h culture of *S. aureus*. 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. Bacterial growth inhibition was assessed by measuring optical density using an ELISA reader, and inhibition percentages were calculated.

2.7 Statistical analysis

All experiments were performed in triplicate, and the results were expressed as mean ± standard error. Statistical analysis was performed using appropriate tests (ANOVA), and a P-value is ≤ 0.05 was considered statistically significant.

3- RESULTS AND DISCUSSION

3.1 Isolation and Identification of *S. aureus*

The characteristic bacterial colonies are consistent with the morphological and biochemical features. The isolates ferment Mannitol on Mannitol salt agar, beta-hemolysis on blood agar and positive result on the DNase agar test, exhibiting a transparent halo around the colonies due to DNA lysis by the DNase enzyme. This test is an important differential test for confirming the diagnosis of *S. aureus* (Figure 1).

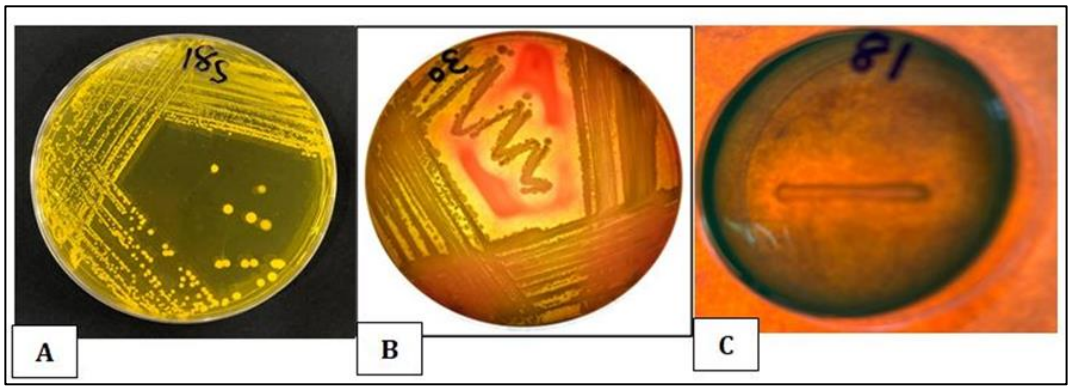

Fig (1): A: *S. aureus* on mannitol salt agar; B: beta-hemolysis on blood agar; C: DNase positive

The distribution of isolates from different clinical samples as shown in the figure 3-2 indicated that *S. aureus* was the most abundant, 32 isolates (30.77%) [22] which is a significant finding in a clinical perspective. This is in line with the latest regional observations. *S. aureus* was, for example, the predominant Gram-positive bacteria involved in UTI's, with 7.6% of the isolated uropathogens in Duhok Province being *S. aureus* after a 12-year retrospective study [23]. The isolation is often a sign of ascending infection from instrumentation or secondary renal seeding after an unrecognized staphylococcal bacteremia. A significant proportion of isolates were from wounds or burns (22 isolates, 9.62% and 13 isolates, 12.5% respectively). Apart from those in abscesses (2 isolates, 1.93%), skin and soft tissue infections (SSTI's) are a significant reservoir for the pathogen. *S. aureus* has unique surface associated adhesions and secreted exotoxins that promote the early colonization of epidermal and dermal tissue damage. A cross sectional study in Basrah city Iraq found that cross sectional (SSTIs) accounted for 40.31% of all SSTIs positive cultures, including an unusually high isolation rate of 50% of positive SSTIs from wounds [24]. *S. aureus* was isolated from blood culture samples [15] 14.42% of which indicate serious invasive systemic infections in the form of blood culture positivity. Its ability to invade the bloodstream in an aggressive fashion is also highlighted by its occurrence in highly sterile and sensitive tissue compartments such as synovial fluid (5, 4.8%), pleural fluid (4, 3.85%) and cerebrospinal fluid (CSF) (3, 2.88%). A noticeable proportion of isolates (11 isolates, 10.58%) were isolated from sputum samples, suggesting lower respiratory tract colonization or pneumonia. Although fewer, mucosal surface recoveries were made, such as from conjunctiva (3 isolates, 2.88%) and high vaginal swabs (HVS) (3 isolates. 2.88%), and ear swabs (2 isolates, 1.93%) [22].

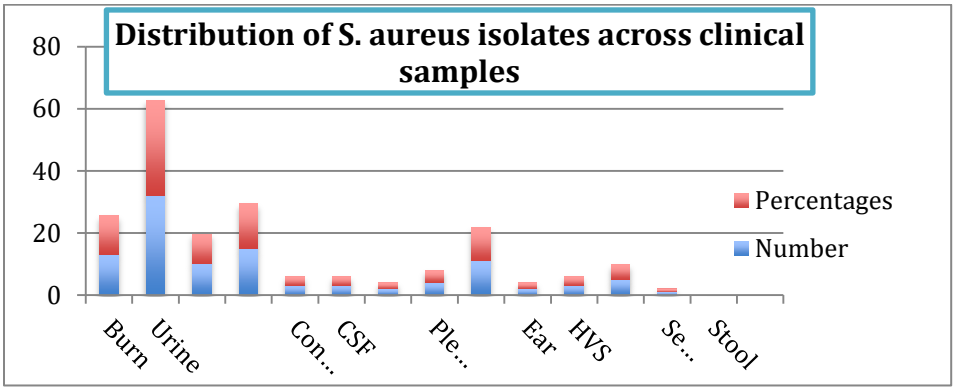

Fig (2): The distribution of *S. aureus* isolates among different clinical sample sites

3.2 Antibiotic Sensitivity

The level of resistance to penicillin's (benzylpenicillin and ampicillin/amoxicillin) was above 90%, reflecting the high MIC values of the isolates and high beta-lactamase activity. This is normal and demonstrates the disadvantages of using these antibiotics clinically with staphylococci. The combination of the cefoxitin and oxacillin tests to detect MRSA (52%) adds even more reliability to the results, because cefoxitin is a very accurate indicator of the presence of the *mecA* gene that causes methicillin resistance. Similar MRSA prevalence and correlation between cefoxitin resistance and presence of the *mecA* gene in clinical isolates have been reported in Iraq [25].

3.3 Detection of *16S rRNA*

The results of the Real-time qPCR (qPCR) technique indicate the successful detection of the *16S rRNA* gene of *S. aureus*, as the fluorescent signals showed a significant increase compared to the negative control. The appearance of this signal confirms the presence of the target genetic material and reflects the high sensitivity of this technique in detecting even low concentrations of bacterial DNA. This has been confirmed in recent studies demonstrating that qPCR is highly sensitive and capable of early detection compared to traditional methods (Figure 3).

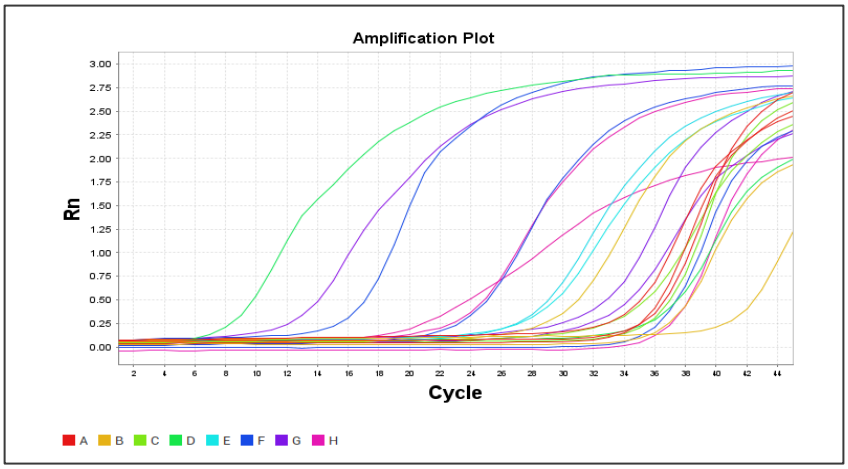

Fig (3): Amplification plot showing fluorescence signals in a Real-Time PCR reaction for the detection of the *16S rRNA* gene

3.4 Qualitative Biofilm Production Assay (Congo red Agar)

From (104) isolates only 80 isolates (76.9%), were biofilm forming, while 24 isolates (23.1%) were non-biofilm-forming (Table 1). These results indicate that more than half of the isolates possess biofilm-forming capabilities, a key virulence factor that promotes bacterial survival within the body and increases antibiotic resistance (Figure 4).

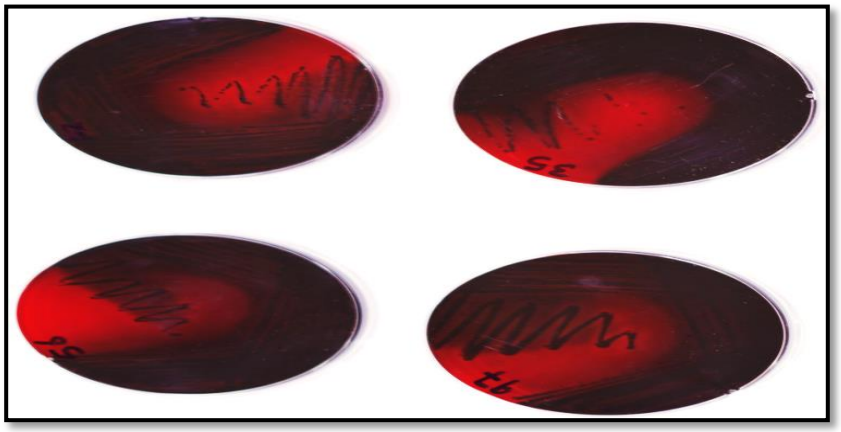

Fig (4): CRA medium detecting biofilm formation

Table (1): Biofilm production distribution of *S. aureus* isolates using the CRA method

Biofilm production	Number of isolates	Percentage (%)
Biofilm producers	80	76.9%
Non-biofilm producers	24	23.1%
Total	104	100%

The results were consistent with the Iraqi study conducted at the University of Kufa, which showed that 91.2% of *S. aureus* isolates were positive for biofilm formation using CRA, a higher percentage than the results of the current study. This may be attributed to the different nature of the studied isolated (multiple resistant isolates MDR) [26]. This difference suggests that antibiotic-resistant isolates often have a higher biofilm formation capacity, which supports the relationship between biofilm formation and antibiotic resistance. The results of this study are consistent with a recent study in Pakistan, which showed a high biofilm-producing rate among isolates using CRA, with approximately 68% of isolates classified as biofilm-forming (strong+moderate) and only a small percentage as non-biofilm-forming [27].

3.5 Quantitative Biofilm Production Assay

The overall capacity of the 104 *S. aureus* clinical isolates to produce biofilms was assessed quantitatively using microtiter plate assays (Figure 5).

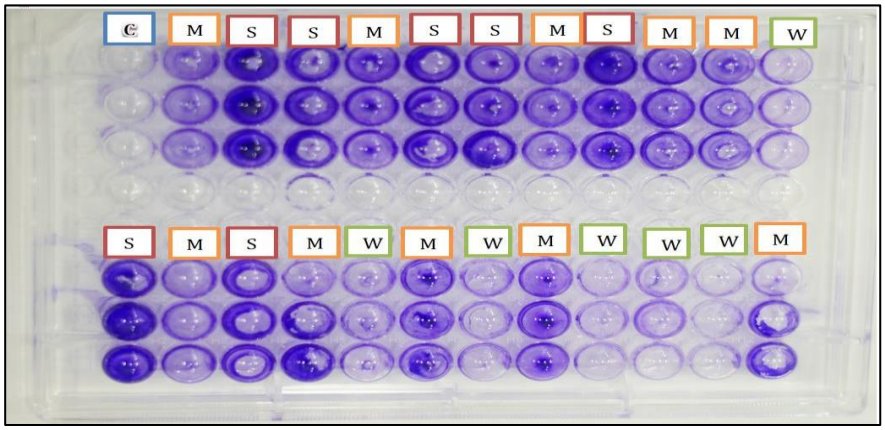

Fig (5): Biofilm production on microplate assay. C: control (-ve, no growth); S: strong biofilm; M: medium biofilm; W: weak biofilm

Table (2): Biofilms cores of *S. aureus* isolates

Score	N	%
Strong	25	24.03
Moderate	39	37.51
Weak	40	38.46
Total	104	100

The results of the analysis show that all of the isolates had the intrinsic physiological mechanisms to produce biofilms. Regional surveys of microbiology also confirmed this universal capacity; for example, a microbiological survey carried out in Diyala province on the clinical isolates of *S. aureus*, revealed that 100% of *S. aureus* isolates were biofilm producers, with 20% classified as weak producers, 44% as moderate producers and 36% as strong producer. Producers [28]. The distribution within the present cohort demonstrated the significant pathogenic capability as 24.03% strong biofilm producer, while 37.51% was moderate biofilm producer, and 38.46% was weak biofilm producer as indicated in (Table 2).

When quantitative biofilm scores are cross tabulated with anatomical sources, a very detailed understanding of *S. aureus* pathogenesis becomes evident. The data set reveals profound adaptations to the environment.

Table (3): Distribution of *S. aureus* isolates according to biofilm scores with types of clinical samples

Clinical samples	Strong	Moderate	Weak	Total
Burn	5 (20%)	7 (17.94%)	2 (5%)	14 (13.46%)
Urine	0 (0%)	6 (15.38%)	24 (57.6%)	30 (28.85%)
Wound	4 (16%)	6 (15.38%)	3 (10.2%)	13 (12.5%)
Blood	8 (32%)	5 (12.82%)	2 (5%)	15 (14.42%)
Conjunctiva	0 (0%)	3 (7.7%)	2 (5%)	5 (4.81%)
Fluid	2 (8%)	1 (2.56%)	1 (2.5%)	4 (3.85%)
Pleural	3 (12%)	3 (7.7%)	1 (2.5%)	7 (6.73%)
Sputum	3 (12%)	3 (7.7%)	1 (2.5%)	7 (6.73%)
Ear	0 (0%)	3 (7.7%)	2 (5%)	5 (4.81%)
HVS	0 (0%)	1 (2.56%)	1 (2.5%)	2 (1.92%)
Synovial	0 (0%)	1 (2.56%)	1 (2.5%)	2 (1.92%)
Total	25 (100%)	39 (100%)	40 (100%)	104 (100%)
F.E.P value		45.77		
P-value		<0.01		

Table 3 showed that the statistical significance of this cross-tabulated distribution is exceptionally high (P-value ≤ 0.01), confirming a powerful deterministic relationship between the specific site of host infection and the phenotypic expression of the bacterial biofilm matrix.

3.6 Antimicrobial activity of *Lactobacillus spp.*

To study the antimicrobial activity of the *Lactobacillus spp.* CFSs using the agar well diffusion method (Figure 6), their pH was neutralized, bacteriocinand treated with trypsin, and catalase added for H_2O_2 identification. The results of this study showed the supernatants of *L. acidophilus* strains were evaluated for the presence of antimicrobial compounds against a number of *S. aureus* biofilm-producing strains as showing in Table 4. Antimicrobial activity against *S. aureus* was due to production of bacteriocin or H_2O_2 and organic acid with more inhibition zone diameters against weak biofilm-producing strains, statistically these differences were non-significant (P-value = 0.28).

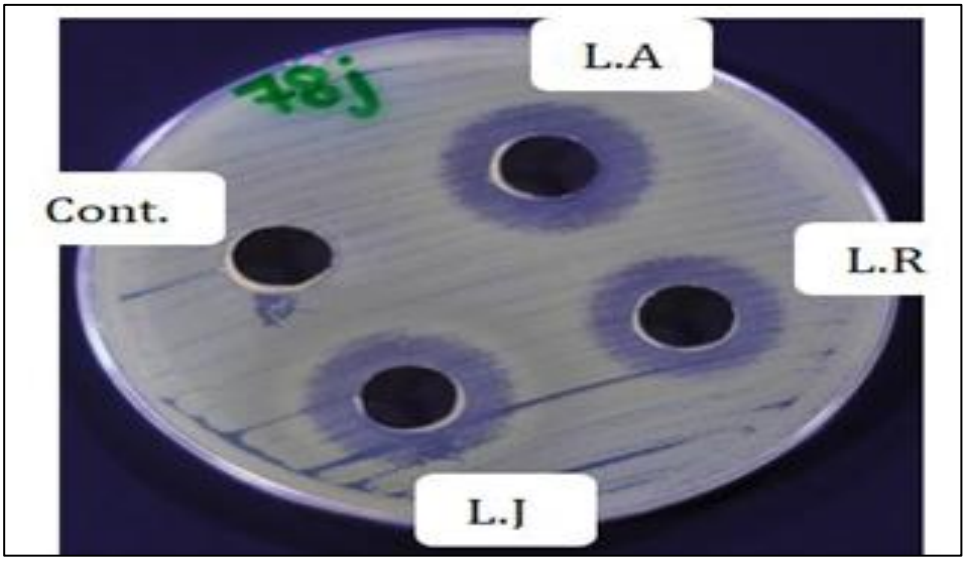

Fig (6): Study the antimicrobial activity of the *Lactobacillus spp.* against *S. aureus* (Cont.: control -ve without CFS; L.A: *L. acidophilus*; L.R: *L rhamnosus*; L.J: *L. johnsonii*)

Table (4): Inhibitory activities of *L. acidophilus* strains against *S. aureus* biofilm-producing strains

Biofilm score	Tryspin treated	Catalase treated	NaOH treated	Untreated	P-value
Strong	-	-	15.83 ± 1.47	20.66 ± 1.36	0.28
Moderate	-	-	17.66 ± 1.96	21 ± 2.19	0.25
Weak	-	-	17.83 ± 3.18	22.50 ± 2.16	

Supernatants of *L. rhamnosus* strains were evaluated for the presence of antimicrobial compounds against a number of *S. aureus* biofilm-producing strains as shown in Table 5. Antimicrobial activity against *S. aureus* was due to the production of bacteriocin, H_2O_2, and organic acid with greater inhibition zone diameters against weak and moderate biofilm-producing strains. Statistically, these differences following neutralization were non-significant for strong and moderate phenotypes (P-value = 0.09), showing only significant reduction against weak producers (P-value = 0.01).

Table (5): Inhibitory activities of *L. rhamonus* against *S. aureus* biofilm-producing strains

Biofilm score	Tryspin treated	Catalase treated	NaOH treated	Untreated	P-value
Strong	-	-	21 ± 2.19	23.33 ± 1.86	0.09
Moderate	-	-	23.83 ± 2.56	27.66 ± 2.50	
Weak	-	-	23.50 ± 2.07	26.16 ± 2.40	0.01

Supernatants of *L. johnsonii* strains were evaluated for the presence of antimicrobial compounds against a number of *S. aureus* biofilm-producing strains as shown in Table 6. Antimicrobial activity against *S. aureus* was due to the production of bacteriocins, H_2O_2, and organic acid, with greater inhibition zone diameters against weak and strong biofilm-producing strains. Statistically, these differences following neutralization were significant (P-value = 0.03).

Table (6): Inhibitory activities of *L. johnsonii* against *S. aureus* biofilm-producing strain

Biofilm score	Tryspin treated	Catalase treated	NaOH treated	Untreated	P-value
Strong	-	-	10 ± 0.89	13.16 ± 1.83	0.03
Moderate	-	-	8.83 ± 1.32	12.66 ± 1.50	
Weak	-	-	11.33 ± 1.96	14 ± 1.0	0.37

Table (7): Comparative the percentages of inhibition growth of *L. acidophilus* extract against *S. aureus* isolates at different serial dilutions

Biofilm score		% of inhibition growth of *L.acidophilus* extract (MIC)									
		1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
Strong (n=6)	**Mean**	72.76	72.76	71.66	70.62	68.35	66.73	64.07	66.86	50	21.59
	SE	1.99	1.43	0.57	1.51	2.08	2.26	0.80	15.02	2.16	6.45
Moderate (n=6)	**Mean**	88.85	88.54	88.20	82.31	72.21	61.17	47.99	35.54	19.89	9.14
	SD	0.68	0.57	1.96	2.66	5.30	8.93	7.46	7.11	8.65	9.39
Weak (n=6)	**Mean**	72.76	72.76	71.66	70.62	68.35	66.73	64.07	66.86	50	21.59
	SD	1.99	1.43	0.57	1.51	2.08	2.26	0.80	15.02	2.16	6.45
P-value		≤0.01	≤0.01	≤0.01	≤0.01	0.122	0.162	≤0.01	≤0.01	≤0.01	0.017

The results of this study showed the antimicrobial action of *L. acidophilus* bacteria against *S. aureus* bacteria was assessed using microtiter plate method following principle of minimal inhibitory concentration. The results observed there was a reduction in the percentages of *S. aureus* bacterial growth at the highest dilution starting from 1/1 to 1/256 titer, while the least percentages of inhibition was observed at the highest dilution 1/512. The results indicated the highest percentages of inhibition growth was against the *S. aureus* bacteria with moderate biofilm score with mean 88.85% at the 1 / 1 titer, while the less value of inhibition growth was observed at the 1/512 titer with mean 9.14% as arranged in Table 7.

Table (8): Comparative the percentages of inhibition growth of *L. rhamnosus* extract against *S. aureus* isolates at different serial dilutions

Biofilm score		% of inhibition growth of *L.rhamonus* extract (MIC)									
		1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
Strong (n=6)	Mean	94.84	94.85	94.97	94.81	93.51	91.47	79.44	70.03	55.43	39.64
	SE	0.32	0.30	0.38	0.41	0.41	1.40	2.91	4.61	6.21	5.65
Moderate (n=6)	Mean	88.75	88.93	88.43	85.67	85.14	79.75	73.50	67.98	43.90	23.55
	SE	0.97	0.71	1.07	4.17	3.26	7.57	10.13	12.05	29.60	20.43
Weak (n=6)	Mean	73.64	73.27	71.29	71.85	69.38	72.77	64.93	51.35	47.03	34.62
	SE	1.62	1.67	1.56	2.36	3.46	1.95	1.23	10.35	6.93	4.182
P-value		≤0.01	≤0.01	≤0.01	≤0.01	≤0.01	≤0.01	0.003	0.008	0.52	0.100

The results of this study showed the antimicrobial action of *L. rhamnosus* bacteria against *S. aureus* bacteria. The results observed that there was a continuous reduction in the percentages of *S. aureus* bacterial growth starting from the 1 / 1 titer down to the extreme dilutions. The results indicated that the highest percentages of inhibition growth were overwhelmingly directed against the *S. aureus* bacteria with a strong biofilm score, reaching a massive mean of 94.84% at the 1/1 titer. The least percentages of inhibition growth were observed at the highest dilution, 1/512, particularly dropping to a mean of 23.55% against moderate biofilms and 34.62% against weak biofilms as showen in Table 8.

The high effectiveness at this level appears to suggest that the bioactive material in the *L. rhamnosus* extract is a highly synergistic mixture of membrane-active biosurfactants, which are organic compounds that are highly diffusible, and their anti-adhesive and matrix-degrading properties are considerable.

Table (9): Comparative the percentages of inhibition growth of *L. johnsonii* extract against *S. aureus* isolates at different serial dilutions

Biofilm score		% of inhibition growth of *L. johnsonii* extract (MIC)									
		1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
Strong (n=6)	Mean	94.89	89.36	80.66	66.29	46.71	36.60	27.97	19.60	16.66	18.41
	SE	0.35	2.67	3.30	6.312	6.25	6.73	6.80	10.70	8.47	9.73
Moderate (n=6)	Mean	89.87	86.32	77.34	63.41	59.72	50.02	28.80	10.72	9.74	5.72
	SE	0.68	4.19	11.69	9.62	11.53	7.91	8.74	7.82	1.36	2.35
Weak (n=6)	Mean	73.64	75.28	64.96	51.32	39.81	25.03	11.38	18.93	18.11	15.78
	SE	1.57	1.52	1.45	4.14	8.56	13.75	7.67	21.33	21.88	22.80
P-value		≤0.01	≤0.01	0.004	0.005	0.006	0.002	0.002	0.51	0.53	0.31

The results of this study showed the antimicrobial action of *L. johnsonii* bacteria against *S. aureus* bacteria. The results observed that there was a rapid, sharp reduction in the percentages of *S. aureus* bacterial growth as dilution increased. The results showed that the bacteria which have a strong score of biofilm showed the highest percentages of inhibition growth, 94.89% at the 1 / 1 titrer. However, the highest dilution (1/512) had the least percentage of inhibition growth at 5.72% against moderate biofilms as seen in Table 3-9, which showed steep threshold dynamics.

In the present study, it is found that *Lactobacillus* species show a remarkable antimicrobial activity against *S. aureus*, and the activity varies according to the probiotic species and the biofilm forming capacity of the isolates. When results were compared, it was observed that *Lactobacillus rhamnosus* had the highest inhibition at all dilutions tested with inhibition percentages of > 94% at lower concentrations. This result is in line with the study that showed *L. rhamnosus* exhibited strong antagonistic activity against Gram-positive pathogens because of the production of high levels of antimicrobial metabolites [29]. *Lactobacillus acidophilus*, on the other hand was found to be moderately inhibitory and maximum inhibition was seen against moderate biofilm-producing isolates (88.85%). As the concentration was reduced, there was a progressive reduction in inhibition, further supporting the concentration dependent effect which has been reported earlier [30] in other studies and supports the concept of the MIC. *Lactobacillus johnsonii* exhibited relatively weak inhibitory activity, especially at higher dilutions, with significant decreases in the inhibition observed. This indicates that its antimicrobial compounds might not be as stable as others *Lactobacillus* species and/or not be produced in as high a quantity. One interesting result in this study was that moderate biofilm producers were more susceptible to inhibition than strong biofilm producers. This could be attributed to the extracellular polymeric matrix that is dense in strong biofilms, and therefore prevents

antimicrobial agents from penetrating the biofilm [31]. Not always emphasized in previous studies, this observation is important. To determine the MICs of cell-free supernatants (CFS) using the broth microdilution method, it was demonstrated that the MICs of the CFS extracts of the *Lactobacillus* spp. against *S. aureus*. The supernatant experiments were also able to show that the antimicrobial effects are primarily due to the presence of organic acids, as neutralization with NaOH decreased inhibitory effects, whereas trypsin and catalase treatments had little effect. This suggests that the role of the bacteriocins and hydrogen peroxide is secondary, again in line with the studies that pointed out the role of acidic metabolites in probiotic activity [32]. Other studies, however, have shown other results, with hydrogen peroxide or bacteriocins as the main antimicrobial agents [33]. Some of these differences could be caused by variations in experimental conditions or strain differences.

Overall, the results of this study highlight the potential of *Lactobacillus* species, particularly *L. rhamnosus*, as effective antimicrobial agents against *S. aureus*, especially in biofilm-associated infections.

4- CONCLUSION

The present study showed that *Lactobacillus* spp. has good antimicrobial activity against *S. aureus* which is dependent on the bacterial spices and biofilm forming ability. *L. rhamnosus* produced the highest inhibitory effect with *L. acidophilus* and *L. johnsonii* being the least effective species. The present results indicate that *Lactobacillus* spp. could be used as potential therapeutic agents to control *S. aureus* associated with biofilms, either as standalone agents or as adjuncts.

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