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Molecular characterization of diabetic foot *Staphylococcus aureus* and inhibitory effects of *Lactobacillus* cell-free supernatant

Noor Subhi Fayyadh [1], Zainab Mohammed Alzubaidy [2], Asmaa Ahmed Chyad [1], Shahrazad Ahmed Khalaf [3*]

[1] Department of biology, college of science, University of Diyala, Diyala, Baqubah, 32001, Iraq.
[2] Department of biology, college of science, Kirkuk University, Kirkuk, Iraq.
[3] Department of Forensic Sciences, College of Science, University of Diyala, Diyala, Baqubah, 32001, Iraq
*Correspondence author: shahrazadah.kh@gmail.com

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of diabetic foot ulcers (DFU) infections. This study investigated the molecular characteristics, virulence factors, biofilm formation, antimicrobial resistance, and the antibacterial and antibiofilm activities of Lactobacillus spp. cell-free supernatants (CFS) against selected *Staphylococcus aureus* isolates. A total of 130 DFU samples were collected from Baqubah City, Iraq. *S. aureus* isolates were identified using biochemical tests, CHROMagar, and the VITEK 2 system. Virulence factors, biofilm formation, antimicrobial susceptibility, and resistance genes (*mecA, icaA, pvl, and fnbA*) were evaluated. The antibacterial and antibiofilm activities of *Lactobacillus spp.* CFS were evaluated against selected biofilm-producing *S. aureus* isolates. Of the 130 samples, 121 (93.1%) showed bacterial growth, with 30 (23.1%) identified *as S. aureus*. Phenotypic testing revealed high virulence: 100% produced hemolysin (83.3% *beta*-hemolysis and 16.7% *gamma-hemolysis*), 66.7% coagulase, 63.3% DNase, and 43.3% gelatinase. MBL and ESBL production were remarkably low (3.3%each). Overall, 93.3% (28/30) of the *S. aureus* isolates demonstrated biofilm-forming capacity, including 12 strong biofilm producers (40.0%) and 16 moderate biofilm producers (53.3%), while two isolates (6.7%) were classified as non-biofilm producers. Disk-diffusion testing showed apparent resistance to teicoplanin in all isolates and 100% resistance to nitrofurantoin, with high resistance to clarithromycin (80%) and tetracycline (60%). Disk-diffusion screening showed high apparent resistance to teicoplanin; however, these glycopeptide susceptibility findings were discordant with subsequent VITEK 2 results and were therefore interpreted cautiously. Among the five selected *S. aureus* isolates examined by PCR, mecA, icaA, and pvl were detected in all five isolates (5/5, 100%), whereas *fnbA* was detected in three isolates (3/5, 60%). Treatment with *Lactobacillus* spp. CFS demonstrated antibacterial activity and reduced biofilm formation by up to 56%.

Keywords: Diabetic foot ulcer, *Staphylococcus aureus*, Biofilm, *Lactobacillus*; Cell-free supernatant.

1. Introduction

The pathogenesis of *S. aureus* in diabetic foot ulcers is associated with skin structure and soft tissue injury. When bacteria enter the affected skin, neutrophils and macrophage cells migrate to the site of infection (Lee *et al.*, 2023). The bacteria cross the lines of defence in various ways, including blocking the action of antibodies and chemical attraction of white blood cells by formation of the capsule and biofilm, thus resisting destruction after ingestion by phagocytes (Dunyach-Remy *et al.*, 2016). In addition to adhesion mechanisms, bacteria have the ability to produce toxins, which represent another virulence factor involved in inflammation (Chen *et al.*, 2022).

MRSA is a major bacterial pathogen and a leading cause of morbidity and mortality worldwide due to antibiotic resistance (Lee *et al.*, 2018). The occurrence of multidrug-resistant *S. aureus* in different environments further demonstrates the widespread distribution and persistence of antimicrobial resistance in this species (Akter et al., 2024). MRSA prevalence and distribution vary substantially between healthcare institutions and countries due to differences in antibiotic prescribing practices, infection control measures and local epidemiology (Alghamdi *et al.*, 2023).

The management of these ulcers is severely complicated by the emergence of MRSA, which utilizes biofilm formation as a primary survival strategy (Abebe *et al.*, 2023). These biofilms act as a physical and chemical shield, rendering conventional antibiotics ineffective and allowing the bacteria to persist in a chronic state. In addition to this, necrotizing toxins such as PVL cause swift destruction of tissues (Goudarzi *et al.*, 2017). It is imperative to genotypically characterize these isolates and identify novel non-antibiotic approaches like probiotic-derived peptides, which can modulate the molecular circuitry previously described to enhance clinical outcomes.

2. Materials and Methods

Study design

A total of 130 clinical specimens were collected from patients with diabetic foot infections between October 2023 and January 2024. Patients of both sexes, aged 33–78 years, were recruited from Baqubah Teaching Hospital and Al-Batool Teaching Hospital in Baqubah City, Diyala Province, Iraq.

Isolation and identification of pathogenic *S. aureus*

The primary methods of identifying the isolates were colony appearance, pigmentation, morphology, Gram staining, and biochemical characteristics. For additional identification, the CHROMagar Base and VITEK 2 System were utilized.

Phenotypic determination of virulence factors

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing of the *Staphylococcus aureus* isolates was performed using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar. Commercial antimicrobial disks were obtained from Mast Group Ltd., United Kingdom. The antimicrobial agents tested were ofloxacin (5 µg), ciprofloxacin (5 µg), clarithromycin (15 µg), teicoplanin (30 µg), gentamicin (10 µg), vancomycin (5 µg), nitrofurantoin (300 µg), chloramphenicol (30 µg), trime-

thoprim–sulfamethoxazole (1.25/23.75 µg), clindamycin (2 µg), rifampin (5 µg), and tetracycline (30 µg). Inhibition-zone diameters for applicable antimicrobial agents were interpreted according to CLSI M100, 32nd edition (2022).

Detection of beta-Lactamase Production

Detection of Metallo-beta-Lactamase (MBLs)

The production of Metallo-beta-Lactamases was detected using the EDTA-Imipenem disc synergy method. Bacterial suspensions were adjusted to 0.5 McFarland turbidity standard using 0.9% normal saline. The inoculum was swabbed onto Mueller-Hinton agar (MHA) plates. Two Imipenem (10 µg) discs were placed on the agar surface at a distance of 20 mm (center to center). Subsequently, 5 µl of 0.5 M EDTA solution was added to one of the discs. After incubation at 37°C for 24 hours, an increase in the inhibition zone diameter of ≥ 7 mm for the Imipenem + EDTA disc compared to the Imipenem disc alone was considered positive for MBL production (Radhika et al.,2022).

Detection of Extended-Spectrum Beta-Lactamase (ESBLs)

The double-disc synergy test (DDST) was employed for the phenotypic screening of ESBL production. Inoculum equivalent to 0.5 McFarland was uniformly spread on MHA plates. An Amoxicillin/Clavulanic acid (20/10 µg) disc was positioned in the center of the plate. Discs of Aztreonam (30 µg), Cefotaxime (30 µg), and Ceftazidime (30 µg) were placed at a distance of 20–30 mm from the central disc. Following incubation at 37°C for 24 hours, a ≥ 5 mm enhancement of the inhibition zone towards the central clavulanic acid disc for any of the peripheral antibiotics was recorded as a positive result for ESBL production (Patel,2017).

Detection of Virulence Factors

Hemolysin Production

The hemolytic activity of *S. aureus* isolates was evaluated using human blood agar plates, inoculated with the test organisms and incubated at 37°C for 24 hours. Following incubation, inspected for the presence and type of hemolytic zones (alpha, beta, or gamma hemolysis) surrounding the colonies, according to the criteria established by Benson (1973).

Gelatinase Production

The proteolytic ability of the isolates to produce gelatinase was determined using gelatin liquefaction medium. The medium was inoculated with *S. aureus* and incubated at 37°C for 24 hours. To assess gelatin hydrolysis, the tubes were subsequently refrigerated at 4°C for 30 minutes. A positive result was recorded for cultures that remained in a liquid state after refrigeration (Cappuccino and Sherman,2005).

DNase Production

Examination of the production of deoxyribonuclease (DNase) was performed by using DNase agar. The medium was inoculated with an overnight culture of the bacterium and incubated at 35°C for 18–24 hr. A distinct halo surrounding the growth of bacteria, together with color change from blue to yellow of the medium, was scored as a positive test for DNase activity (Harley, 2008).

Detection of biofilm formation

The biofilm formation experiment was also done with the aid of the microtiter plate (MTP) method. A 96-well polystyrene plate was loaded with an activated bacterial suspension containing 200µL of activated organism per well, as negative control was used: nutrient broth without bacteria. TSB with 1% glucose, and the incubation was performed at 37 °C for 24

hours after covering the plate. Each well was washed three times with 0.9% NaCl after removing the bacterial suspension carefully. After adding 200µL of methanol, the cells in the wells were fixed for ten minutes; 200 µL of 0.5% crystal violet solution was added to each well, and the biofilms were allowed to stain for 15 minutes, then two or three washes with distilled water were used to get rid of the stain. To remove the excess, 200µL of 95% ethanol was added to each well and left for ten minutes. A spectrophotometer set to measure absorbance at 630nm was used for each well, and the results were compared to the control (Ballah et al., 2022).

Molecular detection of some virulence genes

DNA Extraction: Genomic DNA was extracted from Staphylococcus aureus isolates using the ABIO Pure DNA Extraction Kit according to the manufacturer's protocol. Briefly, the bacterial pellet was resuspended in nuclease-free water and lysozyme solution, followed by incubation at 37°C for 30 min. After centrifugation, the pellet was treated with Proteinase K and lysis buffer (Buffer BL), mixed thoroughly, and incubated at 56°C for 30 min. Ethanol was then added, and the lysate was transferred to a spin column for DNA purification. The column was then washed in sequence with Buffer BW and Buffer TW, followed by low-speed centrifuge the wash buffer residual at high speed. Purified DNA was eluted with 100 µl Buffer AE and collected by centrifugation. The harvested DNA was then quantified and evaluated for quality utilizing a Quantus Fluorometer to confirm its suitability for further downstream molecular analyses. The sequencing of primers used for the detection appeared in Table 1.

Table 1. Primers and thermal conditions used for detection of some virulence genes

Gene	Primer Sequence (5' – 3')	Annealing Tem C° and time	Size (bp)	Reference
mecA	**F: GTAGAAATGACTGAACGTCCGATAA** **R: CCAATTCCACATTGTTTCGGTCTAA**	55C° for 30 sec.	310	(McClure et al.,2006)
icaA	**F:** TCT CTT ACA GTA TTG TAT GTT AGT TCC **R:** ATG GTA CTC CAA TAC TCT GCG	56 C° for 45 sec.	720	(Arciola et al.,2001)
Pvl	**F:** ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC **R:** GCA TCA AGT GTA TTG GAT AGC AAA AGC	55 C° for 30 sec.	433	(Lina et al.,1999)
fnbA	**F:** GCG GAG ATC AAA GAC AAG TA**R:** CAC CAT CTA TAG CTG TGT GG	58 C° for 45 sec.	1278	(Booth er al.,2001)

Agarose Gel Electrophoresis and DNA Loading

Prepared a 1.5% agarose gel using the method of dissolving 1.5 g agarose in 100 mL of 1× TAE buffer with heating until completely dissolved. Then, ethidium bromide (1 µL, 10 mg/mL) was added after slight cooling down; and the solution was poured into a casting tray with a comb and allowed to polymerized at 20°C. After polymerization, the gel was transferred to an electrophoresis chamber containing 1× TAE buffer. Loading dye was mixed with DNA samples before loading into the wells, while PCR amplicons were directly loaded (5 µL per well). Electrophoresis was performed at 100 V for ~60 min, and the DNA fragments were illuminated with ultraviolet (UV) light and photographed using a gel documentation system.

Sources and Diagnostic Determination of the Probiotic Bacterial Isolates

The isolates of *Lactobacillus* spp. were obtained from Salah al-Din University, College of Agriculture, the isolates were recovered from local dairy products and confirmation of the isolates was performed by colony morphology, microscopic examination, follow up with some biochemical tests; Gram staining, catalase, oxidase, growth at different temperatures, sugar fermentation, motility test, endospore formation, milk coagulation activities, 0.4% bacteriostatic phenol tolerance test. As well as the isolates of Lactobacilli were tested for the following features as probiotics: Bile salt tolerance test, Acid tolerance test, NaCl tolerance test, antimicrobial activity of Lactobacillus spp. Isolates, Phenol tolerance test. Based on phenotypic and biochemical characteristics, the probiotic isolates were identified as *Lactobacillus reuteri* and *Lactobacillus plantarum*. Molecular confirmation and strain-level identification were not performed; therefore, the species assignments were based solely on phenotypic and biochemical characterization.

The Antibacterial activity of *Lactobacillus* spp. isolates

The antibacterial activity of *Lactobacillus* spp. was determined by the well diffusion method against MRSA isolates. In brief, MRSA suspensions (10^5–10^7 CFU/mL) were spread onto Mueller–Hinton agar plates. Then, wells (5 mm in diameter) were punched on the agar surface. In vitro bioactivities of cell-free supernatants (CFS) of Lactobacillus spp. The isolates were prepared in MRS broth at 37°C for 48 h before centrifugation (6000 × g, 15 min) and filtration using a sterile cellulose acetate membrane with a pore size of 0.22 µm. CFS was prepared for each well and introduced into wells to diffuse at RT for 1 h before incubation. Then, plates were incubated at 37°C for 24 h, and antibacterial activity was expressed as the diameter of the inhibition zones (mm) (Szczerbiec et al.,2022).

Effect of CFS on Biofilm Formation

Biofilm-forming abilities and anti-biofilm activity were determined in microtiter plates using a crystal violet binding assay. In brief, 200 µL of an overnight *S. aureus* suspension (10^6 CFU/mL) was mixed with 100 µL of CFS in a sterile 96-well microplate and incubated at 37°C for 24 h PDT to give the planktonic cells time to adhere; then, on completion of the incubation period, planktonic cells were washed away by adding sterile distilled water twice before lightly washing three times using PBS. The attached biofilm was fixed in methanol for 15 min, washed with distilled water, stained with 0.1% crystal violet for 5 min, and the excess stain was eliminated through washing. To solubilize the bound dye, absolute ethanol was used, and absorbance measurements were made using ELISA microplate reader at 570 nm. All experiments were performed in triplicate. Biofilm inhibition (%) was calculated according to the following equation: [(ODcontrol − ODtreated) / ODcontrol] × 100, where ODcontrol represents the optical density of untreated bacterial culture and ODtreated represents the optical density of bacterial culture treated with CFS.

Statistical analyses:

Data were analyzed using SPSS software, version 26.0. Categorical variables were summarized as frequencies and percentages. The Chi-square test was used for comparisons between categorical variables where an inferential comparison was appropriate.

3. Results

Distribution of *Staphylococcus aureus* isolates

A total of 130 clinical samples were collected from patients presenting with diabetic foot infections. Following primary culture on selective and differential media and subsequent confirmation via biochemical assays and the VITEK 2 system, bacterial growth was observed in 121 (93.1%) samples, while 9 (6.9%) yielded no growth. Among the positive cultures, 30(23.1%) of *Staphylococcus aureus* isolates were identified, whereas the remaining 91 isolates (70.0%) were identified as other Gram-positive and Gram-negative bacterial species, as shown in Table 2.

Table 2. Distribution Of Bacterial Isolates from Diabetic Foot Infection Samples

Category of Result	Number of Samples (n)	Percentage (%)
Staphylococcus aureus	30	23.1%
Other Gram-positive & Gram-negative bacteria	91	70.0%
No Growth (Sterile samples)	9	6.9%
Total	130	100%

Detection of some virulence factors

The results, as displayed in Table 3, showed that hemolysin production, coagulase activity, DNase activity, gelatinase production, biofilm formation, MBL production, and ESBL production were evaluated among the 30 S*. aureus* isolates. The results showed that all *S. aureus* isolates (100%) demonstrated hemolytic activity on human blood agar. The predominant phenotype was beta-hemolysis, observed in 25 isolates (83.3%), while 5 isolates (16.7%) exhibited gamma-hemolysis (no clear zone). Beta-hemolysis was observed in 25 of the 30 isolates (83.3%), whereas five isolates (16.7%) exhibited gamma-hemolysis. Exoenzyme Production (Coagulase, DNase, and Gelatinase). The isolates showed high frequencies of traditional virulence markers, with 66.7% testing positive for coagulase and 63.3% for DNase. Coagulase activity was detected in 20 isolates (66.7%), whereas DNase activity was detected in 19 isolates (63.3%). Gelatinase activity was detected in 13 isolates (43.3%). Biofilm formation was detected in 28 of the 30 *S. aureus* isolates (93.3%). Of these, 12 isolates (40.0%) were classified as strong biofilm producers and 16 (53.3%) as moderate producers, while two isolates (6.7%) were classified as non-biofilm producers. This phenotype is a critical factor in the chronicity of diabetic foot ulcers, as it provides a protective matrix against both host immune responses and systemic antibiotic therapy. Beta-lactamase profiling (MBL and ESBL): The production of Metallo-beta-Lactamase (MBL) and Extended-Spectrum beta-Lactamase (ESBL) was exceptionally low, with only 1 isolate (3.3%) testing positive for each. MBL and ESBL production were each detected in one of the 30 isolates (3.3%).

Table 3. Prevalence of Virulence Factors and Enzymatic Activity among *S. aureus* Isolates

Virulence factor	Positive n (%)	Negative n (%)
β-hemolysis*	25 (83.3%)	5 (16.7%)
Coagulase	20 (66.7%)	10 (33.3%)

Virulence factor	Positive n (%)	Negative n (%)
DNase	19 (63.3%)	11 (36.7%)
Biofilm formation	28 (93.3%)	2 (6.7%)
Gelatinase	13 (43.3%)	17 (56.7%)
MBL production	1 (3.3%)	29 (96.7%)
ESBL production	1 (3.3%)	29 (96.7%)

The quantitative assessment of biofilm formation among the 30 *S. aureus* isolates revealed that the majority of the strains possessed the ability to form biofilms at varying intensities. Based on the optical density cut-off (ODc = 0.040), the isolates were classified into three categories: Based on the optical density cut-off (ODc = 0.040), 12 isolates (40.0%) were classified as strong biofilm producers, 16 (53.3%) as moderate biofilm producers, and two (6.7%) as non-biofilm producers. Overall, 28/30 isolates (93.3%) demonstrated biofilm-forming capacity.

Table 4. Biofilm Formation Intensity in *S. aureus* Isolates

Biofilm category	Number	Percentage
Strong	12	40.0%
Moderate	16	53.3%
Negative	2	6.7%
Total	**30**	**100%**

Antimicrobial Susceptibility Profile of *S. aureus* Isolates

Table (5) illustrates the high resistance of several classical antibiotics from 30 recovered *S. aureus* isolates from diabetic foot ulcers as a result of susceptibility testing. Disk-diffusion testing showed apparent resistance to teicoplanin in all 30 isolates, while nitrofurantoin showed 100% resistance. High resistance rates were also reported for clarithromycin (24 isolates, 80%), tetracycline (18 isolates, 60%), and trimethoprim-sulfamethoxazole (16 isolates, 53.3%). In contrast, vancomycin (VA) exhibited the highest in vitro activity, with 27 isolates (90%). Good susceptibility was also observed to the following antibiotics: ciprofloxacin 21 isolates, 70.0%), ofloxacin 18 isolates), and rifampicin 18 isolates, 60.0%).

The antimicrobial susceptibility profile showed substantial variation among the tested *S. aureus* isolates. By disk diffusion, teicoplanin and nitrofurantoin showed the highest apparent resistance rates, whereas vancomycin showed 90.0% susceptibility (27/30). Ciprofloxacin and ofloxacin showed 70.0% and 60.0% susceptibility, respectively, while clarithromycin and tetracycline showed comparatively high resistance rates. These findings were interpreted cautiously, particularly for glycopeptides, because the present study did not investigate the molecular mechanisms underlying glycopeptide resistance. By the Kirby–Bauer method, vancomycin showed 10.0% resistance (3/30) and 90.0% susceptibility (27/30), whereas all 30 isolates were classified as resistant to teicoplanin by disk diffusion. However, VITEK 2 confirmation

did not support the glycopeptide resistance observed by disk diffusion. Therefore, these discordant findings should not be interpreted as confirmed teicoplanin or vancomycin resistance.

Table 5. Antimicrobial susceptibility and resistance patterns of *Staphylococcus aureus* isolated from diabetic foot infections.

Antimicrobial Class & Agent	Resistant n (%)	Sensitive n (%)	Intermediate n (%)
Glycopeptides			
Vancomycin (VA)	3 (10.0%)	27 (90.0%)	0 (0.0%)
Teicoplanin (TEC)	30 (100.0%)	0 (0.0%)	0 (0.0%)
Fluoroquinolones			
Ciprofloxacin (CIP)	5 (16.7%)	21 (70.0%)	4 (13.3%)
Ofloxacin (OFX)	12 (40.0%)	18 (60.0%)	0 (0.0%)
Macrolides & Lincosamides			
Clarithromycin (CLA)	24 (80.0%)	6 (20.0%)	0 (0.0%)
Clindamycin (CD)	12 (40.0%)	14 (46.7%)	4 (13.3%)
Aminoglycosides			
Gentamicin (GM)	14 (46.7%)	16 (53.3%)	0 (0.0%)
Tetracyclines			
Tetracycline (T)	18 (60.0%)	10 (33.3%)	2 (6.7%)
Phenicols			
Chloramphenicol (C)	10 (33.3%)	14 (46.7%)	6 (20.0%)
Folate Pathway Antagonists			
Trimethoprim-Sulfamethoxazole (TS)	16 (53.3%)	10 (33.3%)	4 (13.3%)
Ansamycins			
Rifampicin (RF)	12 (40.0%)	18 (60.0%)	0 (0.0%)
Nitrofurans			
Nitrofurantoin (NI)	30 (100.0%)	0 (0.0%)	0 (0.0%)

Note: Teicoplanin and vancomycin disk-diffusion results were discordant with VITEK 2 findings and should not be interpreted as confirmed glycopeptide resistance.

Genotyping detection of *S. aureus* isolates from Diabetic foot infection

Among the five selected *S. aureus* isolates examined by PCR, the *mecA* gene was detected in all five isolates (5/5, 100%). These five *mecA*-positive isolates were therefore molecularly classified as MRSA. This percentage refers only to the PCR-tested subset and should not be generalized to all 30 *S. aureus* isolates.

Table 6. The prevalence of the detected genes in *S. aureus* isolates

Gene	Function / Virulence Role	Expected Amplicon Size (bp)	Prevalence
mecA	Methicillin resistance (PBP2a encoding)	310 bp	5/5 (100%)
icaA	Biofilm formation (Intercellular adhesion)	720 bp	5/5 (100%)
pvl	Panton-Valentine Leukocidin (tissue necrosis)	433 bp	5/5 (100%)
fnbA	Fibronectin-binding protein A (adhesion)	1278 bp	3/5

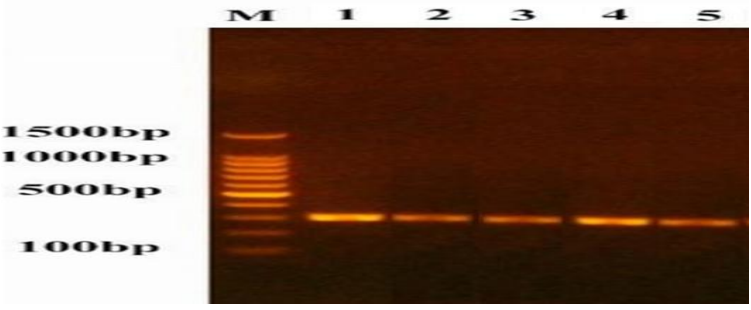

Figure 1. The amplification of *mecA* gene of *S. aureus* samples was fractionated on 1.5% agarose gel electrophoresis stained with EthBr. M: 100bp ladder marker. The amplicon size 310 bp as PCR products

The results showed that *IcaA* gene was identified in 5 isolates (100%), which showed bands of 720 bp as the product size, as in Figure (2). A local study in Al-Najaf by Almayali *et al*, (2018) for *IcaA* and *IcaD* revealed that (97.7%) of *Staphylococcus aureus* isolates tested positive for *IcaA,* while (93.3%) of specimens gave a positive result for *IcaD.*

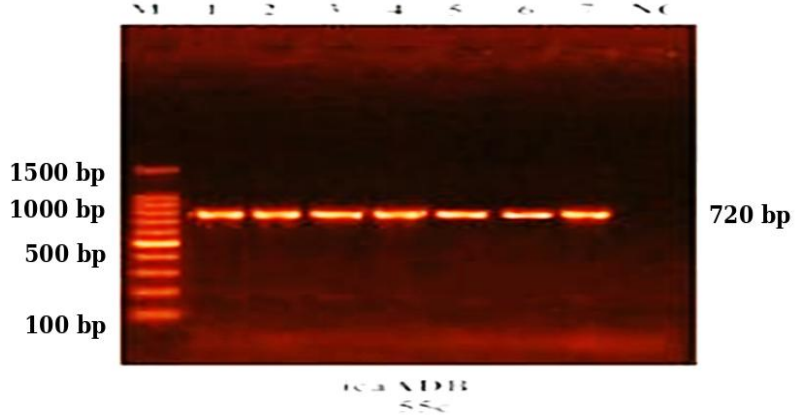

Figure 2. The amplification of *icaA* gene of *S. aureus* samples was fractionated on 1.5% agarose gel electrophoresis stained with EthBr. M: 100bp ladder marker. The amplicon size was 720 bp as PCR product

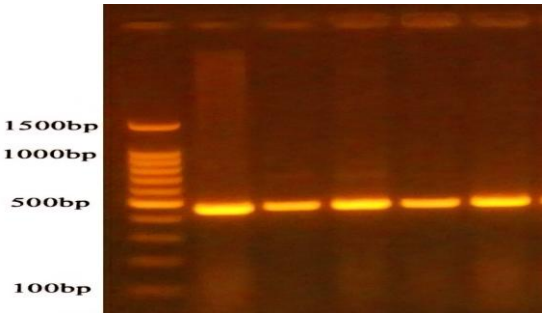

Figure 3. The amplification of *Pvl* gene of *S. aureus* samples was fractionated on 1.5% agarose gel electrophoresis stained with EthBr. M: 100bp ladder marker. The amplicon size 433 bp as PCR products

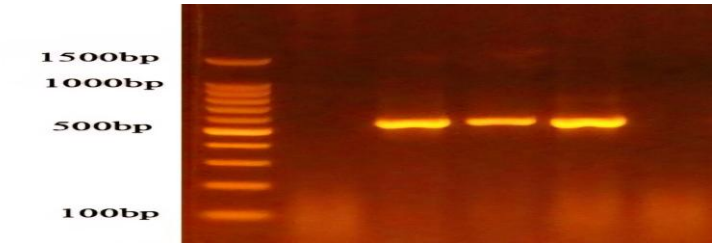

Figure 4. The amplification of *finbA* gene of *S. aureus* samples were fractionated on 1.5% agarose gel electrophoresis stained with EthBr. M: 100bp ladder marker. The amplicon size 1278 bp as PCR products

The antibacterial and antibiofilm of probiotics against isolated bacteria

The inhibitory potential of the tested cell-free supernatant (CFS) was evaluated against four selected biofilm-forming *S. aureus* isolates (SA4, SA12, SA19, and SA20). The antibacterial and biofilm-inhibitory activities of the tested cell-free supernatant (CFS) were evaluated against selected biofilm-forming *S. aureus* isolates. Because bacterial cells were exposed to CFS before biofilm maturation, the assay evaluated inhibition of biofilm formation rather than disruption of an established biofilm.

Table 7. Antibacterial Activity and Biofilm Inhibition Percentage of Cell-Free Supernatant (CFS) against Selected *S. aureus* Isolates.

S. aureus Isolate	Zone of Inhibition (mm)	Biofilm Inhibition (%)
SA4	18	45%
SA12	19	56%
SA19	19	50%
SA20	18	50%
Mean ± SD	**18.5 ± 0.58**	**50.25± 4.50%**

The observed inhibition zones indicate in vitro antibacterial activity of the Lactobacillus-derived CFS. However, the active antimicrobial component(s) and the underlying mechanism of action were not investigated in the present study. The CFS demonstrated measurable in vitro antibacterial activity and inhibition of biofilm formation in the selected isolates. The mean biofilm inhibition was 50.25 ± 4.50%, with the highest inhibition of 56% observed in isolate SA12. However, the active metabolites, contribution of acidity, cytotoxicity, and in vivo efficacy were not evaluated. Therefore, these findings should be considered preliminary and should not be interpreted as evidence of clinical therapeutic efficacy.

4. Discussion

The present study demonstrated a high biofilm-forming capacity among the *S. aureus* isolates recovered from diabetic foot infections, with 28 of 30 isolates (93.3%) classified as biofilm producers. Of these, 12 isolates (40.0%) were strong biofilm producers and 16 (53.3%) were moderate producers. These findings support the importance of biofilm formation in the persistence of *S. aureus* in chronic wound infections.

The antimicrobial susceptibility profile showed considerable variation among the tested isolates. Teicoplanin and nitrofurantoin showed the highest apparent resistance rates by disk diffusion, whereas vancomycin showed 90.0% susceptibility. However, because the glycopeptide susceptibility results obtained by disk diffusion were discordant with subsequent VITEK 2 findings, these results should be interpreted cautiously and should not be considered evidence of confirmed glycopeptide resistance.

Among the five selected isolates examined by PCR, *mecA*, *icaA*, and *pvl* were detected in all five isolates, whereas *fnbA* was detected in three. These proportions apply only to the PCR-tested subset and should not be generalized to all 30 *S. aureus* isolates. The detection of *icaA* is consistent with the high phenotypic biofilm-forming capacity observed in the studied isolates, while the presence of *mecA* in the five selected isolates supports their molecular classification as MRSA.

The Lactobacillus-derived CFS demonstrated measurable in vitro antibacterial and biofilm-inhibitory activity against the selected isolates. Biofilm inhibition ranged from 45% to 56%, with a mean of 50.25 ± 4.50%. However, because the active antimicrobial components were not characterized and the study did not document pH-neutralized CFS or an uninoculated MRS broth control, the inhibitory activity cannot be attributed to a specific metabolite. Therefore, these findings should be considered preliminary in vitro evidence and require further controlled studies before any therapeutic application can be considered. These observations are consistent with the broader evidence that biologically derived products can exert both antibacterial and antibiofilm effects, although their activity and mechanisms vary according to the tested product and bacterial species (Qaralleh et al., 2024).

Limitations

The study did not document pH-neutralized CFS or an uninoculated MRS broth control. Therefore, the observed inhibitory activity cannot be attributed exclusively to specific antimicrobial metabolites, and contributions from acidity or medium-related effects cannot be excluded.

5. Conclusion

The study demonstrated substantial antimicrobial resistance and biofilm-forming capacity among *S. aureus* isolates from diabetic foot infections. Among five selected isolates examined molecularly, *mecA*, *icaA*, and *pvl* were detected in all five, whereas *fnbA* was detected in three. Lactobacillus-derived CFS showed in vitro antibacterial activity and inhibited biofilm formation in selected isolates. These findings demonstrate preliminary in vitro potential and

warrant further characterization of the active components, appropriate controls, toxicity assessment, and in vivo evaluation before any therapeutic application can be considered.

Ethical approval

The study was reviewed and approved by the Committee of Scientific Publishing Ethics, College of Science, University of Diyala, Iraq (Approval Code: 26_ASJ_SCI-191). All participants were informed about the purpose of the study, participation was voluntary, confidentiality was maintained, and oral informed consent was obtained prior to sample collection.

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Author contributions

Noor Subhi Fayyadh: Methodology, investigation, sample collection, laboratory experiments, data curation, formal analysis, and writing—original draft preparation. **Zainab Mohammed Alzubaidy**: Methodology, validation, supervision, interpretation of results, and writing—review and editing. **Asmaa Ahmed Chyad**; Methodology, validation, supervision, interpretation of results, and writing—review and editing. **Shahrazad Ahmed Khalaf**: Conceptualization, study design, supervision, project administration, formal analysis, interpretation of results, writing—review and editing, and correspondence with the journal. All authors read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

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Conflict of interests

The authors declare that they haven't any conflicts of interest regarding publication of this manuscript.

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