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Antibacterial and Anti-Biofilm Formation Using *Lactobacillus plantarum* and *L. retueri* Against MRSA Isolates

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Received: 22 May 2022 **Accepted: 10 July 2022**

DOI: https://dx.doi.org/10.24237/djps.1804.598B

Abstract

The current study was carried out to examine the antibacterial and antibiofilm of local probiotic bacteria *Lactobacillus plantarum* and *Lactobacillus retueri* against methicillin resist *Staphylococcus aureus* (MRSA) isolates. A total of (264) clinical samples were collected from patients with different infections (wounds, burns, urine, vaginal swabs, and blood) admitted to Baquba and Al Batool Teaching Hospitals during the period from September 2021 to April 2022. The results demonstrated that 50(26. 4%) of the isolates were *S. aureus* distributed according to the sources as follows: wound13 (26%), urine11 (22%), vaginal swab10 (20%), blood8 (16%), and burn8 (16%). Some virulence factors were determined and the results showed; ESBL 2%, DNase 62%, gelatinase 42%, Beta-hemolysin 82%, and 2% MBL. Biofilm formation was detected by using the microtiter plate method, the results showed: the percentage of the isolates that strongly produced was 14%, moderate42%, and 44% of the isolates were weakly producer. The result showed 41(82%) multidrug resistance (MDR), 7 (14%) as extensive drug resistance (XDR), and the remaining 2(4%) pan drug resistance (PDR). *mecA* gene was detected in all seven isolates which formed strong biofilm where the percentage was

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(100%). The antibacterial effects of local probiotics *Lactobacillus plantarum* and. *L. retueri* against *S. aureus* growth was done by well diffusion methods and the inhibition zone ranged between (12-17) mm and (16-20) mm. respectively. The antibiofilm formation of *Lactobacillus plantarum* and. *L. retueri* recorded (27%-56%) and (39%-61%) respectively.

Keywords: Antibiofilm, MRSA, *Lactobacillus plantarum*, *Lactobacillus retueri*

تكوين مضاد للبكتيريا ومضاد للبايوفلم باستخدام *Lactobacillus plantarum* و *L. retueri* ضد عزلات MRSA

هند محمد جاسم و زينب محمد الزبيدي

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الخلاصة

أجريت الدراسة الحالية لفحص مضادات البكتيريا والغشاء الحيوي لبكتيريا بروبيوتيك المحلية *Lactobacillus plantarum* و *Lactobacillus retueri* ضد عزلات المكورات العنقودية الذهبية المقاومة للميثيسيلين (MRSA). تم جمع ما مجموعه (264) عينة سريرية من مرضى مصابين بالتهابات مختلفة (جروح ، حروق ، بول ، مسحات مهبلية ، ودم) تم إدخالهم إلى مستشفيات بعقوبة والبتول التعليمية خلال الفترة من سبتمبر 2021 إلى أبريل 2022. وأظهرت النتائج أن 50 (26. 4٪) من العزلات كانت بكتريا *S. aureus* موزعة حسب المصادر على النحو التالي: الجروح 13 (26٪) ، بول 11 (22٪) ، مسحة مهبلية 10 (20٪) ، دم 8 (16٪) ، حروق 8 (16٪)). تم تحديد بعض عوامل الضراوة وأظهرت النتائج ESBL 2 ٪ ، DNase 62٪ ، جيلاتيناز 42٪ ، بيتا هيموليزين 82٪ ، و 2٪ MBL. تم الكشف عن تكوين البيوفيلم باستخدام طريقة صفيحة الميكروتيتر ، وأظهرت النتائج: أن النسبة المئوية للعزلات التي تم إنتاجها بقوة كانت 14٪ ، متوسطة 42٪ ، و 44٪ من العزلات ضعيفة الإنتاج. أظهرت النتيجة 41 (82٪) مقاومة للأدوية المتعددةMDRو7(14٪) كمقاومة واسعة للأدوية (XDR) ، والباقي 2 (4٪) مقاومة للأدوية (PDR). تم الكشف عن جين *mecA* في جميع العزلات السبع التي شكلت غشاء حيوي قوي حيث كانت النسبة (100٪). تم إجراء التأثيرات المضادة للبكتيريا للبروبيوتيك المحلية *Lactobacillus plantarum* و. *L. retueri* ضد نمو *S. aureus* بطرق الانتشار وتراوحت منطقة التثبيط بين (12-17) مم و (16-20) مم على التوالى. أظهر *L. plantarum* نشاطًا مضادًا للغشاء الحيوي مع تأثير واضح على تكوين *S.*

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aureus للغشاء الحيوي بنسبة (27٪ -56٪). بالإضافة إلى ذلك، أظهر تأثير *L. retueri* في دراستنا نطاقًا مضادًا للغشاء الحيوي (39٪ -61٪).

الكلمات المفتاحية: مضاد الغشاء الحيوي،مرسا، *Lactobacillus plantarum, Lactobacillus retueri*

Introduction

Staphylococcus aureus is one of the most important microorganisms in nosocomial and community-acquired infections [1]. It is still predominantly a hospitalized pathogen and was mainly a leading reason for death in hospitals. However, *S. aureus* community infections are increased, and a significant clinical of *S. aureus* include bacteremia, osteoarticular infections, pleuropulmonary infections, skin infections, and infective endocarditis, as well as some diseases, including epidural abscess, urinary tract infections (UTI), toxic shock syndrome, Otitis media, and meningitis [2].

Staphylococcus aureus produces many virulence factors such as enterotoxins, hemolysins, and surface protein adhesins, which are involved in the type and severity of staphylococcal infections [3].

S. aureus creates a complex structure of extracellular polymeric biofilm that provides a fully secure and functional environment for the development of microcolonies, their sustenance, and recolonization of sessile cells after dispersal. *Staphylococcus aureus* biofilm protects the cells against hostile conditions, i. e., changes in temperature, limitations or deprivation of nutrients, and dehydration, and more importantly, protects the cells from antibacterial drugs.

Drugs are rapidly becoming partially or fully inactive against *S. aureus* as they are either less penetrable or impenetrable due to the presence of biofilms surrounding the bacterial cells. Other factors including evasion of innate host immune system, genome plasticity, and adaptability through gene evolution and exchange of genetic material, also contribute to the ineffectiveness

of antibacterial drugs. This increasing tolerance to antibiotics has contributed to the emergence and spread of antimicrobial resistance (AMR) [4].

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most common pathogens linked with an increased antimicrobial resistance [5]. MRSA is usually resistant to a wide range of antibiotics including: macrolides, aminoglycoside, lincosamide, and all β-lactams, and the occurrence of multidrug resistance (MDR) among MRSA makes the treatment is difficult [6;7].

Although MRSA is a frequent colonizer of human skin, wounds, and anterior nares, the intestinal colonization of MRSA has dramatically raised the risk of inducing MRSA-associated colitis as well as creating a favorable environment for horizontal transfer of resistance genes to commensal microorganisms. On the other hand, staphylococcal resistance to last-resort antibiotics has commended the development of novel antimicrobial drugs for the effective decolonization of MRSA. In response, probiotics and their metabolites (postbiotics) have presented therapeutic options. Probiotics exhibit a multitude of anti-MRSA properties (anti-bacterial, anti-biofilm, anti-virulence, anti-drug resistance, co-aggregation, and anti-quorum sensing) by the production of numerous antagonistic compounds such as organic acids, hydrogen peroxide, low molecular weight compounds, biosurfactants, bacteriocins and bacteriocins like inhibitory substances. Furthermore, probiotics regulate the epithelial barrier function and positively impact the host immune system via regulating various signal transduction mechanisms. Preclinical and human intervention studies have suggested that probiotics outcompete by MRSA exhibiting anti-colonization mechanism via protective, competitive, and displacement modes [8]. *Lactobacillus* spp. is one of the most widely used probiotics and can be found in a large variety of food products throughout the world [9]. The genus *Lactobacillus* comprises a large heterogeneous group of Gram-positive, nonsporulating, facultatively anaerobic bacteria which include *L. acidophilus, L. rhamnosus, L. bulgaricus, L. casei* and *L. reuteri,* and *L. Plantarum*. This genus serves a vital function in food fermentation

and can also be found in the GI system of humans and animals at varying levels depending on the species, age of the host, or location within the gut [10].

The present study aimed to Biocontrolling of the growth and antibiofilm effect of probiotics against local clinical isolates of MRSA.

Materials and methods

Collection of Specimens

A total of 264 samples were collected from patients their ages between 7 days and 50 years with various infections, including wounds, burns, vaginal swabs, urine, and blood. These samples were collected appropriately to avoid any possible contamination and prepared for laboratory tests such as catalase and oxidase, cultivation in mannitol salt agar as well as on Bird Parker, as well as detection of their ability to produce coagulase enzyme by adding fibrinogen plasma [11].

Detection of virulence factors

Phenotyping Detection of some virulence factors and Biofilm production

After phenotypically diagnosing isolated bacteria, some virulence factors were detected in them, such as their ability to the production of hemolysin, gelatinase, and DNase enzymes [12; 13].

Detection of Metallo β-Lactamase and Extended-spectrum β-Lactamase production

A bacterial suspension was prepared from *S. aureus* isolates by transferring a single colony to 5 ml of normal saline and comparing it to 0. 5 McFarland (after 24 hours of incubation), the Mueller Hinton agar plates inoculated with this bacterial suspension, and two imipenem discs were placed (10 µg) at a distance of 20 mm on this plate, and 5 µl of EDTA was added to one

of the Imipenem discs and incubated overnight at 37°C [14]. The ability of the isolates to create ESBLs was determined using the combined disk or double-disk diffusion test method [15]. The Mueller Hinton agar plates were inoculated with bacterial suspension (equivalent to 0. 5 McFarland). The antibiotic disk containing a combination of Amoxicillin/Clavulanic acid (30 µg/disk) was placed in the center of the inoculated plate. Then it was surrounded by the antibiotic disk of Aztreonam and the third generation of cephalosporin Cefotaxime and Ceftazidime at a distance of 3 cm from the disk in the center. The plates were incubated for 24 hours at 37 °C. Zone inhibition from 5 mm or more in the presence of Augmentin is suggested as a positive result for the production of ESBL enzyme.

Antimicrobial susceptibility test

The disk diffusion method on Mueller-Hinton agar was used to assess the susceptibility of all isolates to different types of antibiotics, as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2021). table (1)

Table 1: Antibiotics used in the study

Antibiotic	Symbol	Concentration (µg)	Company
Amikacin	AK	30	Mast
Gentamycin	GM	10	Mast
Netilmicin	NET	30	Mast
Azithromycin	ATH	15	Mast
Clarithromycin	CLA	15	Mast
Chloramphenicol	C	30	Mast
Ciprofloxacin	CIP	5	Mast
Levofloxacin	LEV	5	Mast
Ofloxacin	OFX	5	Mast
Clindamycin	CD	2	Mast
Imipenem	IMP	10	Mast
Oxacillin	OX	1	Mast
Nitrofurantoin	NI	300	Mast
Tetracyclin	T	30	Mast
Teicoplanin	TEC	30	Mast
Vancomycin	VA	30	Mast

Detection of *mecA* genes in *S. aureus* isolates by PCR Technique

DNA Extraction

DNA of the isolates was extracted using the ABIO protocol for pure extraction was followed, from 500 µl overnight Brain Heart Infusion Broth, 100 µl of Nuclease-free water, and 100 µl of Lysozyme solution were added to the pellet of *S. aureus* isolates to digest the cell wall. Then for Protein digestion and cell lysis, 20µl of Proteinase K solution (20mg/ml), and 200µl of Buffer BL were added to the sample. Quantus Fluorimeter used to detect the concentration of DNA extracted.

Primers Sequencing

All *S. aureus* isolates were tested for the presence of the 310 base pair (bp) PCR product of *mecA* gene, using the primers (supplied by the Macrogen Company in a lyophilized form). As shown in table (2). The amplification reaction was carried out in 15 µl volume, under the following conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 30 seconds, followed by a final extension at 72°C for 3 min. The amplified products were electrophorized on 1. 5% (w/v) agarose gel, stained using 0. 5 µg/ml ethidium bromide solution, and electrical power was turned on at 100v/mAmp.

Table 2: Primer *mecA* gene sequencing

PRIMER TARGET GENE	SEQUENCE (MACROGEN, KOREA)	ANNEALING TEMP. (°C)	PRODUCT SIZE(BP)	REFERENCE
mecA-F *mec*A-R	5 -TGG CTA TCG TGT CAC AAT CG-3 5 -CTG GAA CTT GTT GAG CAG AG-3	56	310	[16]

Isolation and identification of local probiotic *Lactobacillus* spp.

Sources of *Lactobacillus* spp. isolates

The isolates of Lactobacillus spp. were obtained from Salah Al-Din University, College of agriculture, and isolated from local dairy products. These isolates were confirmed by microscopic examination with colony morphology, motility test, and some biochemical tests, including catalase and oxidase tests, Carbohydrate fermentation, and determination of the growth at 15 and 45˚C [17].

Determination of the properties of the local isolates of *Lactobacillus* spp. as probiotics

Bile salt tolerance test

The detection for bile tolerance of the isolated Lactobacilli, was carried out by growing it in MRS broth containing 0. 1%, 0. 3% bile salt for 24 hours under anaerobic conditions at 37°C. Culture broths with a turbidity of more than 0. 5 units at 560 nm were classified as bile tolerant strains. These isolates were selected for experience with broths containing higher concentrations of 0. 5 and 1. 0% (w/v) of bile salt. Positive results were obtained by the growth of the LAB culture [18].

Acid tolerance test

The isolated Lactobacilli were subjected to the first investigation of acidity tolerance in MRS broth adjusted to pH 2. 5 with 1N HCl for 90 minutes at 37°C. The determination of survival was performed by single streaking on MRS agar plates, and the growth was seen after 24-48 hours of anaerobic incubation at 37°C. Isolates that grew on the agar were considered to be acid tolerant isolates. These isolates were chosen and grown in MRS broth under anaerobic conditions at 37°C. The isolates were inoculated in 10ml of MRS and adjusted to pH 2. 0, 3. 0, 5. 0, and 7. 0 with 1N HCl. Incubating samples at 37°C for 2 hours. Cells were adjusted to serial

dilution by phosphate buffer at pH 7. 0. The dilution was cultured on MRS agar for the determination of viable cells after 48 hours of incubation. Positive results were obtained by the growth of the LAB culture [18].

The Antimicrobial activity of *Lactobacillus* spp. isolates

The antimicrobial activity of *Lactobacillus* spp. was assessed using well diffusion techniques against the growth and antibiofilm of methicillin-resistant *Staphylococcus aureus* isolates. This was done by inoculating approximately 10^5-10^7 CFU/ml of the bacteria on Muller Hinton agar and wells with a 5 mm diameter were done on the surface of cultured media, and the cell-free extracts of *Lactobacillus* spp. obtained by growing in MRS broth, incubated at 37 °C for 48 h, centrifuged at 6000 g for 15 min, the supernatant was filtered through a cellulose acetate filter with a pore size of 0. 22 µm, the wells were filled with 1 ml of the filter, then The dishes were incubated for 2 hours in the refrigerator. The inoculated plates were incubated for 24 hours at 37°C, and the diameter of the inhibition zone was measured with calipers in millimeters [18].

Detection of Biofilm Formation

The biofilm formation was detected by microtiter plate assay according to [19]. The bacteria were inoculated on a Nutrient broth medium at 37°C for 24 hours. Then 200 µl of an isolate suspension (corresponding 0. 5 Macfarland standard) was transferred into each of the three wells of a 96-well flat-bottom polystyrene plate and incubated at 37°C for 24 hours., each well was washed three times using distilled water with rough shaking and later dried thoroughly. The adhering bacterial cells were fixed with 200 µl of absolute methanol. Then, each well was stained with 200 µl of 0. 5% crystal violet for 15 minutes. Repetitive washing was performed to remove the excess stain. Later, the crystal violet bound to the adherent cells was retained with 200 µl of ethanol per well. The test was made in triplicates, and the absorbance of wells filled with bacteria-free Nutrient broth served as a negative control. The amount of crystal violet

removed by 95% ethanol in each well was quantified by measuring the OD 630 nm using an ELISA reader.

Determine the Antibiofilm Effect of Cell-Free supernatant

Biofilm-forming abilities and anti-biofilm activity were determined in microtiter plates using a crystal violet binding assay with modification [20]. The antibiofilm activity was measured as 200µl of 10^6 CFU/ml of the overnight *S. aureus* culture was thoroughly mixed with 100µl of CFS in a sterile microtiter plate and incubated at 37◦C for 24 hours. Following incubation, the non-adherent cells were removed by washing the wells gently 3 times with sterile distilled water. The adherent cells were fixed by using 200 µl of methanol (HiMedia) for 15 min and then the plate was emptied and air dried. The fixed biofilms were stained by using 200 µl of 2% crystal violet (HiMedia) in distilled water for 5 min. Excess stain was removed by washing under running tap water till the color fades away. The stain was extracted from the adherent cells by using 160 µl of 33% glacial acetic acid (HiMedia) in distilled water and OD595 was measured using an ELISA reader. The percent of antibiofilm capabilities was assessed in comparison with the control wells (bacterial wells without CFS).

Statistical analyses

Statistical analysis Fisher's test (GraphPad Prism version 6 software) turned into used for the assessment between samples of the contemporary examination. P-values less than 0. 05 degrees have been considered statistically significant. P-value is as follows: P-value < 0. 01.

Results

Overall 50 isolates of *Staphylococcus aureus* primary were discovered from different clinical sources, distrusted as follows: wounds 13(26%), burns 8(16%), blood 8(16%), vaginal swab 10 (20%), and urine 11(22%). All isolates were grown on blood agar and Mannitol salt agar. Some

isolates create clear β -hemolysis around their colonies in blood agar, whereas others exhibit gamma hemolysis. The result showed that catalase was positive while oxidase was negative. The identification of the isolated bacteria was confirmed by Vitek 2 System. All isolates examined on Baird Parker agar had black, convex, and shiny colonies, indicating the presence of *S. aureus* with a clear zone 34(68%), confirming coagulase-positive, and some isolates proximity 16(32%) without a clear zone confirming coagulase-negative.

Detection of some virulence factors

The results shown in figure (2) appeared some virulence factors such as the production of hemolysin, gelatinase, DNase, biofilm, coagulase, MBL, and β-lactamase enzymes were detected in all fifty isolates of *S. aureus* in this study, table (3).

Table 3: Distribution of virulence factors among *S. aureus* isolates

VIRULENCE FACTOR		% OF POSITIVE RESULT	% OF NEGATIVE RESULT
Gelatinase		42	58
Dnase		62	38
MBL		2	98
Coagulase		68	32
ESBL		2	98
Hemolysin	β-hemolysin	82	-
	γ-hemolysin	18	-
Biofilm		56	44
P *value*		*0. 001	
Chi-square		289. 6	

***P-value < 0. 05 is significant**

Biofilm production

The microtitre plate method using to detect the biofilm formation for all isolates, and the current study results showed that 7(14%) isolates of *Staphylococcus aureus* was strong biofilm and 21(42%) was moderate biofilm while the isolate which weak 22(44%) as appeared figure (1). The prior study by [21]results showed strong biofilm (1%) isolates, moderate (8. 2%) isolates, and weak (54. 1%) isolates, while (36. 7%) of them had no attachment ability.

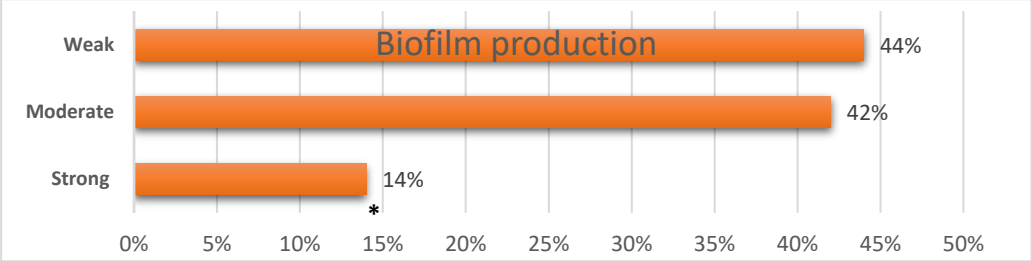

Figure 1: Biofilm production by *S. aureus* isolates. *Significant difference between percentages using Chi-square test (χ 2 -test) at 0. 05 level.

Extended-spectrum β-lactamase and Metallo β- Lactamase enzyme production

It was utilized to detect the isolate's ability to make ESBL enzymes. The results revealed that 1(2%) isolates produced ESBL enzymes. The result of a local study done by [22] showed that 34. 8% ESBLs enzyme producers. According to their relative size and the genes they contain, *S. aureus* plasmids have been divided into three classes: class I, II, and III. These plasmids include a diverse set of genes that code for antibiotic resistance. The majority of Class III plasmids are conjugative plasmids [23]. The *blaZ* gene, which causes penicillin resistance, is carried by a transposable element that can be found either in the core chromosome or as part of a plasmid [24] Detection of the isolate's ability to produce MBLs enzyme; the results revealed that 1(2%) isolates produced MBLs enzyme. This result contradicted the findings of [25] which showed that 50% of isolates were produced.

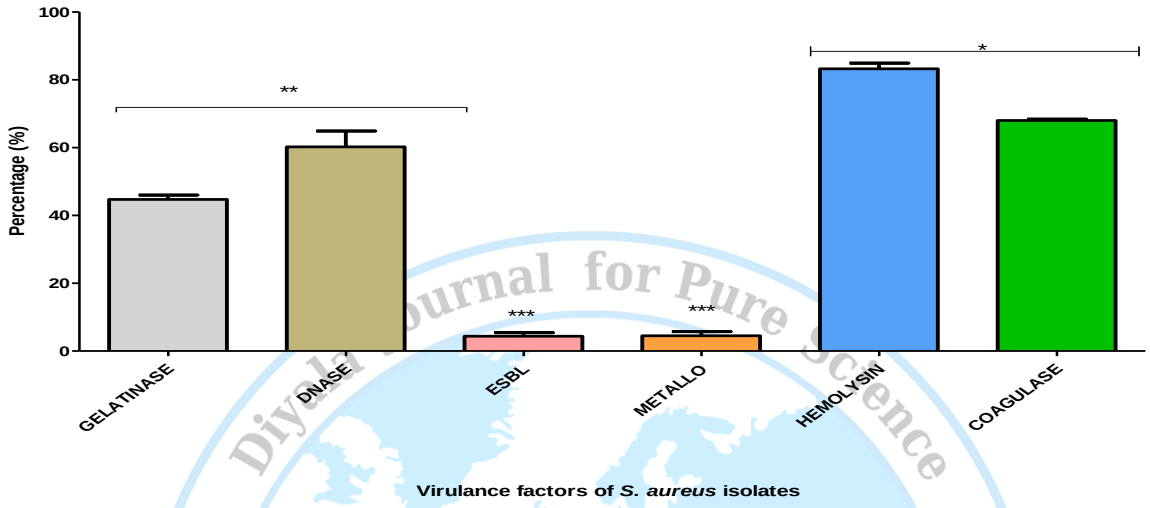

Figure 2: Percentage of Virulence factors of *S. aureus* isolates

Bacterial Resistance to Antibiotics

Fifty *S. aureus* isolates were tested to determine the resistance to 16 antibiotics from various kinds of antibiotics by the Disc Diffusion technique, and it was found that isolates exhibited diverse levels of resistance to different antibiotics (Figure 3). The results showed that 49 (98%) of the isolate appeared as MRSA and 1 (2%) of isolates appeared as MSSA according to complete resistance toward the Oxacillin class of cell wall synthesis inhibitors. This result agreed with the data published by [26] in the Al-Muthanna governorate who recorded that 100% of *S. aureus* isolates are resistant to oxacillin. *S. aureus* develops resistance to beta-lactam antibiotics through two mechanisms: beta-lactam penicillinase and the *mecA* gene. This enzyme degrades the beta-lactam ring in beta-lactam antibiotic structures, rendering them inactive [27]. The second defense mechanism necessitates the acquisition of the *mecA* gene, which encodes the PBP2a protein that aids in bacterial cell wall construction even when beta-lactam antibiotics are present [28]. As well as the current study revealed that the resistance was 100% to Nitrofurantoin, as well as resistance rate to ofloxacin, ciprofloxacin, and levofloxacin was 40%, 42%, and 62%, respectively. Aminoglycosides which include gentamycin, amikacin, and

Netlimicin, the isolates have appeared level of resistance to gentamicin (56%) and Amikacin (52%), while the resistance rate of Netlimicin14% this result agrees with [29] showed Resistant to netilmicin (11. 1%). Macrolides which include azithromycin whose results indicated the percentage of *S. aureus* isolates (78%). Lincosamides which include clindamycin, the percentage resistance that 80% of *S. aureus* isolates were clindamycin resistant. Phenicol includes chloramphenicol classes of protein synthesis inhibitors antibiotics which *S. aureus* isolates were resisted to chloramphenicol by 32%. Tetracyclines include tetracycline the result of the current study detected that 52% of the isolates resisted. Glycopeptides include vancomycin and teicoplanin which is cell wall inhibitors, the study showed that the resistance rate of vancomycin and teicoplanin was 100%, 90% this result agrees with the result of a local study by [30] was mentioned the resistance rate of vancomycin (92. 3%) as well the study by [29] found the percentage resistant 61. 2% to teicoplanin.

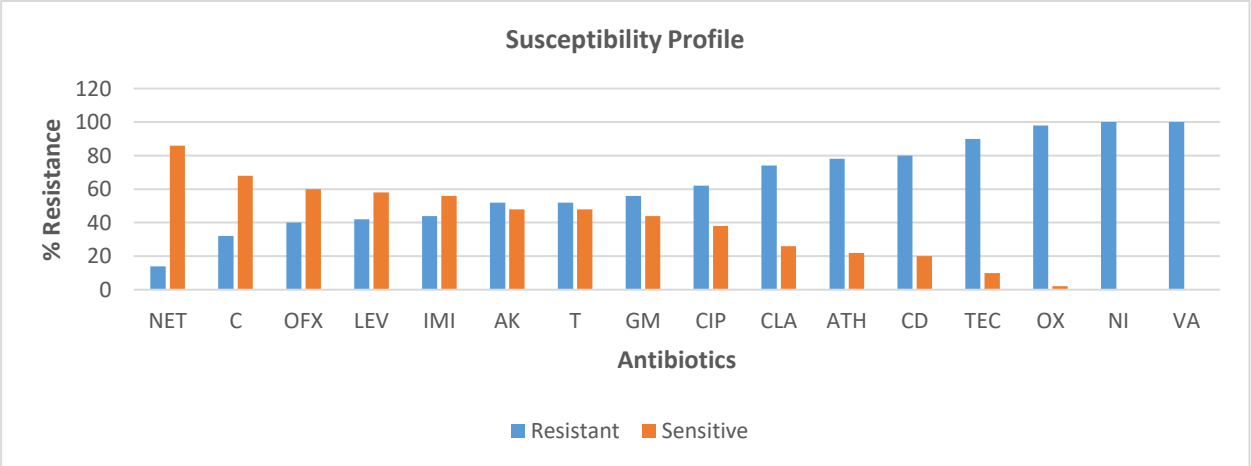

Figure 3: Prevalence and Antimicrobial Susceptibility Profile of *S. aureus* isolate. In general, there is a significant difference between percentages using Chi-square test (χ 2 -test) at 0. 05 level.

Multidrug Resistance Patterns in Isolates

All fifty isolates in the current study saw a multi-drug resistance (MDR, XDR, and PDR) level of resistance to the tested antibiotics, with a rate of 100%. The incidence of Multi-drug resistance (MDR), extensive drug resistance (XDR), and pan-drug resistance (PDR) was investigated among isolates of 50 *S. aureus*. MDR isolates were classified as isolates that showed resistance to antimicrobials from at least three of all antimicrobial categories examined in this study. Extensive drug resistance (XDR) is defined as an isolate that is resistant to all but one or two classes. PDR isolates are resistant to all seven antimicrobial classes tested [31]. Our result showed in figure (4) that 41(82%) were Confirmed as MDR, 7 (14%) of them were confirmed as XDR, and the remaining 2(4%) were PDR isolates. Antibiotic resistance has evolved as a major problem as a result of the extensive and indiscriminate use of these medications in treatment, particularly among *S. aureus* bacteria. This could be due to the creation of biofilms, which increases the pathogenic ability [32].

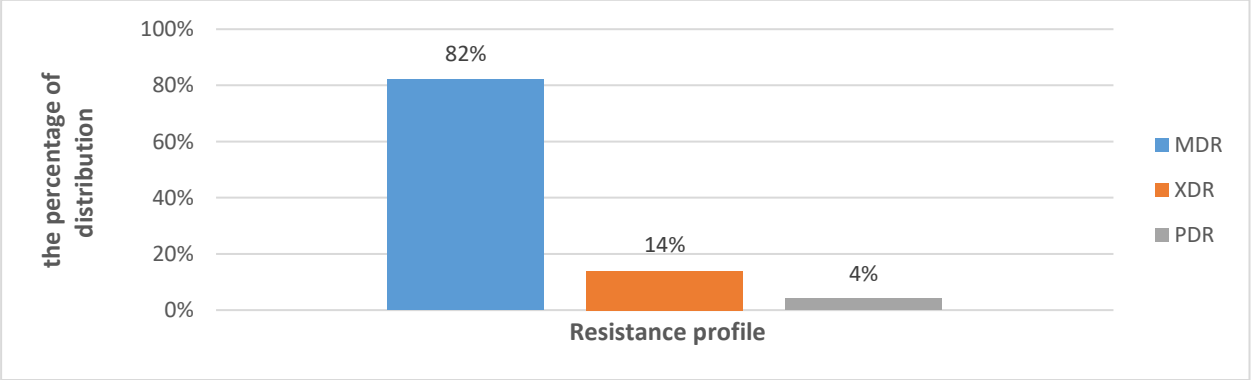

Figure 4: Bar graph of the percentage distribution of multidrug-resistant *S. aureus* (MDR), extended-drug-resistant *S. aureus* (XDR), and pan-drug-resistant *S. aureus* (PDR).

Detection of *S. aureus*

Detection of *mecA* genes by PCR

The result showed that all 7(100%) isolates possessed *mecA* genes, which showed bands of 310 bp as a product size (figure5), these results are in agreement with previous studies as what was revealed by [33] and [34]in Nepal showed (93. 8%), (82. 1%) of isolates had *mecA* gene respectively.

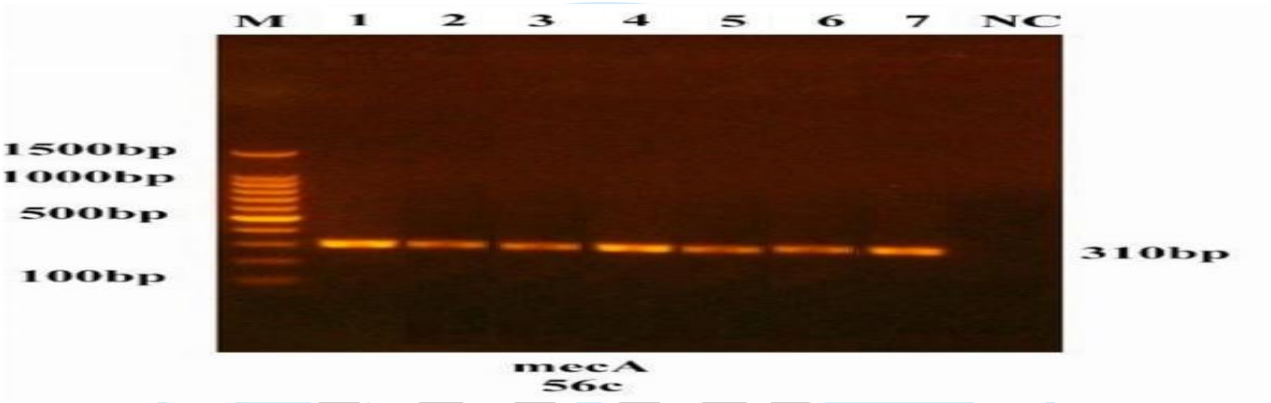

Figure 5: The amplification of *mecA* gene in *S. aureus* was fractionated on 1. 5% agarose gel electrophoresis stained with Eth. Br. M: 100bp ladder marker. Electrical power was turned on at 100v/mAmp for 60min. product pcr 310bp

Isolation and identification of probiotic lactic acid bacteria

Bacteria were isolated from local dairy products in MRS agar media at 37°C below anaerobic conditions for 48 hours. The isolated colonies from local yogurt were named *Lactobacillus* spp. The colonies of LAB on MRS agar were pale, round in shape, soft, mucoid, convex, and surrounded by a clear zone as a result of dissolving $CaCO_3$ which was a result of producing lactic acid that was analyzed as calcium carbonate. The complete findings of microscopic morphology and biochemical characteristics of the isolates were shown in table (4). The isolates appeared as Gram-positive, and catalase and oxidase-negative. All the isolates fermented glucose. *L. Plantarum* isolates also fermented all tested sugar, while *L. reuteri* varies in sugar fermentation.

Table 4: Biochemical test for *Lactobacillus* spp

Tests	*L. reuteri*	*L. Plantarum*
Growth at		
15°C only	-	+
45°C only	+	-
Catalase	-	-
Motility	+	+
Oxidase	-	-
Sugar Fermentation		
Lactose	+	+
Mannose	-	+
Raffinose	-	+
galactose	+	+
Sucrose	+	+
Maltose	+	+
Sorbitol	-	+
Glucose	+	+

The result in a table (5) showed that both *Lactobacillus Plantarum* and *L. reuteri* were more tolerant and resistant to 0. 3% bile salt with a gradual decrease of viable cells in 1. 0% bile salt. Furthermore, these isolates were able to tolerate the increased acidity and could grow well at pH ranging between 3-5.

Table 5: Some characteristics of lactic acid bacteria as probiotics

Characters	*L. reuteri*	*L. Plantarum*
Bile salt tolerance		
0. 1%	+++	+++
0. 3%	++	++
0. 5	++	+
1. 0	+	+
Acid tolerance		
pH 2	++	++
pH 3	+++	+++
PH 5	+++	+++
PH 7	++	++

Antibacterial activity of *Lactobacillus* species against *Staphylococcus aureus*

With the antibacterial activity of lactic acid bacteria as a probiotic against MRSA, it was found that Lactobacillus bacteria have a high efficacy on the S. aureus growth as observed in the table (6). Lactobacillus plantarum was noticed to reach the highest inhibition zone (12-17) mm in most isolates except S6 and S7 which were resistant. The results also showed that the effect of Lactobacillus Plantarum was clear and highly effective in inhibiting S. aureus growth even more than the effectiveness of the antibiotics, which appeared resistant to Vancomycin and Oxacillin. A previous study in Iran by [35] revealed that larger halos were observed against Staphylococcus aureus (15 ± 0. 3), while a result by [36] revealed that the largest zone of inhibition was 13mm with Lactobacillus plantarum[37] showed in their study that L. Plantarum has antimicrobial activity against S. aureus with an inhibition zone of 9 mm.

Lactobacillus reuteri showed an inhibition zone of (16-20) mm in all isolates except S6 which was resistant. In a study carried out in the Basrah governorate, [38] noticed that L. reuteri had (16-20) mm inhibitory action against S. aureus, while another study performed in the Kingdom of Saudi Arabia by Al [39] showed an inhibition zone of (22) mm in Staphylococcus aureus. A previous study in Egypt by [40] revealed that Lactobacillus reuteri showed a higher inhibitory effect on S. aureus isolates.

The antimicrobial impact of Lactobacilli is primarily attributed to the generation of organic acids, such as lactic acid, acetic acid, propionic acid, and occasionally hydrogen peroxide, bacteriocins, and antimicrobial peptides (AMPs) with a wide range of action [41].

Table 6: Antibacterial activity of *L. plantarum* and *L. reuteri* against *S. aureus* isolates

.NO	*LACTOBACILLUS PLANTARUM*	*LACTOBACILLUS REUTERI*
S1	14 mm	17 mm
S2	15 mm	20 mm
S3	17 mm	19 mm
S4	16 mm	16 mm
S5	12 mm	16 mm
S6	R	R
S7	R	19 mm

The antibiofilm activity of *lactobacillus spp*. against Methicillin-resistant *S. aureus* (MRSA) biofilm formation

Lactic acid bacteria (LAB) are known for their capacity to produce lactic acid, among other interesting metabolites with antimicrobial activity. A cell-free supernatant (CFS) is a liquid that contains the metabolites produced by microbial growth and the residual nutrients of the medium used [42]. In our study, it was seen that an anti-biofilm effect of CFS was shown on all the seven S. aureus isolates. Table (7). The adhesion of bacterial pathogens to their host cells is well known as an important virulence factor in pathogenicity.

L. planturum showed a clear effect on S. aureus biofilm formation at a percentage of (27%-56%). The prior study in Iran by [43] observed that biofilm formation by S. aureus strains was inhibited at a range (of 49-66%). [44] in Korea also found that the anti-biofilm action against S. aureus was 44. 45%.

L. retueri showed a range of (39%-61%) anti-biofilm activity. [45] An Iranian study on the inhibition of paenibacillus larvae biofilm formation by Cell-Free Supernatant (at sub-MIC concentration) and revealed a significant reduction of 50% and 37. 29% in 24 and 48-hour exposure to CFS. A study by [46] showed that the effect of antibiofilm against Salmonella typhimurium and Salmonella enteritidis by L. reuteri was (87% and 89%) respectively.

The finding of the current study showed that CFS had different effects on the formation of biofilm. The reasons may be attributed to biofilm structure, CFS concentration, and its mode of action on bacterial cells or penetration through the matrix barrier.

Table 7: Anti-biofilm activity of *Lactobacillus spp*. cell-free supernatants against *S. aureus*

The isolates	*L. planturum* % of inhibition	*L. retueri* % of inhibition
S1	37	39
S2	53	61
S3	56	45
S4	39	50
S5	43	46
S6	30	41
S7	27	58

References

1. L. Miller, J. Fowler, S. Shukla, FEMS microbiology reviews, 44(1), 123-153(2020)
2. T. Rasheed, K. Luti, A. Alaubydi, Iraqi journal of agricultural sciences, 51(5), 1381-1393(2020)
3. G. Cheung, J. Bae, M. Otto, Virulence, 12(1), 547-569(2021)
4. M. Idrees, S. Sawant, N. Karodia, International Journal of Environmental Research and Public Health, 18(14), 7602(2021)
5. Z. Suhaili, N. Putri'Amira Rafee, C. Yeo, Germs, 8(1), 21(2018)
6. S. Mahmoudi, S. Mamishi, M. Mohammadi, Infection and Drug Resistance, 12, 137(2019)
7. K. Alkharsah, S. Rehman, F. Alkhamis, Annals of clinical microbiology and antimicrobials, 17(1), 1-11(2018)
8. B. Nataraj, R. Mallappa, Current microbiology, 78(6), 2194-2211(2021)
9. G. Giraffa, N. Chanishvili, Y. Widyastuti, Research in microbiology, 161(6), 480-487(2010)

10. R. Duar, X. Lin, J. Zheng, *FEMS microbiology reviews*, *41*(Supp_1), S27-S48 (2017)

11. I. BUCUR, V. HERMAN, C. PASCU, Bulletin UASVM Veterinary Medicine, 74, 1(2017)

12. J. Cappuccino, C. Welsh, Microbiology A Laboratory Manual. 12 ed. (Pearson, New York, 2020), 155-191

13. J. Harley, L. Prescott, Laboratory Exercises in Microbiology, 5th. ed., (The McGraw-Hill Companies, New York, 2002)

14. M. Anwar, H. Ejaz, A. Zafar, Journal of pathogens, (2016)

15. A. Patel, A. DiGiandomenico, A. Keller, Nature communications, 8(1), 1-11 (2017)

16. N. Omar, H. Ali, R. Harfoush, International journal of microbiology, (2014)

17. H. Pyer, K. Peh, International Journal of Pharmacy and Pharmaceutical Sciences, 6(1), 189-193(2014)

18. F. Alikhani, H. Dadras, A. Tajehmiri, Journal of Pure and Applied Microbiology, 7(4), 2965-2971(2013)

19. C. Almeida, N. Azevedo, S. Santos, PLoS ONE, 8 (6), 101-371(2013)

20. S. Kaur, P. Sharma, N. Kalia, Frontiers in Cellular and Infection Microbiology, 8, 120(2018)

21. R. Samadi, Z. Ghalavand, B. Nikmanesh, Archives of Pediatric Infectious Diseases, 6(3)(2018)

22. S. Aziz, Kurdistan Journal of Applied Research, 145-151(2020)

23. T. Foster, J. Geoghegan, in Molecular Medical Microbiology, 2nd ed.; Tang, Y. -W., Sussman, M., Liu, D., Poxton, I., Schwartzman, J., Eds (2015), 655-674.

24. M. Vestergaard, D. Frees, H. Ingmer, Microbiology spectrum, 7(2), 7-2(2019)

25. R. Chikkala, N. George, K. Ratnakar, Advances in Infectious Diseases, 2(03), 82(2012)

26. Z. Haneen, W. AL-Hamadany, Annals of Tropical Medicine and Public Health, 22, 130-140(2019)

27. A. Gnanamani, P. Hariharan, M. Paul-Satyaseela, Frontiers in Staphylococcus aureus, 4, 28(2017)

28. L. Harris, S. Foster, R. Richards, Eur Cell Mater, 4(3), 100-20(2002)

29. A. Al-Mayahi, F. Srhan, Jordan Journal of Biological Sciences, 14(4)(2021)

30. A. Albadri, Characterization and Molecular study to detect Multidrug Resistance Bacteria Isolated from Patients with Diabetic Foot Ulcers in Wasit Province, M.sc Thesis, Wasit University, Wasit, Iraq (2021)

31. A. Magiorakos, A. Srinivasan, R. Carey, Clinical microbiology and infection, 18(3), 268-281(2012)

32. F. Gomes, N. Martins, I. Ferreira, Heliyon, 5(5), e01728(2019)

33. O. Angel, P. Kadarko, J. Muazu, GSC Biological and Pharmaceutical Sciences, 7(3) (2019)

34. S. Dhungel, K. Rijal, B. Yadav, Infectious Diseases: Research and Treatment, 14, 11786337211037355(2021)

35. V. Jabbari, M. Khiabani, R. Mokarram, Probiotics and Antimicrobial Proteins, 9(2), 189-193(2017)

36. K. Alebiosu, A. Adetoye, F. Ayeni, West African Journal of Pharmacy, 28(2), 132-142(2017)

37. G. Lashin, E. Tohamy, A. Askora, Bulletin of Faculty of Science, Zagazig University, (1), 61-81(2017)

38. A. Mohammed, N. Hussain, A. Niamah, Journal of Microbiology, Biotechnology and Food Sciences, 2021, 134-139(2021)

39. S. Al Motuirei, A. Saadabi, British Journal of Pharmacology and Toxicology, 5(2), 98-102(2014)

40. L. El-Hosseiny, M. El-Shenawy, H. Baghdadi, Microbiology Research Journal International, 24(6), 1-11(2018)

41. T. Gemechu, African Journal of Food Science, 9(4), 170-175(2015)

42. E. Mani-López, D. Arrioja-Bretón, A. López-Malo, Comprehensive Reviews in Food Science and Food Safety, 21(1), 604-641(2022)

43. H. Zamani, S. Rahbar, S. Garakoui, Pharmaceutical and Biomedical Research, 3(2), 39-44(2017)

44. J. Lee, N. Lee, H. Paik, Food Science and Biotechnology, 30(1), 97-106(2021)

45. R. Babrud, R. Kermanshahi, F. Sede, Iranian Journal of Veterinary Research, 20(3), 192(2019)

46. A. Siddique, S. Azim, A. Ali, Animals, 11(12), 3435(2021)