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RESEARCH ARTICLE OPEN ACCESS

Detection of Some Genes Related with Virulence Factors in the Clinical Isolates of *Staphylococcus aureus* with Potential Effects of Some Probiotics *Lactobacillus* Species

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Abstract

***Staphylococcus aureus* (*S. aureus*) is remarkable pathogen and causative agent of hospital acquired (nosocomial) and community acquired infections. *S aureus* can acquire resistance to most antimicrobial agents (antibiotics). A total 295 *S aureus* isolates were collected from two local hospitals from various sites of body patients, such as urine, pus, sputum, wound, and blood. Patients' ages were ranging from 5-84 years from males and females. The number of identified *S aureus* was 83 detected by traditional and methods and confirmed by molecular method using 16S rRNA gene in polymerase chain reaction (PCR). Antibiotic sensitivity profile was performed to evaluate the resistant and sensitive strains and predominance of methicillin resistant *S aureus* (MRSA). A total 6 (7.3%) from all *S. aureus* isolates was MRSA. MRSA isolates were multi-resistant for all antibiotics, which used in the current study. Several genes that participate in antibiotic resistance were investigated. *SasX* gene was detected in 11 (12.3%) samples which was multi-resistant for many antibiotics. In addition, Furthermore, Exfoliative toxin C and D (*etC* and *etD*) genes. The *etD* gene was detected in 33 (39.75%) samples, but *etC* gene no detected in all of isolates. In addition, probiotic lactobacilli species (spp.) e.g. *L acidophillus*, and *L reuteri* against different isolates of methicillin resistence *S aureus* from different sites of infections observed variance in their inhibitory activity ranging from 9-17mm. In addition, lactobacilli spp. were collected from different sites and different results showed for *S aureus* in culture broth and cell free culture supernatant.**

Keywords: *Staphylococcus aureus*, virulence factors, probiotics, *Lactobacillus* spp.

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INTRODUCTION

Staphylococcu aureus (*S. aureus*) is one of the pathogens causing a wide range of hospital-acquired (nosocomial) and community-acquired infections. *S. aureus* play an important role and causative agent of diseases[1]. The emergence of methicillin-resistant *S. aureus* (MRSA) was a universal healthcare dilemma due to the limitation of curative options[2]. At first, it was showed as healthcare facilities specific problem; newly the community-acquired MRSA has raised[3]. Ness[4] predominant that MRSA are resistant to different β-lactam antibiotics but often the MRSA strains are multidrug resistant due to display resistance to other different antibiotics, such as aminoglycosides, macrolides, tetracycline, fluoroquinolones, and chloramphenicol that are usually used in the medication to different diseases caused by these pathogens. Furthermore, in recent two decades *S. aureus* diseases have become large serious and costly to therapy due to increasing predominance resistivity in *S. aureus* bacteria due to excessive increase use of antimicrobial agents[5]. Bimanand *et al*.[6] demonstrated that *S. aureus* is one of the more common causes of bacteremia and presently carries 20-40% mortality rate monthly. The capability of *S. aureus* strains to produce adhesion and biofilm makes them more resistant to different antimicrobial agents.

On the other hands, *S. aureus* is ability forming of biofilm, and increase resistant antibiotics, particularly in the chronic infections those strains forming biofilm and appearance raised resistance against antibiotics treatment are an important medical issue. It is considered which biofilms give a share in more 80% of all infections in human being[7].

Furthermore, in *S. aureus*, there is several virulence factors related with colonization, adherence, and biofilm formation that controlled by polysaccharide intracellular adhesion (PIA)[8]. *SasX* is a cell wall-anchored protein that is believed to have contributed to large epidemics of nosocomial infections cause by MRSA in Asia. *SasX* gene has been displayed to develop *S. aureus* nasal colonisation in laboratory animals i.e. mouse by encouraging *S. aureus* adherence to the epithelium of nasal cavity[9].

Foster *et al*.[10] stated that the immunization with recombinant *SasX* gene with specific Abs against *SasX* decreased colonization for epithelium nasal cavity.

Moreover, exfoliative toxins (*ETs*) of *S. aureus* are the major reason of human diseases, particularly staphylococcal scalded skin syndrome (SSSS) in infants and young children. There are three types of *ETs*; A, B, C, and D. The *etA* and *etB* are the main causative agents of SSSS. The *etD* toxin produces from *S. aureus* were isolated especially from other cutaneous and soft tissue infections[11]. Moreover, *etC* present only in the animals[12].

Probiotics defined as live benefit micro-organisms (MO) that confer a health benefits for humans and animals when ingested in sufficient quantities[13]. Several strains belonging to various MO, such as bacteria and yeast i.e. *Lactobacillus*, *Bifidobacterium, Saccharomyces boulardii*, and *Saccharomyces cerevisiae*, respectively and others, which are the prevalence of the gut microflora[14]. Probiotics MO together are commensals of the gut and other sites in the body host and differ from MO pathogens in the terms of their actions on the immunity in the gut as they do not stimulate immune system[15]. Yan and Polk[16] stated that the results of evidence based analyses from human being researches and lab animal models have observed the clinical potential of beneficial MO against several diseases. Lactobacilli spp. Produce several substances, such as lactic, and acetic acids, and also to other inhibitory substances e.g. bacteriocins, H_2O_2, CO_2, and others[17].

The aims of this study were to investigate the predominant of *SasX*, *etC*, and *etD* genes in *S. aureus* isolated from samples by polymerase chain reaction (PCR) technique. In addition, estimation effect of some lactobacilli species *in vitro* by different methods.

MATERIALS AND METHODS

Collection of bacterial samples, bacterial maintenance and culturing

A total 295 clinical sample of different infection sites were collected from two local hospitals; Al-Kramah and Al-Zahraa in Wasit city / Iraq. These samples were cultured on the different media, such as blood agar initially and on the selective media i.e. mannitol salt and Staph 110 agars to detect isolates of *S. aureus*. Bacterial cultures were incubated overnight at

37°C for 18-20 hours (hrs). Other techniques, such as traditional and molecular were used to detect different isolates of *S. aureus*.

In addition, in the current study, stander probiotic lactobacilli species were used, such as *Lactobacillus acidophilus* (*L acidophilus*) ATCC 4356, and *Lactobacillus reuteri* (*L reuteri*) ATCC 23272 were prepared and grown on the de Man Rogosa Sharpe (MRS) medium.

Detection of antimicrobial susceptibility test

To conduct of this test, using Bauer-Kirby discs diffusion method to check different isolates susceptibility for different antibiotics. The antibiotics were used in the present study, as the following: Amoxicillin (AMX) 10µg, ampicillin (AMP) 10µg, cefotetan (CTT) 30µg, chloramphenicol (CPL) 30µg, ciprofloxacin (CIP) 5µg, clindamycin (CLI) 2µg, Erythromycin (ERY) 15µg, gentamicin (GEN) 10µg, Nitrofurantoin (NIT) 300µg, oxcacillin (OXA) 1µg, tetracycline (TET) 30µg, and vancomycin (VAN) 30µg. Samples were considered as MRSA, according to the oxacillin antibiotic resistance.

StaphylococcuS. aureus **DNA extraction protocol and primers with PCR conditions**

Genomic DNA in different *S. aureus* isolates was isolated from *S. aureus* using Genomic Kit for DNA Extraction (Geneaid Genomic DNA extraction Kit, U.S.A.). DNA was extracted, according to the manufacturer's instructions. In brief, all samples were centrifuged and pellet was suspended in 0.2 mL GB buffer for 10 minutes. A total 0.2 mL GB buffer was used also for 10 minutes. Then, absolute ethanol (0.2 mL) was added to lysate. 2 ml tube, and used to collect after by GD columns after centrifuge. A W1 buffer added to the GD column and centrifuged. Then, wash buffer was used and elution buffer added and left for 3 minutes to ensure obtaining purified DNA. The different virulence genes responsible for biofilm formation were identified by polymerase chain reaction.

The specific primers, such as 16S rRNA, *SasX*, *etC*, and *etD* were synthesized by Eurofins MWG Operon (MWG, Germany), as outlined in the Table 1.

Table 1. All primer used in the current study, and the specific primer of 16S rRNA gene of *S. aureus*

Primer	Primer's sequence (5'-3')	Product size (bp)	Accession No.
SasX	F TCACCTTTTGCTACACCTGGT	304bp	KU901576.1
	R AATGACTCAAATGTAAGGGGAGT		
etC	F TCCCGCACGGTACTTTTGAA	522	JX298872.1
	R GAATTATGTCCCAGCCCCGT		
etD	F AGTACAATGCAGCCCCTTCC	412	NC_007795.1:277667
	R CCGGATCCAGAATTTCCCGA		8-2777550
16S rRNA	F CTGGAACTGAGACACGGTCC	777	KF678862.1
	R CCCAACATCTCACGACACGA		

Finally, concentration of DNA and quality were evaluated using a NanoDrop spectrophotometer and purity. DNA samples with low purity were discarded, and extractions were repeated if required.

Detection of antagonistic activity using agar spot test method

Overnight cultures of different lactobacilli spp. were spotted onto the surface of MRS agar and incubated for 24hrs. at 37°C to allow colonies to develop. After overnight incubation, the plates were checked for inhibition zones, according to the Klewicka *et al*.[18] method. A clear zone of more than 1 mm around a spot was considered as an indicator of antimicrobial effect[19]. All tests were replicated three times under the same identical experimental conditions.

Statistical analysis

Statistical analysis was used to analyze data according to the Statistical Package for the Social Sciences (SPSS, Version 17.0). As well as, Chi Square used for all isolates with *P* value was less than 0.05 ($P < 0.05$).

RESULTS

Collection of samples

The results of culturing a (295 samples), and taken from different sites of infection in a performance duration extended from February 2018 to February 2019. A total 83 samples of *S. aureus* were obtained from both sexes; male and female with age range 5-84 years, as listed in the Table 2.

Table 2. Percentage and numbers of infected patients, according to the age

Age (Year)	Number	Percentage (%)
5-14	3	3.9
15-24	9	14.5
25-34	12	17.1
35-44	7	7.9
45-54	16	14.5
55-64	21	21.1
65-74	11	15.8
75-84	4	5.3
Total (Mean)	83	100.0

The spread of *S. aureus* was significant (P < 0.05) variable, according to the gender of infected individual. Females were more than males (54.2% and 45.8%), respectively, as outlined in the Table 3.

Table 3. Percentage of infected in both; males and females

Gender	Number	Percentage (%)
Female	45	54.2
Male	38	45.8
Total	83	100.0

Detection isolates of *S. aureus* in different samples

S. aureus were detected using traditional and molecular techniques. In traditional methods, isolates of *S. aureus* were ability to produce β-hemolysis on the blood agar medium, mannitol salt fermentation, production of H_2O_2 from catalase, production of coagulase, and DNase. To confirm diagnosis of *S. aureus*, detection isolates of *S. aureus* by molecular technique using 16S rRNA through Maxime Pre Mix for PCR (I NtRON, Korea), according to the manufacturer's instructions. A total 83 samples were identified as *S. aureus* bacteria. A total 6 samples were identified as MRSA which constitute 7.3% from all isolates of *S. aureus*.

According to type of sample, the samples incidence of *S. aureus* was higher in the pus samples and lower in the burn samples, (39.8% and 6.0%) respectively ($P < 0.05$), as outlined in the Table 4.

Antibiotic susceptibility test

The results for antibiotic susceptibility test were explained in the Fig. 1.

Table 4. Percentage and numbers of *S. aureus* according to the source of infections

Type of sample	Numbers	Percentage (%)
Pus	33	39.8
Wound	15	18.0
Urine	11	13.3
Sputum	10	12.0
Blood	9	10.9
Burn	5	6.0
Total	83	100.0

P < 0.05

P < 0.05

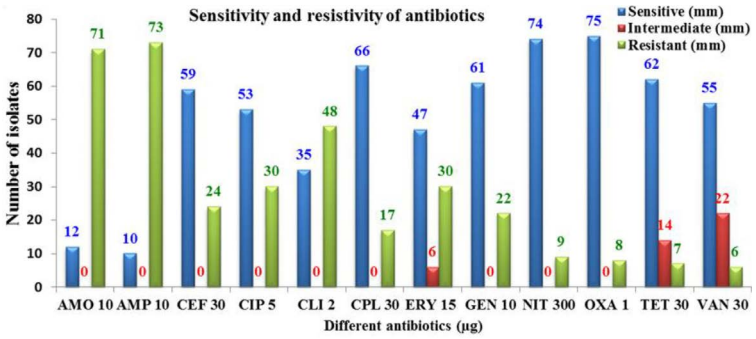

Fig. 1. Antibiotic susceptibility test on the different *S. aureus* isolates

Detection of some biofilm and other genes (*SasX*, *etC*, and *etD*) in *S. aureus* and diagnosis *S. aureus* isolates by 16S rRNA

Diagnosis of different *S. aureus* conducted by 16S rRNA (Fig. 2).

In addition, PCR was used to detect the presence of different genes, such as exofoliative toxins (*etC*, and *etD*) in *S. aureus* samples. The *etD* gene was detected in 33 (39.75%) isolates from all S. *aureus* isolates, as outlined in the Table 5 and the Fig. 3.

On the other hand, *etC* gene no detects in all samples of *S. aureus* samples, as summarized in the Fig. 4.

SasX gene in different isolates of *S. aureus*

Moreover, PCR was used to detect the presence of different genes, such as *SasX* gene in S. aureus samples. In the current study, the high

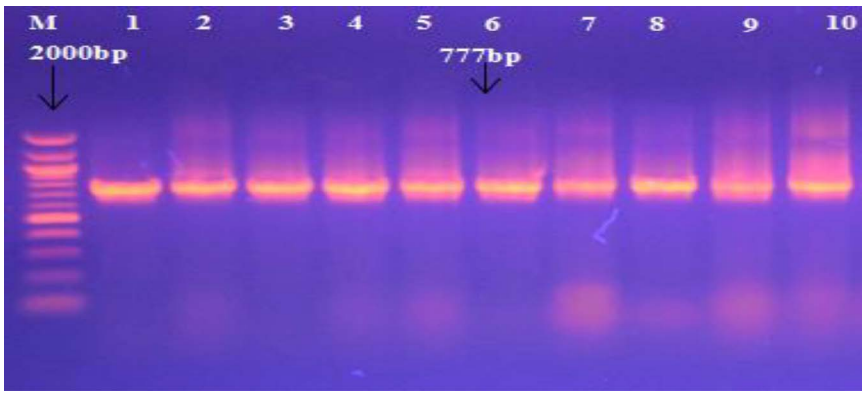

Fig. 2. Agarose gel electrophoresis and Genomic DNA isolated from *S. aureus* showing 16S rRNA gene for *S. aureus* using specific primer, with M: Marker (100-2000bp). All lanes were (1-10) positive PCR amplification at 777bp PCR product size.

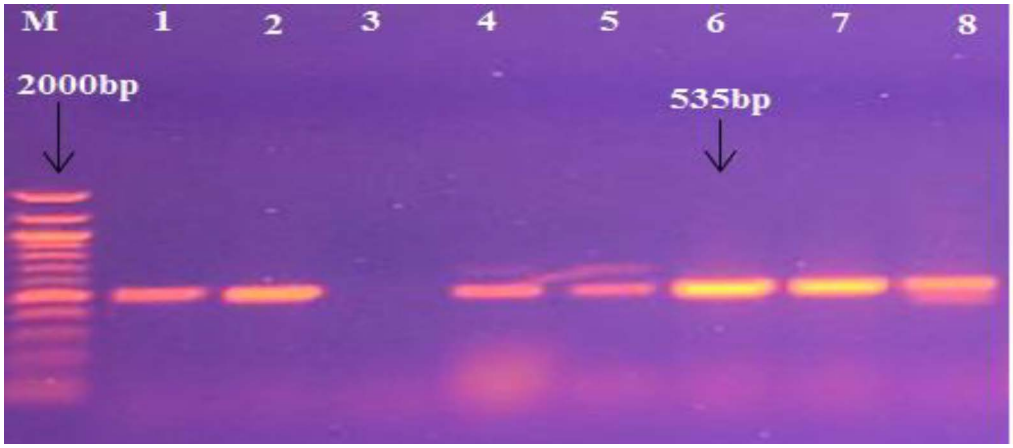

Fig. 3. Agarose gel electrophoresis image that shown the PCR product of *etD* gene in *S. aureus* isolates. Where M: Marker (100-2000bp), lane (1-8) some positive PCR amplification at (535bp) PCR product size.

Table 5. Prevalence of *etD* gene in different samples of *S. aureus*

Sample Gene	Pus	Wound	Burn	Urine	Sputum	Blood	Total
etD No(%)	15(45.5)	10(30.1)	5(15.2)	2(6.1)	1(3.1)	0(0)	33(39.75)

significant ($P < 0.05$) rate was in sputum isolates (50%) and lowest was in urine (9.0%) respectively, as outlined in the Table 6 and the Fig. 5.

Fig. 4. Agarose gel electrophoresis image that shown the PCR product of *etC* gene in *S. aureus* isolates. Where M: Marker (100-2000bp), lane (1-7) negative PCR amplification at (549bp) PCR product size.

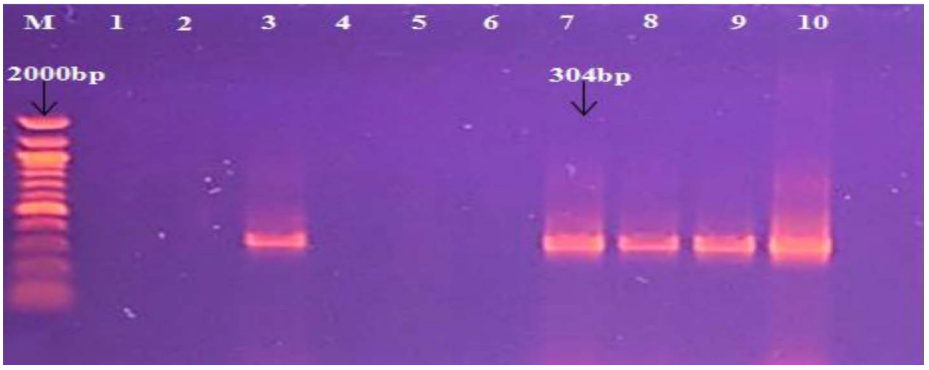

Fig. 5. Agarose gel electrophoresis image that shown the PCR product of *SasX* gene in *S. aureus* isolates. Where M: Marker (100-2000bp), lane (1-10) some positive PCR amplification at (304bp) PCR product size.

Table 6. Prevalence of *SasX* gene in different samples of *S. aureus*

Type	*SasX*		
	Positive No(%)	Negative No(%)	Total No(%)
Pus	4(12.1)	29(87.9)	33(100.0)
Wound	1(6.7)	14(93.3)	15(100.0)
Sputum	5(50.0)	5(50.0)	10(100.0)
Urine	1(9.1)	10(90.9)	11(100.0)
Blood	0(0.0)	9(100)	9(100.0)
Burn	0(0.0)	5(100.0)	5(100.0)
Total No(%)	11(12.3)	72(87.7)	83(100.0)

$P < 0.05$

Table 7. Inhibitory effect of *Lactobacillus* spp. to methicillin resistence *S. aureus* on the MRS agar

MRSA number	*L. reuteri* (mm)	*L. acidophillus* (mm)
1	10	15
2	9	13
3	10	14
4	9	12
5	13	17
6	12	16

Assessment inhibitory effect of probiotic lactobacilli species against different *S. aureus* isolates

The results of lactobacilli species (Standar strains), such as *L acidophillus*, and *L reuteri* against different isolates of methicillin resistence *S. aureus* from different sites of infections observed variance in their inhibitory activity ranging from 9-17mm. Moreover, the results showed that there were variances in the inhibitory effect of the same isolate against antibiotic sensitive and resistant methicillin resistence *S. aureus*.

Agar spot test method

The results with the current study of bacterial culture broth for *Lactobacillus* spp. activity test were displayed obvious activity against methicillin resistence S. aureus.

L acidophilus express inhibition zone of 12-17 mm for *S. aureus* sensitive to antibiotics mentioned above, whereas the results with L reuteri were 9-13 mm, as outlined in the Table 7.

DISCUSSION

S. aureus is the major problem which causes a wide spread of diseases, such as bacteremia and nosocomial infections[20]. Tosas *et al*.[21] demonstrated that *S. aureus* is has been a worldwide health problem causing high rates of infection and death cases. In the present study, demonstrated there were predictive of high significant variance ($P < 0.05$) in spread of S. aureus in varies ages of patients which were variable and high percent of infected patients and ranging between 40-59 years.

The majority cases were patients that infected with wound of surgical operations, as summarized in the Table 4. Furthermore, Radhakrishnan *et al*.[22] stated that *S. aureus* infections distributed between various groups of age similarly, while there were study conducted by Sdougkos *et al*.[23] who reported that the ages between 15-60 years were high susceptible to be infected with *S. aureus*.

Moreover, the all of results with sensitivity test were significant ($P < 0.05$). The majority of these *S. aureus* samples were sensitive to OXA (75mm) and lowest sensitivity was with AMO (12mm), as outlined in the Fig. 1.

Martinez and Baquero[24] mentioned that the processes of resistance occur naturally or acquired by mutations or plasmid transportation. On the other hand, the resistance to beta-lactamase was highest with all antibiotics that used in the current study e.g. AMP (73mm) and AMO (71mm) and lowest resistivity was with VAN (6mm), TET (7mm), OXA (8mm), and NIT (9mm), respectively (Table 1). Abdullahi and Iregbu[25] stated that the results were resistant as to the occurrence of β-lactamase generating of *S. aureus* isolates in the health centres environment due to the usage of the β-lactamase treatments for the handling, presenting chance for the selective colonizing for more resistant β-lactamase *S. aureus*.

With regard to virulence factors, the fact that the existence of *SasX* gene accompanied with other genes responsible for adherence, colonization, and yield of biofilm and their high resistant to antibiotic, and similar to other researcher like Fahimeh *et al*.[26]. The *SasX* protein has remarkable role in the nasal colonization and epidemic wave of MRSA and effect as a virulence factor on the aggregation of bacteria[9]. Moreover, the prevalence of *SasX* isolates were compatible with found Li *et al*.[9] when who reported that *SasX* gene existence has a role in the enhancing nasal colonization and adhering, abscess formation and lower respiratory tract diseases. The strains of *S. aureus* which carry *SasX* gene have been found to spread rapidly in many studies in east China, as reported in Kong *et al*.[27] study. In the present study, 6 (7.3%) were considered isolates as MRSA according to their resistance to OXA, and most of the isolates were isolated from pus and wounds samples. Also, *SasX* gene has a role in the epidemics of MRSA in Asia, and who stated that *SasX* gene increase *S. aureus* colonization, aggregation and evade immune system responses, and lately leads to prolonged survival in blood of human[9]. In the present study, the percent was higher than results in another study that conducted by Dhiman *et al*.[28] in Shanghai (33.3%).

The *etD* is a 27 KDa protein and have structural similarity to other exfoliative toxins, was first detected in 2002 by Nishifuji *et al*.[29]. The predominant of *etD* was linked to the cutaneous

diseases, and that statement agreed with study which conducted by Mohseni *et al*.[11]. The prevalence of *etD* was linked to the cutaneous diseases, and compatible with found of Bukowski *et al*.[30] study.

Furthermore, Sato *et al*.[12] reported that the molecular mass of another gene of exofoliative C gene (*etC*) is a 27 kDa, heat labile, and origins *etC* in the animals, such as chickens and mice. Moreover, *etC* isolated from horse. In the present study we noticed that there is no presence of *etC* gene in all samples of human (Fig. 4). These results of current study compatible with found of Nishifuji *et al*.[29] when who reported that *etC* was not related to human diseases, and identified only in the animal, especially with horse infections.

Probiotic is beneficial intestinal microorganisms have abundant and significant functions, such as they produce several nutrients for body host, inhibit and prevent diseases, which caused by gastrointestinal pathogens, and modulation of immune system response[15]. For probiotic effect, the results were significant (Table 7) with the current study, and were agreed with found of Dowarah *et al*.[31] who suggested that the inhibitory action of probiotics have main related to statement that lactic acid bacteria (LAB) decrease pH and/or secrete organic acids (lactic and acetic acids). The results of *S. aureus* culture broth in the current study showed observable inhibitory effect in the resistant and sensitive to isolates of *S. aureus*. The reason may be due to the production of antimicrobial substances i.e. organic acids which generally the chief end product for LAB. Riviere *et al*.[32] described that the ability to destroy inulin-type fructans of LAB isolates and Prabhujeshwar *et al*.[33] who reported that the ability of organic acids by lactobacilli in various media stated related results. Furthermore, other substances also were noticed to express effect on bacterial growth, such as bacteriocins, and H_2O_2[34]. The results of the current study were compatible with found of Khanafari and Porgham[35], and Vieco-Saiz *et al*.[36].

CONCLUSIONS

The percentages of *Staphylococcu aureus* were increased in pus and wound samples 33 (39.8%) and 15 (18.0%) respectively, and the lowest was in the burn sample 5 (6.0%). Furthermore, MRSA isolates constitute 6 (7.3%) from all S. aureus isolates, and methicillin sensitive *S. aureus* (MSSA) were 92.7 % from all S. aureus samples. MRSA were multiresistant for numerous antibiotics i.e. ampicillin (73%), amoxicillin (71%) and clindamycin (48%). Furthermore, molecular techneque analysis had seen that the found of adherence, biofilm, and exofoliative genes, especially *SasX*, *etC*, and *etD*, that an increase antibiotic resistance to *S. aureus*. Moreover, the increase rates of resistance to β-lactam antibiotics in such large rates, and indicates that the misuse of antibiotic represent a serious problem need to be more investigated.

Finally, the current study demonstrated that the probiotic spp had a significant antimicrobial effect against different isolates of *S. aureus*, especially MRSA, and in several mechanisms and various sources of infections. *S. aureus* culture broth had a highest effect on the *S. aureus* than cell-free culture supernatants.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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