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MOLECULAR STUDY TO DETECT OF PREVALENCE BIOFILM GENES AND EFFECT OF PROBIOTIC ON THE *STAPHYLOCOCCUS AUREUS* ISOLATES IN AL-KUT CITY, IRAQ

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Abstract

Staphylococcus aureus is an opportunistic bacteria and a leading cause of nosocomial and community infections. It can acquire resistance to all the antibiotics. A total 313 *Staphylococcus aureus* isolates were collected from two local hospitals from various sites of infection like pus, wound urine, sputum and blood. Patients were ranging from 1-74 years from both sexes. The number of identified *S. aureus* was 76 detected by traditional biochemical methods and confirmed by 16S rRNA gene in polymerase chain reaction (PCR). Antibiotic sensitivity profile was performed to assess the resistant strains and prevalence of methicillin-resistant *S. aureus* (MRSA). MRSA was identified in 9.2% of isolates, the highest was in pus samples (5.3%) and lowest was in sputum isolates (1.3%). MRSA was also multi-resistant to other antibiotics like nitrofurantoin, vancomycin and ciprofloxacin. Several genes that participate in antibiotic resistance were investigated. Biofilm genes like intercellular adhesion (*ica*), such as *icaA*, *icaB* and *icaC*, and detected in different rates, as for *ica A* it was 15.8%, *icaB* 17.1% and *icaC* 31.6%. In addition, high number of *icaA* carrying isolates were multi-resistant. Probiotics *Lactobacillus* species, such as *Lactobacillus fermentum* and *Lactobacillus casei* were tested against *S. aureus* for their inhibitory effect ranging 14-20mm.. *Lactobacillus* species were collected from different sites and various results noticed for bacterial culture broth and cell free culture supernatant.

Key words *: Staphylococcus aureus*, virulence factors, biofilm, probiotic.

Introduction

Staphylococcus aureus (*S. aureus*) is one of the Gram-positive pathogens causing a wide range of nosocomial infections and an important causative agent for many health associated problems (Steven, 2015). Otto, (2012) mentioned that the main problem facing *S. aureus* treatment is antibiotic resistant from medical point of view. The emergence of methicillin resistant *Staphylococcus aureus* (MRSA) was a global healthcare problem due to the restriction of therapeutic options (Jarvis *et al*., 2012). Initially, it was revealed as healthcare facilities exclusive problem, recently the community acquired-MRSA has increased (Frazee *et al*., 2005). Melzer *et al*., (2013) predominant that the number of infections caused by MRSA increased via the recent years and these are more frequently associated with mortality than infections caused by others. *S. aureus* is one of the most common causes of bacteremia and currently carries 20-40% mortality at 30 days. The ability of *S. aureus* to produce biofilm and adhesion makes them more resistant to antibiotics (Bimanand *et al*., 2018). *S. aureus* characterized by biofilm. A biofilm derived sessile community, and typified by cells that are attached to the substratum, interface, or to each other, are embedded in a matrix of extracellular polymeric substance (EPS), and exhibit an altered phenotype with regard to growth, gene expression and protein production (Donlan and Costerton, 2002). *S. aureus* also use a biofilm layer as protecting mechanism against antimicrobial agents which enhance antibiotic resistance and immune system response evasion (Yarwood *et al.*, 2004). Biofilm is hard to penetrate by antibiotics, antibiotic degrade elements are present in their matrix (Neupane *et al*., 2016). Moreover, provide important environment for horizontal genetic spread of resistance and virulence markers (Soto, 2014).

In *S. aureus*, there is several virulence factors related

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with biofilm that controlled by polysaccharide intracellular adhesion (PIA), which is encoded by the *ica* operon (*icaABCD*). Alkiyama *et al*., (2003) stated that two *ica A* and *D* genes in the operon encode this enzyme. The other genes in this operon include *icaB, icaC* and *icaR* (the regulatory gene). Systemic and intravenous staphylococcal isolates have been shown to harbor *ica* genes astwice as the normal flora of healthy volunteers twice as the microflora of healthy people (Satorres and Alcaraz, 2007).

The products are also involved in the synthesis of an extracellular polysaccharide matrix which can be destroyed by available antibiofilm enzymes (Chung and Toh, 2014). Almost the majority of studies mentioned that the *icaABCD* operon that produced PIA (Ammendolia *et al*., 1999).

Furthermore, probiotics term is defined as a live microorganisms that when administered in sufficient amounts confer a health benefit on the body host (WHO). Morita *et al*., (2006) reported that probiotics microorganisms together with other beneficial microbes are commensals of the gastrointestinal tract (GIT) and other sites in the body host and differ from pathogens in the terms of their actions on the immune system in the GIT as they do not stimulate the proliferation cells of immune system.

The main aims of the current study were to investigate the ability of clinical isolates of different *S. aureus* to form biofilm and the presence of *icaABC* genes in these isolates. In addition, estimation of lactobacilli species effect *in vitro* on these isolates in different methods.

Materials and Methods

Collection of *S. aureus* samples and bacterial cell culture

A total 313 clinical sample of different infection sites were collected from two local hospitals in the Al-Kut city/Iraq. These samples were cultured on the blood agar initially and on mannitol salt agar to detect suspected *S. aureus* isolates. Other microbiological techniques were used for detection of *S. aureus*, such as gram stain, catalase coagulase, DNase tests Analytical Profile Index (API) Staph, and polymerase chain reaction was used for accurate detection.

Antibiotic susceptibility test

Using Bauer-Kirby disc diffusion method for check isolates susceptibility for different antibiotics. The antibiotics used in the present study were listed in the Table (1.1). Samples were considered as MRSA, according to the oxacillin resistance.

Table 1.1 : Antibiotics used against different *S. aureus* isolates in the current study.

Antibiotics	Concentration (µg)
Nitrofurantoin	300
Vancomycin	30
Ampicillin	10
Amoxicillin	10
Erythromycin	15
Gentamicin	10
Ciprofloxacin	5
Clindamycin	2
Cefotetan	30
Chloramphenicol	30
Tetracycline	30
Oxcacillin	1

Extraction DNA of *S. aureus* and primers with PCR conditions

Genomic DNA was isolated from *S. aureus* strains using Genomic Kit for DNA Extraction (Geneaid Genomic DNA extraction Kit, USA). The DNA was extracted, according to the manufacturer's instructions. Briefly, the samples were centrifuged and the pellet was suspended in 200µl GT buffer for 10 min. 200 µl GB buffer was then used also for 10 min. then, absolute ethanol (200 µl) were added to the lysate. 2 ml tube was used to collect the flow after using GD columns after centrifuge. W1 buffer added to the GD column and centrifuged. then wash buffer was used and the elution buffer added and left for 3 mints to ensure obtaining purified DNA. The different genes responsible for biofilm formation were identified by PCR. The primers specific for the *icaA*, *icaB*, and *icaC* synthesized by Eurofins MWG Operon (MWG, Germany).

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

Statistical analysis

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

Results

Collection of samples

The results of culturing a (313 sample), and taken from different sites of infection in a performance duration extended from December 2017 to June 2018, and 76 samples of *S. aureus* were obtained from both sexes

Table 2: All primer sets used in the current study, and the specific primer of 16SrRNA gene of *S. aureus*.

Primer		Sequence	Product size (bp)	Accession No.
sasX	F	TCACCTTTTGCTACACCTGGT	304bp	KU901576.1
	R	AATGACTCAAATGTAAGGGGAGT		
icaA	F	TGTCGATGTTGGCTACTGGG	522	JX298872.1
	R	GCGACAAGAACTACTGCTGC		
icaB	F	TTGCCTGTAAGCACACTGGA	412	NC_007795.1:277667
	R	CTTCCCCAACATGACCTGTGA		8-2777550
icaC	F	TGTCATTTTTGGTACACCTTGCT	566	AP014942.1:2732815-
	R	AGTCTCCATTTGCTAACGCA		2733867
16S	F	CTGGAACTGAGACACGGTCC	777	KF678862.1
rRNA	R	CCCAACATCTCACGACACGA		

Table 3.1 : Percentage and numbers of infected patients, according to the age.

Ages	Frequency numbers	Percentages(%)
1-10	3	3.9
11-20	11	14.5
21-30	13	17.1
31-40	6	7.9
41-50	11	14.5
51-60	16	21.1
61-70	12	15.8
71-80	4	5.3
Total	76	100.0

Table 3.2 : Percentage of infected in both; males and females.

Gender			
		Frequency No.	Percentage (%)
Valid	Male	35	46.1
	Female	41	53.9
	Total	76	100.0

with age range 1-72 years (Table 3.1). The prevalence of *S. aureus* was significantly ($P > 0.05$) variable, according to the gender of infected individual. Females were more than males, 53.9 and 46.1%, respectively (Table 3.2).

Detection of the different *S. aureus* isolates

S. aureus were detected by traditional techniques via their ability to produce β hemolysis on blood agar, mannitol salt fermentation, and production of catalase, coagulase and DNase. Lately, detection of different isolates *S. aureus* by molecular technique using 16SrRNA via Maxime Pre Mix for PCR (I NtRON, Korea). A total 76 samples were identified as *S. aureus*. A total 7 samples were identified as MRSA which represent 9.2% of all samples.

According to type, the samples incidence of *S. aureus* was higher in pus samples and lower in burn samples,

Table 3.3 : Percentage and numbers of *S. aureus* according to the source of infection.

Type of sample	Frequency	Percentages (%)
Pus	29	38.2
Wound	17	22.4
Blood	11	14.5
Urine	8	10.5
Sputum	9	11.8
Burn	2	2.6
Total	76	100.0

(38.2% and 2.6%) respectively ($P < 0.05$), as summarized in the Table 3.3.

Antibiotic susceptibility test

The results for antibiotic susceptibility test are illustrated in the Table 3.4**.**

Detection of some biofilm genes (*sasX*, *icaA*, *icaB*, and *icaC*) in *S. aureus* and diagnosis of different *S. aureus* isolates using 16SrRNA

The diagnosis of different *S. aureus* by 16SrRNA (Fig. 3.1). As well as detection of biofilm contributing in biofilm formation was done by specific primers in PCR, as summarized in the Table 3.5 and the Figs. 3.2-3.5.

PCR was used to detect the presence of *Sasx* gene in S. aureus samples. *Sasx* gene was detected in 8 (10.5%) samples which was multi-resistant for many antibiotics. 3 (42.9%) samples that carries *Sasx* gene were MRSA.

Biofilm genes (*icaA*, *icaB*, and *icaC*) and antibiotic sensitive

All samples were tested for some important genes responsiple for antibiotic resistance and leasion formation during infection, as summarized in the Table 3.6.

Biofilm genes and antibiotic resistance

The present results noticed that when biofilm

Table 3.4 : Antibiotic susceptibility test in different *S. aureus* isolates.

Antibiotics (Dosage)	Resistant		Sensitive		Intermediate	
Nitrofurantoin (300µg)	6	7.9%	70	92.1%		
Vancomycin (30µg)	6	7.9%	51	67.1	19	25%
Ampicillin (10 µg)	68	89.5%	8	10.5%		
Amoxicillin (10µg)	65	85.5%	11	14.5%		
Erythromycin (15µg)	28	36.9%	44	57.9%	4	5.2%
Gentamicin (10µg)	20	26.3%	56	73.7%		
Ciprofloxacin (5µg)	27	35.4%	49	64.6%		
Clindamycin (2µg)	44	57.9%	32	42.1%		
Cefotetan (30µg)	19	25%	57	75%		
Chloramphenicol (30µg)	15	19.7%	61	78.9%		
Tetracyclin (30µg)	12	15.7%	58	76.3%	6	8%
Oxcacillin (1µg)	7	9.2%	69	90.8%		

Table 3.5 : Biofilm genes detection in the different *S. aureus* isolates.

icaA		**Frequency**	**Percent (%)**
Valid	Positive	12	15.80
	Negative	64	84.20
	Total	76	100.00
icaB		**Frequency**	**Percent (%)**
Valid	Positive	13	17.10
	Negative	63	82.90
	Total	76	100.00
icaC		**Frequency**	**Percent (%)**
Valid	Positive	24	31.6
	Negative	52	68.4
	Total	76	100.0

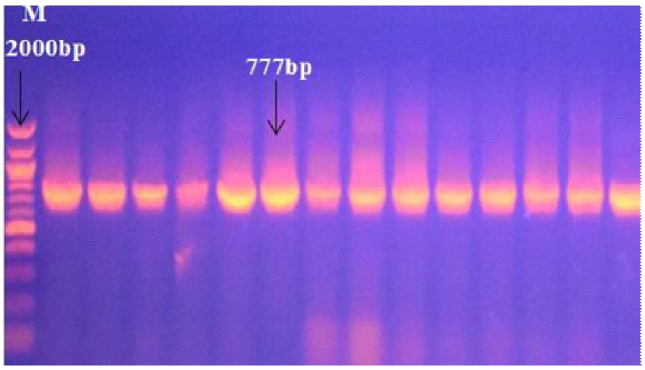

Fig. 3.1 : Gel electrophoresis, Genomic DNA isolated from *S. aureus* showing 16S rRNA gene for *S. aureus* using specific primer, with M: Marker (100-2000bp).All lanes positive PCR amplification at 777bp PCR product size.

formation *icaA* gene is present, antibiotic resistance increase, as shown in the Table 3.7.

Estimation of probiotic bacteria inhibitory activity against *S. aureus* isolates

The results of *Lactobacillus* spp. bacteria against *S. aureus* bacteria isolated from different sites of infections showed variance in their inhibitory effect ranging from 14-20 mm. In addition, the results noticed variance in the inhibitory effect of the same isolate against antibiotic sensitive and resistant *S. aureus*. As in *L. casei* that show obvious inhibitiory effect against cirprofloxacin, cefotetan, tetracyclin, gentamicin and chloramphinicol sensitive to *S. aureus* with an inhibition zone of 14 mm and 18 mm for the multi-resistant *S.aureus*, except cefoxitin.

L. fermentum express inhibibition zone of 20 mm for *S. aureus* sensitive to antibiotics mentioned above and 15 mm for the multi-resistant S.*aureus* except cefoxitin, as outlined in the Table 3.8.

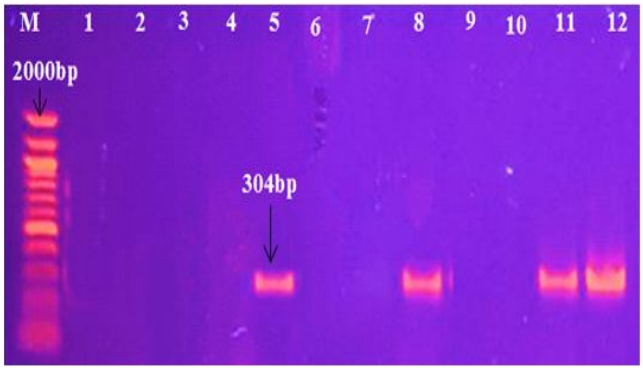

Fig. 3.2 : Agarose gel electrophoresis image that shown the PCR product of *sasX* gene in *Staphylococcus aureus* isolates. Where M: Marker (100-2000bp).Lane (1-12) some positive PCR amplification at 304bp PCR product size.

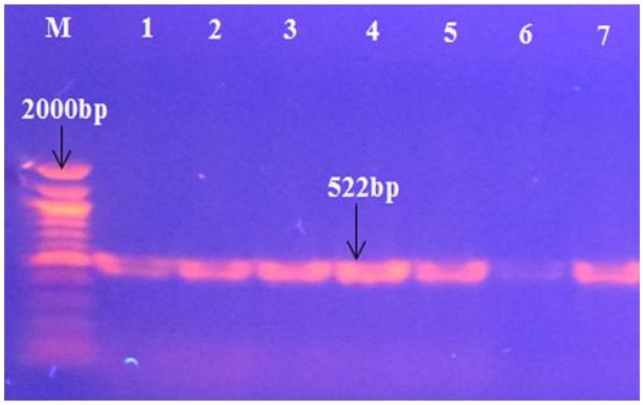

Fig. 3.3 : Agarose gel electrophoresis image that shown the PCR product of *icaA* gene in *Staphylococcus aureus* isolates. Where M: Marker (100-2000bp), Lane (1-7) some positive PCR amplification at 522bp PCR product size.

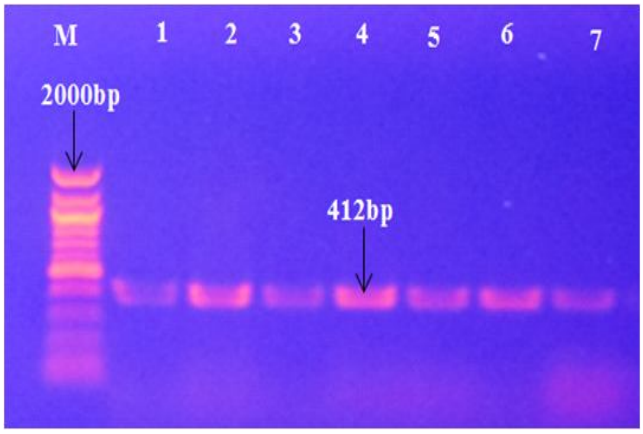

Fig. 3.4 : Agarose gel electrophoresis image that shown the PCR product of *icaB* gene in *Staphylococcus aureus* isolates. Where M: Marker (100-2000bp), Lane (1-7) some positive PCR amplification at 412bp PCR product size

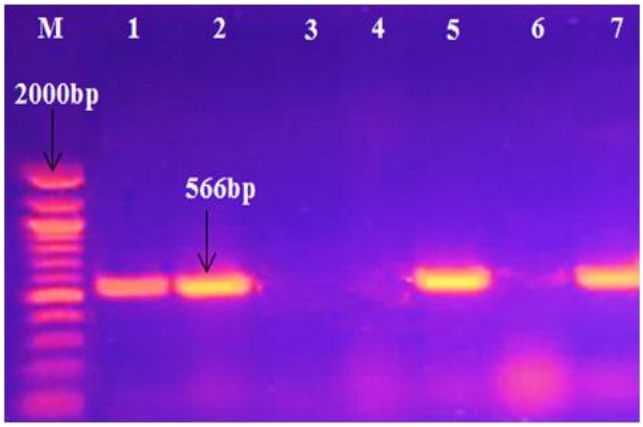

Fig. 3.5 : Agarose gel electrophoresis image that shown the PCR product of *icaC* gene in *Staphylococcus aureus* isolates. Where M: Marker (100-2000bp), Lane ane (1-7) some positive PCR amplification at 566bp PCR product size.

Table 3.6 : Prevelance of biofilm coding genes in the different isolates of *S. aureus*.

Gene	No. and % of +ve isolates (%)	No. and % of -ve isolates (%)	Total(%)
Ica A	12(15.8)	64(84.2)	76(100)
Ica B	13(17.1)	63(82.9)	76(100)
Ica C	24(31.6)	52(68.4)	76(100)

Table 3.7 : Biofilm genes and antibiotic resistancein the different isolates of *S. aureus*.

Multi resistant	*icaA*		*icaB*		*icaC*	
	+ve (%)	-ve (%)	+ve (%)	-ve (%)	+ve (%)	-ve (%)
Positive	12(15.8%)	3(3.9%)	7(9.2%)	8(10.5%)	10(13.2%)	5(6.6%)
Negative	0(0%)	61(80.3%)	6(7.9%)	55(72.4%)	14(18.4%)	47(61.8)%

Table 3.8 : Inhibitory effect of *Lactobacillus* spp. to *S. aureus* on MRS agar.

Inhibition zone (mm)		
S.aureus	**L. fermentum**	**L. casei**
Multiresistant *S. aureus* (except cefoxitin)	15	18
Sensitive *S. aureus* to antibiotics	20	14

Table 3.9 : Inhibitory effect of bacterial culture brothfor *Lactobacillus* spp. On the *S. aureus* isolates

Inhibition zone (mm)		
S.aureus	**L. fermentum**	**L. casei**
Multiresistant *S. aureus* (except cefotilan)	20	19
Sensitive *S. aureus* to antibiotics	22	18

Well diffusion method

The results with the current study of bacterial culture broth for *Lactobacillus* spp. activity test were displayed obvious activity against *S. aureus*. However, the inhibition zone was ranging from 18-20 mm for multi-resistant *S. aureus* except nitrofurantoin and cefotetan, and 18-22 mm for sensitive *S. aureus* to antibiotics (Table 3.9).

Discussion

S. aureus is has been a worldwide health problem causing high rates of infection and death cases which reach 11000 in different world (Tosas *et al*., 2018). In the current study shows there were significant differences (P=0.05) in prevalence of *S. aureus* in the different ages of patients which were variable and the higher percent of infected patients and ranging between 51-60 years. Most of them were diabetic and surgical wounded, as outlined in the Table (3.1). Moreover, Hafeez *et al*., (2004) stated that *S. aureus* infections distributed between various groups of age similarly, while Sdougkos*et al*. (2008) stated that the ages between 15-60 years were high susceptible to be infected with *S. aureus* (Table 3.2).

In addition, the results with sensitivity and resistivity test were significant (P = 0.05). Most of these bacterial samples were sensitive to nitrofurantoin (97.3%) and lowest sensitivity to ampicillin (10.5%) and sensitive to other antibiotics in the different percentage, as summarized in the Table (3.4).

The processes of resistance occur naturally or acquired by mutations or plasmid transportation. (Jose *et al*., 2016).

The resistance to beta-lactamase was highest of all antibiotics, such as ampicillin (89.5%) and amoxicillin (85.5%). The results were resistant due to the occurrence of beta-lactamase generating *S. aureus* in hospital environment and 'selection pressure' due to the usage of the beta-lactam medications for the handling, presenting chance for the selective colonizing for more resistant beta-lactamase bacteria (Kitara *et al*., 2011`). The current results agreed with Al-Zoubi *et al.,* (2015). *S. aureus* sensitivity to gentamicin in the present study compares favorably with reports published by some researchers' liker Nordip *et al*., (1997).

The fact that the presence of *icaA* gene accompanied with other genes responsible for production of slime and biofilm layers and their elevation of antibiotic resistance is similar to other researchers like Vasudevan *et al*., (2003) and Fahimeh *et al*., (2016).

In the current study, 7 (9.2%) were considered isolates as MRSA according to their resistance to cefoxitin most of the isolates were isolated from pus and wound infections (Table 3.7).

As virulence factors, *S. aureus* mechanism for biofilm production in are not fine recognized, a few number of searches about the genes related to biofilm formation. Production of biofilm is documented as a vital bacterial virulence factor for *Staphylococcus* biofilm production may be the fundamental reason for the increasing antibiotic resistance of *S. aureus* strains (Fox *et al*., 2005).

Li *et al.,* (2012) reported that *sasX* gene has a role in MRSA epidemics in Asia and they also mentioned that *sasX* enhance bacterial aggregation and evade immune responses which leads to prolonged survival in human blood. In current study the percent was higher than Song *et al*., (2010) in Shanghai and Dhiman *et al*., (2017), 36.7% and 33.3% respectively.

The samples that carried *sasX* gene and harbor *icaA* gene were MRSA and showed multi-resistance to many antibiotic. The isolates that harbor the biofilm encoding genes showed resistance to many antibiotics, this statement agreed with (Fox *et al*., 2005). The cause increase of resistance is the protective role played by biofilm (Neupane *et al*., 2016). MRSA strain is multi-resistant and this requires more attention and diagnosis to limit their spread and slow their resistance progression. The misuse and random usage of antibiotic contribute in a great manner in increasing resistance. The rapid diagnosis of MRSA is required also for its limitation and PCR represent the ideal technique due to specificity and accuracy (Bengyun *et al*., 2018).The prevalence of *ica* genes are variable in a great manner among various researches (Campoccia *et al*., 2009).

The latent researches about formation of biofilm, the *ica* genes were mentioned as essential for the production and development of biofilm. The *icaABCD* genes produced by *S. aureus* as molecules of intercellular adhesion act as main factors in staphylococcal biofilm formation, Moreover, their presence increase resistance to antibiotic in *S. aureus* (Resch *et al*., 2005).

For probiotic effect, the results were significant (Table 9) with the present study were agreed with found of Annuke *et al*., (2003); and Tejero-Sariñena *et al*., (2012). Suggesting that the inhibitory action has mostly been linked to statement that lactic acid bacteria (LAB) decrease pH and/or secrete organic acids.

The results with the current study of bacterial culture broth for *Lactobacillus* spp. activity test were displayed obvious activity against *S. aureus*. However, the inhibition zone was ranging from 18-20 mm for multi-resistant *S. aureus* (except nitrofurantoin and cefotetan), and 18-22 mm for Sensitive *S. aureus* to antibiotics (Table 3.8).

The results of bacterial culture broth in the current study showed observable inhibitory effect in resistant and sensitive *S. aureus* isolates. The reason for that could be due to production of inhibitory substances, such as Lactic acid which was generally the chief end product. Makras *et al*., (2005) described that the capability to damage inulin-type fructans of lactobacilli isolates and Khalil *et al*., (2018) 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 (Yang*et et al*., 2014. These results were agreed with found of Majeed (2008), and Tejero-Sariñena (2012)

Conclusions

The highest incidence of *S. aureus* isolates was in pus and wound samples 38.2% and 22.4%, respectively, and the lowest was in burn sample (2.6%). In addition, MRSA was present in pus, wound and sputum isolates 5.3%, 2.6% and 1.3 %, respectively and MSSA represent 90.8 % from all samples. MRSA were multi-resistant for several antibiotics, such as ampicillin, amoxicillin and gentamicin. Furthermore, genetic analysis had shown that the presence of biofilm encrypting genes specially *ica* An increase antibiotic resistance of *S. aureus*. In addition, the high rates of resistance to B-lactam antibiotics in such huge rates like ampicillin and amoxicillin 89.5%, and 85.5%, respectively, and indicates that the misuse of antibiotic represent a serious problem need to be more

investigated.

On the other hand, probiotics have a high capability to inhibit *S. aureus* growth in different mechanisms and various sources of infections. bacterial culture broth had a greatest effect on *S. aureus* than cell-free culture supernatants.

Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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