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Gene Expression of *clfA* , *clfB*, and *SdrC* Genes of *Staphylococcus aureus* Under Probiotic Stress

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Abstract

The significant factor that participated in virulence and pathogenesis for Gram-positive pathogen *Staphylococcus aureus* is biofilm development. In hospital settings, biofilm construction on medical equipment is quite concerning since it is a permanent source of infection. The infection results from *S. aureus* biofilm, which was considered a serious problem due to the difficulty in treating it with traditional antibiotics. In recent years, the medical community has been using probiotic bacteria to suppress biofilm development. The current work aims to investigate the impact of cell-free supernatant (CFS) of *lactobacillus acidophilus* on biofilm generation and gene expression of some key adhesion genes (*clfA, clfB*, and *SdrC)* in *S. aureus* clinical isolates. The CFS was extracted from *Lactobacillus. acidophilus,* and minimum inhibitory concentration was estimated. The colorimetric method was carried out to detect the impact of sub-minimum inhibitory concentrations of the CFS extracts on biofilm construction in thirty-three *S. aureus* isolates. Moreover, the expression level of some associated biofilm genes (*clfA, clfB*, and *SdrC*) was determined quantitatively before and after CFS treatment using real-time PCR for three isolates forming a strong biofilm. The result indicated that most isolates formed mostly strong and moderate biofilm with 42.42% and 48.48%, respectively, while 6.06% and 3.03% of isolates were weak and non-biofilm former. Regarding the *clfA* gene, gene expression was only detected in two isolates by which one isolate showed downregulation with a fold change value (0.0883) and another isolate showed upregulation with a fold change value (5.7134) after treatment of isolates with CFS. However, *clfB* and *sdrC* revealed a decrease in expression in two isolates, while the third isolate showed upregulation after treatment of isolates with CFS. The above result indicates that *clfA, clfB, and sdrC* expressed downregulation under the effect of CFS. In conclusion, the ability to produce biofilm plays a significant role in pathogenicity and antibiotic resistance in *S. aureus* isolates, especially when most isolates produce strong and moderate biofilm. Furthermore, the highly inhibitory effect of CFS from *lactobacillus acidophilus* in the reduction of biofilm activity and the drop in expression of key biofilm genes in *S. aureus* isolates highlights the capacity to use CFS as an alternative strategy to eliminate infection related to biofilm in this pathogen.

Keywords: Biofilm, probiotic, *Staphylococcus aureus*, gene expression.

التعبير الجيني لجينات *clfA* و *clfB* و *SdrC* للمكورات العنقودية الذهبية تحت ضغط البروبيوتيك

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الخلاصة

تطور الأغشية الحيويةهوالعامل المهم الذي شارك في ضراوة ومرضية المكورات العنقودية الذهبية إيجابية لصبغة جرام . في المستشفيات، يعد بناء الأغشية الحيوية على المعدات الطبية أمرًا مثيرًا للقلق لأنه مصدر دائم للعدوى. تم اعتبار العدوى الناتجة عن الأغشية الحيوية للمكورات العنقودية الذهبية مشكلة خطيرة بسبب صعوبة علاجها بالمضادات الحيوية التقليدية. في السنوات الأخيرة، كان المجتمع الطبي يستخدم البكتيريا الحيوية لقمع تطور الأغشية الحيوية. يهدف العمل الحالي إلى التحري عن تأثير الراشح الخالي من الخلايا لبكتيريا العصيات اللبنية الحمضية على تكوين الأغشية الحيوية والتعبير الجيني لبعض جينات الالتصاق الرئيسية *clfA* و *clfB* و *SdrC*في العزلات السريرية للمكورات العنقودية الذهبية. تم استخلاص الراشح الخالي من الخلايا لبكتيريا العصيات اللبنية الحمضية وتم تقدير التركيز المثبط الادنى له. تم استخدام الطريقة اللونية للكشف عن تأثير التركيزالمثبط دون الحد الأدنى للراشح على بناء الأغشية الحيوية. علاوة على ذلك، تم تحديد مستوى التعبير لبعض جينات الأغشية الحيوية (*clfA* و *clfB*و *SdrC*)كميًا قبل وبعد المعاملة بالتركير المثبط دون الحد الادنى من الراشح باستخدام تفاعل البوليميراز المتسلسل في الوقت الحقيقي لثلاث عزلات تنتج أغشية حيوية قوية. أشارت النتيجة إلى أن معظم العزلات شكلت أغشية حيوية قوية ومتوسطة في الغالب بنسبة 42.42٪ و 48.48٪ على التوالي بينما كانت 6.06٪ و 3.03٪ من العزلات ضعيفة وغير مكونة للأغشية الحيوية. فيما يتعلق بجين *clfA*، تم الكشف عن التعبير الجيني في عزلتين فقط أظهرت إحداهما انخفاضًا في التنظيم وأظهرت عزلة أخرى ارتفاعًا في التنظيم بعد المعاملة بالراشح الخالي من الخلايا ومع ذلك، اظهرجين *clfB* و *sdrC* انخفاض في التعبيرالجيني في عزلتين بينما أظهرت العزلة الثالثة ارتفاعًا في التعبير بعد المعاملة بالراشح الخالي من الخلايا . تعطي النتيجة المذكورة أعلاه إشارة إلى ان جينات *clfA* و*clfB* و*sdrC* اظهرت انخفاض بالتعبيرالجيني تحت تأثير الراشح الخالي من الخلايا .

في الختام، تلعب القدرة على إنتاج الأغشية الحيوية دورًا هامًا في الإمراضية ومقاومة المضادات الحيوية في عزلات المكورات العنقودية الذهبية، خاصةً أن معظمها يُنتج أغشية حيوية قوية ومتوسطة. علاوة على ذلك، فإن التأثير الراشح الخالي من الخلايا لبكتيريا العصيات اللبنية الحمضية في تقليل نشاط الأغشية الحيوية وانخفاض التعبير الجيني لجينات الأغشية الحيوية الرئيسية في عزلات المكورات العنقودية الذهبية يُبرز إمكانية استخدام الراشح الخالي من الخلايا كاستراتيجية بديلة للقضاء على العدوى المرتبطة بالأغشية الحيوية في هذا المُمرض.

Introduction

Gram-positive pathogen *Staphylococcus aureus* results in relatively unusual urinary tract infections with a rate range from 0.5 % to 6% in the general population [1]. However, it can be distributed in specific groups of people, for instance, elderly catheterized patients or people infected with S. aureus bacteremia [2]. This infection acts as a sophisticated life-threatening condition unless treated. Abroad range of secreted virulence factors are produced from this bacterium [3].

The construction of biofilm is one of the most crucial properties that assist the development of drug resistance in *S. aureus* [4]. Compared to planktonic cells, biofilm has been shown to give considerable survival benefits to microbial communities, with strain resistance rising by up to 1500-fold [5]. Biofilm protects bacterial cells from extreme conditions such as high heat, desiccation, starvation, and antibacterial drugs, so it works as a hindrance to keep the interior environment for bacterial activity steady[6, 7].

Antibiotic tolerance has led to the appearance of antimicrobial resistance, a serious issue that has caused increased morbidity and mortality in the community as well as significant losses in the global economy[8]. Multiple genes must co-express for biofilm development to occur, which is a complicated process. *S. aureus* can express different biofilm-associated genes, such as *clfA,clfB, and sdrC* [9]. The ability to form a biofilm with a high rate of distribution of *S.*

aureus encourages infections, and the emergence of antibiotic-resistant strains makes it essential to discover and create novel antimicrobial agents and potent medications to treat illnesses caused by multidrug-resistant MDR bacteria like *S aureu*s [10, 11].

Probiotics such as probiotic *Lactobacillus* strains are one of the suggested treatments for antibiotic resistance and biofilm-related infection resulting from *S. aureus.* probiotic have many forms to display their benefits such as dropping growth of *S. aureus* by secretion of hydrogen peroxide and bacteriocins [12]. Due to a lack of research focusing on the capacity of *Lactobacillus* cell-free supernatant CFS to reduce biofilm generation in clinical isolates of *S. aureus,* this work was conducted to estimate the capacity of CFS of *Lactobacillus* to minimize the capability of *S. aureus* to develop strong biofilm and reduce gene expression of some biofilm-related gene.

Methodology

Detection of S. aureus isolates

A total of 33 *S. aureus* isolates were identified depending on culture characteristics and biochemical tests from urine samples at different hospitals in Baghdad. The samples were cultured directly on various types of medium, such as mannitol salts agar and blood agar, for culture characteristics detection. Biochemical tests such as catalase, oxidase, and coagulase were utilized for confirmed identification [13]. Moreover, the further identification of isolates was achieved by the Vitek 2 Compact System. This work is achieved under reference number CSEC/1023/0093 for ethics.

Biofilm generation assay

Biofilm development was detected in all isolates under the study using the colorimetric plate technique [14]. Sterile Tryptic soy broth with 1% Glucose (TSBG) was inoculated with 0.5 ml of 18 hrs *S. aureus* growth with 0.5 McFarland turbidity. After that, 1: 100 dilution was prepared, and a volume with 150 μl was poured into each well of the 96-flat bottom plate. The negative control was prepared by loading 150 μl of TSBG without *S aureus* growth. The plate was left for 24 hrs at 37°C. After that, the broth of the wells was discarded, and the plate was carefully washed by applying 150 µl of phosphate buffer saline (PBS) to remove all planktonic cells. The adherent *S. aureus* cells were fixed by methanol and stained with150 μl of crystal violet (0.1%). The plates were inverted onto sterile filter paper and air-dried for 60 minutes. A volume of 0.1 ml of 96% ethanol for 30 minutes was added to solubilize fixed crystal violet. The assay was performed in triplicates for each isolate. The plate was subjected to a microtiter plate reader at 590 nm to estimate the optical density for each control and isolate (ODI). The optical density of cut-off value (ODC) = average OD of negative control + 3x standard deviation (SD) of negative control. The isolates were divided into four main groups: did not create biofilm(ODI < ODC), weak biofilm development isolates (ODI < 2 ×ODC), moderate biofilm development isolates (ODI < 4×ODC), and strong biofilm development isolates (ODI > 4×ODC) [15].

Cell-free supernatant (CFS) extraction

The preparation of cell-free supernatant (CFS) of *Lactbacillus acidophilus* was assessed by transferring 3-4 well isolates colonies to 10 ml of Man,Rogosa and Sharpe (MRS) broth and put in an incubator under anaerobic conditions for 24 hrs at 37°C. The culture was centrifuged at 4000rpm for 15 minutes at 4°C. The supernatant was collected and passed through a 0.45 millipore filter, and then 0.1ml of CFS was cultured on MRS agar to ensure the absence of cells. The stock solution of CFS was used to examine their inhibitory effect against *S. aureus* biofilm production and gene expression [16].

Determination of minimum inhibitory concentration of CFS

The two-fold dilution method was followed to estimate the minimum inhibitory concentration of CFS. Sterile Muller Hinton broth was used to prepare the concentrations (1/2,1/4,1/8,1/16, and 1/32) of CFS, and the volume was completed in each tube to be 2ml. Finally, a volume of 0.2 ml from overnight growth *S. aureus* with turbidity equal to 0.5 McFarland standard was added. The tube contains only *S. aureus* growth, which acts as a positive control, while the tube contains only Muller Hinton broth, which acts as a negative control. The lowest concentration of CFS, in which there is no turbidity detection was considered as MIC values. The MIC was determined in triplicate [12].

The impact of CFS on biofilm formation

To detect the impact of CFS on the biofilm formation of *S. aureus*, five strong biofilm producer isolates with multi-drug resistance were chosen to examine this assay (10, 14,18, 29, and 33). As previously mentioned, a quantitative assay for biofilm formation was performed. Before the staining step, the wells were treated with 150 µl from the sub-inhibitory concentration of CFS, and the plat was re-incubated for 24 hrs at 37°C. The OD for control and isolates was determined using a microtiter plate reader at 590 nm, and the data was analyzed according to Al-Maeni [15]. Moreover, the Reduction % was calculated as mentioned by Abd-allah *et al.,*[17].

$$\text{Reduction\%} = [(OD_{\text{before treated}} - OD_{\text{after treated}})/OD_{\text{before treated}}] \times 100.$$

Detection of the change in expression of clfA, clfB, and SdrC genes *under Lactobacillus acidophilus stress*

The impact of CFS on the expression level of some biofilm-mediated genes was examined in three *S. aureus* isolates (strong biofilm producer and multi-drug resistance). Briefly, two colonies of *S. aureus* isolates were cultured in 9 ml of fresh TSBG and left for 24 hrs at 37°C. Then, the bacterial suspension was diluted to achieve turbidity equal to 0.5 MacFarlane standard. The suspension was divided into tubes treated with a sub-inhibitory concentration of CFS 1/4 (Sub-MIC) and control tubes not exposed to sub-MIC. The bacterial suspension in both treated and control tubes was loaded to 6 wells plate and left for 24hrs at 37°C. The content of the plates was discarded, and PBS was applied to remove all non-adherent cells [12]. After collecting the cells in wells, the bacterial RNA was isolated by following the guidelines of TRIzol™ Reagent(Thermo Scientific, USA). A Quantus Fluorometer was utilized to estimate the concentration of isolated RNA. The difference in the expression level for biofilm-generated genes was measured using the QRT-PCR technique. The components of one-step real-time PCR(Promega, USA) included the following: 5 µl qPCR Master Mix, 0.25 µl RT mix, 0.25 µl $MgCl_2$, 0.5 µl forward primer, 0.5 µl reverse primer, 2.5 µl nuclease-free water and 1µl of RNA. All specific primers with their information and q RT-PCR steps were mentioned in Tables 1 and 2. Finally, the expressions of target genes were calibrated with relative housekeeping genes, and the QRT-PCR results for both treated and untreated tubes were analyzed according to the ΔΔCT Method [18].

Table 1: The specific primers used in QRT-PCR

Gene	Primers sequence	Annealing temperature	Size of product bp	Reference
16SrRNA	16S rRNA forward TGTCGTGAGATGTTGGG 16S rRNA reverse CGATTCCAGCTTCATGT	50°C	253	NCBI
clfA	*clfA* forward AGTGCGCCTAGAATGAGAGC *clfA* reverse TAAGCGGGCATGGTCAAAGT	60°C	389	Designed
clfB	*clfB* forward AGCTGTTGCTGAACCGGTAG *clfB* reverse TTTAGGTGCCTTTGCTCGGT	60°C	415	Designed
sdrC	*sdrC* forward AAAAGGCATGATACCAAATCGA *sdrC* reverse AATTCTCCATTCGTATGTTCTG	53°C	144	[19]

Table 2: Steps of real-time PCR

Steps	Temperature	m: s	No of Cycles
reverse transcriptase Enzyme Activation	37°C	15:00	1
Initial Denaturation	95 °C	05:00	1
Denaturation	95 °C	00:20	40
Annealing	54 or 60 or 63 °C	00:20	40
Extension	72 °C	00:20	40

Statistical analysis

The proportion of the data under the study was achieved by calculating the P-value depending on the Chi-squared test [20].

Results

Biofilm generation assay

The results of the biofilm construction assay showed that 96.96 % of *S. aureus* isolates demonstrated a positive result for biofilm generation. In contrast, 3.03% of isolates did not create biofilm, with significant differences (p = 0.0001) between the two groups. As shown in Figure 1, the capability to create biofilm for positive isolates demonstrated various levels of biofilm construction. Only two isolates (6.06%) were classified as weak biofilm formers isolates, whereas 48.48 % of isolates were indicated as moderate biofilm formers and 42.42% were recorded as strong biofilm formers.

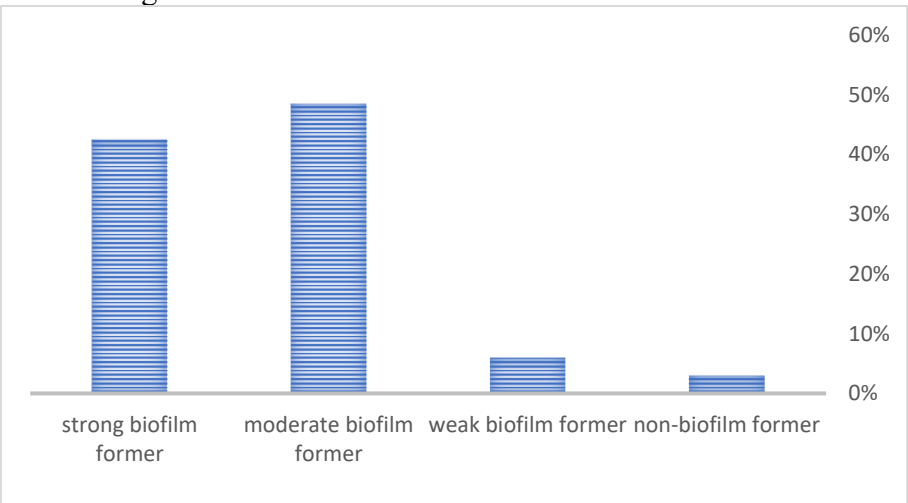


Figure 1: Result of biofilm generation assay

Influence of L. acidophilus -CFS on biofilm formation in S. aureus

The impact of *L acidophilus*-CFS on biofilm formation in *S. aureus* was examined, and the findings showed that the treatment with sub-MIC of CFS significantly reduced the optical density values of five isolates (Table 3). The CFS caused a decrease in biofilm thickness for four isolates (14, 33,10, and 29), and their capability to create biofilm shifted from strong biofilm to the absence of biofilm and weak biofilm. In contrast, the remaining isolate (18) became a moderate producer.

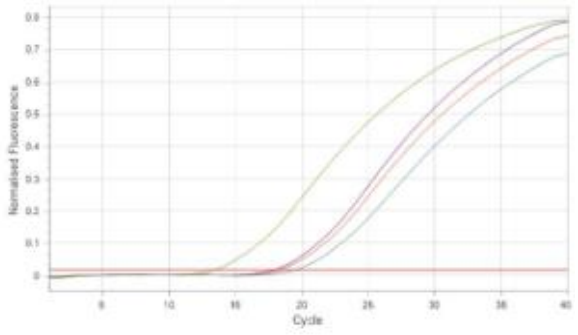
Table 3: The OD values of *S. aureus* isolates before and after exposure to CFS

Isolates	OD mean before treatment	Group of isolate	OD mean after treatment	Group of isolate	Reduction %
10	0.732	strong	0.131	Weak	82.1%
14	0.994	strong	0.058	Non-producer	94.16%
18	0.341	strong	0.173	Moderate	49.26%
29	0.489	strong	0.124	Weak	74.64%
33	0.408	strong	0.056	Non-producer	86.27%

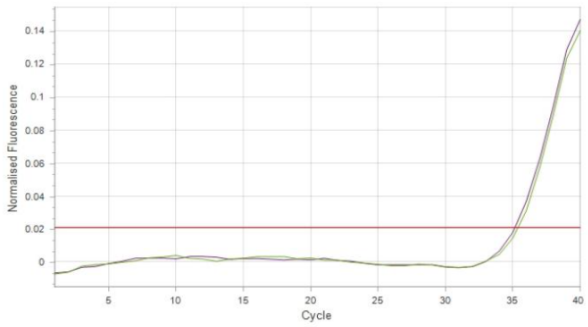
Note; ODC=0.084, 2ODC = 0.1684 , ODC=0.336 Reduction mean 77.28%

Genes expression

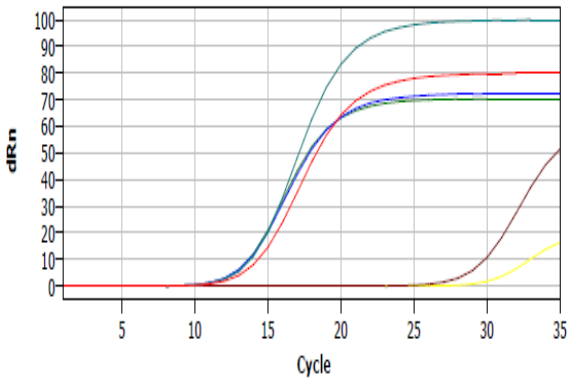
The exposure of *S. aureus* to sub-MIC of CFS results in a decrease in the expression level for *clfB and SdrC* genes in two isolates of *S. aureus* in comparison to the non-exposure isolate (Figure 2). In contrast, the folding change for the *clfA* gene displayed upregulation for isolate number 10 after incubation in the presence of CFS, while isolate number 18 revealed downregulation (Table 4).



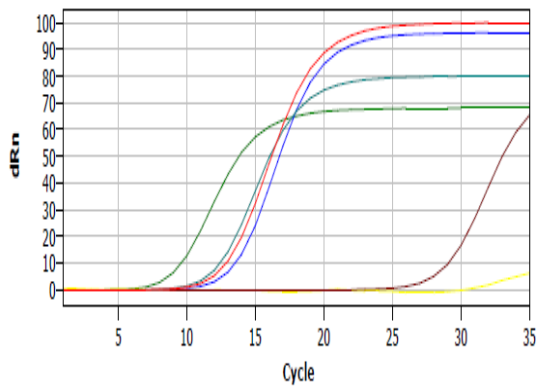
A: Illustrating values of CT of *sdrC* gene for isolates 10 and 33 .Green line and blue lines: indicates control of isolate 10 and isolate33, orange line and purple line: treated of isolate 33 and 10



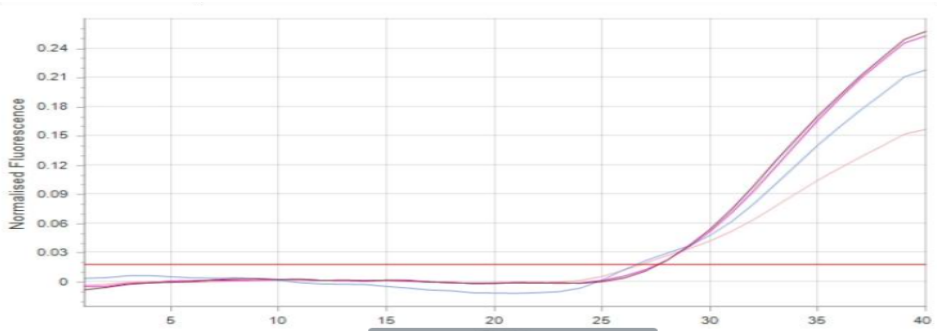
B: Illustrating values of CT of *clf A* gene for isolates number 10.green line and purple lines: indicates control and treated of isolate 10



C: Illustrating values of CT of *clfA* .*clfB*, *sdrC* genes of control for isolate 18. Blue line: *sdrC,* yellow line: *clfA*, brown line *clfB*:



D: Illustrating values of CT of *clfA* .*clfB*, *sdrC* genes of treated for isolate 18. Blue line: sdrC, yellow line: *clfA*, brown line *clfB*:



E: Illustrating values of CT of *Clf B* gene for isolate 10 and 33. blue and .pink lines: indicate control and treatment of isolate 10.Brown and purple lines indicate the control and treatment of isolate 33.

Figure 2: Values of CT for different genes in isolates 10, 18, and 33 with their control

Table 4: Impact of CFS on gene expression of *clfA ,clfB and sdrC* genes

Isolates	16sRNA	*clfA*	ΔCT	ΔΔCT	Folding	Result
C10	11.22	35.38	24.15	00	1	Up-regulation
T10	13.51	35.15	21.64	-2.51	5.7134	
C18	11.95	30	18.05	00	1	Down-regulation
T18	11.32	32.87	21.55	3.5	0.0883	
Isolates	**16sRNA**	***clfB***	**ΔCT**	**ΔΔCT**	**Folding**	**Result**
C10	11.22	26.58	15.36	00	1	Up-regulation
T10	13.51	26.79	13.28	-2.07	4.21	
C18	11.95	27.21	15.26	00	1	Down-regulation
T18	11.32	27.33	16.01	0.75	0.594	
C33	13.67	27.61	13.94	00	1	Down-regulation
T33	12.55	27.54	14.99	1.05	0.48	
Isolates	**16sRNA**	sdrC	**ΔCT**	**ΔΔCT**	**Fold change**	**Result**
C10	11.22	13.41	2.18	0.00	1.00	Down-regulation
T10	13.51	17.99	4.47	2.29	0.20	
C18	11.95	11.35	-0.6	0.00	1.00	Down-regulation
T18	11.32	12.09	0.77	1.37	0.38	
C33	13.67	19.44	5.77	0.00	1.00	Up-regulation
T33	12.55	18.25	5.71	-0.07	1.05	

Note :c: control T: Treated

Discussion

Deposit *S. aureus* is considered a non-pathogenic bacterium in some parts of the body, such as the skin and nose [21], it may cause a wide range of serious diseases that are linked with its capability to create biofilm [22]. The significant finding in our work was that the majority of isolates formed biofilm mostly strong and moderate biofilms, with 42.42% (14/33) and 48.48%(16/33), respectively, while 6.06% (2/33) and 3.03% (1/33)of isolates were weak and non-biofilm former. Aniba*et al*., Mohammed *et al.,* and Khutade *et al.,* [1, 23] results were in consenting with our result by which the strong biofilm ranged between (35 to 46%), and moderate were (43 to 55%). However, Han *et al.,* [24] showed only 8% of isolates were able to form strong biofilm, and 19% were moderate producers. A high percentage of isolates formed strong and moderate biofilm in the current study, which may illustrate the contribution of biofilm in the pathogenicity of *S. aureus* since these isolates enrolled with disease in patients causing urinary tract infection (UTI). The impact of CFS on biofilm construction was carried out phenotypically by colorimetric method, and the result revealed that the OD of four isolates with strong biofilm former were dropdown, converting these isolates to weak and non-biofilm former. In addition, the average reduction in biofilm formation in five isolates was 77.28%. This result was in agreement with Abd-allah *et al.,* [25], who showed an 87% reduction in biofilm formation under the effect of CFS. However, Mao *et al* . [26] showed that only 22% of cases were reduced under the effect of CFS. The high percentage of reduction in biofilm may indicate that CFS can be applied as an antibiofilm agent to eliminate *S. aureus* mediating biofilm formation. Further investigation was performed to investigate the impact of CFS on gene expression of *clfA,B, and sdrC* for three isolates forming a strong biofilm. Regarding the *clfA* gene, gene expression was only detected in two isolates, by which one isolate showed

downregulation and another isolate showed upregulation after treatment of isolates with CFS. However, *clfB* and *sdrC* revealed a decrease in expression in two isolates, while the third isolate showed upregulation after treatment of isolates with CFS. The above result indicates that *clfA, clfB, and sdrC* expressed downregulation under the effect of CFS. The genetic result is compatible with a phenotypic result by which both showed CFS can diminish the percentage of biofilm creation through a decrease in the expression of genes forming a biofilm which are *clfA,B* and *sdrC* that are used to attach cells to the surface of solid substances. In comparison with other studies, there were no studies that showed the effect of CFS on *clfA, clfB,* and *sdrC*. However, other studies upregulated under the effect of interleukin-1b and downregulated under the effect of adamantane derivative [26, 27]. *clfA, clfB,* and *sdrC* were downregulated under the effect of benzimidazole derivative while Peptides DLL37-1 and LL37-1 decreased the expression of only *clfA* and *sdrC* [28].

Conclusion

The ability to produce biofilm plays a significant role in pathogenicity and antibiotic resistance in *S. aureus* isolates, especially when most isolates form strong and moderate biofilm. In addition, the highly inhibitory effect of CFS of *lactobacillus acidophilus* in decreasing biofilm activity and down-regulated expression of biofilm-related genes in this pathogen highlights the capacity to use CFS as an alternative strategy to overcome infection associated with biofilm production in these pathogenic bacteria.

Conflict of Interest

The authors state that they have absolutely no conflicts of interest.

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