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Research Article

Expression of *sfa* and *afa* Genes in Uropathogenic *Escherichia coli* Under Probiotic Effect

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Abstract

Background: Urinary tract infections (UTIs) are primarily caused by *Escherichia coli*. Various genes in UPEC isolates are considered virulence factors that cause illness in humans. ***Objective***: To identify the adhesive genes *sfa* and *afa* in UPEC strains isolated from UTI patients that form biofilm and to investigate the impact of probiotics (*Lactobacillus acidophilus*) on their expression. ***Methods***: Between October and December 2023, 140 samples were obtained from Urinary Tract Infection (UTI) patients and urinary catheter specimens at Al-Yarmouk Hospital and Ghazi Al-Hariri Hospital for Surgical Specialties locations in Baghdad, Iraq. All bacterial isolates were identified using the VITEK-2 system and standard biochemical testing. The isolates were subsequently subjected to PCR to detect adhesive genes *sfa* and *afa* using primers that were specifically designed. The MRS broth was centrifuged, and *Lactobacillus acidophilus* extract was obtained as a result. Then, isolates that contained two genes were subjected to a *Lactobacillus acidophilus* extract test. ***Results***: The formation of biofilm of UPEC isolates was (33.33%) a strong biofilm-former, (61.90%) a moderate biofilm, and (4.76%) a weak biofilm. The *sfa* and *afa* genes were present at a frequency of 80.95% and 14.28%, respectively. At least one adhesive gene was present in most isolates. ***Conclusions***: Most of the isolates showed a decrease in the expression of sfa and afa genes because of the effect of *L. acidophilus* on gene expression.

Keywords: *afa*, Probiotics, *sfa*, Uropathogenic *E. coli,* UTIs.

تقييم التعبير الجيني لجينات *sfa* و *afa* في البكتيريا القولونية المسببة لأمراض المسالك البولية تحت تأثير البروبيوتيك

الخلاصة

الخلفية: تحدث التهابات المسالك البولية في المقام الأول بسبب الإشريكية القولونية. تحتوي عزلات UPEC على جينات مختلفة تعتبر عوامل ضراوة تسبب المرض لدى البشر. **الهدف:** تحديد الجينات اللاصقة *sfa* و *afa* في سلالات UPEC المعزولة من مرضى التهاب المسالك البولية التي تشكل أغشية حيوية وللتحقق من تأثير البروبيوتيك (*L. acidophilus*) على تعبير هذه الجينات مما يؤدي إلى تأثير تكوين الأغشية الحيوية. **الطرق:** بين أكتوبر وديسمبر 2023، تم الحصول على 140 عينة من مرضى التهاب المسالك البولية وعينات القسطرة البولية في مستشفى اليرموك ومستشفى غازي الحريري للتخصصات الجراحية في بغداد، العراق. تم تحديد جميع العزلات البكتيرية باستخدام نظام VITEK-2 والاختبارات الكيميائية الحيوية القياسية. تم إخضاع العزلات بعد ذلك لتفاعل البوليميراز المتسلسل للكشف عن الجينات اللاصقة *sfa* و *afa* باستخدام بادئات تم تصميمها خصيصًا. تم طرد مرق MRS بالطرد المركزي وتم الحصول على مستخلص *L. acidophilus* نتيجة لذلك. بعد ذلك، تم إخضاع العزلات التي تحتوي على جينين لاختبار مستخلص *L. acidophilus*. **النتائج:** كان تكوين الأغشية الحيوية لعزلات UPEC (33.33٪) عبارة عن تكوين غشاء حيوي قوي، (61.90٪) عبارة عن غشاء حيوي متوسط، و (4.76٪) شكل غشاء حيوي ضعيف. كانت الجينات *sfa* و *afa* موجودة بتردد 80.95٪ و 14.28٪ على التوالي. كان هناك جين لاصق واحد على الأقل موجودًا في غالبية العزلات. **الاستنتاجات:** تم تحديد تأثير *L. acidophilus* على التعبير الجيني، مما أدى إلى انخفاض في التعبير عن الجينات *sfa* و *afa* في غالبية العزلات.

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INTRODUCTION

Prevalent among 150 million people worldwide each year, urinary tract infections (UTIs) are a major contributor to morbidity [1]. Inflammatory diseases, such as UTIs, are the result of a variety of pathogens that disrupt the proper functioning of the urinary system [2]. Symptomatic urinary tract infections (UTIs) are classified into three severity levels: urosepsis syndrome, pyelonephritis (an upper UTI characterized by infection in the kidney), and cystitis (a lower UTI characterized by bacteria in the bladder). The term "UTI" designates the presence of a specific quantity of bacteria in the urine, typically exceeding 105/ml [3]. *Escherichia coli* is the predominant pathogen, accountable for more than 80-90% of urinary tract infections acquired in the community and 30-50% of those acquired in hospitals [4]. The capacity of UPEC to invade, grow, ascend, and persist in the uroepithelium is contingent upon its capacity to form biofilms and utilize various virulence

factors [5]. Unique virulence determinants are frequently used to identify UPE isolates. Chromosomal gene clusters on "pathogenicity islands" in the UPEC encode adhesins, such as S pili and fimbrial adhesins, as well as other virulence genes [6]. Extraintestinal infections are frequently associated with *E. coli* strains that express S-fimbrial adhesins. The adhesin family is composed of S-fimbriae (*sfa*), F1C-fimbriae Foc, and S/F1C *sfa* [7]. The expression of operons from the *afa* family by pathogenic *E. coli* strains that are linked to intestinal and extraintestinal infections in humans and animals has been documented [8]. Encoded by the *afa*-1 operon, the fimbrial adhesin is an X-binding adhesin that is mannose-resistant and P-independent. It has the capacity to facilitate the specific binding to human erythrocyte receptors and uroepithelial cells. Specific subtypes are more prevalent in pyelonephritis and other UTIs, as indicated by *Afa* operon data, which suggests a substantial degree of heterogeneity among uropathogenic E. coli isolates [9]. Biofilms, which are microcolonies that are present on the surface of urethral catheters and the mucosa of the urinary bladder, are generated by UPEC. To protect bacteria from the host immune response and antimicrobial therapy, biofilm encapsulates them in an extracellular matrix. Additionally, the biofilm's bacteria are capable of transmitting resistance determinants between one another with ease because of their close association [10]. This will exacerbate drug resistance and confound the treatment of urinary tract infections. The emergence and dissemination of novel antibiotic-resistant isolates has contributed to the rise in popularity of antibiotic-free treatments in recent years. Probiotics show great potential as an alternative therapy methodology for the management of urinary tract infections (UTIs). Specific probiotics have been purported to possess the capacity to attach to uroepithelial cells and hinder the growth of harmful bacteria. Furthermore, lactobacilli can colonize these germs in the urinary system by being administered orally after colonizing the intestines [11]. Probiotics are live microorganisms that, when administered in sufficient quantities, offer health benefits to their recipients [12]. Probiotic microorganisms, including lactic acid bacteria (LAB), have a long history of safe use in both fermented and non-fermented foods. Even though a significant number of probiotic microorganisms are commercially available worldwide, there is still a significant industrial interest in the identification of novel strains [13]. Consequently, *L. acidophilus* is widely regarded as possessing probiotic properties and is one of the most frequently recommended microorganisms for dietary use [14]. The resistance of *L. acidophilus* to both acid and bile salt is superior to that of many other probiotics. *L. acidophilus*'s survival and proliferation in the severe environment of the gastrointestinal tract are facilitated by these characteristics. Further opportunities for its products to function effectively in the human body are provided by its capacity to endure these conditions. Health promotion can be accomplished when the volume of *L. acidophilus* reaches a specific threshold. *L. acidophilus* has a variety of effects on the human body, such as the regulation of intestinal flora balance, nutrition, immunity, anti-cancer and age-delaying effects, and cholesterol reduction [15,16]. The objectives of this study were to detect *sfa* and *afa* genes in UPEC isolates that were isolated from UTI patients and catheters and study the effect of *L. acidophilus* as a probiotic on the expression of these genes.

METHODS

Isolation of bacteria

In the period from October to December 2023, 140 different specimens, including midstream urine and urinary catheter swabs, were collected from patients at Al-Yarmouk Hospital and Ghazi Al-Hariri Hospital for Surgical Specialties. All samples were cultured on MacConkey agar and EMB (eosin-methylene blue) agar, and the isolates were then incubated at 37 ºC for 24 hours. *E. coli* isolates were characterized using different biochemical methods. Also, the VITEK-2 was used to identify UPEC isolates, which utilizes a sophisticated colorimetric approach. A Gram-negative (GN) card was employed to identify *E. coli* with a probability of 93–99%.

Biofilm assay

The Crystal Violet Binding Assay, which O'Toole adapted, was utilized to examine the biofilm formation of *E. coli* strains [17]. *E. coli* strains were subcultured using this approach for an entire night at 37°C in the Brain Heart Infusion Broth. Following the incubation time, these cultures were divided into 96-well polystyrene plates at a ratio of 1:100, and they were incubated for a full day at 37 °C. After that, 1% crystal violet was used to stain the wells. Following this, 96% ethanol was added to each well to solubilize the bound crystal violet. Lastly, at 595 nm, the absorbance of solubilized crystal violet for every well was determined. The experiment was performed in triplicate. The biofilm forms of *E. coli* strains were used to categorize them into four groups: ($OD \leq ODc$) None Biofilm Former (NBF), ($ODc < OD \leq 2 \times ODc$) Weak Biofilm Former (WBF), ($2 \times ODc < OD \leq 4 \times ODc$) Intermediate Biofilm Former (IBF), and ($ODc > 4 \times ODc$). Strong Biofilm Former (SBF).

Inhibition of biofilm formation

Lactobacillus acidophilus was cultured in MRS broth for 16 hours at 37°C before being centrifuged at 9,000 g for 10 minutes at 4°C. Finally, CFS (cell-free supernatant) from Lactobacillus acidophilus was obtained to prevent biofilm formation [18]. The effluent was sterilized using syringe filters with a pore size of 0.2 µm and kept at 4°C until use. Subsequently, the serial

dilution approach was used to determine the appropriate concentration of Lactobacillus acidophilus to prevent UPEC biofilm formation.

Molecular detection of sfa and afa

DNA was extracted from the *E. coli* isolates using the Presto™ Mini gDNA Bacteria Kit processed by Geneaid, Taiwan. The extracted DNA was detected by gel electrophoresis. In this study, conventional PCR was used for the detection of these virulence factors in UPEC isolates from patients with urinary tract infections in Baghdad, Iraq. Table 1 shows the primers used for the detection of UPEC virulence genes, and these primers were designed by the Primer 3 program, and Table 2 demonstrates the PCR program. PCR was performed in a total volume of 25 µl, and components are shown in Table 3.

Table 1: Primers sequences used in this study

Target genes	Primer name	Sequence (5´–3´)	Product size (bp)	Reference
sfa	F	'5-GAGTCAGCCCTCCGTTTTCA-3'	176bp	Primer 3 program
	R	'5-TCTGGGTGAATGTCAAGGCC-3'		
afa	F	'5-TTACCECCACCTITCAGCAT-3'	215bp	
	R	'5- AAGCAGTTTGAGGCAGAGCT-3'		
GAPDH	F	'5-ACTTACGAGCAGATCAAAGC-3'	200bp	
	R	'5-AGTTTCACGAAGTTGTCGTT-3'		

Table 2: PCR amplification program (New England Biolabs)

Gene	Step		Temperature (°C)	Time (M:S)	Cycles
sfa	1	Initial Denaturation	95	3 min	1
	2	Denaturation	95	30 sec	35
	3	Annealing	55	30 sec	
	4	Extension	68	1 min	
	5	Final Extension	68	5 min	1
afa	1	Initial Denaturation	95	3 min	1
	2	Denaturation	95	30 sec	35
	3	Annealing	52	30 sec	
	4	Extension	68	1 min	
	5	Final Extension	68	5 min	1
GAPDH	1	Initial Denaturation	95	3 min	1
	2	Denaturation	95	30 sec	35
	3	Annealing	53	30 sec	
	4	Extension	68	1 min	
	5	Final Extension	68	5 min	1

RNA extraction

The RNA of three *E. coli* isolates was extracted in the absence and presence of probiotics (*Lactobacillus acidophilus*). The RNA of treated and not-treated bacterial cells was extracted according to the manufacturer's directions using the GENEzol TriRNA Pure Kit (Geneaid, Thailand) as follows: For the expression of *sfa* and *afa* before and after treatment with probiotics, qRT-PCR was used. Three isolates were chosen to determine the effect of probiotics. These isolates were selected based on their biofilm formation. The primers used for the expression of *sfa* and *afa* were the same as those used for conventional PCR. The housekeeping gene (*GAPDH*) primers used are listed in Table 1. Furthermore, the components of quantitative qRT-PCR are shown in Table 4. The quantification of *sfa* and *afa* gene expression was carried out using the $\Delta\Delta C_T$ calculation, and the fold of gene expression was given as $2^{-\Delta\Delta CT}$.

Statistical analysis

To identify the impact of distinct groups on study parameters, the Statistical Analysis System- SAS (2018) program was implemented. Significant comparisons between percentages (0.05 and 0.01 probability) were conducted using the Chi-square test.

Table 3: Components of the conventional PCR reaction

Component	Volume (µl)
Taq 2x Master Mix	12.5
Forward primer (10 µM)	1.0
Reverse primer (10 µM)	1.0
Nuclease free water	8.5
Template DNA	2.0
Final volume	25

Table 4: Components of qRT-PCR

Component	Volume (µl)	Final conc
Luna Universal One-Step Reaction Mix(2X)	10	1X
Luna WarmStart® RT Enzyme Mix (20X)	1	1X
Forward primer (10 pmol/µl)	0.8	0.4
Reverse primer (10 pmol/µl)	0.8	0.4
Template RNA	variable	20 ng\µl
Nuclease Free Water	To 20	

RESULTS

Out of 140 (100%) specimens, 21 (15%) showed positive bacterial growth on MacConkey Agar (Figure 1A) and generated pink/red colonies that ferment lactose. Plates were incubated at 37 ˚C for 24 hours.

Former colonies were then changed to EMB (eosin-methylene blue) agar and incubated for another 24 hours. The green metallic sheen colonies indicate the presence of UPEC (Figure 1B).

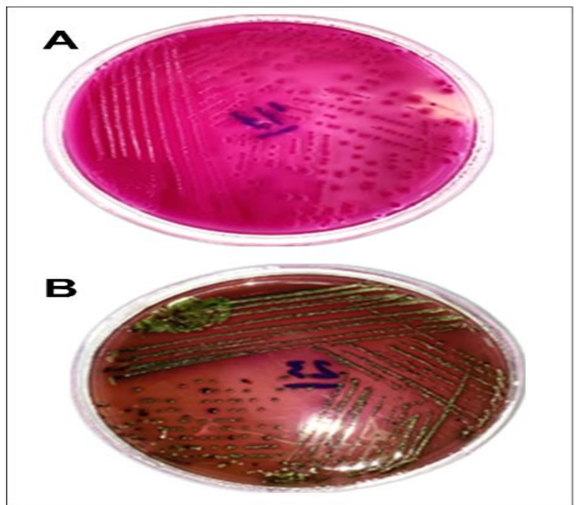

Figure 1: Isolation of UPEC. A) UPEC colonies on MacConkey agar; B) UPEC colonies on EMB agar.

The 21 isolates were identified as *E. coli* by biochemical testing, which yielded positive results for indole and methyl red but negative results for voges-proskauer, urea, and citrate. The housekeeping gene (*GAPDH*) amplification was utilized to establish the bacterial isolates' identity as *E. coli*. A total of 21 *E. coli* isolates were evaluated for biofilm formation using a microtiter plate assay. The findings indicated that 7 isolates (33.33%) developed strong biofilms, 13 isolates (61.90%) formed intermediate biofilms, and 1 isolate (4.76%) created weak biofilms. The chi-square test showed significant differences ($p < 0.05$) among the biofilms formed. Table 5 displays the results of biofilm development in all UPEC isolates.

Table 5: The Strength and Value of biofilm formation in all UPEC isolates

Isolates	Biofilm strength	Biofilm value
E1	weak	0.1533
E2	moderate	0.25
E3	moderate	0.300
E4	moderate	0.296
E5	strong	0.324
E6	strong	0.320
E7	moderate	0.199
E8	strong	0.467
E9	strong	0.346
E10	strong	0.3197
E11	moderate	0.235
E12	moderate	0.224
E13	strong	0.253
E14	moderate	0.187
E15	moderate	0.217
E16	strong	0.332
E17	moderate	0.251
E18	moderate	0.161
E19	moderate	0.268
E20	moderate	0.170
E21	moderate	0.167

To prevent the biofilm formation of UPEC isolates, a 0.25 dilution of *L. acidophilus* extract was chosen, which is the sub-MIC (minimal inhibitory concentration) determined by the serial dilution approach. The PCR technique was employed to detect sfa and afa genes, and gel electrophoresis revealed the bands, as shown in Figure 2. PCR was performed on three isolates before and after probiotic administration, and the findings are given in Tables 6 and 7—regulation of *sfa* and *afa* gene expression.

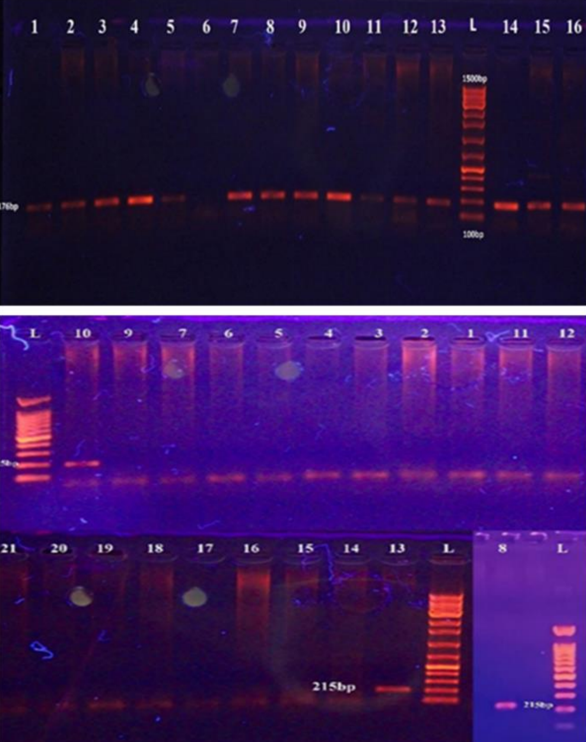

Figure 2: Agarose gel electrophoresis of PCR of the *sfa* and *afa* genes (176 bp and 215 bp, respectively) of *E. coli* isolates.

DISCUSSION

Differences in host sensitivity to pathogens are responsible for the diversity of microorganisms that cause urinary tract infections. This is due to both biological and environmental factors that promote diversification in hosts, diseases, vectors, and societal factors, such as people's efforts to manage disease [19]. The widespread dispersion of *E. coli* isolates can be attributed to the presence of a variety of virulence factors that enable UTI infection, such as secreted and surface virulence factors. Surface virulence factors, such as fimbria (adhesive molecules), and this type of organelle contribute to virulence through separate methods. The methods under consideration include direct activation of host and bacterial cell signaling pathways, encouragement of bacterial invasion, and facilitation of the transport of additional bacterial products [20,21]. This study was done to find UPEC virulence factors (*sfa* and *afa* genes), which are agents that contribute to biofilm development and cause a variety of illnesses.

Table 6: Regulation of *sfa* expression

Isolate code	pre			post			ΔΔCt	Fold change	Result
	hkg	*sfa*	ΔCt	hkg	*sfa*	ΔCt			
1	11.385	23.015	11.63	12.435	27.05	14.615	2.985	0.126	Decrease
2	12.745	20.2	7.455	10.705	21.73	11.025	3.57	0.084	Decrease
3	9.645	11.565	1.92	10.495	13.015	2.52	0.6	0.660	Decrease

Table 7: Regulation of *afa* expression

Isolate code	pre			post			ΔΔCt	Fold change	Result
	hkg	*afa*	ΔCt	hkg	*afa*	ΔCt			
1	11.385	24.225	12.84	12.435	21.8	9.365	-3.475	11.119	Increase
2	12.745	12.645	-0.1	10.705	13.12	2.415	2.515	0.175	Decrease
3	9.645	22.765	13.12	10.495	23.715	13.22	0.1	0.933	Decrease

In recent years, suppression of biofilm formation by pathogenic bacteria has emerged as a prospective therapeutic target [22]. UPEC is known for its ability to form a strong biofilm on the surface of bladder epithelial cells and urethral catheters [10]. The present study also demonstrated the biofilm-forming ability of UPEC isolates in 96-well microtiter plates. Biofilm development has a significant effect on uropathogenicity [23]. Biofilms are also resistant to antimicrobial treatments, which are mostly effective against planktonic bacteria. As a result, probiotic isolations with antibacterial and anti-biofilm capabilities against UPEC may be clinically useful [13]. Lactobacilli strains have previously been tested for their antibiofilm activity against pathogenic bacteria. According to reports, the CFS of fecal lactobacilli isolates prevented the formation of *Vibrio cholerae* biofilms by more than 90% [18]. Lactobacillus plantarum (*L. plantarum*) and *Lactobacillus pentosus* (*L. pentosus*) CFS were also found to have antibiofilm activity against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* [24]. In this investigation, biofilm formation of 21 UPEC isolates was found, and the ratio was (33.33%) a strong biofilm-former, (61.90%) a moderate biofilm, and (4.76%) a weak biofilm, while the p-value for biofilm is 0.0055, indicating a significant. In addition, a PCR program was utilized to detect the presence of sfa and afa genes, which are thought to be responsible for biofilm development. The results show that the *sfa* and *afa* alleles were present at frequencies of 80.95% and 14.28%, respectively. The p-values for *sfa* and *afa* were 0.0046 and 0.0011, respectively ($p < 0.05$). As a result, the presence of the afa gene correlates positively with biofilm formation capacity, whereas the presence of the *sfa* gene correlates negatively with biofilm formation ability. Nonetheless, the *sfa* and *afa* gene combination in the UPEC isolates included in this study is consistent with earlier findings, which suggest that this gene combination was either lacking or poorly characterized [25-27]. Then, *Lactobacillus acidophilus* extract was tested for antibacterial activity against UPEC isolates. Three UPEC isolates were evaluated, and all could form robust biofilms. *Lactobacillus acidophilus* extract reduced the ability to generate biofilm by acting as an antibacterial agent and downregulating the expression of the *sfa* gene. Two of the three isolates had decreased *afa* gene expression due to the antimicrobial activity of *Lactobacillus acidophilus* extract, whereas one had increased *afa* gene expression. The increase in gene expression for this isolate could be due to bacteria experiencing probiotic stress and increasing their expression to counteract the effect of the probiotic, or it could be due to the experimental conditions, or it could be that the expression was in its early stages and did not progress to protein formation.

Conclusion

The current study showed that *L. acidophilus* has a remarkable impact on the gene expression of *sfa* and *afa* genes, which handle biofilm formation. Additionally, *L. acidophilus* is capable of inhibiting biofilm formation in UPEC isolates and causing a decrease in the expression of *sfa* and *afa* genes in most of them. *L. acidophilus* may be a promising probiotic for the prevention and treatment of UTIs, according to our findings. Nevertheless, the potential implementation of these microorganisms as probiotics requires in vivo investigations.

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Conflict of interests

No conflict of interest was declared by the authors.

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Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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