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Correlation between Biofilm Production and Bacterial Urinary Tract Infections: New Therapeutic Approach

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BIOFILMS production during bacterial urinary tract infections (UTIs), being responsible for persistence and relapses. Bacterial producing biofilms are difficult to be eradicated as they reveled antibiotics resistant phenotype that correlated to provided protections by biofilms. The present study revealed that the gram negative bacteria (G-ve) were the most common uropathogenic bacteria causing UTIs with 64.28% biofilm producing ability especially *Escherichia coli* and *Klebsiella pneumoniae*. While, gram positive bacteria (G+ve) represented 23.63% of UTIs with 57.69% biofilm producing ability. The isolated uropathogenic bacteria demonstrated a high and widespread resistance (50% to 95 %) to all used antibiotics except Nitrofurantoin and Imipenem. The most antibiotics resistant uropathogenic isolates were biofilm producers and some of them revealed haemolytic activity. The present study indicated the importance of studying biofilm producing ability of uropahogenic bacteria as it represented 62.72% during UTIs. The anti-biofilm activity of cell free supernatant of *Lactobacillus plantarum* subsp. plantarum DSMZ 20174 and *Lactobacillus acidophilus* DSMZ 20079T against biofilms of both G-ve and G+ve uropathogenic bacteria beside their acid and bile salt tolerance gave potentiality for their use during UTIs as a beneficial tool for dissociation or prevention of biofilms and enable antimicrobial drugs from eradication of infections.

Keywords: Urinary tract infection, *Escherichia coli*, Antibiotic resistance, Biofilm production

Introduction

Urinary tract infection (UTI) is one of the most common infection worldwide and causes serious health problem affecting millions of people each year (Stamm & Norrby, 2001 and Delcaru et al., 2016). Infections occurring in both males and females of all ages, although women tend to have higher occurrence. Urinary tract infections begin in either the urethra or the kidney and eventually migrate to the urinary tract (Grabe et al., 2013). Most infections are caused by bacteria. The recurrent rate is high and often causes chronic with many infections. Many bacteria causing UTIs are associated with biofilm formation. Bacterial biofilms develop on both living surfaces and artificial implants (Donlan, 2002).

The majority of UTIs (95%) resulted from a single bacterial species. *E. coli* is the most frequent infecting bacteria in acute infection (Jellheden et al., 1996 and Ronald, 2002) it is usually found in the lower intestine of humans and present in fecal matter and transferred into the urethra in females. *Enterococcus faecalis* is another common bacteria inhabiting urinary tract infections, it is similar to *E. coli* as it inhabits the gastrointestinal tract of humans. Also, this Gram-positive (G +ve) bacteria are resistance to varieties of antibiotics including penicillin-derivatives, and cephalosporins, this makes its treatment very difficult., *Pseudomonas aeruginosa, Klebsiella, Enterococcus* spp., and *Serratia* spp. being most frequently involved in UTIs (Brede & Shoskes, 2011). G+ve and -ve bacteria have the ability to form biofilms and commonly involved with *Enterococcus faecalis, Staphylococcus aureus, Streptococcus viridans, Staphylococcus epidermidis, Escherichia coli, Klebsiella pneumoniae* , *Proteus mirabilis* and *Pseudomonas aeruginosa* (Donlan, 2001). Biofilm can be defined as a microbiologically derived sessile community characterized with irreversible attach of cells to a substratum or interface and

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embedded in their produced matrix (Flemming & Wingender, 2010) of extracellular polymeric substances (polymeric matrix is made from the combination of exopolysaccharides, proteins, enzymes, teichoic acids, and extracellular DNA). The transition from planktonic growth to biofilm cause changes in the surface molecules expression, virulence factors, and metabolic status and enable bacteria to survive during unfavorable conditions (Klebensberger et al., 2009) Biofilm forming bacteria cause a serious problem during UTIs as they involved in up to 80% of all infections (Stowe et al., 2011) . The importance of controlling biofilm producing uropathogeic bacteria is related to protection provided by biofilm. A biofilm develops on the uroepithelium, bacteria enter this biofilm, which protects them from the mechanical flow of urine, host defenses, and antibiotics, making bacterial elimination difficult and become responsible for colonization and persistence (Jacobsen & Shirtliff, 2011 and Niveditha et al., 2012) . Treatment of biofilm-associated uropathogenic bacteria requires new strategies due to the high levels of antibiotic resistance conferred by biofilm structures (biofilm can be up to 1000-fold more resistant to antibiotics than planktonic cells (Lewis, 2010) especially exopolysaccharides in biofilm matrix. The challenge in treatment of uropathogenic biofilm forming bacteria is the antimicrobial resistance shown by biofilms. Probiotics are defined as live microorganisms with health benefit on the host when administered in adequate amounts (Bhan et al., 2005). The use of food based probiotics for nutritional and therapeutic purposes have assumed great significance recently (Das et al., 2013). Lactobacilli gained significant importance in antimicrobial therapy due to their production of different antimicrobial materials such as bacteriocins and biosurfactants (BSs), which affected on formation of biofilms (Gupta & Garg, 2009). Cell free supernatants of *Lactobacillus* spp. might control and disperse mature established biofilm of *S. aureus* with matrix degrading enzymes such as deoxyribonucleases, glycosidases and proteases (Kaplan, 2010). The treatment of uropathogenic bacteria with antibiotics is commonly used, but recurrence, persistence and antibiotic resistance have been associated mainly with biofilm producing bacteria. So, the current study aimed to determine the most frequent uropathogenic bacteria and estimated their abilities to produce biofilm and resist antibiotics, consequently find new therapeutic agents able to dissociate formed biofilms and transfer bacterial growth into planktonic mode to facilitate eradication of infection via antimicrobial drugs.

Material and Methods

Sampling

About 130 urine samples were kindly provided by Tanta University Hospital, Egypt, and private analytic labs in Tanta, Egypt during summer of 2015. The urine samples were collected from persons suffering from UTIs aging 40-60 years, transferred immediately to lab and cultured.

Bacterial colony count in urine samples of UTI and biochemical identification

Presence of single potential pathogen or two during UTIs could be observed with colony count equal or more than 10^4 CFU/ml and interpreted as positive UTIs, a less than 10^2 CFU/ml was interpreted as negative UTIs (Schneider & Riley, 1996). Urine specimens were cultured for isolation of the bacterial agents of UTI on blood agar and MacConky agar media. All the bacteria isolated from urine in this study were identified using Gram reaction, the G-ve isolates were subcultured on MacCkongy, Eosin methylene blue and Cetrimide agar. Gram +ve cocci were cultured on Manitoal salt agar. The bacterial isolates were biochemically identified by the following tests Haemolysin production (Pavlov et al., 2004), Methyl red (MR) (MacFaddin, 1985) and Voges-Proskauer (VP) (MacFaddin,1980). Also, catalase (Koneman et al., 1992) test was performed to Gram +ve cocci. The identification of Gram-negative, was confirmed by API 20E consists of 27 wells using strip system (BioMereux).

Susceptibility of uropathogenic isolates to different antibiotics

The antibiotic susceptibility test was conducted according to Kirby Bauer disc diffusion method against the following antibiotics: Oxacillin (1 µg), Amoxicillin (10 µg), Cephlothin (30 µg), Cefuroxime (30 µg), Clindamycin (2 µg), erythromycin (15 µg), Nitrofurantion (300 µg), Vancomycin (30 µg), Chloramphenicol (30 µg) Cefoperazone (75 µg), Cefepime (30 µg), Imipenem (10 µg), Gentamicin (10 µg), Rifampin(15 µg), Levofloxacin (5 µg), ciprofloxacine (5 µg), (Sulphamethoxazole/ Trimethoprim) (25 µg), and doxycycline (30 µg). The entire surface of the Muller Hinton agar plate with 2% NaCl was covered with bacterial inoculums, then antibiotics discs were laid

on the dried surface. The plates were incubated at 35°C for 24 h. The diameter of the zone of inhibition was compared according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2010)

Assay of biofilm production

Congo red agar medium (CRA)

The Brain heart infusion agar was supplemented with 5% sucrose and after sterilization Congo red (0.08 g/l) stain which sterilized separately was added to cooled media (55°C). The CRA was mixed well and distributed into plates (Greenberg et al., 1995). Then plates were inoculated and incubated aerobically for 24 to 48 h at 37°C. Presence of black colonies with a dry crystalline revealed that bacteria are biofilm producing and non-producers remain pink (Freeman et al., 1989 and Blanco et al., 2005)

Microtiter plate (MtP) assay

The test was performed according to Christensen et al. (1982). The Overnight bacterial cultures were diluted 1:10 with Trypticase Soy Broth, 96-well microtiter plates were used and seeded with 200 µL per well. The plates were incubated at 37°C for 24 h. After four washes in phosphate buffered saline solution (pH 7.2), biofilms formed by adherent bacteria in plate were fixed with sodium acetate (2%) and stained with crystal violet (0.1% w/v). Finally, the microtiter plates were rinsed under running tap water and the dye bound to the walls of the wells was re-solubilized with 33% (v/v) glacial acetic acid. The absorbance or optical density (OD) was measured at 630 nm using ELISA reader (Hossain & Uddin, 2014). Control was prepared with crystal violet binding to well exposed only to the culture medium (TSB) without bacteria. OD < 0.120 considered as negative producers and > 0.240 considered as biofilm producing bacteria.

Lactobacillus spp.

Lactobacillus acidophilus DSMZ 20079T and *Lactobacillus plantarum* subsp. plantarum DSMZ 20174 were kindly provided by bacteriological Lab, Botany Department, Tanta University.

Dissociation of uropathogenic biofilms by cell free supernatants of Lactobacillus spp. using MtP assay

Overnight *Lactobacillus* cultures contained 10^7 (CFU/ml) were grown in De Man Rogosa Sharpe (MRS) broth at 37°C for 24 h. Cell free supernatants (CFS) were filter-sterilized (0.22 µm Minisart; Sartorius, Germany) to evaluate their effect on produced biofilm of uropathogenic biofilm producing bacteria using MtP assay. Biofilms were allowed to perform for 24 h at 37°C to allow cell attachment as described before, following the incubation period; media were poured out from wells and washed with sterile phosphate-buffered saline. Cell free supernatant (200 µl/ well) were added. The plates were incubated for another 24 h. before the crystal violet staining was performed (Chaieb et al., 2007). Following incubation, biofilm was assessed. Control was prepared with the addition of (200 µl/ well) MRS only as a culture medium without bacteria.

Acid and bile tolerance

MRS broth either with different pH (2.0-6.0 adjusted with HCl) was prepared (Pereira & Gibson, 2002) or supplemented with different concentrations of bile salts (0.1, 0.3 and 0.5% of ox-bile salts, Oxoid) according to Gilliland et al. (1984), inoculated with 1% inoculums (1×10^9 CFU) and incubated overnight. Samples were drawn, plated on MRS plates and incubated at 37ºC for 24 h. Growth on the plates indicated their tolerance to acid or bile salts. The control comprised normal MRS.

Analysis

SPSS 16 version and Excel 2007 were used to analyze collected data. Results were expressed as mean of three replica ± stander deviation.

Results and Discussion

Incidence and classification of bacteria causing UTI

Out of 130 urine samples were investigated, 110 (68 females and 42 males) gave positive cultures with bacterial counts more than 104 CFU/ml. The gram-negative bacilli (Gm –ve) isolates represented 76.36% of isolated bacteria and their biochemical characteristics were illustrated in Table 1 and 2; *Echerichia coli* gave M R +ve ,VP-ve with green metallic sheen on eosin methylene blue agar (presented in 52 of samples and represented 47.27% of UTI), *Klebsiella pneumoniae* gave VP+ve and MR-ve (presented in 22 of samples and represented 20% of UTI), *Pseudomonas aureginosa* gave MR - ve and VP- ve with green colored colonies on cetrimide agar (presented in 10 of

samples and represented 9.09% of UTI). While, gram positive (Gm +ve) cocci gave yellow color on manitol salt agar with β, α haemolysis and represented 23.64% of isolated bacteria; *Staphylococcus aureus* gave VP+, MR +ve and catalase +ve (presented in 12 of samples and represented 10.9% of UTI) and *Enterococcus faecalis* gave VP+ ve, MR - ve and catalase - ve (presented in 14 of samples and represented 12.7% of UTI). Also, percentage of bacterial UTIs in female was 61.81% and 38.18 % in male as illustrated in Table 1

TABLE 1. Incidence and classification of bacteria causing urinary tract infections.

Bacterial isolates	***E. coli***	***K. pneumonia***	***P.aureginosa***	***S. aureus***	***E. faecalis***
Percentage(%)	47.27	20	9.09	10.90	12.72
Sex					
femal	32	14	7	6	9
male	20	8	3	6	5
MR	+	-	-	+	-
VP	-	+	-	+	+
Haemolysis					
Non	20	12	6	2	4
β	22	-	-	5	6
α	10	10	4	5	4

TABLE 2. API 20E biochemical characteristics of Gram-negative bacteria causing urinary tract infections.

Identification	***Pseudomonas aeruginosa***	***Klebsiella pneumonia***	***Escherichia coli***
ONPG	-	+	+
ADH	+	-	-
LDC	-	-	-
ODC	-	-	-
CIT	+	+	-
H2S	-	-	-
URE	-	+	-
TDA	-	-	-
IND	-	-	+
VP	-	+	-
GEL	-	-	-
GLU	+	+	+
MAN	-	+	+
INO	-	+	-
SOR	-	+	+
RHA	-	+	+
SAC	-	+	-
MEL	-	+	+
AMY	-	+	-
ARA	-	+	+
OX	+	-	-
NO2	-	+	+
N2	-	-	-
MOB	+	-	+
MC	+	+	+
OF-O	+	+	+
OF-F	-	+	+

The current study revealed that the most common bacteria causing UTIs were G-ve bacilli with percentage 76.36%, the infection was more common in females (61.81%) than males. The responsible uropathogenic bacteria for infection were *E. coli* followed by *K. pneumonia*, *E. feciales*, *S. aureus* and *P. aeruginosa*, the biofilm producing ability of isolated bacteria was 68.45%. In the same connection, Behzadi et al. (2010) found that the G-ve bacteria especially, *E. coli* and *K. pneumoniae* were the most common uropathogenic bacteria causing UTI.

Antibiotic resistance and biofilm production (CRA) of isolated uropathogenic bacteria

Bacterial biofilms play an important role in UTIs as they responsible for persistent of infections and leading to recurrences and relapses (Delcaru et al., 2016). In addition to antibiotics resistance and biofilms producing abilities, the isolated uropathogenic bacteria revealed hemolytic activity and the highest ratio appeared with isolates of *S. aureus*, *E. faecalis* and *E. coli* (83.3, 71.4 and 61.5 %, respectively), offering another virulent criteria to them. Johnson (1991) discussed the importance of recognizing virulence factors during UTI which included adhesions, haemolysin, K capsule, and resistance to serum killing. Also, presence of two virulence factors (haemolysin and biofilm) in combination during UTI, allowing biofilm producing bacteria to be more resistant to therapy (Akhter et al., 2012).

The results as illustrated in Table 3 revealed that *E. coli* which represented the most common uropathogenic bacteria were presented as 7 isolates with different antibiotic resistant patterns (RP) ranging from 50-95% to all used antibiotics. However, 44.23 of isolated *E. coli* were non biofilm producer and 55.77 were able to produce biofilm. In the same context, Olorunmola et al. (2013) found that *E. coli* isolated during UTI demonstrated a high and widespread resistance (51.1 % to 94.3 %) to all the antibiotics used except Nitrofurantoin. The four different isolates of *K. pneumonia* according to RP (50-92 %) were able to produce biofilm with 72.72% and the percentage of biofilm production of two isolates of *P. aeruginosa* was 60%. However, among 4 isolates of *S. aureus*, 75% were able to produce biofilm and their resistance to antibiotics ranging from 67-92%.

Three isolates of *E. faecalis* were able to produce biofilm with 42.85% and their resistance to antibiotics ranging from 50-83%.

TABLE 3. Antibiotic resistance (AR) and biofilm production (CRA) abilities of isolated uropathogenic bacteria.

Bacterial species (presence frequency of isolates)	% Resistance	Biofilm production
E. coli (7)	75	biofilm
E. coli (3)	83	biofilm
E. coli (10)	92	non
E. coli (7)	67	biofilm
E. coli (9)	95	biofilm
E. coli (10)	50	non
E. coli (6)	58	biofilm
K. pneumonia (4)	75	biofilm
K. pneumonia (6)	83	non
K. pneumonia (8)	92	biofilm
K. pneumonia (4)	50	biofilm
P. aureginosa (6)	82	biofilm
P. aureginosa (4)	77	non
S. aureus (2)	75	biofilm
S. aureus (2)	67	biofilm
S. aureus (5)	92	biofilm
S. aureus (3)	85	non
E. faecalis (4)	50	non
E. faecalis (6)	83	biofilm
E. faecalis (4)	75	non

The most effective antibiotics against isolated G-ve bacilli were Nitrofurantoin, followed by Imipenem and ciprofloxacin. While, Imipenem followed by Nitrofurantoin were the most effective against G +ve cocci. The G+ve bacteria were resistant to Cefepime followed by Cephalothin and Erythromycin. While, G-ve bacteria were resistant to Amoxicillin followed by tetracyclin and Trimethoprim/Sulfamethoxazole. Flores-Mireles et al. (2015) found that the high degree of antibiotic resistances was exhibited by all the biofilm producer uropathogenic bacterial isolates compared with non biofilm producer. Nitrofurantoin was found to be a reliable oral drug for treatment of most of the uropathogens (Ahmed et al., 2015).

The obtained results illustrated that, among 5 different species of isolated uropathogenic bacteria, the most antibiotic resistant bacteria were biofilm producer (*E. coli* with 95% AR, *K. pneumonia* with 92% AR, *P. aeruginosa* with 82% AR, *S. aureus* with 92% AR and *E. faecalis* with 83 % AR). So, the most antibiotic resistant biofilm producer isolates were chosen for ingoing experiment.

In-vitro production of biofilm was significantly more frequent among strains causing relapse (Soto et al., 2006). Consequentially, the current study estimated biofilm as a helpful tool to develop a new therapeutic strategy for eradication of persistent

biofilm-producing uropathogenic bacteria and prevent subsequent relapses. The present study investigated the anti-biofilm ability of cell free supernatant of *L. plantarum* and *L. acidophilus* against most antibiotics resistant biofilm producing uropathogenic isolates, the obtained results revealed significant ability of investigated supernatants in dissociation of formed biofilm of both G-ve and +ve uropathogenic isolates. The greatest anti-biofilm activity was recorded against biofilms of *S. aureus* and *E. coli.* The reduction ability of *L. plantarum* and *L. acidophilus* cell free supernatants was varying from 60.6 to 75% and 54.54 to 82.14% against biofilms of investigated bacteria, respectively as illustrated in Table 4. In the same context, Rao et al. (2016) discussed the ability of controlling the pathogenic biofilms using cell free supernatant of *Lactobacillus* and found that the use of supernatant of *L. platarum* MYS94 to treat the *Klebsiella* biofilm resulted in 52.02 % inhibition of biofilm. Similar results of anti-biofilm activities were reported by Slama et al. (2013) and Rao et al. (2015) who used supernatant of *L. plantarum* and *L. pentosus* in controlling of biofilm production which supported the present work. The anti-biofilm ability of *Lactobacillus* supernatants could be related to production of inhibitory compounds such as organic acids, bacteriocins or other associated compounds as adopted by Rao et al. (2016). Khiralla et al. (2015) demonstrated the *in vitro* anti-biofilm effect of cell free supernatants of *Lactobacillus* sp. against both pathogenic strains of *B. cereus* and *P. aeruginosa*. Also, many researchers reported antibacterial activity of *L. plantarum* and *L. acidophilus* which may be attributed to production of bacteriocins (Vuotto et al., 2014 and Shahandashti et al., 2016) and other antimicrobial compounds.

TABLE 4. Absorbance of *E. coli* biofilm (Mtp) in response to treatment with *L. plantarum* or *L. acidophilus.*

Biofilm producing uropathogenic bacteria	**control**	***L. plantarum***	**Percentage of reduction**	***L. acidophillus***	**Percentage of reduction**
E. coli	0.45±0.04	0.15± 0.0*	66.66	0.12 ± 0.0*	73.3
E. coli	0.31±0.12	0.09 ± 0.0*	70.9	0.08 ± 0.0*	74.19
K. pneumonia	0.33±0.09	0.13 ± 0.0*	60.6	0.15 ± 0.0*	54.54
S. aureus	0.28±0.01	0.07± 0.0*	75	0.05 ± 0.0*	82.14

All data represented mean of three replica± stander deviation, * Significant effect (P = 0.0001) of cell free supernatants of *Lactobacillus* .spp. on biofilm production of investigated bacteria

The acid and bile tolerance of *L. plantarum* and *L. acidophilus* suggested their potential use as therapeutic probiotics during UTI. Bile tolerance is an important criteria during selection of bacteria for probiotic therapeutic uses (Suskovic et al., 2000). It enables the probiotic bacteria to survive, grow and exert its action in the small intestine (Gilliland et al., 1984 and Rao et al., 2015) indicating the tolerance of *L. plantarum* MYS94 to low pH, which is considered as an important characteristic of probiotic bacteria. The bacterial growth during the present study was increased with increasing pH values. The growth was recovered at pH 4 and continued to increase with increasing pH degrees. *L. plantarum* and *L. acidophilus* were tolerated to acidity as bacterial growth reduced up to 10% at pH 3. Also, they tolerated bile salt as the tested levels of salts did not influence growth or survival. There was no difference in cell yield between cultures of control and those with investigated concentrations of bile salt. The present study suggesting the potentiality of using *L. plantarum* subsp. plantarum DSMZ 20174 and *L. acidophilus* DSMZ 20079T in controlling biofilm formation during UTIs to transfer uropathogenic bacteria into planktonic mode and manipulated infections regarding to their anti-biofilm ability, antibacterial activity and bile salt and acidity tolerance.

Conclusion

Biofilms constitute an important contribution to the high incidence, recurrence, and complications of UTIs, thus requiring efficient prevention and control. So, the current study concerned with studying the incidence of resistance, detection of most frequently uropathogenic bacteria and their biofilm producing abilities to better understanding of the bacterial infection subsequently developing new strategy for prevention and treatment. An ideal choice will include a combination of anti-biofilm with effective antibacterial drugs.

Recommendation

Biofilm estimation may be helpful during UTIs to detect patients who require an effective anti-biofilm like *Lactobacillus* sp. within their therapeutic strategy to eradicate persistent biofilm-forming uropathoenic bacteria, enable antibacterial drugs (as Nitrofurantoin or Imipenem) to be effective and prevent subsequent relapses.

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الارتباط بين العدوى البكتيرية للمسالك البولية و إنتاج البيوفيلم: اتجاه علاجي جديد

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تكوين البيوفيلم خلال العدوى البكتيريه للمسالك البولية يكون مسئولا عن استمرار وتكرار العدوى.ولقد وجد ان البكتريا المنتجه للبيوفيلم يصعب القضاء عليها نظرا لمقاومتها للمضادات الحيويه نتيجه الحماية التى تتوفر لها بواسطه البيوفيلم وكشفت الدراسة الحالية أن البكتيريا سالبة الجرام هى الأكثر شيوعا فى عدوى المسالك البولية و إنتاجا للبيوفيلم بنسبه64.28٪ وخاصة بكتريا **ايشرشيا كولاى والكلبسيلا**.بينماكانت نسبه العدوى بالبكتريا موجبه الجرام 23.63٪ ونسبه انتاجها للبيوفيلم57.69 ٪. واظهرت البكتيريا المعزولة مقاومة عالية واسعة الانتشار (50٪ إلى 95٪) لجميع المضادات الحيوية المستخدمة ما عدا نيتروفورانتوين و إيميبينم. وكانت معظم العزلات الاكثر مقاومة للمضادات الحيوية منتجه للبيوفيلم وبعضهم اظهر القدره على انتاج هيموليسين. كما اشارت الدراسه إلى اهميه تعيين قدره البكتريا المتسببه فى عدوى المسالك البوليه على انتاج البيوفيلم نظرا لانها مثلت نسبه 62.72% من البكتريا المعزوله.واظهرالرشيح لبكتريا **لاكتوباسيلوس أسيدوفيلوس و لاكتوباسيلوس بلانتاروم** نشاط مضاد للبيوفيلم المنتج بواسطه كل من البكتريا موجبه وسالبه الجرام , كما اظهرت تلك البكتريا مقدره على مقاومه درجات الحامضيه وتركيزات ملح العصارة الصفراوية مما أعطى إمكانيه لاستخدامها خلال عدوى المسالك البولية نظرا لقدرتها على تحليل البيوفيلم المنتج او منع تكونه وبالتالى تمكين المضادات الحيويه من القضاء على العدوى.