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ORIGINAL ARTICLE

Effects of *Lactobacillus acidophilus* and *Lactobacillus helveticus* on biofilm formation by ESBL-producing uropathogenic *Escherichia coli*

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ABSTRACT

Background*: Uropathogenic E. coli (UPEC) are important clinical pathogens and are considered as one of the major causes of urinary tract infection. Unfortunately, the treatment of UPEC infections is becoming more difficult because isolates are increasingly resistant to commonly used antimicrobial agents. The potential ability of UPEC to form biofilm might explain its outstanding antibiotic resistance and survival properties.* ***Objectives:*** *We aimed to assess the effect of cell free supernatants (CFS) of Lactobacillus acidophilus La-5 and Lactobacillus helveticus B-734 on the ability of ESBL-producing UPEC isolates to form biofilm in vitro and to eradicate the already formed biofilm of UPEC isolates.* ***Methodology:*** *A total of 50 isolates of ESBL-producing UPEC, were recovered from different private laboratories, then subcultured on MacConkey's medium and identified by conventional microbiological methods. Antibiotic susceptibility testing was performed by Kirby-Bauer disc diffusion method and confirmed for ESBL production. The ability of UPEC to produce biofilm was determined using Congo red agar (CRA) method and tissue culture plate (TCP) method. CFS of L. acidophilus La-5 and L. helveticus B-734 were prepared and used to assess its effect on the ability of ESBL-producing UPEC isolates to form biofilm in vitro and the eradication of the already formed biofilm.* ***Results:*** *Biofilm formation was found in 28% of the isolates. Furthermore, the strong biofilm-forming category was detected in 10% of the isolates, whereas the moderate biofilm-forming category was detected in 18%. CFS of L. acidophilus La-5 was more effective (91.12%) in inhibiting the formation of E. coli biofilm than CFS of L. helveticus B-734 (76.44%), while it was almost the same efficacy (83%) regarding eradication of E. coli biofilm. All ESBL- producing UPEC isolates were sensitive to cefoxitin (100%) followed by nitrofurantoin (98%) and imipenem (94%).* ***Conclusion:*** *L. acidophilus La-5 and L. helveticus B-734 may be a new therapeutic options for UTI caused by UPEC.*

INTRODUCTION

E. coli is a common cause of both community-acquired and hospital-acquired urinary tract infections. The rate of ESBL production among these pathogens has accelerated dramatically and has reached pandemic scale[1]. Among the different virulence factors of *E. coli*, is the ability to produce biofilm which is related to their high degree of antibiotic resistance[2]. Biofilm is a complex mixture of microbes which are predominantly attached to hard surfaces. They are often enclosed by thick polysaccharide matrix[3]which is responsible for cell-to-cell and cell-to-surface interactions needed for the formation and stabilization of biofilm[4]. There is a great interest towards development of novel, safe and natural strategies to counteract the establishment of pathogenic biofilms. In this regard, lactic acid bacteria (LAB) as *Lactobacillus acidophilus* and *Lactobacillus helveticus* strains commonly used as probiotics have gained attention as a promising means[5,6]. *Lactobacillus* cell-free supernatants (CFS) represent a potential and safe alternative to synthetic antibiotics for inhibiting the biofilm through presence of some compounds such as bacteriocins, organic acids and hydrogen peroxide produced by *Lactobacillus* and secreted into CFS[7]. The aim of the current study was to assess the effect of CFS of *L. acidophilus* La-5 and *L. helveticus* B-734 on the ability of ESBL-producing UPEC isolates to form biofilm *in vitro* and to eradicate the already formed biofilm of UPEC isolates. It also attempted to detect their antibiotic resistance pattern and to investigate the impact of biofilm formation on antibiotic resistance.

METHODOLOGY

This study was conducted on fifty ESBL-producing *E. coli* isolates recovered from urine specimens obtained from different private laboratories. The study was conducted at the Medical Microbiology and Immunology Department, Faculty of Medicine, Cairo University during the period from October 2019 to April 2020. The study protocol was approved by the ethical committee, Faculty of medicine, Cairo University.

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Culture and identification of *E. coli* isolates:

Isolates were subcultured on MacConkey's medium (Oxoid, UK) and incubated aerobically at 37 °C for 24 hours. Lactose fermenting colonies on MacConkey's medium were identified by conventional methods including colony morphology, Gram-stained smear and biochemical reactions.

Detection of ESBL-producing *E. coli*:

All *E. coli* isolates were screened for extended spectrum β-lactamase (ESBL) production according to Clinical and Laboratory Standards Institute (CLSI) guidelines using Ceftazidime (CAZ; 30µg), Ceftriaxone (CRA; 30µg) and Cefotaxime (CTX; 30µg) as screening agents by disc diffusion Kirby-Bauer method. A decrease in susceptibilities to one or more antibiotics tested may indicate production of ESBLs. Positive screened ESBL-producing *E. coli* isolates were confirmed by combined disc diffusion method using Ceftazidime 30µg and Ceftazidime/Clavulanic 30µg/10µg discs. A greater than or equal to 5 mm diameter difference between the antibiotic zone alone and the combined disc with clavulanate confirmed an ESBL producing organism[8].

Antibiotic susceptibility testing:

All confirmed ESBL-producing UPEC isolates were tested for antibiotic susceptibility using the antibiotic discs (Himedia, India); Cefoxitin (FOX; 30µg), Cefepime (FEP; 30µg), Ertapenem (ERT; 10µg), Meropenem (MRP; 10µg), Imipenem (IPM; 10µg), Gentamicin (GENT; 10µg), Amikacin (AK; 30µg), Tobramycin (TOB; 10µg), Tigecycline (TGC; 15µg), Ciprofloxacin (CIP; 5µg), Levofloxacin (LEV; 5µg), Nitrofurantoin (F; 300µg) and Trimethoprime-Sulfamethoxazole (SXT; 1.25/23.75µg). Results were interpreted according to CLSI standard inhibition zone diameters[8].

Biofilm formation testing:

ESBL-producing UPEC isolates were screened for biofilm formation by both Congo Red Agar (CRA) (qualitative method)[9] and tissue culture plate method (TCP) (quantitative method)[10]. This quantitative assay is considered to be the gold-standard method for biofilm detection[10,11,12].

In CRA method, results were interpreted as follows[9]:

- Black colonies are considered strong biofilm-forming
- Greyish colonies are considered moderate biofilm-forming
- Pink colonies are considered non-biofilm-forming

In TCP method, results were interpreted as follows[10]:

- Mean OD values > 0.24 are considered as strong biofilm-producers.
- Mean OD values 0.12 to 0.24 are considered as moderate biofilm-producers.
- Mean optical density (OD) values < 0.12 are considered as non-biofilm-producers

Effects of CFS of *L. acidophilus* La-5 *and L. helveticus* B-734 on both production and eradication of biofilm of ESBL-producing *E. coli*:

Preparation of cell-free supernatants:

The *Lactobacillus* strains (provided by Dairy Department, Faculty of Agriculture, Cairo University) were grown anaerobically in Man Rogosa Sharpe (MRS) broth for 48 hours at 37˚C. Supernatant was obtained by centrifugation at 4000 rpm for 15 min at 20˚C, then passing through a sterile 0.22 µ-pore-size filter unit (Millex GS Millipore).The filtrate (mixture of metabolites) was collected and then kept at 4˚C[13].

***The co-culture method*[14]: Inhibitory effect of CFS of *L. acidophilus* La-5 and *L. helveticus* B-734 on biofilm-producing uropathogenic *E. coli* was determined *in vitro* as follows:**

E. coli isolates were grown on TSB at 37 °C for 24 h. The turbidity of broth was adjusted to match the 0.5 McFarland standard, then diluted to 1:100 adding fresh TSB. A volume of 100 µL of the bacterial suspension was added together with 100 µL of CFS of *Lactobacillus* strains to a 96-well microtiter plate and incubated 24 h at 37 °C. Positive controls were prepared by inoculating TSB with the *E. coli* strains alone.

***The post-incubation method*[15]: For determining the inhibitory effect of CFS on the already formed biofilm the following was done:**

Biofilms of the pathogenic bacteria were allowed to form in micro-titer plates for 24h. Then, 100 µL of the CFS was added to each well. The plates were incubated for a further 24 h at 37 ˚C. Positive controls were prepared by inoculating TSB with the E. coli strains alone.

***In both methods*, the steps were continued as follows:**

After incubation, the medium was discarded from each well, rinsed with PBS three to four times. Biofilm formation was fixed with 2% Na acetate, and quantified using 0.1% crystal violet method. Finally, 200 µL of 33% glacial acetic acid was added to solubilize the dye. Then optical density was measured at 570 nm. The anti-biofilm activity (%) was calculated using the following formula:

(Control OD570 nm − Test OD570 nm / Control OD570 nm) × 100, where 'Control' represents the optical density values with unchallenged pure culture of test pathogen, and 'Test' represents the values under treatment conditions.

Statistical analysis:

Data was coded and entered using the statistical package for social sciences (SPSS) version 26 (IBM Corp., Armonk, NY, USA). Data was summarized using mean, standard deviation in quantitative data and using frequency (count) and relative frequency (percentage) for categorial data. For comparison of serial measurements within each isolate, the non-parametric Wilcoxon signed rank test was used. For comparing categorial data, Chi square (x^2) test was performed.

Exact test was used instead when the expected frequency is less than 5. *P* values less than 0.05 were considered as statistically significant.

RESULTS

Biofilm-formation:

According to the results of biofilm formation by CRA method, 9 (18%) isolates were biofilm-forming (these 9 isolates were also biofilm-forming by TCP method); 5 isolates were strong producers, while 4 isolates were moderate producers, whereas the remaining 41 isolates (82%) were non-biofilm-forming.

While 14 (28%) isolates were biofilm-forming by TCP method; 5 isolates (10%) were strong biofilm-forming (the same isolates detected by CRA method), 9 isolates (18%) were moderate biofilm-forming, while the remaining 36 (72%) of the isolates were non-biofilm-forming.

Detection of anti-biofilm effect of CFS of *L. acidophilus* La-5 *and L. helveticus* B-734:

Co-culture method: (table 1)

Table 1: Optical density of the biofilm-forming isolates under the effect of CFS of *L. acidophilus* La-5 and *L. helveticus* B-734 by co-culture method.

Isolate No	OD before	Co-culture method OD	
		With CFS of *L.acidophilus* La-5	With CFS of *L.helveticus* B-734
2	0.244	0.014	0.083
3	0.176	0.077	0.037
4	0.162	0.078	0.161
7	0.153	0.063	0.034
8	0.132	0.054	0.083
12*	1.263	0.088	0.065
17*	1.634	0.089	0.061
19	0.172	0.008	0.009
20	0.165	0.006	0.008
22*	1.121	0.094	0.087
24*	1.424	0.324	0.753
33*	3.359	0.441	0.523
36	0.221	0.092	0.032
37	0.154	0.085	0.143
Mean OD	1.035	0.108	0.247
SD OD	0.801	0.123	0.403
P value		0.001**	0.001**
Mean% reduction		91.12%	76.44%
P value between both strains	0.33		

* *Strong biofilm-forming isolates; isolates no.12, 17, 22, 24, 33*

** *P value is significant if < 0.05*

- Under the effect of CFS of *L. acidophilus* L-a5, 12 out of 14 biofilm-forming isolates showed marked reduction (OD < 0.12) in biofilm formation (became non-biofilm-forming). The two remaining strong biofilm-forming isolates (no. 24, 33) were greatly reduced; however, they remained strong biofilm-forming (OD > 0.24).
- Under the effect of CFS of *L. helveticus* B-734, 10 out of 14 biofilm-forming isolates showed marked reduction (OD < 0.12) in biofilm formation, while 2 isolates (no. 4, 37) were not affected. The two remaining strong biofilm-forming isolates (no. 24,33) were greatly reduced; however, they remained strong biofilm-forming (OD> 0.24), similar to the effect of CFS of *L. acidophilus* La-5.
- CFS of *L. acidophilus* L-a5 was more effective (mean reduction 91.12%) than CFS of *L. helveticus* B-734 (mean reduction 76.44%) in preventing biofilm formation; however, this difference was not statistically significant *(P* value 0.33).

Post-incubation method: (table 2)

Table 2: Optical density of the biofilm-forming isolates under the effect of CFS of *Lactobacillus acidophilus* La-5 and *Lactobacillus helveticus* B-734 by post-incubation method.

Isolate No	OD before	Post-incubation method OD	
		With CFS of *L.acidophilus* La-5	With CFS of *L.helveticus* B-734
2	0.240	0.065	0.125
3	0.176	0.074	0.098
4	0.162	0.085	0.068
7	0.153	0.109	0.090
8	0.132	0.087	0.102
12*	1.263	0.075	0.128
17*	1.634	0.114	0.082
19	0.172	0.079	0.074
20	0.165	0.084	0.072
22*	1.121	0.081	0.072
24*	1.424	0.205	0.154
33*	3.359	0.341	0.329
36	0.221	0.102	0.092
37	0.154	0.086	0.091
Mean OD	1.035	0.113	0.113
SD OD	0.801	0.074	0.067
P value		0.001**	0.001**
Mean% reduction		83.39%	82.57%
P value between both strains		0.55	

Strong biofilm-forming isolates; isolates no.12, 17, 22, 24, 33
*** P value is significant if < 0.5*

- Under the effect of CFS of *L. acidophilus* L-a5, 12 out of 14 already formed biofilms were eradicated (OD < 0.12), while the two remaining strong biofilms formed by isolates no. 24, 33 were greatly reduced (OD 0.205, 0.341 respectively) but not completely eradicated.
- Under the effect of CFS of *L. helveticus* B-734, 10 out of 14 already formed biofilms were eradicated (OD < 0.12). The remaining 4 biofilms (one moderate and three strong) formed by isolates no. 2, 12, 24 and 33, respectively were reduced (OD 0.125, 0.128, 0.154 and 0.329, respectively), but not completely eradicated.
- The efficacy of CFS of *L. acidophilus* La-5 and CFS of *L. helveticus* B-734 was almost the same (mean reduction 83.39% and 82.57%, respectively).
- Mean OD after addition of CFS of *L .acidophilus* La-5 and *L. helveticus* B-734 were greatly reduced compared to mean OD before and this reduction was statistically significant (*P* value 0.001) by either co-culture method or post incubation method.

Antimicrobial susceptibility profile:

The antibiotic sensitivity pattern of the 50 uropathogenic ESBL-producing *E. coli* isolates revealed that among orally administered antibiotics; nitrofurantoin showed 98% susceptibility followed by trimethoprim-sulfamethoxazole 76%. Regarding cephalosporins; cefoxitin revealed 100% susceptibility followed by cefepime 54%. Among carbapenems; imipenem showed 94% susceptibility followed by meropenem and ertapenem 84% and 82%, respectively. Tigecycline achieved 90% susceptibility. Among aminoglycosides gentamicin showed 78% susceptibility followed by amikacin and tobramycin 76%. Quinolones showed the least susceptibility; ciprofloxacin 36% and levofloxacin 52%.

Correlation between biofilm-formation and antibiotic susceptibility pattern:

Pearson Chi-Square was used to assess the correlation between biofilm-formation and antibiotic susceptibility patterns of the ESBL-producing *E. coli* isolates as shown in figure (1). Biofilm-forming isolates were more resistant to antibiotics than non-biofilm-forming; however, this difference was not statistically significant

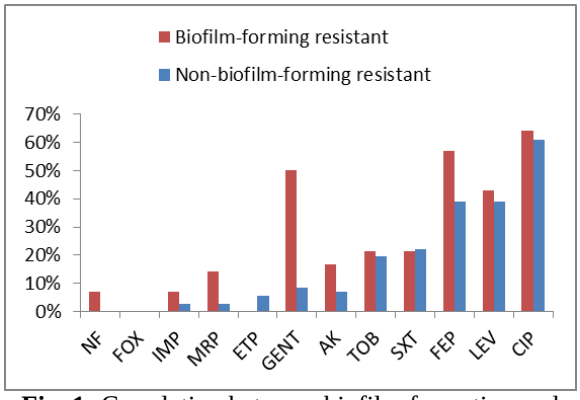

Fig. 1: Correlation between biofilm formation and antibiotic resistance

DISCUSSION

Bacterial biofilms play an important role in UTIs as they are responsible for persistent infections, recurrences and relapses[16]. Recent evidence indicates that probiotics have opened a new horizon to fight with infectious biofilms. Since probiotics cannot induce the strong selective pressure on resistant isolates as conventional antibiotics and also they are less cytotoxic, they can be considered as ideal option for biofilm combating[17]. Several lactobacilli strains showed inhibitory activity of UPEC with different antibiotic susceptibilities[18].

The present work aimed to study the biofilm formation by ESBL-producing UPEC isolates using CRA and TCP methods and to investigate the impact of CFS of *L. acidophilus* La-5 and *L. helveticus* B-734 on both prevention and eradication of biofilm formation. The study also attempted to determine the antibiotic resistance pattern among these isolates.

In the present study, biofilm-forming *E. coli* isolates were 9 out of 50 (18%) by CRA method, while they were 14 out of 50 (28%) by TCP method. Noteworthy, *Karigoudar et al.*[19] reported 49%, 69% of UPEC isolates were biofilm producers by CRA and TCP methods, respectively. In a study by *Sevanan et al.*[20] CRA method showed 59.4% of UPEC isolates to be biofilm producer. *Suman et al.*[21] and *Sharma et al.*[22] reported a higher rates of biofilm production of (92%, 67.5%, respectively) among UPEC isolates by TCP method. All of these studies showed higher rates than ours which could be attributed to the involvement of larger sample size of hospitalized patients. Moreover, the low sensitivity of CRA compared to TCP which is a more sensitive method in biofilm detection could be attributed to the requirement of subjective visual interpretation in the former method[23]. Also in the latter method the supplementation of sugar as sucrose in the media was found to be essential for biofilm formation. Sugar stimulates the fermentation reaction, resulting in anaerobic condition that favors the production of adhesins and consequently increasing biofilm production[24].

In the present study, the effect of CFS of *L. acidophilus* La-5 and *L. helveticus* B-734 on biofilm formation by ESBL producing UPEC was assessed. The mean OD after addition of CFS of *L. acidophilus* La-5 and *L. helveticus* B-734 were greatly reduced compared to OD before and this reduction was statistically significant (*P* value 0.001). In the same context, *Allam*[25] observed a statistically significant reduction (*P* value 0.0001) of OD of biofilm formed by UPEC after addition of CFS of *L. acidophilus* DSMZ 20079T. Similarly, though on a different biofilm-forming organism; CFS of *L. helveticus* 27058 showed significant inhibition (*P* value < 0.01) of biofilm production of *S. aureus* compared to OD before[26]. On the other hand, CFS of a different *Lactobacillus* spp. (*L. paracasei*) had a negative inhibitory activity against biofilm of UPEC strains[27]. The anti-biofilm ability of *Lactobacillus* supernatants could be related to production of inhibitory compounds such as surfactants which affect the expression of biofilm-related genes (*cidA, sarA, icaA, dltB, sortaseA, and argA)*, bacteriocins which induces pore-formation on the bacterial cell surface leading to ATP efflux, exopolysaccharides (EPS), organic acids, lactic acid, fatty acids, enzymes (amylase, lipase) and hydrogen peroxide[17].

The present study revealed that regarding co-culture method; the efficacy of CFS of *L. acidophilus* L-a5 and *L. helveticus* B-734 ranged from 76.44% to 91.12%. This is in accordance with *Abdelhamid et al.*[28] who reported that CFS of *L. acidophilus* and *L. helveticus* inhibited the biofilm formation of UPEC by 70-80%. However, lower inhibition rates were reported by *Li et al.*[29] who stated that CFS of *L. helveticus* MB2-1 significantly inhibited 33.5% of biofilm formation by UPEC.

In the current study, comparing the effects of CFS of *L. acidophilus* L-a5 and *L. helveticus* B-734 on biofilm formation by co-culture method, CFS of *L. acidophilus* L-a5 was more effective (efficacy 91.12%) than CFS of *L. helveticus* B-734 (efficacy 76.44%). Similarly, *Abdelhamid et al.*[28]and *Satpute et al.*[30] have shown that CFS of *L. acidophilus* inhibited the biofilm formation of UPEC by 80% and 90% whereas the inhibition was 70% and 34% when UPEC isolates were exposed to CFS of *L. helveticus*, respectively. The difference in efficacy between different *Lactobacillus* strains on biofilm of UPEC could be explained by the fact that *L. acidophilus* is higher producer of hydrogen peroxide, biosurfactant, and high levels of acids especially acetic acid than *L. helveticus*, all are secreted in supernatant and have antibiofilm activity[31].

In post-incubation method; the efficacy of CFS of *L. acidophilus* La-5 and CFS of *L. helveticus* B-734 to eradicate the already formed biofilm of UPEC was

almost the same (efficacy almost 83%). Similarly, *Abdelhamid et al.*[28] found that CFS of *L. acidophilus* and *L. helveticus* were able to eradicate the already formed biofilm of UPEC by almost 60%. While studies by *Allam*[25] and *Zamani et al.*[32] observed that the reduction ability of a different *Lactobacillus* spp. *(L. plantarum)* on already formed biofilms of UPEC were 37% and 60%, respectively. All of these studies showed lower rates than ours.

Our results showed that the efficacy of CFS of *Lactobacillus* by co-culture method (efficacy reached 91.12%) was greater than the post incubation method (efficacy almost 83%). It seems to be clear that prevention of biofilm formation is easier than eradication of the already formed biofilm. It could be attributed to the increased expression of resistance genes such as quorum sensing (QS) genes and multidrug efflux genes within the biofilm as well as the diffusion limitations of the extracellular polymeric substances that have made biofilm bacteria resistant to eradication[22].

ESBL-producing UPEC isolates show resistance to different antibiotics as penicillins, first, second, third, fourth generations cephalosporins, monobactams, quinolones, cotrimoxazole and aminoglycosides[33]. In our study, 62% of ESBL-producing UPEC isolates were resistant to ciprofloxacin, while 44% were resistant to cefepime by standard disc diffusion method. *Makled et al.*[34] reported similar resistance rates to ciprofloxacin (60%) but lower to cefepime (20%). Whereas in studies conducted by *Ankur et al.*[35] and *Zhao et al.*[36]on clinical isolates of ESBL-producing UPEC, higher resistance rates to ciprofloxacin (93.8%, 94%, respectively) and slightly lower to cefepime (35.55%, 33%, respectively) were shown.

In the present study, the antibiotic resistance among biofilm producing *UPEC* was found to be higher than that among non-biofilm producers; however, this difference was statistically insignificant (*P* value > 0.05). This is found to be in agreement with studies conducted by *Poovendran et al.*[37] and *Karigoudar et al.*[19], which revealed that resistant isolates were comparatively higher (64%, 72%, respectively) among biofilm producers than non-biofilm producers (36%, 28%, respectively) and there was a significant correlation between biofilm production and resistance to multiple antibiotics (*P* value < 0.05). Similarly, *Sharma et al.*[22] observed that biofilm producers were more resistant to antibiotics (70%) than non-biofilm producers which was statistically significant (*P* value < 0.05). This supports that biofilm adds to the virulence profile of microorganism[21]. It could be explained by the presence of exopolysaccharides as the main components in creating the diffusion barrier for the antibiotics in addition to activation of QS genes and multidrug efflux pumps[38].

CONCLUSIONS

CFS of *L. acidophilus* and *L. helveticus* may have a role in prevention and eradication of UPEC biofilm and can be used as a future alternative preventive or therapeutic method. However, further studies on larger sample size and wider scale including catheterized patients are recommended to assess this role. Moreover, studies of different species of lactobacilli to test their effect against biofilm formation by UPEC are recommended.

Conflicts of interest:
The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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