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ORIGINAL ARTICLE

The Role of *Lactobacillus* against *Pseudomonas aeruginosa* infection caused by urethral catheter in Assiut Urology Hospital

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ABSTRACT

Key words:
Lactobacillus acidophilus, Pseudomonas aeruginosa, Biofilm, resistance of Antibiotic

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Background: *The emergence of antibiotic-resistant Pseudomonas aeruginosa has renewed efforts to identify safe and natural substituent agents such as probiotics.* ***Objective:*** *In the present study, we evaluated the role of the Lactobacillus acidophilus isolated from commercial capsules (Puritan Pride, USA) against Pseudomonas aeruginosa and its biofilm isolated from long-term urethral catheter.* ***Methodology:*** *Samples were collected from 120 patients admitted to Assuit Urology Hospital. Bacterial identification was done by cultivation on selective media and by biochemical tests. Antibiotic susceptibility Testing was performed. Biofilm assay was conducted by two methods: Congo red method and Microtiter plate assay. L. acidophilus was isolated and incubated anaerobically in MRS broth at 37ºC for 24h, then centrifugation for 15 minutes at 10000 rpm. The inhibition effect of probiotic on P. aeruginosa strains was carried out by two methods: Agar well diffusion and Microtiter plate assay.* ***Results:*** *Only 37 out of 120 isolates were identified as P. aeruginosa. Antimicrobial susceptibility tests included 15 antibiotics; P. aeruginosa isolates showed multi-drug resistance for antibiotics. According to Microtiter Plate assay, 100% of isolates were biofilm forming. Antimicrobial effect of L. acidophilus using Agar well Diffusion Method showed 81% inhibition rate. The highest zone of inhibition by L. acidophilus was 25 mm. By Microtiter plate assay, Lactobacillus acidophilus was able to inhibit 91.8% of biofilm formed.* ***Conclusions:*** *Although biofilm produced by P. aeruginosa is hardly killed by various antibiotics, L. acidophilus isolated from commercial capsules has shown anti-biofilm activity that can be used as antimicrobial agent after appropriate in vivo testing.*

INTRODUCTION

The urinary system is one of the primary routes via which the human body excretes liquid waste. Using urinary catheter becomes necessary when issues emerge in the lower urinary tract [1]. However, these devices are notoriously prone to Bacterial infection [1]. Catheter-associated urinary tract infection (CAUTI) is one of the most frequent care-associated diseases worldwide, accounting for over 80% of all nosocomial UTIs, with all patients infected by Day 30[2]. Uropathogenic gram negative bacteria such as *Pseudomonas aeruginosa* colonize catheter, and develop biofilm causing nosocomial infections. In comparison to bacteria in planktonic conditions, microorganisms in biofilms have 1000 times higher resistance to antibacterial agents. [3]. Furthermore, overuse of antimicrobials may disrupt the balance of the bladder's naturally present microflora, contributing to pathogenesis. Multi-drug resistant bacteria are the root cause of a wide range of clinical issues around the world [4]. Infectious diseases caused by resistant microorganisms are responsible for rising health-care costs as well as high morbidity and mortality, particularly in developing countries. Antibiotic resistance stimulates wide efforts for finding safe and natural antibiotic alternative agents such as probiotics [5]. Recently, the use of probiotics as safe, and natural live microorganisms against antibiotic-resistant has been reconsidered as an alternative to antibiotics. Probiotics are microorganisms that have a beneficial effect on the host's health, and some of them have antibacterial properties[6]. Probiotics such as *L. acidophilus* can act as microbial barriers against pathogens [7].

METHODOLOGY

Ethical Statement and Study Setting:

Specimens were collected from 120 Adults preoperative, and postoperative patients, female, and male, with long-term standing urethral catheter, more than one week, in Assuit Urology Hospital, Assuit, Egypt, starting from october,2020 until May,2021. An informed written consent was obtained from each patient, and the research was approved by the ethical committee in the Faculty of Medicine, Assuit university. The samples were transferred to the laboratory, and worked upon immediately.

All of the participants were subjected to the following

- Full history.
- Administration of antibiotics or not
- The duration and cause of catheter insertion
- Patient smoker or not
- Demographic data: Age, Name, Sex, Location, Phone number.
- Creatinine and Hemoglobin level in the blood
- The regular exchange of catheter in patients
- Presence of ureteral stent

Identification of *Pseudomonas aeruginosa* isolated from Urethral Catheter:

After the catheter was removed, it was cut and rinsed in phosphate buffered saline before being cultured in Brain Heart Infusion broth[3]. Identification was made by cultivation in different media, morphological characteristics, microscopic examination, and biochemical tests[8,9].

Antimicrobial susceptibility test:

Antibiotic sensitivity of isolates was determined using the Kirby-Bauer disc diffusion method on Muller Hinton agar (*HiMedia*™)[10] *P. aeruginosa* was classified as resistant or sensitive based on the zone of inhibition [11]

Detection of biofilm for *P aeruginosa by* Congo red agar method:

This method is a Qualitative assay for detecting biofilm produced by microorganisms as a result of color change in colonies inoculated on CRA medium [12].

Detection of the action of *L. acidophilus* on *P aeruginosa* by Agar Well diffusion method:

L. acidophilus capsules were suspended in De Man, Rogosa and Sharpe broth (*HiMedia*™). Strains of *Pseudomonas* were cultivated in Muller Hinton Agar. With a sterile metal cylinder, wells 6 mm in diameter were cut into agar plates, and 50µl of cell free supernatant was placed into each well then incubated before antibacterial activity. The clear inhibition area of 3 mm diameter was reported to be positive[13].The supernatants were then neutralized (pH = 7) and the experiment was repeated to know whether the inhibitory effect of the culture supernatants was solely due to their acidic pH or due to other mechanisms[7]. Control for each zone was made using un-inoculated sterile MRS broth as negative control and acetic acid (33%) as positive control [14]. All tests were carried out in triplicate under same conditions.

Detection of biofilm formation by *P aeruginosa* and the action of *L. acidophilus* on *P aeruginosa* biofilm

Microtiter plate method:

Firstly, *P aeruginosa* was cultured in Brain Heart Infusion Broth supplemented with 1% glucose. Culture supernatants were diluted and inoculated with sterile brain heart infusion broth (as controls) into sterile 96 wells of Microtiter plate, covered and incubated. After incubation, each well's bacterial culture broth was removed. Each well was washed three times with sterile PBS to remove bacteria that were not adhered to the wells and shaken. Biofilms adhering to the walls of the wells were fixed with 250µl 96% ethanol per well for 15 minutes, then removed and the plate was left to dry for 15 minutes. Each well was stained with 0.2ml of 0.1% crystal violet for 15 minutes at room temperature, and excess removed by washing three times with distilled water. After air drying, the dye bound to the cells was re-solubilized with 200µl of 33 % acetic acid in water per well, incubated for 15 minutes, and measuring absorbance at 600nm using an ELISA Reader [7]. All tests were performed triplicate.

Secondly, to know the role of *Lactobacillus acidophilus* in biofilm production [7]. *P. aeruginosa* strains were grown as before, then inoculated 100µl per well.

For test wells: 100µl of 0.5 McFarland's standard free cell supernatant of *L. acidophilus* in MRS broth was added to the *P. aeruginosa strains*.

For control wells:

For negative controls of the P. aeruginosa: 200µl of un-inoculated BHIB with 1% glucose.

For negative control for mixed solutions of P. aeruginosa and L. acidophilus strains: 100µl of BHIB with 1% glucose + 100µl of un-inoculated MRS medium supplemented with 0.2% sucrose.

For negative controls of the L. acidophilus: 100µl of un-inoculated MRS medium supplemented with 0.2% sucrose.

***For positive control for biofilm growth*:** 200µl of diluted *P. aeruginosa.*

After incubation, contents of the wells were decanted, washed three times with distilled water, and dried. Then dyed with crystal violet. The dye attached to the cells was re-solubilized using 200µl of 30% acetic acid in water per well after air drying[7]. Using an ELISA reader, the optical density (OD) of each well was measured at 630 nm. Each test was performed triplicate. The cutoff value (ODc) can be used to classify isolates as biofilm producers or non-producers. (ODc) is three standard

deviations (SD) higher than the negative control's mean OD[7]. To know *P aeruginosa* were forming biofilm or not, we used the calculation of cut off value (ODC) according to Stepanović [15]:

Table 1: Shows the Mean OD Value and Biofilm Formation:

Mean OD value	Biofilm Formation
OD ≤ ODc	No biofilm production
OD < OD ≤ 2 * ODc	Weak biofilm production
2 * ODc <OD ≤ 4* ODc	Moderate biofilm production
4 * ODc < OD	Strong biofilm production

Statistical Analysis:

All experiments were carried out in triplicate. The results were expressed by standard deviation (SD). We used a negative and a positive control for each experiment. IBM SPSS statistic software (SPSS Inc. No. 22) was used for statistical analyses, level of confidence 95% and α= *5%. We compared means of paired samples (p-value<0.05).*

RESULTS

Clinical Isolates:

Out of 120 patients, 100% were positive for bacteria. (45%) of catheter in patients were regularly changed, and (55%) weren't. According to the demographic data, gender for all bacterial isolates were 81(67.5%), and 39(32.5%) males and females, respectively. Regarding to *P. aeruginosa*, 26(70.2%), and 11(29.7%) were male and female, respectively. Age ranges were from 20 to 90 years old. Out of 120 isolates, a total of 37(30.8%) *P aeruginosa* isolates were obtained, (table 2, and figure 1). To confirm samples of *P aeruginosa,* we used a control of *P aeruginosa* in its identification. Out of 37 *P. aeruginosa:* 19 (51.3%) were administered antibiotics and 18 (48.6%) weren't. Among patients, Creatinine level in blood ranges were from 0.64-2.9mg\dl, and hemoglobin level was from 9.1-15g\dl. Also, 30% of patients had ureteral stent, and 70% had not, which also cause bacterial infection.

Table 2: The effect of P. aeruginosa on different media

Culture media	Characteristic
Blood agar	White to gray color colonies, and presented β-hemolysis.
MacConkey agar	Colonies were small pale pink, and non-lactose fermenting.
Cetrimide agar	Colonies mucoid ,fruity odour, fluorescentgreen,andcreamy pigments.
Nutrient agar	greenish coloration, and bluish-green coloration

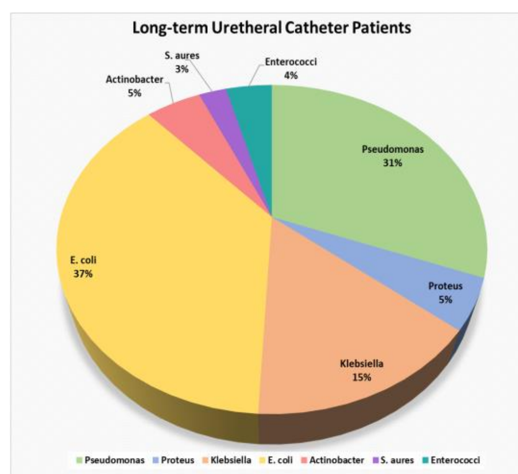

Fig. 1: Shows Different Bacteria isolated from urethral catheter

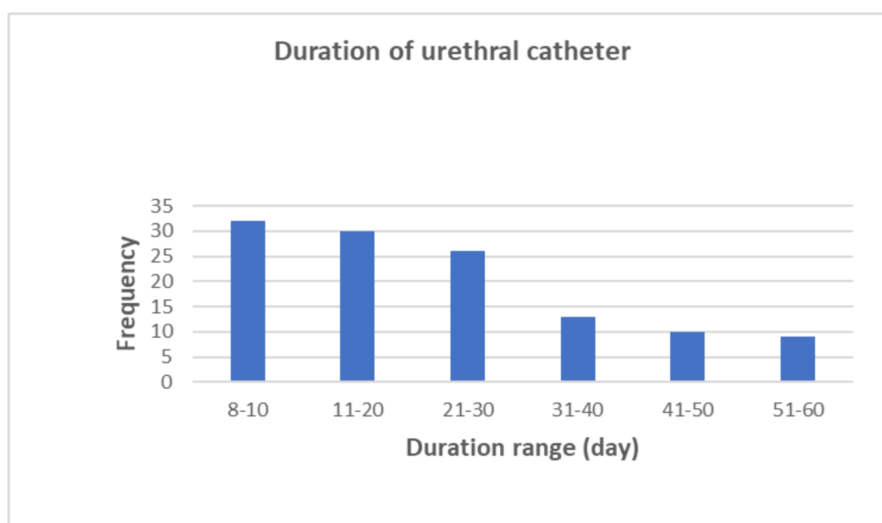

Fig. 2: Shows Duration range of Urethral catheter in 120 clinical isolates.

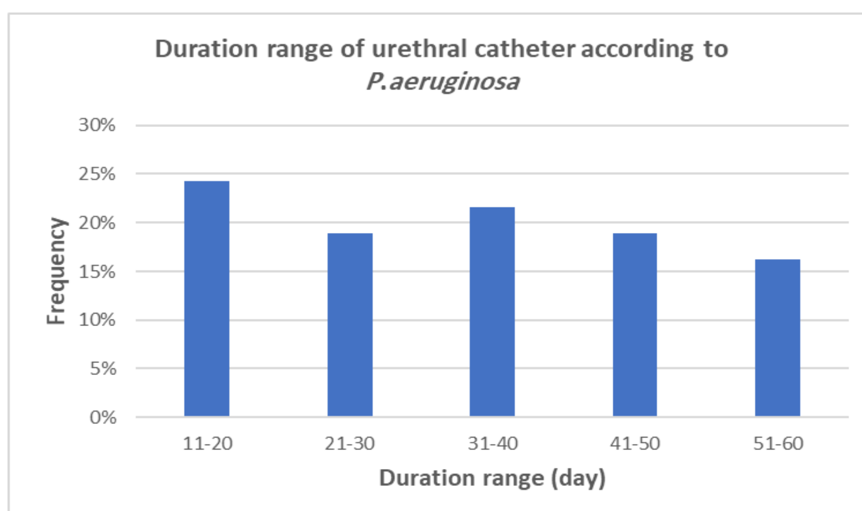

Fig. 3: Shows Duration range of urethral catheter according to P. aeruginosa isolates.

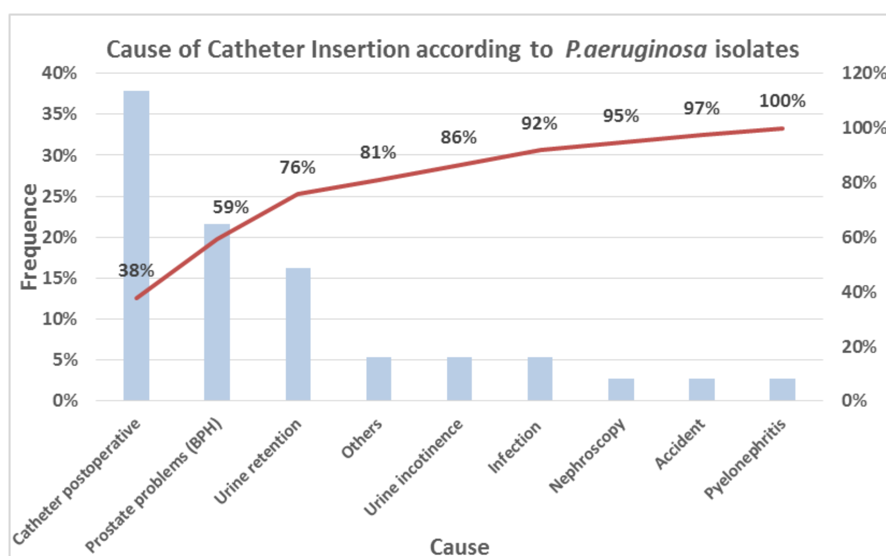

Fig. 4: Shows that about 80% of UTIs in patients belongs to about 20% of the causes observed. Other causes including use of catheter in the delivery of medicine directly into the bladder, and in determining the etiology of various genitourinary conditions in women.

Antibiotics susceptibility test*:*

A total 37 *P aeruginosa* isolates were exposed to different antibiotics. All isolates of *P aeruginosa* were fully resistance (100%) to Nalidixic acid. Additionally, a total 94.5%, 94.5%, 94.5%, 92%, 91.8%, 89% and 86.7% of the isolates exhibited resistance to Ceftriaxone, Cefixime, Amikacin, Ampicillin, Kanamycin, Co-trimoxazole, Tetracycline, respectively. The isolates of P aeruginosa demonstrated different degree of resistance to each antibiotic and the percentage of resistant with piperacillin/tazobactam (72.9%), Meropenem (73%), Gentamicin (81%), Aztreonam (75.6%), streptomycin (78.13%), Imipenem (78.3%), Chloramphenicol (78.3%), summarized in (Figure 5, and 6).

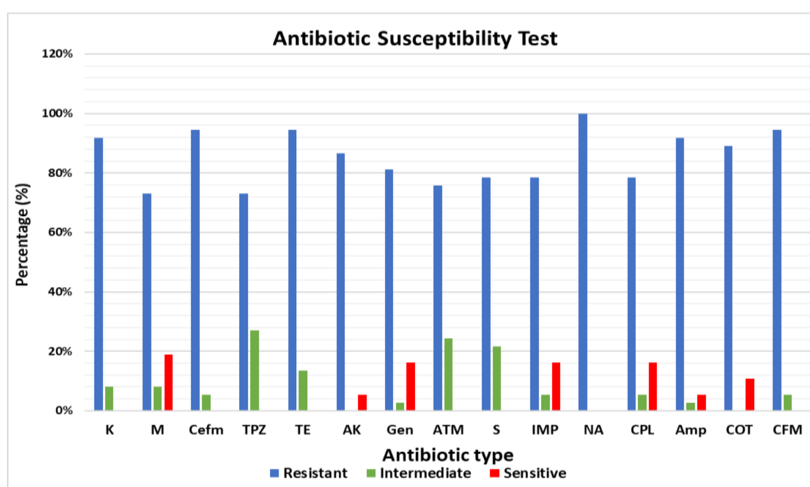

Fig. 5: Shows Antibiogram of P. aeruginosa detected in the studied clinical samples.

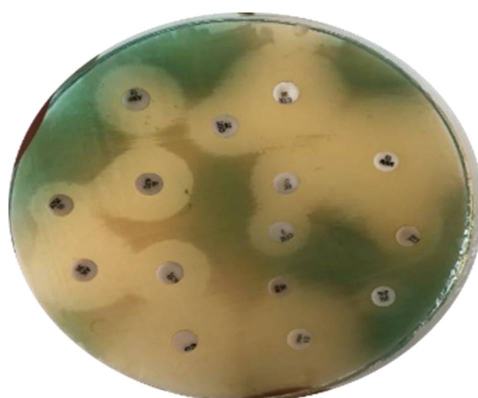

Fig. 6: Showing Pyoverdine pigments and multidrug resistance to Antibiotics

Detection of biofilm production:

Through Microtiter Plate Assay, the results showed that 100% of *P aeruginosa* isolates are biofilm producer, 4(10.8%) isolates of *P aeruginosa* are weak biofilm producer, 5(13.5%) isolates are moderate biofilm producer and 28(75.6%) isolates are strong biofilm producer (Figure 7,8, and table 3). By Congo Red, the results revealed that 32 (86%) isolates of *P aeruginosa* are biofilm producers and 5 (14%) are not producers (figure 9).

Fig. 7: Microtiter plate before crystal violet staining.

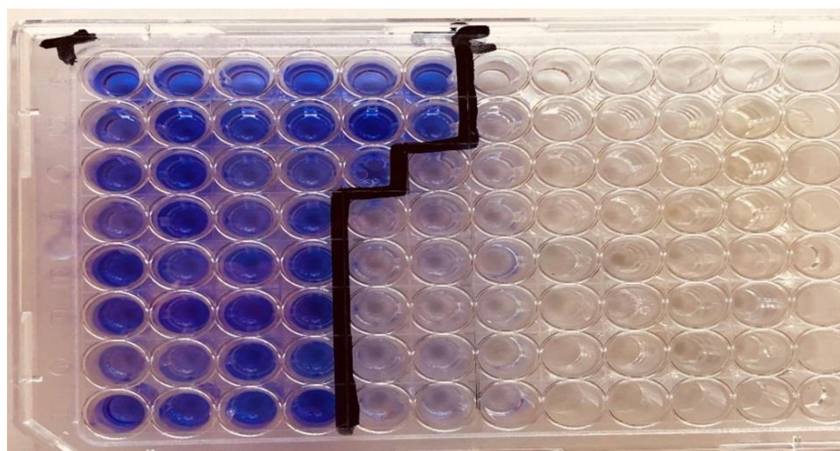

Fig. 8: After Incubation and staining. T: for tested *P. aeruginosa* isolates and C: for control wells (BHIB only).

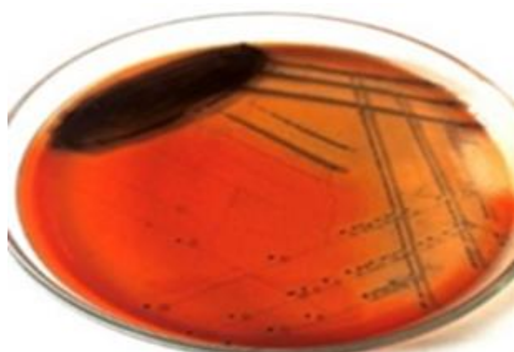

Fig. 9: Showing Black colonies in CRA indicate biofilm production.

Table 3 Optical Density of Biofilm forming wells and Control Wells:

OD of Biofilm forming wells	OD of Control wells
Mean ± SD	Mean ± SD
0.7 ± 0.197	0.1 ± 0.018

Activity of *Lactobacillus acidophilus* against *P aeruginosa*:

Antimicrobial effect of *L. acidophilus* against *P. aeruginosa* using agar well diffusion was selected due to its ability to produce biofilm, and summarized in (table 4, and figure 10).

Table 4: Antimicrobial effect of *L. acidophilus* using Agar Well Diffusion Method:

No. of *P. aeruginosa* isolates	Activity of *L. acidophilus*			
	Inhibition zone (11-25mm)		No inhibition zone	
	No.	%	No.	%
37	30	81	7	19

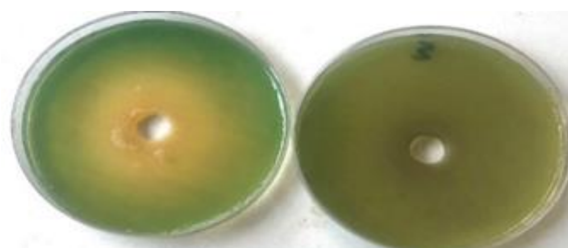

Fig. 10: Plates Show green-yellow pigment of P. aeruginosa. The first plate shows 25mm inhibition zone and the second plate shows no inhibition zone

Activity of *Lactobacillus acidophilus* against *P aeruginosa* and its biofilm:

Cell-free supernatants of *L. acidophilus* were mixed in wells of Microtiter plate with the suspensions of the 37 biofilm-forming *P. aeruginosa* isolates, 91.8% inhibition of biofilm occurred (table 5, and figure 11 and 12). The results were statistically significant (P value < 0.05).

Table 5: Shows OD before and after L. acidophilus:

OD before adding *L. acidophilus* (Pre-Treat)	**OD after adding *L. acidophilus*(post-Treat)**	**Standard error mean**	**% Of Reduction of Biofilm**	**Paired t-test**
Mean ± SD	Mean ± SD	Pre-treat: 0.324		T: 6.975
0.701 ± 0.197	0.46 ± 0.07	Post-treat: 0.11	91.8%	P-value [significance (2-tailed)] $P < 0.05$

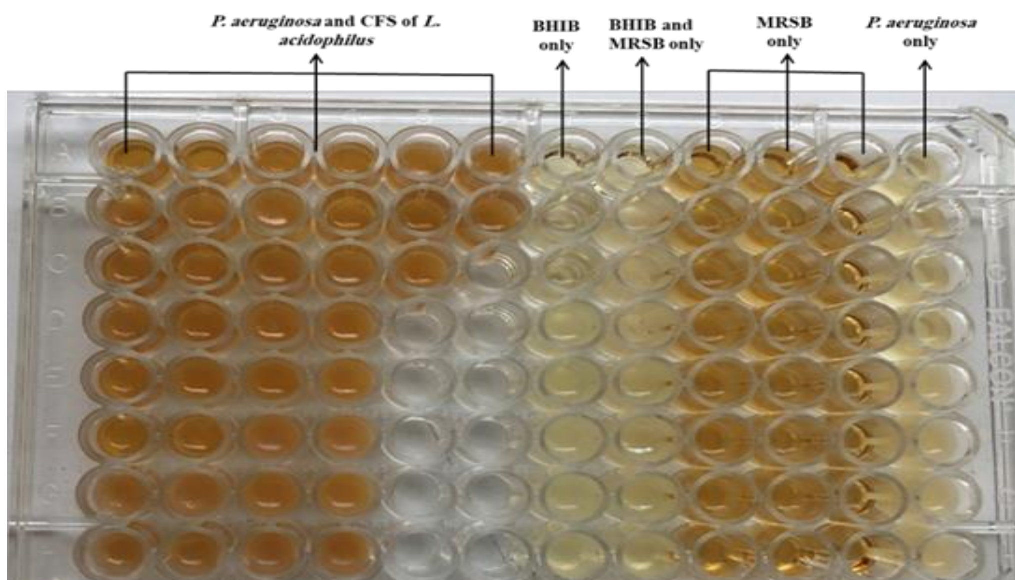

Fig. 11: Microtiter plate before staining with crystal violet.

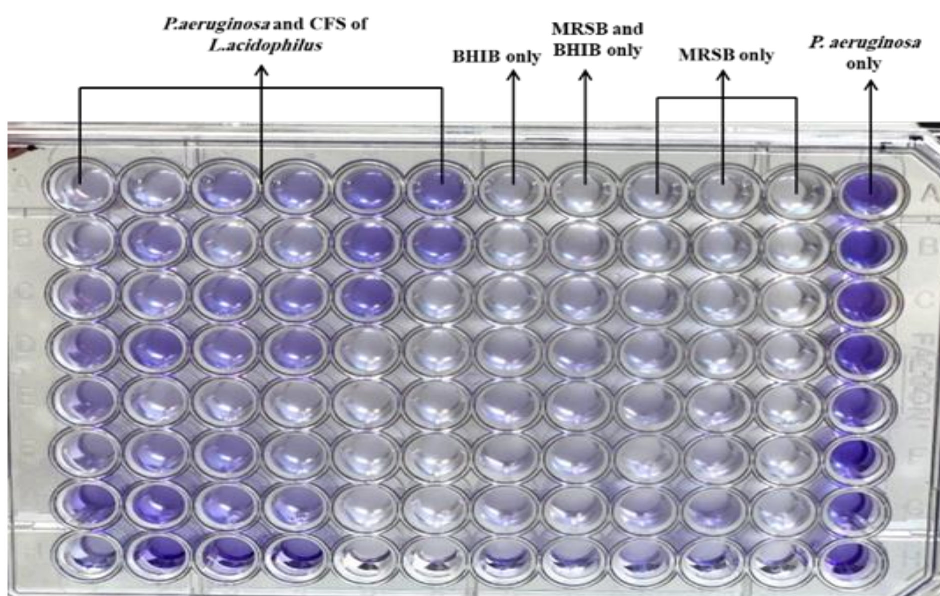

Fig. 12: Microtiter plate after staining with crystal violet showing the role of L. acidophilus against biofilm production of different P. aeruginosa isolates upon incubation.

DISCUSSION

Uropathogenic *P. aeruginosa* is a gram-negative bacterium which is responsible for a high percent of nosocomial infection worldwide. The increasing incidence of disease caused by *P. aeruginosa*, the associated costs, and the burgeoning problems associated with the emergence and spread of multidrug-resistant strains of *P. aeruginosa* indicate that an effective strategy against it is urgently needed[16]. In the present study, out of 120 isolates, 100% were positive for a certain bacterium [17], which indicates that the longer catheter is inserted the more susceptible the patient to get bacterial infection.

By MTP method for Biofilm detection, 100% of isolates of *P. aeruginosa* were biofilm producer, 4(10.8%) *P aeruginosa* isolates produced weak biofilms, 5(13.5%) isolate produced moderate biofilms and 28(75.6%) produced strong biofilms. Also, Forbes *et al.*[8] stated 42 (84%) were strong biofilm producers, 4(8%) isolates were moderate biofilm producer and other 4(8%) isolates produced weak biofilm. The results were also similar to Shokri *et al.*[7]who showed 95% were biofilm producers. In CRA method for detection of biofilm formation, the results observed that 32 (86%) isolates of *P aeruginosa* were biofilm producers and 5 (14%) isolates were not, which is similar to that of with Rewatkar *et al.* [18], who found 27(90%) isolates were biofilm producers using CRA.

The most sensitive, precise, and reliable screening approach for detecting biofilm formation was the microtiter plate method[19]. Biofilm production has been found to be a key determinant of pathogenicity in *P. aeruginosa* infections. Biofilm formation facilitates chronic bacterial infections and reduces the impact of antimicrobial therapy [20].

In the present study, resistance of *P. aeruginosa* strains to Kanamycin, and Tetracycline were 91.8%, and 94.5%, respectively, similar to results of Wahaab *et al.*[21] study, who recorded resistance to those antibiotics were 100%. Resistance of *P. aeruginosa* strains to Aztreonam, Meropenem, Cefepime, Piperacillin/Tazobactam, Gentamicin, and Imipenem were 75.6%, 73%, 94.5%, 72.9%, 81%, and 78.3%, respectively, similar to results of Shokri *et al.* [7] study, who recorded resistance to those antibiotics were 64.5%, 85.4%, 85.4%, 74%, 79.2%, and 84.4%, respectively. Regarding Amikacin, 86.7% of the strains were resistant, in accordance with Dund *et al.* [22] study which reported 85.13% of strains were resistant. Regarding Streptomycin, the resistance was recorded among 78.13% of the strains, similar to results of Al Saimary *et al.*[23] who reported 85% of strains were resistant. Conversely, Chandra *et al.* [24] recorded that 40.7% strains were resistant to Streptomycin.

Moreover, 100% of *P. aeruginosa* strains were resistant to Nalidixic acid, similar to the results of Al Samarrae *et al.* [25]. Regarding Chloramphenicol, the resistance was 78.3%, in accordance with Imanah *et al.*[26] ,and Mohammadpour *et al.* [27] who recorded resistance to Chloramphenicol were 80%, and 97%, respectively. Regarding Ampicillin, the resistance was recorded among 92% of the strains, similar to results of Rewatkar *et al.* [18] and Chandra *et al.*[24] that recorded resistance to Ampicillin by 90% and 100%, respectively, and different from Al Saimary *et al.*, and Kumar *et. al*[23,28] , who recorded resistance to Ampicillin by 65% and 55%, respectively. Furthermore, *P. aeruginosa* strains showed 89% resistance for Co-trimoxazole, similar to results of Sharma et al. [29]that recorded resistance by 86.25%, and different from Thomas *et al*.[30] who recorded 43.2%.

The misuse of antibiotics by health care professionals or non-skilled practitioner and by the public lead to decreasing rate of susceptibility of *P. aeruginosa* to many broad-spectrum antibiotics. It was observed that 52% of patients were administered antibiotic in hospital and 48% were not, which suggests that the high resistance pattern is probably due to weakly guided antibiotic prophylaxis and empiric therapy, and high bacterial rate is probably improper administration to antibiotics, according to [22]. With the fast-developing potential of lactic acid bacteria as bio-control agents against diseases, new prospects as alternatives to antibiotics are already emerging [31].

In the present study, we used *L. acidophilus*, and its antibacterial and anti-biofilm effects were evaluated against *P. aeruginosa* by Agar well diffusion method, and Microtiter plate assay. According to Agar well diffusion method, it was shown that 81% of strains showed inhibition zone between 11-25mm, and 19% did not show inhibition. The results were compatible with study conducted by Al Malkey *et al.* [14], which stated that the inhibition zone by cell free supernatant was between 15-25mm. Shokri *et al.*[7] study recorded similar results which stated 88.6 % of strains were inhibited. We found no inhibitory activity against *P. aeruginosa* strains after neutralizing the acidic pH of the Lactobacillus supernatant. According to Shokri *et al.* [7], this could be attributed to the production of antibacterial organic acid molecules such as lactic, acetic, and formic acid, as well as bacteriocins that were only active in acidic conditions. By Microtiter plate Assay, *L. acidophilus* was able to remove 91.8% of biofilm formed by *P. aeruginosa* strains. This was highly statistically significant (p-value <0.05) to the results which were recorded by a study conducted by Shokri *et al.* [7] that stated CFS of *L. acidophilus* where able to remove 100% of biofilms formed by *P. aeruginosa* strains (P-value < 0.01).

CONCLUSIONS

Our research study aimed at developing a new antimicrobial agent from lactic acid bacteria that is less expensive, uses renewable substrates, produces higher yields, and can be used as bacteriostatic or bactericidal agents. We discovered that including probiotic bacteria into treatment techniques can be extremely beneficial in preventing and/or treating hospital-acquired illnesses. Although in vitro and in vivo safety studies of chosen *Lactobacilli* are required, their extensive biofilm inhibition/removal activity, in addition to their broad antibacterial impact, suggests that they could be used as a biocontrol agent for antibiotic-resistant *P. aeruginosa* strains. We can conclude that although *P. aeruginosa* biofilm is hardly killed by various antibiotics, *L. acidophilus* isolated from commercial capsules has an anti-biofilm activity that can be used as a therapeutic agent after appropriate in vivo testing.

This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

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