Novel Research in Microbiology Journal (2019), 3(4): 428-439
Research Article

(Print) (ISSN 2537-0286)
(Online) (ISSN 2537-0294)
https://nrmj.journals.ekb.eg/
DOI: 10.21608/nrmj.2019.44951

The effect of *Lactobacillus acidophilus* as a probiotic against *Pseudomonas aeruginosa* growth and biofilm formation

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Received: 20 July, 2019; Accepted: 12 August, 2019; Published online: 17 August, 2019

Abstract

The emergence of antibiotic-resistant biofilm producing microorganisms such as *Pseudomonas aeruginosa* has pushed efforts to find safe alternatives to antibiotics; such as probiotics. Lactobacilli are one of these promising probiotics, with reported antibacterial and anti-biofilm activity against many different pathogenic microorganisms. This study aimed to study the potential antibacterial and anti-biofilm effect of *Lactobacillus acidophilus* ATCC 4356, against the growth and biofilm formation of pathogenic *P. aeruginosa*. Cell free supernatant of *L. acidophilus* was tested to inhibit the growth; biofilm formation, and on preformed biofilms by 35 different clinical strains of *P. aeruginosa*, using agar well diffusion and microtitre plate assays. *L. acidophilus* ATCC 4356 recorded powerful growth inhibition against 88.6% of the *P. aeruginosa* strains. Moreover; it significantly inhibited biofilm formation of the strains by 68.52%, and removed already preformed biofilms with 43.8 % activity. Finally; *L. acidophilus* showed a potent inhibitory potential against the growth and biofilm formation by *P. aeruginosa* strains, thus could be used as a powerful probiotic for the bio-control of infections caused by antibiotic resistant and biofilm producing *P. aeruginosa*.

Keywords: *Lactobacillus acidophilus*, Probiotics, *Pseudomonas aeruginosa*, Biofilm

1. Introduction

Bacterial biofilms are complex adherent structured communities of bacterial cells enclosed in a self-produced polymer matrix, which is attached to biotic and/or abiotic surface(s). Bacteria within biofilms differ from their planktonic counterparts; as they resist the host immune responses, and become much less susceptible to disinfectants and antibiotics. That's why diseases associated with biofilms require great efforts for their prevention, diagnosis and treatments (Tremblay *et al*., 2014).

P. aeruginosa is responsible for many worldwide life-threatening diseases especially in

immunocompromised patients. Ha and O'Toole, (2015) reported that its ability to form biofilms; resist wide range of antibiotics together with many other virulence factors, made its clearance by immune system and antibiotic treatments extremely difficult.

New strategies are arising for combating these antibiotic resistant bacteria by using probiotics. Fijan, (2014) defined probiotics as live nonpathogenic microorganisms; that when administered into human body in suitable amounts, confer beneficial health effects. Lactobacilli are one of the safe and natural living bacteria that can be used to antagonize other bacteria as described by Florou-Paneri *et al*., (2013). *L. acidophilus* can act as a microbial barrier against several pathogens through competition for binding sites; enhancement of the host's immune response, and production of antimicrobial substances including; acids, bacteriocins and bacteriocin-like compounds (Shokri *et al*., 2018). The objectives of the current study were to investigate the antibacterial and anti-biofilm potency of *L. acidophilus* ATCC 4356 strain, against different clinical strains of *P. aeruginosa*.

2. Material and methods

2.1. Isolation of *P. aeruginosa* strains, their identification and antibiotic susceptibility assay

Thirty-five clinical isolates of *P. aeruginosa* were isolated from urine samples of patients admitted to intensive care units of Ain Shams University hospitals; Cairo, Egypt, and were suffering from attacks of urinary tract infection (UTI), starting from May, 2018 for a period of 12 months. An informed consent was obtained from each patient or patient's next of kin, and the research was approved by the Faculty of Medicine, Ain Shams University Ethical Committee.

Identification of the isolated *P. aeruginosa* was done according to Collee *et al*., (1996); Cheesbrough, (2006) based on colonies morphology; microscopic examination of Gram stained films, and biochemical assays of the isolated strains. Antibiotic susceptibility testing was done according to the Clinical and Laboratory Standards Institute. (2018), to insure that these isolates belonged to different strains.

2.2. Detection of antibacterial potential of *L. acidophilus* on the growth of *P. aeruginosa* strains

Assessment of antibacterial activity of *L. acidophilus* ATCC 4356 (KWIK-STIK™, France) on the growth of the isolated *P. aeruginosa* strains was carried out using agar well diffusion assay, according to Jamalifar *et al*., (2011); Shokri *et al*., (2018). Briefly, *L. acidophilus* was cultivated in De Man, Rogosa and Sharpe (MRS) broth medium (Merck Company, Germany) for 24 h at 37°C. Cell-free supernatant was obtained by centrifugation of the broth culture at 5000g for 20 min., and then filter sterilized using 0.20 µm Millipore filter (Nalgene Thermo® scientific syringe filter). Muller Hinton agar (MHA) (Oxoid, England) plates were inoculated with cultures of the *P. aeruginosa* strains in substitution to MRS agar plates in reference to Shokri *et al*., (2018); as when MRS plates were used, no growth of any of our *P. aeruginosa* strains was detected. A well (3 mm in diameter) was cut in the middle of each agar plate using sterile cork borer, and then 50 µl of the *L. acidophilus* cell free supernatant was pipetted into each well. Plates were then incubated for 24 h at 37°C. The antibacterial activity was recorded by measuring the diameter of the inhibition zone around each well. A minimum diameter of inhibition zone about 3 mm was considered positive. Three plates were used for each *P. aeruginosa* strain, and the assay was repeated three times. To ensure that the inhibitory potential of the supernatant was not due to its acidic pH; it was neutralized to (pH= 7) using NaOH, and the assay was repeated twice.

2.3. Inhibitory potential of *L. acidophilus* on biofilm formation by *P. aeruginosa* strains

The effect of *L. acidophilus* ATCC 4356 on biofilm formation by the isolated *P. aeruginosa* strains was tested using microtiter plate (MTP) assay, as described by Wu *et al*., (2015); Shokri *et al*., (2018). *P. aeruginosa* strains were cultured statically in

Tryptic soy broth (TSB) medium (Oxoid, England), containing 0.25 % glucose for 24 h at 37°C. Culture supernatants were adjusted to obtain optical turbidity comparable to that of the 0.5 McFarland standard (~10^8 cells/ ml) by dilution of TSB, and then inoculated into sterile 96 wells of polystyrene MTP (100 µl per each well). One hundred µl of 0.5 McFarland's standard free cell supernatant of *L. acidophilus* in MRS medium was added onto the tested *P. aeruginosa* strains. For control wells; a)- 200 µl of un-inoculated TSB with 0.25% glucose was used to serve as negative controls of the *P. aeruginosa* suspension (as a spectrophotometric blank), b)- 100 µl of TSB with 0.25% glucose + 100 µl of un-inoculated MRS medium supplemented with 0.2% sucrose, as negative control for mixed solutions of *P. aeruginosa* and *L. acidophilus* strains (to be used as a spectrophotometric blank), c)-100 µl of un-inoculated MRS medium supplemented with 0.2% sucrose, d)- 200 µl of diluted *P. aeruginosa* suspension (as positive control for biofilm growth), were added to separate wells. The inoculated microplates were then incubated at 37°C for 24 h.

After incubation; the contents of the wells were decanted, washed three times with 300 µl of dist. water, and then dried. Wells were then stained with 200 µl of 0.1% crystal violet for 15 min. at room temperature. Excess stain was aspirated, and the plates were washed three times with dist. water. After air drying, the dye bound to the cells was re-solubilized with 200 µl of 30 % acetic acid in water per well. The optical density (OD) of each well was measured at 620 nm using an ELISA reader. Each strain was tested in triplicate. The cut off value (ODc) was detected, defined as three standard deviations (SD) above the mean OD of the negative control. For easier interpretation of the results, strains were divided into the following categories as set by Stepanović *et al*., (2007):

1. No biofilm producer: OD < ODc

2. Weak biofilm producer: ODc < OD ≤ 2 ODc

3. Moderate biofilm producer: 2 ODc < OD ≤ 4ODc

4. Strong biofilm producer: OD> 4 ODc

2.4. Inhibitory potency of *L. acidophilus* on the preformed biofilms of *P. aeruginosa* strains

P. aeruginosa strains were allowed to grow first in wells of MTP for 24 h to form biofilms (100 µl/ well), and then cell free supernatant of *L. acidophilus* was added to these wells (100 µl/ well), and then incubated for additional 24 h at 37°C. Crystal violet assay and OD measurements were used to assess biofilm formation as described by Shokri *et al*., (2018). Each strain was tested in triplicate.

2.5. Statistical analysis

Quantitative data were described by using mean values and standard deviation (SD). A negative and a positive control samples were used for each assay. Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS) version 22. Paired t-test was used to compare between related samples (pre- and post-treatment). P value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Antibiotics susceptibility pattern of the 35 clinically isolated *P. aeruginosa* strains

The *in-vitro* antibiotic susceptibility testing of isolated *P. aeruginosa* strains to different tested antimicrobials is illustrated in (Fig. 1, 2). The most effective antibiotic against these strains was aztreonam as they were all susceptible to it. Thirty-two strains (91.4%) were resistant to cefepime and tobramycin; 30 strains (85.7%) showed resistance to imipenem and piperacillin/ tazobactam; 29 strains (82.9%) presented significant resistance to ciprofloxacin, levofloxacin and meropenem; 27 strains (77.1%) were resistant to ceftazidime; 24 strains (68.6%) demonstrated resistance to amikacin; and 18 strains (51.4%) were resistant to gentamycin.

P. aeruginosa strains isolated from catheterized patients showed increased resistance to imipenem and piperacillin/ tazobactam (P value <0.05), compared to strains isolated from midstream urine samples as clear in (Table 1.).

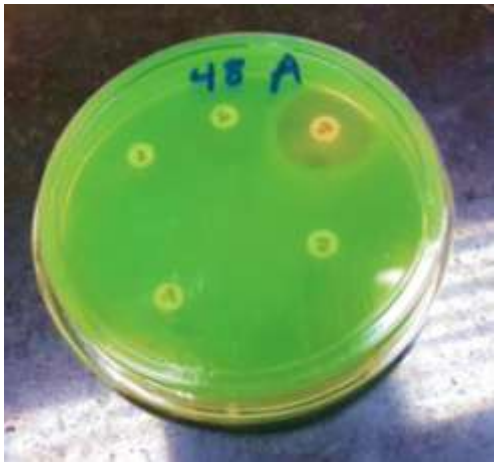


Fig. 1: A Muller-Hinton agar plate seeded with *P. aeruginosa* showing multidrug resistance to Imipenem, Amikacin, Meropenem, Piperacillin/ Tazobactam, but sensitive to Aztreonam antibiotic

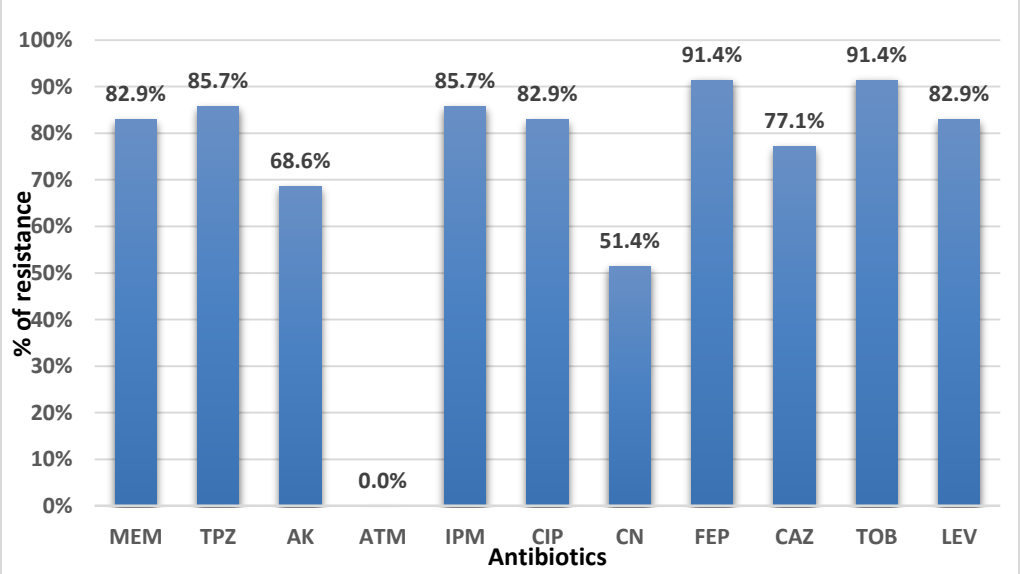


Fig. 2: Percentages of antibiotics resistance pattern of the isolated *P. aeruginosa* strains.

Table 1: Statistical analysis of antibiotic susceptibility of *P. aeruginosa* strains isolated from urinary catheter

Antibiotics		Catheterized				Test value	P-value	Sig.
		Negative		Positive				
		No.	%	No.	%			
LEV	Sensitive	4	30.80%	2	9.10%	2.704	0.1	NS
	Resistant	9	69.20%	20	90.90%			
CIP	Sensitive	4	30.80%	2	9.10%	2.704	0.1	NS
	Resistant	9	69.20%	20	90.90%			
TOB	Sensitive	2	15.40%	1	4.50%	1.225	0.268	NS
	Resistant	11	84.60%	21	95.50%			
CN	Sensitive	8	61.50%	9	40.90%	1.392	0.238	NS
	Resistant	5	38.50%	13	59.10%			
AK	Sensitive	6	46.20%	5	22.70%	2.081	0.149	NS
	Resistant	7	53.80%	17	77.30%			
ATM	Sensitive	13	100.00%	22	100.00%	NA	NA	NA
	Resistant	0	0.00%	0	0.00%			
CAZ	Sensitive	5	38.50%	3	13.60%	2.856	0.091	NS
	Resistant	8	61.50%	19	86.40%			
MEM	Sensitive	4	30.80%	2	9.10%	2.704	0.1	NS
	Resistant	9	69.20%	20	90.90%			
IPM 10 mg	Sensitive	4	30.80%	1	4.50%	4.589	0.032	S
	Resistant	9	69.20%	21	95.50%			
FEP	Sensitive	2	15.40%	1	4.50%	1.225	0.268	NS
	Resistant	11	84.60%	21	95.50%			
TPZ 10 mg	Sensitive	4	30.80%	1	4.50%	4.589	0.032	S
	Resistant	9	69.20%	21	95.50%			

3.2. Antibacterial potential of *L. acidophilus* on the growth of *P. aeruginosa* strains

Upon using MRS medium to test the antibacterial activity of cell free supernatants of the *L. acidophilus* ATCC 4356 against *P. aeruginosa* strains, no growth of these strains were detected at all. However; on using MHA medium in substitution to MRS medium, inhibition zones diameters of about 10-15 mm against 31 (88.6%) *P. aeruginosa* strains were recorded (Fig. 3). After neutralization of the pH of the *L. acidophilus* supernatant, no inhibition zones were detected against all the tested *P. aeruginosa* strains.

3.3. Inhibitory effect of *L. acidophilus* on biofilm formation by *P. aeruginosa* strains

With regard to level of biofilm formation, out of the 35 isolated *P. aeruginosa* strains; 33 (94.3%) were biofilm producers: 12 strains (34.3%) of them were strong biofilm producers, 12 (34.3%) were moderate biofilm producers, and 9 (25.7%) were weak biofilm

producers, while 2 strains (5.7%) did not form biofilm under the described conditions, thus were excluded from further research (Fig. 4).

Mixing the cell-free supernatants of *L. acidophilus* strain in wells of MTP with the suspensions of the 33 biofilm forming *P. aeruginosa* strains led to reduction of the total biofilm mass by 68.52% (Fig. 5a, b), with mean OD reading of 0.72 ± 0.44 as clear in Table 2. These results were statistically significant (P value < 0.001).

Fig. 3: A Muller Hinton agar plate showing inhibition zone of *P. aeruginosa* growth, on adding cell free supernatant of *L. acidophilus* ATCC 4356

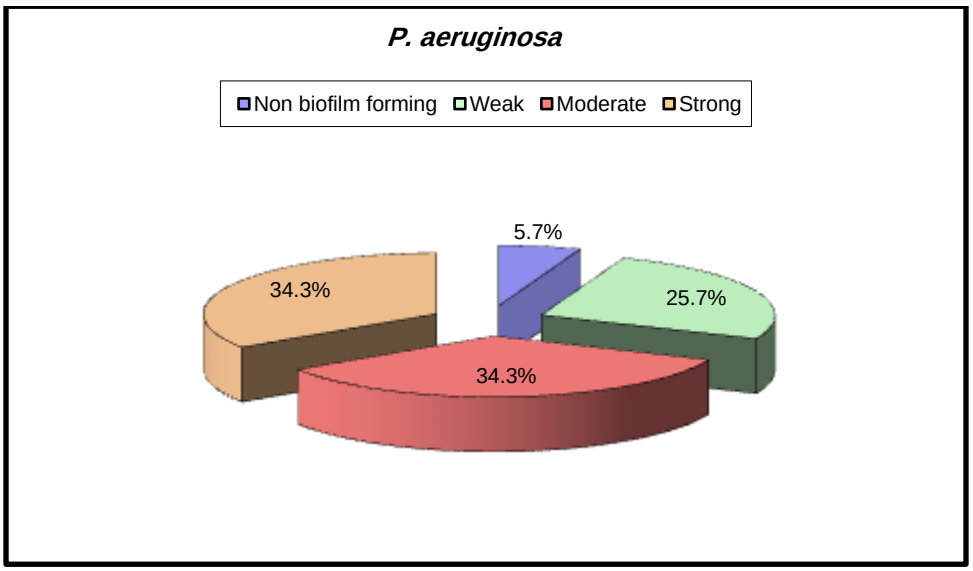
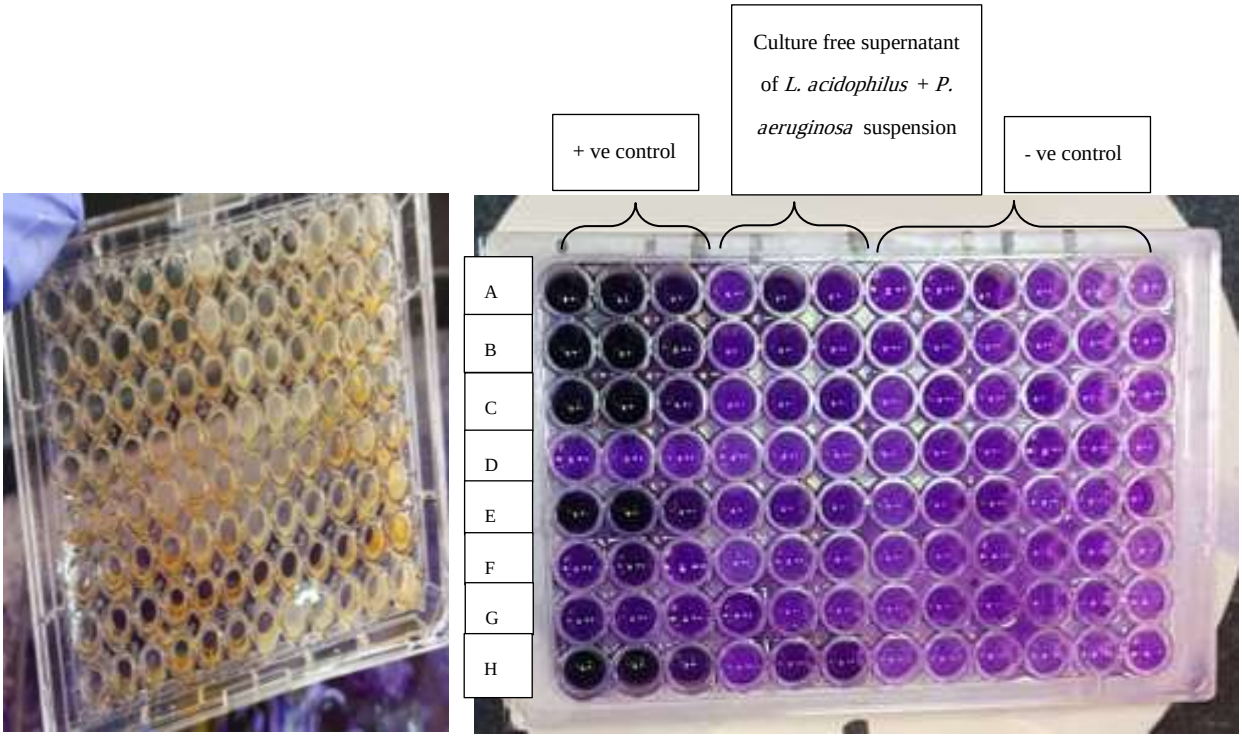


Fig. 4: Degree of biofilm formation of by the clinically isolated *P. aeruginosa* strains



(a) **(b)**

Fig. 5: Microtitre plate before (**a**), and after crystal violet staining (**b**); showing the effect of *L. acidophilus* against biofilm formations of different *P. aeruginosa* strains upon incubation at same time. Row A, B, C, E, H: transforming *P. aeruginosa* strains from strong to weak biofilm producers; Row D: transforming *P. aeruginosa* strain from weak to non-biofilm producers; Row F, G: transforming *P. aeruginosa* strains from moderate to non-biofilm producers.

Table 2: Effect of *L. acidophilus* ATCC 4356 on biofilm formation by *P. aeruginosa* strains

	OD before adding *L. acidophilus*	**OD after adding *L. acidophilus***	**Paired difference**	**% of reduction**	**Paired t-test**	
	Mean ± SD	**Mean ± SD**	**Mean ± SD**		**T**	**p-value**
P. aeruginosa	2.28 ± 0.92	0.72 ± 0.44	-1.56 ± 0.53	68.52%	16.926	< 0.001

3.4. Effect of *L. acidophilus* on the preformed biofilms by *P. aeruginosa* strains

The cell free supernatants of *L. acidophilus* strain removed the already preformed biofilms by the 33 *P. aeruginosa* strains, with reduction percentage of the total biofilm mass of 43.8 % (Fig. 6). The mean OD reading was 1.28 ± 0.65 (Table 3). Recorded results were statistically significant (P value < 0.001).

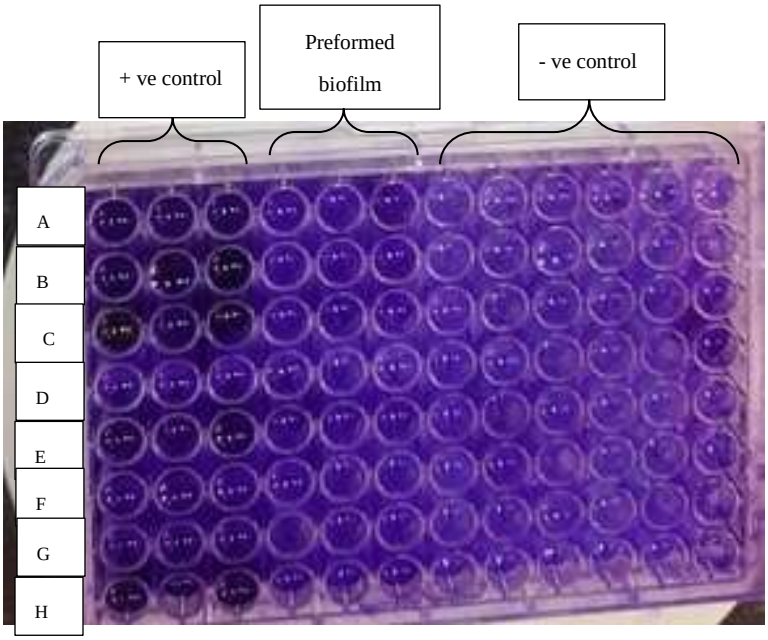


Fig. 6: The effect of *L. acidophilus* against preformed biofilms of different *P. aeruginosa* strains after staining. Row A, B, C, E, H: transforming *P. aeruginosa* strains from strong to moderate biofilm producers; Row D: transforming *P. aeruginosa* strains from weak to non-biofilm producers; Row F, G: transforming *P. aeruginosa* strains from moderate to weak producers.

Table 3: Effect of *L. acidophilus* ATCC 4356 on the preformed biofilm of *P. aeruginosa* strains

	OD before adding *L. acidophilus*	OD of preformed biofilms after adding *L. acidophilus*	Paired difference	% of reduction	Paired t-test	
	Mean ± SD	Mean ± SD	Mean ± SD		T	p-value
P. aeruginosa	2.28 ± 0.92	1.28 ± 0.65	-1.00 ± 0.45	43.80 %	12.836	< 0.001

4. Discussion

P. aeruginosa is a nosocomial pathogen that continues to be problematic from the clinical perspective. It causes opportunistic infections in humans, and is associated with a wide spectrum of multidrug-resistance phenomenon. Gomila *et al*., (2013); Méndez-Vilas, (2013) reported that these untreatable antibiotics resistant strains caused exertion of great efforts, in to order to find alternative approaches for their treatments.

Lactic acid producing bacteria (*Lactobacillus* spp.) inhibit the growth of several bacterial pathogens by producing antimetabolites such as; lactic acids, acetic acid and hydrogen peroxide (Bilkova *et al*., 2011). In the current study; *P. aeruginosa* strains showed resistance to cefepime and tobramycin (91 %), in accordance with similar study of Shokri *et al*., (2018), who reported that resistance to cefepime and tobramycin were 89.6% and 90.6%, respectively. Conversely, a lower resistance to tobramycin (33.3%) was detected earlier by Jamalifar *et al*., (2011). Resistance of *P. aeruginosa* to Imipenem and Piperacillin/ tazobactam in our study was 85.7%; comparable to what was reported by Shokri *et al*., (2018), who stated that a resistance of their isolated *P. aeruginosa* strains to Imipenem was about 84.5%, whereas a lower resistance was reported against Piperacillin/ tazobactam (74 %). Currently; resistance of *P. aeruginosa* strains to meropenem, ciprofloxacin and levofloxacin was 82.8% for each, similar to results of Shokri *et al*., (2018) study, who recorded resistance to the same antibiotics by 85.4% ,79.1% and 75%, respectively.

Regarding ceftazidime; the resistance was recorded among 77% of the strains, in accordance with Chandrahausan *et al*., (2012) who reported comparable results, as 75 % of *P. aeruginosa* strains isolated from non-HIV patients exhibited resistance to ceftazidime, while a higher resistance (82.2%) was recorded among *P. aeruginosa* strains isolated from HIV patients. With regards to amikacin; resistance of *P. aeruginosa* strains was 68.5% in the present study, closely similar to results (64.5 %) reported by Shokri *et al*., (2018). Al-Mathkhury *et al*., (2011) reported lower resistance percentage (50%) to the same drug. Resistance of *P. aeruginosa* strains to Gentamycin was 51.4%, which was very close to what was reported by Jamalifar *et al*., (2011). In the current study; all *P. aeruginosa* strains were sensitive to Aztreonam, while Shokri *et al*., (2018) reported a resistance of 67.7% in their isolates.

In the current study; *P. aeruginosa* strains isolated from catheterized patients showed increased resistance to different antibiotics, compared to strains isolated from midstream urine samples. This result was similar to that of Dund *et al*., (2015). They attributed this high resistance pattern to the weakly guided antibiotic prophylaxis after catheterization and empiric therapy.

On using the MRS medium to test the antibacterial potency of the cell free supernatants of the *Lactobacillus* strain as described by Shokri *et al*., (2018), no growth of *P. aeruginosa* strains were detected at all. This could be attributed to the presence of sodium acetate and ammonium citrate in the medium, to prevent the growth of the contaminating microorganisms. Thus, MHA was used in substitution to MRS medium as described by Jamalifar *et al*., (2011); Chen *et al*., (2019). The cell free supernatants of *L. acidophilus* ATCC 4356 showed high antibacterial potential against 88.6 % of the isolated *P. aeruginosa* strains, assessed by agar well diffusion assay, in accordance with the study of Shokri *et al*., (2018). After neutralization of the acidic pH of the *Lactobacillus* supernatant, we observed no inhibitory activity against *P. aeruginosa* strains. According to Shokri *et al*., (2018), this could be attributed to the production of antibacterial organic acid molecules including; lactic, acetic and formic acid or bacteriocins that were active under acidic conditions only. On the other hand, an earlier study of Valdéz *et al*., (2005) reported that neutralized culture supernatants of *L.*

plantarum showed significant reduction in *P. aeruginosa* viable count by 97 %. This indicated that the antibacterial activity of the Lactobacilli against bacterial pathogens was multifactorial, and included much more than antibacterial organic acid molecules and\ or bacteriocin-like molecules. Chen *et al*., (2019) added other mechanisms proposed for Lactobacilli inhibitory activity such as; the production of hydrogen peroxide and unknown non-lactic acid molecules, competition for nutrients, inhibition of adhesion pathogens to surfaces, and simulation of the immune system.

The current study recorded that 94.3% of *P. aeruginosa* strains were biofilms producers, which coincides with other reports of Jabalameli *et al*., (2012); Shokri *et al*., (2018). On studying the inhibitory potency of the cell free supernatants of *L. acidophilus* ATCC 4356 on biofilm formation by *P. aeruginosa* strains; a decrease in total biofilm mass by 68.52% was noted, as the OD was decreased compared to the OD of the positive control. This was highly statistically significant and matched the findings of Shokri *et al*., (2018). Meanwhile; Al-Mathkhury *et al*., (2011) attributed the effect of Lactobacilli on biofilm formation to their ability to secret cytotoxic compounds, and diminish the quorum signals produced by *P. aeruginosa* necessary for biofilm formation.

On studying the ability of the cell free supernatants of the *L. acidophilus* strain to remove biofilms formed by *P. aeruginosa* strains; results showed a decrease in total biofilm mass by 43.80 %. This was highly statistically significant (P-value< 0.001), but was less than its ability to inhibit biofilm formation from the start. This indicated that *L. acidophilus* might have a more powerful role in preventing biofilm formation by *P. aeruginosa*, than a curable role in infections where biofilm has already established. However, Shokri *et al*., (2018) reported that two of the Lactobacilli strains in their study were able to remove biofilms formed by all *P. aeruginosa* strains with 100 % activity.

Conclusion

The present work illustrated that although *P. aeruginosa* was hardly eradicated by antibiotics; *L. acidophilus* ATCC 4356 has the potency to inhibit bacterial growth, biofilm formation, and remove the pre-formed ones. Accordingly; this strain may be considered as a promising probiotic for bio-control of antibiotic resistant, and biofilm producing *P. aeruginosa* strains.

Conflict of interest

The authors declare that they have no financial or non-financial conflict of interests related to the current manuscript.

Acknowledgements

The authors are grateful to the Department of Medical Microbiology and Immunology, and the Department of Anesthesia, Intensive Care and Pain Management, Faculty of Medicine, Ain Shams University, Cairo, Egypt, for their facilitations and assistance.

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