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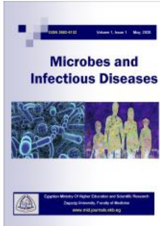

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Original article

Assessment of antibacterial and anti-biofilm activity of probiotic *lactobacillus* against biofilm-forming *Proteus mirabilis* clinical isolates

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ABSTRACT

Background: Emergence of biofilm-producing *P. mirabilis*, especially those resistant to conventional antibiotics, necessitates safe and natural alternatives like probiotics. We investigated the potential antimicrobial and anti-biofilm activities of *L. acidophilus* and *L. reuteri* against *P. mirabilis* clinical isolates. **Methodology:** *P. mirabilis* colonies were identified by traditional methods. Antimicrobial susceptibility and biofilm formation were assessed by disk diffusion and microtiter plate assay (MTP) respectively. *L. acidophilus* and *L. reuteri* were incubated in De Man, Rogosa and Sharpe broth, then centrifuged to get cell free supernatant. Antimicrobial and anti-biofilm effect of supernatant against *P. mirabilis* were tested using agar well diffusion and MTP methods, respectively. **Results:** About 61.3% of *P. mirabilis* isolates were biofilm producers with 92% and 30.7% for ampicillin and imipenem resistance, respectively. Multi-drug resistance represented 42.7%. Supernatants of *L. acidophilus* and *L. reuteri* had 100% antimicrobial inhibition for untreated and 56% and 68% respectively for treated one with mean inhibition zone diameters 18.65±1.05 and 18.32±1.08 respectively for untreated and 8.44±7.54 and 10.0±6.93 respectively for treated supernatant. *L. acidophilus* supernatant showed significant reduction of biofilm formation by ≥78% and ≥75% for *L. reuteri*. Treated and untreated supernatant showed significant eradication of maturely formed biofilm by 37% - 70% for *L. acidophilus* and 40%- 66% respectively for *L. reuteri*. **Conclusion:** *L. acidophilus* and *L. reuteri* demonstrated antibacterial and anti-biofilm activity that with further in vivo and in vitro testing can be employed as a novel antimicrobial agent against *P. mirabilis.*

Introduction

Proteus mirabilis (*P. mirabilis*) is a gram-negative bacterium frequently found in soil and water. It is one of intestinal flora in human. On the opposite side, it could be opportunistic pathogen causing serious community- and hospital-acquired infections of wounds, respiratory, urinary, biliary and blood systems [1].

When *P. mirabilis* comes in contact with solid surfaces (like medical equipment), its virulence factors especially; flagellae facilitate adhesion and motility enabling it to build biofilms and colonize humans [2]. Biofilm formation is essential for protecting it against antibiotics and host defense mechanisms, resulting in multidrug-resistant (MDR) status [3].

Although antibiotics have a fundamental role in treatment of infections, adverse side effects are associated with them e.g. hypersensitivity, toxicity, super-infection, gastrointestinal upset due to gut flora imbalance (e.g., nausea, vomiting, abdominal pain, diarrhea). Nowadays, several researchers are concerned with alternative

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combination therapies. Natural bioactive compounds such as probiotics can work as an adjuvant of antibiotics with immunomodulatory effects and marvelous outcomes [4].

Probiotics are officially described by the World Health Organization as "live bacteria that, when administered in appropriate proportions, have a health benefit on the host". *Lactobacillus* and *Bifidobacterium* are common examples of probiotics that are now used as well as their derivatives to treat infections of various pathogens like *Proteus spp., Enterococci* and *Klebsiella* [5].

There are numerous species of the probiotic genus *Lactobacillus*, including *L. acidophilus, L. reuteri* and *L. crispatus*. They were isolated from various sources as gastrointestinal and genitourinary tract as well as fermented food. These microorganisms display antagonistic activity against different uro-pathogens via production of several antibacterial substances added to their immunomodulatory effects. The antibacterial compounds produced by *Lactobacillus spp.* include lactic acid, bacteriocins, bio-surfactants, hydrogen peroxide as well as many organic acids [6].

Lactobacillus exhibited strong ability to adhere to human epithelial cell lines. Previous studies demonstrated that the adhesion molecules, exopolysaccharides, on the cell walls were involved in adherence ability of *Lactobacillus* strains. Moreover, various adhesion factors of *Lactobacillus* strains are loosely bound to the epithelial cells surface by noncovalent interaction that inhibit pathogen adhesion and biofilm formation [7].

To enhance better management of *Proteus mirabilis* infections, we investigated their antimicrobial susceptibility with the evaluation of potential antimicrobial and antibiofilm activities of *L. acidophilus* (ATCC 4356, DSM 20079) and *L. reuteri* (DSM 20016) against different *P. mirabilis* clinical isolates.

Methods

This cross-sectional study was conducted in microbiology lab, Faculty of medicine, Menoufia University. Clinical isolates were collected over a period of 12 months. Full history taking and appropriate clinical assay were done. Menoufia University ethical approval (IRB approval number 2023 MICR 9-2) and informed consents were obtained.

Specimen collection and processing

Various clinical samples were collected including urine, wound swabs and respiratory specimens. All samples were collected under complete aseptic conditions. They were all obtained from patients with hospital acquired infections apparent 48 hours or more after admission. They were immediately transported to microbiology laboratory for processing. Collected specimens were cultured on blood agar, MacConkey agar and CLED for urine samples (Oxoid, UK) then incubated at 37°C for 24-48 hours. All isolates were processed according to the following methodology flow chart **(Figure 1).**

P. mirabilis identification

Identification of obtained colonies was conducted using Gram staining, colony shape, characteristic swarming and biochemical reactions [8] and then confirmed by VITEK2 compact system (Biomeriuex, France).

Antibiotic susceptibility testing of *P. mirabilis* isolates

The modified Kirby–Bauer disk diffusion method on Muller Hinton agar (MHA) (Oxoid, UK) was used to test antimicrobial susceptibility of all isolates. Used antibiotic disks (Oxoid) included: ampicillin (10µg), amoxicillin-clavulanic acid (AMC; 20/10µg), imipenem (IMP; 10 µg), cefoxitin (FOX; 30 µg), ceftazidime (CAZ; 30 µg), ciprofloxacin (CIP; 5 µg), cefotaxime; (CTX; 30 µg), and amikacin (AK; 30 µg). Quality control *Escherichia coli* (ATCC 25922) strain was used. The CLSI guidelines were used to interpret the diameters of inhibition zones [9].

Isolates that were either intermediate (I) or resistant (R) to at least one drug in at least three antimicrobial categories were chosen as being MDR.

Phenotypic detection of biofilm formation by *P. mirabilis* isolates

Microtiter tissue culture plate method (TCP)

This quantitative method is considered the gold standard for biofilm detection. It was performed in accordance with **Shokri et al.** [10] as follows; Fresh colonies of isolates were inoculated in trypticase soya broth (TSB) supplemented with 1% glucose, then incubated for 18–24 hours at 37°C. About 200 µL of diluted culture solution (inoculum concentration of approximately 5x 10^5 CFU/ml which is 0.5 McFarland bacterial suspension diluted

to 1:100) was incubated in polystyrene microtitre tissue culture plate wells for 18–24 hours at 37°C. After that, we gently tapped the plate to remove any free-flowing bacteria, washed it four times with phosphate buffered saline (PBS), air-dried while inverted, fixed with freshly made sodium acetate then stained with 0.1% crystal violet for 10 minutes and finally PBS was used to remove excess stain from the plate. Crystal violet retained stain was interpreted as biofilm formation. Following air-drying, 95% ethanol was used to re-solubilize the dye that had been bound to the cells, which was then incubated for 15 minutes at room temperature. Using a microtitre plate reader Infinite (Tecan, Korea) at wavelength 620 nm; optical density (OD) was measured to classify the biofilm strength. All tests were run in triplicate. The following formula was used: $OD \leq ODc$ (OD of the negative control) = non-biofilm producer, $ODc < OD \leq (2 \times ODc)$ = weak biofilm producer, $(2 \times ODc) < OD \leq (4 \times ODc)$ = moderate biofilm producer and $(4 \times ODc) < OD$ = strongly biofilm producer [11].

Cell-free supernatant (CFS) preparation from *Lactobacillus* strains

Two strains of *Lactobacilli* probiotics (*L. reuteri,* ATCC 23272, DSM 20016) and (*L. acidophilus* ATCC 4356, DSM 20079) were obtained from Microbiological Resources Centre (MIRCEN), Faculty of Agriculture, Ain Shams University, Cairo, Egypt. *Lactobacilli* were cultivated for 24 hours at 37˚C in De Man, Rogosa and Sharpe broth (MRS) broth, TM 147-Media B2E3AW01) and growth turbidity was adjusted to 0.5 McFarland standards.

Lactobacillus culture was centrifuged at 6000 rpm for 15 min for separation of cells. Supernatant was then filtered through a 0.22 µm membrane filter (Millipore, Billerica, MA, United States). The cell free supernatant was stored at -20 °C and given the label "untreated supernatant" (U). Because lactic acid was produced, the *Lactobacillus* supernatant had a high acidity. To determine whether the culture supernatants' inhibitory effect was caused solely by acidic products or by other products, NaOH was added adjusting the supernatant pH to 6.5–7.0, stored at -20 °C and given the label treated supernatant (T). Stock solutions of treated and untreated supernatants were prepared in different concentrations 1/2, 1/4, 1/8, 1/16, and 1/32 [12].

Detection of antibacterial action of *Lactobacillus* on *P. mirabilis* using agar well diffusion method

P. mirabilis isolates were cultured for 24 h at 37 °C in Muller Hinton broth (Oxoid Ltd., UK). The inoculum was adjusted to 0.5 McFarland then used to inoculate MHA. A sterile metal cylinder was used to cut wells into agar plates that were 6 mm in diameter, 100µl of undiluted and different dilutions (1/2, 1/4, 1/8, 1/16, 1/32) of CFS (T and U) preparations were put into each well and incubated at 37 °C for 24 h. The presence of an inhibition zone, which is measured in millimeters, was considered an indicator of antimicrobial action. All tests were performed in duplicate. Plain MRS broth was used as negative control [13].

Assessment of antimicrobial effect of CFS of tested *Lactobacilli* in combination with ciprofloxacin

Two ciprofloxacin antibiotic disks (one for each tested *lactobacillus*) were impregnated with about 20µl of untreated-undiluted CFS of the tested *lactobacillus* to form combination disks to be placed on the surface of MHA plates swabbed with ciprofloxacin resistant isolates. After over-night incubation, diameters of inhibition zones around combination disks were measured to be compared with those produced previously by plain ciprofloxacin disk and *lactobacilli* CFS (20µl vs 100µl) separately [4]

Detection of anti-biofilm activity of *lactobacillus* CFS on *P. mirabilis*

We tried to evaluate anti-biofilm activity of treated and untreated supernatants of *lactobacillus*. For test wells preparation, 100µl of previously prepared undiluted and different dilutions (1/2, 1/4, 1/8, 1/16, 1/32) of *Lactobacillus* CFS (T and U) were added to 100µl of *P. mirabilis* suspension. After incubation, the previously mentioned steps (under the heading phenotypic detection of biofilm formation by *P. mirabilis* isolates) were repeated. The OD of each well was measured at 620 nm. Obtained results were compared with positive control (200µl of growth suspension of previously identified strong biofilm forming *P. mirabilis*) and negative control (200µl of plain TSB). Each test was performed in triplicate [14].

The following formula was used to determine the percentage OD reduction caused by the addition of lactobacillus CFS:

Percentage of biofilm reduction in test wells = (C-B) - (T-B) / (C-B) *100

Where C is OD620 nm of positive control wells, B is OD of negative control and T is OD of wells treated with CFS [15].

Evaluation of inhibitory effect of *Lactobacillus* CFS on the preformed biofilms

Biofilm producing *P. mirabilis* broth suspensions were again incubated in microtitre plate wells to promote biofilm formation as previously mentioned. After removal of planktonic cells, the adherent cells were gently washed. Non diluted treated and untreated supernatants of *Lactobacillus* were subsequently added to the wells and the plates were incubated at 37°C for 24 h. Biofilm strength was reassessed as early mentioned [15].

Statistical analysis

Data were tabulated & analyzed by SPSS version 20 (SPSS Inc., Chicago, IL, USA). Continuous variables were expressed as mean and SD. Categorical variables were expressed as number and percent. Chi-square was used to study association between qualitative categorical data while, students` t test and Mann-whitney were tests of significance used for comparison between two independent groups with quantitative variables. ANOVA and Kruskal Wallis were tests of significance used for comparison between more than two independent groups with quantitative variables. Paired t and Wilcoxon were tests of significance used for comparison between two related groups with quantitative variables. A significance level of $p<0.05$ was used in all tests.

Results

A total of 75 (10.1%) *P. mirabilis* isolates were obtained from 742 different clinical samples taken from patients diagnosed with hospital acquired infections. Most strains were isolated from male (52%) patients aged 30-60 years old (66.7%) with no statistically significant impact to age and gender on the degree of biofilm formation among isolates.

By TCP method, 46 (61.3%) isolates were biofilm producers with 39.1%, 52.2% and 8.7% as strong, moderate and weak biofilm producers respectively. The antibiotic susceptibility pattern of tested isolates is shown in **table (1)**. The highest resistance among *P. mirabilis* isolates was against ampicillin (69/75; 92%) and amoxicillin-clavulanic acid (66/75; 88%), while lowest resistance was against imipenem (23/75; 30.7%). MDR was detected among (32/75; 42.7%) of isolates. Antibiotic resistance was significantly (***p*** value ≤ 0.001) higher among strong and moderate biofilm producers compared to weak and non-biofilm producing isolates **(Table 1)**.

Antimicrobial activities of *L. acidophilus* and *L. reuteri* against *P. mirabilis* Isolates

The undiluted untreated supernatants of *L. acidophilus* and *L. reuteri* had inhibitory effect against all tested *P. mirabilis* isolates with mean inhibition zone diameters 18.65±1.05 and 18.32±1.08 respectively. The range of zone of inhibition of undiluted untreated CFS were 17.0 – 22.0 mm for *L. acidophilus* and 17.0 – 21.0 mm for *L. reuteri*. Undiluted treated supernatants of *L. acidophilus* and *L. reuteri* had inhibitory effect on 56% and 68% of tested isolates with mean inhibition zone diameters 8.44±7.54 and 10.0±6.93 respectively as shown in **figure (2)**. There was a high statistically significant difference (***p*** value <0.001) between antimicrobial activities of treated and untreated supernatants of both *lactobacilli*. No statistically significant difference was found between antimicrobial activities of *L. acidophilus* and *L. reuteri* against *P. mirabilis* isolates (***p*** value = 0.06 - 0.73). Strength of biofilm formation was not significantly (***p*** value = 0.15 – 0.76) correlated to antimicrobial activities of *L. acidophilus* and *L. reuteri* against *P. mirabilis* isolates. Mean inhibition zone diameters for MDR isolates were smaller than susceptible isolates but with no statistically significant (***p*** value = 0.09 - 0.95) difference. Concentration 1/2 of treated supernatants of *L. acidophilus* and *L. reuteri* retained its antimicrobial activity against 44.0% and 58.7% of isolates respectively while that of untreated supernatants of *L. acidophilus* and *L. reuteri* had activity against 76.0% and 73.3% of isolates respectively with smaller inhibition zone diameters compared to that of undiluted concentration. Lower concentrations showed no inhibitory effect against *P. mirabilis* isolates. Regarding, testing of antimicrobial effect of undiluted untreated CFS of *Lactobacilli* in combination with ciprofloxacin, no significant change in dimeters of inhibition zones was detected (***p*** value = 0.67- 0.7) **(Table 2, figure 3)**.

Anti-biofilm activity of *lactobacillus* CFS on *P. mirabilis*

Undiluted treated and untreated CFS showed significant reduction of biofilm formation

by 78% and 85% respectively for *L. acidophilus* as well as 75% and 80% respectively for *L. reuteri*. The inhibitory effect of the untreated supernatants on biofilm formation had decreased by further dilution of supernatant. The 1/2 and 1/4 of treated and untreated CFS respectively retained its activity and significantly reduced biofilm formation **(Table 3, 4)**.

Effect of *Lactobacillus* CFS on preformed mature biofilms (biofilm eradication)

Undiluted treated and untreated CFS showed significant eradication of maturely formed biofilm by 37% - 59% and 46% - 70% respectively for *L. acidophilus* and 40%- 63% and 44% - 66% respectively for *L. reuteri*.

Table 1. Clinical characteristics, antimicrobial susceptibility and biofilm Formation of *P. mirabilis* isolates

	Total (n=75) N (%)	**Strong biofilm producer (n=18) N (%)**	**Moderate biofilm producers (n=24) N (%)**	**Weak biofilm producers (n=4) N (%)**	**Non-biofilm producers (n=29) N (%)**	**χ2**	***p* value**
Specimen type:							
Urine	30 (40.0)	9 (50.0)	9 (37.5)	1 (25.0)	11 (37.9)	19.0	0.08
Diabetic foot swab	16 (21.3)	4 (22.2)	6 (25.0)	1 (25.0)	5 (17.2)		NS
Surgical wound swab	14 (18.7)	5 (27.8)	6 (25.0)	0 (0.0)	3 (10.3)		
Respiratory samples	10 (13.3)	0 (0.0)	3 (12.5)	2 (50.0)	5 (17.2)		
Ear discharge	5 (6.7)	0 (0.0)	0 (0.0)	0 (0.0)	5 (17.2)		
Antibiotic resistance:							
Ampicillin (10μg)	69 (92.0)	18 (100.0)	24 (100.0)	4 (100.0)	23 (79.3)	10.34	0.01 S
Amox-clav (20/10μg),	66 (88.0)	18 (100.0)	24 (100.0)	4 (100.0)	20 (69.0)	16.22	0.001 HS
Imipenem (10 µg)	23 (30.7)	18 (100.0)	5 (20.8)	0 (0.0)	0 (0.0)	56.38	<0.001 HS
Cefoxitin (30 µg)	36 (48.0)	18 (100.0)	13 (54.2)	0 (0.0)	5 (17.2)	34.55	<0.001 HS
Ceftazidime (30 µg)	45 (60.0)	18 (100.0)	22 (91.7)	0 (0.0)	5 (17.2)	50.12	<0.001 HS
Ciprofloxacin (5 µg)	52 (69.3)	14 (77.8)	24 (100.0)	4 (100.0)	10 (34.5)	29.55	<0.001 HS
Cefotaxime; (30 µg)	45 (60.0)	18 (100.0)	22 (91.7)	0 (0.0)	5 (17.2)	50.12	<0.001 HS
Amikacin (30 µg)	44 (58.7)	16 (88.9)	21 (87.5)	0 (0.0)	7 (24.1)	34.94	<0.001 HS
MDR	32 (42.7)	15 (83.3)	12 (50.0)	0 (0.0)	5 (17.2)	23.33	<0.001 HS

χ2: Chi-square test; NS: not significant; S: significant; HS: highly significant

Table 2. Antimicrobial activities of *L. acidophilus* and *L. reuteri* against *P. mirabilis* isolate

	Inhibition Zone Diameter (mm)				Test of sig.	**p** value
	L. acidophilus		*L. reuteri*			
	Treated CFS	Untreated CFS	Treated CFS	Untreated CFS		
Mean±SD Range Median	8.44±7.54 14.0 – 16.0 14.0	18.65±1.05 17.0 – 22.0 18.0	10.0±6.93 13.0 – 16.0 14.0	18.32±1.08 17.0 – 21.0 18.0	U=0.34[a] t=1.90[b]	0.73[a] NS 0.06[b] NS
Wilcoxon test	7.61		7.58			
P value	<0.001 HS		<0.001 HS			
Percent of antimicrobial activity reduction after CFS treatment	54.74%		45.41%			
Number and percent of isolates showing inhibition zone	42 (56.0)	75 (100.0%)	51 (68.0)	75 (100.0)		
Biofilm forming Strong Moderate Weak Lack	 7.55±7.78 6.20±7.51 11.25±7.54 10.44±7.15	 18.61±0.77 18.58±1.05 19.25±1.89 18.65±1.11	 8.94±7.38 9.79±7.10 7.0±8.12 11.24±6.48	 18.27±1.12 18.16±1.04 18.25±0.50 18.48±1.15		
Test of sig.	K=5.27	F= 0.45	K=2.54	F= 0.38		
P value	0.15 NS	0.71 NS	0.46 NS	0.76 NS		
MDR Positive Negative	 8.43±7.57 8.44±7.61	 18.43±0.61 18.81 ±1.27	 9.28±7.33 10.53 ± 6.67	 18.15±1.01 18.44 ±1.11		
Test of sig.	U=0.22	t=1.68	U=0.05	t=1.13		
P value	0.82 NS	0.09 NS	0.95 NS	0.26 NS		
	Ciprofloxacin disk	Combined ciprofloxacin with untreated CFS	Ciprofloxacin disk	Combined ciprofloxacin with untreated CFS		
Mean±SD Range	11.20±6.99 0.0 – 22.0	11.80±6.56 1.0 – 22.0	11.20±6.99 0.0 – 22.0	11.70±6.70 0.0 – 22.0		
Mann-whitney test	0.41		0.38			
P value	0.67 NS		0.70 NS			

t: students` t test; **U**: Mann-whitney test; F: ANOVA; K: Kruskal Wallis test. [a]: comparison of treated supernatant of *L. acidophilus* and *L. reuteri.*[b]: comparison of untreated supernatant of *L. acidophilus* and *L. reuteri*

Table 3. Anti-biofilm activities of *L. acidophilus* against *P. mirabilis* isolates

	Optical density				**Test of sig.**	***p* value**
	Before		**After adding CFS to biofilm forming isolates**			
	Biofilm forming (N=46)	**Non-biofilm forming (N=29)**	**Treated (N=46)**	**Untreated (N=46)**		
Mean±SD **Range**	0.67±0.20 0.14 – 0.95	0.047±0.02 0.01 – 0.08	0.14±0.04 0.03 – 0.21	0.10±0.03 0.02 – 0.14	Paired t=21.92[a] Paired t=21.92[b]	<0.001[a] HS <0.001[b] HS
Test of sig.	t=20.13		Paired t= 21.92			
P value	<0.001 HS		<0.001 HS			
Percent of biofilm reduction			78%	85%		
Biofilm forming N (%) Strong Moderate Weak Lack	18 (24.0) 24 (32.0) 4 (5.3) 29 (38.7)		0 (0.0) 0 (0.0) 40 (87.0) 6 (13.0)	0 (0.0) 0 (0.0) 27 (58.7) 19 (41.3)	**χ2**=77.45[a] **χ2**=78.06[b]	<0.001[a] HS <0.001[b] HS
χ2			**χ2**=9.28			
P value			0.002 S			
Concentration 1/2 Mean OD (Percent of biofilm reduction)			0.28±0.08 (58.0%)	0.18±0.05 (73.0%)	Paired t=21.92[a] Paired t=21.92[b]	<0.001[a]HS <0.001[b]HS
Concentration 1/4 Mean OD (Percent of biofilm reduction)			0.40±0.12 (39.0%)	0.30±0.09 (55.0%)	Paired t=21.92[a] Paired t=21.92[b]	<0.001[a]HS <0.001[b]HS
Concentration 1/8 Mean OD (Percent of biofilm reduction)	0.67±0.20		0.67±0.20 (0.0%)	0.44±0.13 (34.0%)	NA[a] Paired t=21.92[b]	NA[a] <0.001[b]HS
Concentration 1/16 Mean OD (Percent of biofilm reduction)			0.67±0.20 (0.0%)	0.67±0.20 (0.0%)	NA[a] NA[b]	NA[a] NA[b]
Concentration 1/32 Mean OD (Percent of biofilm reduction)			0.67±0.20 (0.0%)	0.67±0.20 (0.0%)	NA[a] NA[b]	NA[a] NA[b]

[a]: comparison of biofilm forming (before) with treated supernatant of *L. acidophilus*
[b]: comparison of biofilm forming (before) with untreated supernatant of *L. acidophilus*
NA: not applicable

Table 4. Anti-biofilm activities of *L. reuteri* against *P. mirabilis* isolates

	Optical density				**Test of sig.**	***p* value**
	Before		**After adding CFS to biofilm forming isolates**			
	Biofilm forming (N=46)	**Non-biofilm forming (N=29)**	**Treated (N=46)**	**Untreated (N=46)**		
Mean±SD	0.67±0.20	0.047±0.02	0.16±0.05	0.13±0.04	Paired t=21.92[a]	<0.001[a] HS
Range	0.14 – 0.95	0.01 – 0.08	0.04 – 0.24	0.03 – 0.19	Paired t=21.92[b]	<0.001[b] HS
Test of sig.	t=20.13		Paired t= 21.92			
P value	<0.001 HS		<0.001 HS			
Percent of reduction of biofilm formation			75%	80%		
Biofilm forming N(%)					$\chi 2$=77.39[a]	<0.001[a] HS
Strong	18 (24.0)		0 (0.0)	0 (0.0)	$\chi 2$=77.64[b]	<0.001[b] HS
Moderate	24 (32.0)		0 (0.0)	0 (0.0)		
Weak	4 (5.3)		42 (91.3)	35 (76.1)		
Lack	29 (38.7)		4 (8.7)	11 (23.9)		
$\chi 2$			$\chi 2$=3.90			
P value			0.04 S			
Concentration 1/2 Mean OD (Percent of biofilm reduction)	0.67±0.20		0.26±0.08 (60.0%)	0.18±0.05 (73.0%)	Paired t=21.92[a] Paired t=21.92[b]	<0.001[a]HS <0.001[b]HS
Concentration 1/4 Mean OD (Percent of biofilm reduction)			0.39±0.12 (41.0%)	0.28±0.09 (58.0%)	Paired t=21.92[a] Paired t=21.92[b]	<0.001[a]HS <0.001[b]HS
Concentration 1/8 Mean OD (Percent of biofilm reduction)			0.67±0.20 (0.0%)	0.42±0.13 (37.0%)	NA[a] Paired t=21.92[b]	NA[a] <0.001[b]HS
Concentration 1/16 Mean OD (Percent of biofilm reduction)			0.67±0.20 (0.0%)	0.67±0.20 (0.0%)	NA[a] NA[b]	NA[a] NA[b]
Concentration 1/32 Mean OD (Percent of biofilm reduction)			0.67±0.20 (0.0%)	0.67±0.20 (0.0%)	NA[a] NA[b]	NA[a] NA[b]

Figure1. Methodology flow chart

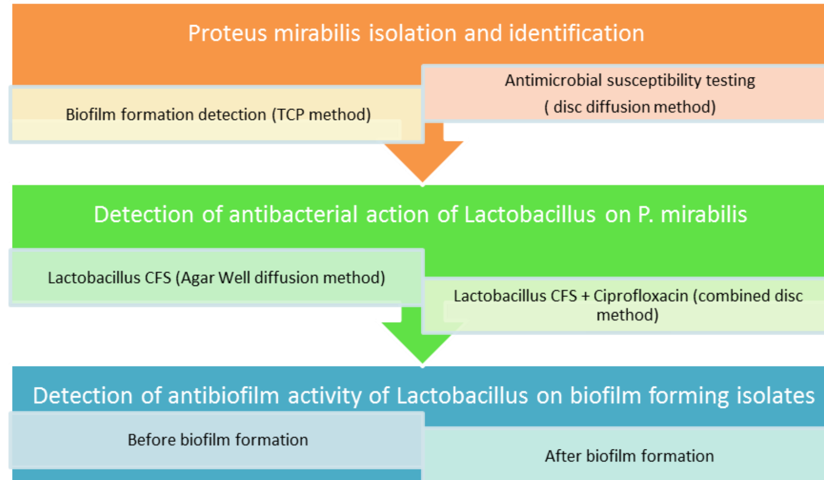


Figure 2. Antimicrobial activities of *L. acidophilus* (A) and *L. reuteri* (B) against *P. mirabilis* isolates

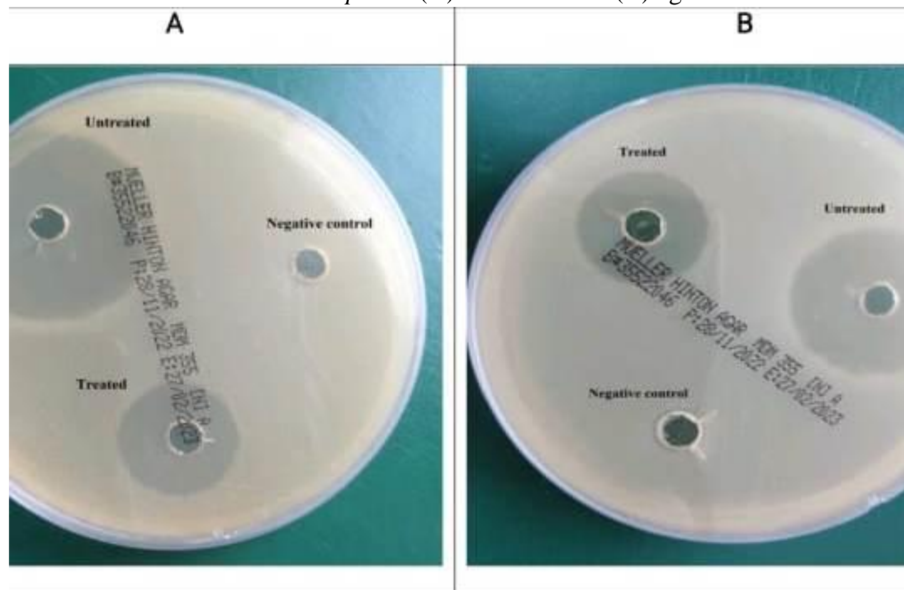
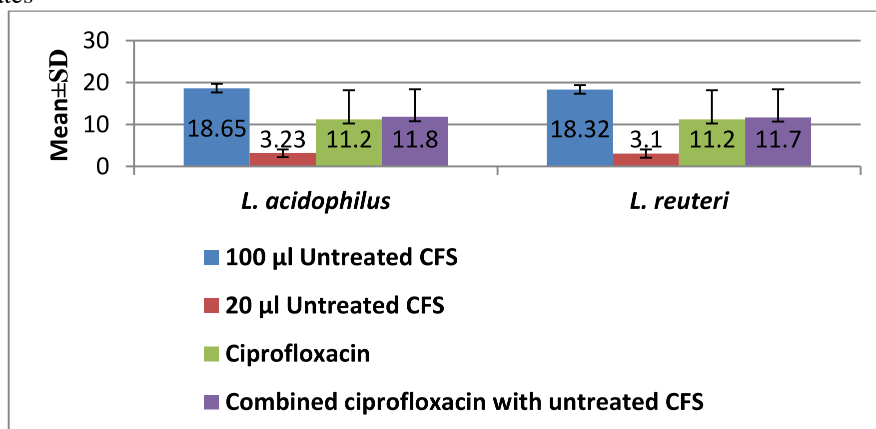


Figure 3. Antimicrobial activities of *L. acidophilus* and *L. reuteri* in combination with ciprofloxacin against *P. mirabilis* isolates



Discussion

P. mirabilis was established as one of the prevalent pathogens linked to nosocomial infections mostly because of biofilm formation. Most biofilm forming microbes exhibit antibiotic resistance with limited spectrum for available treatment regimens. Probiotics are a new and innovative treatment approach that can replace antimicrobial drugs. They are strongly advocated as a more efficient, non-toxic treatment option [16].

In current study, *p. mirabilis* was obtained from 10.1% of clinical samples; mostly from urine, this is comparable to Egyptian study conducted by **Serry et al.** [17] with prevalence rate of 11.75%. Of note that **Kadhim** [18] isolated *P. mirabilis* by higher rates (28.49%) contrasted with lower predominance rates (2%) by **Senthamarai et al.** [19].

The antibiotic susceptibility pattern of the isolates in this study is nearly matched with **Mishu et al.** [20] findings, in which 25% of *P. mirabilis* displayed resistance to imipenem. Nevertheless, 72.73% were resistant to ceftriaxone, 70.45% were resistant to ciprofloxacin. However, lower resistance was detected by **Serry et al.** [17]; piperacillin and amikacin (25.5% each), aztreonam (14.9%), imipenem (8.5%) and meropenem (6.4%). Also **Li et al.** [21] had documented MDR among *Proteus mirabilis* isolates by 46% approximating our finding (42.7%).

In the current study, 46/75 (61.3%) isolates were biofilm producers. 39.1%, 52.2 and 8.7% of which were strong, moderate and weak biofilm producers respectively. This data coincides with **Zaman et al.** [22] with 32% strong, 40% moderate, 16% weak biofilm producers. However, **Kwiecińska-Piróg et al.** [23], and **Oliveira et al.** [24] reported that all the tested strains were biofilm producers; 24.0% weak, 26.0% moderate, and 50.0% strong in the former and 73.2% strong, 25.7% moderate and 1.1% weak biofilm producer in the latter.

Regarding association between degree of biofilm formation and the resistance to tested antibiotics, the prevalence of antibiotic resistance was statistically significantly (p<0.05) higher in strains that were strong and moderate biofilm producers than in those that were weak and non-biofilm producers. In agreement, **Wasfi et al.** [25] and **Mishu et al.** [20], revealed that bacteria trapped in crystalline biofilms develop significant levels of resistance to both the immune system and common antimicrobials.

In the present study, the untreated supernatants of *L. acidophilus* and *L. reuteri* showed inhibitory effect on all tested *P. mirabilis* isolates including MDR strains with mean inhibition zone diameters 18.65±1.05 (17.0 – 22.0 mm) and 18.32±1.08 (17.0 – 21.0 mm) respectively. While, the treated supernatants of *L. acidophilus* and *L. reuteri* showed inhibitory effect only on 56% and 68% of tested isolates with lower mean inhibition zone diameters 8.44±7.54 mm and 10.0±6.93 mm respectively. Our data were similar to those reported by **Shaaban et al.** [13]. In accordance, the study conducted by **Jaber and Almiyah** [5] documented that *L. acidophilus* had a significant inhibitory impact on *P. mirabilis* isolates, with almost similar inhibitory zones after 24 hours of incubation. Additionally, **Shehab et al.** [26], revealed 100% activity of both treated and untreated CFS of *lactobacillus* against *proteus* isolates.

This suggested that the *Lactobacilli* antibacterial action against bacterial pathogens was complex and multifactorial, and comprised more than just organic acid molecules. In agreement, **Torzewska et al.** [6] stated that untreated supernatants of all *Lactobacillus* strains could suppress *P. mirabilis* growth. *Lactobacillus* non-acidic products can suppress harmful bacteria even after acidity had been neutralized. Also, an earlier study of **Valdéz et al.** [27], demonstrated that *P. aeruginosa* viable count was significantly reduced by 97% in treated CFS of *Lactobacillus*. Other mechanisms for *lactobacilli* inhibitory action were added by **Chen et al.** [28], including the production of hydrogen peroxide, competition for nutrition, suppression of pathogen attachment to surfaces and immune system simulation. On the other hand, **Elbadri et al.** [29] found no evidence of pathogen-inhibitory action of neutralized *Lactobacillus* supernatant.

The concentration 1/2 of treated and untreated supernatants of *L. acidophilus* and *L. reuteri* retained its inhibitory activity against most *P. mirabilis* isolates with reduced inhibition zone diameter. In recent study conducted by **Jaber & Almiyah** [5], only concentrations of ≥ 40% revealed antibacterial effects. Our findings are also coinciding with old study done by **Pfeiffer and Radler** [30], which stated an association between inhibition zone diameter and inhibitory substances

concentration. Probiotics are thought to be quite safe with no harmful or noticeable adverse effects [31]. In accordance, **Al-Mathkhury et al.** [32] mentioned that bacteriocin generated by *L. acidophilus* isolated from yogurt significantly inhibit growth of antibiotic resistant gram-negative bacilli. Moreover, **Perez et al.** [33] demonstrated that the next-generation antibiotic that may be utilized to combat the MDR gram-negative infection is crude CFS containing bacteriocin from lactic acid bacteria.

Ciprofloxacin is the most widely used oral antibiotic for treatment of proteus mirabilis associated infections in adults. Quinolones are almost used from the start of injectable regimens or used as an oral continuation therapy. Nowadays, high resistance to ciprofloxacin is encountered which endangers its future use. Combination therapy of probiotics and antibiotics could enhance drug susceptibility, reducing their commonly prescribed doses, improving drug safety and patient outcomes. In current study we tried to evaluate in vitro antimicrobial effect of CFS of untreated *Lactobacilli* in combination with ciprofloxacin on MDR isolates. Unlikely, our results were non-encouraging parallel to reports of **Abdelhalim et al.** [34]. In contrast, study conducted by **Acharjee et al.** [4] detected enhancing effect of these combinations. However, used methodologies and CFS doses were different from ours. An old study of **Kamberi et al.** [35] documented that activity of ciprofloxacin was lower with decreased pH which could explain our results. We think further integrated physiological and pharmaceutical focusing studies are needed.

In the present study, there was significant reduction of biofilm formation by treated and untreated CFS by about 78% and 85% respectively for *L. acidophilus* and 75% and 80% respectively for *L. reuteri* matching results of **Shokouhfard et al.** [36] and **al-jeboury** [37] which revealed that *lactobacillus* filtrate could minimize adhesion of *P. mirabilis* to the uro-epithelial cells. **Chan et al.** [38] explained this by the inhibitory compounds present in *Lactobacillus* filtrates and acidic pH that impact gram negative bacteria by modifying some surface structures (pili); preventing adhesion of bacterial cells. Many previous studies had reported that *lactobacilli* could reduce biofilm formation in Gram negative and Gram positive bacteria [12, 13, 39, 40].

On studying the capacity of *L. acidophilus* CFS to eradicate preformed biofilms; **Elbadri et al.** [29] showed the total mass of the biofilms was significantly reduced by 43.80%. However, this was less than its capacity to prevent the onset of biofilm development. This supported our findings and suggested that *L. acidophilus* may have a more potent action in avoiding biofilm formation than a curable role when biofilm has already developed. However, **Shokri et al.** [10] reported that two *Lactobacilli* strains were able to totally eliminate biofilms formed by various pathogenic strains.

Conclusion

There is a critical need for non-antibiotic safe methods of treating and preventing biofilm-related drug resistant infections. For better treatment and eradication of *P. mirabilis* infections in the future, we advise using potent preparations of *L. acidophilus* (ATCC 4356, DSM 20079) and *L. reuteri* (DSM 20016). Our study recommends future in vivo and in vitro funded studies on larger scale analyzing the interactions between antibiotics and probiotics.

Conflict of interest

Not declared.

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