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Effect of *Lactobacillus fermentum* filtrate on *Pseudomonas aeruginosa* adhesion at biotic and abiotic surfaces

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Abstract

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen which is a serious source of various hospital acquired diseases. Current study aimed to determination of inhibition ability of the probiotic bacteria (*Lactobacillus fermentum*) to inhibit *P. aeruginosa* adhesion. Forty-five isolates of *P. aeruginosa* were obtained from clinical samples, the isolation frequency was equal to 50.56 %. Hydrophobicity results found that 28(62.2%) isolates were strong biofilm producer while 17(37.7%) isolates were moderate biofilm producer. The findings of inhibitory effect of *L. fermentum* filtrate revealed that (1/8) dilution inhibited the bacterial growth. Biofilm formation inhibition test on abiotic surfaces found that *Lactobacillus* filtrate able to inhibit biofilm formation on stainless steel more than glass then plastic. Also results of biofilm inhibition test on biotic surfaces found that that *L. fermentum* filtrate have ability to inhibit biofilm on uroepithelial cells,,approximately (15-20) bacterial cell adhered to epithelia cell without *Lactobacillus* filtrate while there are a very few cells adhered to epithelial cell (0-2) in presence of filtrate.

Recent study may add an additional information obout *L. fermentum* filtrate ability to inhibit biofilm formation by *P. aeruginosa* on abiotic and biotic surface which may be useful in understanding of probiotic role as alternative antibacterial and antibiofim agent at clinical and environmental level

Keywords: biofilm, filtrate, Hydrophobicity, coupons, lactic acid

INTRODUCTION

P. aeruginosa is a Gram negative opportunistic pathogens which is an serious source of various hospital acquired diseases. it is usually hard to treated or remove with conventional antibiotic treatment, especially when settled as biofilm [1] *P. aeruginosa* seldom affects healthy population, but it is more pleasant to affect individuals whose skin, mucous membranes or immune system are impaired; such as, cystic fibrosis patients, burn sufferers or cancer patients treated with chemotherapy [2] In addition this pathogen serves as one of the greatest medicinally biofilm-forming producer, which is commonly linked to hospital acquired of the skin and urogenital tract [3]. Also cystic fibrosis patients suffer from colonization of their lungs by this species [4].

Lactic acid bacteria is one of the greatest species of probiotic bacteria, they are non-pathogenic, sufficient to industrial techniques, acid toleration, bile toleration and make antimicrobial agents such as lactic acid, bacteriocins and hydrogen peroxide, these substances, which have important biomedical benefits, also it produce bio-surfactants [5, 6]. Filtrate of *Lactobacillus* have materials that suppress several of virulence factors of *P. aeruginosa*, like agents capable to prevent sensing molecules from work then causing of quorum sensing inhibition [7].

MATERIALS AND METHODS

Isolation and Identification of *P.aeruginosa*

P.aeruginosa was isolated from different (eighty-nine) clinical samples(wound and urin). Different isolates were cultured on cetrimide agar, then identification was achieved according to [8], by biochemical tests, then by using VITEK 2 compact**.**

Isolation and Identification of *Lactobacillus*

Eight yogurt samples were obtained from commercial shop, streaked on the MRS-CaCO3 agar [9], incubated anaerobically at 37°C for 48 hrs., colonies were selected according to the specific characteristic of *Lactobacillus,* then were submitted to further identifications such as microscopic morphology, catalase, oxidase, VITEK 2 compact and by 16S rRNA sequencing.

Identification of *Lactobacillus* by using 16S rDNA

Extraction of genomic DNA was achieved as mention in manufacturer's guidance, concentration of extracted DNA was detected by Quantus Florometer, DNA visualization was performed by gel electrophoresis (agarose 1%) [10]. Primer were used in current study is: (F-AGAGTTTGATCCTGGCTCAG, R-TACGGTTACCTTGTTACGACTT) has product size 1400 bp [11], PCR reaction was achieved with 25 µl as final volume: template genomic DNA (2 µl), forward and reverse primer 10 pmol/ µl (one µl) of each primer, Go Taq green Master mix 1X (12.5 µl), de-ionized distilled water D.D.W (8.5 µl). The Thermal cycle conditions are: one cycle for initial denaturation (five minutes at 95°C) and thirty cycle for each Denaturation (30 sec, at 95°C), annealing (one mim. at 62°C) and extension (one minute at 72°C), one cycle for each final extension (7 minutes at 72°C) and hold (10 min. at 10°C). PCR product was subjected to sequencing by Sanger sequencing (ABI3730XL automated DNA sequencer) - Korea, results of recent study were obtained by email then examind by using genious software**.**

Biofilm formation

-Hydrophobicity assay

This test was previously described by [12] by using of xylene. The adherence cells percentage was afterward measured by: % Adherence = (1-OD2/OD1) ×100 where OD_1 is the optical density (OD) was determined at 400 nanometers of the bacterial suspension before mixing and OD_2 is the optical density (OD) after mixing. The classification of bacterial categories were dependent on [13] that declared strongly strain hydrophobic (>50%), moderate hydrophobic (20–50%) while low hydrophobic (<20%).

-Polystyrene micro-titter plates assay

Biofilm production was analyzed by polystyrene micro-titter plates according to [14]. Determination of biofilm degree was calculated thusly: OD ≤ ODc no biofilm producer, ODc < OD ≤ 2 × ODc is weak biofilm producer, 2 × ODc < OD ≤ 4 × ODc is moderate biofilm producer, 4× ODc < OD is strong biofilm producer.

Determination of Minimal Inhibitory Concentration of Levofloxacin against stronger biofilm producer *P. aeruginosa* isolate.

This part was performed as described by [15] by using agar dilution method, the MIC breakpoint used for Levofloxacin were those proposed by the clinical and laboratory standards institute criteria [16]

Productien of *L. fermentum* filtrate

Filtrate was prepared according to [17], MRS medium was inoculated with 0.5 McFarland of *L. fermentum* broth, incubated at 37℃ for 48hrs in anaerobic condition, centrifugation at 5000rpm for 15min., the supernatant was taken and filtered by using millipore filter (0.22 nm pore size), loop-full of the filtrate was streaked on the MRS agar plate and incubated for 48hrs. at 37℃ in anaerobic condition to ensure the filtrate is sterile, then was stored at 4℃ untiluse.

Determination of the Minimal Inhibitory Concentration (MIC) of *L. fermentum* filtrate against *P. aeruginosa*:

This Protocol was done according to [18] with some modifications by using serial dilutions of filtrate(1/2, 1/4, 1/8, 1/16, 1/32) with sterile Muller Hinton broth (as Diluent) and complete volume to 2ml. Each tube was inoculated with 100µl of 0.5 McFarland *P.aeruginosa*, then incubated for 24 hrs. at 37℃.

Determination Minimum Biofilm Eliminating Concentration (MBEC) of of *L. fermentum* on Polystyrene plate:

This Protocol was done according to [19] with some modifications,by using serial dilutions of *L. fermentum* filtrate(1/2, 1/4, 1/8, 1/16, 1/32) with sterile tryptic soy broth (as Diluent) in micro-titer plate and the volume was completed to 100µl. Each well was inoculated with 0.1ml of 0.5 McFarland of *P. aeruginosa.,*after the incubation period(24-48) hrs. at 37℃, 1% crystal violet stain was used, absorption was determined at 492nm by ELISA Reader.

Inhibition effect of *L. fermentum* filtrate against *P. aeruginosa* adhesion on coupons

Three types of coupons as abiotic surfaces (stianless steel, glass and polystyrene (plastic)) were used in this test, prepared according to [20, 21] which cut into uniform size with dimensions of 1cm x1 cm. Biofilm formation test by using crystal violet was conducted according to [22] with some modifications. In short, The sterile different types of coupons were added to tubes containing of 2.5 ml of sterile tryptic soy broth with 2.5 ml of *L. fermentum* filtrate, which was previously inoculated with 0.5 McFarland of *P. aeruginosa* suspensions(each tube was triplicated), after the incubation period, the tubes were washed by phosphate buffer saline to eliminate loosely attached bacteria (2-3) times, 2.5 ml of 1 % crystal violet stain was add to each tube for 10 min.

The staining solutions were discharged and coupons were washed off three times with (PBS), after drying, the bounded crystal violet to coupons were solubilized with 2.5 ml of glacial acetic acid for 15 minute, concentration

of crystal violet was calculated by determining O D of destaining solution at 492nm.

Inhibition effect of *L. fermentum* filtrate against *P. aeruginosa* adhesion on uroepithelial cells

Urinary sediment uro-epithelial cells(from human female) were prepared, adherence to epithelial cells was tested as previously described by [23, 24, 25], Overnight cultures of *P. aeruginosa* was suspended to 10^8 cells/ml in phosphate buffered saline (PBS). Equal volumes of the *P. aeruginosa* suspensions and uro-epithelial cells with *L. fermentum* filtrate were mixed and incubated at 37 °C for 30 min. After incubation period, the suspensions were washed with PBS, cells were collected by centrifugation (1000g for 10 min) then placed on microscope slides, fixed with ethanol, and 1% crystal violate was used for staining. As a positive control of adherence, uro-epithelial cells incubated with bacteria free *L. fermentum* filtrate.The number of attached bacteria was calculated in 40 random uro- epithelial cells.

RESULTS AND DISCUSSION

Isolation and Identification

Forty-five isolates of *P.aeruginosa* were obtained from urine and wound samples. Isolates were identified according to their morphology and biochemical characteristics, also VITEK were used to confirmation bacteria as *P. aeruginosa*.

Also Five isolates of *Lactobacillus* spp. that isolate form yogurt sample gave a creamy appearance on MRS media and was confirmed the identification by biochemical test and 16S rRNA sequencing. Figure 1

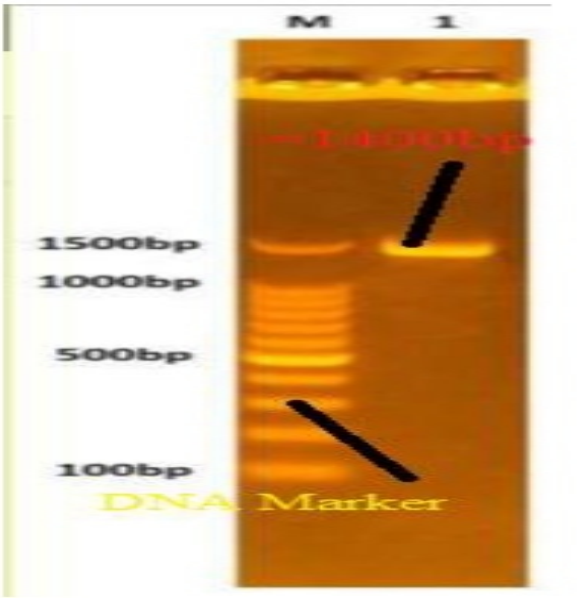

Figure 1. 16s RNA gene of *lactobacillus spp.* were separated on 1% agarose gel electrophoresis stained with ethidium bromide.

After sequencing, data were analyzed by using blast website, the outcome result allowed to determine *lactobacillus* at species level. Results data appear that the bacterial isolate (LB4) belong to following species *Lactobacillus fermentum*.

Lactobacillus alignment with universal isolate recorded on BLAST showed 97% identification, figure 2.

The frequency of *P. aeruginosa* isolation was equal to 50.56 % is nearly similar to [26] who stated that the isolation percentage of *P. aeruginosa* from wound and burn swabs was around (52.37%). Nevertheless, the results disagree to some extent with the findings of [27, 28] who declared that the isolation percentages of *P. aeruginosa* was (32.3%) and (27%) respectively.table 1.

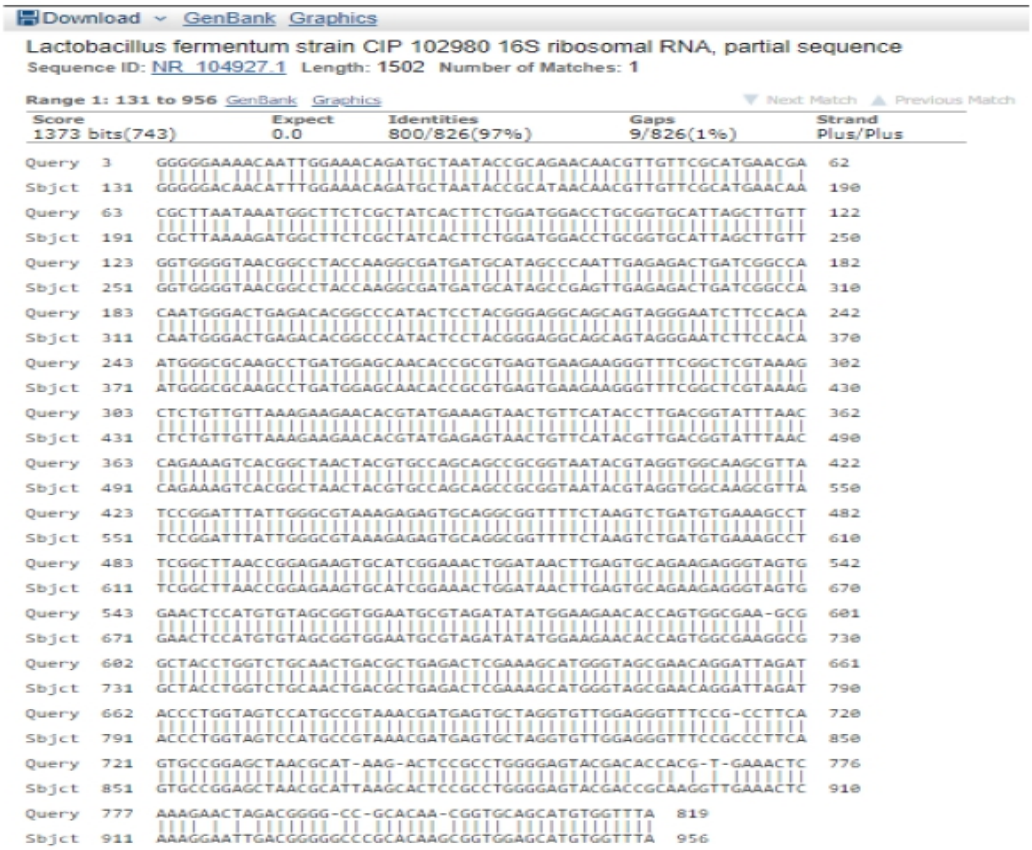

Figure 2. *Lactobacillus* alignment with16S ribosomal RNA of *Lactobacillus fermentum* sequence

Table 1. prevalence of *P. aeruginosa* in urine and wound sample.

Sample source	No. of sample	No.of *P.aeruginosa*	%
Wound	**41**	**26**	**57.7**
Urine	**48**	**19**	**42.2**
Total	**89**	**45**	**99.9**

The table show *P. aeruginosa* isolate percentage obtained from wound sample is higher than isolated from urine as [28] who stated the highest percentage of *P. aeruginosa* was form wound sample also [29] who found *P.aeruginosa* is the most common bacteria isolated from burn and wound infection.

Biofilm formation

- Hydrophobicity assay

The test shows there are 28(62.2%) isolates were strong biofilm formation while there is 17(37.7%) isolates were moderate biofilm producer. The evaluation of bacterial hydrophobicity was achieved by microbial adhesion to solvent test [30]. It consisted of assessing the attraction of the cells towards a polar solvent (Xylene). Moreover, Hydrophobicity of bacterial surface is important to determining the adherence and colonization of bacteria ability to both living (epithelial mucous tissues) and nonliving (medical devices) surfaces [31].

- Polystyrene titer plate method:

Twelve *P. aeruginosa* isolates were tested by Micro titer plate and the results revealed that 5 (41.6%) isolates were strong biofilm producers while 6 (50%) were moderate biofilm producers, only one (8.3%) isolate was weak biofilm producers, table (2, figure 3), its nearly similar to results of [32] who found that there are (41%) isolates was moderate biofilm formation, *the most efficient biofilm producers* isolate (P7 isolate) was selected for other experiments in recent study.

Table 2. O.D values of biofilm formation on microtiter plate

Isolate Name	Mean	Category
Control	0.0886	
P1	0.3364	Moderate
P2	0.355	Moderate
P5	0.4598	Strong
P7	0.749	Strong
P18	0.3556	Moderate
P21	0.2586	Moderate
P28	0.4458	Strong
P33	0.4028	Strong
P37	0.3616	Moderate
P39	0.142	Weak
P41	0.288	Moderate
P44	0.726	Strong

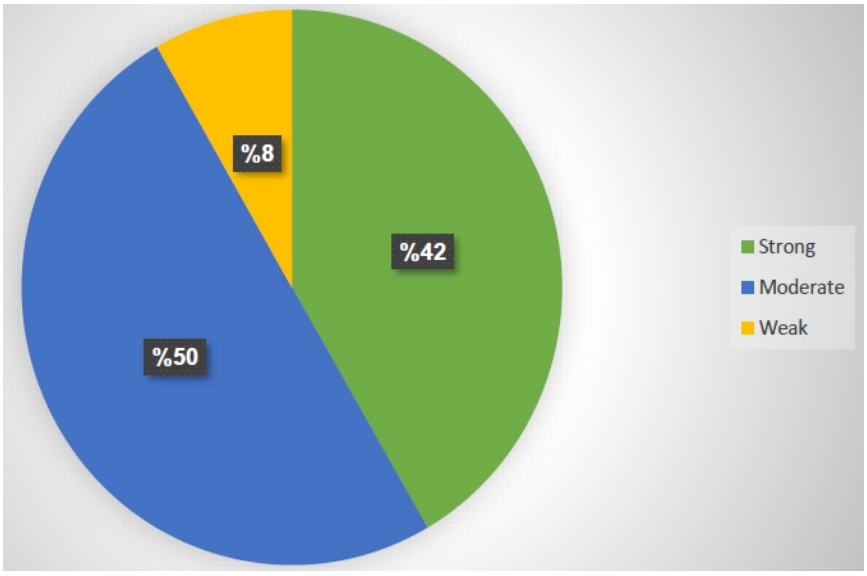

Figure 3. Percentage of biofilm producers *P. aeruginosa* on microtiter plate

Determination of levofloxacin minimal inhibitory concentration

The results revealed that P7 isolate was inhibited at relatively high concentration of Levofloxacin with MIC value of 128 µg/ml therefore, this isolate was subjected to further experiments.

Determination the Minimal Iinhibitory Concentration (MIC) of *L. fermentum* filtrate against *P. aeruginosa*

This experiment is conducted to determine the lowest dilution of *L. fermentum* filtrate to inhibit the growth of *P. aeruginosa* P7 isolate. The findings of this experiment revealed that (1/8) dilution inhibited the bacterial growth (figure (4).

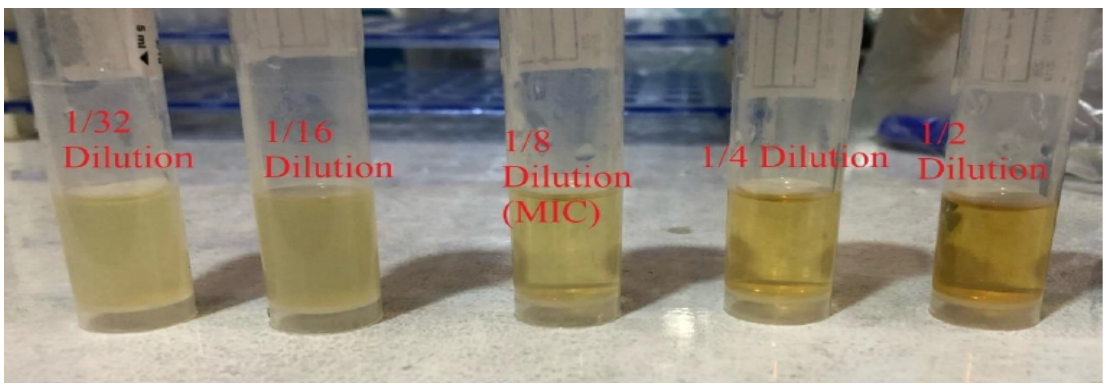

Figure 4. Minimal inhibitory concertation *of L. fermentum* filtrate against *P. aeruginosa* P7

Determination Minimum Biofilm Eliminating Concentration (MBEC) of *L. fermentum* filtrate on Polystyrene plate:

The test was useful to determine the potential impact of

sub-inhibitory dilutions of *L. fermentum* filtrate to inhibit the biofilm formation by *P. aeruginosa* P7 isolate on polystyrene micro titer plate, the result revealed that 1/16 dilution significantly ($P < 0.05$) decreased the biofilm formation process. However, lower dilutions have no influence in inhibiting the biofilm formation**,** table 3**.**

Table 3. Effect of *L. fermentum* filtrate on Biofilm formation

Dilution	1/16	1/32	1/64	0	P-value	MSE
R1	0.408	0.59	0.6	0.59	3.43E-07	0.001
R2	0.4	0.57	0.58	0.57		
R3	0.41	0.56	0.585	0.574		
Mean	0.406	0.573	0.588	0.578		

R: Replication

Several studies showed that antibacterial mechanisms of *Lactobacillus* spp. may be due to a number of factors including the production of bio-surfactants, that inhibit pathogen adherence, decreasing pH levels, production of hydrogen peroxide (H2O2), lactic acid and bactericidal or bacteriostatic substances, including di-acetyl and small heat stable inhibitory peptides (bacteriocins) [33].

Inhibition effect of *L. fermentum* filtrate against *P. aeruginosa* adhesion on coupons

This test is used for determined ability of biofilm formation inhibition by *Lactobacillus* filtrate on the glass, plastic and stainless steel coupons. The results found that the filtrate able to inhibit biofilm formation of *P. aeruginosa* (P7) isolate on stainless steel more than glass and more that plastic (polystyrene) (table 4). This result may depend on several factor such as stainless steel considered as non-pore surface, that make steel smooth surface than other materials, therefore. Furthermore; adhesion force that generated between bacterial cell and stainless steel surface was weaker than force with other surface so it can remove easily.

Surface roughness of the attachment surface is an essential agent which can act on the removal of bacterial cells. The rougher is the surface, the more deep crevices present on the surface, the high retention of the bacterial cells during rinsing process may be due to the possible catch of microorganisms in surface crevices, because these crevices provide place to hide the attached bacteria from shear force [34].

Table 4. Effect of *L. fermentum* filtrate on biofilm formation by *P. aeruginosa* on coupons

Coupons	Plastic	Control	Glass	Control	Steel	Control
R1	0.476	0.589	0.148	0.368	0.379	0.709
R2	0.525	0.600	0.191	0.363	0.451	0.744
R3	0.468	0.536	0.185	0.254	0.437	0.733
Mean	0.489	0.594	0.174	0.328	0.422	0.728
	P-value = 0.0326		P-value = 0.01776		P-value= 0.000229	

R: Replication

Inhibition effect of *L. fermentum* filtrate against *P. aeruginosa* adhesion on uroepithelial cells

This test was used for determine the action of *L. fermentum* filtrate to prevent the adhesion of *P. aeruginosa* P7. Figure (5- B) showing there is approximately (15-20) bacterial cell adhesion to epithelia cell while in figure (5 – A) there are very few cells adhered (0-2) after adding of *L. fermentum* filtrate. This result may be explained by the action of the inhibitory materials found in *L. fermentum* filtrate and to the effect of acidic pH on growth of the gram -negative bacteria by changing several structures of surface such as pili, causing of prevent adhesion of bacterial cells to uroepithelial cells. So biosurfactant as produced by Lactobacillus may help in the development of antiadhesive biologic coating [35, 36, 37].

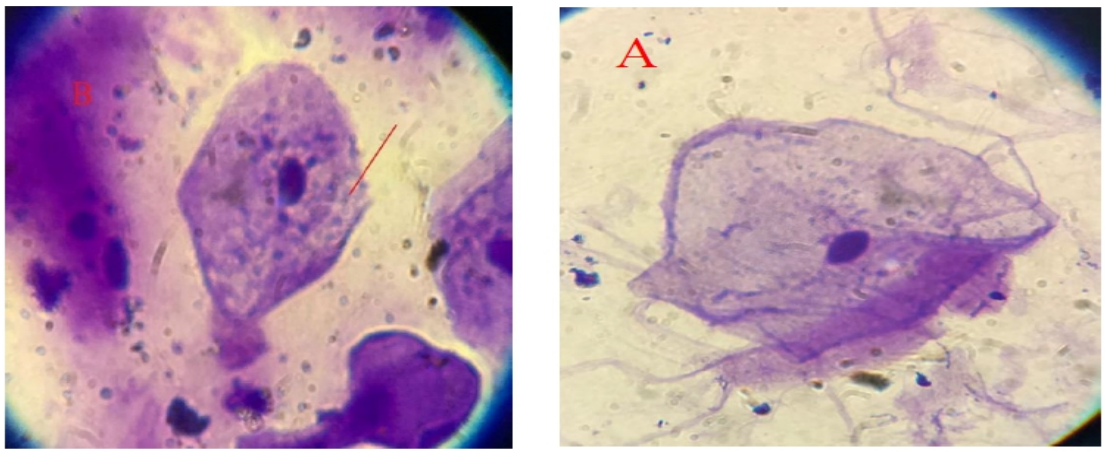

Figure 5: A: Effect of *L. fermentum* filtrate on adhesion on *P. aeruginosa*, B: *P. aeruginosa* adhesion on Epithelial cell before adding of filtrate

CONCLUSION

Recent study concern on inhibitory action of *Lactobacillus* against biofilm formation by *P. aeruginosa* on abiotic and biotic surface which may be useful in understanding of probiotic role as alternative antibacterial and antibiofim agent at clinical and environmental level.

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