

EFFECT OF PROBIOTICS ON SOME VIRULENCE FACTORS OF *PSEUDOMONAS AERUGINOSA* ISOLATED FROM CLINICAL SAMPLES

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ABSTRACT

This study aimed to detect the inhibitory effect of some probiotics against pyocyanin and lipases activity produced by *Pseudomonas aeruginosa*, and comparing this effect with that of the commonly used antibiotics. From a total of 209 bacterial isolates collected from different clinical sources (Urine, ear, sputum, congenital disorder fluids, pus, and wounds) of two hospitals in Baghdad, 16 of them were identified as *P. aeruginosa*. When the sixteen isolates were examined for pyocyanin and lipases production on glycerol supplemented nutrient agar and tween 80 agar, respectively, all isolates, except one,

were positive for pigment and enzymes production. Four probiotic microorganisms namely (*Lactobacillus acidophilus*, *Lb. fermentum*, *Lb. plantarum* and *Saccharomyces boulardii*) were propagated in their media. After incubation, their fermented aliquots were centrifuged and filtered which were considered as unconcentrated filtrates. From these filtrates, one, two and three-fold concentrated filtrates were prepared. Antibiotic solutions of Amikacin, Ceftazidime, Imipenem and Tobramycin were also prepared at a concentration of 10µg/ml for comparison. Results revealed that the three-fold concentrated filtrate of *Lb. acidophilus* was able to inhibit pyocyanin activity strongly (almost entirely) and lipases moderately, followed by those filtrates of *Lb. fermentum* and *Lb. plantarum* which were less strongly in their inhibition. On the other hand, the three-fold filtrate of *S. boulardii* was weakly effective in this regard. Regarding the antibiotics used in this study, Ceftazidime was the most effective one, followed by Imipenem and Tobramycin with moderate effect, then Amikacin with the least effect, while neither Imipenem nor Tobramycin were unable to show any effect on lipases produced by *P. aeruginosa*. From the results, it can concluded that among all

probiotics and antibiotics used in this study, the three-fold concentrated filtrate of *Lb. acidophilus* was the superior in its ability to inhibit the *Pseudomonas aeruginosa* virulence factors included in this study (pyocyanin pigment and lipase enzymes), while the antibiotic Ceftazidime was the most effective compared to the other antibiotics used.

KEYWORD: Lipases, Pyocyanin, *Lactobacillus*, *Saccharomyces boulardii*, Virulence factors of *P. aeruginosa*.

1. INTRODUCTION

Pseudomonas aeruginosa bacteria is widespread in nature, inhabiting soil, water, plants, and animals (including humans). It is an opportunistic pathogen, that rarely causes disease in healthy persons, but its infections is multifactorial and complex. In addition to its pathogenicity, this bacterium has minimal nutritional requirements and can tolerate a wide variety of physical conditions (Koenig 2012).

An important virulence factor of *P. aeruginosa* is pyocyanin, synthesis of pyocyanin is primarily controlled by the quorum sensing process (cell-cell signal communication). *P. aeruginosa* strains which are unable to synthesize pyocyanin can still benefit from its effects if the strain has co- infected the lung with wild type strains which can produce pyocyanin. Denning *et al.*, (2003) suggested that Biosynthesis of pyocyanin can be impaired by disrupting pathway responsible for the synthesis of chorismic acid from shikimate. Pyocyanin inactivates catalase so superoxide radical generated can inhibit cytokines such as IL-4 and IFN- γ which usually upregulate NADPH oxidase. When the lung is confronted with pyocyanin, an increased concentration of catalase and superoxide dismutase is seen in order to deal with the barrage of radicals being produced (Huimin and Hassett 2003).

Another virulence factor of *P. aeruginosa* is lipase, which hydrolyzes esters of glycerol with preferably long-chain fatty acids. They act at interface generated by a hydrophobic lipid substrate in a hydrophilic aqueous medium (Jaeger *et al.*, 1993). Tielen *et al.* (2013) found that the extracellular lipase LipA of *P. aeruginosa* interacts with the polysaccharide alginate in the extracellular biofilm matrix via electrostatic interactions suggesting a role of this interaction for enzyme immobilization and accumulation within biofilms, this represents a physiological advantage for cells.

Probiotics is a term describes the use of live microorganisms as food supplements improving the intestinal microbial balance of the host (Salminen *et al.*, 1999). Their strategies devised the use of probiotics as an alternative therapy for treatment and prevention of bacterial infections (Fooks and Gibson, 2002; Bomba *et al.*, 2006). A growing interest in probiotics as a safe therapeutic agent through their ability to enhance non-specific and specific immune responses, suppress intestinal infections, and anticarcinogenic activity (Grajek *et al.*, 2005).

Doron and Gorbach (2006) found that probiotics have several mechanisms to exert their beneficial effects; they prevent colonization, cellular adhesion, invasion by pathogenic organisms, they have antimicrobial activity, and they modulate the host immune response.

2. MATERIAL AND METHODS

2.1 Isolation of *p. aeruginosa*

A total of 209 bacterial isolates were obtained from various clinical samples (Urine, ear, sputum, congenital disorder fluids, pus, and wounds) from two hospitals in Baghdad and were screened by biochemical tests and Api20E system to isolate *p. aeruginosa*.

2.2 Probiotic organisms and antibiotics

Sugar fermentation test was performed to four previously already identified probiotic microorganisms namely (*Lactobacillus acidophilus*, *Lb. fermentum*, *Lb. plantarum* and *Saccharomyces boulardii*) and four antibiotics solutions of Amikacin, Ceftazidime, Imipenem and Tobramycin were also prepared at a concentration of 10µg/ml.

2.3 Laboratory prepared media

A) Glycerol supplemented nutrient broth: (Rahman *et al.*, 2009)

The medium (GSNB) was prepared by dissolving 25 g nutrient broth powder in 50 ml glycerol and 950 ml D.W. After agar was added, the medium was distributed into 250 ml conical flasks (50 ml each) and autoclaved, before keeping at 4°C until use.

B) Glycerol supplemented nutrient agar

The medium (GSNA) was prepared as in previous item but with the addition of 15g agar. Then, autoclaved and kept at 4°C until use.

C) Tween 80 agar: (Gopinath *et al.*, 2005)

It was prepared from two solutions as follow:

Solution A: Prepared by dissolving 10.0g Peptone, 5.0g NaCl, 0.1g CaCl₂.2H₂O and 15.0g agar 900 D.W. pH was adjusted to 7. Solution B: Prepared by dissolving 10.0g Tween 80 in 100 ml D.W., after the two solutions were autoclaved separately and cooled to about 50°C, they were mixed before distributing in Petri dishes and keeping at 4°C until use.

D) Lipolytic medium: (Zouaoui *et al.* 2012)

It was prepared by dissolving 3 g yeast extract, 1 g peptone, 10 ml olive oil, 0.7 g K₂HPO₄, 0.3 g KH₂PO₄, 0.5g MgSO₄, 0.1g MnCl₂, 0.25 g (NH₄)₂SO₄ and 0.1g CaCl₂, 20 g dextrose in 990 ml D.W. The pH of the medium was adjusted to 7.2 before autoclaving. Then, the medium was distributed into 500 ml conical flasks (100 ml each) and kept at 4°C until use.

2.4 Detection of *P. aeruginosa* pyocyanin and lipases by plate method:

A) Detection of pyocyanin

Glycerol supplemented nutrient agar (GSNA) was poured into the plates to a depth of 3 to 4 mm. After solidification, the bacterial culture was inoculated onto the medium in the plates. Pyocyanin producing isolates were identified on plates after incubation for 72 h at 28°C. The growth on the substrate causes the formation of bluish green halos around bacterial colonies visible to naked eye. Diameters of the colored zone (production of pyocyanin) by the isolates were measured in mm.

B) Detection of lipases

All lipases-producing bacterial isolates were screened on Tween 80 agar medium, then inoculated onto the middle of solid tween 80 agar medium and incubated for 48 h at 35°C. Presence of precipitate which forms a clear zone around the colonies indicates lipases production. The diameter of clear zone was measured for each colony in mm. (Kumar *et al.*, 2012).

2.5 Probiotic preparation

A) *Saccharomyces boulardii*

-Preparation of unconcentrated filtrate

Yeast isolates possessing antimicrobial activity were determined by growing the isolates in SDB, then the medium was distributed in 250 ml conical flasks (each contained 100 ml) before sterilized by the autoclave. The flasks were inoculated with 2 ml of each yeast isolate suspension that already grown for 48 h, and incubated at 28°C for 24 h. After incubation, yeast suspension was centrifuged at 3000 rpm for 15 min, then filtered through autoclaved

Whatman filter paper No.1 (Patil and Nair 2014). This filtrate is considered as the unconcentrated filtrate.

-Preparation of concentrated filtrate

A volume of 100 ml of the unconcentrated filtrate was concentrated to one-fold filtrate by putting in the vacuum oven at (40-45) °C until the volume decreased to (50 ml). The experiment was repeated on the one-fold concentrated filtrate to obtain the two-fold concentrate filtrate (25 ml) and same thing was done for the three-fold concentrate filtrate (12.5 ml) (Izgü and Altınbay, 1997).

B) *Lactobacilli*: Preparation of filtrates

Filtrates of *L. acidophilus*, *L. plantarum* and *L. fermentum* were obtained by growing the isolates in MRS broth with pH 6 and 2% inoculum, then incubated anaerobically at 37°C for 24 h in MRS broth. After that the isolates were centrifuged at 6000 rpm for 10 min. The suspension was taken and filtrated through autoclaved Whatman filter paper No.1 (Moncada *et al.*, 2012). This filtrate is considered as the unconcentrated filtrate. One, two and three-fold filtrates were prepared as in above.

2.6 Effect of probiotics and antibiotics on *P. aeruginosa* virulence factors

A) Pyocyanin extraction

P. aeruginosa was cultured into brain heart broth and incubated overnight then 2% inoculum was inoculated into 50 ml GSNB, After 72 h incubation in GSNB at 28°C (Saha *et al.*, 2008), 50 ml culture broth was centrifuged at 60000rpm for 10 min at 4°C (Contreras *et al.*, 2013). Then, the cell free filtrate was filtered through Whatman filter paper No.1 before mixing with 0.5 total volume chloroform by vortex for 30 sec. After allowed to settle for 10 min, the bottom bluish layer was drained and mixed with 0.2 total volume of 0.1N HCl by vortex for 30 sec. The aqueous pink layer was obtained after 10 min, then pH was adjusted to 7. The acidified balanced water was used as a blank and preserved at 4°C. The absorbance was measured at 520 nm, OD₅₂₀ is multiplied by 17.072 to obtain µg/ml (Essar *et al.*, 1990).

B) Lipases turbidimetric assay

For the turbidimetric assay Tigerstrom and Stelmashuk procedure (1989) was followed with slight modifications: After incubation of 6% inoculum (0.6 at OD₆₀₀) in lipolytic medium for 48 h the culture broth was centrifuged at 6000 rpm for 10 min at 4°C. Then the cell lysate were filtered through Whatman filter paper No.1., and 100 ml of esterase crude filtrate was

added to 2% (v/v) Tween 20 in 20 mM Tris-HCl (1.2 ml), with pH 8.0, and 120 mM CaCl₂ (33 ml). The reaction was stopped after 30 min by placing the reaction tubes into a cold bath as mentioned by (Thakur *et al.*, 2014) to determine the absorbance at OD₅₀₀.

Lipases activity was calculated according to the equation stated by Oliveira *et al.* (2014), as in the following: Activity (U/L) = (Abs* V_S)/ (ε*T*p).

Abs= absorbance at OD₅₀₀, V_S = volume of substrate solution (ml), ε = molar extinction coefficient of substrate, T= temperature (°C), P= volume of sample (ml), U/L = μmol/min/L.

D) Effect of antibiotics on pyocyanin and lipases activity

Each antibiotic was incubated at 1:1 ratio with each of the pyocyanin extract and the lipases adding lipases to the substrate mixture for 15 min and 30 min respectively (Manago *et al.*, 2015; Borkar *et al.*, 2009), then the activity was measured by employing the turbidity reduction assay for pyocyanin and lipases, using D.W. as a control multiplying final result by two for pyocyanin.

E) Effect of probiotics on pyocyanin activity

To investigate the effect of each probiotic, unconcentrated and concentrated filtrates of each of:

Lactobacillus acidophilus, *Lactobacillus plantarum*, *Lactobacillus fermentum* and *Saccharomyces boulardii* was added separately at 1:1 ratio, to the pyocyanin extract and incubated at 37°C for 15 min, Then equal additions and aliquots were used to re-extract pyocyanin as in pyocyanin extraction procedure without PH neutralization, using centrifuged filtered MRS as a control.

F) Effect of probiotics on lipases activity

To investigate the effect of each probiotic, unconcentrated and concentrated filtrates of each of:

Lactobacillus acidophilus, *Lactobacillus plantarum*, *Lactobacillus fermentum* and *Saccharomyces boulardii* was added separately at 1:1 ratio to the lipases enzyme reaction mixture with its substrate and incubated for 30 min, then the activity was measured by employing Lipases turbidimetric assay , using centrifuged filtered MRS as a control and subtracting OD₅₀₀ of each probiotic mixture from the final reading.

3. RESULTS AND DISCUSSION

3.1 Detection of virulence factors

Sixteen isolates were identified as *P. aeruginosa*. The two isolates (Ps1 and Ps4) gave the largest coloration zones of pyocyanin (15 mm each) after 72 h incubation on GSNA, both of the isolates were obtained from urine samples, and they were chosen for the subsequent experiments in this study.

Similarly, Ozyurek *et al.* (2016) found that the highest pyocyanin production can be obtained from *P. aeruginosa* of urine samples other than those of other clinical samples.

Isolates Ps8 and Ps16 were the most efficient two isolates to produce lipases when their hydrolyzed zones reached 9 and 8 mm, respectively. So, they were chosen for the subsequent experiments in the study.

Diameters of lipases production by *P. aeruginosa* on tween 80 agar in this study were rather closed to those reported by Zouaoui *et al.* (2012) who found that *P. aeruginosa* isolates when grown on tween 80 agar gave hydrolyzing zone diameters ranging between 5 to 12 mm.

3.2 Antibiotics effect pyocyanin and lipases activity

All four antibiotics showed various levels of inhibition against pyocyanin activity. Ceftazidime was the most effective one, Imipenem (a carbapenem antibiotic) and Tobramycin (an aminoglycoside antibiotic) followed Ceftazidime in their inhibitory effect on pyocyanin activity. Both antibiotics gave much closed inhibitory effect values, Amikacin was the least effective antibiotic from the four used in the study.

Alatraktchi *et al.* (2016) declared that pyocyanin concentrations of the control were a little less than their highest expected concentrations in seriously infected human body since the two control concentrations rate was 24.35 µg/ml, while pyocyanin concentrations may be as a bit as 0.54 µg/ml and reach up to 27 µg/ml in the septum of infected human. In the optimization study by Abo-Zaid *et al.* (2015), the pyocyanin concentration was elevated up to 26 µg/ml.

Results show that from the four antibiotics used in the current study, only two of them, namely Ceftazidime and Amikacin were effective on the lipases activity. Moreover, Ceftazidime was more effective than Amikacin when it reduced the productivity by about

half, compared to the amikacin which only reduced it to about 1/3 of the initial activity. Adversely, both Imipenem and Tobramycin were non-effective in this regard.

Bisht *et al.* (2012) found that lipases activity of the control samples varied from 250U/L and 450U/L, while lipases activities of the control of *P. aeruginosa* varied between 320U/L to 365.4U/L.

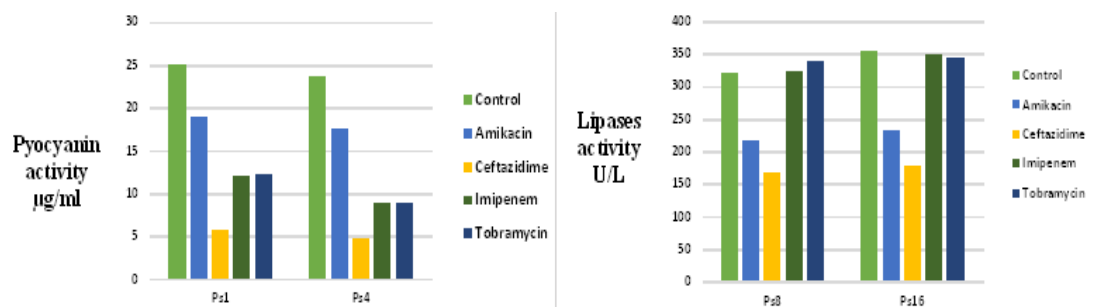


Figure (1) Effect of antibiotics on pyocyanin activity produced by *Pseudomonas aeruginosa* isolates Ps1 and Ps4.

Figure (2) Effect of antibiotics on lipases activity produced by *Pseudomonas aeruginosa* isolates Ps8 and Ps16.

Adding Cefixime to the growth media was found to decrease pyocyanin activity to 50% (Sweedan, 2010). Both Ceftazidime and Cefixime (belong to the third generation of cephalosporins), also Cephalexin (a first generation of cephalosporins) decreased pyocyanin activity to 38%. Bala *et al.* (2011) suggested that using sub MIC of azithromycin (a macrolide antibiotic) gave raise to *P. aeruginosa* virulence factors attenuation in UTI infected mice model since quorum sensing, biofilm formation and motility were substantially prohibited *in vitro*.

The addition of Lincomycin and Clindamycin to the growth media caused inhibition of the production of lipases by *Propionibacterium* spp. with little effect on growth rates as stated by Sheila and Curtis (1982).

Akamatsu *et al.* (2002) concluded that one mechanism of Roxithromycin (semi-synthetic macrolide antibiotic) in *Propionibacterium acnes* treatment is the inhibition of bacterial lipases production, A study by Lu *et al.* (2015) uncovered that inactivation and sub-lethal injury of some pathogens including *Escherichia coli* and *Pseudomonas aeruginosa* may produce oligosaccharides and potentially other components in response to stress. Apparently, living *P. aeruginosa* respond in different ways to abiotic stress depending on the effecting factor. The best chosen therapy would be spontaneously the one that act synergistically

against survival of *P. aeruginosa*, virulence factors production, as well as their activities, Probiotics may be preferably, the candidates as alternatives for these conditions.

3.2 Probiotics effect pyocyanin and lipases activity

Lb. acidophilus filtrates were the most effective against pyocyanin activity showing a concentration dependent effect (CDE). The three-fold concentrated filtrate of this probiotic bacteria was the highly efficient one in inhibiting pyocyanin activity. When compared to all four antibiotics used in this study, the three-fold concentrated filtrates of *Lb. acidophilus* was found to be more efficient in reducing activity of pyocyanin produced by both isolates (Ps1 and Ps4) of *P. aeruginosa*, *Lactobacillus fermentum* (in its three-fold concentrated filtrate) was less effective against pyocyanin than *Lb. acidophilus* with the same filtrate concentration. Furthermore, the three-fold concentrated filtrate of *Lb. fermentum* was less effective than the Ceftazidime in reducing activity of pyocyanin produced by the two isolates of *P. aeruginosa*, but it was more efficient than antibiotics Imipenem, Tobramycin and Amikacin.

Lb. plantarum filtrates were the least effective among *Lb. spp* under study. Compared to the four antibiotics used, the three-fold concentrated filtrate of *Lb. plantarum* gave more effect in reduction of pyocyanin activity than only the Amikacin, very similar to those of Imipenem and Tobramycin, but less than that of Ceftazidime. the three-fold concentrated of *S. boulardii* was still slightly more effective than the Amikacin, the weakest antibiotic under study.

Though being weaker than all *Lactobacillus spp.*, in the study, both inhibitory effects of *S. boulardii* three-fold concentrated filtrate and Amikacin could not reach half of the control activity. For each of the probiotics filtrates, it was not a serious divergence between the two isolates in pyocyanin reduction values, this may be due to the fact that pyocyanin had the same chemical structure in all *P. aeruginosa* strains.

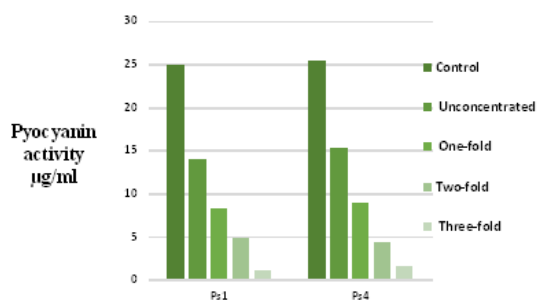


Figure (3) Effect of *Lactobacillus acidophilus* filtrates (Unconcentrated, One-fold, Two-fold and Three-fold) on pyocyanin activity produced by *Pseudomonas aeruginosa* isolates Ps1 and Ps4.

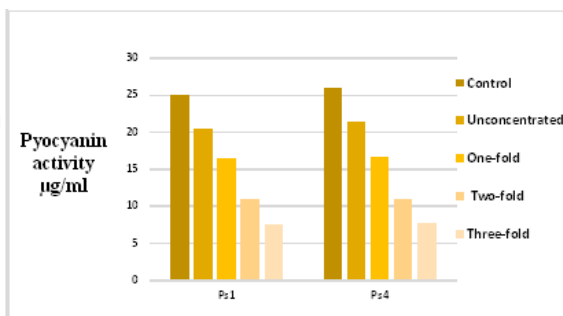


Figure (4) Effect of *Lactobacillus fermentum* filtrates (Unconcentrated, One-fold, Two-fold and Three-fold) on pyocyanin activity produced by *Pseudomonas aeruginosa* isolates Ps1 and Ps4.

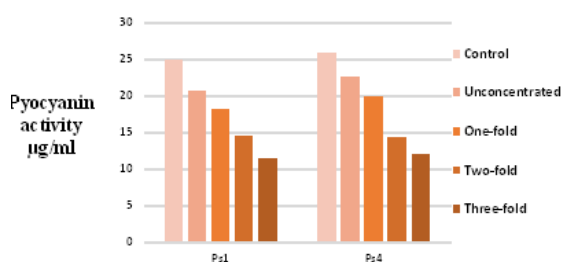


Figure (5) Effect of *Lactobacillus plantarum* filtrates (Unconcentrated, One-fold, Two-fold and Three-fold) on pyocyanin activity produced by *Pseudomonas aeruginosa* isolates Ps1 and Ps4.

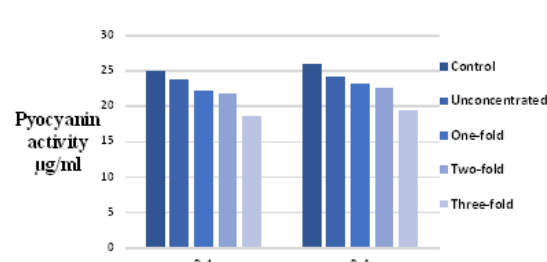


Figure (6) Effect of *Saccharomyces boulardii* filtrates (Unconcentrated, One-fold, Two-fold and Three-fold) on pyocyanin activity produced by *Pseudomonas aeruginosa* isolates Ps1 and Ps4.

All the four probiotics under study showed partial inhibition effects in a CDE and also a strain dependent effect (SDE) for *S. boulardii* filtrates solutions only. All the three *Lb. spp. filtrates* showed similar partial inhibition in such way that the filtrates of *Lb. acidophilus* were the most effective ones followed by *Lb. fermentum* and then *Lb. plantarum*.

The *Lb. spp.* three-fold concentrated filtrate for each of the three probiotics was more effective than any of the antibiotics compromised in this study.

In a similar way of the effects of probiotics on pyocyanin activity, *S. boulardii* filtrates were also the least effective ones on lipases activity.

Among those antibiotics under study, Ceftazidime was the most effective one. However, this antibiotic was weaker in its inhibitory effect on lipases activity than all species of *Lactobacillus* used. Moreover, even it was less effective than *S. boulardii* three-fold concentrated filtrate in one of the pathogenic isolate.

However, these results along with the outcomes of other researchers' efforts suggest a promising benefits of probiotics for the treatment of *P. aeruginosa* infections.

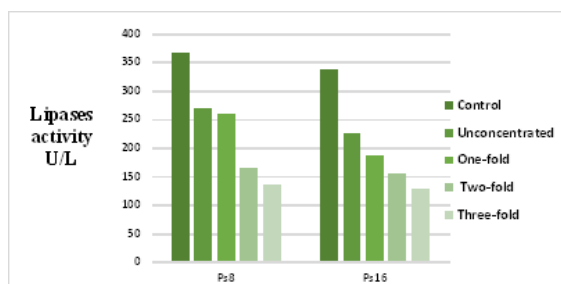


Figure (7) Effect of *Lactobacillus acidophilus* filtrates (Unconcentrated, One-fold, Two-fold and Three-fold) on lipases activity produced by *Pseudomonas aeruginosa* isolates Ps8 and Ps16.

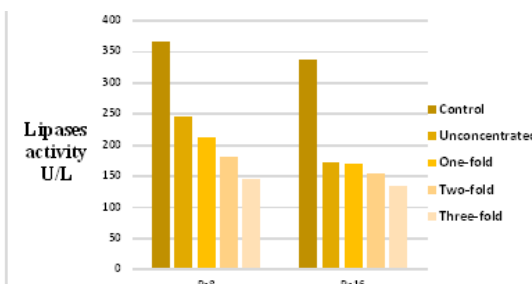


Figure (8) Effect of *Lactobacillus fermentum* filtrates (Unconcentrated, One-fold, Two-fold and Three-fold) on lipases activity produced by *Pseudomonas aeruginosa* isolates Ps8 and Ps16.

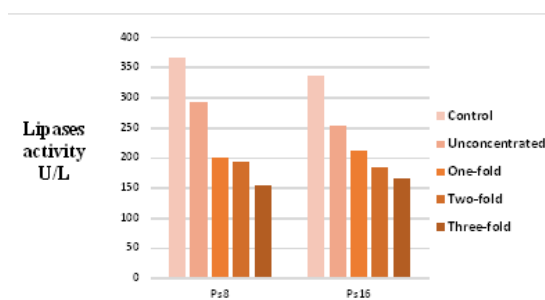


Figure (9) Effect of *Lactobacillus plantarum* filtrates (Unconcentrated, One-fold, Two-fold and Three-fold) on lipases activity produced by *Pseudomonas aeruginosa* isolates Ps8 and Ps16.

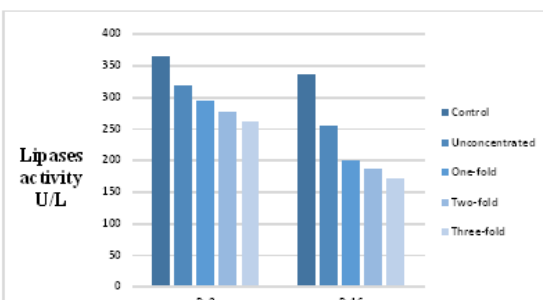


Figure (10) Effect of *Saccharomyces boulardii* filtrates (Unconcentrated, One-fold, Two-fold and Three-fold) on lipases activity produced by *Pseudomonas aeruginosa* isolates Ps8 and Ps16.

Sharma and Chauhan (2014) pointed out that *P. aeruginosa* was most susceptible to growth inhibition by *Lb. acidophilus* and different antibiotics combinations rather than antibiotics alone. *Lactobacillus fermentum* demonstrated anti-adherent activity on *P. aeruginosa* slightly less than of *Lb. acidophilus* according (Hafez *et al.*, 2013).

Lb. fermentum isolated from human colonic mucosal biopsy samples indicated that *Lb. fermentum* produced antimicrobial compounds and cell surface associated proteins to inhibit the growth and adhesion of enteropathogens, such as *P. aeruginosa* (Varma *et al.* 2010). Different *Lb. fermentum* strains filtrates varied in their prevention of elastase activity of *P. aeruginosa* ranging from 53% to 95% inhibition (Alexandre *et al.*, 2014).

In a study by Valdez *et al.*, (2005), the CFU/1g of *P. aeruginosa* isolated from infected burns was reduced only from 10^8 to 10^6 after treatment with *Lb. plantarum*. In addition to its effects against pyocyanin and lipases produced by *P. aeruginosa*, a study by Sharafi *et al.* (2013) suggested occurrence of a change in the cytoplasmic composition of *E. coli* treated by antibacterial compounds purified from *Lb. plantarum*.

The *S. boulardii* filtrates features revealed agreement with Alwan *et al.* comparative study (2014), since it was found that *Lb. acidophilus* filtrates shown up larger zone of inhibition against *Staphylococcus aureus* than *S. boulardii* filtrates, also *Lb. acidophilus* filtrates exhibited inhibitory effect on *S. boulardii*. a study by Sharma and Upadhy (2015) found that the inhibitory effects of *S. boulardii* against *P. aeruginosa* were weaker than those against *E. coli*.

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