ORIGINAL ARTICLE

SYNERGISTIC ANTIBIOFILM ACTIVITY OF PROBIOTIC *LACTOBACILLUS ACIDOPHILUS* AND *PUNICA GRANATUM L.*, AGAINST *PSEUDOMONAS AERUGINOSA* BIOFILM

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Background: Antibiotic resistance is one of the most urgent public health concerns. Biofilm formation is well linked with chronic wounds, chronic obstructive pulmonary disease, urinary tract infections, and cystic fibrosis. Our goal was to assess the biofilm activity of *P. aeruginosa* and the individual and combined anti-biofilm forming activity of probiotic *Lactobacillus acidophilus* and Pomegranate peel extract *Punica granatum L.,* against *P. aeruginosa*. **Methods:** A total of 150 swabs of urine, blood, pus, and CSF were collected from PNS Shifa Hospital Karachi, and *P. aeruginosa* was isolated and identified according to standard bacteriological methods. The ability of *P. aeruginosa* to form biofilms was assessed using a microtiter plate assay. **Results:** The anti-biofilm forming activity of pomegranate peels extract against *P. aeruginosa was* 29.26±19.09 whereas the anti-biofilm forming activity of *Lactobacillus acidophilus* against *P. aeruginosa* was 0.5×10^6. When used in combination, there was significant synergistic activity between Punica granatum L. (pomegranate peel extract) and *Lactobacillus acidophilus*. **Conclusion**: The unique synergistic mixture of natural product extracts and probiotics has demonstrated more efficiency against rapidly evolving pathogens, serving as promising candidates for developing biofilm inhibitors and perhaps proving as possible environmentally friendly agents against bacteria that produce antibiotic-resistant biofilms.

Keywords: Biofilm; *pseudomonas aeruginosa*; Punica Granatum *L., lactobacillus acidophilus*; 96 well microtiter plate synergism

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INTRODUCTION

The rise of antibiotic-resistant pathogens poses a severe risk to morbidity and death globally.[1] Multidrug resistance (MDR) bacteria have long been designated a worldwide priority for investing in novel treatments by the World Health Organization (WHO) as they are an alarming public health concern.[2] In 2016, the WHO created a priority list of antibiotic-resistant bacteria to stimulate research and development of effective medications and other options that can aid in the elimination of MDR bacteria.[3] Biofilms are indeed a natural concern because of their capacity to damage the surfaces on which they grow while also causing infections.[4] Moreover, biofilms are linked to 65% of illnesses and 80% of chronic medical-associated conditions.[5] *P. aeruginosa* is a gram-negative, aerobic, rod-shaped bacterium that can be isolated from soil, plants, and human tissues and is involved in a vast biofilm matrix comprising exopolysaccharides (EPSs), nucleic acids, and proteins.[6,7] The development of biofilms in *P. aeruginosa* depends on a varied mix of exopolysaccharides that use its significant adhesion factors, like flagella and pili. This bacteria can survive on water, diverse surfaces, and surgical devices.[8] As a result, *P. aeruginosa* is common in natural and manufactured habitats, such as lakes, hospitals, and domestic sink drains, becoming a significant source of nosocomial infections in clinical environments and antibiotic resistance.[8] *P. aeruginosa* has been classified as one of the 10th most prevalent antibiotic-resistant bacteria for over a decade due to the prevalence of antimicrobial-resistant strains that induce life-threatening consequences.[9,10] *P. aeruginosa* biofilms have a higher level of antibiotic resistance for several factors, including moderate or insufficient drug penetration, a changed oxidation state within the biofilm, and cellular proliferation in a biofilm.[1] All these processes occur because of the multilayered architecture of biofilms, resulting in antibiotic resistance of the biofilm matrix and treatment strategy failure. Treatment of biofilm infections has already become extremely difficult due to

significant levels of resistance to the majority of mainstream medicines.[11,12] Currently, conventional antibiotic prophylaxis is mainly used to treat *P. aeruginosa* infections; however, increased antibiotic use may result in multidrug-resistant strains of *P. aeruginosa* and failure of traditional antimicrobial therapy.[13,14] Due to the increasing prevalence of life-threatening bacterial infections, traditional natural products and probiotics have been researched, making them a critical success in drug discovery for infectious diseases.[15] These products contain molecules that have low toxicity, specific activity, and high bioavailability. Research has shown that the fruit of *P. granatum* is abundant in anthocyanidins, flavonoids, and phenolic combinations, which are known for their organic activities (antioxidant, anti-inflammatory, anti-biofilm, and antimicrobial).[16] The commonest probiotics belong to the genera *Lactobacillus* spp. As probiotics provide numerous health benefits, recent research highlights the antibiofilm activity of the *Lactobacillus* genus. Osama *et al*. in 2017 and Elbadri et al. in 2019 demonstrated the antimicrobial and antibiofilm action of *Lactobacillus* strains against *Pseudomonas aeruginosa*. These pathogens are commonly involved in persistent infections associated with biofilm formation.[17,18] The efficiency of P. granatum against *P. aeruginosa* has also been measured. However, the soxhlet extraction method for P. granatum has rarely been used. On the other hand, the synergistic anti-biofilm activity of P. granatum and *Lactobacillus Acidophilus* against *P. aeruginosa* biofilm has not been explored yet. Hence, this study aimed to assess the synergistic antibiofilm activity of *Lactobacillus acidophilus* and Punica granatum L. against *Pseudomonas aeruginosa* biofilms.

MATERIAL AND METHODS

The study was conducted between the duration of September 2021 and May 2022. Ethical permission was taken from the Institutional Ethical Review Committee ERC approval no 83/2021. Informed written consent was obtained from all the patients. Parental consent was taken from individuals aged <18. Age, gender, and number of patients were recorded on specially designed subject evaluation proforma. A non-probability convenient sampling technique was utilized. Different samples were collected from PNS Shifa Hospital Karachi, and the number of samples was 150; *P. aeruginosa* was isolated from these swab samples. Specimens that were received in the lab were inoculated on Blood agar and MacConkey's agar culture plates. Culture plates were inoculated at 37 ℃ in an incubator for 24 to 48 hours. Identification of *P. aeruginosa* was done by colony morphology, gram staining, TSI and different biochemical test analyses such as citrate, oxidase, OF glucose, and arginine dihydrolase. After identification, a suspension equal to 0.5 McFarland turbidity standard of all the samples was processed on a 96-well microtiter plate for biofilm production and serial dilution methods for antibiofilm activity.

Inclusion criteria: Patients who consented to provide samples of urine, blood, pus, CSF and respiratory specimen and age group of 15 to 50 years.

Exclusion criteria: Repeated samples, antibiotic use, HIV-infected and malignancy-associated patients.

To prepare the pomegranate peel extract samples, the seeds were manually separated from the peel, then the separated peels were cut into small pieces, and then air-dried at room temperature until a uniform volume was obtained. Air-dried peels were then homogenized with a household electric grinder until a fine powder was obtained. A constant amount of peel powder of (15g) was used to extract the natural bioactive ingredients when placed in a beaker containing (150ml) methanol and left at room temperature for 72 hrs. After preparing the solution, it was filtered with 0.45 m filter paper, then concentrated and evaporated to dry under vacuum using a mini rotary evaporator at 40°C (Soxhlet apparatus) until almost all solvent vaporized. The dry extract obtained from the solvent was stored at -20°C for further use in various tests.[17]

Isolates from freshly cultured plates were again inoculated in Tryptone soy broth (TSB) containing 1% glucose for 24 hours at 37 degrees Celsius before diluting (1:100) with bacterial suspension. Individual wells of sterile polystyrene flat-bottom tissue culturing plates were then filled with 0.2 ml aliquots of the diluted cultures. The broth served as a control to ensure sterility and non-specific media adhesion.

The culture plates were incubated at 37°C for 24–72 hours. Following incubation, the contents in each well were gently removed by tapping the surfaces of the 96-well microtiter. In order to eradicate the free-floating planktonic bacterium, the wells were rinsed four times with 0.2 ml of phosphate buffer solution (PBS pH 7.2). The residual adhering bacterial biofilms were stained with 25 ml of 1% solution of crystal violet dye inserted into each well (this dye stains the cells but not the polystyrene plates).

The plates were then kept at room temperature for about 15 minutes before being

thoroughly cleaned using distilled water. Crystal violet dye was utilized to stain adhering bacterial stained cells that established biofilm throughout the sides of the wells. The dyed biofilms were then solubilized in 200 ml of 95% ethanol (to remove the dye's purple color); 125 ml of these biofilms were loaded into a fresh polystyrene 96-well microtiter plate, which was then read. The optical densities (OD) of 570 nm-stained adherent bacteria were evaluated using a micro ELISA auto reader (model 680, Bio rad). The OD 570nm estimate wavelength was examined to determine which microorganisms adhered to the surface and generated biofilms. The experiments were done in triplicate for each strain.[19]

The following equation is usually used to calculate the FIC index: FIC is calculated as (Ac/A) + (Bc/B), where Ac and Bc represent the combined inhibitory biofilm concentration of the compounds, and A and B represent the respective individual biofilm inhibition concentrations of the compounds.

All experiments were conducted at least in triplicate. All the data was entered on SPSS v.26 (IBM, Chicago, United States) was utilized to summarize patients' demographic data and conduct descriptive statistics. Pearson chi-square test was used for analysis. The significance level was taken as $P < 0.05$. Quantitative data were expressed as mean ± standard deviation.

RESULTS

The gender distribution of the participants is presented in Table 1 showing male predominance, 96 (64%) versus 54 (36%) females.

In Figure 1, the frequency distribution of *P. Aeruginosa* is established according to age groups. Lowest predominance was in the age group <18 (13%) versus highest predominance in the age group 35-50, (58%) subjects.

Figure-2 represents the frequency distribution of biofilm formers and non-biofilm formers of *Pseudomonas aeruginosa*. Among 150 patients 123(82%) were biofilm formers versus 27(18%) non biofilm formers.

Table-2 represents the anti-biofilm forming activity of pomegranate peel extract (PPE) against *Pseudomonas aeruginosa*. The anti-biofilm concentration is shown in mean and standard deviation values (29.26±19.09).

Table no 3 represents the anti-biofilm forming activity of *Lactobacillus* against *Pseudomonas aeruginosa*. The anti-biofilm concentration is given in mean and standard deviation values 0.5x10^6 (CFU/ml).

Table no. 4 presents the combined synergistic effect of Pomegranate Peel Extract (PPE) and *Lactobacillus acidophilus* against *Pseudomonas aeruginosa*. Individual PPE values are shown in mean and standard deviation 29.26±19.09; combined PPE values were 3.60±2.27 in mean and standard deviation. Whereas the individual *Lactobacillus* anti-biofilm concentration shows the value of 0.5x10^6 CFU/ml, combined *Lactobacillus* anti-biofilm concentration shows the value of 0.5X10^5 CFU/ml. Hence, the relationship has been termed as synergistic.

The checkerboard approach defines synergism as a Fractional Inhibitory Concentration (FIC) index of 0.5, portrays synergistic impact as an FIC index of >0.5 and 1, indifference effect as an FIC index of >1 and 2, and antagonism effect as an FIC index of >2 or >4. Concentrations within the FIC index were such that the inhibitory concentration of each compound and *lactobacillus* acidophilus is in the range of concentrations tested.[20]

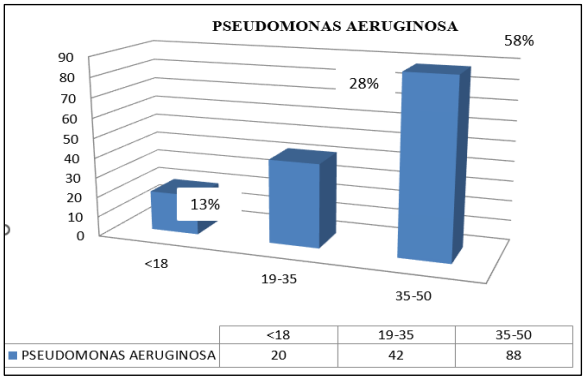

Figure -1: Frequency distribution of *Pseudomonas aeruginosa* according to age groups (n=150)

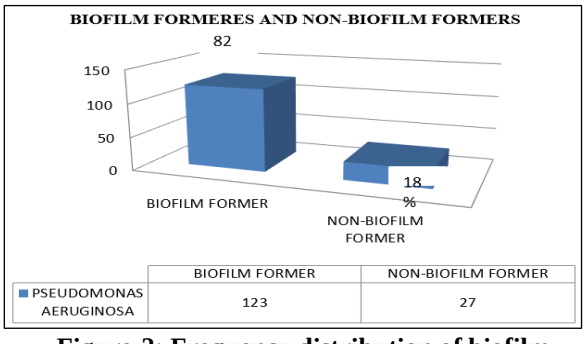

Figure-2: Frequency distribution of biofilm former and non-biofilm former among *Pseudomonas aeruginosa* (n-150)

Table-1: Frequency distribution of genders (n-150)

Gender	Frequency (%)
Males	96(64%)
Females	54(36%)
Total	150

Table-2: Anti-biofilm forming activity of pomegranate peel extract (PPE) against *Pseudomonas aeruginosa*

Culture	compound	Antibiofilm concentration (ppe) mg/ml
Pseudomonas Aeruginosa	**Pomegranate peel extract (PPE)**	**29.26±19.09**

Table-3: Antibiofilm forming activity of *lactobacillus* against *pseudomonas aeruginosa*

Culture	Culture	Antibiofilm activity of L*actobacillus acidophilus*
Pseudomonas aeruginosa	***Lactobacillus acidophilus***	**0.5x10^6 (CFU/ml)**

Table-4: combined effect of pomegranate peel extract and *Lactobacillus* acidophilus against *Pseudomonas aeruginosa.*

Culture	PPE Individual (mg/ml)	PPE Combine (mg/ml)	Lactobacillus Individual (CFU/ml)	Lactobacillus Combine (CFU/ml)	Relation
Pseudomonas aeruginosa	29.26±19.09	3.60±2.27	0.5x10^6 (CFU/ml)	0.5x10^5 (CFU/ml)	Synergism

DISCUSSION

Pseudomonas aeruginosa is an important opportunistic pathogen with strong virulence and an invasive nature. We found a higher incidence of *Pseudomonas aeruginosa* among males than females. This difference could be because *P. aeruginosa* is often linked with surgical infections and wounds, commonly found in men, considering their occupation.[21] Our study shows the maximum prevalence of *P. aeruginosa* in the age group of 35–50 years, which is almost consistent with the results of a study conducted in Gujranwala, illustrating a higher infection rate in the 45–66 age group.[22] This finding could be ascribed to the senior group's higher rate of comorbidities, lower immunity, and ongoing medical treatments. *P. aeruginosa* is a microorganism that is known to form potent bacteria.[23] Biofilms of *P. aeruginosa* create serious complications in immunocompromised people, such as those with cystic fibrosis or wound infection.[24] Furthermore, the unusual biofilm features impede infection clearance, leading to persistent infections. In our study, a higher number of biofilm formers were found; this finding was very close to a research conducted in India where 30 isolates were found to be *P. aeruginosa* positive out of 60.[25] It is also in accordance with an Iranian study where 87% of the clinical isolates were bio-film producers.[26] However, our findings contradict an Egyptian survey where P. aeruginosa was found in only 45% of the isolates.[27] In another research in Punjab, Pakistan, out of 200 samples, 52 (26 %) were affected with *P. aeruginosa* on blood agar. In that study, the Microtiter plate assay (MPA) detected 49 (94.23 %) isolates as biofilm producers.[1] We believe that the difference in biofilm production in these studies could be because of the different culture methods.

The failure of conventional antibiotic therapies indicates that biofilm treatments need auxiliary up-gradation.[28] Natural anti-biofilm agents selectively exterminate the persistent biofilms and allow the diffusion of bioactive constituents into the biofilm matrix. These natural extracts target various phases of the biofilm cycle to degrade the biofilm matrix and finally inhibit cell growth. Pomegranate peels contain Punicalagins and ellagic acid that can be extracted on large scales as they significantly inhibit in vitro biofilms produced by *P. aeruginosa*.[29,30] We have established the anti-biofilm forming activity of Pomegranate Peel Extract (PPE) against P. aeruginosa. Our results are almost similar to an in vitro study conducted in Turkey, which showed that the antibiofilm mass inhibition of Punica Granatum was 30-20%.[31] Our results are also comparable to a Brazilian study that chose a hydroalcoholic extraction method for pomegranate residues and established inhibition of *P. aeruginosa* biofilm by 45%.[32]

It should be noted that the antibiofilm ability of Punica Granatum L. has not been explored much, especially concerning the biofilm of *P. aeruginosa*. One study revealed the antibiofilm capacity of methanolic extract of pomegranate against biofilms produced by *Candida albicans*, *Staphylococcus aureus*, methicillin-resistant *S. aureus*, and *Escherichia coli*. Ellagic acid was the main component that inhibited the growth of these bacteria.[33] An Iranian study also successfully evaluated the antibiofilm of pomegranate peel extracts with silver nanoparticles against *P.aeruginosa*.[34]

We also demonstrated the anti-biofilm forming activity of a probiotic (*Lactobacillus acidophilus*) against *P. aeruginosa*. Probiotic bacteria are microbial food supplements with beneficial properties on human health. *Lactobacillus* is part of the normal intestine flora, and its probiotic efficiency has been explored.[35,36] It plays a vital role in human health and stimulates the immune system. Our results regarding the *Lactobacillus* activity were consistent with a study conducted in Egypt where it was shown that Lactobacillus acidophilus could remove 91.8% of biofilm formed by *P. aeruginosa* strains.[37] Likewise, in another Egyptian study evaluated the probiotic effect of *Lactobacillus Acidophilus*, a decrease of 68.52% (inhibition) and 43.80 % (removal) was seen in the total biofilm mass of *P. aeruginosa*.[16] Shokri et al.'s study

revealed an 80-100% inhibition effect by *Lactobacillus* fermentum regarding biofilm produced by *P. aeruginosa*.[38] Similarly, in research, seven *Lactobacillus* strains were tested against *P. aeruginosa* clinical isolates. They found a 100% inhibitory effect on the isolates.[39] Hence, bacterial-mediated therapy may be deemed as a successful treatment modality.[40]

Natural sources nowadays are a research focus due to their antibiofilm activity.[41] Our study has evaluated the synergistic effect of pomegranate peel extract and *Lactobacillus acidophilus* against *P. aeruginosa*. The synergistic activity has never been assessed, and this combination showed a significant synergistic effect apart from their individual anti-biofilm activity. Our findings have determined that the combined impact is more effective than the individual effect; hence, it is proven that the inhibition of biofilm formation by pomegranate peel extract and *Lactobacillus acidophilus* with a synergistic combination can play an essential role in the treatment and prevention of biofilm-associated infections. More bacteria apart from *P. aeruginosa* should be researched for biofilm formation and antibiofilm forming activity of other natural product extracts must be evaluated.

CONCLUSION

The ineffectiveness of present antibiotics for managing biofilm-related illnesses is a significant setback. This is due to the layers of protection created by bacteria in the biofilm. Our research study tried to develop a new anti-biofilm agent from Pomegranate peel extract and probiotic *Lactobacillus acidophilus* that is inexpensive, uses waste resources, and can be used as a biofilm inhibitory agent. If natural substances and extracts are examined for desired medicinal benefits, tremendous progress in discovering novel antibiotics, therapies, and other valuable medications will be produced.

Ethics approval and consent to participate: The study was performed in accordance with the Declaration of Helsinki. Ethical permission was taken from Bahria University and Medical College's Institutional Ethical Review Committee ERC with the approval no. 83/2021. Informed written consent was obtained from all participants before administering the questionnaire. Parental consent was taken from individuals aged <18. All protocols were carried out in accordance with university guidelines and regulations.

Consent for publication: Not Applicable

Availability of data and materials: The data sets used and/or analyzed during the current study are available from the corresponding author on request.

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AUTHORS' CONTRIBUTION

Hadia Khursheed is responsible for the conceptualization, planning and writing of the manuscript. Rimsha Qasim wrote, analyzed and critically reviewed the manuscript. All authors have read and approved the final manuscript.

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