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The impact of Nitrogen and Carbon Sources on the Biofilm Formation of *Micrococcus luteus*

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Abstract

This study is conducted to show the influence of different media on the extent and pattern of biofilm formation. Trends of newly emerging pathogens continue steadily. *Micrococcus luteus* is one of those emerging pathogens. Incidental isolations of this bacteria have been recorded from patients with urinary tract infection and/or immunocompromised conditions. Biofilm formation on the surfaces of wound drainage and urinary catheters has been reported to be the source of recurrence and colonization of the pathogen in those patients. The current study's approach assesses the role of nutrient availability on the patterns of attachment till detachment and dispersion of the biofilms. Different species of bacteria are used to correlate their biofilm formation trend. *Micrococcus luteus* was chosen in the study due to its emerging pathogenic potential. Validation of biofilm formation is provided by involving *Proteus mirabilis*; which is an ideal biofilm producer, in parallel with *Micrococcus luteus* throughout the entire experimental settings. The findings of this study confirm statistically significant differences in biofilm formation patterns when nutritionally different culture media have been utilized to resemble possible environments for the pathogen. *Micrococcus luteus* has been found to possess the highest potential to produce biofilm in peptone water media where it over paced *Proteus mirabilis*. Results of the study reveal that both availability and scarcity of carbon and nitrogen sources can influence both positively and negatively on the patterns of biofilm formation by different strains of bacteria and incubation time. Biofilm assessment is an inevitable technique for nosocomial infections due to the complications of antibiotic susceptibility trends that prolong the hospitalization process, which limits treatment capacity.

Introduction

Hospital-acquired diseases are among the most critical implications of post-surgical recovery procedures. Once the infection colonizes through biofilm formation, the patient's recovery may need considerable extensions before the patient's discharge. Biofilm is the number of microorganisms enclosed within an extracellular polymeric substance (EPS) and the cells cooperating or attaching to a surface. It is the favored mode of almost every microorganism, while planktonic microbial increase; virtually rarely exists in nature ***[1-3]***. The microbial cells developing into a biofilm are physiologically and genetically more secure than the same organism's planktonic cells. Existence within a biofilm offers microorganisms a shielding environment that effectively

minimizes attacks of antimicrobials, biocides, detergents, or mechanic stresses ***[4-6]***. There is adequate evidence indicating that biofilm formation leads to an expanded resistance compared to planktonic cells ***[712]***. Biofilms are abundant in nature, aggregates of cells trapped in EPS. The EPS comprises 97% water, less than 1-2% nucleic acids, 1-2% polysaccharides, and less than 1-2% proteins. Microbial biomass takes about 2-5% of the sessile biofilm structure ***[3, 13-18]***. Shifting from planktonic to sessile growth mode is a complicated process in which gene expression and adaptation to available resources result in biofilm formation ***[16, 19-21]***. Surface Attachment is the initial step where cells adhere to the hydrophilic, rough, and supportive solid surface as they slow down their flow with the liquid such as water and blood ***[5, 22]***. Post-attachment chemical signals lead to bacterial multiplication, called Micro-Colony formation, followed by the activation of exopolysaccharide production. Activations of genes related to biofilm formation result in the formation of EPS. Once water fills the channels of the EPS, the three-dimensional (3D) structure will be established ***[4, 22-25]***. Patterns of the biofilm detachment are either natural (i.e., programmed detachment) or due to mechanical stress from the surrounding environment ***[26-28]***. Lytic activities of enzymes to digest alginate, termination of EPS production by microorganisms, and quorum sensing are amongst the other mechanisms that lead to the detachment of the biofilm. Quorum sensing of microbial communication enables microbes to coordinate their gene expression in response to the density of instantaneous local populations. Interaction of microbes includes same as well as different species. Biofilm formation/dispersion, environmental stress (i.e., antibiotics, disinfectants & presence of competing species), and food depletion trends are all coordinated by utilizing quorum sensing systems ***[29-31].*** Post biofilm, planktonic microorganisms might continue exhibiting the acquired traits of the biofilm, such as antibiotic resistance ***[32-34]***. Both availability and scarcity of nutrients can activate transcriptional regulatory mechanisms for the production of EPS. Thus, it is worth investigating for different bacterial strains ***[35]***.
The involvement of *Proteus mirabilis* in this study is due to its ability to form biofilm and the clinical picture presented by the pathogen. *Proteus mirabilis* is frequently isolated from urinary tract infection patients. The presence of urease enzyme is their diagnostic biochemical activities which enables them to hydrolyze urea to ammonia. Consequently, it leads to an increase in the pH of the urine, which ends with precipitations and accumulation of crystals and stones and the inflammation and discomfort of the patient during urination ***[2, 36-40]***.

Micrococcus luteus, on the other hand, has been recently introduced as an emerging pathogen in immunocompromised patients and post-operational hospitalized patients, especially in urological surgeries. Despite their small size at the cellular level, they can generate large biomasses and, more importantly, form biofilms; on the one hand. On the other hand, *Micrococcus luteus* is urease positive. Thus, its long-term threat to the urinary system is not less than what *Proteus mirabilis* is known for ***[2, 35, 41, 42]***.

The most complicated issue with clinical conditions of former biofilm microbes is their ability to resist the antibiotic. It has been recorded that per antibiotic exposure of the microbes in biofilm, only an outermost layer can be influenced. The layer that is close to the surface lining on which the biofilm is formed is not harmed. For an emerging pathogen, it is worth investigating all possible details about its virulence factors and antibiotic susceptibility patterns, especially in the biofilm mode of growth. This study is only to focus on biofilm patterns of the bacteria ***[22, 26, 36, 38]***.

Therefore, this research aims to determine the type(s) of the media that offer better support for biofilm formation, assessing the ability of *Micrococcus luteus* to produce biofilm and examining the incubation time needed for the formation/dispersion of biofilm.

Methods and Materials

A. Biofilm Formation

In this study, four different times and five different media were used for biofilm formation screening. First, the assessment of biofilm formation was carried out by Microtiter Plate (96-Well Plate) Assay. This technique observes bacterial adherence to an abiotic surface possible. 96-Well Microtiter Plates was used to produce biofilm in four batches for 1-, 2-, 3- and 5-day intervals, then the absorbance of released crystal violet was read by BioTek ELISA reader and CECIL 7500 spectrophotometer at 590nm.

Bacterial isolates were obtained from UTI patients and diagnosed by VITEK 2 Compact at Komar Research Laboratory facilities. Biofilm study was commenced with producing (0.6 McFarland) of bacterial suspension from a single pure isolated colony which is equivalent to (1×10^7 CFU mL^{-1}) bacterial suspension, then pipetting 150 µL of different bacteria's suspensions (*Micrococcus luteus* and *Proteus mirabilis*) in different media (Brain Heart Infusion Broth [Oxoid/England], Peptone Water [Neogen/UK], Normal Saline 0.85% w/v Sodium chloride (NaCl), Distilled Water and Nutrient Broth [Neogen/UK] solutions into the wells of sterile 96-well flat-bottomed microtiter plates. The incubation temperature was set at 37ºC. Each bacterial suspension was transferred into 12 separate wells of the 96-well plates; thus, 12 replicas were provided to represent the biofilm formation patterns for each experimental setting.

Bacterial growth was terminated according to the experimental settings in four batches, i.e., after 1st, 2nd, 3rd, and 5th-day intervals. This is done by rinsing off the media residuals following their complete drainage. Microtiter plates were dried then covered, ready for the quantitative crystal violet method. After finishing the 5th-day batch, 200 µL 0.5 % (v/v) of crystal violet stain was added to each well for 15 minutes at room temperature. Crystal Violet drained, and the wells were rinsed by tap, PBS, and air-dried.

The biofilm stain was released by carefully adding 225 µL of 30% acetic acid to the wells for 20 minutes with gentle tabbing. The absorbance of the released strain was recorded from readouts of the CECIL 7500 [Canada] spectrophotometer at 590 nm.

B. Statistical Analysis

GraphPad Prism was utilized to analyze all data. Three-way ANOVA and Tukey's multiple comparisons tests were utilized for the statistical analysis. A *p*-value of less than 0.05 ($p<0.05$) was regarded statistically significant; *p* values of less than 0.0001 ($p<0.0001$) was considered as statistically highly significant.

Results and Discussion

Results of 2 different strains of bacteria cultured in 5 other media for four different durations are elaborated as follows. Both *Proteus mirabilis* and *Micrococcus luteus* were inoculated into Peptone Water (PW), Nutrient Broth (NB), Brain Heart Infusion (BHI), Normal Saline (NS), and Distilled Water (DW). Figure 1 illustrates the absorbance at 590 nm by the released crystal violet stain, which is directly proportional to the biofilm biomass produced by each bacterium per each experimental setting. From the fore-mentioned figure, biofilm production by *Micrococcus luteus* in peptone water has recorded the highest absorbance at 590 nm; this can be revealed visually. Though, statistical analysis is required to confirm this finding. The observations showed that different incubation times have been influential on the trend and pattern of biofilm formation.

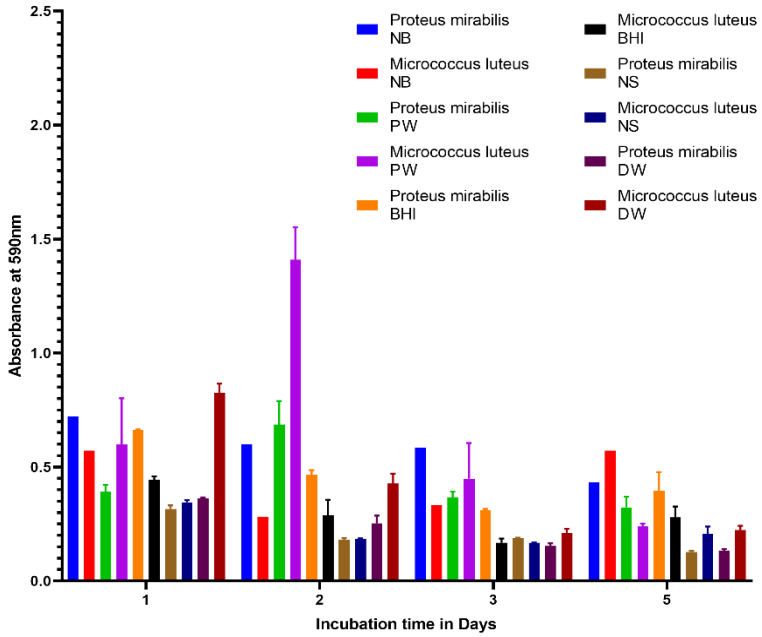
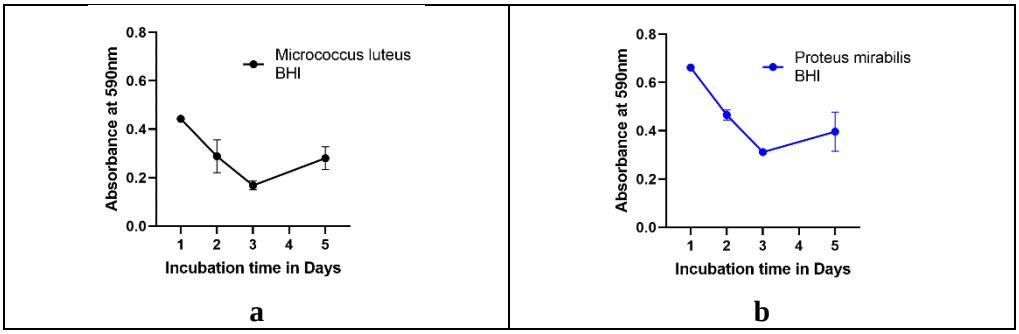


Figure 1. Overall absorbance records at 590nm per bacterium per media per time.

Patterns of attachment to detachment for each bacterium have been recorded (Figure 2, a-j), illustrates each bacterium's biofilm formation pattern. Both strains exhibited a similar pattern of attachment and detachment in (BHI broth, PW, and DW) media suspensions (Figure 2, a-f); which infers that the availability of nutrients and osmolarity have a similar influence on the patterns of biofilm formation. Namely, when both strains are suspended in BHI (Figure 2, a-b), they both form the biofilm efficiently on the first day of incubation. Then detachment started until the third day. On the 5th-day, biofilms were rebuilt for the second time. Whereas their biofilm formation pattern in the suspension of PW (Figure 2, c-d); started moderately, then reached its peak on the second day of incubation, followed by a gradual detachment of the biofilm. Distilled water suspensions of both bacteria (Figure 2, e-f); started with built up of the biofilm, then followed by gradual detachment previous studies have shown that additional glucose to the media; which is the case of BHI broth; possess a negative influence on the biofilm formation when the incubation intervals are similar to the current study ***[43]***. In contrast, the results of other studies might lead to an extension of confusion, yet incubation intervals of those studies are in hours, not days ***[44]***.

However, when they were incubated on NB and NS (Figure 2, g-j), their biofilm patterns diverged. At the same time, *Micrococcus luteus* suspension in NB (Figure 2, g-h); attached on the first day and started detachment until the third day. It started reattachment on the fifth day; the *Proteus mirabilis* trend started with similar early attachment then detached gradually. Directions of both strains in NS suspensions agreed with those of NB, yet with milder fluctuations (Figure 2, i-j). This could be linked to their different genetic adaptation and/or physiological behavior, as reported by other studies ***[35].***



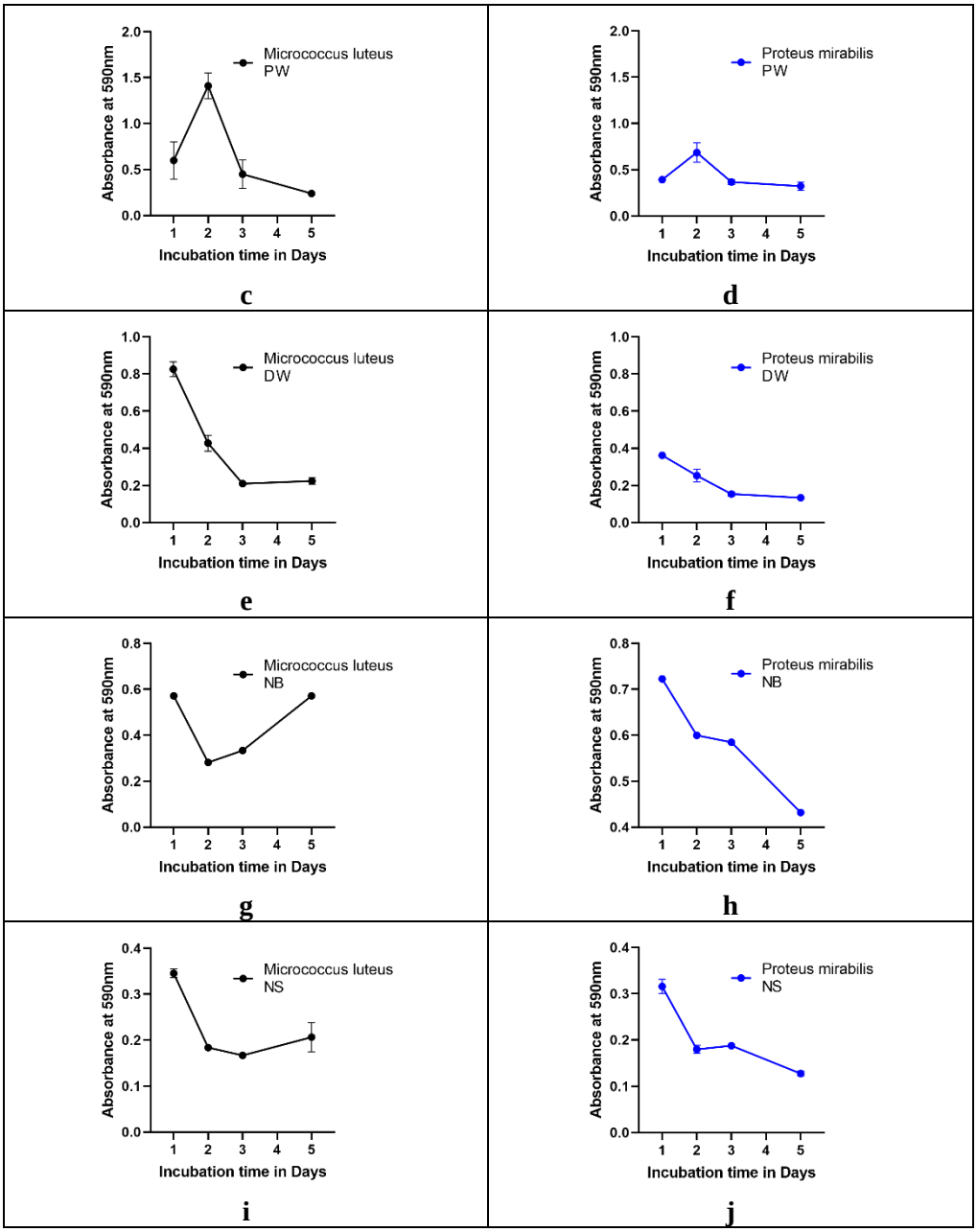


Figure 2 (a-j). Patterns of biofilm formation by each bacterium per media throughout the incubation period.

Statistical analysis has shown the significance of the use of different bacteria and media types and incubation time; Table 1 shows the *p*-value per listed conditions after Three-way ANOVA by GraphPad Prism. It can be noticed that in all experimental settings, time plays a statistically highly significant role in the buildup and dispersion pattern of the biofilm formation, *p*-value <0.0001.

Table 1. *p* values of Three-way ANOVA.

Source of Variation	P -value	P-value summary	Culture Media
Time in DAYS	<0.0001	****	NS vs. DW
Culture Media	<0.0001	****	
Bacterial Strains	<0.0001	****	
Time in DAYS x Culture Media	<0.0001	****	
Time in DAYS x Bacterial Strains	<0.0001	****	
Culture Media x Bacterial Strains	<0.0001	****	
Time in DAYS x Culture Media x Bacterial Strains	<0.0001	****	
Time in DAYS	<0.0001	****	PW vs. NB
Culture Media	0.1231	ns	
Bacterial Strain	0.1393	ns	
Time in DAYS x Culture Media	<0.0001	****	
Time in DAYS x Bacterial Strain	0.0177	*	
Culture Media x Bacterial Strain	<0.0001	****	
Time in DAYS x Culture Media x Bacterial Strain	<0.0001	****	
Time in DAYS	<0.0001	****	PW vs. BHI
Culture Media	<0.0001	****	
Bacterial Strain	0.274	ns	
Time in DAYS x Culture Media	<0.0001	****	
Time in DAYS x Bacterial Strain	0.0023	**	
Culture Media x Bacterial Strain	<0.0001	****	
Time in DAYS x Culture Media x Bacterial Strain	0.0007	***	

It can be noticed that each bacterial strain's attachment to detachment pattern was changing in different media used in this study. Therefore, numerically and statistically significant differences dominate the experimental settings represented in table-1 whereas Figure 3 (a-c) visually illustrates the arrangement of data per each analysis of three-way ANOVA.

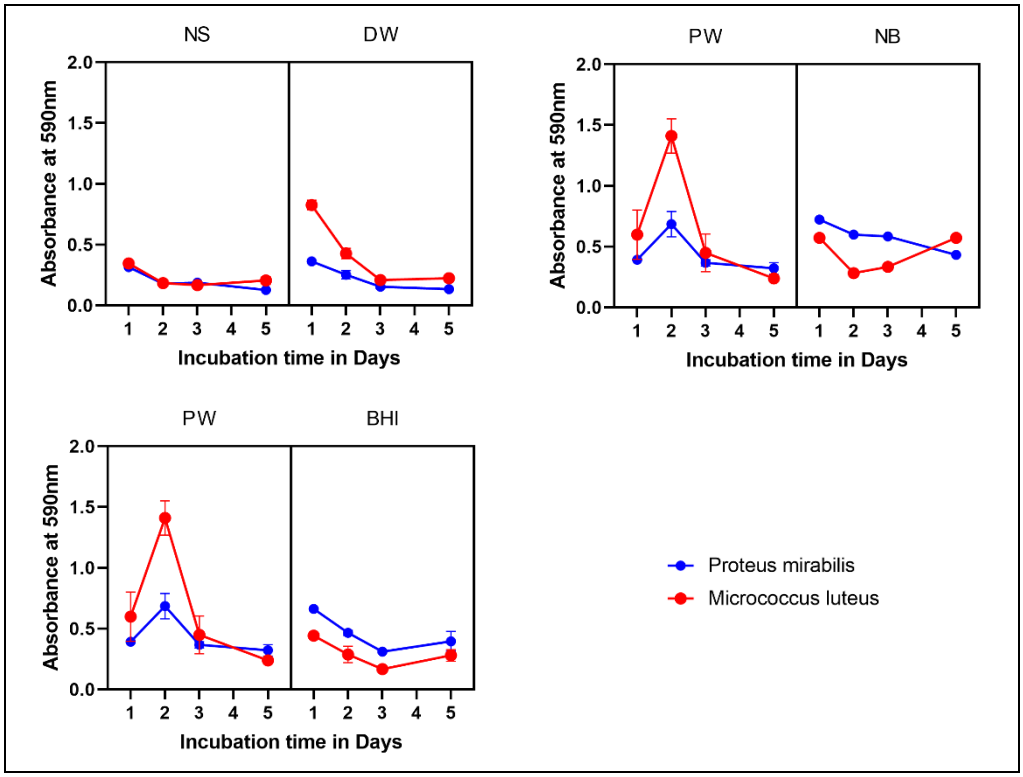


Figure 3 (a-c). Visualizing the patterns of biofilm formation for three-way ANOVA; *p* values are presented in Table-1.

The biofilm produced after two days of incubation by *Micrococcus luteus* in peptone water showed a statistically significant difference compared to the other settings of the experiment that were conducted by this study. Figures 4, 5, 6, and 7 graphically show biofilm formation ability by both bacterial strains. The emphasis is on the significance of Peptone Water Suspension of the bacterial strains on the second day's incubation.

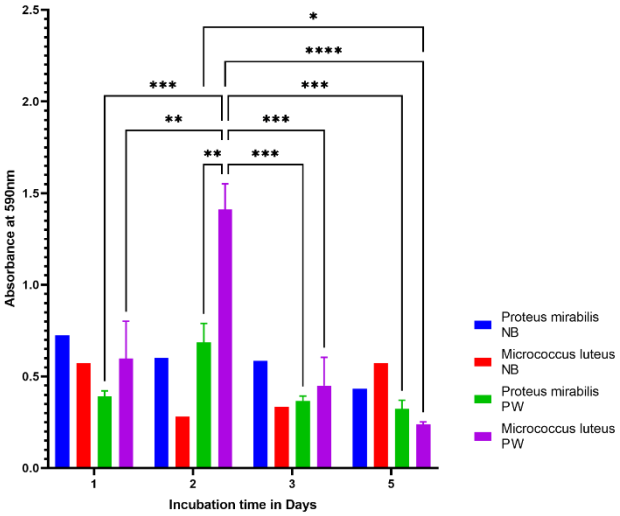


Figure 4. Patterns of biofilm formation by each bacterium in Nutrient broth versus Peptone water throughout the incubation period.

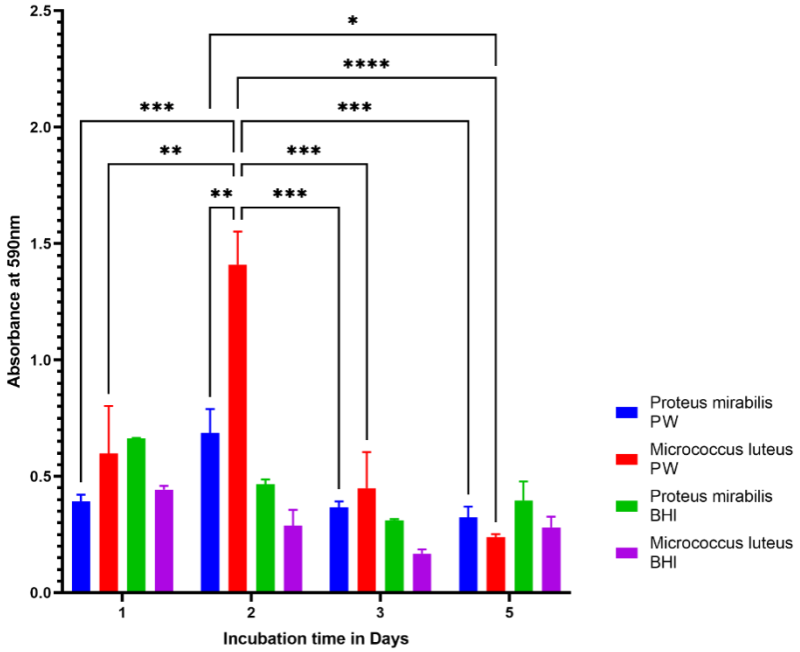


Figure 5. Patterns of biofilm formation by each bacterium in Peptone water versus Brain Heart Infusion throughout the incubation period.

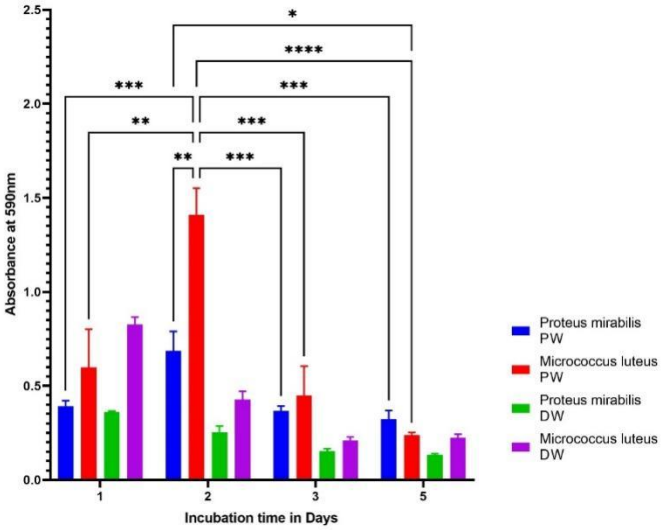


Figure 6. Patterns of biofilm formation by each bacterium in Peptone water versus Distilled Water throughout the incubation period.

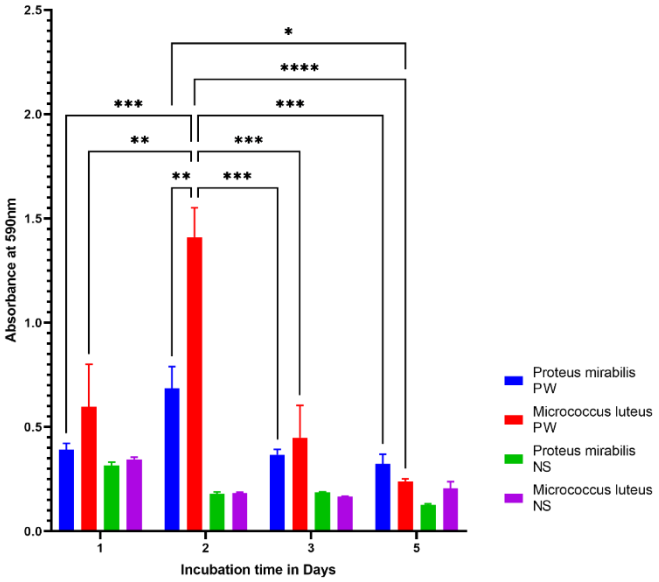


Figure 7. Patterns of biofilm formation by each bacterium in Peptone water versus Normal Saline throughout the incubation period.

The biofilm formation results of this study reveal the different potentials of each isolate to form biofilm under different experimental settings. Biofilm formations were carried out by utilizing different incubation periods and media.

Proteus mirabilis and *Micrococcus luteus* have built up biofilm in nutrient broth. However, when assessments were done for brain heart infusion broth on day five, biofilm production was decreased, which was due to the depletion of resources in this media. *Proteus mirabilis* and *Micrococcus luteus* in day one formed biofilm in distilled water due to their ability to modify against osmolarity. The increase in biofilm formation in distilled water and normal saline in producing biofilm, especially in both *Proteus mirabilis* and *Micrococcus luteus*, due to the capacity of these bacteria to produce anti-osmolarity that means the bacteria which can prevent the cell wall or outer membrane from damage, as well as the ability of some bacteria such as *Micrococcus luteus* to thrive in a low level of the nutrient environment due to their possible oligotrophic nature ***[45]***. Furthermore, a psychological situation that is influencing the levels of hormones has been reported to increase the susceptibility of individuals to biofilm formation. Invitro studies have shown that High concentrations of epinephrine are in favor of biofilm formation by *Micrococcus luteus* ***[46]***.

In recent studies, septic arthritis, prosthetic valve endocarditis ***[47]***, and recurrent bacteremia cases were reported to be associated with *Micrococcus luteus* infection. They have shown that the antibiotic activity of pomegranate rind extract (PRE), Zn (II), is efficient against *Micrococcus luteus* and other bacteria ***[48]***. *Micrococcus luteus* biofilm formation can be prevented enzymatically by introducing DNaseI ***[49]*** and NucB lysozyme to the medium or using surfactants of microbial origin such as rhamnolipid ***[50].***

The characteristic carotenoid pigment of *Micrococcus luteus* can be used in skincare and cosmetics as it possesses antibacterial, antifungal activities and the ability to absorb Ultra Violet rays ***[51].***

Conclusion and Recommendations

The findings of this study conclude that complications of colonized infection with *Micrococcus luteus* UTI patients are inevitable because it possesses a similar and occasionally more competent capacity to form biofilm than *Proteus mirabilis*. At this point, the study results demand further cautiousness with *Micrococcus luteus* infections. Early diagnosis and antibacterial treatment are the keys to efficient infection control. Also, ingredients of the used media play a prominent role in biofilm formation. Media supplied with carbon and nitrogen sources support the production of biofilm as they are going to be assimilated by the microbes to produce the structure of the biofilm. However, additional amounts of glucose in the media can influence the pattern of biofilm production differently, i.e., short-term to long-term contract. *Micrococcus luteus* has a significant capacity to produce biofilm in all experimental settings, especially in Peptone Water, where it formed more biomass *Proteus mirabilis* in the 96-wells as well as incubation time has a significant influence on the formation and dispersion patterns of the biofilm.

We recommend the following points for future studies:

- Time intervals for incubation periods to be modified, especially for sharp fluctuations, so that more accurate details are collected. This can be done by fractioning days into hours or minutes.
- The osmolarity of the media was designed as serial dilutions of solutes, i.e., suspensions of bacteria in gradually decreased specific gravities.
- Shifting from complex media (which contain extracts) to defined media for such studies enables traceability of resource utilization.
- Serial dilutions of carbon and nitrogen sources were set in combinations, i.e., finding the most supportive ratio of carbon source to nitrogen source to form biofilm.
- Different incubation temperatures in addition to 37^{o}C
- Along with standard 96-well plates, surfaces of other tools and materials of real hospital life settings to be involved such as; metal, glass, rubber, and silicon. The best nominates to start with are sections of known surface area from wound drainage and urinary catheters made of different materials (Silicon, rubber, and PVC or Polyvinyl Chloride).
- Utilizing scanning electron microscopy to visualize and characterize biofilm structures.

References

[1] H. Kanematsu and D. M. Barry, *Biofilm and Materials Science*. Cham: Springer International Publishing, 2015.

[2] L. Hall-Stoodley, J. W. Costerton, and P. Stoodley, "Bacterial biofilms: from the Natural environment to infectious diseases," *Nat. Rev. Microbiol.*, vol. 2, no. 2, pp. 95–108, Feb. 2004, doi: 10.1038/nrmicro821.

[3] M. Jamal, U. Tasneem, T. Hussain, and S. Andleeb, "Bacterial Biofilm: Its Composition, Formation and Role in Human Infections," *Res. Rev. J. Microbiol. Biotechnol.*, vol. 4, no. 3, pp. 1–14, 2015.

[4] J. W. Costerton, "Bacterial Biofilms: A Common Cause of Persistent Infections," *Science (80-.).*, vol. 284, no. 5418, pp. 1318–1322, May 1999, doi: 10.1126/science.284.5418.1318.

[5] C. Vuong *et al.*, "Polysaccharide intercellular adhesin (PIA) protects Staphylococcus epidermidis against major components of the human innate immune system," *Cell. Microbiol.*, vol. 6, no. 3, pp. 269–275, Mar. 2004, doi: 10.1046/j.1462-5822.2004.00367.x.

[6] J. C. NICKEL, M. OLSON, R. J. C. McLEAN, S. K. GRANT, and J. W. COSTERTON, "An Ecological Study of Infected Urinary Stone Genesis in an Animal Model," *Br. J. Urol.*, vol. 59, no. 1, pp. 21–30, Jan. 1987, doi: 10.1111/j.1464-410X.1987.tb04573.x.

[7] V. Adetunji, "Crystal violet binding assay for assessment of biofilm formation by isolates of Listeria monocytogenes and Listeria spp from a typical tropical abattoir on wood, steel and glass surfaces.," *Glob. Vet.*, vol. 6, pp. 6–10, 2011.

[8] P. Gilbert, J. Das, and I. Foley, "Biofilm Susceptibility to Antimicrobials," *Adv. Dent. Res.*, vol. 11, no. 1, pp. 160–167, Apr. 1997, doi: 10.1177/08959374970110010701.

[9] C. Vinodkumar, S. Kalsurmath, and Y. Neelagund, "Utility of lytic bacteriophage in the treatment of multidrug-resistant *Pseudomonas aeruginosa* septicemia in mice," *Indian J. Pathol. Microbiol.*, vol. 51, no. 3, p. 360, 2008, doi: 10.4103/0377-4929.42511.

[10] M. Okada *et al.*, "Structure of the Bacillus subtilis quorum-sensing peptide pheromone ComX," *Nat. Chem. Biol.*, vol. 1, no. 1, pp. 23–24, Jun. 2005, doi: 10.1038/nchembio709.

[11] F. G. Sauer, H. Remaut, S. J. Hultgren, and G. Waksman, "Fiber assembly by the chaperone–usher pathway," *Biochim. Biophys. Acta - Mol. Cell Res.*, vol. 1694, no. 1–3, pp. 259–267, Nov. 2004, doi: 10.1016/j.bbamcr.2004.02.010.

[12] R. D. Waite, A. Papakonstantinopoulou, E. Littler, and M. A. Curtis, "Transcriptome Analysis of Pseudomonas aeruginosa Growth: Comparison of Gene Expression in Planktonic Cultures and Developing and Mature Biofilms," *J. Bacteriol.*, vol. 187, no. 18, pp. 6571–6576, Sep. 2005, doi: 10.1128/JB.187.18.6571-6576.2005.

[13] S. RAJAN, "Pulmonary infections in patients with cystic fibrosis," *Semin. Respir. Infect.*, vol. 17, no. 1, pp. 47–56, Mar. 2002, doi: 10.1053/srin.2002.31690.

[14] L. E. C. Rivera, A. P. Ramos, and C. Padilla Desgarennes, "Péptidos antimicrobianos: antibióticos naturales de la piel Artículo de revisión," *Dermatología Rev Mex Vol.*, vol. 51, no. 2, pp. 57–67, 2007, [Online]. Available: http://www.medigraphic.com/pdfs/derrevmex/rmd-2007/rmd072d.pdf.

[15] R. Baselga, I. Albizu, and B. Amorena, "Staphylococcus aureus capsule and slime as virulence factors in ruminant mastitis. A review," *Vet. Microbiol.*, vol. 39, no. 3–4, pp. 195–204, Apr. 1994, doi: 10.1016/0378-

1135(94)90157-0.
[16] M. R. Parsek and P. K. Singh, “Bacterial Biofilms: An Emerging Link to Disease Pathogenesis,” *Annu. Rev. Microbiol.*, vol. 57, no. 1, pp. 677–701, Oct. 2003, doi: 10.1146/annurev.micro.57.030502.090720.
[17] Hastyar najmuldeen, “Assessment of Chlorine Resistance Enterobacter cloacae Isolated from Water Storage Tanks in Sulaimaniyah City-Iraq,” *Passer*, vol. 3, no. 1, 2020, doi: 10.24271/psr.17.
[18] P. Naves, “Effects of human serum albumin, ibuprofen and N-acetyl-l-cysteine against biofilm formation by pathogenic Escherichia coli strains,” *J Hosp. Infect*, vol. 76, pp. 165–170.
[19] L. S. Havarstein, G. Coomaraswamy, and D. A. Morrison, “An unmodified heptadecapeptide pheromone induces competence for genetic transformation in Streptococcus pneumoniae.,” *Proc. Natl. Acad. Sci.*, vol. 92, no. 24, pp. 11140–11144, Nov. 1995, doi: 10.1073/pnas.92.24.11140.
[20] J. R. Govan and V. Deretic, “Microbial pathogenesis in cystic fibrosis: mucoid Pseudomonas aeruginosa and Burkholderia cepacia.,” *Microbiol. Rev.*, vol. 60, no. 3, pp. 539–574, 1996, doi: 10.1128/mr.60.3.539-574.1996.
[21] P. Sreenivasan, “Clinical response to antibiotics among children with bloody diarrhea,” *Indian Paediatr.*, vol. 50, pp. 340–341, 2013, [Online]. Available: https://www.indianpediatrics.net/mar2013/mar-340-341.htm.
[22] S. Khan, P. Singh, M. Ansari, and A. Asthana, “Isolation of Shigella species and their resistance patterns to a panel of fifteen antibiotics in mid and far western region of Nepal,” *Asian Pacific J. Trop. Dis.*, vol. 4, no. 1, pp. 30–34, Feb. 2014, doi: 10.1016/S2222-1808(14)60309-1.
[23] C. A. Fux, J. W. Costerton, P. S. Stewart, and P. Stoodley, “Survival strategies of infectious biofilms,” *Trends Microbiol.*, vol. 13, no. 1, pp. 34–40, Jan. 2005, doi: 10.1016/j.tim.2004.11.010.
[24] L. Ma, M. Conover, H. Lu, M. R. Parsek, K. Bayles, and D. J. Wozniak, “Assembly and Development of the Pseudomonas aeruginosa Biofilm Matrix,” *PLoS Pathog.*, vol. 5, no. 3, p. e1000354, Mar. 2009, doi: 10.1371/journal.ppat.1000354.
[25] E. L. Larson, C. Gomez-Duarte, L. V. Lee, P. Della-Latta, D. J. Kain, and B. H. Keswick, “Microbial flora of hands of homemakers,” *Am. J. Infect. Control*, vol. 31, no. 2, pp. 72–79, Apr. 2003, doi: 10.1067/mic.2003.33.
[26] T.-F. C. Mah and G. A. O’Toole, “Mechanisms of biofilm resistance to antimicrobial agents,” *Trends Microbiol.*, vol. 9, no. 1, pp. 34–39, Jan. 2001, doi: 10.1016/S0966-842X(00)01913-2.
[27] J. B. Lyczak, C. L. Cannon, and G. B. Pier, “Lung Infections Associated with Cystic Fibrosis,” *Clin. Microbiol. Rev.*, vol. 15, no. 2, pp. 194–222, Apr. 2002, doi: 10.1128/CMR.15.2.194-222.2002.
[28] R. P. Novick and E. Geisinger, “Quorum Sensing in Staphylococci,” *Annu. Rev. Genet.*, vol. 42, no. 1, pp. 541–564, Dec. 2008, doi: 10.1146/annurev.genet.42.110807.091640.
[29] D. Hogan, “Why are bacteria refractory to antimicrobials?,” *Curr. Opin. Microbiol.*, vol. 5, no. 5, pp. 472–477, Oct. 2002, doi: 10.1016/S1369-5274(02)00357-0.
[30] K. Poole, “Mechanisms of bacterial biocide and antibiotic resistance,” *J. Appl. Microbiol.*, vol. 92, pp. 55S-64S, May 2002, doi: 10.1046/j.1365-2672.92.5s1.8.x.
[31] C. A. Gordon, N. A. Hodges, and C. Marriott, “Antibiotic interaction and diffusion through alginate and exopolysaccharide of cystic fibrosis-derived Pseudomonas aeruginosa,” *J. Antimicrob. Chemother.*, vol. 22, no. 5, pp. 667–674, 1988, doi: 10.1093/jac/22.5.667.
[32] C. Vuong and M. Otto, “Staphylococcus epidermidis infections,” *Microbes Infect.*, vol. 4, no. 4, pp. 481–489, Apr. 2002, doi: 10.1016/S1286-4579(02)01563-0.
[33] L. Foulston, A. K. W. Elsholz, A. S. DeFrancesco, and R. Losick, “The Extracellular Matrix of Staphylococcus aureus Biofilms Comprises Cytoplasmic Proteins That Associate with the Cell Surface in Response to Decreasing pH,” *MBio*, vol. 5, no. 5, pp. 01667–14, Sep. 2014, doi: 10.1128/mBio.01667-14.
[34] J. C. Nickel and J. W. Costerton, “Bacterial localization in antibiotic-refractory chronic bacterial prostatitis,” *Prostate*, vol. 23, no. 2, pp. 107–114, 1993, doi: 10.1002/pros.2990230204.
[35] R. Van Houdt and C. W. Michiels, “Biofilm formation and the food industry, a focus on the bacterial outer surface,” *J. Appl. Microbiol.*, vol. 109, no. 4, pp. 1117–1131, 2010, doi: 10.1111/j.1365-2672.2010.04756.x.
[36] K. Lewis, “Riddle of Biofilm Resistance,” *Antimicrob. Agents Chemother.*, vol. 45, no. 4, pp. 999–1007, Apr. 2001, doi: 10.1128/AAC.45.4.999-1007.2001.
[37] R. Edwards and K. G. Harding, “Bacteria and wound healing,” *Curr. Opin. Infect. Dis.*, vol. 17, no. 2, pp. 91–96, Apr. 2004, doi: 10.1097/00001432-200404000-00004.
[38] J. N. Sims *et al.*, “Visual Analytics of Surveillance Data on Foodborne Vibriosis, United States, 1973-2010,” *Environ. Health Insights*, vol. 5, p. EHI.S7806, Jan. 2011, doi: 10.4137/EHI.S7806.
[39] N. J. Trengove, M. C. Stacey, D. F. McGechie, N. F. Stingemore, and S. Mata, “Qualitative bacteriology and leg ulcer healing,” *J. Wound Care*, vol. 5, no. 6, pp. 277–280, Jun. 1996, doi: 10.12968/jowc.1996.5.6.277.
[40] D. E. Bradley, “The Length of the Filamentous Pseudomonas aeruginosa Bacteriophage Pf,” *J. Gen. Virol.*,

vol. 20, no. 2, pp. 249–252, Aug. 1973, doi: 10.1099/0022-1317-20-2-249.
[41] K. Matsuura, Y. Asano, A. Yamada, and K. Naruse, "Detection of micrococcus Luteus biofilm formation in microfluidic environments by pH measurement using an ion-sensitive field-effect transistor," *Sensors (Switzerland)*, vol. 13, no. 2, pp. 2484–2493, 2013, doi: 10.3390/s130202484.
[42] A. Tahmourespour, "Biofilm formation potential of oral streptococci in related to some carbohydrate substrates," *African J. Microbiol. Res.*, vol. 4, no. 11, pp. 1051-1056, 2010.
[43] X. Chen, Y. Xu, H. Winkler, and T. R. Thomsen, "Influence of biofilm growth age, media and antibiotics exposure time on Staphylococcus aureus and Pseudomonas aeruginosa biofilm removal in vitro," pp. 1–11, 2020, doi: 10.21203/rs.3.rs-20023/v1.
[44] G. H. Bowden and L. Y, "Nutritional influences on biofilm development," *Adv Dent Res*, vol. 11, pp. 81–99.
[45] L. C. Gomes, J. M. R. Moreira, M. Simões, L. F. Melo, and F. J. Mergulhão, "Biofilm Localization in the Vertical Wall of Shaking 96-Well Plates," *Scientifica (Cairo).*, vol. 2014, pp. 1–6, 2014, doi: 10.1155/2014/231083.
[46] N. D. Danilova, T. V. Solovyeva, S. V. Mart'yanov, M. V. Zhurina, and A. V. Gannesen, "Stimulatory Effect of Epinephrine on Biofilms of Micrococcus luteus C01," *Microbiol. (Russian Fed.*, vol. 89, no. 4, pp. 493–497, 2020, doi: 10.1134/S0026261720040049.
[47] G. Rodriguez-Nava, A. Mohamed, M. A. Yanez-Bello, and D. P. Trelles-Garcia, "Advances in medicine and positive natural selection: Prosthetic valve endocarditis due to biofilm producer Micrococcus luteus," *IDCases*, vol. 20, p. e00743, 2020, doi: 10.1016/j.idcr.2020.e00743.
[48] V. Celiksoy, R. L. Moses, A. J. Sloan, R. Moseley, and C. M. Heard, "Synergistic In Vitro Antimicrobial Activity of Pomegranate Rind Extract and Zinc (II) against Micrococcus luteus under Planktonic and Biofilm Conditions," *Pharmaceutics*, vol. 13, no. 6, p. 851, 2021, doi: 10.3390/pharmaceutics13060851.
[49] J. T. Blakeman *et al.*, "Extracellular DNA Provides Structural Integrity to a Micrococcus luteus Biofilm," *Langmuir*, vol. 35, no. 19, pp. 6468–6475, 2019, doi: 10.1021/acs.langmuir.9b00297.
[50] Y. Jiang, M. Geng, and L. Bai, "Targeting biofilms therapy: Current research strategies and development hurdles," *Microorganisms*, vol. 8, no. 8, pp. 1–34, 2020, doi: 10.3390/microorganisms8081222.
[51] H. Z. Majeed, "Antimicrobial activity of Micrococcus luteus Cartenoid pigment," *Al-Mustansiriyah J. Sci.*, vol. 28, no. 1, p. 64, 2017, doi: 10.23851/mjs.v28i1.314.