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RESEARCH NOTE

Assessment Assays for Biofilm-forming Capacity and Pathogen Inhibition of Lactic Acid Biofilm-forming Bacteria (LABFB)

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This study aimed at isolating lactic acid biofilm-forming bacteria from in-dwelling catheters and at comparing assays for evaluating biofilm-forming capacity (BFC) and pathogen inhibition (PI) of the isolates against biofilm-forming pathogens. Lactic acid biofilm-forming bacterial isolates were evaluated using the improvised tube and microplate assays for BFC. Crystal violet and triphenyl tetrazolium chloride (TTC) were compared as stains for spectrophotometric analysis in the two assays. The isolate (Isolate 2) with the highest BFC was tested for PI on biofilm-forming pathogens using the same assays. Results showed that Isolate 2, characterized as *Pediococcus pentosaceus* (accession number ATCC 25745), exhibited PI against *Escherichia coli* and *Chryseobacterium indologenes*. For BFC, the improvised tube was the optimal assay ($p = 0.000$); for PI, both assays were comparable ($p = 0.066$). TTC was the ideal stain in both assays ($p = 0.001$).

Keywords: biofilm, lactic acid biofilm-forming bacteria, *Pediococcus*

INTRODUCTION

Lactic acid bacteria (LAB), an important part of natural microbiota, have established beneficial effects and are recognized as potential probiotics through the production of various antimicrobial agents such as organic acids, hydrogen peroxide, bacteriocins, and adhesion inhibitors such as biosurfactants (Alameri *et al.* 2022; del Rio *et al.* 2019).

Several *Lactobacillus* species have been isolated as human microflora from the gut (Yang *et al.* 2012), the female urogenital tract (Ventolini 2015), and the respiratory tract (Du *et al.* 2022); that has been proven responsible for maintaining a healthy balance of microflora in the aforementioned human systems. While biofilm formation of opportunistic pathogens is one of the leading causes of nosocomial infections in hospitals, biofilm formation of non-pathogenic bacteria can be advantageous since aside from maintaining ecological stability, establishment, and long-term permanence of protective bacteria from intrusive pathogenic bacteria can be achieved (Gómez *et al.* 2016). This leads to the concept that if a layer of protective probiotic biofilm such as LAB can be established in nasopharyngeal and urinary catheters, the growth of pathogenic or opportunistic pathogens in in-dwelling catheters of patients under intensive care can be minimized or prevented. Since biofilm-associated infectious diseases are one of the leading problems in nosocomial infections, prevention of their formation in in-dwelling devices such as catheters is a matter of importance in public health.

The goal of this study was to obtain lactic acid biofilm-forming bacterial isolates from nasopharyngeal and urinary catheters and to test such against known pathogens. Moreover, it also aimed to determine the optimal assay for assessing biofilm formation since a method for

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assessment for biofilm formation in actual catheters under simulated conditions is presently lacking. The existing assay, the microplate assay for the determination of biofilm formation, as presented by Cannon *et al.* (2020) and Christensen *et al.* (1985) utilizes microtiter plates, which are not of similar material as the catheter tubes used in the actual setting (Allkja *et al.* 2020). Also, the common dyes, *i.e.* crystal violet and 2,3,5-triphenyl tetrazolium chloride (TTC) (Haney *et al.* 2018) used in the microplate assay have varied results in most studies (Brown *et al.* 2013; Sabaeifard *et al.* 2014). Therefore, it is also essential to determine which dye can be optimal when reading the results.

MATERIALS AND METHODS

Isolation of Potential Lactic Acid Biofilm-forming Bacteria (LABFB) From Urinary and Nasogastric Catheters

LABFB were isolated from sample catheters (used catheters extracted from ICU patients) obtained from a medical institution in coordination with the ICU Head Nurse and Infectious Control Nurse, with the approval of the Head of the Medical Institution and the Institutional Ethics Committee. Three samples of each of the used urinary and nasogastric catheters were cut into 50.8 mm pieces (in three replicates) and were suspended in sterile *Lactobacillus* Mann Rogosa Sharpe (MRS) broth (TM 147, TM MEDIA®, INDIA). These were incubated under anaerobic conditions in an air-tight container using the candle jar method at 7 °C for 72 h and were centrifuged at 6000 x *g* for 15 min. The cut pieces were then aseptically removed, and the supernatant was decanted. The precipitate formed at the bottom was resuspended in sterile phosphate-buffered saline (PBS), serially diluted up to the 10th dilution, and even-numbered dilutions were plated in sterile MRS agar (M641I, HIMEDIA®, INDIA) and incubated under anaerobic conditions for 24 h. Colonies showing variable morphology, color, and dimension were picked and isolated using the streak plate technique. To ensure the purification of the colonies, re-isolation was repeated three times. Purified colonies were stored in MRS agar (M641I, HIMEDIA®, INDIA) slants overlaid with sterile mineral oil under refrigerated conditions.

Evaluation of Biofilm-forming Capacity (BFC) of Lactic Acid Biofilm-forming Isolates

Microplate (96 well) assay. A loopful of isolated LABFB was inoculated in 10-mL MRS Broth (TM 147, TM Media®, India), incubated under anaerobic conditions for 24 h, and standardized to 0.5 MacFarland standard. The process of Christensen *et al.* (1985) was adopted for the microplate method. The negative control was 200-µL MRS Broth (TM 147, TM Media®, India), also done in triplicate. Separate preparations were made for crystal violet and TTC dyes. The microplates were incubated under anaerobic conditions at 37 °C for 24 h. The excess broth was aseptically aspirated, and the plates were rinsed three times with sterile PBS. The microplates were infused with crystal violet (C.I. No. 425555, Lobachemie, India) and TTC (1083800010, Merck Millipore, Germany), separately and were washed with sterile PBS three times after 5 min of dye exposure. The dyed microplates were allowed to dry and were analyzed for BFC using a microplate reader (Multiskan FC, Thermo Scientific, Torrace, CA, USA) at 570 nm.

Improvised tube assay. A loopful of isolated LABFB was inoculated in 10-mL MRS Broth (TM 147, TM Media®, India) and standardized at 0.5 McFarland standard. A 10-mm-long sterile catheter fragment was aseptically inoculated into the standardized broth. After 24-h incubation, the catheter fragments were removed and washed with sterile PBS three times and transferred to Petri dishes filled with crystal violet (C.I. No. 425555, Lobachemie, India). The same method was followed to prepare tubes stained with the TTC (1083800010, Merck Millipore, Germany) dye. After 5 min of staining, catheter fragments were washed with sterile distilled water and analyzed for BFC based on absorbance reading using a UV-Vis spectrophotometer (Implen C40, Germany) at 570 nm.

Evaluation of Pathogen Inhibition (PI) against Selected Pathogens

The LABFB isolate with the highest mean BFC (LABFB Isolate 2) was challenged-tested on six biofilm-forming pathogens, *Staphylococcus epidermidis* PNCM 10098, *Candida albicans* (CA) USTCMS 1201, *Escherichia coli* (EC) PNCM 1634, *Aspergillus niger* PNCM 3080, *Pseudomonas aeruginosa* PNCM 1335, and *Chryseobacterium indologenes* (CI) WVSU 01 (biofilm-forming isolate from water pipe); using the microplate assay and the improvised tube assay.

Microplate (96-well) assay. LABFB Isolate 2 was inoculated in the wells of polystyrene 96-well plates (in triplicates) using the same method for biofilm infusion by (Christensen *et al.* 1985). The biofilm-infused wells were then incubated at anaerobic conditions in an air-tight container using the candle jar method at 37 °C for 24 h and were aseptically washed three times with sterile PBS. These were then inoculated with 180 µL of 0.5 McFarland standardized cultures of the aforementioned test pathogens for the challenge test. The negative control was 200-µL MRS broth (TM 147, TM Media®, India). Each treatment was done in triplicate. The plates were incubated under anaerobic conditions at 37 °C for 24 h. The plates

were then dyed with crystal violet (C.I. No. 425555, Lobachemie, India) (C.I. No. 425555, Lobachemie, India) and TTC (1083800010, Merck Millipore, Germany) (separate preparations were made for each dye) and were analyzed for PI using a microplate reader (Multiskan FC, Thermo Scientific, Torrace, CA, USA) at 570 nm.

Improvised tube assay. Ten (10) mm sterile catheter fragments were infused with LABFB Isolate 2 using the same procedure for assessment of BFC. After incubation, the catheter fragments were washed with sterile PBS and aseptically transferred to 10 mL of 0.5 MacFarland standardized cultures of the test pathogens. The set-ups were incubated for 24 h. The catheter fragments were washed with sterile PBS and infused with crystal violet for 5 min. The same method was followed for catheter fragments to be stained with TTC dye. The catheter fragments were then washed with sterile distilled water and subjected to absorbance reading using a UV-vis spectrophotometer (Implen C40, Germany) at 570 nm.

Characterization of Lactic Acid Biofilm-forming Isolate with Anti-biofilm Activity against Pathogens

The bacterial isolate that showed significant BFC and PI was subjected to DNA isolation using gPURE DNA Isolation Kit (IBI Scientific). The 16S rRNA gene was amplified by PCR from the DNA isolated from the LABFB isolate. These were the primers used for the amplification of the gene:

forward 27F–5'-AGAGTTTGATCMTGGCTCAG-3'
reverse 1492R – 5'-CGGTTACCTTGTTACGACTT-3'

The PCR temperature conditions were as follows: 5 min at 94 °C, 60 s at 94 °C in 35 cycles, 45 s at 53 °C, 90 s at 68 °C, and 10 min at 68 °C; 4 °C was set as hold temperature to keep amplicons safe for the next use. Electrophoresis of the amplified PCR product was done on a 1 kb DNA ladder with 1% agarose gel and TAE as a buffer, which was subsequently visualized by staining with ethidium bromide. The results of the DNA Sequence were evaluated using the basic local alignment search tool (BLAST NCBI) and phylogenetic analysis using MEGA 11 software based on the 16S rRNA sequence was done by the maximum likelihood tree with 1000 bootstrap replications.

Data Analysis

Initial characterization of isolates with biofilm-forming capacity (BFC). Isolates were morphologically characterized through Gram staining to have an initial perspective that the isolates were Gram-positive cocci.

Assessment for biofilm-forming capacity (BFC) and pathogen inhibition (PI). The total mean absorbance values of the isolates for both stains and assays were used to determine BFC. PI was computed by obtaining the mean difference between the absorbance values of the biofilm-forming isolates and the final absorbance values after the introduction of the pathogen.

Statistical analysis of data. Statistical analysis was conducted using IBM SPSS Statistics Version 23 at a 0.05 significance level. Two-way analysis of variance with least significant difference (LSD) *post hoc* test was used to determine significant differences in the stains (TTC and CV) and the assays (96 well and improvised tube assays) in detecting the BFC of LABFB isolates. For comparison of PI of Isolate 2 against selected pathogens, t-tests were used to determine the more efficient assay and stain.

RESULTS AND DISCUSSION

Morphological Characteristics of Lactic Acid Biofilm-forming Bacteria (LABFB) Isolate

A total of six LABFB isolates were obtained, but the isolate with the highest BFC was Isolate 2. This isolate was a gram-positive coccus from a circular, opaque, and non-pigmented colony with an entire margin and a shiny appearance.

Biofilm Forming Capacity (BFC) of Lactic Acid Biofilm-forming Bacteria (LABFB) Isolate 2

Figure 1 shows that crystal violet in microplates consistently showed the lowest absorbance values, whereas the improvised tube method using TTC showed the highest absorbance values. Statistics showed a significant difference in the BFC of the LAB isolates as to material (p = 0.000; F = 5.439; df = 5; partial eta squared = 12.4%); and as to stain (p = 0.000; F = 16.830; df = 5; partial eta squared = 30.5%). As to material, the effect size was moderate at 12.4%; whereas to stain, the effect size was large at 30.5%. This indicates that the effects were because of the treatments. *Post hoc* tests confirm that of the six isolates, Isolate 2 had the highest BFC (0.95 ± 0.634). Isolate 2 was molecularly classified as *Pediococcus pentosaceus* ATCC 25745*,* which is further discussed in the latter part of the paper. A study by Barros *et al.* (2001) reported several *Pediococcus* strains isolated from human clinical sources, thereby implying that *Pediococcus* can be isolated from human sources, including in-dwelling devices like catheters. Also, it was reported by Nguyen *et al.* (2020) and Tatsaporn and Kornkanok (2020) that species of LAB can form biofilm in static conditions. Moreover, LAB species like *Pediococcus pentosaceus* were reported to have the ability to form biofilms, which can prevent the colonization of EC O157 (Cisneros *et al.* 2021). Also, a study by Mahgoub *et al.* (2022) reports that probiotic LABs have the potential to be used as a means for preventing pathogens from colonizing and forming biofilm in urinary

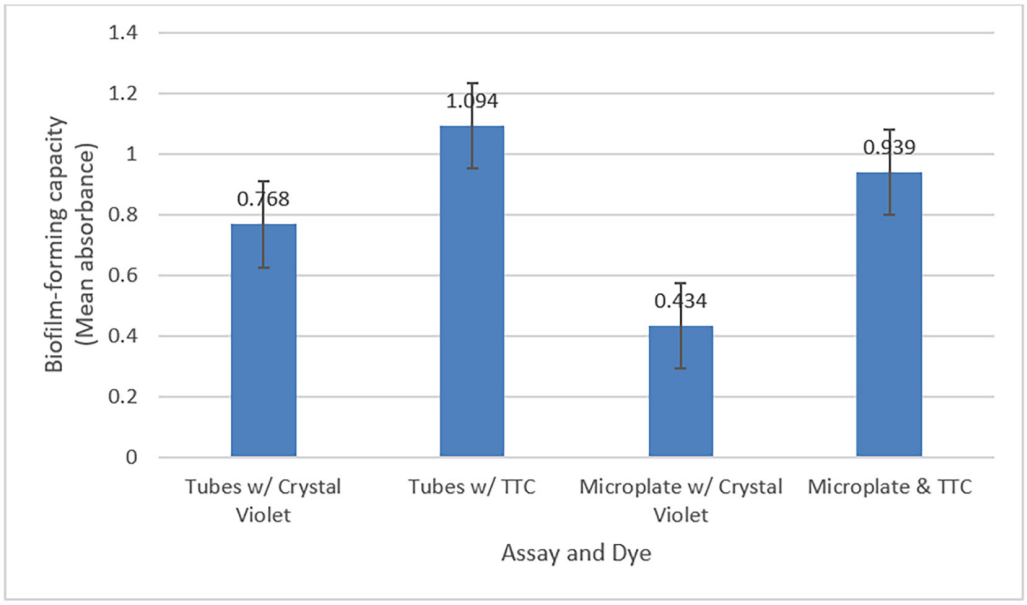

Figure 1. Biofilm-forming capacity compared as to assay and stain used.

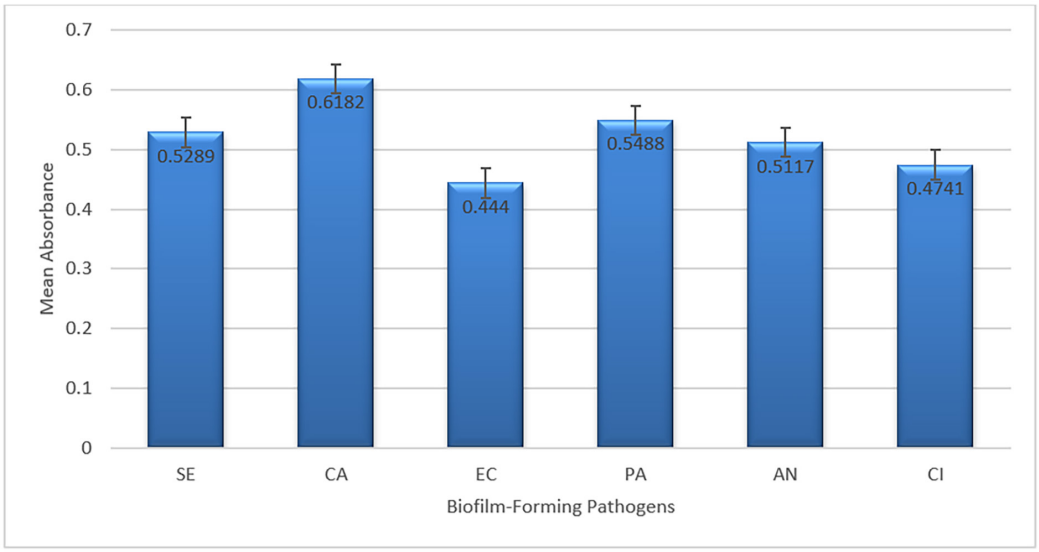

Figure 2. Pathogen inhibition of Isolate 2 against biofilm-forming pathogens*: Staphylococcus epidermidis* (SE), *Candida albicans* (CA), *Escherichia coli* (EC), *Pseudomonas aeruginosa* (PA), *Aspergillus niger* (AN), and *Chryseobacterium indologenes* (CI).

catheters pre-coated with LAB. This therefore supports the premise that catheters pre-coated by a probiotic biofilm can inhibit pathogens from forming biofilm.

Pathogen Inhibition (PI) of Lactic Acid Biofilm-forming Bacteria (LABFB) Isolate 2

Results in Figure 2 showed that LABFB Isolate 2 exhibited PI against all six biofilm-forming pathogens, with EC as most inhibited (0.444 ± 0.629), followed by CI (0.474 ± 0.536), and CA as least inhibited (0.6182 ± 0.618). For comparison of materials and stains, TTC and microplate assay showed the highest values for PI, whereas crystal violet and improvised tube assay showed the lowest values, thereby implying that the improvised tube assay and TTC were most efficient in demonstrating PI for all biofilm-forming pathogens (Figure 3). Statistically, there was no significant difference between the improvised tube and the microplate assays in determining PI (p = 0.066; t = 0.947; df = 143). For the stain used, TTC was more efficient in determining PI over crystal violet (p = 0.001; t = 2.472; df = 143). This indicates that either the microplate or the improvised tube assays can be used to determine

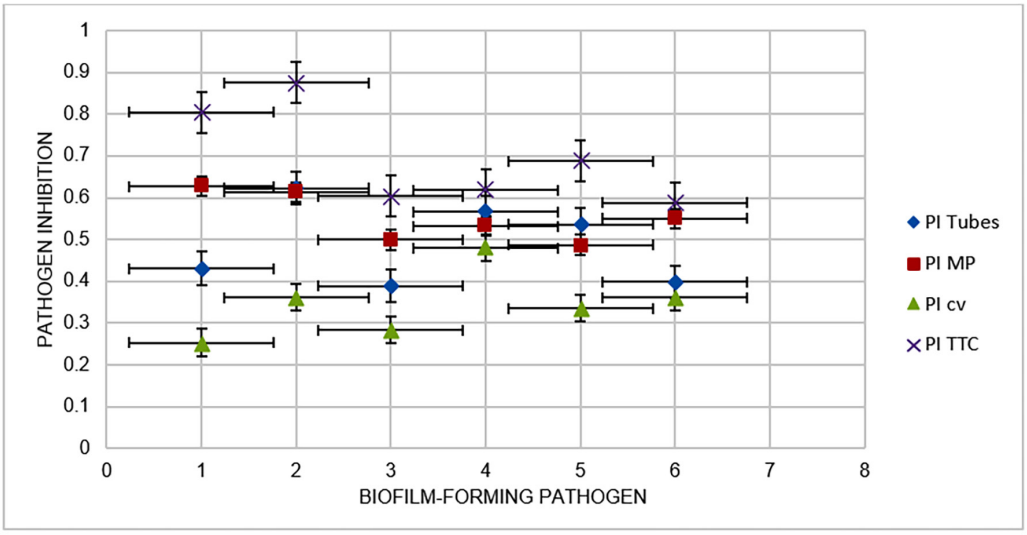

Figure 3. Pathogen inhibition of Isolate 2 against biofilm-forming pathogens: *Staphylococcus aureus* (1), *Candida albicans* (2), *Escherichia coli* (3), *Pseudomonas aeruginosa* (4), *Aspergillus niger* (4), and *Chryseobacterium indologenes* (6) as to assay and stain.

PI if TTC is used as the stain. PI by LABFB Isolate 2 can be supported by a study by Jang *et al.* (2015), which discusses that *Pediococcus* spp contains a T1-derived antimicrobial substance with a LysM domain that hydrolyzes peptidoglycan. Because of this, *Pediococcus* spp. is known to inhibit other pathogens such as EC, *Staphylococcus aureus*, and *Listeria monocytogenes.* In addition, a major bacteriocin – namely pediocin extracted from *Pediococcus* spp. – was reportedly anti-bactericidal and is known to inhibit food-borne pathogens, *Listeria monocytogenes* and *Staphylococcus aureus* of different strains (Choi *et al.* 2023; Loying *et al.* 2022; Soltani *et al.* 2022). The parallel efficacy of the improvised tube and the microplate assays in determining PI and the efficacy of the improvised tube assay in determining BFC can be supported by a study by Shrestha *et al.* (2018), which reported that biofilm formation was detected by tube adherence, Congo red agar, and tissue culture plate method (microplate), thus demonstrating that all assays (microplate, tube assay, Congo red) were effective in detecting biofilm-producing CNS strains. The efficacy of TTC as a stain for assessing biofilm formation is supported by a study by Sabaeifard *et al.* (2014), which reported that TTC assay was more sensitive in detecting low amounts of biofilm formed by different concentrations of bacteria in contrast to crystal violet, suggesting that TTC is more sensitive and less expensive than other vital staining methods. This also further supports the sensitivity of TTC in detecting PI in this study, as the binding of biofilm is greatly reduced through PI. Moreover, a study by Xu *et al.* (2016) further supports this observation as 2,3-bis(2-methoxy-4-nitro-5-sulfophenly)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) was found to be efficient in assessing metabolic activity while determining PI.

Characterization of Lactic Acid Biofilm-forming Bacteria (LABFB) Isolate 2

The bacterial isolate was identified as *Pediococcus pentosaceus* through the semi-quantitative culture technique. This was subjected to 16S rRNA gene sequencing, with the following result being the sequence obtained from the sample:

The bacterial isolate was identified as *Pediococcus pentosaceus* through the semi-quantitative culture technique. This was subjected to 16S rRNA gene sequencing, with the following result being the sequence obtained from the sample:

GTGCCGTCGGCGTGCTATACATGCAGTCGAACGAACTTCCGTTAATTGAT

TATGACGTACTTGKACTGRTTGRGATTTTAACACGAAGTGAGTGGCGAAC

GGGTGAGTAACACGTGGGTAACCTGCCCAGAAGTAGGGGATAACACCTGG

AAACAGATGCTAATACCGTATAACAGAGAAAACCGCATGGTTTTCTTTTA

AAAGATGGCTCTGCTATCACTTCTGGATGGACCCGCGGCGTATTAGCTAG

TTGGTGAGGTAAAGGCTCACCAAGGCAGTGATACGTAGCCGACCTGAGAG

GGTAATCGGCCACATTGGGACTGARACACGGCCCARACTCCTACGGGAGG

CAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCC

GCGTGAGTGAARAAGGGTTTCGGCTCGTA-AAGCTCTGTTGTTAAAGAAGA

AKGTGAAAGCCTTCGGCTCAACCGAAGAAGTG-CATTGGAAACTGGGAGAC

ACGTGGGTAAGAGTAACTGTTTACCCAGTGACG-GTATTTAACCAGAAAGC

TTGAGTGCAGAAGAGRACAGTGGAACTCCATGT-GTAGCGGTGAAATGCGT

CACGGCTAACTACGTGCCAGCAGCCGCGGTA-ATACGTAGGTGGCAAGCGT

AGATATATGGAAGAACACCAGTGGCGAAGGCG-GCTGTCTGGTCTGCAACT

TATCCGRATTTATTGGGCGTAAAGCGAGCGCAG-GCGGTCTTTTAAGTCTA

GACGCTGAGGCTCGAAAGCATGGGTAGCGAA-CAGGATTAGATACCSTGGT

Table 1. BLAST reference for *P. pentosaceus.*

Description	Scientific name	Max score	Total score	Query cover	E value	Per. ident	Acc. len	Accession
Pediococcus pentosaceus strain CP000422.1 16S ribosomal RNA gene, partial sequence	*Pediococcus pentosaceus*	1899	1899	95%	0	97.05	1509	ATCC25745
Pediococcus pentosaceus strain FB058 16S ribosomal RNA gene, partial sequence	*Pediococcus pentosaceus*	1888	1888	92%	0	96.03	1245	MF436194.1
Pediococcus pentosaceus strain SA14 16S ribosomal RNA gene, partial sequence	*Pediococcus pentosaceus*	1866	1866	94%	0	95.02	1213	ON103325.1
Pediococcus pentosaceus strain FB145 16S ribosomal RNA gene, partial sequence	*Pediococcus pentosaceus*	1858	1858	94%	0	94.87	1257	MF945626.1
Pediococcus pentosaceus strain TMPC 62322 16S ribosomal RNA gene, partial sequence	*Pediococcus pentosaceus*	1840	1840	94%	0	94.56	1241	OM617975.1
Pediococcus pentosaceus strain TMPC 4G926 16S ribosomal RNA gene, partial sequence	*Pediococcus pentosaceus*	1836	1836	93%	0	94.89	1210	OM617968.1

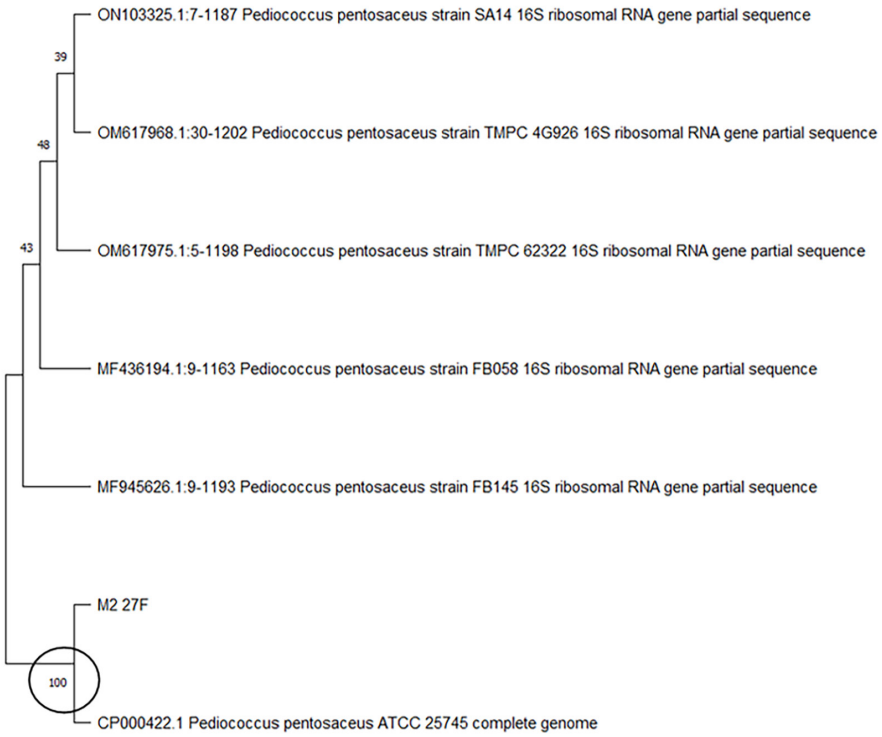

Figure 4. Phylogenetic tree generated using maximum likelihood. The percentage of trees in which the associated taxa clustered together in 1000 bootstrap replicates is shown next to the branches. Nodes with more than 95% bootstrap values applied are indicated with a black circle.

AGTCCRTGCCGTAAACGATGATTACTAAGTGTTG-GAGGGTTTCCGCCCTT

CAGTGCTGCAGCTAACGCATTAAGTAATCCGCCT-GGGGAGTACGACCGCA

AGGTTGAAACTCAAAGATTGACGGGGCCCGCA-CAGCGTGAGCATGTGTTT

ATTCGAAGCTACGCGAGACTTACAGGTCTGA-CATCTCTGACAGTCTAGAG

ATAGAGTCCCTCGGGACGATGACAGTGTGCAT-GTGTCGTCAGCTCKGTCT

G A A T G T G G G T A G T C C G C A C G R G S C A C - TATACTAGTGCAGCATAGTGGGAM

TCWATGAACTGCGKACAACGAGAAGTGGGGAC-GACGTCATMTYATGCCTA

TGACTGTCACACCGYTCATGGWGGTCMACAGTC-CGGACCGAGTTARCGTA

TCTCTCTTAGACACTCTAG

Based on BLAST reference, LABFB Isolate 2 has a sequence that was 97% identical to the partial gene sequence of 16S rRNA of *Pediococcus pentosaceus* ATCC 25745 (highlighted in yellow in Table 1). Figure 4 shows the phylogenetic genetic tree generated using maximum likelihood, which confirms the identity of the strain of the isolate as *Pediococcus pentosaceus* ATCC 25745. This strain of *Pediococcus* is established as LAB with probiotic and antagonistic properties against pathogens (Lin *et al.* 2019).

CONCLUSION

In conclusion, LABFB; specifically *Pediococcus pentosaceus* isolated from used nasopharyngeal and urinary catheters, can form biofilm and can reduce pathogen binding. It is also concluded that in this study, the improvised tube assay is more effective than the conventional microplate assay in assessing the binding capacity of LABFB, whereas both assays are efficient in assessing the PI. Also, TTC is the ideal stain for both assays.

ACKNOWLEDGMENTS

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STATEMENT ON CONFLICT OF INTEREST

The authors of this study declare no conflicts of interest.

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