International Research Journal of Biological Sciences _____ ISSN 2278-3202
Vol. **7(1),** 1-5, January **(2018)** *Int. Res. J. Biological Sci.*

Antagonistic effect of bacteriocin-like substances against biofilms formed by *P. aeruginosa*, *K. pneumonia,* and *P. vulgaris*

Sanchita Kar, Md. Nuruddin Mahmud, Kamrun Nahar Islam and Mohammad Seraj Uddin*
Department of Microbiology, University of Chittagong, Chittagong-4331, Bangladesh
seraj.mbio@cu.ac.bd

Available online at: www.isca.in, www.isca.me
Received 21st August 2017, revised 30th December 2017, accepted 5th January 2018

Abstract

Biofilms are frequently associated with many diseases including urinary tract infections. Structural organization and chemical properties of these assorted growths of microorganisms confer them the ability to resist antimicrobial therapy and thus infections caused by these microorganisms are becoming increasingly difficult to contain. Urinary catheters are typically involved in urinary tract infections due to their susceptibility to biofilm formation. Therefore, inhibition and/ or reduction of biofilms is critical to reduce catheter-associated infections. In this study, we used bacteriocin like substances produced by Lactobacillus coryniformis and Lactobacillus casei to reduce and/ inhibit biofilms formed by several pathogenic bacteria that are frequently associated with urinary tract infections. We isolated and identified eleven biofilm-forming bacterial isolates from urinary catheters collected from hospitalized patients: five Pseudomonas aeruginosa, three Klebsiella pneumoniae, and three Proteus vulgaris. Biofilm-forming capacity of the isolates was examined using microtitre plate method and three isolates showing the strongest biofilm forming capacity were selected for further investigation. To determine the effects of bacteriocin like substances, culture free supernatants (CSF) were prepared from Lactobacillus casei and Lactobacillus coryniformis and partially purified to remove non-proteinaceous substances. The CFS and partially purified substances (PPPs) were examined against biofilms formed by the bacterial isolates. Both the CFS and PPPs reduced biofilms formed by the isolates tested in our study. Our results indicate that biofilms formed by the P. aeruginosa, K. pneumoniae, and P. vulgaris isolates were significantly reduced upon treatment with bacteriocin like substances from the lactobacilli species used in our study.

Keywords: Bacteriocin, lactobacillus *coryniformis, lactobacillus casei, pseudomonas* sp.*,* urinary tract infection, urinary catheter.

Introduction

Biofilms play vital roles to cause infections as the structural organization and chemical properties of the biofilms provide them selective advantages to survive, spread, and resist antimicrobial therapy[1]. Although biofilms are formed on a variety of surfaces including cooling towers, ship hulls, paper mills, tabletops, dishcloths, computer keyboards, faucet handles etc., biofilms on medical devices (MDs) have attracted special attention as they have been linked to many infectious diseases. Many medical devices are prone to biofilm formation and significant numbers of morbidity and mortality have been attributed to chronic persistent infections resulting from biofilm-associated infections. Urinary catheter associated urinary tract infection (UTI) has been attributed to be responsible for majority of the nosocomial infections and these infections are mainly caused by persistent infections due to pathogens in the biofilms formed on urinary catheters.

Therefore, therapeutics targeting biofilm-associated infections are important to reduce biofilm-associated infections. Owing to their phenotypic and biochemical characteristics, microorganisms in biofilms often develop resistance to antimicrobial therapy. Urinary catheters are frequently associated with urinary tract infections (UTIs). Microorganisms form biofilms on the inner and outer surfaces of urinary catheters and planktonic cells from these biofilms enter into bladder causing UTIs. Studies have indicated that ~30% of all nosocomial infections are caused by (UTIs) and around 75% of all nosocomial UTIs are associated with urinary catheters[2-4]. Therefore, catheter associated biofilms are a major source and reason for UTIs. However, catheter associated urinary tract infections are difficult to treat as the microorganisms in these biofilms are usually resistant to antimicrobial therapy.

The predominant pathogens associated with catheter associated urinary tract (CAUTs) infections include *Escherichia coli*, Enterococci, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Candida albicans*, *Enterobacter*, *Proteus mirabilis* and coagulase-negative Staphylococci[5]. Antimicrobial agents introduced either systemically or directly into the catheter are not effective in preventing bacterial colonization[6]. Moreover, many bacteria have become resistant to antibiotics commonly used to treat UTIs[7]. Thus, antimicrobial drug therapy is not an effective treatment for UTIs. Lactic acid bacteria (LAB) are potential repertoires of antibacterial compounds[8]. Antimicrobial

compounds from LAB have been reported to delay the onset of biofilm on catheters[9]. We postulated that LAB harbor potent antibacterial substances that are effective against biofilm-forming bacteria. We report here that bacteriocin like substances produced by two lab, *Lactobacillus casei* and *L. coryniformis*, reduce and / or remove preformed biofilms and these proteinaceous substances have significant potential to reduce biofilm-associated infections.

Materials and methods

Collection of samples: To collect biofilm-forming bacteria, urinary catheters were withdrawn from patients presented with urinary tract infections at Chittagong Medical College Hospital and University of Science and Technology Hospital, Bangladesh. Each catheter was inserted into individual sterile polybag and transported to the laboratory at 4°C within 6 hours of collection. The catheters were cut into pieces of ~1 cm in length, rinsed with sterile phosphate buffered saline (PBS, pH 7.4), and 1 ml of the PBS was transferred to 5 ml of sterile Brain Heart Infusion broth (BHIB) medium. The medium was incubated at 37°C for 24 hours.

Isolation and identification of bacteria: One loop-full suspension from BHIB was streaked on Cetrimide agar to select *P. aeruginosa*, Casein Enzyme Hydrolysate (CLED) agar to select urinary pathogen, MacConkey agar and Eosin Methylene Blue (EMB) to select gram negative organisms. The plates were incubated at 37°C for 24hr. Bacterial isolates were selected on the basis of colony characteristics. The bacterial isolates were identified using standard morphological, cultural, and biochemical tests according to the standard description of "Bergey's Manual of Determinative Bacteriology".

Quantitative detection of biofilm formation: For quantitative analysis of biofilm formation, each bacterial isolate was inoculated into 10 mL of trypticase soy broth with 1% glucose and incubated at 37□ for 24hours. Then each culture was diluted 100 times in fresh trypticase soy broth medium at room temperature. and 125 µL from each diluted culture was transferred to individual wells of sterile 96-well flat bottom polystyrene tissue culture treated plates (Sigma Aldrich, Costar, USA).After incubation at 37^{o} for 24 hours, contents of each well were removed by gentle tapping.

Each well was washed four times with 0.2 mL of phosphate buffer saline (pH 7.2) to remove the free floating bacteria from the well. The wells were treated with sodium acetate (2%) and stained by crystal violet (0.1%). The wells were rinsed with deionized water and allowed to dry at room temperature. Optical density (OD_{630}) of the stained adherent biofilm was determined by using micro ELISA autoreader (model 680, Biorad, UK) and analyzed for calculating biofilm-forming capacity of the bacterial isolates. The experiment was performed three times in triplicates and the results were interpreted according to the criteria as described by Stepanovic et al.[10].

Preparation of cell free supernatant (CFS): *L. coryniformis* and *L. Casei* used in this study were donated by the Bangladesh Council of Scientific and Industrial research (BCSIR), Chittagong. The bacteria were grown in 50 ml MRS (DE MAN, ROGOSA, SHARPE) at 30^{0}C with shaking for 24 h. Then 25 ml of actively growing culture was inoculated in 225 ml fresh medium containing 500 ml conical flask at 30^{0}C with shaking for 48 h. Then the cultured broth was centrifuged at 10,000 rpm for 15 minutes at 4°C.

The supernatant was then filtered through Whatman No. 1 filter paper to remove residual cells[11]. Thereafter, the culture free supernatant (CFS) was stored at 4°C until the assay. Partial purification of the CFS was done by solvent (chloroform) extraction method[12]. Briefly, equal amounts of chloroform and CFS were mixed and stirred vigorously using a magnetic stirrer for 20 min. The aqueous layer, organic layer and the layer between (interfacial layer) the aqueous and organic phase were harvested separately. The interfacial layer was centrifuged at 13,000 rpm for 20 min and sediments were separated from the remaining chloroform and medium. The sediments were air dried and dissolved in 100 µl of 20 mM PBS and stored at 4°C until use. Inhibitory activity was assayed by agar well diffusion method (50 µl in each well)[13].

Determination of biofilm susceptibility: To determine the susceptibility of cell free supernatant (CFS) and partially purified substances (PPPS) to remove pre-formed biofilms, individual isolates were added to wells in microtiter plates containing growth medium and incubated at 37°C for 24 hours. After incubation, planktonic cells were discarded and the wells were washed again with PBS to remove the weakly adherent cells. The biofilms formed on the wells were then treated separately with CFS (200 µl) and PPS (200 µl). The residual biofilm on each well was estimated using crystal violate assay[14]. All experiments were carried out three times independently. The data obtained from three replicates were analyzed by a one-way ANOVA. In all cases, the level of statistical significance was $P < 0.05$.

Results and discussion

Isolation and identification of biofilm-forming *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Proteus vulgaris* from urinary catheters: To isolate biofilm-forming *Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Proteus vulgaris* from urinary catheters, we collected urinary catheters from hospitalized patients. Then bacterial colonies attached to the urinary catheters were isolated using selective media. The identity of the isolates was confirmed on the basis of their morphological, cultural, biochemical, and microscopic characteristics. Subsequently, the biofilm forming capacity of the isolates was examined using microtiter plate method. We found that nine of the eleven isolates exhibited biofilm formation (Table-1). Although majority of the isolates exhibited biofilm formation, variability in biofilm formation existed

among the isolates. We categorized the biofilm-forming capacity of the isolates according the criteria as described by Stepanovic et al.[10]. Our analysis revealed that varying degree of biofilm forming capacity existed among the isolates. The biofilm-forming capacity of these bacteria are similar to that of previous studies[15-17]. Three isolates (PA3, KP2, and PV3), one from each species that exhibited strongest biofilm formation, were selected for further study.

Susceptibility of bacteriocin like substances against preformed biofilms: Microorganisms in biofilms are generally resistant to antimicrobial compounds due to the structure and behavior of microorganisms in these environments. Moreover, inappropriate and indiscriminate use of antimicrobial compounds have contributed to the increase of resistance to these compounds. This necessitates the development of alternative agents and methods to contain antibiotic resistance. As microorganisms in biofilm environment are equipped with additional strategies to resist antimicrobial compounds, they are more cumbersome to control. However, recent studies showed that lactic acid bacteria can defer the commencement of biofilm formation on urinary catheters[9].

This led us to postulate that LAB produces substances that can reduce or inhibit the formation of biofilms on urinary catheters. To examine the effects of substances produced by *Lactobacillus coryniformis* and *Lactobacillus casei,* against biofilms formed by our isolates, we obtained culture free supernatant (CFS) from the overnight growth of the lactobacilli and tested against preformed biofilms using microtiter plate method. We found that the CFS produced by both lactobacilli significantly reduced biofilm formation of all the isolates (Figure-1).

Table-1: Screening of biofilm forming isolates.

Isolates[a]	OD_{630}[b]	Remarks[c]
PA1	0.03±0.04	-
PA2	0.08±0.03	+
PA3	0.30±0.01	+++
PA4	0.24±0.01	++
PA5	0.23±0.01	++
KP1	0.07±0.03	+
KP2	0.23±0.01	++
KP3	0.06±0.02	-
PV1	0.12±0.04	+
PV2	0.10±0.03	+
PV3	0.21±0.01	++
PA	0.30±0.01	+++

[a]bacterial isolates: *P. aeruginosa*(PA), *K. pneumonie*(KP), *P. vulgaris*(PV); [b]OD_{630} obtained for each isolate; [c]Interpretation of the results according to the method as described by Stepanovic et al.[10]. The symbols "+", "++" "+++" and "-" represent weak, moderate, strong and no biofilm formation, respectively.

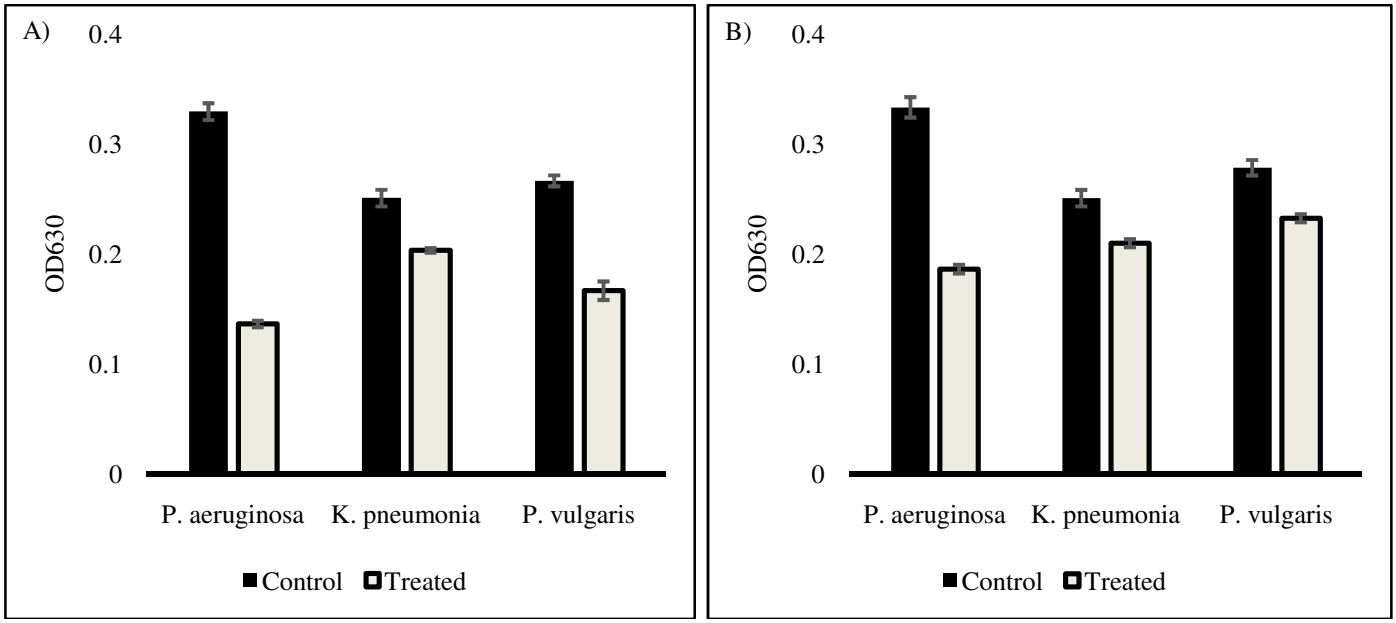

Figure-1: Susceptibility of preformed biofilms to CFS of *L. coryniformis* (a) and *L. casei* (b). Preformed biofilms were treated with CFS, incubated at 37°C for 24 hours, rinsed with PBS and the residual biofilms were quantified at 630 nm using the crystal violet assay[14]. The reduction of biofilm was expressed as a percentage of negative control.

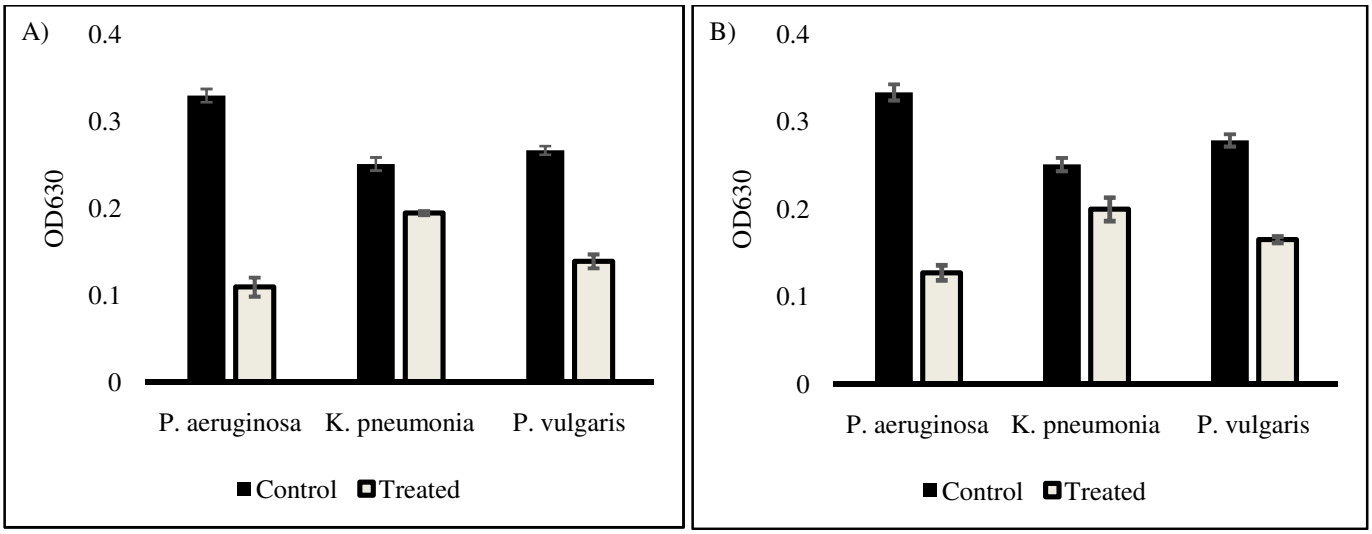

Figure-2: Susceptibility of preformed biofilms to PPPS of *L. coryniformis* (a) and *L. casei* (b). Preformed biofilms were treated with PPPs, incubated at 37°C for 24 hours, rinsed with PBS and. the residual biofilms were quantified at 630 nm using the crystal violet assay[14]. The reduction of biofilm was expressed as a percentage of negative control.

Because CFS contains non-proteinaceous substances, we partially purified the CFS to concentrate proteinaceous bacteriocin-like substance(s) using solvent (chloroform) extraction method[12]. We also examined the susceptibility of preformed biofilms to the partially purified proteinaceious substances (PPPs). Treatment of the preformed biofilms with PPPs led to significant removal of preformed biofilms (Figure-2). These results indicate that biofilms formed on biomedical devices can be reduced and / or removed by treatment with bacteriocin like substances produced by the two lactobacilli used in our study. This is consistent with a previous study in which biofilms formed by *Pseudomonas aeruginosa* were shown to be inhibited by proteins from *B. licheniformis*[11]. Similarly, bacteriocin from *L. coryniformis* and *L. casei* was previously used to inhibit foodborne pathogens[18,19].

Conclusion

Our results indicate that bacteriocin like substances from *L. Coryniformis* and *L. casei* can reduce and/or remove biofilms formed by *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Proteus vulgaris*. We conclude that biofilms formed on artificial surfaces can be reduced upon treatment with bacteriocin like substances from the two lactobacilli used in our study. Further investigation might reveal the mechanism of biofilm reduction and potential application of these substances to reduced / inhibit biofilm formation in medical devices and thereby reduce biofilm-associated infections.

References

1. Donlan R.M. (2002). Biofilms: microbial life on surfaces. *Emerging infectious diseases,* 8(9), 881-890.

2. Saint S. and Chenoweth C.E. (2003). Biofilms and catheter-associated urinary tract infections. *Infect Dis Clin North Am,* 17(2), 411-432.

3. Weber D.J., Sickbert-Bennett E.E., Gould C.V., Brown V. M., Huslage K. and Rutala W.A. (2011). Incidence of catheter-associated and non-catheter-associated urinary tract infections in a healthcare system. *Infection control and hospital epidemiology,* 32(8), 822-823.

4. Singha P., Locklin J. and Handa H. (2017). A review of the recent advances in antimicrobial coatings for urinary catheters. *Acta biomaterialia,* 50, 20-40.

5. Emori T.G. and Gaynes R.P. (1993). An overview of nosocomial infections, including the role of the microbiology laboratory. *Clinical microbiology reviews,* 6(4), 428-442.

6. Trautner B.W. and Darouiche R.O. (2004). Role of biofilm in catheter-associated urinary tract infection. *American journal of infection control,* 32(3), 177-183.

7. Pollard J., Wright J., Feng Y., Geng D., Genberg C. and Savage P.B. (2009). Activities of ceragenin CSA-13 against established biofilms in an in vitro model of catheter decolonization. *Anti-Infective Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Infective Agents)*, 8(4), 290-294.

8. Parada J.L., Caron C.R., Medeiros A.B.P. and Soccol C.R. (2007). Bacteriocins from lactic acid bacteria: purification, properties and use as biopreservatives. *Brazilian Archives of Biology and Technology*, 50(3), 512-542.

9. Heinemann C., van Hylckama Vlieg J.E., Janssen D.B., Busscher H.J., van der Mei H.C. and Reid G. (2000). Purification and characterization of a surface-binding protein from Lactobacillus fermentum RC-14 that inhibits adhesion of Enterococcus faecalis 1131. *FEMS microbiology letters,* 190, 177-180.

10. Stepanovic S., Vukovic D., Hola V., Di Bonaventura G., Djukic S., Cirkovic I. and Ruzicka F. (2007). Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *APMIS : acta pathologica, microbiologica, et immunologica Scandinavica,* 115, 891-899.

11. Dusane D.H., Damare S.R., Nancharaiah Y.V., Ramaiah N., Venugopalan V.P., Kumar A.R. and Zinjarde S.S. (2013). Disruption of microbial biofilms by an extracellular protein isolated from epibiotic tropical marine strain of Bacillus licheniformis. *PloS one,* 8, e64501.

12. Breukink E. (2006). A lesson in efficient killing from two-component lantibiotics. *Molecular microbiology,* 61(2), 271-273.

13. Dusane D.H., Pawar V.S., Nancharaiah Y.V., Venugopalan V.P., Kumar A.R. and Zinjarde S.S. (2011). Anti-biofilm potential of a glycolipid surfactant produced by a tropical marine strain of Serratia marcescens. *Biofouling*, 27(6), 645-654.

14. Dusane D.H., Rajput J.K., Kumar A.R., Nancharaiah Y.V., Venugopalan V.P. and Zinjarde S.S. (2008). Disruption of fungal and bacterial biofilms by lauroyl glucose. *Letters in applied microbiology*, 47(5), 374-379.

15. Kwiecinska-Piróg J., Bogiel T., Skowron K., Wieckowska E. and Gospodarek E. (2014). Proteus mirabilis biofilm-Qualitative and quantitative colorimetric methods-based evaluation. *Brazilian Journal of Microbiology*, 45(4), 1423-1431.

16. Al-Mathkhury H.J., Ali A.S. and Ghafil J.A. (2011). Antagonistic effect of bacteriocin against urinary catheter associated Pseudomonas aeruginosa biofilm. *North American journal of medical sciences,* 3(8), 367-370.

17. Macleod S.M. and Stickler D.J. (2007). Species interactions in mixed-community crystalline biofilms on urinary catheters. *Journal of medical microbiology*, 56(11), 1549-1557.

18. Lü X., Yi L., Dang J., Dang Y. and Liu B. (2014). Purification of novel bacteriocin produced by Lactobacillus coryniformis MXJ 32 for inhibiting bacterial foodborne pathogens including antibiotic-resistant microorganisms. *Food Control*, 46, 264-271.

19. Lü X., Hu P., Dang Y. and Liu B. (2014). Purification and partial characterization of a novel bacteriocin produced by Lactobacillus casei TN-2 isolated from fermented camel milk (Shubat) of Xinjiang Uygur Autonomous region, China. *Food control,* 43, 276-283.