Prevalence and Factors Influencing Biofilm-producing Bacteria on the Toothbrushes of Health Sciences Undergraduate Students

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Article History:
Received on March 25, 2024
Accepted on July 1, 2024
Published on July 29, 2024

Abstract:

Introduction: Biofilm is the formation of microorganisms that adhere to surfaces, producing a matrix that makes them resistant to harsh conditions such as antimicrobial therapies. This resistance can result in prolonged and challenging infection treatment. Common items like toothbrushes can harbour microbes that produce biofilm, thus contributing to adverse effects for the users. However, the factors that might interplay with the occurrence of biofilm on toothbrushes are scarce. Hence, this study aimed to understand the prevalence and potential causes of biofilm-producing bacterial contamination of toothbrushes among undergraduate students of a health sciences campus. **Methodology:** The study included 36 respondents, selected through convenience sampling. Each respondent completed a questionnaire that addressed demographic information, toothbrush characteristics, and oral hygiene practices. Their toothbrushes were tested by soaking the bristles in distilled water for 30 minutes, and then cultivating the water on Congo Red Agar plates. **Results:** Biofilm-producing bacteria were identified by the black and dry colony growth on the plates. Bacterial growth was observed on all toothbrushes, with five identified as biofilm producers. The study found no significant association (p>0.05) between toothbrush characteristics or oral hygiene practices and the presence of biofilm-producing bacteria on the toothbrushes. **Conclusion:** The findings suggest that toothbrushes can harbour bacterial growth, indicating the need for further research to understand the potential health implications.

Keywords: Biofilm, toothbrush, hygienic practices

Introduction:

Biofilms are complex communities of microorganisms that are ubiquitous and can develop in diverse environments, from natural habitats like ant bodies (Actinobacteria) to industrial areas like ship

hulls (Lo'pez, et al. 2010). In medical settings, they can adhere to devices like catheters and stents, with growth rates varying based on device type and implantation duration (Su, et al. 2022). The formation of biofilms poses challenges in health management due to their ability to trigger various diseases. Susewind, et al. (2015) observed that biofilm formation on oral equipment like dentures could harbour *Candida albicans*, leading to oral health complications and systemic diseases. Additionally, orthodontic appliances can exacerbate biofilm growth, resulting in tooth decay and white spot lesions (WSLs) (Ren et al. 2014). Furthermore, their resilience against various threats including host defenses, environmental stresses, and notably antibiotics, elevates concerns about biofilm-related infections in healthcare communities. Their ability to withstand antibiotics is particularly alarming, with their tolerance potentially increasing by 10-1000 times compared to planktonic cells (Rumbaugh and Sauer, 2020).

While extensive data exists regarding bacterial contamination on common everyday items, such as toothbrushes, limited studies focus on the detection of biofilm producers among these contaminants (Karibasappa et al., 2011; Saini and Kulkarni, 2013; Asumang et al., 2019). Moreover, there is a lack of accessible studies focusing on the population of health science students, who could serve as key advocates for promoting awareness of oral hygiene within the broader community. Therefore, the present study aims to detect biofilm-producing microorganisms, highlighting their potential to establish resilient communities on toothbrush surfaces among health science undergraduate students. In addition to detecting their presence, another crucial aspect to emphasize is the factors that influence their establishment. As reported by Chao et al. (2014), host-bacteria interactions such as quorum sensing, and environmental factors such as nutrient availability, pH, and temperature, heavily influence biofilm activities. By understanding the behavior of these biofilm producers concerning other influencing factors, the present study sought to determine the possible association between biofilm producers and toothbrush characteristics, as well as host oral hygiene practices.

Methodology:

Ethical Approval

The study received ethical clearance from the Kulliyyah of Allied Health Sciences Postgraduate and Research Committee (KPGRC) International Islamic University Malaysia (IIUM) Research Ethics Communities (IREC) (IREC 2023-KAHS/DBMS12). The participant's personal information was kept private at all times.

Questionnaires Preparation and Distribution

The survey employed in this study adapted and modified questionnaires used in previous studies by Khamuani, et al., (2018) and Nadar, et al. (2021). The questionnaire consisted of three parts: Part 1 was about participants' consent and demographic details including gender, program, and year of study, Part 2 focused on toothbrush characteristics, such as type and material; and Part 3 addressed oral hygiene practices, including brushing frequency and duration, toothbrush storage, and the use of other oral care products. The questionnaire was distributed to participants who volunteered and met the inclusion criteria, which included being active undergraduate students who had a used toothbrush. Recruitment efforts utilized verbal and written messages, as well as through online channels such as social media. Those who agreed to participate received additional face-to-face clarification from the researchers. Each participant was assigned a code to replace the real name to ensure the confidentiality of their data. A similar coding system was employed for toothbrush analysis.

Preparation of Congo Red Agar plate

Congo Red Agar (CRA) is a specialized agar medium used to selectively isolate and differentiate bacteria based on their capability to produce extracellular polysaccharides, particularly those involved in biofilm formation. The interaction of red dye in the agar with the compound of the extracellular polymeric substances (EPS) or its intermediate changes the red colour to dark red or black, which can be easily observed in the colony growth on the agar (Rajkumar et al., 2016). The agar was prepared by mixing 47g of Brain Infusion Heart, 5g of glucose, 10g of Technical Agar No. 2, and 0.8g of Congo Red Dye with 1L of distilled water before autoclaving at 121°C for 15 minutes (Bose et al., 2009). The poured into plates to solidify. The solidified plates were then incubated overnight to inspect for contamination. The agar plates were stored at 2 - 8°C before use.

Sampling and Biofilm Detection

A total of 36 toothbrushes were collected from participants and transported to the lab in a sterile pouch. The bristles of each toothbrush were immersed in sterile distilled water for 30 minutes. Subsequently, the water was thoroughly swirled before transferring one loopful onto Congo Red Agar (CRA) plates for culturing. These plates were then incubated at 37°C

for 24 - 48 hours under aerobic conditions. Any growth of bacterial colonies was recorded, with the presence of black colonies indicating biofilm producers. Alongside the experimental samples, *Streptococcus mutans*, a biofilm-producing clinical strain obtained from another biofilm-unpublished study, was cultivated as a positive control. Agar plates without bacterial culture was incubated together to ensure the sterility of the plates.

Statistical Analysis

The occurrence of biofilm-producing bacteria in the samples was analysed using descriptive analysis. Meanwhile, the association between these bacteria and variables like demographic data, toothbrush characteristics, and oral hygiene habits was assessed using the Chi-square test. A *p*-value less than 0.05 was considered statistically significant.

Results:

Demographic data of participants

Out of the 36 toothbrushes collected from the undergraduate students, the majority, 31 (86.11%), belonged to females while five (13.89%) belonged to the male respondents. A significant portion, 28 students (77.78%), were in their third year, with only one student (5.56%) from the second year and five students (13.89%) in their fourth year. The majority of respondents were enrolled in the allied health sciences program (32, 88.89%) while the remainder were from the medical, dentistry, and science-based programs. None of the respondents were smokers.

Biofilm-producing bacteria on toothbrushes

Upon completing the laboratory analysis, all toothbrushes revealed the presence of bacterial growth, five of which were identified as biofilm producers. The count and percentage of toothbrush samples positive for biofilm-producers are illustrated in Figure 1. Black colonies on the plates signified positive results for biofilm-producing bacteria (Figure 2).

Distribution of biofilm-producing bacteria and its association with evaluated variables

Regarding the material of the bristles, 28 students (77.78%) used non-charcoal bristles, while eight students (22.22%) used charcoal bristles. Two toothbrushes with charcoal bristles and three with non-charcoal bristles were found to be harboured by biofilm-producing bacteria, accounting for 25% and 10.7% respectively. Intriguingly, the majority of biofilm producing bacteria were found on the soft bristle samples (four out of five bacteria) equating to 25% of the total of 16 soft bristle toothbrushes. While one biofilm producer was detected on a medium bristle toothbrush, none were found on the hard bristles. Nevertheless, none of these distributions were significantly different (Table 1).

In terms of toothbrush usage duration, 11 students (30.56%) had used their toothbrushes for three months. Five students (13.89%) reported usage for one month, and another five for less than a month (13.89%). Three students (8.33%) had been using their toothbrushes for over four months, and two students (5.56%) for exactly four months. Biofilm-producing bacteria was detected on a toothbrush with one-month usage and on two toothbrushes each from the two and three-month usage categories. However, this distribution did not show any significant variance (Table 1).

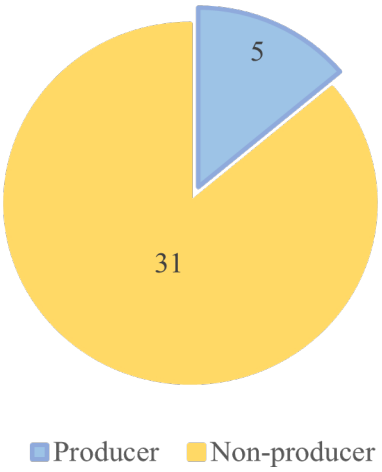

Figure 1: Occurrence of biofilm producing bacteria on toothbrushes. Total number of samples, n=36.

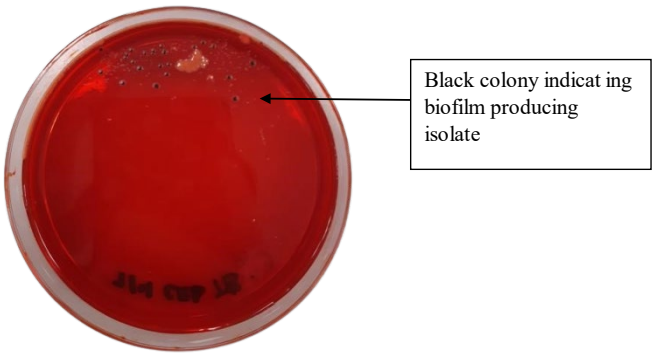

Figure 2: Black colony on Congo Red Agar plate indicating biofilm producing bacteria.

Table 1: Distribution of biofilm-producing bacteria and its association analysis with the variables

Variable	Isolated bacteria, N (%)		x^2 (df)	*p*-value
	Biofilm producer	Non-biofilm producer		
Bristle materials				
Charcoal	2 (25)	6 (75)	0.000 (5)	1.000
Non-charcoal	3 (10.7)	25 (89.3)		
Type of toothbrush				
Soft bristle	4 (25)	12 (75)	2.919 (3)	0.529
Medium bristle	1 (6.2)	15 (93.8)		
Hard bristle	0 (0)	1 (100)		
Unknown	0 (0)	3 (100)		
Toothbrush usage duration				
<1 month	0 (0)	4 (100)	2.842 (3)	0.562
1 month	1 (20.0)	4 (80)		
2 months	2 (18.2)	9 (81.8)		
3 months	2 (18.2)	9 (81.8)		
4 moths	0 (0)	2 (100)		
> 4 months	0 (0)	3 (100)		
Brushing duration				
<1 minute	0 (0)	2 (100)	2.842 (3)	0.562
1 minute	2 (9.1)	20 (90.9)		
2-5 minutes	3 (27.3)	8 (72.7)		
>5 minutes	0 (0)	1 (100)		
Brushing frequency in a day				
Once a day	0 (0)	2 (100)	1.699 (2)	0.549
Twice a day	4 (12.9)	27 (87.1)		
>Twice a day	1 (66.7)	2 (33.3)		
Type of toothpaste				
Fluoride	4 (1.54)	22 (84.6)	0.416 (2)	1.000
Fluoride-free	0 (0)	3 (100)		
Unknown	1 (14.3)	6 (85.7)		
Use of mouthwash				
Yes	0 (0)	7 (100)	0.000 (1)	0.559
No	5 (17.2)	24 (82.8)		
Toothbrush storage				
Bedroom	4 (15.4)	22 (84.6)	2.834 (5)	1.000
Toilet	1 (16.7)	5 (83.3)		
Other	0 (0)	4 (100)		

N=number of participants

A majority of respondents, 22 (61.11%), spent approximately one minute brushing their teeth. Next, 13 participants (30.56%) took between two to five minutes, while a single respondent (2.78%) brushed for more than five minutes. In terms of daily brushing frequency, the prevalent habit was twice a day (86.11%). Only two participants (5.56%) reported brushing once daily, and a few, 8.33%, brushed more than twice. Furthermore, 23 participants opted to replace their toothbrush quarterly, whereas eight changed it once a year (22.22%). The presence of biofilm-producing bacteria on toothbrushes varied based on brushing duration, with two brushes from the one-minute group and three from the 2 - 5 minute group. Moreover, four out of the five biofilm producers were found on brushes that were used twice daily (Table 1).

As for toothpaste preferences, 26 participants (72.22%) used fluoride toothpaste, and just three (8.33%) opted for fluoride-free variants. In the field of additional oral care, 29 participants (80.56%) did not use mouthwash. Interestingly, toothbrushes that used fluoridated toothpaste harbored four out of the five biofilm-producing bacteria. Furthermore, when considering the factor of mouthwash usage, all five-biofilm producers were detected on the toothbrushes of individuals who did not practice the use of mouthwash (Table 1).

In relation to toothbrush storage, the bedroom was the primary location for 26 participants (72.22%), where four biofilm producers were detected from toothbrushes which were kept in this space. Six (16.67%) stored their toothbrushes in the bathroom, while the rest had alternative storage spots. Yet, no significant association was identified for this distribution (Table 1).

Discussion:

Biofilms commonly develop on clinical equipment and devices such as catheters, orthopaedic implants, dental prosthetics, and breast implants. Their presence can cause severe infections in humans and exhibit antibiotic resistance, potentially leading to reinfections and chronic inflammation. Furthermore, biofilms can form on common items used like mobile phones, doorknobs, and clothing (Dixit, et al., 2023). This situation increases the risk of getting infected by biofilm pathogens which is harder to treat with the standard antimicrobial therapies. The occurrence of biofilm producers on toothbrushes, the most common daily used tools in oral health care, increases the risk for oral-associated disease. This research revealed that toothbrushes could harbour biofilm-producing bacteria, albeit at a low number, in five out of the 36 samples (13.89%). Though the percentage is low, it still suggests that toothbrushes can be considered as conducive environments for microbial growth. This is supported by research from Saini and Kulkarni (2013) who identified that toothbrushes can be primary habitats for various microorganisms, particularly bacteria. The space between the bristles of toothbrush can trap food particles, providing nourishment for microbes and enabling them to thrive and proliferate. These findings support the hypothesis that toothbrushes can be ideal breeding grounds for bacteria.

The Chi-Square test analysis revealed no significant association between all examined variables (characteristics of toothbrushes and hygienic practices of the participant) and the presence of biofilm-producing bacteria. These findings challenge the initial hypothesis suggesting these variables as potential contributors to biofilm development on toothbrushes. For instance, with regards to charcoal bristles, AlDhawi, et al. (2020) observed that charcoal bristles show a lower colony forming unit (CFU) count on blood agar plates than non-charcoal ones. Considering the characteristics of activated charcoal, renowned for its ability to adsorb toxins and eliminate bacteria, it was predicted that toothbrushes containing charcoal infusion could harbour fewer bacteria compared to those without charcoal (Silberman, et al., 2023; Thamke, et al., 2018). This reduction in bacterial presence may result in fewer cells available to initiate biofilm formation, consequently leading to a lower quantity of detected biofilm in the examination. This justification could be supported by the *in vitro* analysis done by Panariello, et al., (2020) who reported that charcoal-based dentifrices demonstrated inhibition of *Streptococcus mutans* biofilm, albeit the reduction was not statistically significant. Since very minimal studies focus on this scope, further study is needed to clarify the exact association and mechanism that may exist.

Although this study did not identify a substantial association between the variables investigated and the formation of biofilm, there were indications of increased growth of biofilm-producing bacteria under certain circumstances. For example, toothbrushes featuring soft bristles exhibited a higher incidence of biofilm compared to those with medium or hard bristles. It could be speculated that the ability of soft bristles to retain moisture for extended periods might create an environment conducive to bacterial growth, including stages of bacterial colonization essential for biofilm development. The pliability of soft bristles may form deeper recesses and spaces where bacteria can shelter, especially if the bristles bunch up when damp. Even though these factors might directly influence bacterial growth, the propensity for biofilm formation likely depends on the level of bacterial contamination on the brush. The precise mechanisms through which soft bristles might offer an ideal surface for biofilm-forming cells to begin attachment and progress through subsequent stages remain elusive. Further studies would certainly provide a more detailed understanding.

Brushing teeth multiple times, a day appears to increase the likelihood of biofilm-producing bacteria residing on toothbrushes compared to brushing just once daily. Kim, et al. (2018) noted that toothbrushes used three times a day harboured a higher count of general bacteria and coliform than those used less frequently. This suggests that the more a toothbrush is exposed to the oral environment, the greater the transfer of oral bacteria to the brush. While frequent brushing may reduce oral bacteria, including biofilm-producing ones in the mouth, they might persist on the bristles without proper cleaning measures. However, frequency isn't the only influencing factor; brushing techniques, as highlighted by Chhaliyil, et al. (2020), can also play a role in this phenomenon.

Initially, it was hypothesized that fluoride toothpaste could diminish bacterial viability in oral biofilms (Naumova, et al., 2019). This assumption stems from the knowledge that fluoridated toothpastes that contain sodium fluoride can permeate bacterial cell membrane, thereby interrupting bacterial metabolism. Amine fluoride, another compound known for its cationic antimicrobial properties, can attach to the bacterial cellular surface, thus leading to poor integrity of the cell membrane. Such interactions could diminish bacteria transfer from the oral cavity to the toothbrush leading to decreased biofilm development. Surprisingly, our findings showed a higher presence of biofilm-producing bacteria on toothbrushes used with fluoridated toothpaste. A potential reason might be other factors at play, such as toothbrush cleaning habits. Conversely, our observation aligned with the initial assumption regarding the impact of mouthwash. Mouthwash, an oral antiseptic has been shown to reduce biofilm growing on the surface of glass and Teflon after just a 30-second rinse. The foams of mouthwash prevent colonization and cariogenic action of acid-producing bacteria (Jones, et al., 2018). Among the biofilm-producing bacteria samples in this study, none of the participants used mouthwash, indicating its potential role in reducing biofilm formation.

The storage location for toothbrushes was initially thought to influence bacterial contamination. For instance, a moist bathroom environment might boost bacterial growth due to factors like aerosol from flushing. Contrarily, four out of five biofilm-positive samples in this study were stored in bedrooms. While humid conditions can support bacterial growth (Kim, et al., 2018), Okafor (2016) suggested that storage location might not significantly affect oral health.

Students with backgrounds in health sciences, medicine, and science, particularly dentistry are well-informed about the importance of oral hygienic practices. Their knowledge may contribute to proper oral cleaning habits, including the correct use and storage of their toothbrush and toothpaste. The awareness and practice of these respondents likely resulted in lower contamination rates, highlighting the significance of education in oral hygiene

Despite the significance of the study for future healthcare, particularly in oral health, it faces a lack of participant diversity and inadequate number of toothbrush samples. This imbalance, including the gender and academic program of respondent, characteristics of toothbrush materials and other variables may affect the study's conclusions. A larger-scale study is recommended to provide a clearer understanding and more robust conclusions.

Conclusion:

In summary, the study identified the potential for biofilm formation on toothbrushes, with certain variables possibly influencing this occurrence, even if not deemed significant in this research. It is imperative to delve deeper into these potential risk factors to enhance prevention strategies in oral healthcare. Additionally, this research emphasizes the need to heighten awareness about oral hygiene practices, especially among health sciences students, to ward off potential oral health challenges in the future.

Acknowledgements:

We would like to acknowledge all laboratory personnel in Department of Biomedical Science, Kulliyyah of Allied Health Sciences for their direct and indirect helps during the study.

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