Pesquisa Brasileira em Odontopediatria e Clínica Integrada 2021; 21:e0257
https://doi.org/10.1590/pboci.2021.107
ISSN 1519-0501 / eISSN 1983-4632

ORIGINAL ARTICLE

Sucrose, Lactose, and Xylitol Exposures Affect Biofilm Formation of *Streptococcus mutans*

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Academic Editor: Burak Buldur

Received: 07 December 2020 / **Review:** 19 February 2021 / **Accepted:** 02 March 2021

How to cite: Nuraini P, Kriswandini IL, Ridwan RD, Soetjipto. Sucrose, lactose and xylitol exposures affect biofilm formation of *Streptococcus mutans*. Pesqui Bras Odontopediatria Clín Integr. 2021; 21:e0257. https://doi.org/10.1590/pboci.2021.107

ABSTRACT

Objective: To determine the level of biofilm formation of *S. mutans* after being exposed to 5% sucrose, 8% lactose, or 1% xylitol. **Material and Methods:** This research was a laboratory-based experimental study with post-test only control group design. *S. mutans* was grown in test tubes containing tryptose soy broth (TSB) medium supplemented with 1% glucose. They were incubated at 37° C for 24 hours to grow the biofilms. The culture was then exposed to 5% sucrose, 8% lactose or 1% xylitol, incubated for 24 hours at 37° C, and examined using ELISA at a wavelength of 625 nm. The statistical analysis was performed using a one-way analysis of variance followed by the least significant difference test (α=0.05). **Results:** There were some differences in the biofilm formation of *S. mutans* after exposure to 5% sucrose, 8% lactose, or 1% xylitol ($p<0.05$). An LSD test indicated significant differences among the biofilm formations after exposure to 5% sucrose and 8% lactose and between 5% sucrose and 1% xylitol. In comparison, there were no significant differences ($p>0.05$) between 8% lactose and 1% xylitol. **Conclusion:** Sucrose, lactose and xylitol can form biofilms and the formation of lactose biofilms is the same as xylitol.

Keywords: Biofilms; Dental Plaque; *Streptococcus mutans*; Disaccharides; Sugar Alcohols.

Introduction

Epidemiology of dental caries worldwide, as established by the World Health Organization (WHO), showed average DMF T (Decay Missing Filling) index values at 12 years old ranging from 1.7–2.4. The DMF-T index in Southeast Asia, according to the WHO report, showed an average of 1.95 (± 1.24). The minimum and maximum values were 0.50 and 3.94, respectively [1].

The prevalence of child dental caries in Indonesia was part of Basic Health Research in 2018 conducted by Ministry of Health of Indonesia and is 88.8%. In children 3–4 years old, 81.5% suffered with dental caries with the average of dmf-t is 6.2, while in children 5–9 years old the prevalence is 92.6% with a DMF-T of 0.7. Note that the dmf-t index describes the severity of tooth decay in deciduous teeth and the DMF-T index describes the severity of tooth decay permanent teeth. The severity of dental caries in permanent teeth rises along with age. The average DMF-T index at 12 years is 1.9 [2].

Streptococcus mutans is a commensal bacterium in the human oral cavity and is a well-known cariogenic pathogen. *S. mutans* plays a key role in the formation of biofilms (i.e., dental plaque), which underlies several major oral diseases and tooth decay. The oral bacteria produce glucosyltransferases (GTFs) that are involved in the production of a water-insoluble, sticky glucan, using sucrose as the sole substrate. This insoluble glucan is responsible for biofilm formation [3].

Dental caries is caused by acid formation by cariogenic bacteria such as *S. mutans* and results from the interaction of *S. mutans* and other related bacteria by the production of biofilm on tooth surfaces [4]. Biofilm consists of many types of bacteria and their extracellular matrix (ECM) products. The major ECM component in dental plaque is the glucan produced by *S. mutans*. Biofilm formation is largely affected by the environment, and the mechanisms by which the gene expression of individual bacterial cells affects biofilm development have attracted interest. The environmental factors determine the cell's decision to form or leave a biofilm [5].

Previous research on the effectiveness of oral health education on oral hygiene and dental caries in school children stated that traditional oral health education, including brushing your teeth every day, effectively reduces plaque [6]. Basic health research report of the Ministry of Health in Indonesia on 2018 children aged 5–9 years brush 93.2% every day but the correct time to brush their teeth is only 1.4%. Likewise, the consumption of sweet foods and drinks with a frequency of more than once per day was 59% and 66.50% [7]. The World Health Organization in 2003 emphasized Oral Health Education (OHE) on behavior and strategies that improve oral health or reduce oral risk disease; health promotion in schools should encourage daily brushing, supervised brushing, fluoride use, and promotion of good nutrition, among other strategies.

Biofilm formation one of the most successful strategies for survival *S. mutans* in the dental environment. *S. mutans* is a bacterium that can metabolize various sugars into organic acids, which can cause cariogenic damage to the tooth surface. The types of sugars consumed by children from the disaccharide type are in addition to sucrose and lactose. Thus, the formation of dental biofilms can lead to the development of oral infectious diseases, including dental caries. Several clinical studies have focused on the effects of sucrose and lactose on *S.mutans*, but the results are debatable [8,9].

Substituting nutritive sweeteners for cariogenic sugars is an important measure for caries prevention in oral hygiene care. The sugar substitutes used are usually some sugar alcohol, such as mannitol, sorbitol, or xylitol. However, there has never been a study on the effect of lactose and xylitol on the biofilm formation of S. mutans bacteria. Therefore, the purpose of the study was to determine the biofilm formation of *Streptococcus mutans* bacteria after being exposed to sucrose, lactose or xylitol.

Material and Methods

Study Design and Ethical Clearance

This research was a laboratory-based experimental study with post-test only control group design. This study received ethical clearance from the Bioethics Committee of Faculty of Dental Medicine of Airlangga University Surabaya (044/HRECC.FODM/II/2019). The study was conducted at the Laboratory of Microbiology, Brawijaya of School of Medicine, Indonesia.

S. mutans Culture

Bacterial culture was carried out to multiply *S. mutans* stock bacteria (ATCC 25175), which was obtained from the Research Centre of Faculty Dental Medicine Airlangga University, by inoculating 1 ose pure culture of *S. mutans* bacteria into tryptose soy broth (TSB) media and then incubated it at 37° C for 24 hours.

Biofilm Formation Test

A total of 150 µL of *S. mutans* bacteria (10^5–10^6 CFU ml^{-1}) had been cultured in Brain Heart Infusion Broth (BHIB). *S. mutans* cells were cultivated for 24 h in four different media: BHIB (control), BHIB medium + 5% sucrose (S), BHIB medium + 8% lactose (L) and BHIB medium +1% xylitol (X) for one night at 37° C, were placed in a microtiter. Then, the bacteria were incubated for 24 hours at 37° C; after incubation, the media and the cells that were not attached to the microtiter were removed. Next, the planktonic cells were rinsed with sterile water. The cells were fixed by adding formalin (37%, 1:10 dilution) and 2% sodium acetate in wells containing attached cells (biofilms). Each well was stained with 200 µL of 0.1% crystal violet for 15 minutes at room temperature. Then, 100 µL of 95% alcohol was used to remove the dye after rinsing twice in sterile water. Microplates were placed in a shaker and then shaken for 10 minutes. The amount of biofilm formed was measured by measuring the optical density of the suspension formed by using a microplate reader (Zenix Microplate Reader) at a wavelength of 630 nm [10].

Scanning Electron Microscopy (SEM)

Biofilm formation was also observed by scanning electron microscopy (SEM). The cells were grown on sterile glass coverslips by immersing them in 12-well cell culture plates. The wells were inoculated and incubated at 37 °C for 24 h. The coverslips were removed after 24 h and washed three times in sterile PBS. The resultant samples were fixed in 2.5% glutaraldehyde in PBS (pH = 7.4) with 2% formaldehyde overnight. Post fixing, samples were rinsed thrice with PBS and dehydrated in absolute ethanol. The samples were then completely dried, coated with gold, and observed using SEM Inspect-S50 (FEI Company, Hillsboro, Oregon, USA) at 25,000 times magnification.

Statistical Analysis

The statistical analysis was performed using a one-way analysis of variance followed by the least significant difference test at a significance level of $p = 0.05$. The data were presented as mean and standard deviation. The difference between groups was estimated using a one-way ANOVA test ($p=0.00$), followed by a multiple comparison post-hoc Tukey HSD test conducted to compare multiple means ($p=0.00$). P-values were considered significant when <0.05. The statistical analysis was performed using SPSS 21 Software (IBM SPPS Inc., Armonk, NY, USA).

Results

Biofilm formation by microorganisms depends on the phase of growth, nutritional availability, and environmental conditions. Biofilm formation of S. mutans exposed to 5% sucrose, 8% lactose or 1% xylitol was observed after the staining procedure from microplate reader readings with a wavelength of 625 nm with the absorbance value or optical density (OD). Higher OD values indicate the growth of bacterial biofilms (Figure 1).

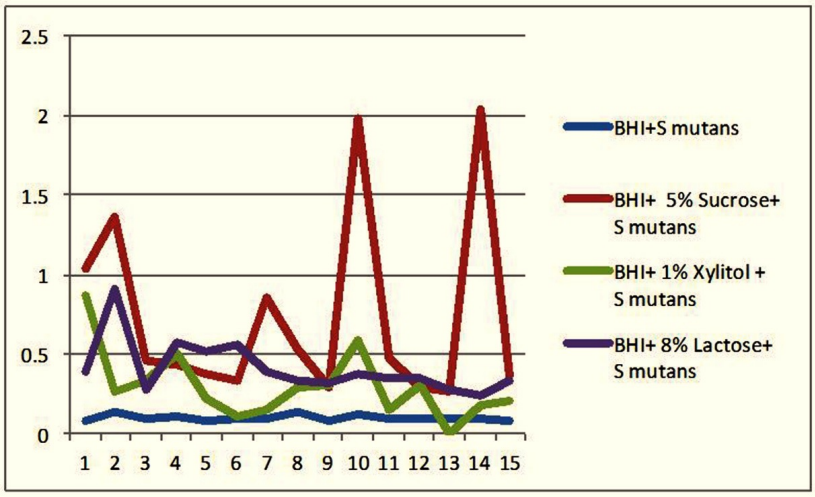

Figure 1. *S. mutans* biofilm formation after being exposed to 5% sucrose, 8% lactose and 1% xylitol and BHI + *S. mutans* as a control.

The ANOVA test indicated some significant differences between substrates ($p<0.05$). The average and standard deviation of *S. mutans* biofilm formation after exposure to 5% sucrose (0.80069 ± 0.163460) was higher than after exposure to 8% lactose (0.40950 ± 0.164126) also after exposure to 1% xylitol (0.30250 ± 0.196727) and control (0.09956 ± 0.019415). There were significant differences in biofilm formation *between S. mutans* exposed to 5% sucrose and 8% lactose, as well as 5% sucrose and 1% xylitol ($p<0.05$), while *S. mutans* exposed to 8% lactose and 1% xylitol showed no significant difference ($p>0.05$). Biofilm formation induced by sucrose, lactose and xylitol was analyzed by SEM. The SEM results image showed that biofilm formation induced by sucrose was characterized by densely clustered microcolonies (Figure 2).

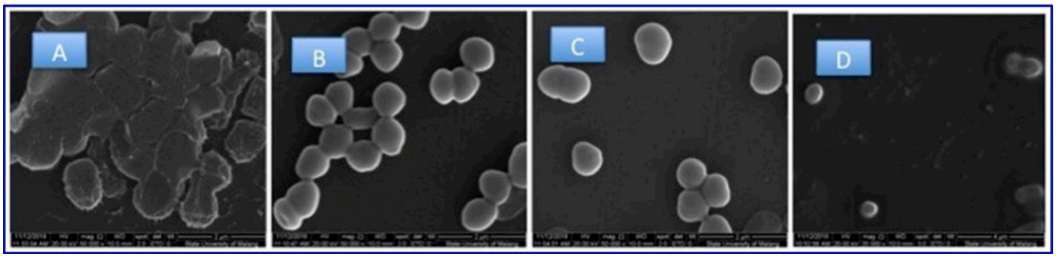

Figure 2. Electron microscopy, SEM of *S. mutans* biofilm formed after exposure to Sucrose (A), Lactose (B), Xylitol (C) and Control (D).

Discussion

Dental biofilm formation is highly dependent on the human diet. Both sucrose and lactose are types of sugar that children often consume. In a previous study, biofilm formation by analysis of the vitality of *S. mutans*

bacteria, which was induced by 5% sucrose by confocal laser scanning microscopy, was increased compared to 1% xylitol. Previous studies with different concentrations between sucrose and lactose reported that the formation of *S. mutans* biofilms induced by lactose showed the same results as sucrose and another research showed that the lactose-induced *S. mutans* bacteria showed lower biofilms than sucrose [8,9,11]. Therefore, the concentration of sucrose, lactose and xylitol as a substitute for sugar used in this study was based on the daily consumption of food, namely 5% sucrose, 8% lactose and 1% xylitol.

The 5% sucrose concentration used in this study is related to the concentration of sucrose to form cariogenic biofilms, which cause an acidic environment and demineralization of enamel, 8% concentration of lactose in this study refers to macronutrients breastfeeding varies within the mother, estimated at 6.7 to 7.8%, while 1% xylitol concentration is based on several previous clinical studies, which analyzed a final xylitol concentration of 1% in saliva for 10 minutes after use of a product containing xylitol such as chewing gum or toothpaste. Sucrose and lactose are fermentable disaccharides that can act as substrates to synthesize extracellular polysaccharides, which is considered important for accelerating biofilm formation.

Sucrose is recognized as the most cariogenic sugar. When in contact with the oral cavity biofilm, sucrose is rapidly metabolized by the bacterial consortium and used as a substrate to produce large amounts of organic acids, which causes a decrease in pH in the biofilm [12]. Xylitol is a natural sweetener approved by the US Food and Drug Administration (FDA) and the American Academy of Pediatric Dentistry and is used to substitute sucrose. However, xylitol polyols cannot be metabolized to acid by microorganisms in the oral cavity [6].

Lactose is found in milk and many dairy products and can be quickly fermented by the oral bacteria, including the highly cariogenic bacteria *S. mutans*. Therefore, the population of industrialized countries consumes a diet rich in milk because it contains all the basic components needed for the development and maintenance of human life [13]. In this study, biofilm formation of *S. mutans* bacteria was obtained after exposure to 5% sucrose, 8% lactose and 1% xylitol. These results are supported by several other researchers to prove the occurrence of biofilm formation after exposure to sucrose [5,7,13,14].

Biofilms are a structured community of microbial cells attached to a surface and forming a three-dimensional (3D) extracellular matrix [9,10]. This matrix consists of various substances such as extracellular polymers, exopolysaccharides, proteins, lipids, nucleic acids, and lipooligosaccharides [11]. These matrices are important for biofilm development and can express the virulence of some pathogenic bacteria [12]. The ability of biofilm formation tested on several carbohydrate diets indicated that more extensive biofilm formation was closely related to the presence of sugar. Previous authors have shown the role of sugar in the etiology of dental caries and the importance of sugar as the main dietary substrate that drives the caries process [15].

In this study, biofilm formation of *S. mutans* bacteria was obtained after exposure to 5% sucrose, 8% lactose and 1% xylitol. These results were supported by several other researchers to prove the occurrence of biofilm formation after exposure to sucrose [8,9,11,13,14]. Furthermore, after exposure to 5% sucrose, biofilm formation was higher than that of 8% lactose and 1% xylitol, while the formation of *S. mutans* bacteria exposed to 8% lactose was not significant compared to 1% xylitol.

Conclusion

It was found that sucrose, lactose and xylitol can form a biofilm of *S. mutans* bacteria. Sucrose and lactose are disaccharide sugars in the mouth that are fermented by enzymes found in bacteria. The presence of these two types of sugar is needed by both the body and bacteria, but in high concentrations, it can increase

bacterial biofilms. This study showed that the biofilm formed from lactose was lower than sucrose and was not different from xylitol as a sugar substitute. The response of lactose to biofilms was the same as xylitol, which was used as a sugar substitute. Subsequent research, the content contained in sucrose, lactose and xylitol influenced the formation of biofilms.

Authors' Contributions

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All authors declare that they contributed to critical review of intellectual content and approval of the final version to be published.

Financial Support

This research was fund by Ministry of Research, Technology and Higher Education (Grant Number: 122/SP2H/PTNBH/DRPM/2018).

Conflict of Interest

The authors declare no conflicts of interest.

Data Availability

The data used to support the findings of this study can be made available upon request to the corresponding author.

Acknowledgements

The authors thank to Airlangga University for facilitated this research.

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