**PROBIOTICS VS. BACTERIA: HOW LACTOBACILLUS CASEI BATTLES STREPTOCOCCUS MUTANS BIOFILM (IN VITRO)**

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**Keywords:**

*Biofilm Thickness; Probiotics Lactobacillus casei; Streptococcus mutans.*

**ABSTRACT**

***Background:****Caries formation is influenced by several factors such as teeth, microorganisms, biofilm, and also time. The initial bacteria that play an important role in biofilm formation is Streptococcus mutans. Probiotics made from these bacteria are considered capable of overcoming dental and oral health problems because they are able to suppress the biofilm which will coat and protect the mucosa from invasion by cariogenic bacteria such as caries.*

***Purpose:****To determine the effect of the probioticLactobacillus casei in reducing Streptococcus mutans biofilm growth (in vitro).*

***Method:****The research design carried out was experimental research with a post test only control group design. The research samples were divided into 3 groups, namely the chlorhexidine, distilled water and probiotic groups. Biofilm thickness testing was carried out by OD (Optical density) using an ELISA-reader. Parametric statistical test analysis using the Kruskal Wallis Test.*

***Results:****The results of the Kruskal Wallis statistical test showed P= 0.000 there is a significant difference in the 3 Streptococcus mutans thickness test groups.*

***Conclusion:****The probioticLactobacillus casei has the effect of reducing the thickness of the Streptococcus mutans biofilm*

**INTRODUCTION**

 Dental caries is a dental disorder that often occurs in society. Based on the 2023 Riskesdas, 56.9% of the population aged over 3 years experienced dental and oral health problems, and only11.2% of them went to the doctor for treatment. This needs serious attention because there has been no significant change in the last 5 years when compared to the results of the 2018 Riskesdas. The 2023 Riskesdas also stated that Indonesian people experience dental and oral health problems with a caries prevalence of 43.6%(RISKESDA, 2023). Dental caries occurs due to a process of demineralization of the tooth structure caused by microorganisms in a colony called a biofilm. Microorganisms produce acid which forms cavities in the tooth structure, namely on the surface of enamel, dentin, or cementum.(Sibarani,2014). They can live and reproduce in an acidic environment and adhere to the tooth surface. Streptococcus mutans is one of the microorganisms responsible for the formation of dental caries.(Komansilan et al., 2015).

The cause of tooth decay begins with the presence of sucrose and bacteria that stick to the surface of the teeth and then form a thin layer in the form of a biofilm.(Listrianah et al., 2019). Biofilm is a thin layer that occurs due to colonization of bacteria and is coated by an extracellular polysaccharide matrix that supports bacterial survival. Streptococcus mutans is the main colonizer of caries formation because it is cariogenic. This bacteria is acidogenic, resistant to low pH, and also stores polysaccharide reserves such as glycogen.(Fina Maghfirah, Dewi Saputri, 2017).

The right way to reduce biofilm accumulation on the tooth surface is by mechanical means or toothbrush and chemically using mouthwash.(Karyadi & Roza, 2021). If only using a toothbrush, the fissure, buccal pit and interproximal gingival margin area cannot be reached with a toothbrush alone, then it can be helped by using mouthwash to clean the parts that cannot be reached with a toothbrush (Karyadi, 2020). Research by(Kumar et al., 2013)said that Chlorhexidine (CHX) is the 'gold standard' mouthwash and is most widely used to help clean areas that cannot be reached with a toothbrush. Long-term use of chlorhexidine can cause stains on teeth, taste disorders, ulceration in the oral cavity, and also swelling of the parotid glands both unilaterally and bilaterally(Kumar et al., 2013). The use of chlorhexidine also has side effects such as discoloration of the teeth, changes in tooth color, sensations, and unpleasant tastes.(Kumar et al., 2013).

From this, an alternative is needed that can be used to reduce microorganisms that cause caries and hard tissue diseases in teeth, namely by using probiotics. Probiotics are a collection of microorganisms that when given in sufficient quantities will provide benefits to the host and are able to fight infections.(Fina Maghfirah, Dewi Saputri, 2017). Probiotics made from bacteria are considered capable of overcoming dental and oral health problems such as caries, gingivitis, periodontitis, and halitosis.(Sugiman, 2014). One example of probiotic bacteria is Lactobacillus cassei which is found in fermented milk drinks, namely yakult and yogurt.

Study in (Lindawati&Simanjuntak,2020) showed that chlorhexidine and probiotic mouthwash lactobacillus casei have antimicrobial effects on plaque accumulation of Streptococcus mutans. This study explains that chlorhexidine, sodium fluoride, and probiotic mouthwash reduce the levels of Streptococcus mutans. Probiotic mouthwash is effective and equivalent to chlorhexidine and sodium fluoride mouthwash, but chlorhexidine mouthwash has some side effects on dental and oral health.(Lindawati & Simanjuntak, 2020). Other research by(Fina Maghfirah, Dewi Saputri, 2017)also explains the formation of Streptococcus mutans and Candida albicans biofilm mass given probiotic drinks containing Lactobacillus casei proven to show the thickness of Streptococcus mutans biofilm decreasing, on the other hand this study is still uncertain which bacteria can reduce the number of Streptococcus mutans colonies among the bacteria in yakult. So it is necessary to conduct research on the effect of one of the bacteria in yakult. Therefore, in this study the author chose to try to use Lactobacillus casei bacteria in reducing the growth of Streptococcus mutans biofilm (in vitro).

Considering this background, the author wanted to conduct a study to determine the effect of probiotic Lactobacillus casei in reducing the growth of Streptococcus mutans biofilm.

Based on Islamic teachings, Allah SWT teaches us to maintain cleanliness, as according to the following hadith:



Meaning: It was narrated from Sa'ad bin Abi Waqas, from his father, that Rasulullah SAW said: "Indeed, Allah SWT is Most Holy and loves purity, He is Most Clean and loves cleanliness, He is Most Noble and loves glory, and He is Most Beautiful and loves beauty. Therefore, clean your places." (HR. Tirmizi)..

Based on this hadith, we can conclude that Allah SWT encourages us to maintain our health and cleanliness, both spiritually and physically, because Allah SWT loves people who maintain their health and cleanliness, and cleanliness is part of faith.

**RESEARCH METHODS**

This study used a laboratory experimental design with a posttest-only control group design, involving three treatment groups: probiotic Lactobacillus casei (K1), 0.2% chlorhexidine as a positive control (K2), and aquades as a negative control (K3).

The independent variable in this study was the administration of Lactobacillus casei probiotics, while the dependent variable was the thickness of Streptococcus mutans biofilm. Operational definitions include measuring biofilm thickness using the microtiter plate biofilm assay method with reading the results using an ELISA Reader at a wavelength of 620 nm.

The research process includes preparation of tools and materials, biofilm making, and formation of probiotic and test bacterial suspensions. Sterilization of tools was carried out using an autoclave at a temperature of 121°C with a pressure of 2 atm for 15 minutes. Streptococcus mutans biofilm was cultured in TYS20B media under anaerobic conditions for 48 hours at a temperature of 37°C. Lactobacillus casei probiotics were cultured using MRS Broth media supplemented with pure milk and incubated for 48 hours at the same temperature. Chlorhexidine 0.2% and distilled water were prepared as controls.

This study involved taking saliva from respondents using the spitting out method after stimulation with xylitol chewing gum. Respondents aged 18-25 years were selected based on inclusion and exclusion criteria, and were asked to sign an informed consent. The study procedure involved mixing Streptococcus mutans solution and saliva in a well plate, administering test solutions (probiotics, chlorhexidine, or aquadest), incubation, staining using gentian violet crystals, and reading OD results using an ELISA Reader.

Data processing was carried out through normality tests (Shapiro-Wilk) and homogeneity (Levene's Test). If the data is normally distributed and homogeneous, parametric statistical analysis was performed using the One Way ANOVA test. Otherwise, the nonparametric Kruskal-Wallis test was used. Data analysis was performed using SPSS software.

This study was conducted in September and October 2024 at the Microbiology and Biology Laboratory of the Faculty of Medicine, Sultan Agung Islamic University, Semarang. Ethical clearance for this study was obtained from the Research Ethics Committee of the Faculty of Dentistry, Sultan Agung Islamic University. The study was conducted in accordance with the procedures and ethical principles of scientific research.

**RESEARCH RESULT**

This study was conducted to determine the effect of giving probiotics Lactobacillus casei, 0.2% chlorhexidine, and distilled water on the thickness of Streptococcus mutans biofilm. Biofilm making is done by culturing Streptococcus mutans bacteria assisted by pure patient saliva, the function of saliva here is used to help stimulate the formation of biofilm so that the biofilm can form and stick to the well plate wall. After that, three control group influences will be given, namely Lactobacillus casei, 0.2% chlorhexidine, and distilled water, then reading and measuring the thickness of the biofilm will be carried out using an ELISA-reader (Indriyanti et al, 2019)**.**

The results of the Streptococcus mutans biofilm thickness test show the average value of the number of Streptococcus mutans with 3 treatment groups given Lactobacillus casei probiotics, aquades, and the treatment group given 0.2% chlorhexidine in table 1.

**Table1** Mean and standard deviation of thicknessStreptococcus mutans

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Group | N | Mean | Standard Deviation |
| Probiotics | 9 | 0.07515 | ± 0.001771 |
| Chlorhexidine | 9 | 0.07726 | ± 0.002881 |
| Aquadest | 9 | 0.09332 | ± 0.007246 |

The lowest average thickness of Streptococcus mutans biofilm was in the Lactobacillus casei control group, while the highest thickness of Streptococcus mutans biofilm was in the aquadest control group.

The data was tested using the Shapiro-Wilk test to determine its normality and also tested using the Lavene Statistic test to determine its homogeneity. The results of the normality test and homogeneity test can be seen in table 2.

**Table2** Normality Test and Homogeneity Test

|  |  |  |  |
| --- | --- | --- | --- |
|  | Group | Normality | Homogeneity |
| Probiotics | 0.178 | DataNormal | 0.028 | Non-Homogeneous Data |
| Chlorhexidine | 0.726 |
| Aquadest | 0.077 |

Based on table 2 shows the results of the normality test all data are normally distributed (p ≥ 0.05). The data was then tested for homogeneity using the Levene Statistic test. The homogeneity test in table 2, obtained a value of 0.028 (p <0.05) which indicates that the thickness data of Streptococcus mutans is not homogeneous.

Based on these data, it is known that the data is normally distributed and not homogeneous, so the next stage is the Kruskal Wallis parametric test to determine the difference in the thickness of the Streptococcus mutans biofilm in each group, then continued with the Mann Whitney post-hoc test.

**Table 3** TestPost-hoc Mann Whitney and Kruskal Wallis Tests

|  |  |  |
| --- | --- | --- |
|  | Mann Whitney Post-hoc Test | Kruskal Wallis Test |
| *Chlorhexidine-casein* | 0.342 | There is no significant difference | 0,000 |
| *Chlorhexidine-aquades* | 0,000 | There is a significant difference |
| *casei-Aquades* | 0,000 | There is a significant difference |

 **(a) (b) (c)**

The results of the significance number in the Kruskal Wallis Test shown in the table are 0.000 (p < 0.05) so it can be concluded that there is a significant difference between the chlorhexidine, aquades, and probiotic groups on the absorbance value of Streptococcus mutans biofilm. To find out which group has the most significant difference, a Mann Whitney post-hoc test was conducted. In the Mann Whitney post-hoc test above, it can be concluded that there is a significant difference between the chlorhexidine-aquades and casei-aquades groups, while chlorhexidine-casei has no significant difference on the absorbance value of Streptococcus mutans biofilm with a p value> 0.05.

Based on the results of the statistical analysis above, it can be said that there is a significant difference between the administration of Lactobacillus casei, Chlorhexidine, and aquades on the thickness of Streptococcus mutans biofilm.

**DISCUSSION**

The results of the study showed that there was a significant difference between the administration of Lactobacillus casei bacteria, 0.2% chlorhexidine, and distilled water on the average optical density of the thickness of Streptococcus mutans biofilm. Table 4.1 shows the lowest average value of Streptococcus mutans biofilm thickness in the Lactobacillus casei control group, while the highest Streptococcus mutans biofilm thickness was in the distilled water control group. Based on research fromFahruddin et al. (2019), explains that the lower the optical density value, the lower the thickness of the Streptococcus mutans biofilm formed.

Further post hoc Mann Whitney test showed that there was no significant difference in the thickness of mutans biofilm between Lactobacillus casei bacteria and 0.2% chlorhexidine. This is because both control groups can influence each other in inhibiting the thickness of s. mutans biofilm. In the analysis of the results of this study, it was found that casei was proven to be more able to reduce the thickness of s. mutans biofilm compared to chlorhexidine, but on the other hand, it should be remembered that this study only used s. mutans biofilm and L. casei probiotics here are only effective as an alternative to reduce microorganisms that cause caries, not as a substitute for chlorhexidine. Because between chlorhexidine and L. casei probiotics there are differences in mechanisms and how to use them is also different. Chlorhexidine works by disrupting the bacterial cell membrane and inactivating important proteins in cells, while probiotics function more to balance the microbiota and prevent the growth of pathogens than by directly destroying existing biofilms.(Widani et al., 2015).

In the Mann Whitney post hoc analysis test, it also showed that there was a significant difference between L. casei and chlorhexidine against aquades. Based on research from Anatje et al, (2022) it was stated that aquades as a negative control did not form a clear zone in the well. This clear zone or bacterial inhibition zone is a clear area that forms around the well of bacterial growth media that is not overgrown with bacteria. This is because aquades does not have antibacterial properties. The reason for using aquades is as a comparison to see whether or not there is an effect of the solvent on bacterial growth. Unlike L. casei and chlorhexidine which can inhibit biofilm growth, L. cassei with its protective mechanism of action while chlorhexidine with its antibacterial properties(Aritonang, N et al., 2019). Simple use of distilled water can still have an effect on maintaining dental and oral health because one of the main benefits of distilled water or plain water is its ability to help maintain pH balance in the mouth (Anatje et al, 2022). These three control groups, namely L. casei, Chlorhexidine, and distilled water, have their own characteristics, meaning that in the three control groups of this study they have different functions, methods of use, times of use, and mechanisms of action.

Biofilm growth can be inhibited by using a probiotic solution of Lactobacillus casei which produces organic acids which are bacteriostatic against Streptococcus mutans biofilms.(Jothika et al. 2015). This study shows that probiotic Lactobacillus casei is effective in reducing the thickness of Streptococcus mutans biofilm. This occurs because the content of probiotic lactobacillus casei produces compounds that can inhibit the growth of Streptococcus mutans. Probiotics produce antagonistic substances such asbiosurfactants, bacteriocins, exopolysaccharides (EPS), organic acids (lactic acid, acetic acid, propionate and succinate), hydrogen peroxide which can inhibit the activity of pathogenic bacteria and their adhesion to the surface and the formation of unfavorable environmental conditions for pathogenic bacteria such as decreased pH and nutrient competition*(Barzegari et al.,*2020). The mechanism of probiotics by producing biosurfactants will disrupt biofilms because the adsorption of biosurfactants to the substrate can disrupt the adhesion and desorption processes of microbes. Biosurfactants have bactericidal behavior by disrupting the cytoplasmic membrane of pathogens or disrupting biofilm proteins, causing cell lysis, and metabolite leakage. Biosurfactants can disrupt the cell division process and affect the expression of biofilm-related genes, thereby disrupting the release of signaling molecules in the quorum sensing (QS) system and subsequent biofilm formation.(Carvalho et al., 2021).

Chlorhexidine 0.2% solution was used as a positive control because it has bacteriostatic and bactericidal effects against various types of microbes. Chlorhexidine works effectively in inhibiting and killing Gram-positive and Gram-negative bacteria because its molecules are positively charged (cations), while bacteria have a negative charge (anion)(Pratiwi et al., 2022). This molecular interaction allows chlorhexidine to penetrate bacterial cells and form a strong bond with their cell membranes. As a result, the permeability of the bacterial cytoplasmic membrane is disrupted, allowing intracellular compounds to exit the cell. This damage renders the bacterial cell dysfunctional, allowing chlorhexidine to penetrate the entire plaque surface more effectively.(Panesa et al., 2018).

*Chlorhexidine*and probiotic Lactobacillus casei can be used to control bacterial biofilms, but with different mechanisms. Chlorhexidine works by disrupting the bacterial cell membrane and inactivating important proteins in the cell, and has the ability to penetrate the biofilm and kill bacteria, making it more effective in reducing biofilms.(Sinaredi et al., 2014)Meanwhile, probiotics work by competing with pathogenic bacteria for resources and adhesion sites, and can produce antimicrobial substances.(Aritonang, N et al., 2019).

The limitation of this study is that it did not use other types of bacteria, so it is less similar and less representative of the environment in the oral cavity.

**CONCLUSION**

Based on the results of this study it can be concluded:

1. Administration of the probiotic Lactobacillus casei has been shown to have an effect on the thickness of Streptococcus mutans biofilm.
2. The lowest thickness of Streptococcus mutans biofilm was in the influence of the Lactobacillus casei control group, while the highest thickness of Streptococcus mutans biofilm was in the influence of the aquadest control group.
3. There was a significant difference between the chlorhexidine-aquades and casei-aquades groups, whereas chlorhexidine-casei did not show a significant difference in the thickness of the Streptococcus mutans biofilm.

We would like to express our gratitude to Allah SWT for His grace, so that this research can be completed properly. We would like to express our gratitude to Dr. drg. Yayun Siti Rohmah, Sp.BM, Dean of the Faculty of Dentistry, Sultan Agung Islamic University, Semarang, as well as to the first supervisor, Dr. drg. Hj. Rahmawati Sri Praptiningsih, M.MedEd, and the second supervisor, drg. Rina Kartika Sari, Sp.PM, who have provided guidance, support, and valuable input in the preparation of this paper. We would also like to thank the Microbiology Laboratory of the Faculty of Dentistry, UNISSULA, which has facilitated this research. We would also like to express our gratitude to our family, friends, and colleagues at the Faculty of Dentistry who have provided encouragement, prayers, and motivation. Hopefully, this work will be useful for the development of science, especially in the field of dentistry.

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