EFFECT OF THE PROBIOTIC STREPTOCOCCUS SANGUINIS ON THE FORMATION OF STREPTOCOCCUS MUTANS BIOFILM IN ARTIFICIAL SALIVA

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ABSTRACT

Background: Dental caries is a disease of the hard tissues of the teeth that most often occurs with a prevalence rate of about 45.3%. Dental caries is occurring because of the interaction between biofilms and carbohydrates from the food on the tooth surface (host). The caused by the biofilm formation is the presence of colonies of Streptococcus mutans bacteria. To reduce the accumulation of biofilms, mechanical and chemical methods can be used, namely using a toothbrush and mouthwash. Probiotics are widely used in dentistry because they have anti-plaque activity and form H2O2 which can reduce biofilm formation.

Purpose: The purpose of this study was to determine the effect of the probiotic Streptococcus sanguinis on the formation of Streptococcus mutans biofilm in artificial saliva.

Method: This research method was experimental analytic with post test only group design. The samples were 9 and divided into 3 groups, namely the probiotic group, Streptococcus sanguinis, the positive control group chlorhexidine and the negative placebo control group. Each group incubated in adhesion phase for 4 hours at 37°C. The formation of biofilms is measured by calculating the average results of Optical Density using an ELISA-reader. Data analysis was performed using the test One-way Anova.

Results: The average values of optical density in the S. sanguinis, chlorhexidine and placebo groups, in sequence, were 0.374, 0.414, and 0.420. One-way Anova test obtained a significant value (p) of 0.014 (p<0.05) which indicates that there are significant differences in the probiotic group Streptococcus sanguinis, the chlorhexidine group, and the placebo group.

Conclusion: This study showed that the probiotic Streptococcus sanguinis had a significant impact on the formation of Streptococcus mutans biofilm in artificial saliva.

Keywords: Biofilm, Optical Density, Probiotic, Streptococcus mutans, Streptococcus sanguinis

INTRODUCTION

Dental caries are a pathological disease in the oral cavity characterized by demineralization of tooth enamel. The aetiology of caries is multifactorial, namely host, time, substrate, and bacteria. The initial cause of the formation of dental caries is caused by the presence of sucrose and bacteria that adhere to the tooth surface and form a thin layer, namely a biofilm layer.

Biofilm is a thin layer formed due to the colonization of bacteria and is covered by a polysaccharide extracellular matrix that supports bacterial survival. One of the bacteria that forms a biofilm is a strain of Streptococcus mutans. Streptococcus mutans is considered as the main colonization in biofilm formation because it is cariogenic. Biofilm formation occurs in several phases, namely adhesion, maturation and dispersion. In the initial phase of biofilm formation, reversible and irreversible adhesion occurs on the surface so that it will form microcolonies, in this phase it lasts for 2-4 hours. The next phase is the maturation phase, which is divided into 2 stages, namely, involving communication between cells and producing autoinducer signals as well as increasing the size and thickness of microcolonies into macrocolonies. The dispersion phase is the phase that occurs when bacteria attached to the surface will move from one surface area to another to get nutrients back.

The right way to reduce biofilm accumulation is to brush your teeth and use a mouthwash in the form of chlorhexidine. The use of a toothbrush and chlorhexidine has disadvantages such as the interproximal part is not accessible, then the use of chlorhexidine for a long time can cause staining of the teeth. Another alternative to chlorhexidine is using probiotics.
Probiotics are live microorganisms that are given in sufficient quantities/dose to provide benefits to the host. The use of probiotics in the oral cavity can improve oral health without having a negative impact on oral flora or microbiota. The local effect of probiotics when interacting with other bacteria is to inhibit the growth of pathogens by producing H₂O₂ or hydrogen peroxide, bacteriocins and organic acids. The example of a probiotic is a strain of *Streptococcus sanguinis* which is a commensal bacteria in the oral cavity. *Streptococcus sanguinis* is considered to be able to inhibit the growth of *Streptococcus mutans* forming biofilms by producing H₂O₂.

The purpose of this study was to determine the effect of the probiotic *Streptococcus sanguinis* on the formation of *Streptococcus mutans* biofilm in artificial saliva.

**METHOD**

This research was conducted based on Ethical Clearance No. 301/B.1-KEPK/SA-FKG/11/2021. This research is included in the experimental research method with a post test only group design. The sample size was calculated based on the Federer formula, the number of samples obtained was 9 in the three treatment groups. In this study, the samples were divided into 3 groups, namely the probiotic group, *Streptococcus sanguinis*, the positive control group (+) chlorhexidine, and the negative control group (-) placebo so that the total number of samples was 27 samples.

**Bacterial Suspension Method**

Preparation of suspension of *Streptococcus mutans* and *Streptococcus sanguinis* bacteria using Brain Heart Infusion (BHI) media. Pure isolates from Streptococcus mutans and *Streptococcus sanguinis* were put in BHI media and 1% sucrose was added. The growth of *Streptococcus mutans* and *Streptococcus sanguinis* was adjusted to the standard McFarland turbidity of 0.5 so that the resulting bacterial suspension was 1.5 x 10⁹ CFU/ml. The bacteria were incubated at 37°C for 24 hours.

**Sampling on Media**

The study sample was prepared by forming a pellicle on each well-plate of artificial saliva from the lab of FK Unissula with McDougall method (NaHCO₃ 9.8 gr, Na₂HPO₄ 8 gr, KCl 0.56 gr, NaCl 0.47 gr, MgSO₄ 0.12 gr, CaCl₂ 0.04 gr) as much as 100 µl and incubated for 90 minutes. Saliva that did not adhere to the well-plate was rinsed with 100 µl phosphate buffer saline (PBS). The suspension of *Streptococcus mutans* ATCC 25175 was added to well-plate media along with the probiotic *Streptococcus sanguinis* ATCC 10556, control (+) chlorhexidine (Minosep), and control (-) placebo as much as 50 µl. Samples were incubated for 4 hours (adhesion phase) at 37°C. Rinse the well-plate with PBS 3 times and dry for 5 minutes. Drops of 1% gentian violet crystals on a 150 µl well-plate for 15 minutes and rinse with PBS 2 times, dry for 5 minutes. Ethanol 96% as much as 200 µl added to the well-plate and let stand for 15 minutes. Reading the results by measuring the optical density with a wavelength of 540 nm using an ELISA-reader.

**RESULT**

The results of the comparison of the average optical density of *Streptococcus mutans* biofilm:

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probiotic</td>
<td>0.374 ± 0.041*</td>
</tr>
<tr>
<td>Chlorhexidine</td>
<td>0.414 ± 0.120*</td>
</tr>
<tr>
<td>Placebo</td>
<td>0.420 ± 0.220*</td>
</tr>
</tbody>
</table>

Table 1. The mean value of optical density showed a significant difference between the three treatment groups.

The results of the normality test and homogeneity test showed a significance value (p) of 0.885 in the probiotic group of *Streptococcus sanguinis*, 0.880 in the positive control group, and 0.605 in the negative control group (sig. (P) > 0.05) indicating that the data were normally distributed. The results of the homogeneity test obtained a significance value (p) of 0.571 (sig. (P) > 0.05) so it can be concluded that the distribution of the data in the three groups was declared homogeneous.

The results of the One-way Anova analysis showed a significant result (p) of 0.014 (sig. (P) < 0.05), it can be concluded that there were significant differences in the probiotic group *Streptococcus sanguinis*, the chlorhexidine group and the placebo group.

**TREATMENT OF THE PROBIOTIC, CHLORHEXIDINE AND PLACEBO GROUPS**

The suspension of *Streptococcus mutans* ATCC 25175 was added to well-plate media along with the probiotic *Streptococcus sanguinis* ATCC 10556, control (+) chlorhexidine (Minosep), and control (-) placebo as much as 50 µl. Samples were incubated for 4 hours (adhesion phase) at 37°C. Rinse the well-plate with PBS 3 times and dry for 5 minutes. Drops of 1% gentian violet crystals on a 150 µl well-plate for 15 minutes and rinse with PBS 2 times, dry for 5 minutes. Ethanol 96% as much as 200 µl added to the well-plate and let stand for 15 minutes. Reading the results by measuring the optical density with a wavelength of 540 nm using an ELISA-reader.

**Table 2. Bonferroni Post Hoc Test**

<table>
<thead>
<tr>
<th>(I) Treatment</th>
<th>(J) Treatment</th>
<th>Sig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probiotic</td>
<td>Chlorhexidine</td>
<td>0.049*</td>
</tr>
<tr>
<td>Placebo</td>
<td>Probiotic</td>
<td>0.029*</td>
</tr>
<tr>
<td>Chlorhexidine</td>
<td>Placebo</td>
<td>0.033*</td>
</tr>
<tr>
<td>Placebo</td>
<td>Probiotik</td>
<td>0.029*</td>
</tr>
<tr>
<td></td>
<td>Chlorhexidine</td>
<td>0.033*</td>
</tr>
</tbody>
</table>

Table 2. The Bonferroni Post Hoc test found that the significance value between the probiotic *Streptococcus sanguinis* and chlorhexidine was 0.049 (sig. (P) < 0.05) and the probiotic *Streptococcus sanguinis* with placebo was 0.029 (sig. (P) < 0.05) so it
can be concluded that there are significant differences in value between the probiotic Streptococcus sanguinis with chlorhexidine and the probiotic Streptococcus sanguinis with placebo. There was a significant difference between chlorhexidine and placebo with a value of 0.033 (sig.(p)<0.05).

DISCUSSION

The results of this study were the average optical density value of the probiotic group of Streptococcus sanguinis was lower than the control group (+) chlorhexidine and the control group (-) placebo. In this study, it was found that the probiotic *Streptococcus sanguinis* could inhibit the formation of the *Streptococcus mutans* biofilm. The lower the optical density value, the lower the biofilm formed.  

_**Streptococcus sanguinis** is a commensal bacteria in the oral cavity._ When both bacteria (*S. mutans* and *S. sanguinis*) were inoculated together, it was proven that _Streptococcus sanguinis_ could inhibit the growth of _Streptococcus mutans_. In addition, _Streptococcus sanguinis_ also has antibacterial properties against pathogenic bacteria.  

_**Streptococcus mutans**_ biofilm growth can be influenced by other types of Streptococcus bacteria, one of which is _Streptococcus sanguinis_. Oral Streptococcus strains compete for attachment sites on tooth surfaces that are coated with saliva and are capable of producing antimicrobial compounds such as bacteriocins and H$_2$O$_2$.  

_Streptococcus sanguinis_ produces H$_2$O$_2$ through SpxB which is encoded by the SpxB gene as an antimicrobial compound that can affect the initial formation of biofilms by inhibiting bacteria _Streptococcus mutans_. H$_2$O$_2$ produced by pyruvate oxidase (SpxB) in the reaction that converts pyruvate to acetyl phosphate. This reaction requires oxygen with H$_2$O$_2$ and CO$_2$ generated during the catalytic process, H$_2$O$_2$ produced will most likely diffuse out of the cell and exert an antimicrobial effect on susceptible species H$_2$O$_2$.  

The initial attachment process in biofilm formation occurs when the oxygen pressure (O$_2$) high enough to allow respiration and production of H$_2$O$_2$, so that species that are susceptible to H$_2$O$_2$ will lose to compete with _Streptococcus sanguinis_.  

H$_2$O$_2$ is ROS (reactive oxygen species) which can cause damage to cellular macromolecules in the form of proteins and DNA. Besides, H$_2$O$_2$ can also kill bacteria directly and modulate the expression of virulence genes of bacteria _Streptococcus mutans_. Another consequence of the formation of H$_2$O$_2$ is the release of extracellular DNA (eDNA) that supports the initial formation of biofilms and H$_2$O$_2$ is the only agent required to induce eDNA release. Probiotic _Streptococcus sanguinis_ has an effect on the formation of _Streptococcus mutans_ biofilm in artificial saliva.

**REFERENCES**