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VIABILITY OF DUAL-SPECIES BIOFILM OF STREPTOCOCCUS MUTANS AND LACTOBACILLUS ACIDOPHILUS AFTER APPLICATION OF MAULI BANANA STEM GEL

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ABSTRACT
Introduction: *Dual-species biofilm of Streptococcus mutans and Lactobacillus acidophilus in dental caries has a higher viability or ability to survive in comparison to mono-species biofilm, consequently it is more resistant to antibiofilm. Mauli banana stem gel (MBSG) contains isoleucine, cinnamic acid, and another bioactive constituents that may work as antibiofilm, hence it is able to decrease the viability of dual-species biofilm of S. mutans and L. acidophilus. This research aims to analyze the effect of MBSG on viability of dual-species biofilm of S. mutans and L. acidophilus.* ***Material and methods:*** *This research is a true experimental research with post test only with control group design which divides dual-species biofilm of S. mutans and L. acidophilus into 12 groups with 4 replications. Group 1 are given 0.2% chlorhexidine gel (positive control), group 2 are not given any treatment (negative control), group 3 are the media control, and group 4-12 are given MBSG concentration of 6.25%, 12.5%, 25%, 37.5%, 50%, 62.5%, 75%, 87.5%, and 98%. Each group is incubated for 72 hours, afterward is given MTT reagent and read using a microplate reader.* ***Results:*** *The result of Kruskal-Wallis (p=0,00) and Mann-Whitney test showed significant difference between all treatment groups and control groups.* ***Conclusion:*** *MBSG concentration of 6.25%, 12.5%, 25%, 37.5%, 50%, 62.5%, 75%, 87.5%, and 98% are able to decrease the viability of dual-spesies biofilm of S. mutans and L. acidophilus.*

Keywords: Biofilm, *lactobacillus acidophilus*, mauli banana stem gel, *streptococcus mutans*,viability
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INTRODUCTION

Dental caries is a result of tooth structure demineralization which the incidence reaches 36% of worldwide population.[1] The prevalence of caries in Indonesia in 2018 (88.8%) increased by 16.2% from 2013 (72.6%).[2,3] South Borneo ranked as the second highest province with dental caries in Indonesia (86.9%).[4] The city in South Borneo that has the highest caries incidence rate is Banjarmasin City (60.5%).[5] These data indicate that caries still requires optimal prevention. The gold standard for caries prevention is generally using 0.2% chlorhexidine (CHX).[6]

Chlorhexidine is a broad-spectrum antibacterial that causes lysis of bacteria, including non-pathogenic commensal bacteria, by destroying the stability of the bacterial cell wall.[6] Chlorhexidine is available in form of mouthwash, gel, toothpaste, and varnish.[7] Research by Sicca et al (2016) on fluoride as a dentrifice stated that gel is able to prevent caries better than mouthwash and paste.[8] This statement is supported by Soekanto et al (2019) which states that gel has high stability, a fast release mechanism of active ingredients, and a long onset of action in preventing caries.[9] The use of CHX causes side such as tooth discoloration, taste disturbances, mucosal irritation, swelling of the parotid gland, and an increase in supragingival calculus.[10] Therefore, alternative material with optimal functions and minimal side effects is needed, one of which is using herbal ingredients. The phytochemical constituents of herbal ingredients is proven to be able to control and eliminate pathogenic bacteria from the oral cavity.[11] Local herbal ingredients that are currently being developed and have the potential as antibacterial are mauli banana stems.[12]

Mauli banana is a member of *Musa sp* which is often consumed by the people of South Borneo. Mauli banana stem has several secondary metabolites such as tannins, saponins, alkaloids, and flavonoids which are antibacterial.[13] In a preliminary study, it was found that the highest content of mauli banana stem were isoleucine (Ile) and cinnamic acid (CA). Both compounds have the potential as antibiofilms by lysing bacteria, hence they can intervene the pathomechanism of dental caries.[14] The caries pathomechanism is initiated by the activity of the glycosyltransferase enzyme of *S. mutans* as the initial bacteria in dental caries that breaks down sucrose into glucans.[15] Glucan is the biofilm framework that *L. acidophilus* will use as other cariogenic bacteria for colonization. Both will interact and form a dual-species biofilm.[16] Dual-species biofilm is more resistant to antimicrobials and is a source of nutrition for bacterial viability, so that lactic acid from sucrose metabolism will continue to form, as a result the pH of the oral cavity will become acidic and cause caries.[17]

A study by Apriasari et al (2013) stated that mauli banana stem extract at a concentration of 25% was able to inhibit the growth of *S. mutans.*[18] However, the effect of mauli banana stem on the viability of dual-species biofilms of *S. mutans* and *L. acidophilus* is remain unknown. Viability is the ability of a microorganism to survive after being given an exposure.[19] The process of maturation and adhesion of the dual-species biofilm *S. mutans* and *L. acidophilus* takes 72 hours.[20] Intervention on bacterial viability will result in reduced metabolite ability and bacterial viability, hence primary initiation will be inhibited and caries can be prevented.[17] Based on this background description, it is necessary to conduct a study to analyze the effect of mauli banana stem gel (MBSG) on the viability of dual-species biofilms *S. mutans* and *L. acidophilus.*

MATERIALS AND METHODS

This research is a true experimental research with a post test only with control group design which has been declared ethically feasible by the Health Research Ethics Commission, Faculty of Dentistry, Lambung Mangkurat University with the register number of No.039/KEPKG-FKGULM/EC/III/2021. The study population was the dual-species viability of the biofilm *Streptococcus mutans* (ATCC 25175) and *Lactobacillus acidophilus* (ATCC 4356) obtained from the Microbiology Research Center Laboratory of Faculty of Dentistry, Airlangga University, Surabaya, Indonesia. The study population was divided into 12 groups with a minimum of 4 replications. Group 1 are given 0.2% chlorhexidine gel (positive control), group 2 are not given any treatment (negative control), group 3 are the media control, and group 4-12 are given MBSG concentration of 6.25%, 12.5%, 25%, 37.5%, 50%, 62.5%, 75%, 87.5%, and 98%.

Extraction of Mauli Banana Stem

Prior to the extraction procedure, a determination test was carried out on the plants to ensure that the plants used were the correct mauli banana species (*Musa acuminata*). Three medium-sized mauli banana stems were obtained from SMK-PP Banjarbaru, Indonesia. The process of making mauli banana stem extract was carried out by the maceration method. The banana tree trunk used is taken 10 cm from the root tuber, then washed and cut into smaller pieces. The banana stem pieces were dried in the oven for 3 days. After drying, the pieces of banana stem were crushed into simplicia using a blender, then soaked in 70% ethanol for 3x24 hours. The simplicia obtained are approximately 2.5 kg. The immersion results were evaporated using a rotatory evaporator at a temperature of 40°-50°C to obtain a thick mauli banana stem extract with a total weight of 116.75 grams. The viscous extract that was obtained was then tested free of ethanol using acetic acid (CH_3COOH) and sulfuric acid (H_2SO_4), then heated. The extract can be declared free of ethanol if there is no smell of ester.

Formulating Mauli Banana Stem Gel

After getting the results of mauli banana stem extract from the rotatory evaporator and it was declared free from ethanol, the next step was processing the extract into a gel with concentrations of 6.25%, 12.5%, 25%, 37.5%, 50%, 62.5 %, 75%, 87.5%, 98%. MBSG is made by mixing mauli banana stem extract with gel-based ingredients which are propylene glycol, glycerin, Na-CMC, nipagin, and aqua ad. Each gel concentration that has been formed is subsequently dissolved with dimethyl sulfoxide (DMSO) for testing the antibiofilm on the viability of *S. mutans* and *L. acidophilus*.

Tabel 1: Ingredients of MBSG

	Ingredient	Function	Gel (%) (grams (g))								
			F1	F2	F3	F4	F5	F6	F7	F8	F9
1	Mauli banana stem extract	Active compounds	6.25% (1.25)	12.5% (2.5)	25% (5)	37.5% (7.5)	50% (10)	62.5% (12.5)	75% (15)	87.5% (17.5)	98% (29.4)
2	Propylene Glycol	Maintain the viscosity	5% (1)	5% (1)	5% (1)	5% (1)	5% (1)	5% (1)	5% (1)	5% (1)	5% (1)
3	Glycerin	Emollient and humectant	10% (2)	10% (2)	10% (2)	10% (2)	10% (2)	10% (2)	10% (2)	10% (2)	10% (2)
4	Na-CMC	Gelling agent	10% (2)	10% (2)	10% (2)	10% (2)	10% (2)	10% (2)	10% (2)	10% (2)	10% (2)
6	Nipagin	Preservative	0.1% (0.02)	0.1% (0.02)	0.1% (0.02)	0.1% (0.02)	0.1% (0.02)	0.1% (0.02)	0.1% (0.02)	0.1% (0.02)	0.1% (0.02)
7	Aqua ad	Solvent	20 ml	20 ml	20 ml	20 ml	20 ml	20 ml	20 ml	20 ml	20 ml

Note :
F1: MBSG concentration of 6.25%; F2: MBSG concentration of 12.5%; F3: MBSG concentration of 25%; F4: MBSG concentration of 37.5%; F5: MBSG concentration of 50%; F6: MBSG concentration of 62,5%; F7: MBSG concentration of 75%; F8: MBSG concentration of 87,5%; F9: MBSG concentration of 98%.

Culture Conditions of Microorganism

The microorganisms used in this study were *S. mutans* and *L. acidophilus*. *S. mutans* was cultured anaerobically (10% H_2, 80% N_2, 10% CO_2) in triptic soy broth (TSB) media with 1% sucrose nutrient supply at 37°C for 18 hours, while *L. acidophilus* was cultured anaerobically (10% H_2, 80% N_2, 10% CO_2) on Man Rogosa Sharp Broth (MRSB) medium under acidic conditions at 37°C for 24 hours.

Saliva Coating

Saliva in this study was obtained from artificial saliva of McDougall's solution by Fusayama Meyer's method which was dissolved in PBS. Then, 200 µL of saliva was taken to be applied to a 96-well plate and incubated at 37°C for 1 hour.

Formation of Dual-Species Biofilm

The results of bacterial culture for 18 hours in the previous process were harvested by centrifugation for 10 minutes at 4°C at a speed of 5000 rpm. The pellets formed were diluted with BHIB + 8% sucrose, then measured by UV-Visspectrophotometry to OD600 = 0,1. The microorganisms were taken as much as 100 µL and inoculated in the well, then the well-plate was incubated under anaerobic conditions (10% H_2, 80% N_2, 10% CO_2) for 90 minutes. The supernatant formed after incubation was replaced with 200 µL of MBSG solution which had different concentrations and dissolved with DMSO. After that, the well-plate was reincubated for 72 hours under anaerobic conditions (10% H_2, 80% N_2, 10% CO_2). The reincubated supernatant was aspirated, subsequently the wells were rinsed twice with PBS. Prior to the MTT assay procedure, microscopic examination with gram staining of the dual-species biofilm sample was carried out to ensure the quality and purity of the sample was properly formed by *S. mutans* and *L. acidophilus*.

Analysis of Viability od *S. mutans* and *L. acidophilus* with *MTT assay*

The wells that had been rinsed with PBS were added with 50 µL of 5 mg/mL MTT solution, then the plate was incubated for 3 hours at 37°C. After that, the acidified isopropanol solution was given and placed in an orbital shaker for one hour.

The results of the MTT assay in the form of optical density (OD) were observed using a microplate reader (490 nm). Viability is expressed as a percent and is obtained by inserting OD into the following formula:

$$\frac{\text{OD of Treatment Group}}{\text{OD of Negative Control}} x\ 100\%$$

Analysis of Statistic

Viability of dual-species biofilm data were then statistically analyzed using the Kolmogorov-Smirnov normality test, then continued with the Kruskal Wallis test and the Mann-Whitney follow-up test.

RESULTS

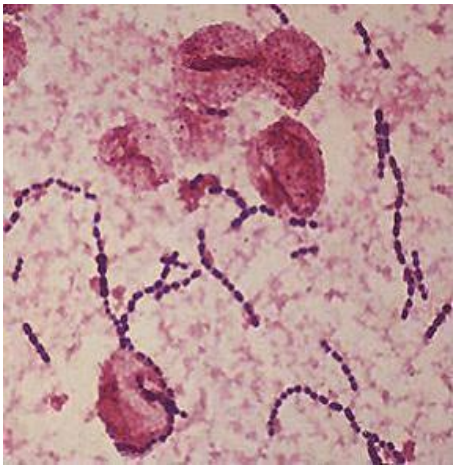

Figure 1: Binocular digital microscopic image of *Streptococcus mutans* and *Lactobacillus acidophilus*

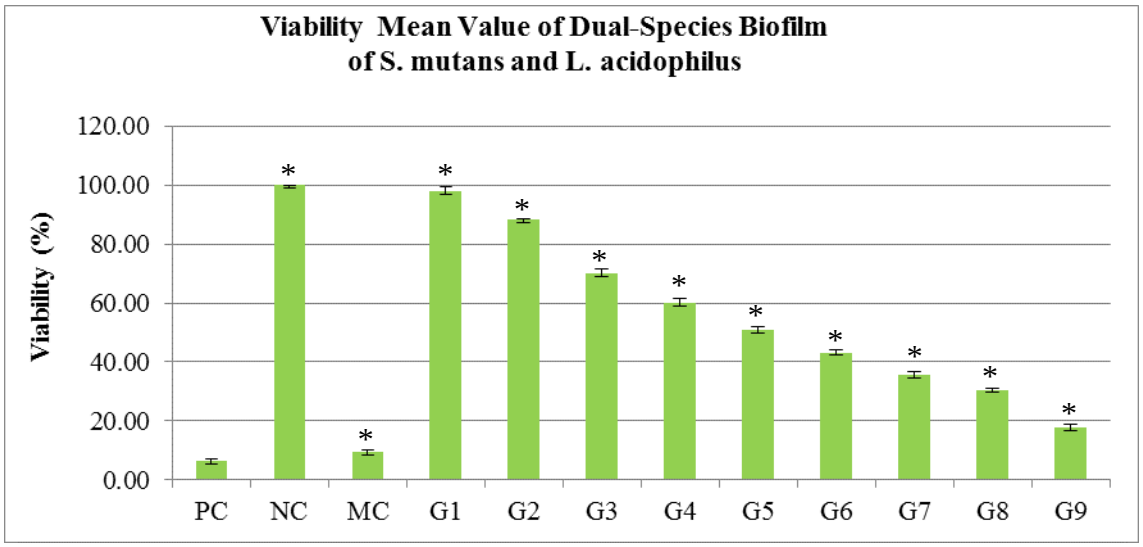

Figure 2: Viability Mean Value of Dual-Species Biofilm of *S. mutans* and *L. acidophilus*

Note :

*:There is a significant difference with the PC; PC: Positive control (0.2% chlorhexidine gel); KN: Negative control; KM: Media control; G1: MBSG concentration of 6.25%; G2: MBSG concentration of 12.5%; G3: MBSG concentration of 25%; G4: MBSG concentration of 37.5%; G5: MBSG concentration of 50%; G6: MBSG concentration of 62,5%; G7: MBSG concentration of 75%; G8: MBSG concentration of 87,5%; G9: MBSG concentration of 98%.

The highest average of dual-species biofilm viability was in the negative control group (100 ± 0.00) and the lowest average was found in the positive control group of 0.2% chlorhexidine gel (6.27 ± 0.67). The viability calculation were then analyzed statistically. The results of the Kolomogorov-Smirnov normality test on the viability of dual-species biofilms showed a significant value ($p>0.05$) in all groups, except for the negative control group, which means the data were not normally distributed. Data analysis was continued with the Kruskal Wallis nonparametric test. The significance value in the Kruskal Wallis nonparametric test for the viability of dual-species biofilm was $p=0.000$ ($p<0.05$), which indicated that there was a significant difference. Data analysis was continued with a post-hoc test using the Mann-Whitney test to see which groups had significant differences. The results of the Mann-Whitney test on the viability of the dual-species biofilm *S. mutans* and *L. acidophilus* showed that there was a significant difference between all study groups in the form of a decrease in the viability of the dual-species biofilm of *S. mutans* and *L. acidophilus* with different results for each group. These results indicate that MBSG concentrations of 6.25%, 12.5%, 25%, 37.5%, 50%, 62.5%, 75%, 87.5%, 98% are able to reduce the value of dual-species biofilm viability of *S. mutans* and *L. acidophilus*, but have not been able to compete with 0.2% chlorhexidine gel.

DISCUSSION

Viability is the ability of cells to recover after got exposured to foreign objects or pathogens and is stated in percentage.[19] The intervention by applying a gel on the viability of dual-species biofilms will cause disruption of bacterial metabolism and affect the bacterial viability.[17] Application of chlorhexidine gel 0.2% and MBSG concentrations of 6.25%, 12.5%, 25%, 37.5%, 50%, 62.5%, 75%, 87.5%, 98% against dual-species biofilm *S. mutans* and *L. acidophilus* showed a decrease in the viability of the dual-species biofilm. Dual-species biofilms have properties that are more resistant to antibiofilm agents than mono-species biofilms, this is in line with the research of Mei et al (2013) using silver diamine fluoride.[21] The results of the study by Mei et al (2013) showed that the formation of dual-species Biofilms of *S. mutans* and *L. acidophilus* that cause dental caries can be decreased even though they are more resistant. Dual-species biofilms have better adaptability and survival than mono-species biofilms due to the commensalism association between bacteria through cell-to-cell interactions or quorum sensing. The presence of secondary colony bacteria has an influence on the composition and viscosity of the extracellular polysaccharide matrix, so that in addition, dual-species biofilms can survive environmental changes by continuously producing lactic acid, carrying out conjugative plasmid transfer, and complementing survival mechanisms through enzyme interactions from each microorganism on a dual-species biofilm.[21]

The results of the study on the dual-species biofilm group of *S. mutans* and *L. acidophilus* that were not given any interventions (negative control) showed a viability value of 100%. The high viability value indicates the ability of the dual-species biofilm metabolites to work well because there are nutrients for the development of *S. mutans* and *L. acidophilus*. *Streptococcus mutans* as the initiator bacteria in the caries pathomechanism requires a substrate of sucrose for metabolism.[22] The metabolic products of *S. mutans* will be catalyzed by the glycosyltransferase enzyme into a biofilm structure and utilized by *L. acidopilus* to co-aggregate to form a dual-species biofilm of *S. mutans* and *L. acidophilus*.[15,16] Dual-species biofilm that formed without any interventions leads bacterial metabolism to continue, so that the conditions become more acidic and there is no decrease in viability.[23]

Mauli banana stem gel groups showed a variable decrease in the viability of dual-species biofilms. The more volume of mauli banana stem extract mixed in the gel, the higher the decrease in viability of the dual-species biofilm *S. mutans* and *L. acidophilus*. Gel with a higher volume of mauli banana stem extract showed a higher content of active compounds. The active compounds in the mauli banana stem which have antibiofilm properties are the main compounds, which are isoleucine (Ile) and cinnamic acid (CA), as well as several other secondary metabolites such as alkaloids, phenols, tannins, saponins, and flavonoids in the mauli banana stem gel.[13,22,24]

Isoleucine is a type of branched chain amino acid (BCAA) that can act as an antimicrobial peptide (AMP).[25] Antibiofilm activity by AMP was more common in gram-positive bacteria than in gram-negative bacteria, although in both types of bacteria AMP caused the same effect.[26] Antimicrobial peptides can act as antibiofilms with different mechanisms, such as the destruction of the initial structure forming the biofilm which is extracellular polymeric matrix, inhibition of biofilm adhesion, and downregulation of bacterial quorum sensing factors.[27] Isoleucine as AMP is able to penetrate bacterial cell walls due to the electrostatic activity between the positive ionic charge on the peptide and the negative ionic charge on the bacterial cell wall.[26] When AMP penetrates the bacterial cell membrane, the synthesis of peptidoglycan will be inhibited because the peptidoglycan precursor will be bound by AMP. The binding of peptidoglycan results in impaired bacterial permeability and other components in the cytoplasm will be degraded. Degradation of cytoplasmic components will cause a decrease in nucleotide acid biosynthesis so that the ability of bacterial metabolites decreases and bacteria will lyse.[26] In addition, AMP causes a decrease in the regulation of biofilm-forming genes due to the inhibition of a system called alarmone that is responsible for the bacterial response to foreign objects.[28] Another ingredient in the mauli banana stem gel that affects the viability of the dual-species biofilm *S. mutans* and *L. acidophilus* is cinnamic acid (CA).

Cinnamic acid is a polyphenolic compound capable of penetrating bacterial cell membranes by passive diffusion with a MIC value above 5 mm and a topological polar surface area (TPSA) value of 37.30 Å, where a TPSA value <40 Å is an indication of a compound capable of good diffusion.[29] Cinnamic acid has been proven to have a fine ability to inhibit the growth of gram-positive bacteria.[30] Cinnamic acid will inhibit the synthesis of the H+-ATPase enzyme so that it will cause cytoplasmic acidification.[29] Cytoplasmic acidification causes bacterial proteins to be denatured and results in decreased viability to a lysis bacteria.[17] The content of another secondary metabolites such as alkaloids, tannins, saponins, and flavonoids also affects the antibiofilm activity of MBSG.

Alkaloids cause respiratory and enzyme disorders in bacteria, this will damage cell membranes and reduce bacterial virulence.[13] Tannins act as antibiofilms by interfering with cell wall growth through the destruction of peptidoglycan synthesis so that regulation of protein composition will be inhibited.[31] Saponins are capable of damaging cell membranes and inhibiting protein synthesis that will lead into bacterial cell death.[13,31] The antibiofilm

properties of flavonoids occur through the mechanism of inhibition of the DNA gyrase enzyme and the destruction of the lipid layer on the bacterial cell membrane which causes intramembranous leakage.[31] The leakage will interfere with bacterial aggregation in biofilm formation. The mechanism of action and reaction of all the active compounds in the mauli banana stem gel as mentioned above is a factor causing the decreased viability of the dual-species biofilm formation of *S. mutans* and *L. acidophilus.*

The results of this study showed that GBPM concentrations of 6.25%, 12.5%, 25%, 37.5%, 50%, 62.5%, 75%, 87.5%, 98% are able to reduce the viability of dual-species biofilms of *S. mutans* and *L. acidophilus*, but have not been able to match the ability of 0.2% chlorhexidine gel which causes a decrease in the viability of dual-species biofilms up to 93%. The results of this study are in line with the research of Haghoo et al (2017) which compared chlorhexidine and *Cyperus rotundus* with the diffusion method in inhibiting the growth of *S. mutans* and *L. acidophilus* which causes dental caries.[32] The high viability reduction by chlorhexidine gel is due to its strong bacteriostatic and bactericidal properties. The positive ion charge in chlorhexidine will interact with the bacterial cell wall which is negatively charged so that the permeability of the bacterial cell will be disturbed.[32] Penetration of chlorhexidine into the cell membrane lead an osmotic condition between chlorhexidine and cytoplasmic fluid which causes the stability of the cell wall to be disturbed. Disruption of the bacterial cell wall results in the precipitation of macromolecules into the cytoplasm and results in the rupture of the bacterial cell wall.[32] The MBSG concentration of 6.25%, 12.5%, 25%, 37.5%, 50%, 62.5%, 75%, 87.5%, and 98% are able to decrease the viability of dual-spesies biofilm of *S. mutans* and *L. acidophilus*.

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