



Antibacterial and Antibiofilm Activity of Pineapple Jelly Candy Sweetened with Stevia Against *Streptococcus mutans*

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Abstract

Introduction: *Streptococcus mutans* is the main cause of dental caries. Pineapple and stevia exhibit antibacterial activity against *S. mutans*. Their combination can be formulated into a non-cariogenic jelly candy. **Purposes:** To investigate the antibacterial and antibiofilm effects of jelly candy formulated with pineapple and stevia against *S. mutans*. **Methods:** This study used an in vitro experimental design with a post-test only control group design. The antibacterial activity was assessed using the agar well diffusion method, while the antibiofilm activity was evaluated using the microtiter plate method. The experimental groups consisted of pineapple and stevia jelly candy, stevia jelly candy without pineapple, and a negative control group (jelly candy without pineapple and stevia). Antibacterial activity was determined by measuring the diameter of the inhibition zone, whereas antibiofilm activity was assessed by measuring absorbance or optical density values. Data from each test were analysed using Welch's ANOVA and One-Way ANOVA tests. **Results:** Welch's ANOVA analysis of inhibition zone diameters among groups demonstrated a significant difference ($p < 0.05$). The largest inhibition zone was observed in the pineapple and stevia jelly candy group (10.72 mm). The One-Way ANOVA results also revealed significant differences in absorbance values among all groups ($p < 0.05$). Pineapple and stevia jelly candy group exhibited the lowest absorbance value compared with other groups. **Conclusion:** Pineapple and stevia jelly candy possessed antibacterial and antibiofilm activities against *S. mutans*.

Keywords: Antibacterial; antibiofilm; pineapple jelly candy; *Streptococcus mutans*; stevia

Introduction

Pineapple (*Ananas comosus*) is a tropical plant that has long been utilized as a traditional medicinal plant and is widely distributed throughout Indonesia. Previous studies in the field of oral health have reported that nearly all parts of the pineapple plant, including the fruit flesh, peel, core, and leaves, exhibit antibacterial activity.¹ The antibacterial properties of pineapple are attributed to the presence of several natural bioactive compounds, particularly in the flesh, peel, and core. These active compounds



include bromelain, flavonoids, and tannins. Bromelain is a proteolytic enzyme capable of disrupting bacterial cell membranes and inhibiting intracellular bacterial enzymes, thereby suppressing bacterial growth.² Flavonoids act by disrupting bacterial cell walls and interfering with cell membrane function. Meanwhile, tannins exert antibacterial effects by precipitating bacterial cell walls, resulting in disruption of bacterial growth and enzymatic activity.¹

In dentistry, *Streptococcus mutans* is recognized as one of the primary bacteria associated with dental caries. Previous studies have demonstrated that pineapple juice effectively inhibits the growth of *S. mutans*, even at low concentrations.³ In addition to its antibacterial activity, bromelain contained in pineapple has also been reported to exhibit antibiofilm properties by degrading proteins and glycoproteins in saliva, thereby reducing bacterial adhesion and plaque formation on tooth surfaces.⁴ Flavonoids and tannins can inhibit enzymes involved in exopolysaccharide synthesis, such as glucosyltransferases. This results in decreased biofilm matrix formation.⁵ Dental plaque or biofilm is a thin, adherent, and colourless layer formed on the tooth surface, consisting of a complex community of bacteria embedded within a salivary matrix. The presence of *S. mutans* in dental plaque contributes to acid production from retained food debris, which subsequently leads to demineralization and damage to the tooth structure.⁶

Jelly candy is a cariogenic food commonly consumed by children due to its sweet and adhesive properties.⁷ Generally, jelly candy is formulated using hydrocolloid components such as gelatin, fruit extracts, and sucrose as the main sweetener.⁸ Modification of jelly candy composition may reduce its cariogenic potential.⁹ The selection of fruit extracts with antibacterial and antibiofilm properties in jelly candy formulations is expected to reduce the cariogenic effects of the product. Pineapple may be considered a suitable fruit extract ingredient for non-cariogenic jelly candy formulations. The cariogenic properties of jelly candy are also attributed to the presence of sucrose as a sweetening agent; therefore, a non-cariogenic natural sweetener alternative is required, one potential alternative is stevia.¹⁰

Stevia is a plant from the *Asteraceae* family that is widely used as a low-calorie natural sweetener. In addition, stevia has been reported to possess antibacterial and antibiofilm activities due to the presence of bioactive compounds, including stevioside, alkaloids, flavonoids, and tannins. Previous studies have shown that stevia extract



demonstrates anticariogenic potential through its antibacterial activity against *S. mutans* and its influence on salivary pH.¹¹ Based on these findings, this study aimed to evaluate the antibacterial and antibiofilm activities of jelly candy containing pineapple and stevia against *S. mutans*.

Methods

This study was an in vitro laboratory experimental study using a post-test only control group design to evaluate the antibacterial and antibiofilm activities of pineapple jelly candy sweetened with stevia against *S. mutans*. Antibacterial activity was assessed by measuring the diameter of the inhibition zone, while antibiofilm activity was evaluated by measuring the absorbance or optical density (OD) of biofilms formed in microplate wells. Sample size calculation for each assay was determined using Federer's formula.

For the antibacterial activity assay, the samples were divided into three groups: (1) jelly candy containing pineapple and stevia, (2) jelly candy containing stevia without pineapple, and (3) a negative control consisting of jelly candy without pineapple and stevia. Each group was tested in nine replicates. For the antibiofilm activity assay, the samples were divided into four groups: (1) jelly candy containing pineapple and stevia, (2) jelly candy containing pineapple without stevia, (3) jelly candy containing stevia without pineapple, and (4) a negative control consisting of jelly candy without pineapple and stevia. Each group was tested in six replicates.

The subject of this study was *Streptococcus mutans* strain ATCC 25175 obtained from the Microbiology Laboratory, Research Center, Faculty of Dental Medicine, Universitas Airlangga. The research object was pineapple obtained from a pineapple plantation located in Karang Jaya Village, East Prabumulih District, Prabumulih City, South Sumatra. The inclusion criteria were Queen variety pineapples with a maturity age of 5 months, characterized by approximately 50–75% yellow skin coloration. The exclusion criteria included overly soft and excessively watery fruit flesh, as well as overly yellowish-brown skin colour.¹²

Ethical clearance for this study was obtained from the Medical and Health Research Ethics Committee, Faculty of Medicine, Universitas Sriwijaya. Jelly candy preparation was carried out at the Biochemistry Laboratory, Faculty of Medicine, Universitas Sriwijaya. The antibacterial activity assay against *S. mutans* was conducted

at the Microbiology Laboratory, Research Centre, Faculty of Dental Medicine, University of Airlangga, while the antibiofilm activity assay was performed at the Microbiology Laboratory, University of Muhammadiyah Palembang.

Preparation of Pineapple Jelly Candy

The prepared pineapples were peeled, the pineapple eyes were removed, and the fruits were washed thoroughly under running water. The pineapple flesh was cut into pieces and blended using a juicer to separate the filtrate from the pulp. A total of 100 mL of pineapple filtrate was heated to 80–100°C, after which the heat source was turned off, and the filtrate was allowed to cool to room temperature (20–25°C). Subsequently, 85 mL of cooled pineapple filtrate was mixed with 15 mL of stevia solution prepared by diluting 15 drops of stevia (Beeru^R) with distilled water to a final volume of 15 mL. The pineapple filtrate and stevia mixture was heated at 45–50°C, followed by the addition of 35 g gelatin powder. The mixture was stirred for approximately 10 minutes until the solid ingredients dissolved completely and the jelly mixture thickened. The thickened jelly mixture was poured into silicone molds and allowed to stand at room temperature for 1 hour (Figure 1).



Figure 1. Jelly candy in Silicone Molds

The jelly candies were then stored in a refrigerator at 5°C for approximately 24 hours. Before removal from the molds, the candies were kept at room temperature for 1 hour to normalize the temperature. The jelly candies were then removed from the molds and packaged using plastic clip bags. Four different jelly candy formulations were prepared: (1) jelly candy containing pineapple and stevia, (2) jelly candy containing pineapple without stevia, (3) jelly candy containing stevia without

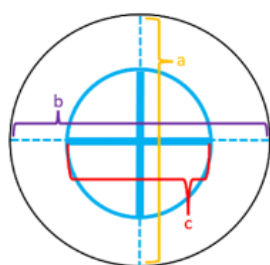
pineapple, and (4) jelly candy without pineapple and stevia. The omitted ingredients in each formulation were replaced with distilled water in equivalent proportions.

Preparation of *Streptococcus mutans* Suspension

Streptococcus mutans was cultured on Mueller Hinton Agar (MHA) and incubated at 37°C for 24 hours. A loopful of *S. mutans* colonies was collected using a sterile inoculating loop and suspended in sterile 0.9% NaCl solution in a test tube. The bacterial suspension was homogenized and adjusted to the turbidity standard of 0.5 McFarland (1.5×10^8 CFU/mL) using a densitometer.¹⁰

Antibacterial Activity Assay

A sterile cotton swab was immersed in the *S. mutans* suspension and pressed against the inner wall of the tube to remove excess liquid. The swab was evenly spread onto the surface of Mueller Hinton Agar (MHA) plates using the swab technique and allowed to dry for 5 minutes. Antibacterial activity was evaluated using the well diffusion method. A total of 2 g of jelly candy was weighed, crushed using a mortar and pestle, and dissolved in 6 mL distilled water using a vortex mixer. Three wells with a diameter of 5 mm were made on each inoculated MHA plate using a pipette tip. Each well was filled with a paper disc and 20 µL of jelly candy solution using a micropipette. The plates were incubated at 37°C for 24 hours. After incubation, the inhibition zones, indicated by clear areas surrounding the wells, were measured using a calliper with an accuracy of 0.01 mm. The inhibition zone diameters were measured vertically and horizontally (Figure 2), and the results were recorded in millimeters (mm). The inhibition zone diameter was calculated using the following formula:



$$\frac{(a - c) + (b - c)}{2}$$

Description:

a = Vertical diameter
b = Horizontal diameter
c = Diameter of the well

Figure 2. Measurement of The Bacterial Growth Inhibition Zone.¹⁰



The inhibition zone diameter was measured three times to minimize measurement errors. The mean value of the three measurements was calculated and recorded as the inhibition zone diameter.

Antibiofilm Activity Assay

A 96-well microplate consisting of 12 columns and 8 rows was prepared. A total of 200 μ L of bacterial culture suspension was added into each well according to the treatment groups and incubated at 37°C for 24 hours to allow biofilm formation. After incubation, the culture medium was discarded, leaving a biofilm layer attached to the bottom surface of the wells, which was subsequently washed using phosphate-buffered saline (PBS).^{12,13} A total of 2 g of each jelly candy formulation was crushed and dissolved in 6 mL distilled water. Subsequently, 200 μ L of the jelly candy solution was added into the designated wells according to the treatment groups.¹³ The microplate was then incubated again at 37°C for 24 hours. After incubation, the solutions in the wells were discarded and the wells were washed twice with phosphate-buffered saline (PBS). Next, 200 μ L of 0.5% crystal violet solution was added to each well and allowed to stand for 15 minutes. The crystal violet solution was then removed, and the wells were washed twice with phosphate-buffered saline (PBS), followed by the addition of 200 μ L of 96% ethanol.¹² The 96-well microplate was subsequently placed into a microplate reader, and the absorbance or optical density (OD) values were measured at a wavelength of 492 nm.^{12,13}

Results

The results of the inhibition zone test of pineapple jelly candy (*Ananas comosus*) against the growth of *S. mutans* using the well diffusion method demonstrated the presence of a clear zone surrounding the wells in the pineapple and stevia jelly candy group (Figure 3).

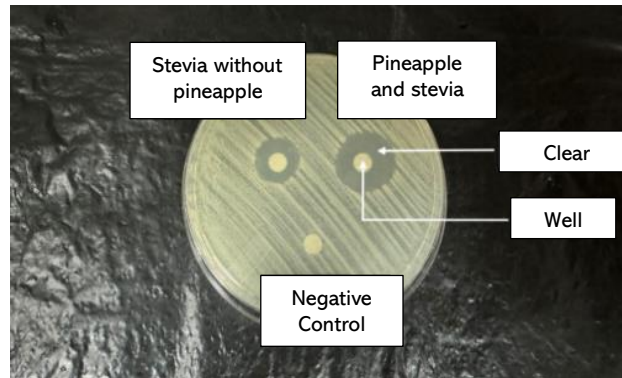


Figure 3. Diameter of the Inhibition Zone

The diameter of the resulting clear zone was measured, and the results are presented in Table 1.

Table 1. Inhibition Zone Diameters of Jelly Candy Against *S. mutans*

Jelly candy groups	Repetition									Mean (mm)
	I (mm)	II (mm)	III (mm)	IV (mm)	V (mm)	VI (mm)	VII (mm)	VIII (mm)	IX (mm)	
Pineapple and stevia	10,95	10,80	10,75	10,80	10,90	10,60	10,40	10,55	10,75	10,72
Stevia without pineapple	8,90	8,60	8,55	8,40	8,60	8,55	8,20	8,35	8,50	8,51
Negative control	0	0	0	0	0	0	0	0	0	0

The Shapiro–Wilk normality test showed that all experimental groups had normally distributed data ($p > 0.05$). However, Levene’s homogeneity test indicated that the data were not homogeneous ($p < 0.05$); therefore, data analysis was continued using the parametric Welch’s ANOVA test. The results of Welch’s ANOVA test are presented in Table 2.

Table 2. Welch’s ANOVA Results of Jelly Candy Inhibition Zone Diameter Against *S. mutans*

Jelly candy groups	Inhibition Zone Diameter (mm)	P value
	Mean $\pm$ SD	
Pineapple and stevia	10,72 $\pm$ 0,17	< 0,01*
Stevia without pineapple	8,51 $\pm$ 0,19	
Negative control	0,00 $\pm$ 0,00	

*significant difference was observed ($p < 0.05$)

The parametric Welch’s ANOVA test showed a statistically significant difference ($p < 0.05$); therefore, the Games–Howell post hoc test was performed to identify the groups with significant differences in inhibition zone diameter. The results of the Games–Howell post hoc analysis are presented in Table 3.

Tabel 3. Games–Howell Post Hoc Analysis of Jelly Candy Inhibition Zones Against *S. mutans*

Jelly candy groups	Pineapple and stevia	Stevia without pineapple	Negative control
Pineapple and stevia	-	< 0,001*	< 0,001*
Stevia without pineapple		-	< 0,001*
Negative control			-

*significant difference was observed ($p < 0.05$)

The Games–Howell Post-Hoc Test showed significant differences in the mean inhibition zone diameter among all tested groups. These findings indicate that pineapple and stevia jelly candy exhibited the strongest antibacterial activity compared with the other groups. However, stevia jelly candy without pineapple also demonstrated antibacterial activity.

Antibiofilm activity of the jelly candy against *S. mutans* was evaluated by measuring the biofilm optical density (OD) values in microplate wells using a microplate reader at a wavelength of 492 nm. The results of *S. mutans* biofilm formation are presented in Figure 4.

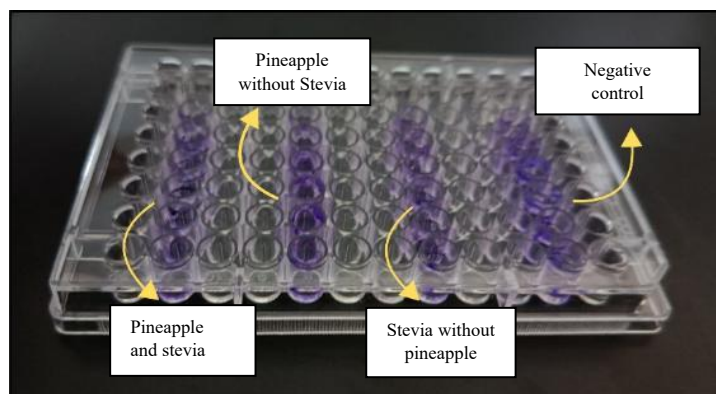


Figure 4. Biofilm Formation in 96-Well Microplates

A ring-like pattern indicating *S. mutans* biofilm formation was observed on the surface of the wells in all groups. The absorbance values were measured using a microplate reader (Figure 5).



Figure 5. Measurement of Absorbance Values Using a Microplate Reader

The absorbance data were analysed using the Shapiro–Wilk normality test and Levene’s homogeneity test. The results showed that all treatment groups were normally distributed ($p > 0.05$) and had homogeneous data of variance ($p > 0.05$). Subsequently, all data were analyzed using a One-Way ANOVA test, the results of which are presented in Table 4.

Table 4. One-Way ANOVA Results of Mean Absorbance Values

Jelly candy group	Absorbance value	<i>P value</i>
	Mean $\pm$ SD	
Pineapple and stevia	0,1280 $\pm$ 0,4532	0,00*
Pineapple without stevia	0,2575 $\pm$ 0,0378	
Stevia without pineapple	0,4005 $\pm$ 0,0456	
Negative control	0,6666 $\pm$ 0,0955	

*significant difference was observed ($p < 0.05$)

Table 4 showed that the negative control group had the highest absorbance value, whereas the lowest absorbance value was observed in the pineapple and stevia jelly candy group. The parametric One-Way ANOVA test demonstrated a significant difference in the mean absorbance values among the treatment groups, as indicated by a significance value of $p < 0.05$. Furthermore, the Tukey Post-Hoc Test was performed to determine the groups with significant differences. The results of the Tukey Post-Hoc Test are presented in Table 5.



Table 5. Post-Hoc Tukey

Jelly candy group	Pineapple and stevia	Pineapple without stevia	Stevia without pineapple	Negative control
Pineapple and stevia	-	0,007*	0,000*	0,000*
Pineapple without stevia		-	0,003*	0,000*
Stevia without pineapple			-	0,000*
Negative control				-

*significant difference was observed ($p < 0.05$)

Table 5 showed significant differences among all groups. These findings indicate that pineapple and stevia jelly candy exhibited the strongest antibiofilm activity against *S. mutans*, as demonstrated by the lowest absorbance value and significant differences compared with all other groups. Pineapple jelly candy without stevia and stevia jelly candy without pineapple also demonstrated antibiofilm activity compared with the negative control group; however, the antibiofilm activity of pineapple jelly candy without stevia was greater than that of stevia jelly candy without pineapple.

Discussion

The results of this study demonstrated that pineapple jelly candy (*Ananas comosus*) sweetened with stevia exhibited antibacterial activity against *Streptococcus mutans*. This antibacterial activity was indicated by the formation of an inhibition zone or clear zone surrounding the wells containing the pineapple and stevia jelly candy solution on the agar medium. The inhibitory effect against the growth of *S. mutans* was presumed to be associated with the addition of pineapple extract to the jelly candy formulation. This finding is supported by an in vitro study conducted by Goudarzi et al., which reported that pineapple fruit extract was able to inhibit the growth of *S. mutans*.¹⁴ Pineapple contains several active compounds, including bromelain, flavonoid and tannin.¹⁵ However, in this study, the bromelain enzyme was inactivated due to the heating process involved in the preparation of the jelly candy. Bromelain is a proteolytic enzyme (protease) capable of hydrolyzing peptide bonds in protein chains, including gelatin, thereby preventing gel formation of jelly candy.¹⁶ To achieve a firm and stable texture bromelain present in pineapple filtrate must be denatured through thermal treatment at temperatures above 60°C.¹⁷ This heating process ultimately inactivates the antibacterial activity of bromelain. Therefore, the antibacterial activity of pineapple jelly candy against *S. mutans* is likely attributable to other bioactive compounds, particularly tannins and flavonoids, which remain relatively stable during thermal



processing at temperatures below 100°C.^{18,19} The inhibition zone diameter formed in the pineapple and stevia jelly candy group in this study was categorized as moderate, with a mean inhibition zone diameter of 10.72 mm. This finding is consistent with a study by Wahyuningsih et al., which reported that pineapple juice mouthwash produced moderate inhibition zone diameters against *S. mutans* at concentrations of 5% (8.6 mm), 10% (9.4 mm), and 15% (9.7 mm).³

The antibiofilm activity of pineapple and stevia jelly candy against *S. mutans* was determined based on the absorbance values obtained from the assay. Higher absorbance values indicate thicker biofilm formation, whereas lower absorbance values indicate thinner biofilm formation.¹³ The results of this study showed that pineapple–stevia jelly candy exhibited the lowest absorbance value compared to the other groups. This lower absorbance indicates that the combination of pineapple and stevia has the most effective antibiofilm activity against *Streptococcus mutans*. This finding is supported by studies conducted by Fitri *et al.* and Haqiqi, which demonstrated that chewing pineapple fruit in school-aged children can reduce the average plaque index.^{20,21} Another study conducted by Rohmaniar *et al.* also demonstrated the antibiofilm activity of pineapple, where rinsing with pineapple juice was shown to reduce plaque accumulation in patients with fixed orthodontic appliances.²²

The stevia jelly candy group without pineapple in this study also demonstrated antibacterial and antibiofilm activity against *Streptococcus mutans*. However, the inhibition zone measurements and absorbance values differed significantly compared to the jelly candy group containing both pineapple and stevia. This effect is presumed to be due to the presence of active compounds in stevia which are capable of inhibiting the growth of *S. mutans*. These findings are supported by a study by Reshimi *et al.*, which demonstrated that stevia leaf extract at concentrations of 2.5%, 5%, and 10% exhibited antibacterial activity against *S. mutans*, with mean inhibition zone diameters of 12 mm, 13 mm, and 18 mm.¹¹

The jelly candy without pineapple and stevia (negative control) showed no inhibition zone and exhibited the highest absorbance value compared to the other groups, indicating the absence of both antibacterial and antibiofilm activity. This result is likely due to its composition, which consists only of water and gelatin, lacking bioactive compounds with antibacterial properties.²³



Conclusion

Pineapple (*Ananas comosus*) jelly candy sweetened with stevia demonstrated antibacterial and antibiofilm activity against the growth of *Streptococcus mutans*. This jelly candy has the potential to be developed as a healthy snack with anticariogenic properties. Further studies are recommended to include organoleptic evaluation, physicochemical analysis, as well as contamination and safety testing to ensure that the product complies with the Indonesian National Standard (SNI).

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