

***Streptococcus mutans* Antibacterial Activity of 0.1%
Lysozyme Tooth Paste as an Alternative
for Children Below 3 Years Old
in Preventing Early Childhood Caries (ECC)
(Experimental Laboratory Study)**

**Aktivitas Antibakteri *Streptococcus mutans* dari Pasta Gigi Lisozim 0,1%
Sebagai Alternatif Untuk Anak Di Bawah 3 Tahun
Dalam Mencegah Karies Anak Usia Dini (PAUD)
(Studi Laboratorium Eksperimental)**

Essie Octiara¹, Heriandi Sutadi², Yahwardiah Siregar³, Ameta Primasari⁴

¹ Department of Pediatric Dentistry,
Faculty of Dentistry, Universitas Sumatera Utara, Jl. Alumni no. 2, Medan 20155, Indonesia
²Department of Pediatric Dentistry, Faculty of Dentistry,
Universitas Indonesia, Jl Salemba Raya no. 4, Jakarta 10430, Indonesia
³Department of Biochemistry, Faculty of Medicine,
Universitas Sumatera Utara, Jl. Dr. T Mansur no. 5, Medan 20155, Indonesia
⁴Department of Oral Biology, Faculty of Dentistry,
Universitas Sumatera Utara, Jl. Alumni no.2, Medan 20155, Indonesia
Email: essie.octiara@usu.ac.id

Abstract

One of the ways of preventing Early Childhood Caries (ECC) is by removing tooth debris through the use of fluoride toothpaste. However, its use by children below 3 years old needs to follow recommended quantity due to the possibility of being ingested, thereby causing fluorosis. Lysozyme has potential to be fluoride substitution as it mediates bacterial aggregation, inhibits adhesion, and activates autolysin by destructing their cell wall. Therefore, this study aims to compare *Streptococcus mutans*' antimicrobial activity of non-fluoride lysozyme toothpaste of various concentrations such as 0.025, 0.05, 0.1, and 0.2 %. This experimental study was conducted using a post-test design and the tested toothpaste was lysozyme-contained with 0.025, 0.05, 0.1, and 0.2% concentrations. Meanwhile, children toothpaste containing fluoride, enzyme, and 0.2% chlorhexidine were used as the positive control. The test of *S. mutans* antibacterial activity used 3 methods, namely disc diffusion, well, and microtiter plate. The analytical test used Anova one way with Bonferroni post hoc and $p < 0.05$ significant level. The results showed that all concentrations of lysozyme toothpaste had an inhibitory effect on the growth of *S. mutans* ($p < 0.05$), while the highest mean was on 0.1 and 0.2% concentrations. This showed that a 0.1% concentration of lysozyme toothpaste can be used as an alternative for children below 3 years old.

Keywords: Early Childhood Caries (ECC), Lysozyme Tooth Paste, Antibacterial Activity, *Streptococcus mutans*

Abstrak

Salah satu pencegahan ECC yang efektif adalah membersihkan gigi dari debris. Penggunaan pasta gigi berfluor pada anak di bawah usia 3 tahun harus dengan jumlah yang disarankan, karena dapat tertelan sehingga menyebabkan fluorosis. Lisozim sebagai zat aktif dalam pasta gigi mempunyai kemampuan memediasi agregasi bakteri dan menghambat perlekatan bakteri dan juga mengaktifkan autolisin bakteri dengan menghancurkan dinding sel bakteri. Tujuan penelitian ini membandingkan daya aktivitas antimikroba *Streptococcus mutans* dari pasta gigi lisozim tanpa fluor dengan berbagai konsentrasi 0,025%; 0,05%, 0,1%, 0,2 % dan 0,4%. Jenis penelitian adalah eksperimen dengan rancangan *post test only*. Pasta gigi yang dicoba adalah pasta gigi lisozim dengan konsentrasi 0,025%; 0,05%; 0,1% dan 0,2%. Pasta gigi anak berfluor, pasta gigi enzim dan chlorheksidin 0,2% merupakan kontrol positif. Uji aktivitas antibakteri *S.mutans* digunakan 3 metode yaitu metode difusi dengan *disc* dan sumuran serta *microtiter plate*. Uji analisis digunakan *Anova one way* dengan *post hoc Bonferoni* dan tingkat kemaknaan $p < 0,05$. Hasil penelitian diperoleh semua konsentrasi pasta gigi lisozim memiliki daya hambat terhadap pertumbuhan *S.mutans* ($p < 0,05$). Rerata aktivitas antibakteri *S.mutans* yang terbesar

dimiliki oleh pasta gigi lisozim konsentrasi 0,1% dan 0,2%. Disimpulkan pasta gigi lisozim konsentrasi 0,1% dapat digunakan sebagai pasta gigi alternatif untuk anak usia di bawah 3 tahun.

Kata kunci: ECC, pasta gigi lisozim, aktivitas antimikroba, *S.mutans*

INTRODUCTION

According to the American Academy of Pediatric Dentistry (AAPD), tooth caries at an early age is the presence of caries lesion on the surface of deciduous teeth (cavities or non-cavities), tooth loss due to caries, or filled teeth on children under 6 years old.¹ Several countries including Indonesia have discovered a high prevalence of ECC cases in 2 years old children with 27.66% in China², Srilanka 32.19%,³ and 68.5% in Northern Thailand⁴. Based on national data, there is an increase in caries prevalence among Indonesian children. According to Riskesdas 2007, the cases were 6.9% in children of the 1-4 years old age group, while in 2013, 10.4% was reported.^{5,6} However, the ECC prevalence was higher than national data, which was 57.7% in Medan.⁷

The high prevalence of ECC on early-age children has drawn health workers' attention, especially the pediatric dentist. Therefore, regular removal of debris from teeth has been found as an effective way of preventing ECC. The habit of brushing teeth is properly carried out after the first tooth eruption.¹ In Lithuania, a study has shown that children who brushed their teeth since the first tooth eruption had lower ECC risk.⁸ Similarly, mothers who brushed their children's teeth since the beginning of eruption had more caries-free children ($p= 0.007$).⁹ In Indonesia, the APPD recommended that fluoride-containing toothpaste use for children below 3 years old needs to be only the size of rice and not exceed the size of a bean for children between the ages of 3 to 6 years.¹⁰

This was intended to prevent the risk of paste ingestion, which might cause fluorosis.¹¹ Children below 1 year of age can also have their teeth cleaned by using a smooth toothbrush without paste or using non-fluoride toothpaste.¹⁰ A previous study on 6-71 months old children showed that more children using fluoride-containing toothpaste were not affected by ECC (85.8%) compared to 14.2% who were affected.¹²

Although toothpaste quantity given to early age children is limited, some parents are still worried about using fluoride-containing toothpaste on children of 2 years old and below because of ingestion. Therefore, the non-fluoride type that still contains

Streptococcus mutans (*S. mutans*) antibacterial active substance can be given as an alternative.

Lysozyme as an innate immunity in saliva became first and prominent body defense in the oral cavity.¹³ It is antibacterial, antiviral, anti-tumor, and immune modulator activity. Furthermore, its activity as antimicrobial was related to lytic action on bacteria by hydrolyzing the β (1-4) bond between N-acetylmuramic and N-acetylglucosamine acid in the peptidoglycan layer of the bacteria cell wall.¹⁴ Glycosidic bond hydrolysis has also led to the formation of small pores in the bacteria cell wall which caused their death.¹⁵

Although the addition of lysozyme enzyme on toothpaste has been spread in Indonesia, it has not been widely used. Moreover, it also contained active substances such as fluoride, amyloglucosidase, lactoferrin, and lactoperoxidase.¹⁶ A previous experimental study of brushing teeth using fluoride toothpaste which also contained lysozyme, lactoferrin, and lactoperoxidase after a week showed a significant *S. mutans* and *L. acidophilus* decrease ($p<0.001$) in children with ECC.¹⁷

Meanwhile, the addition of lysozyme enzyme active substrate as an antimicrobial on toothpaste increases the efficacy of teeth cleaning procedures, controls plaque, and prevents caries on children,¹⁸ which becomes an alternative for children below 3 years old. This is because it does not contain fluoride and is safe when accidentally ingested. Therefore, this study aims to compare *S. mutans* antimicrobial activity of lysozyme toothpaste without fluoride on different concentrations which are 0.025, 0.05, 0.1, and 0.2%

MATERIALS AND METHODS

This experimental study was carried out using a post-test design and the ethical clearance was approved by the Ethical Research Committee Faculty of Medicine Universitas Sumatera Utara (USU) no. 59. It was conducted in the Pharmacy lab of Microbiology Department of Faculty of Science and Integrated Lab of Faculty of Medicine, USU. In this study, the toothpaste tested without fluoride at 0.025, 0.05, 0.1, and 0.2% concentrations, was lysozyme

crystals (hen egg white) from Sigma Aldrich. The positive control used were 0.2% chlorhexidine mouthwash, children's fluoride, and enzyme toothpaste, which are found on markets. Meanwhile, basic substances of tested toothpaste such as calcium carbonate, glycerol, sorbitol, sodium carboxymethyl cellulose, tragacanth, sodium benzoate, flavorings, and distilled water, without additional lysozyme enzyme were used as the negative control.

The tested toothpaste, control, and chlorhexidine mouthwash were analyzed to determine its ability in inhibiting *S. mutans* growth by diffusion method using disc and well and microtiter plate method. The techniques included:

Making bacteria culture and *S. mutans* dilution *S. mutans* were cultured in TYS20B medium and incubated in a 37°C anaerobic jar for 2x24 hours. After 2 days, bacteria suspension was prepared by placing a single colony in BHI (Brain Heart Infusion), vortexing until homogeneous, and comparing the turbidity to standard 10⁸ McFarland solution.¹⁹ When the culture solution was equivalent to the standard, it was diluted by preparing 7 tubes with 9 ml physiological saline solution and each tube contained 5 mL saline solution. Subsequently, 1 mL bacteria were taken from the dilution, placed in the first tube, and vortexed. Approximately 1 mL was also taken from the first tube to be put in the second tube, and until the seventh tube, where 5 mL was taken and placed in the eighth tube (containing 5 mL saline), then vortexed.²⁰

Diffusion method using a disc with toothpaste in solid medium. Approximately 1 ml of diluted *S. mutans* (eight tubes) was put on TYS20B agar petri dishes and the bacteria solution was allowed to spread on the agar surface. The disc containing toothpaste, which was previously soaked for 1 hour was later put on the surface. Subsequently, the petri was incubated in an anaerobic jar under 37°C temperature for 3x24 hours, while the inhibition zone (light zone) appeared around the disc, and was counted in mm by caliper.²⁰

Diffusion method using well with toothpaste in solid medium. Diluted 1ml of *S. mutans* was put on agar petri dishes and the bacteria solution was spread to the surface. The medium was punched by using a perforator with 6 mm diameter, the hole was also filled by toothpaste, and 0.2% chlorhexidine 0.05 mg. After the petri dish was incubated for 24 hours under 37°C temperature, the inhibition zone was measured using caliper in 3 ways, namely horizontally, vertically, and diagonally.²¹

Microtiter plate method Approximately 1-2 colonies of *S. mutans* colonized in TYS20B agar me-

dium were put in a tube containing 30 ml TYS20B liquid medium. Subsequently, the medium was incubated for 24 hours under 37°C temperature, while the remaining was agitated at 120 rpm.

After 24 hours, the 0.5 mL TYS20B liquid medium containing *S. mutans* was moved into the tube of 4.5 mL liquid TYS20B, while dilution was conducted until 10⁴ CFU/mL and homogenized by shaking. In each well, 200 µL liquid TYS20B and lysozyme powder active substances were put, which had been diluted by nuclease-free water to make 50 µL 0.025, 0.05, 0.1, and 0.2% concentrations. The 0.2% chlorhexidine of 50 µL was put in the fifth well as a positive control, while negative controls were 250 µL TYS20B liquid solution with *S. mutans* in the sixth and without in the seventh well. Subsequently, incubation was carried out for 48 hours in the incubator.

The bacteria in each well were removed and the wells were rinsed with 275 µL sterile aqua dest 3 times after 48 hours. The wells were further fixed heat in the oven at 70°C temperatures for 20 minutes. Subsequently, 250 µL sterile violet crystals of 0.1% µL/U concentration were added to the well and soaked for 1 minute. The violet crystals were removed and the well was washed 3 times using 275 µL of 95% ethanol. On the last wash, 95% ethanol was soaked in the well for 2 minutes before being removed. Then, the optical density of the antibiofilm of lysozyme on the *S. mutans* was read by a spectrometer on 575 nm.²² Meanwhile, the analysis test used in this study was Anova one way with Bonferroni post hoc test on p<0.05 level of significance.

RESULTS

The inhibition zone diameter from the disc method in the TYS20B medium showed that the highest diameter mean was from the tested lysozyme toothpaste of 0.1% concentration, although 0.2% concentration was not significantly different. However, this inhibition zone diameter was still lower than the enzyme toothpaste, children's fluoride toothpaste, and 0.2% chlorhexidine (see Table 1).

According to the well method, the highest inhibition zone was discovered on fluoride toothpaste, followed by 0.2% chlorhexidine, as well as 0.1%, 0.05%, 0.2%, and 0.025% lysozyme toothpaste in the last place (p<0,05) (see Table 2).

The microtiter plate method showed that the highest antibiofilm was on chlorhexidine mouthwash, followed by 0.2% lysozyme solution, as well as 0.1%, 0.05%, and 0.025% lysozyme (see Table 3).

DISCUSSION

In this study, the tested *S. mutans* isolated and cultured from children with ECC were the main agent of caries etiology,²³ Their antibacterial substances used in this toothpaste was lysozyme, a strongly cationic protein capable of mediating bacterial aggregation, inhibiting adhesion, and also activating autolysin by destructing their cell wall.^{14,15} Meanwhile, the lysozyme activity remains when the enzyme is absorbed in the pellicle.²⁴

The source of lysozyme used were crystals from hen egg-white, meanwhile, their variety can affect the result. Although human lysozyme is 3-4 times more reactive than hen egg white lysozyme (HEWL), it is very similar to human lysozyme and contains 129 amino acids (14,3 kDa). Since it is not expensive and has a good correlation to human lysozyme, it is often used in different studies as a substitute.²⁵

Based on the results of the disc method, the lysozyme toothpaste in various concentrations had an inhibitory effect on the growth of *S. mutans*. The highest inhibition zone diameter to *S. mutans* was on 0.1% lysozyme toothpaste with a mean of 13.24 mm, followed by 0.2% concentration with 12.6 mm mean 12.6 mm, 0.05% (mean 12.04 mm), and 0.025% concentration (mean 9.06 mm) in the last place (Table 1). According to Indonesian Health Ministry, a substance is said to have antibacterial activity when the inhibition diameter is equal to or higher than 6 mm.²⁶ Also, Davis and Stout²⁷ stated that antibacterial activity criteria with inhibition zone diameter of 5 mm or less are categorized as weak, 5-10mm is medium, 10-20mm is strong, and 20mm or more is very strong. Based on these criteria, the inhibition zone of 0.05, 0.1, and 0.2% lysozyme concentration against *S. mutans* in the toothpaste is categorized strong, while 0.025% concentration is medium.

According to Bonferroni's post hoc result, there was no mean difference of inhibition against *S. mutans* among lysozyme concentrations. Meanwhile, there was a difference in inhibition against *S. mutans* in various concentrations of lysozyme and children's fluoride toothpaste ($p < 0.05$), but no difference when compared to the enzyme type. Inhibition to *S. mutans* of lysozyme was still not comparable to children's fluoride toothpaste on the market (positive control) but to the enzyme toothpaste (Table 1). Moreover, the children's fluoride toothpaste contains Sodium Lauryl Sulphate (SLS) detergent substance,

which is widely used due to its stability, antibacterial aspect, and low surface tension that facilitates paste flow to whole teeth parts.²⁸ This detergent contained fluoride as antibacterial for *S. mutans*, therefore, its inhibition was the highest compared to enzyme toothpaste as a positive control. Although enzyme toothpaste available in the market also contains fluoride, it does not contain SLS detergent, which causes oral cavity mucosal desquamation, ulceration, and dry mouth.²⁹

This was also shown in the well method, where all lysozyme toothpaste concentrations had inhibition of *S. mutans* growth. Based on the results, there was no difference of inhibition to *S. mutans* among various lysozyme toothpaste concentrations compared to the positive control, except for 0.025% concentration ($p < 0.05$). The highest mean of inhibition was in 0.1% concentration (19.23 mm), followed by 0.05% (15.93 mm), and 0.2% (15.23 mm) (Table 2).

According to the microtiter plate method, lysozyme solution of 0.025, 0.05, 0.1, and 0.2% concentrations had inhibited *S. mutans* bacteria ($p < 0.05$). However, 0.025% lysozyme solution inhibited differently compared to 0.2% solution ($p = 0.043$), while there was no different inhibition between 0.05 and 0.1% concentration compared to 0.2% lysozyme concentration. The highest inhibition of *S. mutans* was discovered in 0.2% concentration, followed by 0.1% and 0.05%, therefore, the higher the lysozyme concentration, the higher the inhibition of *S. mutans* (Table 3).

Based on the 3 methods of *S. mutans* anti-bacterial activity examination, 0.05, 0.1, and 0.2 % lysozyme toothpaste could be used as an alternative for children. This is similar to a previous study that showed the use of lysozyme concentration in toothpaste ranged between 0.01-0.1%.³⁰ Similarly, Sing et.al³¹ discovered that the required concentration in inhibiting bio-film formation was in the 50-200 µg/ml. A study by Andrade et.al.¹³ also discovered the bactericidal and bacteriostatic effects of lysozyme against *S. mutans* were 68.5 mg/mL (MBC concentration) and 58.7 mg/mL, respectively. Tonguc-Altin et.al also described that toothpaste containing lysozyme and lactoferrin has the highest inhibitory effect on the Mutans Streptococci and Lactobacili.³²

According to the disc and well method, it is concluded that the mean of *S. mutans* antibacterial activity from 0.1% lysozyme toothpaste was the highest of all three concentrations. However, the microtiter plate method showed that the mean of *S.*

mutans antibacterial activity was the highest in 0.2% concentration. Therefore, during the bulk production of toothpaste, the 0.1% lysozyme can be used as an alternative for children below 3 years old.

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TABLES

Table 1. Inhibition Zone Diameter to *S. mutans* from Various Tooth Pastes and Chlorhexidine Used Disc Method

Test Group	Inhibition zone diameter to <i>S. mutans</i> (mm)					
	Repetition I	Repetition II	Repetition III	Means	SD	p
Lysozyme 0.025% (A)	9.9	10	7.5	9.06	1.18	0.000* ¹
Lysozyme 0.05% (B)	11.4	10	15.3	12.04	1.24	
Lysozyme 0.1% (C)	14.6	10.6	15	13.24	1.21	
Lysozyme 0.2% (D)	15.6	8.6	15.2	12.68	1.4	
Children enzyme toothpaste (E)	10.55	16.9	14.8	14.08	1.27	
Children fluoride toothpaste (F)	28.15	30.4	25.8	28.05	1.08	
Chlorhexidine 0.2%® (G)	19.25	23	25.5	22.43	1.15	
Negative control	0	0	0	0		

* Significant; ¹Anova one-way test (log 10 transformation); Bonferroni Post Hoc Test: A vs F=0.000; A vs G=0.002; B vs F=0.005; C vs F=0.014; D vs F=0.008; E vs F=0.022

Table 2. Inhibition Zone Diameter to *S. mutans* from Various Tooth Pastes and Chlorhexidine Used Well Method

Test Group	Inhibition zone diameter to <i>S. mutans</i> (mm)					
	Repetition I	Repetition II	Repetition III	Means	SD	p
Lysozyme 0.025% (A)	0	6.5	9.2	5.23	4.73	0.03* ¹
Lysozyme 0.05% (B)	14.2	17.8	15.8	15.93	1.80	
Lysozyme 0.1% (C)	23	15.8	18.9	19.23	3.61	
Lysozyme 0.2% (D)	11.2	18.5	16	15.23	3.71	
Children enzyme tooth paste (E)	11.4	14.15	20.2	15.37	4.46	
Children fluoride tooth paste (F)	17.9	35.85	32.2	28.65	9.49	
Chlorhexidine 0.2%® (G)	17.1	22.3	28	22.47	5.45	
Negative control	0	0	0	0	0.00	

*Significant ¹Anova one way test, Bonferoni Post Hoc Test= A vs F=0.002; A vs G=0.026

Table 3. Optical Density (nm) of Anti-biofilm of Lysozyme on the *S. mutans* from Various Concentrations of Lysozyme Solution and Chlorhexidine Used Microtiter Plate Method

Test Group	Optical density (nm) of anti-biofilm of <i>S. mutans</i>					
	Repetition I	Repetition II	Repetition III	Means	SD	P
Lysozyme Solution 0.025% (A)	0.5379	0.5270	0.6246	0.5632	0.05	
Lysozyme Solution 0.05% (B)	0.6201	0.5070	0.3900	0.5057	0.115	
Lysozyme Solution 0.1% (C)	0.3481	0.5744	0.5376	0.4867	0.121	

Lysozyme Solution 0.2% (D)	0.2232	0.3295	0.2892	0.2806	0.054	0.000* ¹
Chlorhexidine 0.2%® (E)	0.0510	0.0506	0.0488	0.0506	0.001	
Negative Control (with <i>S. mutans</i>) (F)	0.5756	0.6569	0.7552	0.6626	0.090	
Negative Control (without <i>S. mutans</i>) (G)	0.5925	0.8521	0.7202	0.7216	0.130	

*Significant ¹Anova one way Test; Bonferroni Post Hoc Test: A vs D= 0.043; A vs E= 0.000; B vs E= 0.001; C vs E=0.001G vs D= 0.001; G vs E= 0.000; F vs D= 0.003; F vs E= 0.000

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