Garlic Extract Effectivity Against the Viability of Biofilms Produced by *Streptococcus mutans* Serotypes C and F in Pediatric Patients with Early Childhood Caries

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Abstract

Early childhood caries (ECC) is characterized by the presence of one or more decayed teeth, the presence of a lesion with or without a cavity, caries-induced tooth loss, or patching of the surface of deciduous teeth from birth to the age of 72 months. *Streptococcus mutans* is the most common microorganism of caries and is present in dental plaques. *S. mutans* is a Gram-positive facultative anaerobic bacteria present in the human oral cavity. Based on serotype-specific polysaccharides, *S. mutans* is classified into three serotypes: c, e, and f. The prevalence of each serotype based on a study conducted in Jakarta was dominated by serotypes f (85.5%), c (74.2%), and e (22.6%). Garlic (*Allium sativum*) is known to inhibit the proliferation of various types of pathogenic bacteria.

This study aims to test the viability of biofilms produced by S. mutans serotypes c and f in response to treatment with garlic extracts at concentrations of 10%, 25%, 50%, and 100%.

Dental biofilm samples were collected from children aged 3-5 years, cultured in TSY20B medium, and confirmed using a conventional PCR technique. Biofilms were established in microwell plates and were incubated for 24 h. Each plate was exposed to garlic extract at concentrations of 10%, 25%, 50%, or 100%, with 0.2% chlorhexidine serving as positive control. The methyl-thiazolyl-tetrazolium assay was used to assess cell viability.

There was a statistically significant decrease ($p \leq 0.001$) in the viability of biofilms produced by *S. mutans* serotypes c and f when exposed to garlic extract concentrations of 50% and 100%. The four concentrations decrease the viability of *S. mutans* serotypes c and f. The decrease in the viability of *S. mutans* serotype c was significant at concentrations of 50% and 100%, while that of serotype f was significant at a concentration of 100%. Garlic extracts can be used as alternative antibacterial oral agents against *S. mutans*

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Introduction

Early childhood caries is defined as the presence of one or more decayed teeth, tooth loss, or patching of the surface of any deciduous teeth in infants and children younger than 6 years.[1,2] Early childhood caries are classified by the number of tooth elements involved: type 1 - moderate effect to the maxillary primary incisors; type 2 – moderate to severe effect to the incisive teeth, molars, and elder caninus; and type 3 - widespread and severe condition involving the mandibular incisors.[3] Dental caries is a multifactorial disease that is characterized by the formation of complex biofilms. *Streptococcus mutans* is a Gram-positive facultative anaerobic bacteria that is commonly found in the human oral cavity and plays a significant role in the development of early childhood caries.[2] *S. mutans* has several antigens (c, e, f, and k) and 70%–100% of colonies isolated from humans are serotype c. The prevalence of serotypes based on a study conducted in Jakarta was dominated by serotypes f (85.5%), c (74.2%), and e (22.6%).[4,5] *S. mutans* possesses acidogenic properties and produces organic acids derived

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from carbohydrates that are known to survive in a highly acidic environments. *S. mutans* also produce dextran, an adhesive polysaccharide that supports the growth and adhesion of other bacteria on the enamel surface of the tooth. However, these bacteria also produce acids that dissolve the tooth.[3] The decrease in environmental pH due to the accumulation of lactic acid produced by *S. mutans* kills off non-cariogenic commensal bacteria. At the critical pH of 5.5, hydroxyapatite becomes soluble and demineralizes the enamel and dentine surfaces. *S. mutans* grows on nutrient-rich complex media, such as the selective medium TYS20B. The bacitracin content in this medium is selective against other bacteria. Furthermore, *S. mutans* is resistant to several antibiotics.[6]

A number of microorganisms adhere to the tooth surface and form biofilms, consisting of an extracellular matrix derived from the cell itself or the surrounding environment.[14] The development of biofilms is divided into three phases: adhesion, active accumulation, and maturation. The adhesion phase is the initial process of biofilm formation and is related to the interaction of oral bacteria with the tooth surface.[7,8] In the accumulation phase, coaggregation occurs, which is the interaction and attachment of genetically different bacterial cells. In this phase, there is a change to the bacteria dominance in biofilms, such as *Streptococci* replaced with *Actinomyces* and various Gram-positive *bacilli*.[8] In the maturation phase, bacterial proliferation in biofilms progresses more slowly because most energy is used to produce extracellular polymeric substances.

Garlic (*Allium sativum*) has been cultivated on the Asian continent as a medicinal plant for more than 6,000 years. *A. sativum* extract has the ability to inhibit the proliferation of various pathogenic bacteria, viruses, and fungi. Garlic contains 65% water, 28% carbohydrates (especially fructose), 2.3% organosulfur, 2% protein (especially allinase), and 1.2% free amino acids. The predominant organosulfur compounds in garlic include S-allyl-cysteine sulfoxides, such as alliin and γ-glutamylcysteine. Alliine is a sulfur-rich amino acid that is responsible for the smell and flavor of garlic, and used as a precursor of allicin. In freshly crushed garlic, alliine and other sulfoxide compounds, except cycloalliin, are converted into thiosulfinic compounds, such as allicin, by alliinase enzymes. Alliine has antibacterial properties.[12] Allicin is able to inhibit the synthesis of DNA, protein, and RNA. In addition, the organosulfur and phenol contents of garlic have antimicrobial activities.

The objective of this study was to determine the *in vitro* effectiveness of various concentrations of garlic extract against the viability of biofilms produced by *S. mutans* serotypes c and f in children with early childhood caries.

Materials and methods

The Ethical Committee of the Faculty of Dentistry of Universitas Indonesia approved the study protocol and written informed consent was obtained from the parents of all the patients prior to the commencement of the study.

The aim of this laboratory experimental study was to test the viability of biofilms produced by *S. mutans* serotypes c and f in response to treatment with garlic extracts at concentrations of 10%, 25%, 50%, and 100%. The study cohort consisted of children aged 3–5 years who visited the Pediatric Dental Clinic of Universitas Indonesia and were diagnosed with early childhood caries. The inclusion criteria were good general health and parental approval to participate in the study. The exclusion criteria included current medical care involving the use of any medication and refusal to undergo an examination.

Plaque samples from the tooth surfaces were collected from the study subjects using sterile toothpicks into Eppendorf tubes containing 1 mL of brain heart infusion broth. The tubes were stored in a cooler and transported to the Oral Biology Laboratory of Universitas Indonesia for incubation.

Cultivation by TYS20B medium

The plaque samples were further vortexed to ensure homogeneity. A total of 20 µL of solution was collected with a pipette and was cultured on TYS20B agar in petri dishes, which were incubated under anaerobic conditions (5% CO_2, 10% H_2, and 85% N_2) at 37°C for 24 h.

Identification of *S. mutans* serotypes c and f by polymerase chain reaction (PCR) analysis

After 24 h of culturing on TYS20B agar, *S.*

mutans cells were identified by PCR. Each reaction mixture contained Dreamtaq Green PCR Master Mix (10 µL), primer 1 (2 µL), primer 2 (2 µL), nuclease-free water (2 µL), and the DNA template (4 µL). The PCR program was set according to the serotype intended to be identified and the quality of the PCR products was confirmed by electrophoresis. The primers used for PCR amplification are listed in the following table: [10,11]

Primer	Sequence (5'–3')
GTFB-F	ACTACACTTTCGGGTGGCTTGG
GTFB-R	CAGTATAAGCGCCAGTTTCATC
Serotipe C SC-F	GGAGTGCTTTTTACAAGTGCTGG
Serotipe C SC-R	AACCACGGCCAGCAAACCCTTTAT
Serotipe F SF-F	CCC ACA ATT GGC TTC AAG AGG AGA
Serotipe F SF-R	TGC GAA ACC ATA AGC ATA GCG AGG

The PCR conditions to identify *S. mutans* with GTFB primers were initial denaturation at 95ºC for 1 min, then 35 cycles of amplification (denaturation at 94ºC for 30 s, annealing at 53ºC for 1 min, and elongation at 70ºC for 2 min), and a final elongation step at 72ºC for 7 min. The PCR conditions to identify serotype c with the Sc primers were initial denaturation at 95ºC for 1 min, then 30 cycles of amplification (95ºC for 30 s, 59ºC for 30 s, and 72ºC for 30 s) and a final elongation step at 72ºC for 4 min. The PCR conditions to identify serotype f with the Sf primers were initial denaturation at 95ºC for 1 min, then 30 cycles of amplification (95ºC for 30 s, 59ºC for 30 s, and 72ºC for 30 s) and a final elongation step at 72ºC for 4 min. Furthermore, electrophoresis was performed using TBE Buffer (1x Tris, 107.78 g/L; disodium ethylenediaminetetraacetate dihydrate, 7.44 g/L, boric acid, 55 g/L) at 60 V and 400 mA for 75 min. The volume of the DNA ladder (GeneRuler 100 bp; Fermentas, Waltham, MA, USA) was 2 µL (100 mg/µL). After completion, a gel documentation system was used to assess the gel. The results of DNA digestion with the use of restriction enzymes were documented on a data sheet or in a computer file.

Preparation of garlic extract

Locally grown garlic was purchased from Pusat Penelitian Bioteknologi-LIPI (Bogor, Indonesia), was stored at 4ºC, and was analyzed within 1 month. Briefly, 40 g of garlic were crushed with a garlic crusher and the garlic juice and debris were collected in a centrifuge tube by pouring 40 mL of the diluents (20% ethanol) in the crusher. After shaking for 30 min, the tube was left for 10 min at room temperature and, then, centrifuged at 9000 rpm for 10 min. Afterward, the contents were filtered using filter paper to separate the supernatants. To obtain concentrations of 10%, 25%, and 50%, dilution was carried out with sterile aquades.

Viability test using the methyl-thiazolyl-tetrazolium (MTT) assay

S. mutans serotypes c and f were propagated in brain heart infusion broth and, then, homogenized using a vortex mixer. Afterward, a 100-µL aliquot of the bacterial suspension was transferred to each well of a 96-well microplate, which was then incubated at 37°C under aerobic conditions for 24 h. Each well was washed with phosphate-buffered saline. Then, the biofilms of *S. mutans* serotypes c and f were exposed to 100 µL of garlic extract at various concentrations. Chlorhexidine (0.2%) served as a positive control and solutions without garlic extract as negative controls. After incubation with garlic extract, each well was washed with phosphate-buffered saline and 50 µL of 5 mg/ml of MTT solution was added and the plate was incubated at 37°C for an additional 3 h. Acidified isopropanol was added and the microplate was placed on an orbital shaker for 1 h. The results of the MTT assay were read using a microplate reader at a wavelength of 490 nm. The optical density (OD) of the treatment and control groups were used in the following formula to calculate the percentage of viable cells.

$$\frac{OD\ treatment}{OD\ negative\ control}\ x\ 100\%$$

Results

S. mutans were identified on TYS20B media and the serotypes of these bacteria were determined by conventional PCR. The product sizes of serotypes c and f were 727 316 bp, respectively. The data were analyzed using the Kruskal–Wallis nonparametric test to observe differences in the viability of biofilms produced by *S. mutans* serotypes c and f against garlic extract concentrations of 10%, 25%, 50%, and 100%. The Mann–Whitney post hoc test was used to

identify differences in the viability of *S. mutans* serotype c in response to treatment with garlic extract concentrations of 10%, 25%, 50%, and 100%, as well as the positive (0.2% chlorhexidine gluconate) and negative controls.

As Tables 1 and 3 show, there were significant differences in the viability of *S. mutans* serotypes c and f against garlic extract concentrations of 10%, 25%, 50%, and 100% ($p < 0.05$, Kruskal–Wallis test). There were significant differences in the viability of biofilms of at least two groups.

Treatment Group	n	Median (min–max) (%)	*p*
Negative control	4	72.69 (63.84–190.79)	<0.001
Postive control (CHX 0,2%)	4	1.99 (1.18–7.81)	
10% Garlic extract	4	24.58 (8.5–46.81)	
25% Garlic extract	4	15 (10.76–18.65)	
50% Garlic extract	4	10.15 (8.25–22.55)	
100% Garlic extract	4	8,78 (7.59–15.41)	

Kruskal–Wallis test, *$p < 0.05$

Table 1. Differences in biofilm viability of *S. mutans* serotype c in response to treatment with garlic extracts (10%, 25%, 50%, and 100%), 0.2% chlorhexidine gluconate (positive control), and a negative control.

Treatment Group	*p*
Negative control vs 10% Garlic extract	0.021*
Negative control vs 25% Garlic extract	0.021*
Negative control vs 50% Garlic extract	0.021*
Negative control vs 100% Garlic extract	0.021*
Postive control (CHX 0,2%) vs 10% Garlic extract	0.021*
Postive control (CHX 0,2%) vs 25% Garlic extract	0.021*
Postive control (CHX 0,2%) vs 50% Garlic extract	0.021*
Postive control (CHX 0,2%) vs 100% Garlic extract	0.043*
10% Garlic extract vs 25%	1.000
10%Garlic extract vs 50%	0.564
10%Garlic extract vs 100%	0.149
25%Garlic extract vs 50%	0.021*
25%Garlic extract vs 100%	0.021*
50%Garlic extract vs 100%	0.083

Mann–Whitney post hoc test, *$p < 0.05$

Table 2. Post hoc analysis of differences in biofilm viability of *S. mutans* serotype c in response to treatment with garlic extracts (10%, 25%, 50%, and 100%), 0.2% chlorhexidine gluconate (positive control), and a negative control.

As the results of the Mann–Whitney post hoc analysis in Tables 2 and 4 show, there were statistically significant differences in the viability of *S. mutans* serotypes c and f between the positive control group and garlic extract groups at concentrations of 10%, 25%, 50% and 100%; the negative control group and garlic extract groups at concentrations of 10%, 25%, 50% and 100%; *serotype c* in the 50% and 100% groups; and serotype f in the 50% group.

Treatment Group	n	Median (min–max) (%)	*p*
Negative control	4	99.36 (37.44–163.84)	<0.001
Positive control (CHX 0,2%)	4	3.42 (0,98–4.28)	
10% Garlic extract	4	23.68 (6.36–38.05)	
25% Garlic extract	4	24.04 (22.57–38.61)	
50% Garlic extract	4	11.93 (8.13–39.46)	
100% Garlic extract	4	9,23 (5,57–13,77)	

Kruskal–Wallis test, *$p < 0.05$

Table 3. Differences in biofilm viability of *S. mutans* serotype f in in response to treatment with garlic extracts (10%, 25%, 50%, and 100%), 0.2% chlorhexidine gluconate (positive control), and a negative control.

Treatment Group	*p*
Negative control vs 10% Garlic extract	0.043*
Negative control vs 25% Garlic extract	0.043*
Negative control vs 50% Garlic extract	0.021*
Negative control vs 100% Garlic extract	0.021*
Positive control (CHX 0,2%) vs 10% Garlic extract	0.021*
Positive control (CHX 0,2%) vs 25% Garlic extract	0.021*
Positive control (CHX 0,2%) vs 50% Garlic extract	0.021*
Positive control (CHX 0,2%) vs 100% Garlic extract Garlic 10% Garlic extract vs 25%	0.043*
10% Garlic extract vs 50%	0.564
10% Garlic extract vs 100%	0.773
25% Garlic extract vs 50%	0.083
25% Garlic extract vs 100%	0.248
50% Garlic extract vs 100%	0.021*
	0.248

Mann–Whitney post hoc test, *$p < 0.05$

Table 4. Post hoc analysis of differences in biofilm viability of *S. mutans* serotype f in response to treatment with garlic extracts (10%, 25%, 50%, and 100%), 0.2% chlorhexidine gluconate (positive control), and a negative control.

As the results of the Mann–Whitney post hoc analysis in Table 5 show, there were no statistically significant differences in the viability of *S. mutans* serotypes c and f following treatment with garlic extract concentrations of 10%, 25%, 50%, and 100%.

Concentration		n	Median (min–max) (%)	*p*
10% Garlic extract	*S. mutans ser. c*	4	24.58 (8.85–46.81)	0.773
	S. mutans ser. f	4	23.68 (6.36–38.05)	
25% Garlic extract	*S. mutans ser. c*	4	15 (10.76–18.65)	0.773
	S. mutans ser. f	4	24.04 (22.57–38.61)	
50% Garlic extract	*S. mutans ser. c*	4	10.15 (8.25–22.55)	0.564
	S. mutans ser. f	4	11.93 (8.13–39.46) 8.78	
100% Garlic extract	*S. mutans ser. c*	4	(7.59–15.41) 9.23 (5.57–13.77)	0.564
	S. mutans ser. f			

Table 5. Differences in biofilm viability of *S. mutans serotype c and f* in response to treatment with garlic extracts at concentrations of 10%, 25%, 50%, and 100%.

Discussion

The samples used in this study were clinical isolates of *S. mutans* collected from the dental plaques of children aged < 6 years with early childhood caries. In accordance with the findings of previous studies, the prevalence of caries in children residing in Jakarta was 81.2%.[1] Good oral health is important in children, as *S. mutans* thrives in the oral cavity. In the oral cavity, *S. mutans* reproduce at a rate of 2–4 faster and produce more acid than under laboratory conditions.[12]

The initial procedure of studies of *S. mutans* obtained from plaques is known as the culture stage. *S. mutans* was cultivated on the selective medium TYS20B, composed of trypticase soy broth, yeast extract, sucrose, and bacitracin to prevent the growth of other bacteria, especially those resistant to antibiotics.[14]

Throughout the experiment, the biofilm was exposed to garlic extracts during the 24-h biofilm formation phase, or the maturation phase. In previous studies, the bacteria were in the stationary phase, characterized by stabilization of bacterial growth.[15] According to another study, to achieve better adhesion of the biofilm to the base of the wells incubation can be continued for more than 18 h.[12]

The results shown in Table 1 confirmed that each increase in garlic extract concentration decreased the viability of *S. mutans* serotype c. These findings were consistent with those of prior studies of the effectiveness of antibacterial agents and confirmed the relationship between the garlic extract concentrations and the effects produced. The maximum effect was achieved at the highest concentration.[15]

Table 3 shows an increase in cell viability at garlic extract concentrations of 10% and 25%. Thus, in addition to concentration relationships, there were also clinical variation factors. For example, individuals with more than one million colony-forming units of *S. mutans* in the saliva were at a greater risk of caries formation.[13]

As the test results of various garlic extract concentrations in Tables 2 and 4 show, there was a significant difference in the viability of biofilms produced by *S. mutans* serotypes c and f at garlic extract concentrations of 50% and 100%. These results imply that the effects of garlic extract were best at concentrations ≥ 50%, which is consistent with the findings of previous studies of the effects of garlic extract at concentrations of 5%, 10%, 20%, and 100%, which demonstrated the best antibacterial effect at higher concentrations.[13]

As the results in Table 2 and 4 show, there were significant differences in the viability of biofilms produced by *S. mutans* serotypes c and f in response to treatment with the garlic extracts versus the negative control, suggesting that various concentrated garlic extracts had effectively killed the bacteria. This finding was in accordance with the results of a previous study in which allicin inhibited the synthesis of both DNA and RNA, which subsequently inhibits the formation of amino acids and proteins, which results in the death of bacteria due to a lack of nutrition.[13]

As Tables 2 and 4 show, there were significant differences in the viabilities of the biofilms produced by *S. mutans* serotypes c and f in response to treatment with the garlic extracts and the positive control (0.2% chlorhexidine gluconate). In fact, the killing ability of 0.2% chlorhexidine gluconate was better than that of the garlic extracts.

According to previous studies, 0.2% chlorhexidine gluconate is effective against both Gram-positive and -negative bacteria. The chlorhexidine molecule is positively charges (cation), whereas most of bacteria have a negative charge (anion), which allows for strong attachment of chlorhexidine to the bacterial cell membrane. Chlorhexidine cause a change in the permeability of the bacterial cell membrane

resulting in the release of the cytoplasm and eventual bacterial death. However, the long-term use of mouthwash containing 0.2% chlorhexidine gluconate stains the teeth and changes the composition of the normal flora in the oral cavity.[14] Previous research has suggested that the long-term use of mouthwash containing chlorhexidine can lead to hypersensitivity and is cytotoxic to the periodontal ligament tissues.[12] Therefore, garlic extracts can be used as an alternative antibacterial oral agent against *S. mutans*.

Conclusions

A 10% garlic extract was found to decrease bacterial viability, while the decrease in the viability of *S. mutans* serotype c was significant at concentrations of 50% and 100%, while that of serotype f was significant at a concentration of 100%.

Declaration of Interest

The authors report no conflict of interest. The authors have no relevant affiliations with the subject matter or materials discussed in the manuscript, such as employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties. No writing assistance was utilized in the production of this manuscript. This study was fully supported and financed by the Directorate of Research and Community Service of Universitas Indonesia.

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