ORIGINAL ARTICLE

Inhibition Effect of Garlic (*Allium sativum*) Extract on *Streptococcus sanguinis* Biofilm Formation Involving Bacterial Motility Mechanism

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ABSTRACT

Introduction: *Streptococcus sanguinis* is a primary colonizer in oral biofilm formation, often implicated in infective endocarditis. Methods to control oral biofilm formation are yet to be developed. Garlic (*Allium sativum*) has shown antimicrobial activities against many pathogen species. We sought to observe the potential of garlic extract to inhibit bacterial adherence to hydroxyapatite (HA) discs as a model of the tooth surface. **Methods:** Susceptibility of *S. sanguinis* ATCC 10556 to garlic extract was examined by minimum inhibitory concentration (MIC) test using broth microdilution method. For bacterial adherence assay, saliva-coated HA discs were incubated with various concentrations of extract then stimulated with *S. sanguinis* ATCC 10556 suspension. Adherent bacteria were stained with 0.1% crystal violet and measured at 595 nm using a microplate reader. A qualitative method to test bacterial motility was performed using Motility Indole Ornithine (MIO) medium. **Results:** The result of minimum inhibitory concentration test showed that MIC value for garlic ethanolic extract was at a concentration of 625 µg/ml. Moreover, garlic extract inhibited bacterial adherence to HA discs starting at concentration of 62.5 µg/ml. The inhibition of bacterial motility can be observed, indicated as limited the diffused growth of bacteria closer to the inoculating line. Observation using SEM confirmed these results. **Conclusion:** This present study suggest that garlic extract has the ability to inhibit *S. sanguinis* adherence to HA discs involving inhibition of bacterial motility, with the optimal concentration being 500 µg/ml.

Keywords: *Streptococcus sanguinis*, Garlic, Biofilm, Bacterial motility

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INTRODUCTION

Streptococcus sanguinis is the earliest species among primary bacterial colonies on tooth surface. These bacteria could attach to both tooth surface and oral epithelium by their cell wall protein component, adhesin. The attachment of these bacteria to the acquired pellicle is the beginning of oral biofilm formation commonly called dental plaque (1). On the tooth surface, it will attach to the hydroxyapatite (HA) surface, the main component of tooth enamel. This attachment occurs through interaction with salivary glycoprotein in the aquired pellicle and will facilitate another Gram-positive bacteria such as *Actinomyces* to Gram-negative bacteria such as *Veillonella, Fusobacterium nucleatum* and *Porphyromonas gingivalis* (2,3). Bacterial motility plays an important role in bacterial attachment on a surface forming biofilm. The planktonic bacteria use their flagella or type IV pili (Tfp) to move toward a surface. Once the bacteria attached, replication and recruitment of another planktonic bacteria will occur, forming microcolonies and then mature biofilms. *Streptococcus sanguinis* does not have flagella. These bacteria move and attach to the surface using their Tfp (4, 5, 6).

Streptococcus sanguinis is a commensal bacteria, that often implicated in infective endocarditis. Among the Viridans group of streptococci, *S. sanguinis* is the most common cause factor of native-valve infective endocarditis. This bacterium can be found in the buccal mucosa and has the ability to aggregate thrombocytes that might predispose to infective endocarditis (7). Poor oral hygiene is a predisposing factor in bacterial endocarditis, therefore, a way to control oral biofilm is important to obtain healthy teeth and oral tissue and prevent systemic bacterial invasion. In healthy people, oral biofilm can be removed easily by brushing the teeth, flossing or using mouthwash. However, these methods are not always effective in inhibiting oral biofilm

formation, especially in the immunocompromised host like hospitalized patients, persons with weakened immune systems and diabetic (8). Therefore, exploration of the substance that can inhibit oral biofilm formation is deemed necessary.

Garlic (*Allium sativum*) is widely used as a flavoring agent. This substance is also recognized as medicinal herbs. Allicin, the water-soluble component of garlic, is known to have antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and oral bacteria such as *Streptococcus mutans, Streptococcus sobrinus* and *Actinomyces oris* (9, 10). The aim of this study was to analyze the potential of garlic extract to inhibit *S. sanguinis* biofilm formation to the HA discs as a model of the tooth surface.

MATERIALS AND METHODS

Sample preparation

Streptococcus sanguinis ATCC 10556 and saliva were prepared as our previous study (11). The bacteria were grown in Brain-Heart Infusion Broth (BHI; Difco Inc., USA) under microaerobic conditions at 37°C. A bacterial stock suspension was prepared at a concentration of $1.5x10^8$ colony forming units (CFU)/ml. Garlic was obtained and confirmed by a botanical expert at a herbal manufacturing company, CV Merapi Farma, Yogyakarta, Indonesia (ref. 20/06/MFH/2019), and was processed into an ethanolic extract using maceration technique at the Integrated Research and Testing Laboratory of Universitas Gadjah Mada Yogyakarta, Indonesia, by modification of the previous study (12). The 350 g of garlic was washed and chopped into the small pieces, then mixed with 1000 ml ethanol 96%. The mixture was stored at 4°C for 24 hours. The macerate was separated by filtration 3 times and concentrated by a vacuum rotary evaporator at 40°C to obtain an ethanolic extract of garlic. The extract in paste form was stored in a refrigerator at 4°C until used. Extract in paste form was dissolved with dimethyl sulfoxide 1% (DMSO, Merck, Germany) solution as a stock in a concentration of 5000 µg/ml for the analysis of bacterial susceptibility and a concentration of 4000 µg/ml for the bacterial adherence assay. The stock of extract was filtered using a syringe filter 0.45 µm (Sartorius, Germany) before diluted with BHI broth.

Human saliva was collected from three healthy volunteers and was prepared and sterilized by modification of the Yamaguchi method (13). Before they pooled their saliva, the volunteers rinsed their mouths with water to minimize bacterial contamination and incubated at 65°C for 30 minutes. Saliva was then centrifuged for 15 min, 3000 g, 4°C. The supernatant was stored at -80°C until used.

The HA discs (10 mm in diameter, 1.2 mm in thickness) were created by putting 500 mg hydroxyapatite powder (was made from cow bone, obtained from Mechanical Engineering Faculty, Universitas Gadjah Mada, Yogyakarta) in a mould and pressed with 120 Mpa. Finally, the discs were sintered for two hours at 1450°C. The sterilization of the hydroxyapatite discs was done by autoclaving the discs for 15 minutes at 100°C (14). Each of HA disc was incubated in 250 µl of saliva on 24-well plate for 30 minutes at 37°C.

Analysis of bacterial susceptibility

Susceptibility of *S. sanguinis* ATCC 10556 to garlic extract was determined by minimum inhibitory concentration (MIC) test using broth microdilution method. Approximately 10 µl of 1.5×10^8 CFU/ml bacterial culture was inoculated into 100 µl of BHI broth containing a garlic ethanolic extract at a concentration ranging from 19.53 to 5000 µg/ml in 96-well culture plate (Iwaki, Japan). The experiments were performed by using 5 replicates. Ciprofloxacin (Sigma-Aldrich, USA) was used as a standard of antibacterial agent at a concentration of 5 µg/ml. The plates were incubated for 24 h at 37°C and observed for the absence of turbidity visually. The MIC of garlic extract was defined as the lowest concentration showing no turbidity (15, 16).

Bacterial adherence assay

Bacterial adherence assay was performed as in the previous study (11). Saliva-coated HA discs in 500 µl Brain-Heart Infusion Broth (BHI; Difco Inc., USA) were incubated with 100 µl garlic ethanolic extract at a concentration ranging from 62.5 to 4000 µg/ml for 30 min at 37°C in 24-well culture plate (Iwaki, Japan). The culture was stimulated with 100 µl of 1.5×10^8 colony forming unit per ml (CFU/ml) *S. sanguinis* suspension and incubated at 37°C for 24 hours. After incubation, the HA discs were rinsed with 300 µl phosphate buffer saline (PBS, Sigma-Aldrich, Germany, pH 7.4) solution and fixed with 250 µl absolute methanol for 15 min. Adherent bacteria were stained with 0.1% (wt/vol) crystal violet for 10 min at room temperature and rinsed with PBS twice. Stained adherent bacteria were extracted from the discs using 96% ethanol and transferred to a fresh 96-well plate. The absorbance of stained adherent bacteria was measured at 595 nm using a microplate reader (Thermo Scientific, USA).

Motility assay

For motility assay, Motility Indole Ornithin (MIO, Laboratorios Conda, Spain) medium was used. One hundred µl of $1.5x10^8$ CFU/ml *S. sanguinis* suspension was incubated with various concentrations of garlic ethanolic extract in 1000 µl Brain-Heart Infusion Broth (BHI; Difco Inc., USA) for 24 hours at 37°C. After incubated, each culture was inoculated using a wire with stab motion to the bottom of MIO medium, then further incubated for 24 hours at 37°C (according to the MIO medium protocol). In MIO medium, bacterial motility was indicated as diffused growth or turbidity extending away from the inoculating line, while non

motile organisms grow along the inoculating line.

Scanning Electron Microscopy (SEM)

Observation with scanning electron microscope (SEM) was done to provide a general overview of the bacterial adherence to the HA disks. Saliva coated HA discs in 500 µl BHI Broth (Difco Inc., USA) were incubated with 100 µl garlic ethanolic extract at a concentration of 0 and 500 µg/ml for 30 min at 37°C in 24-well culture plate (Iwaki, Japan). The culture was inoculated with 100 µl of 1.5 x 10^8 colony forming unit per ml (CFU/ml) *S. sanguinis* suspension and incubated at 37°C for 24 hours. Thereafter, the discs were rinsed twice with sterile PBS and placed in a primary fixative solution (glutaraldehyde 0.15 M 2.5% [vol/vol] in PBS) for 12 h at 4°C. The discs were rinsed with sterile PBS, then treated with the secondary fixative (osmium tetroxide [OsO4 1% w/v]) for 1 hr. The discs were subsequently rinsed with distilled water, dehydrated in an ethanol series (70% for 10 min, 95% for 10 min, and 100% for 20 min) and air dried overnight in a desiccator. The discs were coated twice with platinum vanadium using a sputter ion (Bal-Tec SCD 005; BAL-TEC, Balzers, Liechtenstein), followed by bonding to carbon double-side tape for examination by SEM (Hitachi SU3500, Japan) (17).

Statistical analysis

Bacterial adherence as represented by the OD was analyzed using a One-way ANOVA at a significance level of 0.05. OD values were presented as mean + SD. Bacterial susceptibility, motility, and SEM were determined with visual observation. Experiments were performed in triplicates unless stated otherwise.

RESULTS

Analysis of bacterial susceptibility

The result of minimum inhibitory concentration (MIC) test showed that MIC value for garlic ethanolic extract was at a concentration of 625 µg/ml. It was indicated by no turbidity in the BHI medium like it was at a ciprofloxacin group as a standard of antibacterial agent (Fig. 1). The data were representative value from three times experiments.

Bacterial adherence assay

The bacterial adherence assay demonstrated that inhibition occurred at concentrations of 62.5, 125, 250, 500 and 1000 µg/ml., characterized by a decreased optical density (OD) value compared to the control group. OD value decreased proportionally with increasing extract concentrations, but at a concentration of 2000 and 4000 µg/ml the OD values have the similar values as at a concentration of 1000 µg/ml as seen in Fig. 2. The data were confirmed from three times experiments. Then, the data was analyzed using a One-way ANOVA at a significance level of 0.05 and there was a significant difference between groups (p=0.000). The aim of this study was to inhibit *S. sanguinis* biofilm formation, so that the optimum concentration of Allium extract was at a concentration of 500 µg/ml.

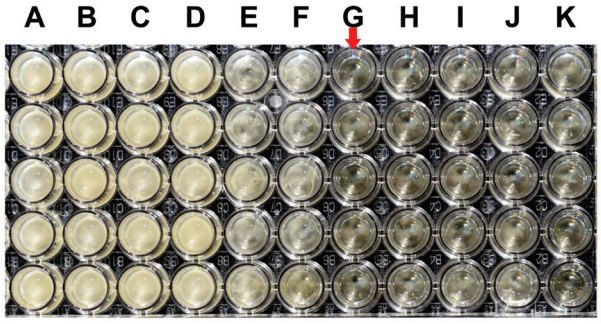

Figure 1: The bacterial susceptibility. No treatment (A), at a concentration ranging from 19.53 µg/ml (B) to 5000 (J) µg/ml. The MIC was at a concentration of 625 µg/ml (G, arrow). Ciprofloxacin was used as a standard of antibacterial agent (K).

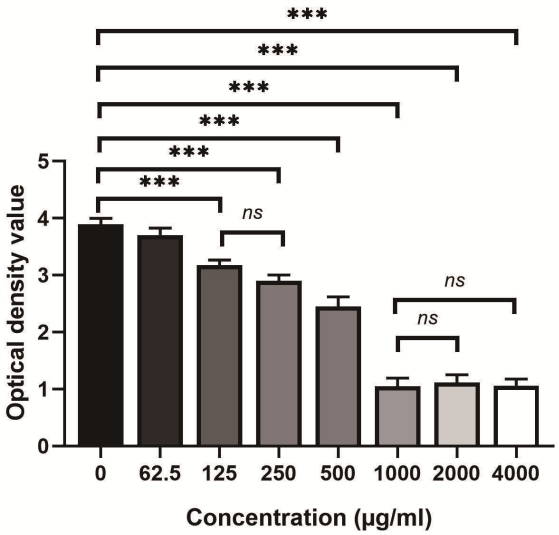

Figure 2: Optical density values of S. sanguinis adherence at various concentrations. (ns: not significant; *: p<0.05; **p<0.01; ***p<0.001)

Motility assay

The inhibition of bacterial motility was started to be observed at the treatment of 62.5 µg/ml extract. The motility inhibitions were greater as the extract concentration increased, indicated by the diffused growth of bacteria closer to the inoculating line. At concentration of 500 µg/ml, there was no bacterial motility observed, seen as bacteria growing along the inoculating line. These bacteria also ferment dextrose to form acid, causing bromocresol purple (the pH indicator in this medium) to change from purple to yellow (Fig. 3).

Scanning Electron Microscopy (SEM)

Observation using SEM confirmed these results as representative of experimental groups. Bacterial cells can be observed either on untreated or extract-treated disks. As presented at Fig. 4, the number of *S. sanguinis*

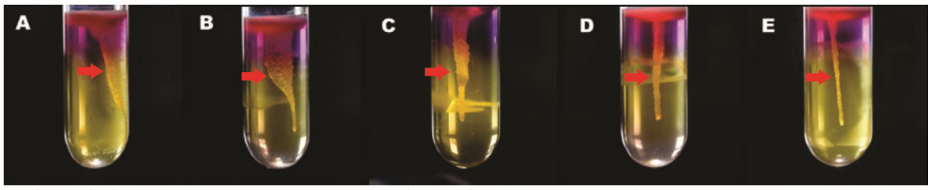

Figure 3: The inhibition of S. sanguinis-treated motility in MIO medium. The bacterial motility (arrow) in a no treatment (A), a treated with 62.5 µg/ml (B), 125 µg/ml (C), 250 µg/ml (D) and 500 µg/ml (E) of Allium extract.

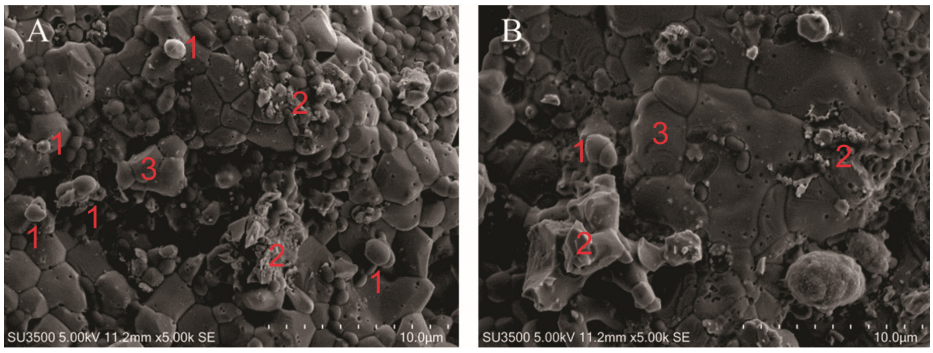

Figure 4: Scanning electron microscopy of HA surface. The number of S. sanguinis colonies (1) were higher in the untreated disk (A) compare to that of the 500 µg/ml extract treated disk (B). The extracellular matrix (2) of the S. sanguinis biofilm also detected in a higher intensity on the HA crystal (3) of the untreated disk (magnification 5,000X)

colonies were higher in the untreated disk compare to that of the 500 µg/ml extract treated disk. The extracellular matrix of *S. sanguinis* biofilm also detected in higher intensity on the untreated group.

DISCUSSION

The bacterial adherence assay demonstrated that garlic extract inhibits biofilm formation by *S. sanguinis* in a concentration-dependent manner. This result is similar to a study conducted by Wu and colleagues in biofilm formation by S. epidermidis using both water and ethanol extracts of allicin and garlic (18). The concentration of garlic extract in this study was higher than they were in the previous study. It is expected that the number of bacteria in this study is higher than it was in the previous study. The inhibition of biofilm formation has still occurred at the treatment of 2000 and 4000 µg/ml, but the OD values at these concentrations had the similar value as at concentration of 1000 µg/ml. This phenomenon might possible because extract concentrations above 1000 µg/ml were in saturation, so that no further adherence inhibition occurred. Analysis of bacterial susceptibility demonstrated that the MIC of garlic ethanolic extract was at a concentration of 625 µg/ml. This result is similar to a study conducted by Bachrach and colleagues which reported that allicin inhibited the growth of Gram-positive oral bacteria such as S. mutans, S. sobrinus and Actinomyces at a concentration of 600 µg/ml or higher (10). Therefore, in this study the optimum concentration of Allium extract to inhibit *S. sanguinis* motility was at a concentration of 500 µg/ml.

Generally, there are three types of bacterial motility: swarming, swimming and twitching motility. In swimming and twitching motility, the bacteria move freely, while for swarming motility flagella is required (19). *Streptococcus sanguinis* does not have flagella, so that there is no swarming motility facilitated in this species. Swimming motility can be detected when bacteria immersed in the semi-solid medium. In this study, swimming motility of these bacteria were detected in semi-solid MIO medium. *Streptococcus sanguinis* produces Tfp to generate force and promote intense surface-associated motility called twitching motility. The Tfp direction of motion is parallel to the long axis of chains of cells, forming a spreading zone around bacterial growth (20). Similar to the flagella in Gram-negative bacteria, Tfp consists of pilins and is synthesized by a machinery consisting of 15 conserved proteins (21. 22). Thus, in this study it was estimated that there was inhibition of twitching motility as well as swimming motility.

The antimicrobial activity of Allium is dominantly contributed by allicin. Other than allicin, several peptides, including flavonoids, alkaloids and saponin also have minor contributions (23). Allicin is not readily present in fresh garlic, but it enzymatically formed from its alliin by alliinase upon damage to the Allium tissue, such as by chopping or chewing. Different extraction processes of Allium resulted in different antimicrobial compounds. Thiosulfinates including allicin are present in freshly crushed Allium preparation. Upon maceration in oil, ajoene is occurred, while upon heating at 121°C, heterocyclic compounds and allyl alcohol is present (24). Thiosulfinates have the properties to inhibit microorganism due to their -S(O)-S- components that will react with the sulfhydryl (SH) components of

microorganism cell wall protein formed mixed disulfides (25). Our study similarly demonstrated an inhibition of *S. sanguinis*-treated motility in a concentration-dependent manner. It is estimated that allicin-S(O)-S- components in garlic extract reacted with sulfhydryl (SH) components of Tfp resulting in motility inhibition. Twitching motility is important for bacterial colonization because it increases the colonization area (26). Therefore, inhibition of twitching motility is important for inhibition of biofilm formation.

Streptococcus sanguinis is a facultative anaerobe bacterium, but in some cases it can be found in periodontal lesions and deep abscess. There is also a diversity of pilus expression among *S. sanguinis* strain (27, 28). It would be interesting to investigate the potential of garlic extract to inhibit *S. sanguinis* biofilm formation among clinical isolate of *S. sanguinis* strain from patients with various diseases such as periodontitis and deep abscess for future study.

CONCLUSION

In summary, this present study showed that garlic extract inhibited *S. sanguinis* biofilm formation on HA discs as a model of the tooth surface by interfering with bacterial motility mechanism. The observed optimal concentration of Allium extract was 500 µg/ml. Thus, we proposed that garlic extract may serve as a potential natural product for oral care medicaments to control dental plaque formation. Future studies may be directed to develop a standardized and safe natural herbal medicine product that may be readily available and applicable for daily use.

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