Original Article

Effect of garlic on bacterial biofilm formation on orthodontic wire

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ABSTRACT

Objective: To examine the effect of garlic extract on the biofilm formation by *Streptococcus mutans* on orthodontic wire and on glucosyltransferase gene expression.

Materials and Methods: Growth inhibition of oral bacteria was tested after 50 μL of garlic extract was placed on an agar plate. The minimum inhibitory concentration (MIC) of garlic extract on *S mutans* growth was first determined. After cultivating streptococci in biofilm medium (BM)-sucrose with garlic extract and orthodontic wire, adenosine triphosphate (ATP) measurement and viable cell counting was performed from the bacteria attached on the wire. Scanning electron microscopy (SEM) analysis of morphology was observed on bacterial cells attached to orthodontic wire. The effect of garlic extract on gene expression was evaluated using quantitative real-time polymerase chain reaction (PCR) of glucosyltransferase.

Results: Though garlic extract had a clear antibacterial effect on all microorganisms, it also enhanced *S mutans* attachment on orthodontic wire. Low concentration of garlic extract also increased glucosyltransferase gene expression of *S mutans.*

Conclusions: Despite its antibacterial function, garlic extract increases biofilm formation by *S mutans* to orthodontic wire, likely through upregulation of glucosyltransferase expression. Garlic extract may thus play an important role in increased bacterial attachment to orthodontic wires. (*Angle Orthod.* 2011;81:895–900.)

KEY WORDS: Orthodontic wire; Bacterial biofilm; Garlic extract; *Streptococcus mutans*; Glucosyltransferase

INTRODUCTION

Dental plaque, the biofilm that forms on the surface of teeth, can induce some of the most common diseases afflicting humankind, including caries, gingivitis, and periodontitis.[1] Bacterial adhesion to biomaterials and the ability of many microorganisms to form biofilm on foreign bodies are well-known steps in the pathogenesis of oral infections.

The insertion of orthodontic wire tends to create new surfaces available for plaque formation and therefore to increase the level of microorganisms in the oral cavity. It has long been suggested that orthodontic bands and wires lead to an increased plaque accumulation and elevated levels of streptococci and lactobacilli.[2] In addition, orthodontic patients with fixed appliances frequently present an abundance of *Streptococcus mutans* in plaque compared with untreated orthodontic patients.[3] Therefore, prevention of bacterial attachment to orthodontic wires is a critical concern for orthodontists.

Garlic (*Allium sativum*), an essential food ingredient worldwide, has long been known to have antibacterial, antifungal, and antiviral effects.[4] The main antimicrobial constituent of garlic, allicin, is generated by the enzyme alliinase when garlic is crushed.[4] Garlic extract has been shown to be an effective agent for controlling methicillin-resistant *Staphylococcus aureus*[5] and oral pathogens such as *S mutans* and *Porphyromonas gingivalis*.[6] However, the effect of garlic on dental

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biofilm formation has not been well studied. Since *S mutans* exists almost exclusively in oral biofilms and is considered the primary etiologic agent of human dental caries,[7] we evaluated the effect of garlic extract on biofilm formation by *S mutans* on orthodontic wire in vitro.

The ability of *S mutans* to induce dental caries is derived in part by its ability to synthesize water-insoluble glucans, this cariogenic property being dependent on the expression of extracellular glucosyltransferases (GTFs).[8] GTF genes are classified in terms of solubility in respect to *S mutans*: insoluble glucan synthesis (gtfB), insoluble/soluble glucan synthesis (gtfC), and soluble glucan (gtfD).[9] Mutations of these GTF family genes reduce the incidence of dental caries in rats, indicating that all three types of GTFs in *S mutans* are responsible for the pathogenesis of dental caries.[8] In the present study, adenosine triphosphate (ATP) assay, viable cell counting, and scanning electron microscopic (SEM) analysis were performed on in vitro growth to investigate the effect of garlic on microbial attachment to orthodontic wire. In addition, quantitative real-time polymerase chain reaction (PCR) for GTF gene expression was carried out following garlic treatment.

MATERIALS AND METHODS

Bacterial Growth and Garlic Extract

Organisms were maintained on brain heart infusion (BHI) agar medium and grown under aerobic conditions. The biofilm assay was performed in biofilm medium (BM) containing 3% sucrose.[10] Fresh garlic (24.19 g) was blended in a sterilized mortar and pressed with gauze. This extract was centrifuged at 12,000 rpm for 10 minutes and filtered with a 0.45-μm filter to get 8.9 g; it was then stored at −20°C until use.

Growth Inhibition by Agar Diffusion Test

Oral bacteria and a fungus tested in this study were: *Streptococcus mutans* (ATCC 25175, KCTC 3065), *Streptococcus sobrinus* (ATCC 27607), *Streptococcus sanguinis* (KCTC 3287), *Streptococcus gordonii* (KCTC 3297), *Enterococcus faecalis* (ATCC 19433, KCTC 3206), and *Candida albicans* (KCTC 7965). Bacterial species were inoculated in BHI broth and incubated for 4–6 hours, to the point when growth is considered to be in the logarithmic phase. The density of the bacterial suspension was adjusted with sterile phosphate buffer saline (PBS) to match the density of McFarland standard 0.5. The bacterial broth suspension was streaked evenly onto the BHI agar plates with a cotton swab. After the inoculum had dried, an 8-mm filter paper disk impregnated with 50 μL of garlic extract was placed onto an agar plate and incubated overnight at 37°C in aerobic condition. Diameters of inhibition zones around specimens were measured at three different points. Three specimens were tested for each variable.

Minimum Inhibitory Concentration Determination

Ninety-six well microtiter plates were used to minimum inhibitory concentration (MIC),[11] each garlic extract concentration being tested in triplicate at serial dilutions of 0, 1, 2, 4, 8, 16, 32, 64, and 128 mg/mL. Columns 1 and 2 were used for garlic extract as a negative control, and columns 11 and 12 were used for positive controls. Each well was filled with 100 μL BHI broth containing garlic extract and 100 μL inoculated broth and incubated overnight at 37°C. To establish the specific MICs, turbidimetric (A_{600}) measurements were carried out using a microplate reader (Infinite 200 NanoQuant, Tecan, Zurich, Switzerland). The mean value A_{600} 0.06 from the wells containing only culture medium was accepted as the breakpoint value denoting MIC.

Bacterial Attachment on Orthodontic Wire

To evaluate the effect of garlic extract on bacterial biofilm formation, we used sterile orthodontic wire (3M Unitek, St Paul, Minn; stainless steel, rectangular, 0.016 × 0.022 inch) for biofilm formation. Several rapid and easy methods for detecting bacteria have been developed, among these, ATP luminescence assay[12] and viable bacterial cell counting were used in this study. ATP assay is based on detection of ATP, a molecule redundant in living cells, including bacteria. *S mutans* was cultured in an Eppendorf tube containing 1.5 mL BM-sucrose broth soaked with 2-cm wire and sub-MIC garlic extract (0, 4, 8, 16 mg/mL). After 40-hour incubation at 37°C under aerobic condition, each wire was washed twice in sterile PBS (pH 7.2) and moved to another sterile Eppendorf tube. For ATP assay, 100 μL of PBS was added and then tubes were sonicated three times for 30 seconds at 30-second intervals. After pipetting this solution into 96-well white plates (Greiner Bio-One CELLSTAR plate, Kaysville, Utah), 100 μL of ATP bioluminescent assay kit solution (Sigma, St Louis, Mo) was added to each well. Luminescence was detected after 5 minutes on a microplate reader (Infinite 200 NanoQuant, Tecan). The average luminescence value of negative control wells containing PBS buffer and ATP assay solution was subtracted from each luminescence value. The relative change in luminescence was calculated as a percentage of control values not containing garlic extract.

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Table 1. Growth Inhibition Zones Against Oral Bacteria by Garlic Extract

	Enterococcus faecalis	*Streptococcus gordonii*	*Streptococcus sanguinis*	*Streptococcus mutans*	*Streptococcus sobrinus*	*Candida albicans*
Growth inhibition zone, mm	16 ± 0.9	17.5 ± 1.2	20 ± 1.7	40 ± 2.3	26 ± 1.7	55 ± 2.5

For viable cell counting, each wire incubated with streptococci and garlic extract in BM-sucrose for 40 hours was sonicated in 1 mL PBS. The PBS was serially diluted to 1/10,000 and each 100 μL was spread on BHI agar plate. After incubation for 2–3 days, bacterial colonies were counted from each plate, and the relative colony-forming units (CFUs) were calculated as a percentage of controls not containing garlic extract. All samples were processed in triple. SEM (FE-SEM, JSM-6700F, Jeol, Tokyo, Japan) analysis was performed on each wire following 10% glutaraldehyde fixation with air drying.

Quantitative Real-time PCR

In order to verify the changes in GTF expression levels under the effect of garlic, real-time PCR was performed. Total RNA was extracted from the cultured *S mutans* treated with garlic extract (control, 8 mg/mL, and 16 mg/mL) using QIAzol solution (QIAGEN, Valencia, Calif). Isolated RNA (1 μg each) was reverse transcribed using the SuperScript synthesis system in the presence of random primers (Invitrogen, Carlsbad, Calif). The resultant cDNA was amplified on a real-time PCR machine (ABI Prism 7000, Applied Biosystems, Carlsbad, Calif) using gene-specific primer pairs with SYBR *Premix Ex Taq* (TaKaRa, Madison, Wis) as described by the manufacturer. The primers for 16S rRNA, gtfB, gtfC, and gtfD were used as described previously.[9] Expression levels were quantified using SDS 2.1 software (Applied Biosystems). The relative expression levels of GTF family genes were normalized to those of 16S rRNA in the same samples.

RESULTS

Table 1 shows the diameter of inhibition zones produced by garlic extract against oral bacteria. The inhibition zone of *S mutans*, the most important bacterium of those involved in early colonization in plaque formation was larger than those of other experimental strains (Table 1). These results show that *S mutans* was the strain most sensitive to garlic extract among the test strains except only C albicans.

The MIC of garlic extract against oral bacteria was determined using microtiter plates. Garlic extract showed the lowest MIC (16 mg/mL) against *Candida albicans*. MICs against *S mutans* and *S sobrinus* were 32 mg/mL and 64 mg/mL, respectively. The other oral bacteria were inhibited by 128 mg/mL of garlic extract (Table 2).

Measuring the effect of garlic on bacterial attachment to orthodontic wire, we interestingly found that the relative luminescence of wire-attached *S mutans* and *S sobrinus* (Figure 1a) increased continuously in a concentration-dependent manner in both bacteria, suggesting more bacterial cells attached to the orthodontic wire in the presence of garlic extract (*S mutans*, $P = .01$ for 16 mg/mL garlic extract; *S sobrinus*, $P = .006$ for 16 mg/mL garlic extract). We further investigated whether this increased luminescence might be directly caused by the garlic, but found no such remarkable effect (Figure 1b), leading us to conclude that the increased chemiluminescence from wire-attached *S mutans* or *S sobrinus* might be solely induced by each bacteria.

Table 2. Minimum Inhibitory Concentration (MIC) of Garlic Extract Against Oral Bacteria

Microorganism	MIC, mg/mL
Enterococcus faecalis	128
Streptococcus gordonii	128
Streptococcus sanguinis	128
Streptococcus mutans	32
Streptococcus sobrinus	64
Candida albicans	16

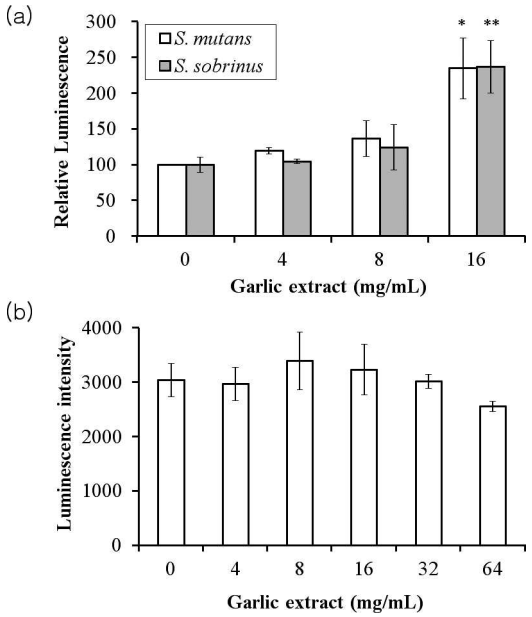

Figure 1. ATP assay to measure bacterial cell growth on orthodontic wires treated with garlic extract. ATP was measured in (a) *S mutans* and *S sobrinus* (* $P = .01$; ** $P = .006$). (b) No remarkable increase of luminescence was detected from garlic extract itself without any microbial inoculation.

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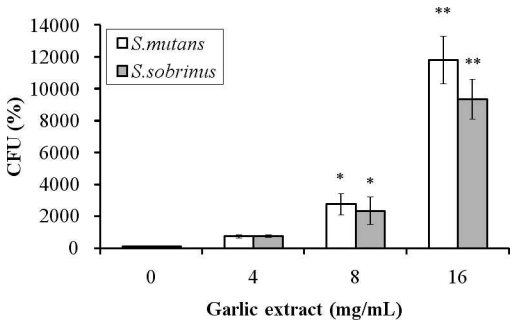

Figure 2. Viable bacterial cell counting to measure bacterial cell growth on orthodontic wire treated with garlic extract (* $P < .005$; ** $P < .001$).

Figure 2 represents the viable cell counting of *S mutans* and *S sobrinus* attached on wire. The number of CFUs increased dramatically in BM-sucrose containing garlic extract when compared with that of the control ($P < .05$ for 8 mg/mL garlic extract; $P < .01$ for 16 mg/mL garlic extract). When 16 mg/mL of garlic extract was added to BM-sucrose, the total CFUs of *S mutans* and *S sobrinus* attached on wire increased up to 110 fold and 93 fold, respectively.

Figure 3 shows SEM images of *S mutans* on orthodontic wire. Compared with negative control of garlic extract, bacterial attachment and aggregation on wire notably increased in the garlic-containing condition.

The mRNA expressions of gtfB, gtfC, and gtfD were significantly upregulated in a dose-dependent manner when the concentrations of garlic extract were lower than MIC (Figure 4). Among these genes, gtfB increased more remarkably than did gtfC or gtfD when *S mutans* was cultured with 8 mg/mL and 16 mg/mL of garlic extract.

DISCUSSION

Garlic extract has a wide spectrum of antibacterial activity, affecting *Escherichia*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Klebsiella*, *Proteus*, *Clostridium*, *Mycobacterium*, and *Helicobacter* species.[13,14] Previous reports have shown a synergistic antibacterial effect

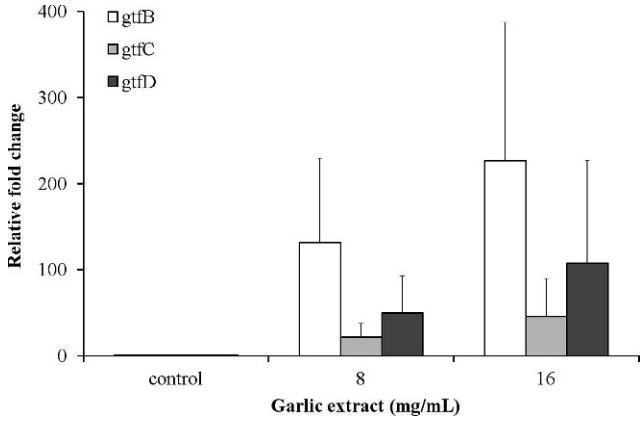

Figure 4. Quantitative real-time PCR for GTF family gene expression. The data are expressed as the means and standard deviations of three biologically independent experiments performed in duplicates.

when garlic extract and antibiotics are combined.[15] Some oral streptococci have been shown to be sensitive to garlic extract, and a mouthwash containing garlic extract effectively reduced the total salivary bacterial and mutans streptococci counts.[16] Shuford et al.[17] demonstrated that fresh garlic extract inhibited growth of *Candida albicans* in its planktonic, adherent, and sessile phases, raising the question of whether garlic has an antifungal effect on *Candida albicans* biofilm, and if so, what the underlying inhibitory mechanism is.

In this study, we tried to uncover the effect of garlic extract on dental biofilm formation using *S mutans* by analyzing attachment on orthodontic wire following garlic extract treatment. Due to garlic's known antibiotic function, we hypothesized that it would likely inhibit bacterial attachment to orthodontic wires via its antibacterial effect. Against expectation, however, garlic extract actually increased bacterial biofilm formation.

In agreement with ATP assay and viable counting of the bacterial cells on the wire, SEM image showed a clear effect of garlic extract on *S mutans* growth in terms of increased attachment. A possible explanation could be that garlic extract actually contains a biologically active substance effective at low doses

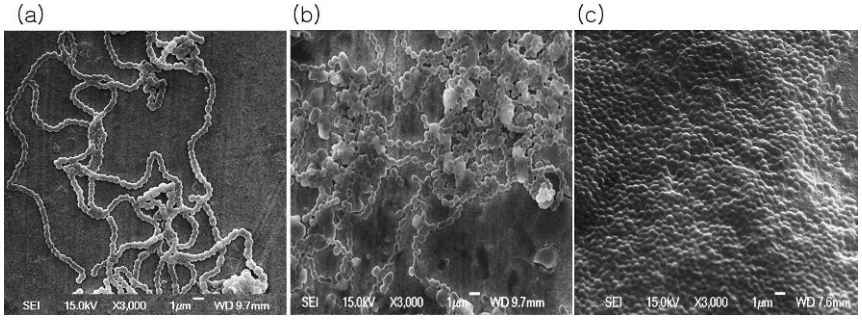

Figure 3. Scanning electron micrographs of *S mutans* on orthodontic wire surface treated with garlic extract at (a) 0 mg/mL, (b) 8 mg/mL, and (c) 16 mg/mL (original magnification 3000×).

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for gene activation prior to bacterial cell growth inhibition.

Bacterial attachment is the initial step in the formation of biofilm communities. The GTFs, in concert with glucan-binding proteins, contribute greatly to initial attachment and to the formation of biofilms.[18] A previous study showed that sub-MICs of allicin may play a role in the prevention of adherence of *Staphylococcus epidermidis* to microtiter plates.[19] *S epidermidis* biofilm formation is known to be associated with the production of the polysaccharide intercellular adhesin (PIA), poly-*N*-acetylglucosamine polysaccharide (PNAG), and recent evidence indicates that staphylococcal accessory regulator (SarA), a central regulatory element that controls the production of *S aureus* virulence factors, is essential for the synthesis of PIA/PNAG and ensuing biofilm development in this species.[20] These results suggest that the enzymes participating in bacterial biofilm formation are specific to bacteria, and that the increase of bacterial attachment by garlic extract through upregulation of GTF family genes expression is therefore very specific to *S mutans*.

Antibacterial poly(D,L-lactic acid) coating on implants showed that coating increased the total amount of *S epidermidis* attachment,[21] suggesting physiochemical characteristics, like surface charge, could influence bacterial attachment. Therefore, some components of garlic extract in our study could have induced effective bacterial biofilm formation to wire. Another possibility is that the increase in biofilm formation by garlic extract was caused by pH change of the medium. Previous research into the regulatory mechanisms of GTF family genes in *S mutans* showed that biofilm acidification or excess metabolizable carbohydrate (glucose or sucrose) can induce GTF gene expression.[22] For this reason, we measured the effect of pH changes during *S mutans* cultivation with garlic extract. However, the pattern of GTF gene expression was not significantly changed (data not shown). We thus concluded that the increase in bacterial attachment on wire due to upregulated GTF expression was induced by garlic extract itself.

Because it is not clear whether it is allicin that activates GTF genes or some other components of the garlic extract, these observations call for further investigation. The present findings may offer fresh insight into garlic-induced GTF expression in *S mutans* at the molecular level, with potential consequences for proper care of orthodontic wire.

CONCLUSIONS

- Garlic extract increases bacterial biofilm formation to orthodontic wire in a concentration-dependent manner.
- The GTF family of genes (gtfB, gtfC, and gtfD) was significantly upregulated compared to MIC at a lower concentration of garlic extract despite garlic's antibacterial effect.
- Garlic extract seems to contain biological materials that promote formation of biofilm via activation of GTFs.

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