

The effect of *Piper betle* and *Psidium guajava* extracts on the cell-surface hydrophobicity of selected early settlers of dental plaque

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Abstract: The adhesion of early settlers of dental plaque to the tooth surface has a role in the initiation of the development of dental plaque. The hydrophobic surface properties of the bacteria cell wall are indirectly responsible for the adhesion of the bacteria cell to the acquired pellicle on the tooth surfaces. In this study, the effect of aqueous extract of two plants (*Psidium guajava* and *Piper betle*) on the cell-surface hydrophobicity of early settlers of dental plaque was determined *in vitro*. Hexadecane, a hydrocarbon was used to represent the hydrophobic surface of the teeth in the oral cavity. It was found that treatment of the early plaque settlers with 1 mg/ml extract of *Psidium guajava* reduced the cell-surface hydrophobicity of *Strep. sanguinis*, *Strep. mitis* and *Actinomyces* sp. by 54.1%, 49.9% and 40.6%, respectively. Treatment of these bacteria with the same concentration of *Piper betle* however, showed a comparatively lesser effect (< 10%). It was also observed that the anti-adhesive effect of the two extracts on the binding of the early plaque settlers to hexadecane is concentration dependent. (J. Oral Sci. 48, 71-75, 2006)

Keywords: early plaque settlers; *Piper betle*; *Psidium guajava*; cell-surface hydrophobicity; hexadecane; bacterial adhesive property

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Introduction

It has been suggested that hydrophobic forces are involved in the adherence of a variety of bacteria to host tissues (1-3). In the development of early plaque, the early settlers will first adhere via the acquired pellicle to the tooth surface. In the adhesion of the early plaque bacteria to the pellicle-covered tooth surface, the hydrophobic surface properties of the bacteria cell wall are indirectly responsible in the formation of hydrophobic bonds between the bacteria cell and the acquired pellicle on the tooth surfaces (4). The hydrophobic characteristic of a bacterial surface is dependent on several factors which include the presence of appendages such as fimbriae on the external surface of the cell wall (2) and/or the presence of components such as the amphiphatic lipoglycan lipoteichoic acid (1) and/or structurally related polypeptides within the cell wall (5). For colonizing bacteria such as the early settlers of the dental plaque, much of their ability to adhere to the tooth surface has been associated with the presence of appendages on their external wall surface. These external appendages possess many hydrophobic cell-surface molecules that consist of non-polar amino acids (6,7).

The adhering ability of microorganisms to the tooth surface determines the developmental stage of dental plaque. With suitable environment and nutrients, cariogenic plaque will subsequently develop. To minimize the development of cariogenic plaque, the adhering ability of the early settlers needs to be controlled.

It has been reported that the aqueous extract of two plants, *Piper betle* and *Psidium guajava* reduced the adherence of early plaque bacteria to an experimental

pellicle (8). Modification of the interacting receptors of the salivary components in the experimental pellicle which may act as binding receptors for the early plaque settlers, *Strep. sanguinis*, *Strep. mitis* and *Actinomyces* sp., was strongly suggested to be the action mechanism of both plant extracts (9). The objective of this study was to determine whether these two plants extracts exhibit an equivalent modifying effect on the cell surface hydrophobicity of these bacteria. The cell-surface hydrophobicity of the bacteria was determined using hexadecane as a hydrocarbon that mimics the hydrophobic nature of the acquired pellicle.

Materials and Methods

Strep. sanguinis, *Strep. mitis* and *Actinomyces* sp. used in this study were isolated from early plaque sampled from the mouth. The isolated microbes were kept as pure culture stocks and stored at -70°C (Hetofrig CL410, Denmark) in 20% glycerol until further use.

Preparation of bacterial cultures for hydrophobicity studies

Individual strains *Strep. sanguinis*, *Strep. mitis* and *Actinomyces* sp. were revived from the pure culture stocks which had been kept at -70°C in 20% glycerol. Once thawed, 500 μl of the culture stock was inoculated into 50 ml-culture tubes containing 40 ml of Brain Heart Infusion (BHI) broth (Oxoid, UK). The bacterial strains were allowed to grow and propagate in an incubator (Memmert, Germany) at 37°C . Following an 18-24 h incubation period, the cells were ready to be harvested for use in the preparation of bacterial cell suspension.

Preparation of bacterial suspensions

The harvested bacteria cells were washed twice with saline before they were used to prepare a standard cell suspension with an $\text{OD}_{600\text{nm}}$ of 0.6 (equivalent to $\times 10^8$ cells/ml) (9).

Determination of cell-surface hydrophobicity

The assay on the relative cell-surface hydrophobicity of the bacterial cells was carried according to the method described by Koga et al. (10). Three ml of each of the bacterial suspension (*Strep. sanguinis*, *Strep. mitis* and *Actinomyces* sp.) was dispensed into the respective clean sterile test tubes. The optical absorbance of the bacterial suspension was measured at 550 nm wavelength using a spectrophotometer (Shimadzu UV160A, Japan). This will give the optical absorbance of the total cell suspension of each bacterium in the absence of hexadecane. Following that, 200 μl of hexadecane (SIGMA, Germany) was pipetted into each of the test tubes. The tubes were vigorously

agitated for 1 min using a vortex mixer before they were allowed to stand for at least 15 min until the immiscible phases were well separated. The lower aqueous phase of the mixture was gently aliquoted out into cuvette using a sterile Pasteur pipette and its optical absorbance (A_{u}) was measured at 550 nm. The percentage of adsorption of the bacteria cells to hexadecane (A_{b}) was calculated as the percentage reduction in optical density relative to that of the total cell suspension in the absence of hexadecane (A_{t}).

$$A_{\text{b}} = \frac{A_{\text{t}} - A_{\text{u}}}{A_{\text{t}}} \times 100$$

Preparation of *Piper betle* and *Psidium guajava* extracts

The *Piper betle* and *Psidium guajava* extracts were made into stocks of 100 mg/ml. These stocks were prepared by dispensing 200 mg of the dried pellet obtained by vacuum-dried method (8) into a sterile 2 ml-microfuge tubes containing 2 ml of sterile distilled water. The microfuge tubes were sonicated (Ultrasonic Selecta CE95) for 5-10 min to break up the pellet and later centrifuged using the microcentrifuge (Jouan A14, France) at 10,000 rpm for 10 min to obtain a clear suspension of the extract. The debris was discarded to eliminate any interference in the reading of the optical absorbance.

The effect of *Piper betle* and *Psidium guajava* aqueous extracts on the cell-surface hydrophobicity of *Strep. sanguinis*, *Strep. mitis* and *Actinomyces* sp.

The effect of *Piper betle* and *Psidium guajava* on the cell-surface hydrophobicity of the early plaque colonizers was determined by measuring the cell-surface hydrophobicity of the bacteria following a 60 sec exposure of the cells to different concentrations of the plant extracts. Three ml of bacterial cell suspension was dispensed into Tube 1 through Tube 5. An appropriate amount of plant extracts (100 mg/ml stock) was added into Tube 2 through Tube 5. Sterile distilled water was then added to each of the test tubes to give a final concentration of 0, 1, 5, 10 and 20 mg/ml in Tube 1 through Tube 5. Following this, 200 μl of hexadecane was pipetted into the respective tubes. The tubes were vigorously mixed using a vortex mixer for one min and later allowed to stand for at least 15 min until the immiscible phases got separated. The optical density of the lower aqueous phase was read at 550 nm using a spectrophotometer. Tube 1 which was devoid of the plant extract serves as the blank control and measures the actual cell-surface hydrophobicity of the bacteria cell in the absence of the extracts. The effect of the plant

extracts on the adsorption of bacteria cells to the hydrocarbon was calculated as the percentage reduction in optical density relative to those recorded of the untreated bacteria cells in Tube 1. In the study chlorhexidine-containing mouthrinse (CHX) was used as a reference, based on its widely accepted antimicrobial activities against oral microorganisms.

Statistical analysis

Statistical analysis was carried out using the one way analysis of variance (ANOVA). The MINITAB 13 for Windows statistical program was used to determine the mean, standard deviation and evaluate the significance of the data obtained in the study. Results were expressed as mean \pm standard deviation from nine determinations ($n = 9$).

Results

Cell-surface hydrophobicity of the early plaque settlers

Among the three bacteria tested, *Strep. sanguinis* was the most hydrophobic followed by *Strep. mitis* and *Actinomyces* sp. (Fig. 1). The percentage of adsorption to the hydrocarbon for *Strep. sanguinis* (at $91.0 \pm 1.0\%$) was significantly higher than *Strep. mitis* and *Actinomyces* sp. The hydrophobic binding affinity of *Strep. mitis* and *Actinomyces* sp. was comparatively lower at $61.3 \pm 2.8\%$ and $57.1 \pm 1.3\%$ ($p < 0.05$), respectively.

The effect of *Piper betle* and *Psidium guajava* on the hydrophobicity of *Strep. sanguinis*, *Strep. mitis* and *Actinomyces* sp.

It was shown that the cell-surface hydrophobicity of the early plaque settlers is strongly affected following treatment with the aqueous extract of *Psidium guajava* (Fig. 2). Exposing the bacteria to 1 mg/ml of the extract drastically reduced their cell-surface hydrophobicity to almost half of the untreated condition (54.1% , *Strep. sanguinis*; 49.9% , *Strep. mitis*; 40.6% , *Actinomyces* sp.). The reduction however was less drastic at the higher concentrations of the extracts (5, 10 and 20 mg/ml). Comparing with 1 mg/ml, the effect of 20 mg/ml extract on the reduction of the cell-surface hydrophobicity of the bacteria was found to be the least. The difference was only 10.6% for *Strep. sanguinis*, *Strep. mitis* and 14.9% *Actinomyces* sp. The extract of *Piper betle* also exhibited similar effect on the cell-surface hydrophobicity of the three early plaque settlers. However, the degree of the effect was relatively less compared to the effect of *Psidium guajava*. At 1 mg/ml, the *Piper betle* extract was found to reduce the cell-surface hydrophobicity by only 10% compared to the

untreated condition (Fig. 2). Among the three bacteria, the effect on *Actinomyces* sp. was the least with only 0.2% reduction of the cell-surface hydrophobicity was observed. With a concentration of 20 mg/ml, the effect of *Psidium guajava* extract on the reduction of cell-surface hydrophobicity of the three early plaque settlers was observed to be three times more effective compared to the *Piper betle* extract.

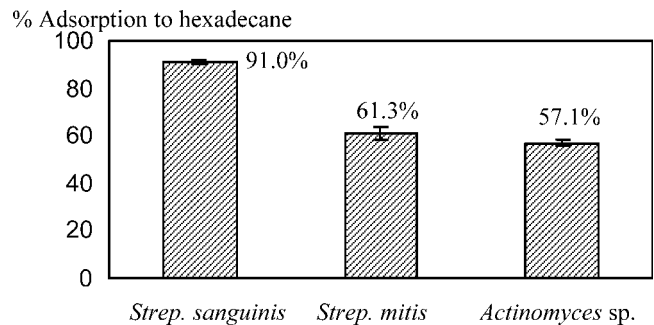


Fig. 1 The cell-surface hydrophobicity of *Strep. sanguinis*, *Strep. mitis* and *Actinomyces* sp. measured by their binding affinities to hexadecane. The values plotted were means \pm standard deviations of nine determinations ($n = 9$; $p < 0.05$).

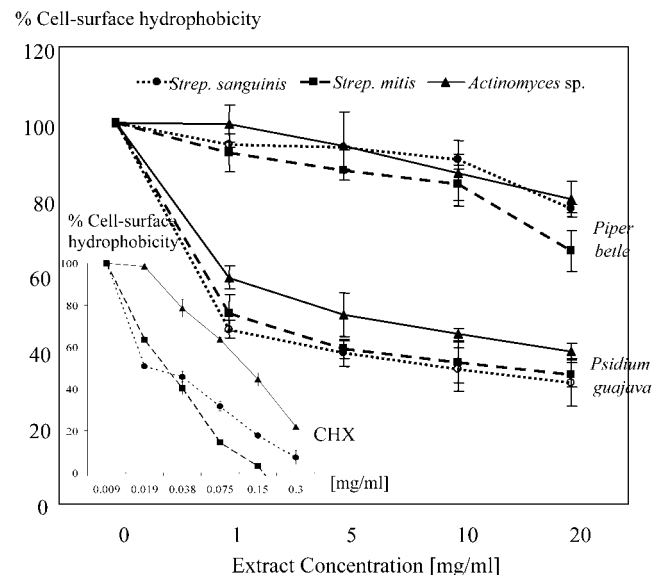


Fig. 2 The effect of *Piper betle* and *Psidium guajava* on the cell-surface hydrophobicities of *Strep. sanguinis* (●●), *Strep. mitis* (■■) and *Actinomyces* sp. (▲▲) as individual strains. Inserted at the lower left of the graph is a lower concentration scale showing reduction of the cell-surface hydrophobicities of the cells following treatment with chlorhexidine-containing mouthrinse (CHX). The percentages were means \pm standard deviations of nine determinations ($n = 9$).

The cell-surface hydrophobicities of the early plaque bacteria were significantly affected following treatment with CHX. The adsorption of *Strep. mitis* to hexadecane was totally inhibited at about 0.15 mg/ml CHX. At the same concentration, the adsorption of *Strep. sanguinis* was reduced by 80% and *Actinomyces* sp. by about 60% (Fig. 2; inserted graph).

Discussion

The adherence of the early plaque bacteria to pellicle-coated surface is assisted and stabilized by non-specific hydrophobic binding (11-13). Any disruption of these bonds may affect the binding capacity of the bacteria. If the cell wall properties are altered, the binding capacity may also be affected and diminished. Olsson et al. (14) had reported that almost no plaque had accumulated on a hydrophobic crown surface. This indicates that the adhering capacity of salivary deposits and consequently, of plaque microorganisms to the tooth surface was affected by the surface properties of the tooth.

In oral cavity, the *Strep. sanguinis*, *Strep. mitis* and *Actinomyces* sp. have a common role as early settlers of the dental plaque. However, each of these bacteria exhibited different hydrophobic characteristics, apart from the different binding affinities to experimental pellicle reported earlier (8). Among the three bacteria tested, *Strep. sanguinis* was shown to be the most hydrophobic with about 91% of its cells adsorbed to the hydrocarbon layer. The cell-surface hydrophobicities of *Strep. mitis* and *Actinomyces* sp. was lesser and about 33% to 34% less hydrophobic than *Strep. sanguinis* (Fig. 1).

The finding that *Strep. sanguinis* is the most hydrophobic among the oral streptococci in this study is in accordance with earlier reports (3,15). Being more hydrophobic, *Strep. sanguinis* adhere strongly to saliva-coated surfaces compared to *Strep. mutans* (4). It has been suggested that the hydrophobic nature of *Strep. sanguinis* is due to the presence of a cell surface protein of molecular mass 16kDa in the cell wall polypeptide (16). Other factors that may contribute to the overall-surface hydrophobicity of a bacterium are the presence of external appendages, lipoteichoic acids (LTA) and hydrophobic proteins on the cell-wall (5,15). *Strep. sanguinis*, *Strep. mitis* and *Actinomyces* sp. possess external appendages that have many hydrophobic domains consisting of non-polar amino acids with which many adhesions are associated with (17). The presence of external appendages increases the probability of hydrophobic interaction with other cells and surfaces (18) as it allows adhesion to occur at a greater distances from the negatively charged surface (19).

It was shown that the hydrophobic properties of *Strep.*

sanguinis, *Strep. mitis* and *Actinomyces* sp. that had been treated with the extracts of *Piper betle* and *Psidium guajava* diminished uniformly with increased concentrations of the extracts and hence, the effect is concentration dependent. This may imply that the extracts of *Piper betle* and *Psidium guajava* have the ability to alter the surface characteristics of the bacteria as demonstrated and thus reduce the adsorption to the hydrocarbon hexadecane. The effect of the *Psidium guajava* on the cell surface hydrophobicity of these bacteria was shown to be three times higher compared to the *Piper betle*.

Conclusion

The cell-surfaces of *Strep. sanguinis*, *Strep. mitis* and *Actinomyces* sp. were found to be very hydrophobic in nature. It was suggested that this property helps the bacteria to interact strongly with the experimental pellicle (8). This may explain why *Strep. sanguinis*, *Strep. mitis* and *Actinomyces* sp. were identified as the earliest bacteria that colonize the pellicle-covered tooth surface (20). Among the three early plaque settlers tested, the cell-surface of *Strep. sanguinis* was the most hydrophobic followed by *Strep. mitis* and *Actinomyces* sp. The extracts of *Piper betle* and *Psidium guajava* reduce significantly the cell-surface hydrophobicity of these bacteria with the *Psidium guajava* extract exhibiting greater effect. The extracts were observed to have the ability to alter and disturb the surface characteristics of the early plaque settlers and make them less adherent. This could account for the significant reduction in the hydrophobic binding capacity of the bacteria when treated with the extracts.

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