The effect of selected commercially available mouth-rinses vs. curcumin photosensitizers in an artificial mouth model mimicking their use before meals on early colonizers single species biofilm

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Abstract. – OBJECTIVE: The aim of the study was to determine the bacterial-adherence to the experimental pellicle pretreated with commercially available oral-rinse/photosensitizer (mimicking use of oral-rinse/photosensitizer before meals).

MATERIALS AND METHODS: An artificial mouth (NAM) system was used for the development of single-species biofilm (Actinomyces viscosus, Streptococcus mitis and Streptococcus sanguinis respectively). Two commercially available oral-rinses containing active ingredients (Essential oils (EO) and Chlorhexidine gluconate (CHX) were used. Curcumin photosensitizer (PS) was used as a photosensitizer against the microbes. For the adherence study, the experimental pellicle on the beads (in the capillary tubes of the NAM system) was pretreated with the oral-rinse and photosensitizer before the inoculation of bacteria; this would resemble the use of oral-rinse/photosensitizer before meals.

RESULTS: It was observed that on treatment with the oral-rinses the bacterial population of S.mitis, S.sanguinis and A.viscosus (adherence) was significantly reduced where the reduction was less for EO-based oral-rinse compared with that of CHX and curcumin PS in the following sequence EO<CHX<PS (p<0.05).

CONCLUSIONS: From the results, it appears that curcumin photosensitizer and oral-rinses contain CHX to be preferably used before-meal and EO after-meal.

Key Words: Bacterial-adherence, Essential oils, Chlorhexidine gluconate, Curcumin photosensitizer.

Introduction

Oral cavity, like other locales of human body, encloses the distinguishing micro-florae which are distinctively distributed in various sites and surfaces of the mouth. For instance, gram-positive streptococci (facultative anaerobes) are among the major component of the flora, residing on the occlusal surfaces of teeth and are predisposed by the saliva. The biological environment is defined by
the metabolism and composition of its microbial populaces which are interactive, metabolically, and structurally well-organized. As a reaction to any change in the oral environment, a momentous relocation will occur in the structure and metabolism among its microflora which live in a dynamic equilibrium with their habitation.

Whole saliva is a clear complex bio-fluid which surrounds the oral tissues and contains a mixture of glandular and non-glandular secretions. Glandular secretions are secreted from salivary glands. Non-glandular secretions include crevicular fluids, which contain oral microorganisms and host cells. Saliva does the conditioning of the oral cavity. It contains proteins such as glycoproteins, phosphor-protein, proline-rich proteins, histidine-rich protein, α-amylase; glucosyltransferases (derived from bacteria). Bacteria adhere to the tooth by adhesion receptor and charge interactions among other mechanisms.

Classically, in the formation of oral biofilm, which is also known as dental plaque, the pioneer bacteria will first adhere to the tooth surface in a reversible manner. This is then followed by an irreversible interaction forming a substratum for the adhesion of secondary bacteria.

In the oral cavity, Streptococcus species like Streptococcus mitis, Streptococcus sanguinis and Actinomyces species like Actinomyces viscosus are among the primary/early colonizers of oral biofilm/dental plaque. They have a primary attachment role by binding to the acquired pellicle on the tooth surface. The primary bacteria further provide the substratum for the attachment of secondary colonizers.

The antimicrobial effect of oral rinse/photosensitizer can be achieved better without compromising the normal oral flora if used at the right time and in the right concentration. Two potential times for oral rinse application were suggested, that is before and after biofilm formation. Before the biofilm started to form, this is related to immediately after tooth brushing. The use of oral rinse/photosensitizer after brushing could act as a protective layer to minimize the adherence of bacteria to the salivary pellicle, a stage considered to have a leading role in biofilm formation. When used after biofilm formation such as after meal, the oral rinse may affect the bacterial population in the plaque by modifying the organization of plaque matrix that may favor bacterial detachment.

The active ingredient(s) in various commercially available oral rinses such as those that contain essential oils (EOs) and Chlorhexidine gluconate (CHX) is/are in concentration that have potential to kill bacteria as claimed by the manufacturers (Colgate, and Listerine). The amount of the respective antimicrobial agent/active ingredient incorporated in the commercially available oral rinses is stated but there is no mention of when to use the oral rinses, (before or after meals). Similarly, the use of laser diodes and photodynamic (PD) therapy has been used for periodontal therapies, root canal disinfection and for eradication of dental caries demonstrating beneficial effects. The photosensitizers used in PD therapies are activated with laser diodes at a specific wavelength which has a potential to convert photosensitizer into a modified condition (triplet state). The activated photosensitizers form reactive oxygen species leading to lysis of bacterial cell wall.

There are many bacterial species in the oral cavity. In this study we chose three species which are among the early colonizers of dental plaque. The normal ecology of the oral cavity should be maintained and, hence, in this study, the antibacterial effect of two commercially available oral rinses (those that contain CHX and EO as active ingredients) in the control of plaque development was investigated and compared with emerging photosensitizer Curcumin. The antibacterial effect of the oral rinses and curcumin PS was investigated in vitro with respect to the bacterial adherence toward oral rinse- / PS -treated experimental pellicle.

Materials and Methods

Preparation of Brain Heart Infusion Broth (BHI Broth)

Brain heart infusion (BHI) broth was used to culture the bacteria (Streptococcus mitis, Streptococcus sanguinis, Actinomyces viscosus) for this study. It was prepared according to the manufacturer’s instruction (Oxoid Ltd, Hampshire, England, United Kingdom). Sterilized BHI broth was then kept in the fridge at 4°C until further use. Prior to use it was normalized to room temperature (25°C).

Preparation of Brain Heart Infusion Agar (BHI Agar)

The BHI agar plates were used to grow bacteria and for the enumeration of the colony forming units (CFU). The agar plates were then stored in the fridge at 4°C until further use. Prior to use

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the plates were normalized to room temperature (25°C).

**Preparation of the Stock Culture of Bacteria**

The respective oral bacterial species (*Streptococcus sanguinis* BAA-1455, *Streptococcus mitis* ATCC 49456 and *Actinomyces viscosus* ATCC 43146) were obtained from the Faculty Dentistry, University of Malaya, Kuala Lumpur, Malaysia. The stock culture of the respective bacteria was prepared according to the following procedure. The sterilized BHI broth (1:100v/v) was first inoculated with the bacteria and then placed in an incubator at 37°C for 18-24 hrs (*Streptococcus mitis* and *Streptococcus sanguinis*) and 24-36 hrs (*Actinomyces viscosus*). Three hundred microliters (µl) of the growth suspension were mixed with two hundred microliters (µl) of glycerol were used as the stock culture and stored in 1 ml Eppendorf tubes at -80°C until further use.

**Preparation of Phosphate Buffered Saline (PBS) Solution**

PBS was prepared according to the manufacturer instructions (Oxoid Ltd, Hampshire, England, United Kingdom). The sterilized PBS solution was stored in the fridge at 4°C until further use. The PBS solution was used as a medium for bacterial serial dilution for bacterial population determination.

**Preparation of Sterile Saliva**

Ethical approval for saliva collection was obtained from the Ethics Committee, Faculty of Dentistry, University Malaya, Kuala Lumpur, Malaysia and Riyadh Elm University, Riyadh, Saudi Arabia. Sterile saliva was prepared according to the method described by De Jong and Van Der Hoeven. About 40 ml of stimulated whole saliva (SWS) was collected for single experiment. Single volunteer T.Z was asked to chew a sugar free gum to stimulate saliva production. The SWS was collected in an ice-chilled test tube. The saliva was collected from a single healthy/medically fit female volunteer (27 years old) to minimize any variation that may arise between individuals. The aggregation of proteins in SWS samples was minimized by adding 1,4-Dithio-D,L-Threitol (DTT) to a concentration of 2.5 mM. Following the addition of DTT, the saliva was stirred slowly for 10 min before it was centrifuged at 864 g for 30 min at 4°C. The supernatant obtained was then filter-sterilized using a disposable syringe filter of 0.2 µm pore size (Minisart, Syringe filters, Thermo Fisher Scientific, Waltham, MA, USA) into sterile test tube. The sterile SWS was kept at -20°C until further use. Prior to use, the saliva was first thawed and then centrifuged (to remove any precipitate). The sterile SWS was used to coat the glass beads. It will mimic as the layer of acquired pellicle on the tooth surface which was referred as the experimental pellicle.

**Preparation of the Bacterial Suspension**

The stock culture of the respective oral bacteria (*Streptococcus sanguinis*, *Streptococcus mitis* and *Actinomyces viscosus*) was thawed at room temperature. The microbes were inoculated into sterilized BHI broth (1:100 v/v) and incubated at 37°C for duration in accordance with the bacterial species (18-24 hrs for *Streptococcus sanguinis* and *Streptococcus mitis* and 24-36 hrs for *Actinomyces viscosus*). The bacterial suspension was adjusted spectrophotometrically at 550 nm at 0.144 absorbance value during their growth phase which is equivalent with 10⁶ cells/ml.

**Determination of the Effect of the Commercially Available Oral Rinses and Curcumin PS Towards Bacterial Adherence in Single Species Biofilms**

The effect of oral rinses was determined on the adhering bacterial population for single species biofilms. In this experiment, the saliva coated glass beads were treated with the respective concentration of oral rinses/photosensitizer prior to the development of 24 hr single species biofilms. The respective oral rinses (CHX and EO) were used, at the original commercially available concentration. The curcumin PS was photo-activated by a diode laser of 400 μm fiber optic tip for 30 s around the tube containing glass beads. The diode laser with a radiation intensity of 1200 mW/cm² was used for photo-activation.

The following formula was applied to calculate the adherence potential of bacteria:

\[
\% \text{ of bacterial adherence} = \frac{\text{AOR}}{\text{AW}} \times 100
\]

where AOR is the mean of bacteria (CFU count) treated with respective oral rinse, Aw is the mean of bacteria (CFU count) treated with deionized distilled water (negative control).

The experiment was carried out using four artificial mouth models (NAM system) running...
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simultaneously, each connected to a peristaltic pump. Sterile saliva was pumped separately into the glass capillary tubes of each of the NAM system at the 0.3 ml/ min for 2 min. This would allow the saliva to coat the glass beads and created the experimental pellicle. This was followed by allowing pumping of oral rinses/ PS to flow for 2 mins in different NAM system a) sterile deionized distilled water; b) oral rinse containing EO; c) curcumin PS followed by photo-activation using diode laser and d) oral rinse containing CHX. Later deionized distilled water was pumped to remove any excess oral rinses/ photosensitizer. Subsequently bacterial suspension of A. viscosus which was standardized first to the absorbance of 0.144 at 550 nm and representing 1x10^6 bacteria population was pumped through the capillary tubes of the respective NAM systems at the same flow rate (0.3 ml/min) for 24 hr at 37°C. After 24 hr, three glass beads (one near the inlet, one at the middle and one near the outlet) were taken out of the capillary tube with care and used for the determination of bacterial population. The remaining four glass beads were stored in 4% glutaraldehyde (Sigma Aldrich, St. Louis, MO, USA) for SEM viewing. Each of the selected beads (inlet, middle and outlet beads respectively) was immersed separately in a micro-centrifuge tube containing 1000 µl phosphate-buffered solution (PBS). This was followed by sonication in an ultrasonicator for 10 s and vortexed for 1 min in order to detach the adhered bacteria into the PBS solution. The PBS solution was then diluted serially up to T6 where T1 (101) was referred to the first tube and T6 (106) the sixth tube of the serial dilutions. One hundred µl of the respective serially diluted bacterial suspensions from the T1 to T6 tubes were pipetted out and streaked onto separate BHI agar plates. The procedure was repeated thrice for each dilution.

Culture plates were incubated for 24 hr at 37°C followed by Colony Forming Unit (CFU) determination. Culture plates of dilution factor which had the CFU number 30-300 were selected and the bacterial population was expressed as Colony forming unit/ml (CFU/ ml). CFU of the bacterial population on every petri dish was determined using a Colony Counter (Rocker, Colony Counter Galaxy 230, New Taipei City, Taiwan). Each individual experiment was carried out in triplicate. The CFU count of the bacterial population on the glass bead located at different positions (inlet, middle and outlet) was determined and analyzed for the percentage difference in the average bacterial population between the different positions.

The above procedure was repeated with other bacteria which in this case were S. mitis and S. sanguinis, respectively. Each of the individual experiment was carried out three times.

**Preparation of Samples for SEM Viewing**

The preparation of glass beads samples for SEM viewing was carried out according to the method described by Lagace et al.19

**Statistical Analysis**

The data obtained from validation of bacterial population on the selected beads and the data obtained from the treated and control group were analyzed and compared by One-way ANOVA and Bonferroni test in post hoc using IBM SPSS statistics software version 22 (SPSS Inc., Armonk, NY, USA). The level of statistical significance was set at $p \leq 0.05$.

**Results**

**Growth Curve of Bacterial Species**

Figure 1 shows the graphic representation of the growth curve for three bacterial species with absorbance value vs. time. For A. viscosus before the growth phase it was the lag phase which remained till the end of the 13th hour. It was followed by the

![Figure 1. Growth curve of A. viscosus ATCC 43146, S. mitis ATCC 49456 and S. sanguinis BAA-1455. The growth of A. viscosus, S. mitis and S. sanguinis was measured in absorbance at 550 nm. The experiments were carried out in triplicate and the growth curve was plotted based on the average of (3 absorbance values) vs. time.](image)
exponential growth phase (log phase) which started at about 14th hour after incubation and remained until 24th hour of incubation. After the log phase it entered the stationary phase showing a consistent value till the 38th hour of incubation. Whereas for S. mitis (Figure 1) it was shown that the exponential growth/ log phase of this bacterium is between 7th hour to about 15th hours of incubation. The lag phase which is the earliest phase of growth was before the 6th hour of incubation. Bacteria continued to multiply till the 15th hour of incubation after that the growth became static (Stationary phase) which remained till the 24th hour of incubation. For S. sanguinis (Figure 1) the log/exponential growth phase started at the 6th hour and continued until the 14th hour of incubation. The lag phase continued until the 15th hour of incubation. The stationary phase began at the 15th hour and remained static up to the 24th hour of incubation.

**Effect of the Commercially Available Oral Rinses Towards the Bacterial Adherence in Single Species Biofilms**

Figure 2A shows the percentage of adherence of A. viscosus towards the experimental pellicle pre-treated with the 100% concentration of two respective oral rinses available commercially and curcumin photosensitizer. The percentage of adherence was observed to be in the following decreasing sequence: EO > CHX > PS. On statistical analysis using a One-way ANOVA, Bonferroni post-hoc test, significant difference was observed between the negative control and the respective treatment groups (p ≤ 0.05). On analyzing the difference in the adherence between the different treatment groups, statistically significant difference was observed between all the three treatment groups (EO, PS and CHX) (p ≤ 0.05).

![Figure 2A](image1.png)

**Figure 2.** A, Percentage of A. viscosus adherence expressed in Mean ± SD towards the experimental pellicle pre-treated with the respective oral rinse (100% concentration)/photosensitizer. The active ingredients were: EO (Essential oils), PS (Curcumin) and CHX (Chlorhexidine gluconate). B, Percentage of S. mitis adherence expressed in Mean ± SD towards the experimental pellicle pre-treated with the respective oral rinses/photosensitizer. C, Percentage of S. sanguinis adherence expressed in Mean ± SD towards the experimental pellicle pre-treated with the respective oral rinses/photosensitizer. Symbols “*” represents statistically significant difference observed between the negative control and the treatment groups (p < 0.05); “a” represents statistical difference between EO- and CPC-treatment groups (p < 0.05); “b” represents significant difference between EO- and CHX-treatment groups (p < 0.05); “c” represents significant difference between CPC- and CHX-treatment groups (p < 0.05). The data were analyzed using One-way ANOVA, Bonferroni post-hoc test.
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Figure 2B shows the percentage of S. mitis adherence on the pre-treated experimental pellicle. The sequence of bacterial adherence was observed to be similar to that of A. viscosus: EO > CHX > PS. On statistical analysis using a One-way ANOVA, Bonferroni post-hoc test, significant difference in bacterial adherence was observed between the negative control (ddH2O) and the treatment groups (p ≤ 0.05). The analysis between the treatment groups showed a statistically significant difference in the bacterial adherence to the pre-treated experimental pellicle with EO compared with that of the other two respective treatment groups (PS and CHX) (p ≤ 0.05). The percentage of adherence potential of S. sanguinis towards the experimental pellicle pre-treated with the respective oral rinses at their commercially available concentration was determined (Figure 2C). The bacterial adherence to the experimental pellicle pre-treated with EO showed the maximum adherence of about 19.24%. A reduction in the adherence was observed for experimental pellicle pre-treated with CHX. A further reduction was demonstrated when curcumin PS was used to pre-treat the experimental pellicle. On performing a statistical analysis using One-way ANOVA, Bonferroni post-hoc test, a significant difference was observed in the adherence of S. sanguinis between the negative control (ddH2O) and the three respective treatment groups (p ≤ 0.05). A statistically significant difference was also observed between EO and other two treatment groups (p ≤ 0.05), similarly the difference between PS- and CHX-treatment groups was also statistically significant (p ≤ 0.05).

On micrographs of SEM, it was observed that the bacterial adherence to the experimental pellicle pre-treated with the respective oral rinse/PS was less as compared to the negative control (Figure 3). Micrographs of all the single species in 24 hr biofilms showed high numbers of bacterial cells present on the glass beads (experimental pellicle) with negative control (ddH2O). Compared to the negative control less adherence of bacteria was allowed by EO treated experimental pellicle. After EO, CHX showed to affect the bacterial adherence and allowed lesser bacteria to adhere. Curcumin PS treatment showed to allow the least adherence of bacteria to the experimental pellicle.

The characteristics of bacterial cells adhering to the saliva-coated glass beads pre-treated with the respective oral rinse observed under SEM in single-species biofilms and presented in Figure 3.

Discussion

The early plaque colonizers play an important role in the development of dental plaque. Therefore, if the bacterial load of dental plaque is not removed in early stage it can lead to various oral diseases. Even on brushing there are various sites of the tooth where bristles of toothbrushes cannot reach to clean (interproximal surfaces, pits and fissures). If the bacterial load of the biofilm on such sites is not reduced, it will undergo maturation resulting in pathogenic bacterial complexes. These complexes will further lead to dental caries, gingivitis and periodontitis20-22. Thus, in addition to mechanical, chemical (oral rinses) cleaning of the oral cavity is also required. Moreover, the PS has emerged having a potent effect in reducing the microbial load when used for various dental therapies23-24. At present there is an increasing interest to investigate the effect of commercially available oral rinses vs. curcumin PS on the microbial residents of the oral cavity. Many of the investigations have been focused on the ability of the active ingredients to either promote the growth of beneficial organisms or inhibit the growth and metabolism of oral bacteria associated with certain diseases. Oral rinses with active ingredients CHX and EO were selected for the study being most commonly used as they are regarded efficient products to reduce the dental plaque load25.

Growth Curve Study

It was necessary to determine the growth phases of bacterial species prior to their use in the study as the bacterial cell size varies at different stages of growth; the bacterial cell is largest during the exponential growth phase whereas, during the lag and the death phase, the bacterial cells are smaller in size. The bacterium is required to be in the exponential growth phase as it has a potential to perform normal metabolic function to grow and flourish. Secondly during growth phase, the bacteria secrete a large amount of extracellular polysaccharides
(water-insoluble) which contribute to the plaque matrix and consolidates attachment of bacterial cells\textsuperscript{20}.

To form the oral biofilm of early plaque colonizers it was necessary to determine the dynamics of the bacterial growth as the time of onset of growth phase varies for the respective bacterial species. The growth phase determined for \textit{A. viscosus} was between 12\textsuperscript{th} -21\textsuperscript{st} hour whereas for \textit{S. mitis} 7\textsuperscript{th}-15\textsuperscript{th} hour and 6\textsuperscript{th}-13\textsuperscript{th} hour for \textit{S. sanguinis}. The possible reason for the variation in time is the size of the respective bacterial species. \textit{S. sanguinis} being the smallest bacterial specie was observed to show growth phase earliest when compared with the other bacterial species. It was followed by \textit{S. mitis} then \textit{A. viscosus}.

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**Figure 3.** Population and characteristics of \textit{A. viscosus}, \textit{S. mitis} and \textit{S. sanguinis} adhering to the saliva-coated glass beads pre-treated with respective commercially available oral rinses and photosensitizer observed under SEM in single-species biofilm.

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SEM micrographs (10,000×) showing population of \textit{A. viscosus} (rod), \textit{S. mitis} (c cocci) and \textit{S. sanguinis} (c cocci) in 24-hour biofilms.
Effect of Oral Rinses/Photosensitizer on Adherence of Single Species in the Biofilm

The determination of bacterial adherence referred to the use of oral rinse before meals (before biofilm formation). Therefore, the experimental pellicle was treated with respective oral rinses followed by pumping the respective bacterial species inoculum for 24-hrs to develop simulated biofilm5,26.

The adhering potential of *A. viscosus* was determined on pretreated biofilm with respective commercially available concentrations of oral rinses. The percentage adherence was found to be highest for biofilm pretreated with negative control. The percentage of adherence decreased for the biofilm pretreated with EO commercially available concentration. Decrease in percentage was further observed for *A. viscosus* pretreated biofilm with commercially available concentrations of CHX followed by PS.

Similar to *A. viscosus*, maximum *S. mitis* percentage of adherence was observed for biofilm pretreated with negative control. The percentage of adherence for *S. mitis* was observed to decrease for the biofilm pretreated with EO, followed by CHX pretreated biofilm. The percentage of adherence further decreased for the biofilm pretreated with oral rinse containing active ingredient PS. The percentage of bacterial adherence observed for *S. mitis* biofilm pretreated with EO was approximately similar to that reported by Fatin-Majdina et al. 5 (22.5%). They compared the effect of *Salvadora persica* on the adherence potential of *S. mitis* using NAM model. They used EO as a positive control and ddH2O as the negative control1. The percentage of adherence for *S. sanguinis* to pretreated biofilm was observed to be similar to that of *A. viscosus* and *S. mitis*. The maximum percentage was observed for the *S. sanguinis* biofilm pretreated with negative control followed by commercially available concentration of EO. Decrease in percentage of adherence was observed for biofilm pretreated with CHX. It was observed that the decrease in the percentage of adherence of *S. sanguinis* to the biofilm pretreated with PS is maximal. The percentage adherence for *S. sanguinis* was observed to be higher on pretreatment with EO as compared to results reported by Fatin-Majdina et al.5. There could be various reasons for the difference in the percentage of *S. sanguinis* adherence, most importantly the bacterial species used in our study were from the American Type Culture Collection (ATCC), whereas they probably used the clinical isolates of *S. sanguinis* which can likely produce the difference. Secondly, the saliva of individual varies from person to person in the amount of protein content which plays an important role for the adherence of the bacterial species to the experimental pellicle. The saliva contains salivary amylase which produces dextrin; this amylase takes part in the formation of acquired pellicle on tooth. It also binds with pili of *S. sanguinis* oral bacteria and promotes bacterial adhesion subsequently for the formation of plaque 27-29.

Conclusions

The adhering capacity of early plaque colonizers bacteria to the experimental pellicle and subsequently their population in the oral biofilm formed is competently reduced by using oral rinse/ PS in effective concentration. The Curcumin PS and CHX rinse when used at their commercially available concentration were found to be very effective formulations against the bacterial adhesion (referred to the usage before meal).

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Authors’ Contribution


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