Plaque Triclosan Concentration and Antimicrobial Efficacy of a New Calcium Carbonate Toothpaste with 0.3% Triclosan Compared to a Marketed 0.3% Triclosan Toothpaste

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Abstract

- **Objective:** To compare the delivery and retention of triclosan in dental plaque, and to compare the antibacterial efficacy of a newly developed toothpaste to a marketed calcium carbonate toothpaste.

- **Methods:** Two clinical delivery/retention studies were carried out to determine the concentration of triclosan in plaque 10 minutes, and two and four hours after brushing with a new triclosan-containing toothpaste with magnesium aluminium silicate or a marketed triclosan-containing toothpaste. Both studies had a double-blind, randomized, complete cross-over design. Supragingival plaque samples (minimum 2 µg) were taken from smooth surfaces of all teeth (1–7) in all four quadrants for the 10-minute plaque measurements and in two randomly allocated quadrants at the two- and four-hour time points. Triclosan concentration was measured by HPLC. Antibacterial efficacy was evaluated in vitro using a biofilm formation approach. Three replicate experiments were carried out to check for repeatability and consistency of the assay. Toothpaste slurries were prepared by stirring one part by weight of each toothpaste with two parts by weight of deionized water. An overnight culture suspension of *Streptococcus mutans* (ATCC 25175) was prepared and then adjusted to give a bacterial count of approximately 10^7 CFU/ml. Sterile HAP discs were used as substrate and treated with the toothpaste slurry before inoculation with the standardized culture suspension of *S. mutans*. Following incubation in brain heart infusion (BHI) broth containing 2% sucrose for four hours, standard Total Viable Count (TVC) procedures were carried out and colonies counted (log_{10} values).

- **Results:** Brushing with the new calcium carbonate/triclosan toothpaste resulted in a higher triclosan concentration in plaque after 10 minutes, and two and four hours compared to a marketed triclosan toothpaste. The increase ranged from 14% to 35% and was statistically significant (p < 0.05). The antibacterial efficacy of the new calcium carbonate/triclosan toothpaste, measured four hours after application, was greater than that of a marketed toothpaste with 0.3% triclosan. The difference was statistically significant (p < 0.05).

- **Conclusion:** The new calcium carbonate toothpaste with 0.3% triclosan and magnesium aluminium silicate demonstrated significantly greater efficacy four hours post-brushing both in terms of in vivo delivery and in vitro antibacterial action compared to a marketed calcium carbonate toothpaste with 0.3% triclosan.

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Introduction

Toothpaste formulations containing antimicrobial agents in addition to fluoride are designed to reduce the levels of plaque and its pathogenic bacteria while maintaining the healthy balance of the oral cavity.1 Triclosan,2 a polychlorophenoxyphenol, is a very important antibacterial agent, marketed for use in numerous oral care products. Triclosan has a broad spectrum of activity which affects Gram positive and Gram negative bacteria, as well as yeasts and fungi.3 Lindhe summarized that triclosan is a useful antibacterial agent to be incorporated into oral products because it has a broad spectrum of activity on oral bacteria, is compatible with the ingredients in oral products, and has a long history of safe use in consumer products.4
copolymers and other agents such as zinc citrate trihydrate in silica-based formulations. The clinical work reported here describes two independent assessments conducted to compare the activity of a new calcium carbonate toothpaste containing 0.3% triclosan and magnesium aluminium silicate (MAS) to a marketed calcium carbonate toothpaste containing 0.3% triclosan. First, the in vivo availability/retention of triclosan in plaque at 10 minutes (Study 1), and then, separately, at two and four hours post-brushing was examined (Study 2). Finally, there was the in vitro antimicrobial efficacy assessment (Study 3) using a hydroxyapatite (HAP) disc Streptococcus mutans biofilm model.

Materials and Methods

In Vivo Delivery and Retention of Triclosan (Studies 1 and 2)
The objective of this work was to compare the concentration of triclosan in plaque directly after brushing (10 minutes, Study 1), and later, with a different panel of subjects, another assessment at two and four hours after brushing (Study 2) with the new triclosan-containing toothpaste with magnesium aluminium silicate was compared to a marketed triclosan-containing toothpaste without the magnesium aluminium silicate.

The toothpaste formulations used in the two independent assessments were:

- **Test paste**: A novel calcium carbonate-containing formulation with 0.3% triclosan, magnesium aluminium silicate, and 0.76% sodium monofluorophosphate (SMFP).
- **Control paste**: A commercial calcium carbonate-containing formulation with 0.3% triclosan and SMFP, marketed in India as Colgate® Dental Cream - Strong Teeth Maximum Cavity Protection (Colgate-Palmolive [India] Ltd).
- **Between assessments**: A standard calcium carbonate fluoride toothpaste with 0.76% SMFP and no other active ingredients served as the toothpaste between treatments.

Study Designs – Studies 1 and 2
The protocols for the studies were reviewed and approved by an independent research ethics committee. The studies were carried out by Intertek Clinical Research Services, Capenhurst, UK.

The concentration of triclosan in plaque was measured directly with one panel after tooth brushing (10 minutes), and then with the second panel the concentration of triclosan in plaque was measured two and four hours after tooth brushing.

Both studies had a double-blind, randomized, complete cross-over design. In each, a minimum of 40 subjects was required to complete the study to meet the pre-set level of power and sensitivity to detect differences in the outcome measures between the test and control toothpastes. Subjects who were withdrawn from the studies were not replaced. To be included, subjects had to be in good general and dental health, have at least 20 natural teeth with at least five in three out of four quadrants. Subjects were excluded if they had untreated caries or significant periodontal disease, any systemic disease or taking medication that would interfere with the data, and sensitivity to oral health products. Subjects were not admitted to the study if they had taken antibiotics one month prior to the beginning of the study, were currently part of another oral product study, or were either knowingly pregnant or breast feeding. No smokers were admitted to the study.

Subjects did not brush their teeth for 12 hours prior to taking the first plaque sample.

**Triclosan Analysis.** Efficacy was assessed by measuring the concentration of triclosan in plaque samples directly after brushing (10 minutes, Study 1), or two and four hours after the tooth brushing sequence (Study 2). Subjects brushed occlusal surfaces with the allocated toothpaste for 60 seconds, rinsed for 30 seconds with the resultant slurry, spat out, and rinsed with tap water (5 ml for five seconds).

Supragingival plaque samples (minimum 2 µg) were taken from smooth surfaces of all teeth (1–7) in all four quadrants for the 10-minute plaque measurements, and in two randomly allocated quadrants at the two- and four-hour time points for that study assessment.

Triclosan extraction from the plaque samples and subsequent analysis followed standard operating procedures. Triclosan was extracted by adding 100 µL of ethanol to each plaque sample. Triclosan concentration was measured in the extract by HPLC (Agilent 1200 Modular HPLC System with DAD) by Intertek Pharmaceutical Services Manchester, Manchester, UK.

**Statistical Analysis.** Data (concentration of triclosan in plaque, ppm) from the 10-minute sampling study were analyzed using an ANOVA mixed model with product as fixed factor and subjects as random factor. The data from two- and four-hour study time points were analyzed using an ANOVA mixed model with timepoint, product, and timepoint*product interaction as fixed factors, and subject as random factor. Before analysis, the data were log transformed. The level of statistical significance was set at p < 0.05.

**Study 3 – In Vitro Antimicrobial Efficacy**
A biofilm formation approach was used to determine the antimicrobial activity of the toothpaste formulations. Three replicate experiments were carried out to check for repeatability and consistency of the assay.

**Toothpaste Treatment Preparation.** The toothpaste formulations used in Study 3 were:

- A novel calcium carbonate-containing formulation with 0.3% triclosan, magnesium aluminium silicate, and 0.76% sodium monofluorophosphate (SMFP).
- A commercial calcium carbonate-containing formulation with 0.3% triclosan and SMFP, marketed in India as Colgate Dental Cream - Strong Teeth with Cavity Protection (Colgate-Palmolive [India] Ltd).
- A standard calcium carbonate-containing fluoride toothpaste with 0.76% SMFP and no other active ingredients.
- Water control.

Dentifrice slurries were prepared by stirring one part by weight of each dentifrice with two parts by weight of deionized water. The dentifrice samples were stirred using a magnetic stirrer for 20 minutes or until entirely homogenous. Toothpaste slurries were centrifuged at 4,600 RPM for 20 minutes to remove all solids. The clear supernatant was then decanted ready for disc treatment.
Saliva Processing. Saliva was collected from eight to 10 subjects on the morning before brushing, with no drinking or eating. Subjects chewed unflavored chewing gum and the resulting saliva was pooled and sterilized through a process of centrifugation followed by heat shock of the supernatant and then again centrifuged. The supernatant was then transferred into sterile tubes and a sample streaked out onto agar to ensure sterility. The above stocks were stored at -20°C and the required stocks defrosted overnight at 4°C prior to use for pellicle-coating the HAP discs.

Bacterial Culture Preparation. A culture suspension of *S. mutans* (ATCC 25175) was prepared in 200 ml brain heart infusion (BHI) broth containing 2% sucrose and incubated at 37°C, 5% CO₂, for 24 hours. Post-incubation, the turbidity of the broth was adjusted to give a bacterial count of approximately 10⁷ CFU/ml ready for inoculation of the HAP discs. Culture purity was confirmed through gram staining.

Disc Treatment. Sterile HAP discs (IFGL Bio Ceramics Limited, Kolkata, West Bengal, India) were placed into 12 well plates. Two ml of the three toothpaste supernatants or 2 ml of sterile distilled water were added to the individual wells containing HAP discs and kept for a duration of three minutes. The treatments were aspirated out and the discs washed with PBS through gentle shaking for 10 seconds and aspirating the remaining PBS from the wells. The washing procedure was repeated twice.

Pellicle Coating and Inoculation. Clarified, sterile saliva was added to wells containing the treated HAP discs then incubated at 37°C, 5% CO₂, for four hours. Following incubation the excess saliva was aspirated out and each well containing a HAP disc inoculated with 1.5 ml standardized culture suspension of *S. mutans* ATCC 25175 was then incubated at 37°C, 5% CO₂, for four hours. At the end of the four-hour incubation the culture was aspirated, discarded, and the discs washed with PBS. The HAP discs were transferred using sterile tweezers into tubes containing 5 ml Butterfield PBS. The tubes were vortexed at 1,000 RPM for five minutes to remove the attached bacteria from the HAP disc. Total Viable Count (TVC) for each disc was enumerated by serially diluting the bacterial suspension, starting with 10⁰, 10¹, and 1⁰, then plating on trypticase soy agar (TSA) using a spiral platter (Interscience). A 100 µl of sample was plated for each dilution in duplicate. Plates were incubated at 37°C, 5% CO₂, for 48–72 hours in a 5% CO₂ incubator. Following the incubation period, colonies were counted and raw counts were converted into log₁₀ values to equalize variance in data.

Statistical Analysis. The mean counts for the four test regimens, including replicate plates, were calculated and the differences between the four regimens were assessed for statistical significance using the Tukey-Kramer test. The level of statistical significance was set at p < 0.05.

Results

In Vivo Delivery to and Retention of Triclosan in Plaque

Plaque Triclosan Concentration at Baseline. In both studies, the baseline samples were taken after subjects brushed with triclosan-free toothpaste for a minimum of five days. The concentration of triclosan in all baseline samples was below the lower detection limit (0.05 ppm). This means that the effective concentration of triclosan in plaque was zero at baseline.

Plaque Triclosan Concentration 10 Minutes After Brushing (Study 1). Fifty-one subjects completed the study with data for analysis. The observed mean triclosan concentrations in plaque 10 minutes after brushing with both study toothpastes are given in Table I.

<table>
<thead>
<tr>
<th>Mean Plaque Triclosan Concentration (ppm) 10 Minutes After Brushing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>New calcium carbonate/triclosan toothpaste with magnesium aluminium silicate</td>
</tr>
<tr>
<td>Marketed calcium carbonate/triclosan toothpaste</td>
</tr>
<tr>
<td><strong>Group Comparison</strong></td>
</tr>
</tbody>
</table>

The statistical analysis of the log-transformed data showed that plaque triclosan concentrations 10 minutes after brushing with the new triclosan formulation with magnesium aluminium was statistically significantly greater than after brushing with the marketed calcium carbonate/triclosan toothpaste (p < 0.05).

Plaque Triclosan Concentration Two and Four Hours After Brushing (Study 2). The study completed with 42 subjects. The observed mean triclosan concentrations in plaque two and four hours after brushing with both study pastes are given in Table II.

<table>
<thead>
<tr>
<th>Mean Plaque Triclosan Concentration (ppm) Two and Four Hours After Brushing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>New calcium carbonate/triclosan toothpaste with magnesium aluminium silicate</td>
</tr>
<tr>
<td>Marketed calcium carbonate/triclosan toothpaste</td>
</tr>
<tr>
<td><strong>Group Comparison</strong></td>
</tr>
</tbody>
</table>

The statistical analysis of the log-transformed data showed that plaque triclosan concentrations two and four hours after brushing with the new triclosan formulation with magnesium aluminium was statistically significantly greater than after brushing with the marketed calcium carbonate/triclosan toothpaste (p < 0.05).

**In Vitro Antimicrobial Efficacy (Study 3).** Table III and Figure 1 show the log₁₀ viable counts of *S. mutans* which are attached to the HAP disc after four hours of incubation post-toothpaste treatment. In this model, lower log₁₀ values indicate...
The efficacy of a new toothpaste formulation. Statistical analysis, comparing the mean counts for the four test regimens using the Tukey-Kramer test, confirmed that all four test regimens were statistically significantly different from each other (p < 0.05).

### Table III

<table>
<thead>
<tr>
<th>Log&lt;sub&gt;10&lt;/sub&gt; Viable Counts of S. mutans</th>
<th>Study 1</th>
<th>Study 2</th>
<th>Study 3</th>
<th>Statistical Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control = Water</td>
<td>4.88</td>
<td>4.35</td>
<td>4.37</td>
<td>A</td>
</tr>
<tr>
<td>Standard fluoride toothpaste</td>
<td>4.36</td>
<td>3.86</td>
<td>3.75</td>
<td>B</td>
</tr>
<tr>
<td>Marketed calcium carbonate/triclosan</td>
<td>3.73</td>
<td>3.76</td>
<td>3.10</td>
<td>C</td>
</tr>
<tr>
<td>New calcium carbonate/triclosan toothpaste with magnesium aluminium silicate</td>
<td>2.74</td>
<td>2.48</td>
<td>2.54</td>
<td>D</td>
</tr>
</tbody>
</table>

In Table III the different letters refer to significant differences between the treatments.

Figure 1. Mean (SD) log<sub>10</sub> viable counts of S. mutans.

In all three replicate experiments, the novel calcium carbonate toothpaste with 0.3% triclosan and magnesium aluminium silicate showed the lowest log<sub>10</sub> TVC values compared to the marketed calcium carbonate toothpaste with 0.3% triclosan, the standard fluoride toothpaste, and the water control. It also demonstrated that the calcium carbonate toothpaste with 0.3% triclosan and magnesium aluminium silicate has a greater antimicrobial effect in this model than the other treatments.

### Discussion

It is widely accepted that the long-term benefit of antibacterial agents and other active ingredients in toothpaste is greatly enhanced when contact time for actives can be extended significantly beyond the relatively short duration during tooth brushing, typically only between 40 seconds and two minutes. Additionally, increased benefits in terms of antibacterial efficacy will be obtained from higher concentrations of actives at the relevant oral sites. Regarding oral bacteria implicated in the mediation of oral diseases such as caries and gum problems, dental plaque is the most relevant site of action. Hence, the in vivo studies reported here were aimed at measuring the concentration and time-profile of triclosan in plaque to evaluate the efficacy of a new toothpaste formulation.

The in vivo studies clearly demonstrate that the use of the new calcium carbonate toothpaste with 0.3% triclosan and magnesium aluminium silicate results in higher triclosan delivery and retention than a marketed calcium carbonate toothpaste with 0.3% triclosan (Colgate Dental Cream - Strong Teeth). This was found when taking samples 10 minutes after brushing, but more importantly was also apparent two and four hours after brushing. This time profile is most important for the antibacterial efficacy of toothpaste because during this post-brushing time cleaned tooth surfaces become re-colonized quickly with pathogenic oral bacteria, and plaque not removed during the brushing process also tends to undergo rapid growth. Hence, if significant levels of antibacterial agents such as triclosan can be shown to be present in plaque during this period after brushing, enhanced efficacy can result.

The antibacterial activity of triclosan on oral bacteria in vitro has been well documented and reported. Published research by Coppi and co-workers have used S. mutans ATCC 25175 during evaluation for bacteriologic effects. Similar in vitro models have been used to test the efficacy of toothpaste with 0.3% triclosan and PVM/MA copolymer for triclosan delivery and efficacy.

S. mutans is a primary etiologic agent implicated in human dental caries, and is particularly effective at forming biofilms on the hard tissues of the human oral cavity. Adherence of streptococci, including S. mutans, to dental surfaces is the first step in the formation of biofilms by these organisms. Mutans streptococci express several surface adhesions that can bind to salivary pellicles formed on the teeth, whereas sucrose-dependent adherence is mediated by glucan-binding proteins and water-insoluble glucans produced from sucrose by glucosyltransferase (GTF) enzymes.

The selection of the four-hour biofilm formation period was based on a published paper by Sreenivasan, where salivary bacteria were assessed post-brushing at the end of two and four hours. Likewise, Nyvad and Kilian established that bacteria from saliva will start to re-colonize tooth surfaces within four hours after cleaning, and recognized this early stage of the plaque biofilm formation as critical for the eventual cariogenic potential of dental plaque. Furthermore, the work of Brecc, et al. indicated that a higher number of microorganisms and a more complex bacterial flora are established during the first four hours of plaque formation in subjects with a preceding period of no oral hygiene.

The results from the in vivo studies presented in this report demonstrated statistically significantly higher levels of triclosan from use of the new calcium carbonate toothpaste with 0.3% triclosan and magnesium aluminium silicate compared to a marketed calcium carbonate toothpaste with 0.3% triclosan (Colgate Dental Cream Strong Teeth). The in vitro antimicrobial efficacy study presented here indicated that at the post-treatment four-hour biofilm formation period, the novel calcium carbonate toothpaste with 0.3% triclosan and magnesium aluminium silicate reduced growth of S. mutans to a statistically significantly greater extent than a marketed calcium carbonate toothpaste with 0.3% triclosan (Colgate Dental Cream Strong Teeth).
Conclusion

The studies presented in this paper demonstrate that the new calcium carbonate toothpaste with 0.3% triclosan and magnesium aluminium silicate provided statistically significantly greater efficacy four hours post-brushing both for in vivo assessments and in vitro antibacterial action compared to a marketed calcium carbonate toothpaste with 0.3% triclosan.

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References