Antibacterial Efficacy of a Propolis Toothpaste and Mouthrinse Against a Supragingival Multispecies Biofilm

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Purpose: To determine in vitro the antibacterial properties of propolis toothpaste and mouthrinse against an in vitro multispecies biofilm model.

Materials and Methods: Six-species biofilms grown anaerobically on pellicle-coated hydroxyapatite disks were fed with glucose/sucrose-supplemented medium 3 times daily for 45 min and incubated in 37°C saliva between feedings for up to 64.5 h. At each interval, biofilms were exposed to six different slurries and solutions, including: 1) toothpaste without propolis, 2) toothpaste with propolis, 3) toothpaste with chlorhexidine, 4) mouthrinse with propolis, 5) mouthrinse with chlorhexidine, 6) saline solution (control). Afterwards, biofilms were harvested and the number of colony forming units were determined (CFU). The results were analysed using ANOVA, followed by the Bonferroni test at a 5% significance level.

Results: The strongest CFU reduction was shown after treatment with 0.12% chlorhexidine (p < 0.0004). When comparing the different toothpastes, there was no statistically significant difference (p < 0.05) in CFU reduction. However, they all showed a significant reduction in CFU of more than one log-step vs the saline control group. Nevertheless, the propolis-containing mouthrinse showed no significant reduction in CFU.

Conclusion: All toothpastes under investigation displayed some growth inhibition in this supragingival biofilm model, which accounted for an approximately 80%–88% linear reduction. However, the propolis mouthwash had no effect.

Key words: antimicrobial activity, biofilm, dentifrices, propolis, S. mutans

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One of the most extensively studied natural biofilms in oral health and disease is supragingival dental plaque. Clinical studies demonstrate that unrestricted accumulations of dental plaque are associated with the initiation and progression of oral disease, including gingivitis, caries and periodontal disease. It has been shown that professional plaque control will prevent disease initiation or stop its progression. Thus, the main objective of prophylaxis and therapy remains the effective control and reduction of the bacterial flora, especially by mechanical means, e.g. toothbrushing. However, it is impossible for most people to completely remove the oral biofilm by brushing only, especially in areas difficult for the bristle ends of a toothbrush to reach, mainly interdental areas, fissures and grooves. Therefore, the latter constitute places where plaque is easily left behind and may also be difficult to remove by other mechanical means. During oral hygiene, the use of a dentifrice has long
been indicated, not only as a cosmetic additive associated with flavour, but also as a preventive and therapeutic agent against oral diseases. Propolis is a natural product produced and used by honeybees to cover hive walls and fill gaps and cracks. Bees collect resin from cracks in the bark of trees and leaf buds. This resin is masticated, salivary enzymes are added and the partially digested material is mixed with beeswax and used by bees to seal holes in their honeycombs and to smooth out the internal walls. Propolis is responsible for the protection of honeycombs especially against microorganisms. The chemical composition of propolis is highly variable and depends on the local flora at the site of collection. It has been proven that propolis has a wide range of biological therapeutic effects, such as antimicrobial, antifungal and antiviral activities. Therefore, propolis may be a good candidate as an antimicrobial adjunct to the treatment or prevention of infectious diseases.

In vivo studies evaluated the antibacterial action of propolis on Streptococcus mutans colonising the oral cavity and demonstrated a significant antimicrobial activity against this species. However, to the best of our knowledge, in vitro evaluations of toothpastes and mouthrinses containing propolis in a multispecies biofilm model are scarce.

Therefore, the aim of this study was to compare the growth-inhibiting efficacy of propolis in an in vitro supragingival biofilm model in comparison to chlorhexidine, a commonly used chemo-preventive agent. The antimicrobial effect of propolis was tested and compared in two different galenic forms, namely toothpaste and mouthrinse. Toothpastes with and without propolis, toothpastes with chlorhexidine (0.12%) and mouthrinses with propolis and with chlorhexidine were used. The control was a saline solution. The hypothesis of this study was that the propolis-containing toothpaste and mouthrinse are as effective as the control counterparts containing chlorhexidine (0.12%) in reducing the bacterial growth as compared to the control treatment with physiological saline solution.

**MATERIALS AND METHODS**

**Biofilm preparation**

Actinomyces naeslundii OMZ 745, Veillonella dispar OMZ 493, Fusobacterium nucleatum OMZ 598, Streptococcus mutans OMZ 918, Streptococcus oralis OMZ 607 and Candida albicans OMZ 110 were used for supragingival biofilm formation. All strains were cultivated on Columbia blood agar (CM 331, Oxoid; Basingstoke, Hampshire, UK) supplemented with 5% whole human blood.

Biofilms were grown in 24-well polystyrene cell culture plates on sintered hydroxyapatite disks (Ø 9 mm; Clarkson Chromatography Products; South Williamsport, PA, USA) that had been preconditioned (pellicle-coated) in 1 ml of processed whole unstimulated pooled saliva and incubated for 4 h at room temperature with shaking (95 rpm).

An overall survey of the time flow of each biofilm experiment is provided in Fig 1. After salivary pellicle formation, saliva was replaced at time 0 h with a mix of 480 μl processed saliva and 1120 μl mFUM containing 0.3% glucose. mFUM corresponds to a well-established tryptone-yeast-based broth medium designated as FUM and modified by supplementing 67 mM Sørensen’s buffer (final pH 7.2). The carbohydrate concentration in mFUM was 0.3% (w/v), consisting of glucose for the first 16.5 h and from then on of a 1:1 (w/w) mixture of glucose and sucrose. At timepoint 0, a volume of 200 μl of a microbial suspension prepared from equal volumes and densities of each strain was added to each well and the culture plate was anaerobically incubated at 37°C for 45 min. Then the disks were dip-washed 3 times in 2 ml of physiological saline (0.9% NaCl) to remove growth medium and free floating cells but not bacteria adhering firmly to the disks. They were then transferred to new wells containing only 1600 μl of fresh processed saliva and incubated anaerobically at 37°C up to time point 16.5 h (period of famine). At timepoints 16.5, 20.5, 24.5, 40.5, 44.5 and 48.5 h, all disks were re-exposed for 45 h.
min to a cell-free mixture of processed saliva (480 μl) and mFUM (1120 μl) with 0.15% glucose + 0.15% sucrose (periods of feast), after which they were dip-washed as described above and transferred back into processed saliva (famine). After the feeding periods starting at 16.5, 20.5, 40.5 and 44.5 h, the disks were put back into the same processed saliva provided before feeding, whereas after the feeding periods starting at 16.5 and 40.5 h, the disks were transferred to new plates with fresh processed saliva. Experiments were stopped and biofilms analysed after 64.5 h of incubation.

**Exposure of biofilms to the different solutions**

Figure 2 provides a schematic overview of the allocation of biofilms to the different treatments within one experiment. In brief, every experiment covered one of the 6 testing solutions and included triplicate biofilms for each treatment. The effect of a toothpaste without propolis (dips 1/2/3), toothpaste with propolis (Propodent, Bio Cumnatura; Rüti, Switzerland; dips 4/5/6; 0.9% propolis), mouthrinse solution with propolis (Mundwasser, Bio Cumnatura; dips 13/14/15; 10% propolis, no alcohol), mouthrinse solution with chlorhexidine (Curasept, Curaden International; Kriens, Switzerland; ADS 212 0.12%, dips 10/11/12), toothpaste with chlorhexidine (Curasept ADS 712, chlorhexidine digluconate 0.12%, dips 7/8/9) and of a physiological saline solution (0.9% NaCl, dips 16/17/18) was evaluated. Each solution had a dilution of 1:3 in physiological saline solution. Every disk was exposed for 1 min (gentle shaking) in test solutions (1 ml), dip-washed 3 times in saline solution and then transferred back to its well for the feeding period, as described above.

**Harvesting and determination of colony forming units (CFU)**

After 64.5 h, the biofilms were dip-washed in saline to remove loosely adhering bacteria. Then the surface of each disk was scraped with a plastic scaler (Kerr Hawe; Bioggio, Switzerland) to remove the entire biofilm, and the cells were collected in 1 ml saline. The cell suspension was then sonicated (5 s, 30 W) and aliquots were plated out using a spiral diluter. Total colony-forming units (CFU) were assessed on Columbia blood agar (CBA) plates, which were incubated anaerobically for 72 h at 37°C. After 72 h of incubation, to allow better prediction of the dilutions, a Live/Dead Test was performed, followed by counting all CFU under a stereomicroscope. This procedure was repeated three times.

**Statistical analysis**

Descriptive statistics of the data were performed with SPSS version 20.0 (SPSS; Chicago, IL, USA) and illustrated with box plots. The log10-transformed data met the requirements for parametric analysis. Hence, differences between treatments were analysed by a one-way ANOVA followed by the Bonferroni test (15 intergroup comparisons, significance level at p < 0.0033).
Table 1  Mean values and standard deviation of the total colony forming units (CFU) following the treatment with different testing solutions

<table>
<thead>
<tr>
<th>Testing solutions</th>
<th>Mean value</th>
<th>N</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toothpaste without propolis</td>
<td>5.14 x 10^7</td>
<td>9</td>
<td>1.10 x 10^7</td>
</tr>
<tr>
<td>Toothpaste with propolis</td>
<td>3.94 x 10^7</td>
<td>9</td>
<td>1.14 x 10^7</td>
</tr>
<tr>
<td>Toothpaste with chlorhexidine</td>
<td>6.49 x 10^7</td>
<td>9</td>
<td>2.55 x 10^7</td>
</tr>
<tr>
<td>Mouthrinse with propolis</td>
<td>3.77 x 10^8</td>
<td>9</td>
<td>1.69 x 10^8</td>
</tr>
<tr>
<td>Mouthrinse with CHX</td>
<td>4.58 x 10^6</td>
<td>9</td>
<td>3.66 x 10^6</td>
</tr>
<tr>
<td>Saline solution / Control</td>
<td>3.36 x 10^8</td>
<td>9</td>
<td>1.94 x 10^8</td>
</tr>
</tbody>
</table>

RESULTS

The mean values and standard deviations of the CFU in the different treatments are given in Table 1. Figure 3 shows the effect of propolis and chlorhexidine on the total CFU of the in vitro supragingival biofilms.

As expected, the mouthwash containing chlorhexidine showed the highest reduction in CFU, by 2-log steps. As seen in the box plots in Fig 3, there were no significant differences in CFU between treatments with the different toothpastes. However, a significant CFU reduction in the groups treated with the three toothpastes was observed when compared to the control group (p < 0.0033), accounting for approximately 1-log step. The CFU of the treatment with the mouthrinse containing propolis was comparable to that of the control, and showed no significant reduction in CFU.

DISCUSSION

Conceivably, antimicrobial agents can influence oral biofilm formation in different ways. For example, this can be achieved by preventing bacterial adhesion to surfaces, affecting bacterial viability or disrupting an existing biofilm.1

Propolis, a resin collected by bees, exhibits a considerable variety of well-established pharmacological activities. Its antimicrobial potential has been widely studied, especially against oral pathogens.7,15,19 The antimicrobial action of propolis is associated with its ability to inhibit glucosyltransferase activity, which is essential for S. mutans to catalyse the formation of soluble and insoluble glycans, as well as to provide adherence.15 Some authors have reported that triclosan is not as effective in inhibiting this activity.15 It has also been shown that a dentifrice containing propolis and aloe vera reduced the contamination of toothbrush bristles by S. mutans.4 However, data on the growth-inhibiting effects using propolis toothpastes and mouthwashes are still scarce, particularly in multi-species biofilm models.

The results observed in the current study did not show a significant difference in the reduction of the CFU, either for the propolis-containing toothpaste or for the mouthwash formulation when tested in the in vitro supragingival biofilm model. The effect was comparable to the chlorhexidine toothpaste or to the toothpaste containing none of these agents. It is therefore plausible that the observed antimicrobial efficacy of the toothpaste (1-log reduction) is due to the common carrier component of these three preparations.

There may be several reasons for these results. In some studies, propolis showed inhibitory effects on the growth of oral bacteria such as Streptococcus mutans, S. sobrinus or Actinomyces naeslundii.5,18 However, these studies were performed using planktonic cultures, where the susceptibility to antimicrobial agents is up to 1000 times higher than in biofilms structures such as dental plaque.6,12,14 Furthermore, inhibitory effects of propolis are often studied using minimal inhibitory concentration (MIC) tests.5,10,16 These tests have limitations, as these set-ups use bacterial cultures
rather than biofilms; therefore, the obtained MIC values do not provide sufficient information concerning the efficacy of antimicrobial agents against infections involving biofilms, which are more resistant to antimicrobial substances. In addition, higher concentrations of the active compounds were used, whereas a dilution of 1:3 was applied in this experiment. Therefore, in vitro-based studies must be interpreted with caution in general and are put into perspective by the present investigation.

It is noteworthy that many studies used ethanol extracts of propolis, which showed a negative effect on the growth of the microorganisms tested.\textsuperscript{10} In the propolis toothpaste, the availability of the active compound is probably much lower due to chemical interactions; its diffusion into the biofilm may be also hampered by the extracellular matrix of the biofilm itself. This would also explain the difference of CFU between the toothpaste and the mouthwash containing the same concentration of CHX.

CONCLUSION

Within the limits of this study, it may be concluded that propolis seems to have no effect in respect to reduction of the CFU in the supragingival biofilm model used, neither when contained in the present toothpaste nor in the present mouthwash. The effect of the propolis toothpaste was similar to that of CHX-containing toothpaste and a toothpaste with no active ingredient, and therefore there is no evidence of a superior effect in this formulation. Therefore, the hypothesis was rejected in this context. In contrast, with regard to mouthwashes, the CHX-containing mouthwash exhibited a standard antimicrobial efficacy, whereas the mouthwash containing propolis exhibited virtually no antimicrobial efficacy in this in vitro biofilm model.

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REFERENCES