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Modulation of *Streptococcus mutans* virulence by dental adhesives containing anti-caries agents

Carolina Bosso André[a,*], *Pedro Luiz Rosalen*[b], *L.C.C. Galvão*[c], *Bruna Marin Fronza*[a], *Glaucia Maria Bovi Ambrosano*[d], *Jack Liborio Ferracane*[g], *Marcelo Giannini*[a]

[a] *Department of Restorative Dentistry, Piracicaba Dental School, State University of Campinas, Piracicaba, SP, Brazil*
[b] *Department of Physiological Sciences, Piracicaba Dental School, State University of Campinas, Piracicaba, SP, Brazil*
[c] *Department of Dentistry, Dom Bosco Graduate School and CEUMA University, São Luis, MA, Brazil*
[d] *Department of Social Dentistry, Piracicaba Dental School, State University of Campinas, Piracicaba, SP, Brazil*
[g] *Department of Restorative Dentistry, School of Dentistry, Oregon Health & Science University, Portland, OR, USA*

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ABSTRACT

Objectives. The aim of this *in vitro* study was to analyze the effect of the incorporation of two anti-caries agents into dental adhesives on the reduction of the virulence of *Streptococcus mutans* and on the adhesion to dentin.

Methods. Apigenin (1 mM) and tt-Farnesol (5 mM) were added separately and in combination to a self-etch adhesive (CS3 — Clearfil S3 Bond Plus) and to an each-and-rinse adhesive (OPT — OptiBond S). Biofilm of *S. mutans* was grown on adhesive-coated hydroxyapatite disks for 115 h and bacterial viability, dry-weight, alkali soluble, water soluble, intracellular polysaccharides and protein were quantified. Bond strength and dentin-adhesive interface were performed to analyze the effects of the incorporation on the physical properties and to identify changes in hybrid layer morphology.

Results. Addition of Apigenin and Apigenin + tt-Farnesol to CS3, and Apigenin or tt-Farnesol to OPT reduced the dry-weight of *S. mutans* biofilm. Insoluble polysaccharide decreased with the addition of Apigenin to CS3 and tt-Farnesol to OPT. Intracellular polysaccharide decreased with addition of Apigenin and Apigenin + tt-Farnesol to CS3. No changes in dentin bond strength, resin–dentin interfacial morphology, total amount of protein and soluble polysaccharide were observed with the additions.

Significance. Biofilms that are less cariogenic around dental restorations could decrease secondary caries formation; in addition, the reduction of virulence of *S. mutans* without necessarily killing the microorganism is more unlikely to induce antimicrobial resistance.

* *Corresponding author at*: Department of Restorative Dentistry, Operative Dentistry Division, Piracicaba Dental School, State University of Campinas, Av. Limeira, 901, Bairro Areião, Piracicaba, SP, Brazil – Zip code: 13414-903. Fax: +55 19 21065218.
E-mail address: carolina.bosso@gmail.com (C.B. André).

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1. Introduction

To overcome the disadvantages of composites restorations, such as contraction gaps and bonding degradation that may lead to secondary caries formation and restoration failure, restorative materials containing antibacterial agents have been developed [1,2]. Substances with broad antimicrobial spectra have been added to these restorative materials with no concern regarding the oral health resident bacteria and the promotion of bacterial resistance. Two compounds derived from Brazilian propolis, Apigenin and *tt*-Farnesol, are described in the literature as potential anti-caries agents [3–5] which do not exert a significant effect on the viability of the oral microbiota population, and have been considered for new approach to antimicrobial therapy [3]. Also, propolis is a non-toxic natural beehive product, and these compounds have shown to be non-mutagenic and non-toxic, *in vitro* and *in vivo* [4,6].

Streptococcus mutans does not always dominate within plaque, however, in the pathogenesis of the disease, it can assemble an insoluble polymeric matrix in the presence of sucrose, the exopolysaccharides (EPS) that act as a supportive framework and barrier to diffusion [7–9] within the oral biofilm structure. The matrix of cariogenic biofilms are recognized as essential virulence factors associated with the initiation of cariogenic biofilm [7], and the main constituents of this matrix are polysaccharides [8,10], mostly glucans synthesized by microbial glycosyltransferases (Gtfs) [5]. At least three Gtfs are produced by this bacterium: GtfB that synthesizes mostly insoluble glucans, GtfC that synthesizes a mixture of insoluble and soluble glucan, and GtfD that synthesizes mostly soluble glucans [6,11,12].

According to Koo et al. [4], Apigenin (4′,5,7-trihydroxyflavone) is a potent inhibitor of Gtfs B and C and has virtually no antibacterial activity against *S. mutans* [4]. *tt*-Farnesol, a natural sesquiterpene (3,7,11-trimethyl-2,6,10-dodecatrien-1-ol), disrupts the proton permeability of the *S. mutans* membrane, affecting the production and secretion of Gtfs and acidurance [4,5]. In addition, the combination of fluoride with these two compounds showed a reduction in the virulence of *S. mutans* [3].

Regarding the effects of these two natural compounds from propolis, the incorporation of both into a dental restorative material could yield benefits in terms of enhanced durability of composite restorations, mainly in areas where biofilms accumulate, such as the interproximal and cervical regions of the teeth. Therefore, the aim of this in *vitro* study was to incorporate two anti-caries agents, Apigenin and *tt*-Farnesol, into fluoride-containing dental adhesives in order to analyze their effect on the virulence of *S. mutans* biofilms, as quantified by polysaccharide production, as well the bonding to dentin.

2. Materials and methods

2.1. Anti-caries agent additions

Two anti-caries agents were added alone or in combination to two commercial dental adhesives containing fluoride [3]. Apigenin (Api, CAS: 520-36-5, lot number: SLBL4733V) was added at 1 mM and *tt*-Farnesol (Far, CAS: 106-28-5, lot number: MKBQ3298V) at 5 mM; both compounds were obtained from Sigma–Aldrich (St. Louis, MO, USA). The same concentrations for both compounds were used when they were combined. These concentrations were chosen based on previous published data [3]. The compounds were added into the bottle of each adhesive and blended with vortex mixer (AP-56, Phoenix Luferco, Araraquara, SP, BR). A total of eight groups were evaluated: (1) Clearfil S3 Bond Plus (CS3) with no addition; (2) CS3 with Api; (3) CS3 with Far; (4) CS3 with Api and Far; (5) OptiBond S (OPT) with no addition; (6) OPT with Api; (7) OPT with Far; and (8) OPT with Api and Far. The lot numbers, compositions and application technique of the adhesives are described in Table 1.

2.2. Biofilm of S. mutans assay

Dental adhesives (30 μL) were applied on hydroxyapatite disks (10 mm diameter × 2 mm thick, Clarkson Chromatography Products Inc., South Williamsport, PA, USA), previous autoclaved, and light activated (Valo, Ultradent Products Inc., South Jordan, UT) with radiant exposure of 15 J/cm^2 on each side, and biofilms of *S. mutans* UA159 were formed on the disks (n = 6). A negative control group, a hydroxyapatite disk with no adhesive applied (control disk), was included to analyze the effect of the adhesive composition itself, with no additions, on biofilm formation. Human whole saliva was collected from one donor (approved by Institutional Ethics committee, CEP-FOP/UNICAMP # 047/2014), clarified by centrifugation (10,000 × *g*, 4 °C, 10 min), sterilized and diluted (1:1) in adsorption buffer (AB — 50 mM KCl, 1 mM KPO4, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$, pH 6.5), and supplemented with the protease inhibitor phenylmethylsulfonyl-fluoride (PMSF) at a final concentration of 1 mmol/L. The disks were placed in a vertical position in 24-well plates and inoculated with approximately 2 × 10^6 CFU/mL in low molecular weight media (buffered ultrafiltered — 10 kDa cutoff membrane; Prep/Scale; Millipore, MA, USA, of tryptone and yeast extract, pH 7.0, with 1% (w/v) sucrose), at 37 °C, 5% CO_2 [4]. The biofilms were grown undisturbed during 24 h, and then the culture media was replaced daily for 5 days (total of 115 h of growth). After this period of time, all the biofilm was collected by scraping it with a sterile spatula into 5 mL of physiological solution and 10 min in an ultrasound bath to remove the adhered bacteria. The removed biofilm was processed to count the viable cells (CFU), to calculate the dry-weight (biomass), to assess the total amount of polysaccharides (alkali soluble, water soluble and intracellular), and protein quantification (Micro BCA™ protein assay kit, Thermo Fisher Scientific, Waltham, MA, USA) using colorimetric assays [4]. Alkali soluble and water soluble were extracted and measured using glucose as standard, and glycogen was used to quantify the intracellular polysaccharides. Additional disks were analyzed using scanning electron microscopy (SEM — JSM-5600, JEOl Inc., Peabody, MA, USA). The 115 h old biofilms were rinsed in sterile 0.9% NaCl then fixed with a 4% glutaraldehyde solution (v/v, in phosphate buffered saline PBS, pH 7.4) for 24 h. Biofilms then were dehydrated in ascending ethanol concentration (50, 70, 90, and 100%), dried for 24 h,

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