



ORIGINAL ARTICLE

Description of *Streptococcus mutans*, *Streptococcus sanguinis*, and *Candida albicans* biofilms after exposure to propolis dentifrice by using OpenCFU method



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KEYWORDS

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Abstract *Context:* Dental caries is a major and chronic dental public health problem, which can usually be prevented by regular oral hygiene. The most common oral hygiene practice is brushing teeth with a dentifrice. Propolis has emerged as a promising anti-cariogenic agent, which is considered to be a good oral antiseptic for prevention of caries. Several studies have shown that the use of C has an influence in the growth of oral biofilms. There are several standard methods used to count bacterial colonies, such as crystal violet and CFU Count assays. OpenCFU method is a technique that can be used to calculate biofilm colonies more faster, precisely, and accurately.

Aim: To compare several methods for evaluating the number of biofilm colonies formed with exposure to a standard dentifrice and propolis.

Methods and materials: Biofilm assays were carried out on 96-well microplates. Reference strains of oral *Streptococcus* species (*S. mutans* ATCC 25175^T and *S. sanguinis* ATCC 10566^T) and yeast (*Candida albicans* ATCC 10231^T) were inoculated into wells, and 200 µL of standard and propolis dentifrice solution were added to each well and incubated for 18 h at 37 °C. Bacteria and yeast were then sub-cultured on respective media and the colony-forming units (CFU) were counted manually. The other wells were stained by crystal violet and incubated for 15 min, followed by observation

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using an inverted microscope and evaluated using crystal violet analysis and the OpenCFU automated method.

Results: The numbers of CFUs determined for all strains were similar in the standard-dentifrice group and propolis-dentifrice group, and were similar among the three methods: crystal violet staining, manual CFU count, and OpenCFU analysis.

Conclusion: OpenCFU analysis can be reliably used as a rapid and a more practical method to analyse the growth of oral microorganism biofilms. However, high digital image quality is required to provide an accurate analysis for colony counting.

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1. Introduction

Tooth brushing plays a considerable role in the prevention of carious lesions (Khurshid et al., 2017; Herrera Mdel et al., 2013; De Lacerda Vidal et al., 2017). The composition of the most standard dentifrices includes abrasives, humectants, binders, water, detergents, flavouring agents, preservatives, sweeteners, colouring agents, and therapeutic agents (Subramanian et al., 2017; Davies et al., 2010; Pipert, 2013). Propolis is a herbal substance produced by bees that exhibits antimicrobial, anticancer, antifungal, antiviral, and anti-inflammatory properties. Propolis consists of 50% resin and balsam, 30% wax, 10% aromatic oil, 5% pollen, 5% organic material, and active compounds such as flavonoids, cinnamic acid, and terpenes (Libério et al., 2009). Siquera et al. (2015) noted that propolis has better fungistatic and fungicidal properties than fluconazole, which is a common ingredient in a standard dentifrice (Siquera et al., 2015; Al-Ani et al., 2018). Indeed, a study of the effect of propolis on *Streptococcus mutans* vulnerability, development of caries, and glycosyltransferase activity in rats suggested that the extract of propolis has cariostatic effects (Libério et al., 2009). Although the precise mechanism of the antimicrobial effect of propolis is unknown, several studies have suggested that apigenin binds through the double bond between C-2 and C-3 to ultimately inhibit glucosyltransferase activities, and tt-farnesol disrupts the bacterial membrane to impair the accumulation and composition of biofilm polysaccharides (Koo et al., 2002; Koo et al., 2003).

Conventional colony counting and crystal violet staining are the standard methods for measuring bacterial colonies. OpenCFU is a new platform that was developed to calculate bacterial cell colonies through image-based data with the main advantage of shortening the time required for the colony count calibration since it can process digital images quickly (Geissmann, 2013).

The aim of the present study was to determine the capabilities of propolis dentifrice in inhibiting the growth of biofilms of typical oral microorganisms, *S. mutans*, *S. sanguinis*, and *C. albicans*, as determined using crystal violet staining, manual colony forming unit (CFU) counting, and the OpenCFU method. This is the first report providing the profiles of the growth of *S. mutans*, *S. sanguinis*, and *C. albicans* biofilms visualized using digital photos of a 96-well microplate stained using crystal violet and counted by the OpenCFU method.

2. Materials and methods

2.1. Preparation of the propolis dentifrice solution, artificial saliva, and strain culture

The propolis and non-propolis dentifrice were provided by Chemical Engineering Laboratory, Universitas Indonesia. The propolis dentifrice was composed of 46% calcium carbonate, 20–40% water, 14% glycerine, 1% sodium alginate, 1% mint, 0.2% sodium saccharine, 0.3% sodium benzoate, and active substances such as 0.15% sodium fluoride and 5% propolis. The composition of the non-propolis dentifrice was similar to that of the propolis dentifrice, except for the addition of propolis. Each dentifrice was prepared as a solution by diluting 10 g of the dentifrice with 10 mL distilled water followed by homogenization. Subsequently, the solution was filtered with a syringe filter (Sartorius Minisart) with a micropore diameter of 0.2 µm to obtain the final sterile dentifrice solution.

Sterilized artificial saliva (provided by the Department of Biochemistry, Faculty of medicine, Universitas Indonesia) was used to allow for initial attachment of the three oral microorganism species. The artificial saliva was composed of 0.4 g/L sodium chloride, 0.4 g/L potassium chloride, 1 g/L urea, 0.795 g/L calcium chloride dihydrate, 0.005 g/L sodium sulphide nonahydrate, and 0.69 g/L sodium phosphate dihydrate.

Two reference strains of oral *Streptococcus* species (*S. mutans* ATCC 25175^T and *S. sanguinis* ATCC 10566^T) and yeast (*C. albicans* ATCC 10231^T) were provided by the laboratory stock at Oral Biology Laboratory, Universitas Indonesia. The bacteria were sub-cultured in a rich medium composed of Bacto™ Brain Heart Infusion (BHI) broth (Difco Laboratories, BD) and incubated anaerobically at 37 °C for 24 h, while the yeast was sub-cultured in Sabouraud dextrose broth (DSB) and incubated aerobically at 37 °C for 24 h. The optical density of the three sub-cultured microorganism strains was measured at 600 nm (OD₆₀₀). The sub-culture medium was diluted until the OD₆₀₀ value reached 0.1.

2.2. Biofilm formation in 96-well microplates

Biofilm assays were carried out on 96-well microplates (TPP, Switzerland) (Azereido et al., 2016; Coffey, 2014). To grow the tested bacteria as a biofilm, artificial saliva was used as a pellicle. Approximately 100 µL of the artificial saliva was

added to each well, shaken for 5 min on a shaker (Certomat U, B Braun, Biotech International) at 60 rpm, and incubated for 60 min at 37 °C. The artificial saliva supernatant was decanted from the 96-well microplates, and then 200 µL of each bacterial sub-cultured medium was inoculated into the wells and further incubated for 90 min at 37 °C in an anaerobic jar containing 80% N₂, 10% CO₂, and 10% H₂. In addition, 200 µL of *C. albicans* sub-cultured medium was inoculated into the wells, and incubated for 90 min at 37 °C in aerobic conditions. An Eppendorf pipette was used to gently remove the supernatant of the microorganism sub-cultured medium from the wells to reveal the formed biofilm at the base of the wells on the bottom of the microplate.

Approximately 200 µL of propolis and non-propolis dentifrice solution was added to each well and incubated for 18 h at 37 °C in the same anaerobic atmosphere containing *S. mutans* and *S. sanguinis*, and in the aerobic atmosphere for *C. albicans* culture described above. Then, the supernatant was removed and the base of the microplate well was gently washed with 200 µL of phosphate-buffered saline (PBS) for biofilm evaluation.

2.3. Biofilm evaluation

The first evaluation was conducted using crystal violet analysis. After crystal violet staining, the absorbance of the eluted solution was measured using a microtiter plate reader (M965 + Microplate Reader-Merck Inc) at 600 nm. The second evaluation was conducted using manual enumeration of CFUs, and the third evaluation was conducted using the OpenCFU method.

For crystal violet staining, after 18 h of biofilm incubation, the entire supernatant was aspirated and the wells were gently washed with 200 µL of PBS (Liu et al., 2017; Pui et al., 2017; Tram, 2013). The biofilms were then allowed to dry at a room temperature, and 200 µL of crystal violet (0.5% v/v) was added to each well and incubated for 15 min. The crystal violet solution was removed, and the microtiter plates were observed using an inverted microscope (PrimoVert, Zeiss). Finally, 96% ethanol was added to the wells to extract the absorbed crystal violet from bacterial cells, and the OD value was measured on a microtiter plate reader.

For the manual CFU count, after the biofilms were washed with PBS and allowed to dry at 25 °C, the formed biofilm layer

at the base of well was collected and diluted ten-fold. Fifty microliters of the dilution solution with concentrations of 10⁻⁴–10⁻⁷ were poured on each BHI agar medium plate and were incubated anaerobically at 37 °C (2 × 24 h), and then the CFU count was performed manually.

In the OpenCFU method, the data were obtained during the crystal violet staining procedure. The photos were taken by a digital camera (Zeiss AxioCam ERc5s) on an inverted microscope after biofilm staining by crystal violet. An overview of bacteria from the results of the photo shoot were transferred to the computer, and the number of bacteria was determined automatically using the OpenCFU program (Geissmann, 2013).

3. Results

For *S. mutans*, *S. sanguinis*, and *C. albicans*, the control group exposed to the standard non-propolis dentifrice and the propolis-dentifrice group showed similar degrees of biofilm formation as assessed with the standard methods (crystal violet analysis and CFU manual count) and OpenCFU analysis. Overall, there was a decrease in the formation of biofilms after exposure to either dentifrice (Table 1).

OpenCFU analysis conducted in bacteria (*S. mutans* and *S. sanguinis*) and yeast (*C. albicans*) was based on the results of photographing the biofilms lining 96-well plates after staining with crystal violet, and the appearance under an inverted microscope. Photographs were taken of the biofilms after exposure to a non-propolis dentifrice, propolis dentifrice, and the control of *S. mutans*, *S. sanguinis*, and of *C. albicans* (Fig. 1).

4. Discussion

S. mutans is a major pathogen causing human dental caries, which normally exists as a regular member of the mature dental biofilm community. However, under certain conditions, it can become dominant, leading to progression of dental caries (Damle et al., 2016). *S. sanguinis* is also a natural member of the resident oral biofilm community, and was the only species identified to be significantly associated with dental health by comparing colony numbers of caries and caries-free samples from children (Kreth et al., 2005; Zhu et al., 2018). *C. albicans*

Table 1 Colony forming units (CFU) of *S. mutans*, *S. sanguinis*, and *C. albicans* determined by the crystal violet absorbance value, manual count, and OpenCFU.

Microorganism	Variable	Crystal violet absorbance value	CFU	Open CFU
<i>Streptococcus mutans</i>	Control	0.490 ± 0.018	427 × 10 ⁸ ± 10.4 × 10 ⁸	31.8 cells/mm ²
	Non-propolis toothpaste	0.389 ± 0.015	403.85 × 10 ⁸ ± 5.8 × 10 ⁸	24.8 cells/mm ²
	Propolis toothpaste	0.365 ± 0.049	329.95 × 10 ⁸ ± 31.4 × 10 ⁸	14.5 cells/mm ²
<i>Streptococcus sanguinis</i>	Control	0.371 ± 0.133	10.28 × 10 ⁸ ± 1.01 × 10 ⁸	117.8 cells/mm ²
	Non-propolis toothpaste	0.272 ± 0.187	5.97 × 10 ⁸ ± 2.67 × 10 ⁸	86.5 cells/mm ²
	Propolis toothpaste	0.257 ± 0.005	6.25 × 10 ⁷ ± 2.05 × 10 ⁷	54 cells/mm ²
<i>Candida albicans</i>	Control	0.597 ± 0.005	19.05 × 10 ⁸ ± 2.48 × 10 ⁸	72.8 cells/mm ²
	Non-propolis toothpaste	0.243 ± 0.007	7.56 × 10 ⁸ ± 0.73 × 10 ⁸	53.8 cells/mm ²
	Propolis toothpaste	0.302 ± 0.040	6.24 × 10 ⁸ ± 1.58 × 10 ⁸	57.3 cells/mm ²

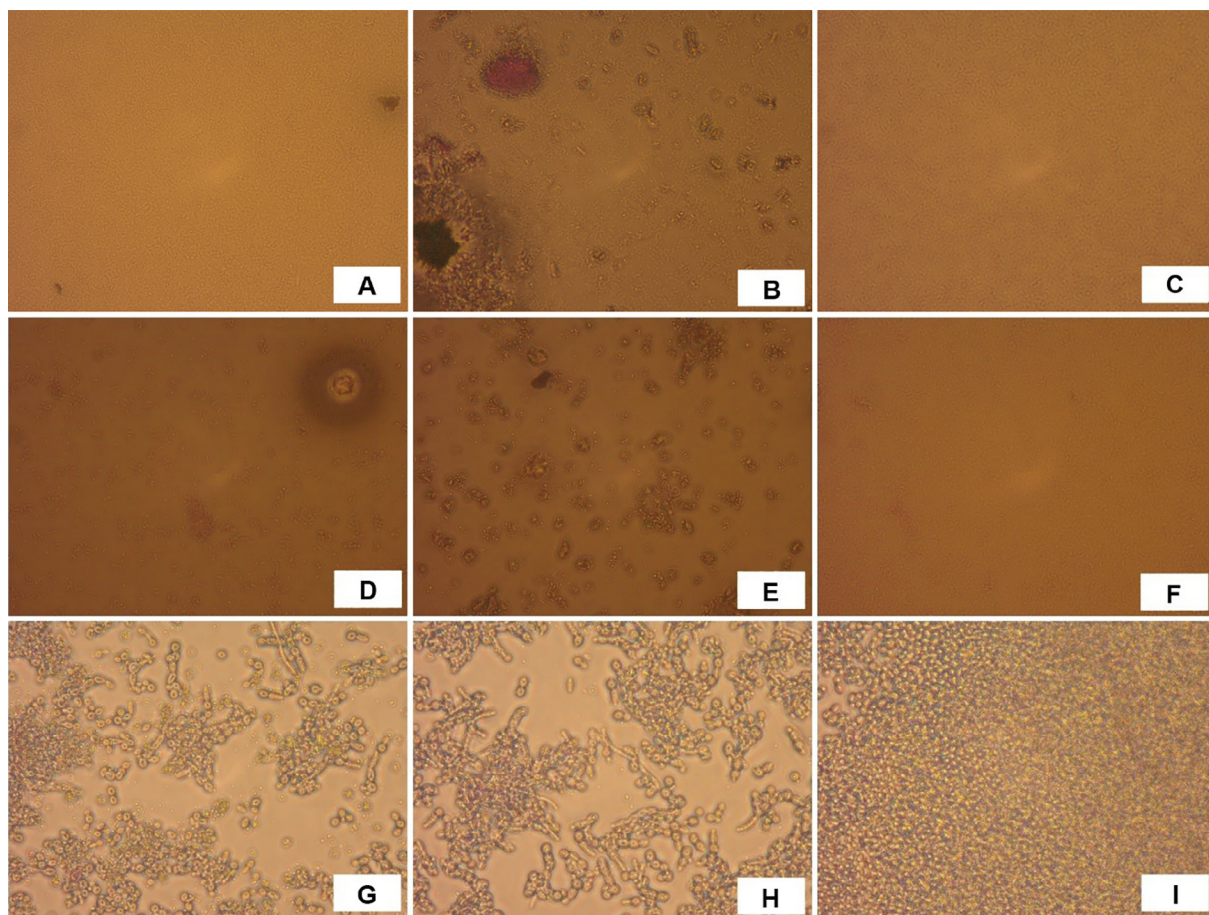


Fig. 1 Microscopic images of *Streptococcus sanguinis*, *Streptococcus mutans*, and *Candida albicans*. A: *S. sanguinis* biofilms exposed by propolis dentifrice, B: *S. sanguinis* biofilm exposed by non-propolis dentifrice, C: Control of *S. sanguinis* biofilms, D: *S. mutans* biofilms exposed by propolis dentifrice, E: *S. mutans* biofilm exposed by non-propolis dentifrice, F: Control of *S. mutans* biofilms, G: *C. albicans* biofilms exposed by propolis dentifrice, H: *C. albicans* biofilms exposed by non-propolis dentifrices, I: Control of *C. albicans* biofilms.

biofilms are also present in the dental biofilm and are associated with dental caries. Regarding its ability to form biofilms, there are several important factors that influence the virulence of *C. albicans*: (i) the morphological transition between yeast and hyphal forms, (ii) expression of adhesions and invasins on the cell surface, (iii) thigmotropism, (iv) phenotypic switching, (v) secretion of hydrolytic enzymes, (vi) resistance to changes in environmental pH, (vii) metabolic flexibility, (viii) a powerful nutrient acquisition system, and (ix) response to oxidative stress (Alonso et al., 2018).

Antimicrobial agents may be used to further inhibit biofilm development and consequently prevent caries. Propolis, as a promising anti-cariogenic agent, can be considered a good oral antiseptic for prevention of caries (Libério et al., 2009; Silva-Carvalho et al., 2015). The biological activities of propolis are attributed to a variety of its major chemical constituents, including phenolic acids, phenolic acid esters, flavonoids, and terpenoids such as Caffeic acid phenethyl ester (CAPE), artepilin C, pinobanksin, pinocembrin, and pinobanksin 3-acetate (Silva-Carvalho et al., 2015; Huang et al., 2014). Some studies have demonstrated the good inhibitory activities of toothpastes and solutions for mouth rinses containing propolis on oral pathogens (Libério et al., 2009; Puchkov, 2016). However, herbal dentifrices have rarely shown significantly greater anti-

plaque activity compared to the conventional dentifrices (Davies, 2010; Mehta et al., 2018).

In this study, we evaluated the OD values of yeast and bacteria after crystal violet staining and incubation for 18 h. Overall, there was higher biofilm production of *S. mutans*, *S. sanguinis*, and *C. albicans* in the control group compared to the dentifrice-exposed groups. The crystal violet staining method for biofilm quantification remains the most frequently used quantification technique in microtiter plate assays. In microtiter plate assays, part of the biomass may stem from cells sedimented to the bottom of the wells, and subsequently embedded by extracellular polymeric substances. These assays stain both live and dead cells as well as some components present in the biofilm matrix, and are thereby well suited to quantify the total biofilm biomass (Azeredo et al., 2016; Coffey and Anderson, 2014).

Based on manual CFU counting, the CFUs of *S. sanguinis* in the control group were about double than those counted in the standard-dentifrice and propolis-dentifrice groups, with similar counts for the two dentifrice groups. This demonstrated that exposure to propolis-dentifrice reduces the growth of *S. sanguinis* biofilms. The most widely used technique to estimate biofilm cell viability is determination of CFUs on agar media. However, this method has some serious drawbacks and

limitations, namely (i) the fraction of detached live cells may not be representative of the initial biofilm population and (ii) a subpopulation of biofilm cells can be viable but non-culturable, and would not be detected by the CFU counting approach. Alternatively, biofilm biomass and viability can be assessed by different methods that rely on microbiological and molecular methods, or can be modified with physical or chemical properties of the biofilm. Using a physical method, the total biofilm biomass can be obtained from dry or wet weight measurements. The analysis of adhered cells to surfaces can easily be performed using microscopy methods. If the surface is transparent, then standard light microscopy can be used (Azeredo et al., 2016).

Currently, there are numerous software packages available that allow for processing confocal image stacks to make a two-dimensional data representation or virtual three-dimensional representations such as animations. There are two main categories of image processing software according to its function: (i) programs for making images for presentations and (ii) programs for obtaining quantitative measurements of biofilm images (Azeredo et al., 2016). All of the data obtained using the OpenCFU analysis method were in line with the results derived from the measurements using crystal violet analysis. OpenCFU is a software that offers faster and more accurate calculations, and is more robust to the presence of usual artefacts than NIST's Integrated Colony Enumerator (NICE). Apart from its efficiency to count bacterial colonies, the program can also be used to enumerate other circular objects such as seeds or pollen (Geissmann, 2013; Puchkov, 2016). OpenCFU was designed to accelerate the calibration phase owing to its fast processing time and by immediately displaying results after parameters are changed (Geissmann, 2013). The program can deliver two different types of results: a summary or a detailed output. In the summary, each row of data contains the name of the analysed image, the number of colonies detected in this image and, if a mask was used, the surface of the mask. In the detailed output, each row of data corresponds to a different colony. Each colony is characterized by the name of the image it comes from and the surface of the mask used for the image. It is also useful for users needing the position (X, Y) of its centre, corrected median values of colour intensity, area, perimeter, and the number of colonies that were in the same cluster as the colony. This latter output is particularly helpful for users needing to perform advanced analyses (Geissmann, 2013; Puchkov, 2016).

The main limitations of this study are the inconsistency of reading due to the limited capability of the microscopic camera to focus on the object, and that no CFU count assays for *S. mutans* or *C. albicans* were conducted.

5. Conclusion

OpenCFU analysis can be used as a novel for analyzing the growth of oral biofilms, with several advantages, such as being a more faster, rapid, and practical than the standard techniques. As it can be seen on crystal violet and CFU manual counting assays, also showed a data similarity to OpenCFU analysis in accordance to the herbal propolis dentifrice impact on the growth of three biofilms species. This study has a drawback since no statistical analysis was performed.

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Declaration of Competing Interest

There are no conflicts of interest.

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