

# Mouthwashes: an in vitro study of their action on microbial biofilms and cytotoxicity to gingival fibroblasts

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This study evaluated the in vitro antibiofilm effect of 5 different commercial mouthwashes (Cepacol Traditional, Colgate Plax Fresh Mint, Listerine Cool Mint, Oral-B Complete, and Sensodyne) on *Candida albicans*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus mutans*, *Escherichia coli*, and *Pseudomonas aeruginosa*. The cytotoxic effect of the mouthwashes on gingival fibroblasts was also analyzed. A colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used to investigate the viability of biofilms after 48 hours and gingival fibroblasts after 24 hours. The biofilms were exposed to the mouthwashes for 2 different lengths of time: T1, the time recommended by the manufacturer (30 or 60 seconds); and T2, double the recommended time (60 or 120 seconds). All antiseptic mouthwashes caused a significant reduction of biofilm ( $P < 0.05$ ) as well as a significant reduction of viable gingival fibroblasts ( $P < 0.05$ ) with both exposure times (T1 and T2). It can be concluded that the commercial mouthwashes demonstrated effective antibiofilm activity; they were more effective on bacteria than on *C. albicans*. A significant cytotoxic effect on gingival fibroblasts was also observed.

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**Key words:** antibiofilm activity, cytotoxicity, gingival fibroblasts, gram-negative, gram-positive, mouthwashes

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**GENERAL DENTISTRY  
SELF-INSTRUCTION**



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**M**outhwashes are widely used as an additional tool for oral hygiene maintenance. These products have compounds (synthetic and/or natural) that act on microorganisms by inhibiting their growth and blocking some of their enzymatic reactions, thus playing an effective role in biofilm control.<sup>1</sup>

Biofilms are structured communities of microbial cells that adhere to a surface and exhibit phenotypic heterogeneity.<sup>2</sup> These microorganisms form a microecosystem where by-products (such as acids) that may affect the integrity of the surface are released. Besides being used to metabolize carbohydrates derived from the diet of the host, these by-products may cause accumulation into a biofilm. When present on the teeth, biofilms can contribute to demineralization and consequently the development of dental caries. One of the microorganisms involved in dental biofilm formation is *Streptococcus mutans*, which easily adheres to the tooth surface due to its synthesis of extracellular proteins.<sup>3</sup>

*Candida albicans*, a commensal yeast that is commonly found in mucosal layers, can be pathogenic in cases of immunodeficiency, such as in patients with human immunodeficiency virus (HIV), cancer, transplants, and/or prolonged hospitalization. In the oral cavity, *C. albicans* is responsible for clinical manifestations of both pseudomembranous and erythematous candidiasis.<sup>4</sup>

Isolates of *Staphylococcus aureus* have been obtained from supragingival and subgingival biofilms in patients with periodontitis as well as in hospitalized patients with respiratory infections.<sup>5,6</sup> *Staphylococcus aureus* is often associated with infections caused by implants or biomaterials.<sup>5</sup>

Another bacterium responsible for periodontal disease is *Enterococcus faecalis*, identified in root canal infections and apical periodontitis.<sup>7</sup> *Enterococcus faecalis* colonizes deeper layers of dentin, and it may have the capacity for tubular invasion, which negatively impacts endodontic treatments.<sup>8</sup>

*Escherichia coli* is a gram-negative bacterium that has a major virulence factor, lipopolysaccharide, present in the outer membrane. This endotoxin can cause endodontic diseases such as pulpal/periapical inflammation and periodontitis.<sup>9</sup>

Another gram-negative bacterium that may cause periodontal disease is *Pseudomonas aeruginosa*, whose presence in subgingival biofilm can induce an aggressive form of periodontitis.<sup>10</sup> When systemically disseminated from the oral cavity, *P. aeruginosa* may cause serious occurrences of primary respiratory infections in hospitalized and/or immunocompromised patients.<sup>11</sup>

Gingival fibroblasts comprise the main cellular lineage of the connective gingival tissue and play an important inflammatory role in periodontal disease.<sup>12</sup> Together with epithelial cells, they form the gingival mucosa. In cases of inflammation, there may

be a loss of integrity of the epithelium and consequent exposure of the underlying conjunctive tissue. Cellular reactions to local microbial attacks may cause inflammation.<sup>13</sup>

It is evident that appropriate control of these microorganisms is important for oral health, as they interfere with the integrity of the oral tissues, and cause significant disease at many sites in the oral cavity, as well as severe complications in other locations in the body due to dissemination. An adequate oral cavity hygiene regimen includes products that effectively remove, control, and/or eliminate these pathogens without causing damage to host tissues, resulting in optimal oral health and the prevention of many local and systemic diseases that may affect the welfare of an individual. The aim of this in vitro study was to evaluate the antibiofilm action of commercial mouthwashes on species of interest to oral health: the yeast *C albicans*; gram-positive bacteria *S aureus*, *S mutans*, and *E faecalis*; and gram-negative bacteria *E coli* and *P aeruginosa*. This study also aimed to evaluate the cytotoxic effects of these products on gingival fibroblasts.

## Materials and methods

### Mouthwashes and experimental groups

In vitro experiments were performed to evaluate 5 different commercial mouthwashes:

- Cepacol Traditional (Sanofi US): alcohol, cetylpyridinium chloride, eucalyptol, and menthol
- Colgate Plax Fresh Mint (Colgate-Palmolive): cetylpyridinium chloride and sodium fluoride
- Listerine Cool Mint (Johnson & Johnson): alcohol, eucalyptol, menthol, and thymol
- Oral-B Complete (Procter & Gamble): cetylpyridinium chloride and sodium fluoride
- Sensodyne (GlaxoSmithKline): cetylpyridinium chloride and sodium fluoride

The experiments were performed taking into consideration the time of use indicated by the manufacturer (T1: 30 seconds for Cepacol, Colgate, Listerine, and Sensodyne; 60 seconds for Oral-B). Because some consumers may use these mouthwashes for longer periods, the manufacturer's recommended time was also doubled (T2: 60 seconds for Cepacol, Colgate, Listerine, and Sensodyne; 120 seconds for Oral-B).

For the test on biofilms, chlorhexidine (0.12%) was used as a positive control and sterile saline (0.9% NaCl) as a negative control. For the test on gingival fibroblasts, chlorhexidine (0.12%) was used as a positive control and phosphate-buffered saline (PBS) was used as a negative control.

### Microbial strains

Standard strains of *C albicans* (ATCC 18804), *S aureus* (ATCC 6538), *S mutans* (ATCC 35688), *E faecalis* (ATCC 4083), *E coli* (ATCC 25922), and *P aeruginosa* (ATCC 15442) were used. *Candida albicans* was cultured in Sabouraud-dextrose agar (Himedia Laboratories) and bacterial strains were cultured in brain-heart infusion (BHI) agar (Himedia Laboratories) at 37°C/24 hours (5% CO<sub>2</sub> for *S mutans*). Strains were kept frozen (–80°C) in BHI with 20% glycerol for the bacteria and in yeast extract peptone dextrose broth with 16% glycerol (Himedia Laboratories) for *C albicans*.

### Biofilm formation

The colonies of *C albicans* were added to 5 mL of a yeast nitrogen base broth (Sigma-Aldrich), and the colonies of bacteria were added to BHI broth. After incubation at 37°C for 24 hours, the cultures were centrifuged (2000 rpm/10 min), the supernatant was discarded, and the pellets were suspended in 0.9% NaCl. Then, this solution was standardized at a concentration of 10<sup>7</sup> colony-forming units per milliliter in a B-582 spectrophotometer (Micronal), with an optical density (OD) variation of ±0.02. The following standards were used: *C albicans*, λ = 530 nm (OD 0.381); *S aureus*, λ = 490 nm (OD 0.477); *E faecalis*, λ = 760 nm (OD 0.385); *S mutans*, λ = 398 nm (OD 0.560); *E coli*, λ = 590 nm (OD 0.362); and *P aeruginosa*, λ = 630 nm (OD 0.090).

Biofilms were formed in wells of microtiter plates with the 5 experimental groups and the positive and negative control groups for each time of application (14 groups total), with 10 replicates in each group. For this purpose, 200 μL of standardized inoculum was added in each well for a period of 90 minutes at 37°C under shaking (75 rpm) to obtain preadhesion. Following this period, the supernatant was discarded, and a broth for biofilm growth was added; the broth was replaced every 24 hours during the incubation at 37°C/75 rpm. After 48 hours, the biofilms were separately exposed to the antiseptic mouthwashes, chlorhexidine, and NaCl for the defined application times (T1 or T2).

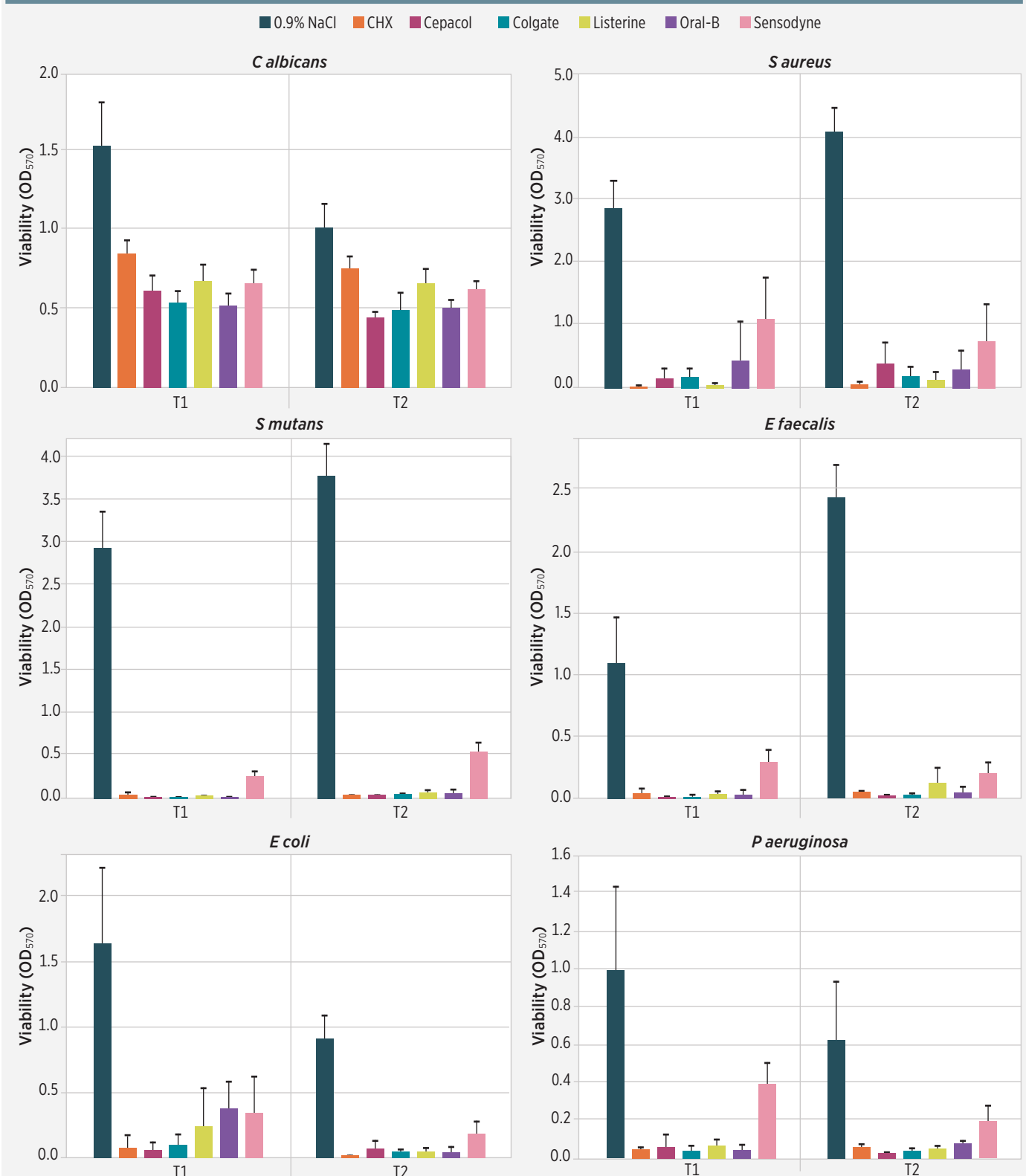
### Quantification of cell viability of microbial biofilms

The percentage of surviving cells after exposure to the antiseptic products was verified by the analysis of metabolism of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich). In each well, 200 μL of MTT solution (0.5 mg/mL in PBS) was added, and the groups were incubated (at 37°C for 1 hour) under light protection. This solution was discarded and dimethyl sulfoxide (Sigma-Aldrich) was added for the solubilization of products derived from biochemical activity promoted by the biofilm viable cells. After 20 minutes (10 minutes of incubation at 37°C plus 10 minutes of shaking), the OD reading (λ = 570 nm) of each well was performed in a microplate spectrophotometer (Bio-Tek). The results were converted into a percentage of cell viability based on the average obtained in the negative control group, which was considered to be the standard for 100% viability.

### Cytotoxicity of the mouthwashes to gingival fibroblasts

Gingival fibroblasts (FMM-1 cells) obtained from the Faculty of Dentistry of the University of São Paulo, Brazil, were used for verification of the cytotoxic profile of the mouthwashes. Cells were cultured in Dulbecco's Modified Eagle medium (LGC Biosearch Technologies) supplemented with 10% fetal bovine serum (Invitrogen) and 1% streptomycin (Gibco) with a humidified incubation (37°C/5% CO<sub>2</sub>). The 5 experimental and 2 control groups for each time of application (14 groups total) were distributed on a microtiter plate (Nunc) with 10 replicates in each group. Then 200 μL of cell suspension containing 2 × 10<sup>5</sup> viable cells, as determined by exclusion using a 0.4% Trypan blue test (Sigma-Aldrich), was added to each well. After incubation (37°C/24 hours and 5% CO<sub>2</sub>) to achieve cell adhesion, the

**Chart 1.** Mean optical density values obtained on biofilms exposed to antiseptic or control mouthwash (n = 10).



**Abbreviations:** CHX, chlorhexidine; NaCl, sodium chloride (normal saline); OD<sub>570</sub>, optical density ( $\lambda = 570 \text{ nm}$ ); T1, exposure time indicated by the manufacturer; T2, exposure time double that indicated by the manufacturer.

**Mouthwashes:** 0.12% CHX; Cepacol Traditional; Colgate Plax Fresh Mint; Listerine Cool Mint; Oral-B Complete; Sensodyne.

Error bars represent the standard deviation.

cultures were exposed to the mouthwash, the positive control agent (chlorhexidine), or the negative control agent (sterile PBS) for the defined time periods (T1 or T2). After the viability of the fibroblasts was measured by the MTT assay and after OD ( $\lambda = 570 \text{ nm}$ ) was obtained in the microplate, the cell viability percentage and rate of reduction after treatment were determined based on the average obtained in the PBS control group, which was considered to be the standard for 100% viability.

### Statistical analysis

The comparison of mean values obtained in the groups treated with antiseptics for each biofilm and culture of gingival fibroblasts was performed with an analysis of variance and a Tukey test ( $P \leq 0.05$ ).

### Results

The evaluated antiseptic mouthwashes, including chlorhexidine, demonstrated significant antibiofilm effects, resulting in significant reductions ( $P < 0.05$ ) in the viability of *C albicans*, *S aureus*, *S mutans*, *E faecalis*, *E coli*, and *P aeruginosa* biofilms compared to the 0.9% NaCl negative control group (Chart 1).

A significant reduction ( $P < 0.05$ ) was observed in cell culture viability of gingival fibroblasts compared to the PBS negative control group after exposure to antiseptic mouthwashes (Chart 2). The mean reduction percentages of microbial biofilms and fibroblasts are listed in Table 1. Table 2 shows the statistical analyses of these results.

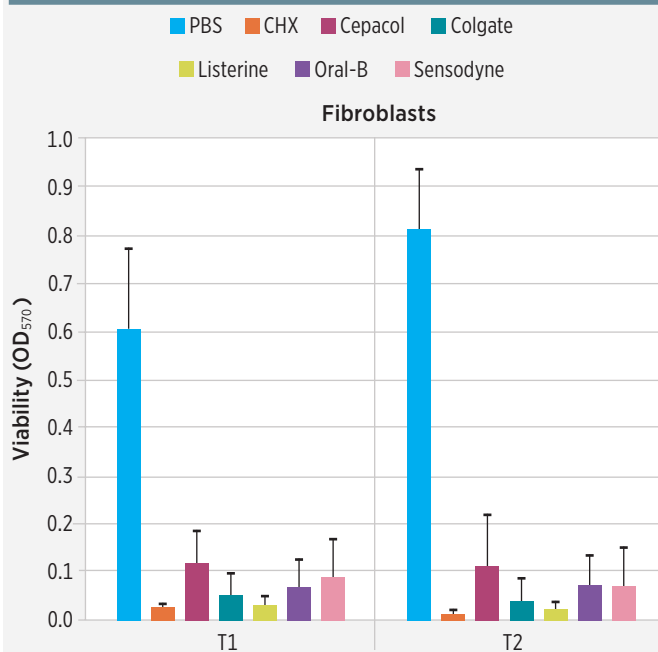
### Discussion

Although the tested mouthwashes differed somewhat as to the extent of in vitro reduction percentages, effective antibiofilm activity was observed with all the experimental groups in the present study. There were significant reductions in the cell viability of each microorganism compared to the 0.9% NaCl negative control group. Significant, high cytotoxic activity on cultures of gingival fibroblasts was observed for all experimental groups when compared to the PBS negative control group. This has been confirmed in other in vitro studies that have demonstrated that antiseptic mouthwashes both reduce biofilms and show cytotoxic activity on gingival fibroblasts.<sup>14-16</sup>

Some of the mouthwashes used in this study contain biocompound formulations of plant origin, such as eucalyptol (Cepacol and Listerine), menthol (Cepacol and Listerine), and thymol (Listerine). Biocompounds of vegetal origin have been used in the formulation of oral care products due to their known effects on the biofilm. These products penetrate the cell wall of a microorganism and settle between the fatty acid chains of the lipid bilayer, causing structural alterations of the cell membrane.<sup>17</sup> Changes in membrane fluidity and permeability directly affect the cell wall of the microorganism, resulting in loss of adhesion to the surfaces of the host.<sup>18</sup>

Eucalyptol (1,8-cineole) can be isolated from the essential oil of plants of the genus *Eucalyptus*.<sup>19</sup> Antimicrobial activity of this biocompound against *S aureus*, methicillin-resistant *S aureus* (MRSA), *P aeruginosa*, *E coli*, and *C albicans* has been confirmed.<sup>20</sup> Menthol is the predominant biocompound in the essential oil of some plants of the genus *Mentha* as well as other plant species such as *Artemisia nelagrica*, *Caesulia*

**Chart 2.** Mean optical density values obtained on gingival fibroblasts exposed to antiseptic or control mouthwash (n = 10).



**Abbreviations:** CHX, chlorhexidine; OD<sub>570</sub>, optical density ( $\lambda = 570 \text{ nm}$ ); PBS, phosphate-buffered saline; T1, exposure time indicated by the manufacturer; T2, exposure time double that indicated by the manufacturer.

**Mouthwashes:** 0.12% CHX; Cepacol Traditional; Colgate Plax Fresh Mint; Listerine Cool Mint; Oral-B Complete; Sensodyne.

Error bars represent the standard deviation.

*axillaris*, *Chenopodium ambrosioides*, and *Cymbopogon citratus*.<sup>21</sup> Its antimicrobial action on dermatophyte fungi, yeasts, and bacteria has been proven in other studies.<sup>22-24</sup> Thymol can be isolated from plants such as *Thymus vulgaris*, *Monarda punctata*, *Origanum vulgare*, and *Lippia sidoides*.<sup>25,26</sup> Thymol negatively impacts the development of streptococcal biofilms (*Streptococcus sanguinis*, *Streptococcus sobrinus*, and *S mutans*), lactobacilli (*Lactobacillus casei*, *Lactobacillus plantarum*, and *Lactobacillus coryniformis*), actinomyces (*Actinomyces odontolyticus* and *Actinomyces naeslundii*), and periodontal pathogens (*Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis*).<sup>27</sup> Its antimicrobial actions have also been observed on *E faecalis* and *S aureus* biofilms, as well as *E coli* in planktonic form.<sup>24-26</sup>

As mentioned previously, the exposure times used in the present experiments were the application time indicated by each manufacturer (T1, 30 or 60 seconds) and double the recommended application time (T2, 60 or 120 seconds). Different results were obtained after treatment with the antiseptics for both times for each microorganism.

Among the microorganisms evaluated, *C albicans* showed the lowest percentages of biofilm reduction. Mean reductions ranged from 44.47% (SD, 5.58%) to 66.05% (SD, 5.20%) for T1 applications and from 25.42% (SD, 7.67%) to 56.29% (SD, 4.23%)

**Table 1.** Mean (SD) reduction percentages of biofilms and gingival fibroblasts after treatment with antiseptic mouthwash (n = 10).\*

Group	Time	Antiseptic mouthwash					
		CHX	Cepacol	Colgate	Listerine	Oral-B	Sensodyne
<i>Candida albicans</i>	T1	44.47 (5.58) <sup>a</sup>	59.79 (6.39)	64.91 (5.11)	55.93 (6.56) <sup>a</sup>	66.05 (5.20) <sup>a</sup>	56.76 (5.41) <sup>a</sup>
	T2	25.42 (7.67) <sup>a</sup>	56.29 (4.23)	51.48 (10.17)	34.85 (9.89) <sup>a</sup>	50.04 (4.94) <sup>a</sup>	38.34 (5.86) <sup>a</sup>
<i>Staphylococcus aureus</i>	T1	99.10 (0.87)	94.73 (6.01)	93.77 (5.36)	98.26 (1.06)	84.49 (22.53)	61.47 (23.77) <sup>a</sup>
	T2	98.42 (1.66)	90.48 (8.36)	95.42 (4.16)	96.83 (3.72)	92.72 (7.51)	81.57 (14.47) <sup>a</sup>
<i>Streptococcus mutans</i>	T1	98.33 (0.67)	99.16 (0.31)	99.25 (0.06)	98.77 (0.52)	99.23 (0.57)	91.08 (2.47) <sup>a</sup>
	T2	98.84 (0.27)	98.81 (0.41)	98.61 (0.69)	98.02 (1.07)	98.31 (1.23)	85.47 (3.01) <sup>a</sup>
<i>Enterococcus faecalis</i>	T1	95.87 (3.83)	98.56 (0.49)	98.55 (1.79)	96.32 (2.76)	97.16 (4.37)	72.61 (9.55) <sup>a</sup>
	T2	97.90 (0.81)	99.22 (0.10)	98.80 (0.48)	94.86 (4.78)	98.22 (2.11)	91.72 (3.77) <sup>a</sup>
<i>Escherichia coli</i>	T1	95.36 (6.45)	96.28 (3.85)	94.10 (5.56)	85.46 (18.46)	76.91 (13.17) <sup>a</sup>	79.15 (17.66)
	T2	97.75 (0.74)	91.97 (6.88)	94.91 (3.11)	94.38 (3.05)	95.34 (4.93) <sup>a</sup>	80.01 (11.04)
<i>Pseudomonas aeruginosa</i>	T1	95.10 (0.94)	93.77 (6.72)	95.93 (2.57)	93.24 (3.58)	95.59 (3.08)	60.28 (11.10)
	T2	90.24 (2.86)	95.34 (1.29)	93.45 (2.69)	91.69 (2.49)	87.19 (2.85)	68.63 (13.49)
Gingival fibroblasts	T1	96.95 (1.86)	97.15 (1.51) <sup>a</sup>	96.02 (3.24)	94.32 (3.39)	97.03 (1.79)	95.70 (1.63)
	T2	93.90 (3.27)	92.41 (4.25) <sup>a</sup>	94.47 (2.50)	94.55 (1.39)	96.60 (1.71)	93.29 (1.96)

\*Reduction percentages are based on comparison to negative control (0.9% sodium chloride for biofilms; phosphate-buffered saline for gingival fibroblasts) considered to represent 100% viability.

**Abbreviations:** CHX, chlorhexidine; T1, exposure time indicated by the manufacturer; T2, exposure time double that indicated by the manufacturer.

**Mouthwashes:** 0.12% CHX; Cepacol Traditional; Colgate Plax Fresh Mint; Listerine Cool Mint; Oral-B Complete; Sensodyne.

<sup>a</sup>Statistically significant difference between treatment times for each microorganism or cell in each treated group (ie, within column) (analysis of variance and Tukey test;  $P \leq 0.05$ ).

for T2 applications. Ramage et al compared the in vitro action of antifungal agents and mouthwashes on clinical strains of *C albicans* and found a greater effect of antifungals on suspensions than on biofilm; however, the mouthwashes demonstrated better results in reducing the viability of the biofilm (approximately 80%).<sup>14</sup> Those researchers used the concentrations and contact times specified by the manufacturers, similar to T1 in the present study. Studies have shown that resistance of candidal species to antifungal agents is related to the extracellular polysaccharide matrix of the biofilm, which restricts the penetration of these agents; a reduced ability of the agents to limit the growth or nutrient supply of the yeast; expression of resistance genes, especially those related to efflux pumps; and the presence of a small population of cells capable of resistance to the action of the antifungal agent.<sup>28</sup>

Significant reductions in the biofilm of *S aureus* were observed in all treated groups. In the present study, the mean reductions ranged from 61.47% (SD, 23.77%) to 99.10% (SD, 0.87%) for T1 and from 81.57% (SD, 14.47%) to 98.42% (SD, 1.66%) for T2. In 2013, Smith et al analyzed the action of mouthwashes on clinical strains of MRSA and found that no product completely eliminated biofilms; reductions were in the range of 70%.<sup>15</sup> In the present study, the action of mouthwashes on biofilms proved to be an important agent of control of this microorganism. The nostrils are major reservoirs of *S aureus*, but it also can be found in the oral cavity and can spread from there to other parts of the body or to other individuals, possibly causing clinical manifestations.<sup>29</sup> Individuals

with periodontitis or prosthetic restorations are more virulent oral carriers of this microorganism than healthy individuals.<sup>29</sup> Although it is more commonly present in the oral cavity than in the gingival sulcus, *S aureus* can be isolated from supragingival and subgingival biofilms.<sup>5</sup> Hospitalized individuals may develop lower respiratory tract infections caused by *S aureus* cells detached from dental and periodontal biofilms.<sup>6</sup> Another study found that detached biofilm cells of *S aureus* from dentures can be transmitted by oral fluids to the lower respiratory tract, causing pneumonia by aspiration.<sup>30</sup> As stated previously, *S aureus* is often associated with infections caused by implants or biomaterials.<sup>5</sup>

A significant reduction of *S mutans* biofilm resulted from the application of all the antiseptics used in this study. The mean reductions of this biofilm ranged from 91.08% (SD, 2.47%) to 99.25% (SD, 0.06%) for T1 and from 85.47% (SD, 3.01%) to 98.84% (SD, 0.27%) for T2. The use of mouthwashes in vitro also contributed to the reduction of *S mutans* in a 2014 study performed by Yang et al, who found significant (between 0.05- and 5.54-log) reductions in biofilms.<sup>31</sup> According to the authors, values above a 5.00-log reduction represented complete elimination of the biofilm. The effectiveness of mouthwashes against *S mutans* may contribute to the decrease of the infections caused by this microorganism. Antimicrobial agents may prevent and treat caries and periodontal disease by impairing the adhesion of the microorganism to surfaces, thereby controlling growth and consequently affecting biofilm formation, which also favor the reduction of clinical symptoms.<sup>32</sup>

**Table 2.** Statistical analyses among the antiseptic mouthwash groups in this study.

Compared mouthwashes		<i>Candida albicans</i>		<i>Staphylococcus aureus</i>		<i>Streptococcus mutans</i>		<i>Enterococcus faecalis</i>		<i>Escherichia coli</i>		<i>Pseudomonas aeruginosa</i>		Gingival fibroblasts	
		T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2
CHX	Cepacol	S	S	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
CHX	Colgate	S	S	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
CHX	Listerine	S	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
CHX	Oral-B	S	S	NS	NS	NS	NS	NS	NS	S	NS	NS	NS	NS	NS
CHX	Sensodyne	S	S	S	S	S	S	S	S	NS	S	S	S	NS	NS
Cepacol	Colgate	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Cepacol	Listerine	NS	S	NS	NS	NS	NS	NS	S	NS	NS	NS	NS	NS	NS
Cepacol	Oral-B	NS	NS	NS	NS	NS	NS	NS	NS	S	NS	NS	S	NS	NS
Cepacol	Sensodyne	NS	S	S	NS	S	S	S	S	S	S	S	S	NS	NS
Colgate	Listerine	S	S	NS	NS	NS	NS	NS	S	NS	NS	NS	NS	NS	NS
Colgate	Oral-B	NS	NS	NS	NS	NS	NS	NS	NS	S	NS	NS	NS	NS	NS
Colgate	Sensodyne	S	S	S	S	S	S	S	S	NS	S	S	S	NS	NS
Listerine	Oral-B	S	S	S	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Listerine	Sensodyne	NS	NS	NS	S	S	S	S	NS	NS	S	S	S	NS	NS
Oral-B	Sensodyne	S	S	S	S	S	S	S	S	NS	S	S	S	NS	NS

**Abbreviations:** CHX, chlorhexidine; NS, not significant; S, significant; T1, exposure time indicated by the manufacturer; T2, exposure time double that indicated by the manufacturer.

**Mouthwashes:** 0.12% CHX; Cepacol Traditional; Colgate Plax Fresh Mint; Listerine Cool Mint; Oral-B Complete; Sensodyne.

Analysis of variance and Tukey test ( $P \leq 0.05$ ).

It is known that the tooth surface is a site for *S mutans* colonization and subsequent biofilm formation. This microorganism also may accumulate in supragingival and subgingival areas and is capable of causing dental caries and periodontal disease together with *Actinomyces* spp and *P gingivalis*, respectively.<sup>13,33</sup>

A significant reduction of *E faecalis* biofilm was observed after treatment with the mouthwashes in the present study. The mean reduction ranged from 72.61% (SD, 9.55%) to 98.56% (SD, 0.49%) for T1 and from 91.72% (SD, 3.77%) to 99.22% (SD, 0.10%) for T2. These reductions demonstrate that the mouthwashes may contribute to the control of this bacterium in the oral cavity. Although it is not an inherent resident of this site, *E faecalis* is usually disseminated through food and other materials prepared with inadequate attention to hygiene; once in the oral cavity, *E faecalis* can reach the root canals and cause persistent endodontic infections as well as apical periodontitis.<sup>7,34</sup> In an in vitro study, Valera et al verified that *E faecalis* biofilm was eliminated from root canals with the application of commonly used irrigants, such as chlorhexidine and sodium hypochlorite.<sup>35</sup> The authors also reported that natural products such as castor oil extract (*Ricinus communis*) and ginger extract (*Zingiber officinale*) showed effective antimicrobial action.<sup>35</sup> Their results were similar to those of the present study, which demonstrated that all the mouthwashes tested (with or without natural antimicrobial agents) were effective in the in vitro reduction of *E faecalis* biofilm.

In all the treated groups, the biofilms of *E coli* showed statistically significant reductions. The mean reductions ranged from 76.91% (SD, 13.17%) to 96.28% (SD, 3.85%) for T1 and from 80.01% (SD, 11.04%) to 97.75% (SD, 0.74%) for T2. Pires et al studied the action of a mouthwash and found that the product (diluted 1:20 in artificial saliva) was enough to completely eliminate *E coli*.<sup>36</sup> The control of this bacterium can contribute to the reduction of endodontic and periodontal diseases, since a constituent of its membrane, lipopolysaccharide, is responsible for endodontic infections such as pulpal and periapical disease.<sup>37</sup>

All the experimental products resulted in significant reductions of *P aeruginosa* biofilm; mean reductions ranged from 60.28% (SD, 11.10%) to 95.93% (SD, 2.57%) for T1 and from 68.63% (SD, 13.49%) to 95.34% (SD, 1.29%) for T2. Baffone et al studied the antibiofilm action of different mouthwashes on adhesion of *P aeruginosa* to a titanium surface, and the inhibitory rates ranged between 40.26% and 100.0%.<sup>38</sup> As stated previously, *P aeruginosa* has the ability to cause periodontal disease.<sup>10</sup> A high prevalence of this bacteria was detected in the subgingival biofilm of patients with HIV who also had chronic periodontitis.<sup>39</sup> It has been shown that the presence of *P aeruginosa* in the subgingival microbiota can increase the chances that an individual will present with aggressive periodontitis.<sup>10</sup> It is known that the interaction between *P aeruginosa* and periodontal pathogens can increase the invasive capacity of *P aeruginosa* in epithelial cells, favoring systemic dissemination.<sup>40</sup> Another result of this

interaction is the adhesion and internalization of respiratory pathogens in epithelial cells; these pathogens cause respiratory infections, especially in individuals with poor oral hygiene and periodontal disease, conditions commonly found in hospitalized and immunocompromised patients.<sup>11,40</sup>

When the action of mouthwashes on cultured gingival fibroblasts was examined, it was found that all the evaluated products caused a significant reduction of the cell viability; mean reductions of this culture ranged from 94.32% (SD, 3.39%) to 97.15% (SD, 1.51%) for T1 and from 92.41% (SD, 4.25%) to 96.60% (SD, 1.71%) for T2. In an in vitro study to verify the cytotoxicity of 2 essential oil-based or chlorhexidine-based mouthwashes on gingival fibroblasts, Tsourounakis et al found that there was a complete elimination of these cells when the concentrations of the tested products were greater than 25% (exposure time 60 seconds).<sup>16</sup> Even at lower concentrations, these mouthwashes resulted in morphologic changes, decreased viability, and loss of cell function.<sup>16</sup>

## Conclusion

All evaluated mouthwashes showed effective antibiofilm activity against *S aureus*, *S mutans*, *E faecalis*, *E coli*, *P aeruginosa*, and *C albicans*, although the antiseptics were more effective on bacteria than on the yeast. All the mouthwashes used were cytotoxic to gingival fibroblasts. These findings demonstrate the importance of consumer awareness about using these types of products correctly with respect to concentration and application time.

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## Disclaimer

The authors report no conflicts of interest pertaining to any of the products or companies discussed in this article.

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