



## Inhibition of Biofilm Formation from *Streptococcus mutans* by Mouthwashes *in Vitro*

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Type of Publication: Original Research Paper

Conflicts of Interest: Nil

### Abstract

Most commercial mouthwashes are claimed to be effective in reducing the accumulation of bacteria and the occurrence of dental decay. This study was aimed to test the antibiofilm formation and antibacterial properties of 4 types of mouthwashes *in vitro*. *Streptococcus mutans* was used in the experiment since it is the primary causative agent of biofilm formation and dental caries. Antibacterial activity of each mouthwash was examined by broth microdilution method. Biofilm formation inhibition test was modified from microtiter dish biofilm formation assay. *S. mutans* which was adjusted to be  $2.0 \times 10^6$  CFU/ml, and was inoculated in Todd Hewitt broth with 3% sucrose (w/v) at 37 °C for 48 hours. Then, the optical density of biofilm was measured by a microplate reader at 570 nm. The results revealed that all 4 mouthwashes were able to inhibit biofilm formation. The MBICs of Betadine Gargle, Listerine, Colgate and C-20 were at the dilution of 1:640, 1:320, 1:320 and > 1:1280, respectively. For the antibacterial examination, the amount of growth in each well was also measured as optical density by a microplate reader at 600 nm. The MIC of Betadine Gargle, Listerine, Colgate, and C-20 were at the dilution of < 1:10, 1:10, 1:160, and 1:640, respectively. In conclusion, these *in vitro* studies demonstrated that all 4 mouthwashes were very effective in antibiofilm formation and some also had ability to inhibit growth of the cariogenic bacteria.

**Keywords:** biofilm formation, mouthwash, minimum biofilm inhibitory concentration, MBIC, MIC, *Streptococcus mutans*

### INTRODUCTION

Dental caries can be found frequently in people at all ages. People sometimes consider this as insignificant pain. However, it can lead to serious health problems and cannot be overlooked. The prevalence of dental caries among 151 Thai children aged 9-18 months evaluated was 32.5%, 15.9% had at least one cavity (cavitated caries) and 16.6% had white lesions (non-cavitated caries) [1]. The most prevalent and consequential oral diseases globally are dental caries (tooth decay), periodontal disease, tooth loss, and cancers of the lips and oral cavity [2]. As with most non-communicable diseases (NCDs), oral conditions are chronic and strongly socially patterned. Children

living in poverty, socially marginalized groups, and older people are the most affected by oral diseases and have poor access to dental care [2]. Their oral hygiene was usually poor, indicated by high levels of dental biofilm and high numbers of mutans streptococci. Because of the failure to incorporate oral health into general health promotion, millions suffer intractable toothache and poor quality of life and end up with few teeth [3]. *S. mutans* is a cariogenic bacterium that plays an important role in the beginning of dental caries, both in fissures and on smooth enamel surfaces [4].

These are all the reasons dental caries should be prevented and treated with caution. Varieties of mouthwash firms and corporations have claimed their products to have various properties, such as anti-cavity, antibacterial, and more. We hope to determine the antibiofilm formation and antibacterial activity of these products and its active ingredients.

## BACKGROUND

*S. mutans* has been implicated as a primary causative agent of dental caries in humans, and one of its important virulence properties is an ability to form biofilm known as dental plaque on tooth surfaces. The bacterium synthesizes adhesive glucan from sucrose by the action of glucosyltransferases (GTFs), then glucans mediate firm adherence of its cells to tooth surfaces. *S. mutans* also produce multiple glucan-binding proteins (Gbp proteins), which are thought to promote adhesion. Furthermore, the cell surface protein antigen c (PAC), a major surface protein of *S. mutans*, is correlated to its virulence in regard to development of dental caries, as it is known to participate in bacterial adherence to tooth surfaces via interaction with the salivary pellicle. Together, these bacterial surface proteins coordinate to produce dental plaque, thus inducing dental caries [5].

*Streptococcus mutans* has been strongly implicated as the principal etiological agent in human dental caries [6]. One of the important virulence properties of these organisms is their ability to form biofilm known as dental plaque on tooth surfaces [7, 8]. Dental plaque is one of the best-studied biofilms [9, 10].

Biofilm microbes are typically surrounded by an extracellular matrix that provides structure and protection to the community. Biofilm-grown microbes are also notorious for their resistance to a range of antimicrobial agents including clinically relevant antibiotics. The microtiter dish assay is an important tool for the study of the early stages in biofilm formation and has been applied primarily for the study of bacterial biofilms. Furthermore, published work indicates that biofilms grown in microtiter dishes do develop some properties of mature biofilms, such as antibiotic tolerance and resistance to immune system effectors [11].

Chlorhexidine gluconate was used as a positive control for the biofilm test as it is proven to be effective against biofilm formation. A study conducted by

Martínez-Hernández M, Reda B, Hannig M. reported that, rinsed with 0.2% chlorhexidine significantly reduced biofilm formation on enamel. Both biofilm colonization and vitality were dramatically impaired. Moreover, a considerable biofilm disruption induced by the chlorhexidine rinses was observed. Remarkably, a single application of chlorhexidine to a 48-h mature biofilm causes biofilm ultrastructure alterations and induces a substantial reduction in biofilm thickness and bacterial vitality [12].

## MATERIALS AND METHODS

Researcher team used quantitative methods to gather data regarding the amount of biofilm formation and the number of bacteria in wells of each solution, both with and without mouthwash. Our experiments were divided into two primary parts, biofilm formation test and antibacterial test. After undergoing all processes of the experiment, the amount of biofilm and the number of bacteria in each well were measured as optical density (OD) at 570 nm and 600 nm by using a microplate reader.

4 Mouthwashes and control:

- Mouthwash 1: Betadine Gargle
- Mouthwash 2: Listerine Cool Mint
- Mouthwash 3: Colgate Plax
- Mouthwash 4: C-20 Blue Sally (0.12% chlorhexidine gluconate, as positive control)
- Negative control: 0.01% DMSO of the media.

### Bacterial stain

Laboratory control strains *S. mutans* (ATCC 25175) were purchased from American Type Culture Collection (Rockville, Md., USA). Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to Mueller-Hinton broth (MHB, Oxoid, Basingstoke, UK) that were incubated under 5% CO<sub>2</sub> for 48 h at 37°C. The bacterial cultures were diluted with the media to achieve optical densities corresponding to 2.0 x 10<sup>6</sup> CFU/ml. Todd Hewitt broth (THB, Oxoid) with 3% sucrose (w/v) was used for antibiofilm formation test but MHB was used for antibacterial activity test.

### Antibiofilm formation

The biofilm inhibition of 4 mouthwashes (Betadine Gargle, Listerine, Colgate and C-20) were determined

using the method modified from O'Toole [11]. Briefly, initial candidate mouthwash was prepared in dimethyl sulfoxide (DMSO, Sigma Aldrich, USA), and subsequent two-fold dilution was performed with 2 ml of DMSO. 20 µl of a two-fold concentration of mouthwash (undiluted, 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640) was pipetted to the prepared 96-well microtiter test plates. Then each well was incubated with 180 µl of suspended *S. mutans* culture in THB with 3% sucrose. C-20 (0.12% chlorhexidine gluconate) was used as positive control. DMSO was used as a negative control. The 96-well plate was incubated under 5% CO<sub>2</sub> for 48 h at 37°C without agitation. The biofilm was washed with phosphate buffer saline pH 7.4 (PBS) and then stained with 200 µl of 0.1% crystal violet for 15 minutes, then rinsed with PBS for 3 times. After that, a decolorizer, 200 µl of 30% acetic acid was added to each well. All experiments were set up in triplicate. The amount of biofilm formation of each well was determined by assessment of turbidity by optical density readings at 570 nm with a Biochrom Asts Expert Plus Microplate Reader. The amount of biofilm in each well was compared with that in the positive biofilm control. The MBIC recorded the lowest concentration of the mouthwash that its OD was lower than that of the next lower concentration at least 2 times.

### Antibacterial Activity

The minimum inhibitory concentrations (MIC) of mouthwashes were determined using the broth microdilution method in 96-well microtiter plates which was modified from Wiegand I, et.al [13]. Briefly, initial candidate mouthwash was prepared in DMSO, and subsequent two-fold dilution was performed with 2 ml of DMSO. 20 µl of a two-fold concentration of mouthwash (undiluted, 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640) was pipetted to the prepared 96-well microtiter test plates. Then each well was incubated with 180 µl of suspended *S. mutans* culture in MHB. C-20 (0.12% chlorhexidine gluconate) was used as positive control. DMSO was used as a negative control. The 96-well plate was incubated under 5% CO<sub>2</sub> for 48 h at 37°C. All experiments were set up in triplicate. The resulting turbidity was observed, and after 48h, MIC was determined by assessment of turbidity by optical density readings at 600 nm with a Biochrom Asts Expert Plus Microplate Reader. The amount of growth in each well was compared with that in the positive

growth control. The MIC recorded the lowest concentration of the mouthwash that its OD was lower than the next lower concentration at least 2 times.

## RESULTS

### Antibiofilm Formation Activity

*Figure 1 : OD Level of biofilm formation in each mouthwash and dilutions*

### Antibacterial Activity

*Figure 2 : OD Level of broth microdilution method in each mouthwash and dilutions*

Data could be read from Figure 1&2:

**OD** or Optical Density is a measure of how much color has been stained. The concentration of color stains is proportional to the amount of pathogen that was present in the sample.

**MBIC** or Minimum Biofilm Inhibitory Concentration is the lowest concentration of an agent that can inhibit biofilm formation.

**MIC** or Minimum Inhibitory Concentration is the lowest concentration of an agent that can inhibit visible growth of the microorganism.

*Table 1 MBIC, MIC and Active ingredients of each mouthwash*

## DISCUSSION

Our biofilm formation study showed that the positive control (C-20) was the best inhibitor as it should be because chlorhexidine was proven to be effective against biofilm formation [12]. C-20 was most effective because its active ingredient was 0.12% chlorhexidine gluconate, which is a germicidal mouthwash that reduces bacteria in the mouth. The MBIC of C-20 could not be determined because even the highest dilution (1:1280) of chlorhexidine could inhibit biofilm formation as high as 76.099%. To collect this data, more serial dilution (1:2560, 1:5120, etc.) have to be done. Excluding positive control, Listerine was the best inhibitor at the dilution of 1:320 and lower. However, at the dilution of 1:640 Betadine Gargle and Colgate inhibited biofilm formation better than Listerine. At the dilution of 1:640, percent biofilm inhibition of Betadine Gargle and Colgate was 91.312% and 65.284%, while that of Listerine was only 19.326%. We could also see that Betadine Gargle could inhibit biofilm formation more effectively than

Colgate. The MBIC of Betadine Gargle, Listerine, and Colgate could be measured as percent biofilm inhibition drops below 70% at the dilution of 1:640, 1:320, and 1:320, respectively. Thus, Betadine Gargle was very effective mouthwash as it has the lowest MBIC. Even though the lower dilution should have higher biofilm inhibition activity than the higher one, the dilution of 1:10 in all mouthwash had lower biofilm inhibition than the dilution of 1:20. This may be because other components in the mouthwash could be able to stain crystal violet color the same as biofilm. Therefore, the presented color could possibly be more than the actual biofilm formed. In our present study, Listerine and Colgate were still able to inhibit more than 70% of biofilm formation even at a minute concentration or high solution. It could be implied that these mouthwashes may have certain active ingredients that could be very effective to inhibit biofilm formation. Additional study of the activity of active ingredients in the aspect of biofilm formation is also required for commercial mouthwash.

The efficiency of antibacterial activity of mouthwashes was also demonstrated. At the dilution of 1:10 and higher, Betadine Gargle seemed to be the least effective bacteria inhibitor as its OD values were almost equal to the OD value of negative control. We could only assume that Betadine Gargle had no effect on *S. mutans* since an experiment on higher concentrations was not conducted. The MIC of Listerine, Colgate, and C-20 was at the dilution of 1:10, 1:160, and 1:640, respectively. Therefore, C-20 was the best inhibitor followed by Colgate, Listerine, and Betadine Gargle in order.

## CONCLUSION

In conclusion, our study illustrated that all tested mouthwashes had the potential to inhibit biofilm formation, even at minute concentrations. The data also showed that most mouthwashes were effective in the growth inhibition of the cariogenic bacteria. Of all these 4 mouthwashes, C-20 exhibited the greatest anticariogenic activity, including antibiofilm formation and antibacterial activity. Further testing of Betadine Gargle in higher concentrations is required to determine its antibacterial activity. We hope our findings may serve as a guide for mouthwash selection as well as contributing to future research.

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antimicrobial substances. Nat Protoc. 2008;3(2):163-75.

**TABLE**

**Table 1: MBIC, MIC and Active ingredients of each mouthwash**

Mouthwash	Betadine Gargle	Listerine	Colgate	C-20
Active ingredient (% w/v)	0.5% povidone-iodine	0.092% eucalyptol 0.042% menthol 0.060% methyl salicylate 0.064% thymol	0.075% cetylpyridinium chloride 0.05% sodium fluoride	0.12% chlorhexidine gluconate
MBIC	1:640	1:320	1:320	>1:1280
MIC	<1:10	1:10	1:160	1:640

**FIGURES**

**Figure 1: OD Level of biofilm formation in each mouthwash and dilutions**

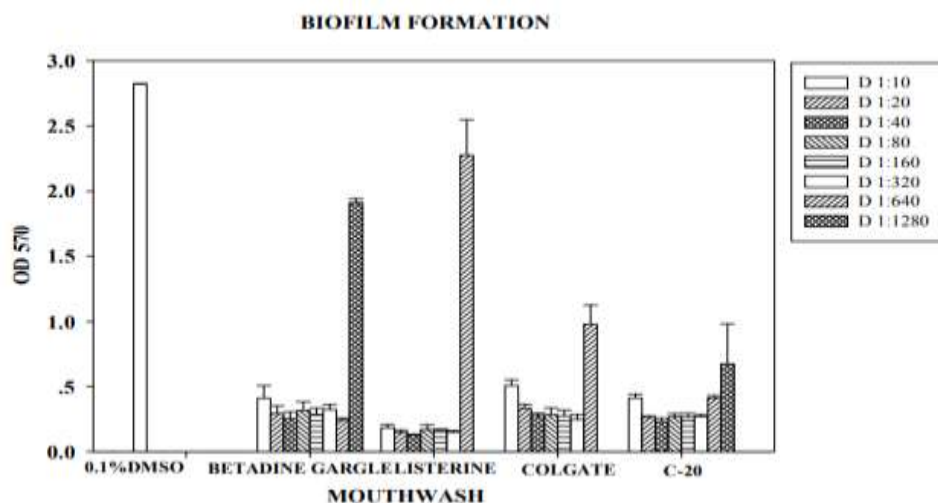


Figure 2 : OD Level of broth microdilution method in each mouthwash and dilutions

