EFFECT OF IN-OFFICE BLEACHING GELS ON BIOFILM FORMATION ON ENAMEL

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ผลของเจลฟอกสีฟันในคลินิกต่อการสร้างแผ่นชีวภาพบนผิวเคลือบฟัน

นางสาวศุทธินี อิทธาถิรุธ

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ศุทธินี อิทธาถิรุธ : ผลของเจลฟอกสีฟันในคลินิกต่อการสร้างแผ่นชีวภาพบนผิวเคลือบฟัน. (EFFECT OF IN-OFFICE BLEACHING GELS ON BIOFILM FORMATION ON ENAMEL) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.อรนาฎ มาตังคสมบัติ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร.พนิดา ธัญญศรีสังข์, 51 หน้า.

วัตถุประสงค์ เพื่อเปรียบเทียบความหยาบพื้นผิวของผิวเคลือบฟัน และการสร้างแผ่นชีวภาพของเชื้อสเตร็ปโตคอคคัสบนผิวเคลือบฟันหลังจากฟอกสีฟันในคลินิกด้วยไฮโดรเจนเปอร์ออกไซด์ความเข้มข้นร้อยละ 25 และ 35 วัสดุและวิธีการ นำฟันหน้าแท้และฟันกรามน้อยแท้ที่ถูกถอนมาตัดขนาด 3x3x2 มม^3 ได้จำนวน 162 ชิ้น แบ่งอย่างสุ่มออกเป็น 3 กลุ่ม กลุ่มละ 54 ชิ้น ด้วยวิธีการสุ่ม คือ กลุ่มที่1 (กลุ่มควบคุม) แช่ในน้ำเกลือ กลุ่มที่2 ฟอกสีฟันด้วยไฮโดรเจนเปอร์ออกไซด์ความเข้มข้นร้อยละ 25 (Zoom2™) และกลุ่มที่3 ฟอกสีฟันด้วยไฮโดรเจนเปอร์ออกไซด์ความเข้มข้นร้อยละ 35 (Beyond™) โดยวัดความหยาบพื้นผิวทั้งก่อนและหลังการฟอกด้วยเครื่องวัดความหยาบพื้นผิว ต่อมาในแต่ละกลุ่มถูกแบ่งอย่างสุ่มเป็น 3 กลุ่มย่อย กลุ่มละ 18 ชิ้น ดังนี้ กลุ่มที่ 1 กลุ่มควบคุม บ่มด้วยอาหารเลี้ยงเชื้อที่ปราศจากเชื้อ กลุ่มที่ 2 บ่มด้วยเชื้อสเตร็ปโตคอคคัส มิวแทนส์ และกลุ่มที่ 3 บ่มด้วยเชื้อสเตร็ปโตคอคคัส แซงกวินิสเป็นเวลา 24 ชม. โดยวัดปริมาณการสร้างแผ่นชีวภาพโดยการย้อมสีด้วยคริสตัลไวโอเลต และตรวจโครงสร้างของแผ่นชีวภาพด้วยกล้องอิเล็กตรอนแบบส่องกราดกลุ่มละ 3 ชิ้น วิเคราะห์ข้อมูลทางสถิติด้วยครัสคาลวัลลิส แมนวิทนีย์ยู และบอนเฟอโรนี่ คอร์เร็คชั่น ที่ระดับนัยสำคัญทางสถิติที่ $p<0.05$ ผลการทดลอง การฟอกสีฟันในคลินิกทั้งสองระบบทำให้ความหยาบของผิวเคลือบฟันลดลงอย่างมีนัยสำคัญทางสถิติ เมื่อเทียบกับกลุ่มควบคุม ($p<0.001$) แต่ไม่มีความแตกต่างกันทั้งสองระบบ ไม่พบความแตกต่างในการสร้างแผ่นชีวภาพของเชื้อสเตร็ปโตคอคคัส มิวแทนส์ ทั้งสามกลุ่ม แต่พบว่าการสร้างแผ่นชีวภาพของเชื้อสเตร็ปโตคอคคัส แซงกวินิส ในกลุ่มที่มีการฟอกสีฟันด้วยไฮโดรเจนเปอร์ออกไซด์ด้วยความเข้มข้นร้อยละ 35 มีค่าสูงกว่าอีกสองกลุ่มอย่างมีนัยสำคัญทางสถิติ ($p<0.001$) สรุป การฟอกสีฟันในคลินิกทั้งสองระบบทำให้ผิวเคลือบฟันเรียบขึ้น แต่เมื่อฟอกด้วยไฮโดรเจนเปอร์ออกไซด์ ที่ความเข้มข้นร้อยละ 35 มีการสร้างแผ่นชีวภาพของเชื้อเตร็ปโตคอคคัส แซงกวินิสเพิ่มขึ้น

สาขาวิชา ทันตกรรมบูรณะเพื่อความสวยงามและทันตกรรมรากเทียม ลายมือชื่อนิสิต .....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5376148532 : MAJOR ESTHETIC RESTORATIVE AND IMPLANT DENTISTRY
KEYWORDS: BIOFILM FORMATION / BLEACHING / ENAMEL / HYDROGEN PEROXIDE

SUTTINEE ITTATIRUT: EFFECT OF IN-OFFICE BLEACHING GELS ON BIOFILM FORMATION ON ENAMEL. ADVISOR: ASSOC. PROF. ORANART MATANGKASOMBUT, Ph.D., CO-ADVISOR: PANIDA THANYASRISUNG, Ph.D., 51 pp.

Objective: To compare the effects of 25% and 35% hydrogen peroxide in-office bleaching systems on surface roughness and streptococcal biofilm formation on human enamel. Materials and Methods: Human anterior teeth and premolars were cut into 3x3x2 mm^3. Enamel specimens (n=162) were randomly divided into 3 groups (n=54 each): control, bleached with 25% hydrogen peroxide (Zoom2™, Discus Dental, Culver City, CA, USA), and bleached with 35% hydrogen peroxide (Beyond™, Beijing, China). The surface roughness was measured by a profilometer before and after treatments. The specimens were then placed randomly into 3 subgroups (n=18 each) and incubated with trypticase soy broth control, *Streptococcus mutans*, or *Streptococcus sanguinis*, for 24 hours. Biofilm formation was quantified by crystal violet assay. The biofilm structure was also visualized by scanning electron microscopy (n=3 each). Data were analyzed by Kruskal-Wallis and Man-Whitney U tests with Bonferroni corrections with significance level at $p<0.05$. Results: Both bleaching systems significantly reduced enamel surface roughness comparing to control ($p<0.001$). Remarkably, *Streptococcus sanguinis* biofilm on enamel bleached with 35% hydrogen peroxide was significantly higher than other groups ($p<0.001$). In contrast, no difference in *Streptococcus mutans* biofilm was observed. Conclusion: Both 25% and 35% hydrogen peroxide caused similar degrees of reduction in enamel surface roughness, but 35% hydrogen peroxide markedly promoted *Streptococcus sanguinis* biofilm formation.

Field of Study:	Esthetic Restorative and Implant Dentistry	Student's Signature	.....
		Advisor's Signature	.....
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CONTENTS

Page

Page

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I
INTRODUCTION

Rationale and significance of the problem

In recent years, vital tooth bleaching has become a popular treatment option for discolored teeth and is often requested by a large number of patients. Several in-office bleaching, home bleaching and over-the-counter bleaching products are available.[1] Hydrogen peroxide (H_2O_2) or carbamide peroxide (CP) agent is used at different concentrations in various systems. High concentrations of bleaching agents, such as 35% CP or 25-35% H_2O_2, is generally used for in-office bleaching techniques[1], whereas home bleaching techniques use 10-20% CP.[2] Due to the higher concentrations, in-office bleaching can be done in short treatment time and is suitable for patients who seek immediate whitening.[3]

Several *in vitro* studies demonstrated that home-bleaching agents using low concentrations of H_2O_2 or CP do not alter the enamel surface morphology.[4-8] However, inconsistent results were observed when the effect of high concentrations of bleaching agents on enamel was examined. An *in vitro* study found no alteration on enamel surface hardness and roughness after bleaching with strip (Crest Whitestrips Supreme, Procter and Gamble, Cincinnati, Ohio, USA), tray (Opalescence 20% PF, Ultradent, South Jordan, Utah, USA), or in-office (Opalescence X-Tra Boost, Ultradent) bleaching systems.[9] Two *in vivo* studies examining epoxy replicas of tooth surfaces showed no significant difference in surface roughness after using 35% CP or 38% H_2O_2.[10, 11] On the contrary, the alteration of enamel surface roughness and demineralization was found after bleaching with 35% CP *in vitro*.[12, 13]

Moreover, one study found that, although enamel surface roughness was not affected by bleaching with 16% CP or 35% H_2O_2 alone, it was increased after brushing suggesting that wear resistance was reduced.[14] These alterations of enamel surface could affect bacterial adhesion and susceptibility to dental caries.

Oral bacteria form biofilm on enamel surface and increase the risk of caries and periodontal disease. Only a few studies investigated biofilm formation on enamel surface after bleaching. A clinical study in Turkey showed that plaque accumulation scores were significantly higher after a 5-day non-brushing period in the group bleached with 35% H_2O_2.[15] An *in vitro* study of bacterial adhesion on enamel after treated with 10% CP found that there was no significant difference in surface

roughness between groups, but there was significantly higher adhesion of *Streptococcus mutans* (*S. mutans*) in the test group.[16] In another *in vitro* study, both surface roughness and adherence of *S. mutans* were increased when enamel was treated with 35% H_2O_2, but no linear correlation was observed between roughness and bacterial adhesion.[17] To our knowledge, no study had previously examined biofilm formation of early colonizers, such as *Streptococcus sanguinis*, on enamel surface after bleaching.

These previous studies suggested that high concentrations of bleaching agents may alter enamel surface and increase bacterial adhesion. Zoom2™ bleaching system (Discus Dental, Culver City, CA, USA), which contains 25% H_2O_2, and Beyond™ whitening kit (Beijing, China), which contains 35% H_2O_2, are widely used in Thailand and around the world. However, information on their effects on enamel surface and biofilm formation is still limited. Therefore, this study aimed to examine enamel surface roughness and biofilm formation of both *S. mutans* and *S. sanguinis* on human enamel after bleaching with 25% and 35% H_2O_2.

Research question

Do in-office bleaching systems containing 25% and 35% hydrogen peroxide alter the enamel surface roughness or affect biofilm formation on enamel?

Objective of the study

The purpose of this *in vitro* study was to compare enamel surface roughness and biofilm formation on enamel after bleaching with 25% and 35% H_2O_2 in-office bleaching gels.

Statement of hypothesis

Null hypothesis

1. There is no significant difference in surface roughness of enamel among the control, bleached with 25% and 35% H_2O_2 groups.
2. There is no significant difference in biofilm formation on enamel among the control, bleached with 25% and 35% H_2O_2 groups.

Alternative hypothesis

1. There is a significant difference in surface roughness of enamel among the control, bleached with 25% and 35% H_2O_2 groups.
2. There is a significant difference in biofilm formation on enamel among the control, bleached with 25% and 35% H_2O_2 groups.

Scope of the study

This study is an *in vitro* experiment to evaluate the alteration of the enamel surface roughness and biofilm formation after bleaching with in-office bleaching system at different concentration of H_2O_2. This study used extracted human anterior teeth and premolars.

Basis assumption

1. The experiments were performed and observed by one examiner.
2. The popular in-office bleaching systems in Thailand were chosen in this study (Zoom2™ and Beyond™).
3. Bleaching procedure was done following the manufacturer instruction.
4. Biofilm formation assay was performed using sterile technique.

Limitations

This is an *in vitro* study examining a single species biofilm. The experimental design did not use flow chamber that simulate oral environment. The data may not fully reflect the situation *in vivo.*

Keywords

Biofilm formation, Bleaching, Enamel, Hydrogen peroxide

Ethical considerations

This study used human anterior teeth and premolars that were extracted due to dental treatment. The teeth were kept in 0.1% thymol. The protocol was

approved by the Ethical Committee No. HREC-DCU 2012-050 on June, 29th 2012 at Faculty of Dentistry, Chulalongkorn University.

Expected benefits of the study

The result of this study will be useful for dentists and patients when choosing tooth whitening methods and could influence oral care after in-office bleaching. If the result shows more biofilm formation after in-office bleaching procedure, it is important that a proper oral hygiene instruction should be done after in-office bleaching. Moreover, the result of this experiment will be useful as preliminary data for further study.

CHAPTER II
Review of Literatures

Vital bleaching systems

Vital bleaching procedures using H_2O_2 or CP are popular for tooth discoloration treatment and for esthetic purposes. H_2O_2 is a colorless liquid with a bitter taste and is a strong oxidizing agent.[18] Its reaction produces free radicals, reactive oxygen and hydrogen peroxide anions.[19] Either organic or inorganic molecules can be affected by cleavage of double bonds in pigment molecules and dissolving them from the tooth resulting in brighter appearance. CP is a form of hydrogen peroxide with urea, which is easily broken down and produces water to release free radicals.[3] The urea will be degraded into carbon dioxide and ammonia resulting in higher pH, which is favorable in bleaching procedure.[20] The penetration of bleaching agent into the pulp chamber can cause tooth sensitivity from reversible pulpitis.[21]

H_2O_2 forms free radicals as demonstrated below;

$$H_2O_2 \longrightarrow 2HO^{\bullet}$$

$$HO^{\bullet} + H_2O_2 \longrightarrow H_2O_2 + HO^{\bullet}_2$$

$$HO^{\bullet}_2 \longleftrightarrow H^{+} + O^{\bullet}_2$$

H_2O_2 produces reactive oxygen molecules and hydrogen peroxide anions;

$$2H_2O_2 \longleftrightarrow 2H_2O + 2[O] \longleftrightarrow 2H_2O + O_2$$

$$H_2O_2 \longleftrightarrow H^{+} + HOO^{-}$$

Sulieman, 2008[3]

There are three methods of vital tooth whitening available: in-office bleaching, home bleaching and over-the-counter bleaching.[1] Over-the-counter technique contains low concentration agents such as 3-6% H_2O_2. These products can be applied twice a day by gum shields, strips or paint-on[22] with no side effect on enamel. In a clinical study, three different bleaching techniques, ie. Whitestrip (over the counter technique), Opalescence PF (home bleaching technique) and Opalescence X-tra boost (in-office bleaching technique), were compared using a visual analog scale for the patients' acceptance and scanning electron microscope for enamel surface roughness. The results showed that Whitestrip had the lowest score of tooth hypersensitivity, but caused the most gingival irritation in the patients, and none of the teeth had enamel surface alteration.[23] This result was similar to

that of an *in vitro* study which compared the effect of Crest Whitestrip Supreme (over-the-counter bleaching), Opalescence 20% PF (home bleaching) and Opalescence X-tra boost (in-office bleaching) that showed no alteration in surface hardness and roughness in enamel.[9]

Home bleaching technique uses 10% CP as a standard regimen.[24] This requires the patients to wear individual trays for 8 hours overnight for 2 weeks.[3] A higher concentration of bleaching agent can be used on any tooth with darker color in same arch, such as canines.[3] An *in vitro* study found that 10% and 16% CP gave a faster whitening result than 5% CP.[25]

A few studies investigated biological effects of home bleaching gels. In a clinical study a 3.5% H_2O_2 gel with a 5% potassium nitrate (FKD, Kin Lab) and a 10% carbamide peroxide–based gel (Opalescence, Ultradent) showed no gingival irritation related to the applied products.[26] Another study found tooth hypersensitivity as assessed by cold water and gingival irritation at the gingival margin which resolved after ending the procedure in patients after bleaching with 7.5% H_2O_2 gel (Visalys whitening) and 20% CP gel (Opalescence PF).[27] This result was similar to another study of side effects during tooth bleaching which reported transient tooth hypersensitivity and gingival irritation after using 7% H_2O_2.[28] However, the tooth sensitivity can be decreased by using desensitizing agents such as potassium nitrate and fluoride.[29] Moreover, one study found that adherence of *S. mutans* was significant higher after treated with 10% CP.[16]

In-office bleaching generally uses high concentration of agents such as 35% CP or 25-35% H_2O_2.[1] They are available in either chemically activated or a dual-activation system. Many forms of curing lights are used, such as halogen curing light, plasma arc lamps and diode lasers with 830 and 980 nm. wavelengths.[3] A review study reported that a light-activated in-office bleaching system showed a better immediate whitening result when lower concentrations (15-20%) of H_2O_2 were used.[30] However, no immediate difference was found when higher concentrations (25-35%) of H_2O_2 were used. Furthermore, light also increases the tooth sensitivity.[30]

In addition, an *in vitro* study demonstrated that, among the four different activation methods: halogen, LED, laser and chemical activation of 38% H_2O_2 (Opalescence Xtra Boost), halogen unit showed not only the highest level of change, but also the highest temperature increase. The author suggested that although light activation can give a better result in the tooth color change, we should be

concerned of the increase in pulp temperature during the procedure.[31] This result is similar to a clinical study that compared four bleaching methods using 35% H_2O_2 (Whiteness HP) with or without light. They found no difference in color change between groups and most patients had sensitivity after bleaching.[32] Another study found that light enhanced immediate color change in the 25% H_2O_2 (Zoom!™) treatment, but it increased tooth sensitivity during treatment and had short-term color rebound.[33]

The effect of bleaching gels on enamel

The effect of bleaching gels on enamel surface morphology has been observed in many studies by using Knoop surface microhardness for surface hardness measurement and using profilometer or scanning electron microscope to examine surface roughness. The home bleaching gels at different concentrations, such as 10-16% CP or 6-7.5% H_2O_2, did not alter the enamel surface hardness and roughness.[4-8] In addition, a decrease in enamel surface roughness was found after using bleaching agents containing low concentrations of hydrogen peroxide associated with amorphous calcium phosphate due to remineralizing effect of saliva.[34]

High concentrations of bleaching agents can promote alterations of enamel surface. An *in vitro* study found that 35% CP showed significantly higher enamel surface roughness than control or using 37% CP.[12] Demineralization of enamel was also observed after bleaching with 35% CP.[13] In contrast, two *in vivo* studies performed by making surface replica and using non-contact profilometer found that there was no significant difference between test and control groups when the enamels were bleached with 35% CP or 38% H_2O_2.[10, 11] However, the surface roughness of bleached enamel can increase more easily than unbleached enamel after brushing when 35% H_2O_2 or 16% CP was used.[14]

The effect of bleaching on bacterial adhesion was evaluated after bleaching with 10% CP in three different brands (Opalescence®, Karisma® and NiteWhite®) for 8 hours per day in a 30-day period.[16] No significant difference in the enamel surface roughness was observed, but there was a significantly higher bacterial adhesion in bleached groups. Furthermore, another *in vitro* study found that both surface roughness and adherence of *S. mutans* were increased when the enamel was bleached repeatedly with 35% H_2O_2 with or without acid.[17]

Oral biofilm

The oral cavity harbors a wide variety of species and ecologies of microorganisms. Oral biofilm can cause major oral diseases such as caries or periodontal diseases when it is in imbalance.[35] The formation of salivary pellicle is the first step of plaque formation and is important in microbial adhesion. The initial colonizers, such as *Streptococcus gordonii* (*S. gordonii*), *Streptococcus oralis* (*S. oralis*), and *Streptococcus sanguinis* (*S. sanguinis*) adhere to salivary pellicle.[36] They are held together by proteins, polysaccharides, and DNA secreted by cells.[37] Biofilm forms when planktonic microorganisms adhere to a surface and follow by colonization, growth and maturation, and detachment of some micro-organisms.[38]

S. mutans plays an important role in cariogenesis.[39] Glucan can be produced by some mutans streptococci from sucrose via glucosyltransferases. It then holds the bacteria together in the dental plaque, which could subsequently lead to dental caries.[40]

A study found a positive correlation between surface roughness and biofilm formation of *S. mutans*.[41] This relationship was also reported in an *in vitro* study which investigated initial bacterial adhesion of *S. sanguinis* on resin, titanium and zirconia.[42] Therefore, the effect of high concentrations of bleaching agents in in-office bleaching gels on enamel could lead to increase bacterial adhesion and increase risk of dental caries. Moreover, a study found that even when the surface roughness was not significantly different, but there was a significantly difference in bacterial adhesion after bleaching.[16] We thus examined the effect of in-office bleaching gels on enamel surface roughness and bacterial adhesion of *S. mutans* as a representative of cariogenic bacteria and of *S. sanguinis* as a representative of initial colonizers.

Measuring methods for total amount of biofilm

There are 3 methods for quantification and visualization of biofilm adhesion, which are determination of CFUs (Colony forming units), high-resolution microscopic technique, and staining technique.[43] First, the determination of CFUs is the traditional method for quantifying viable cells by desorption of biofilm using ultrasonication or agitation and plating the cells on agar plates.[44] However, measuring for viability may not represent the total amount of adherent cells and could lead to incorrect conclusion.

Scanning electromicroscopy (SEM) is a high-resolution microscopic technique. The samples need to be fixed, dehydrated and coated with a conductive substrate. It is difficult to quantify the bacterial adherence, but it can provide the details of ultrastructure of bacterial and its surrounding matrix.[43] We used this technique to view the extent and structure of biofilm on the enamel surface.

Staining techniques can be used to measure biofilm in microtitre-plates using different staining assays.[43] For example, crystal violet staining reflects the total amount of biofilm adhesion and can be quantified by using a microplate reader.[42] Resazurin reduction or Alamar Blue Assay is used for quantification of viable cells. These assays are analyzed in a fluorometer.[45] In this study, we used the crystal violet assay to measure the total amount of biofilm on enamel surface.

CHAPTER III
MATERIALS AND METHODS

Research design

This is an *in vitro* study. Extracted human permanent anterior teeth and premolars were collected for the experiments.

Sample description

In our pilot study, *S. mutans* biofilm formation was analyzed using 8 specimens per group. The result is shown in the table 1. The optical density of each specimen in the *S. mutans* biofilm groups was subtracted with the mean OD of the respective groups without bacteria (background staining) and the mean and SD of each group was calculated.

Table 1 The optical density value ($OD_{595\ nm}$) of *S. mutans* biofilm formation in pilot study

Group	Optical density value ($OD_{595\ nm}$) (mean ± SD)
25% H_2O_2 with *S. mutans*	0.150± 0.113
35% H_2O_2 with *S. mutans*	0.218 ± 0.174
Untreated with *S. mutans*	0.120 ± 0.188

Sample size calculation for balanced ANOVA was performed using a web-based tool at http://homepage.stat.uiowa.edu/~rlenth/Power/. When the power is set at 80% and significance level at 5%, the calculated sample size is equal to 75 per group. Due to limitation of tooth collection, we are capable of testing only 15 per group, which would give our study an 80% power to detect a difference of at least 0.23.

Specimen preparations

1. Sound permanent anterior teeth and premolars extracted due to periodontal problem or orthodontic treatment without caries and stored in 0.1% thymol were used.[34]

2. The teeth were polished with pumice and rubber cup, mounted in acrylic block by sticky wax and cut into 3x3x2 mm^3 pieces at the middle of the buccal and lingual surfaces by ISOMET 1000 (figure 1 and 2). The specimens were marked at incisal surface when they were cut in incisal axis by permanent pen and drilled marked spot by diamond round bur. Finally, 162 specimens were stored in normal saline.

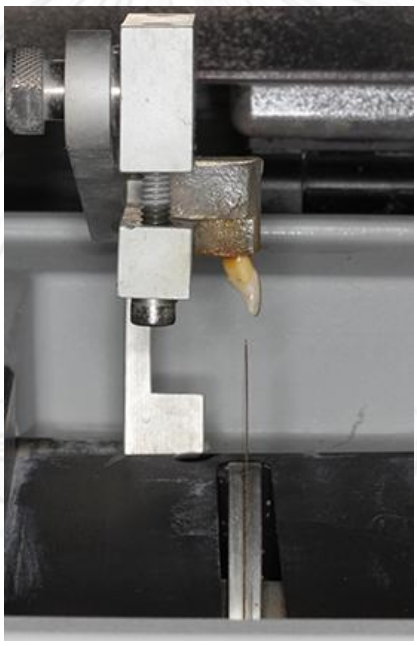

Figure 1 Cutting a central incisor by Isomet 1000

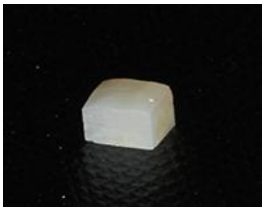

Figure 2 Prepared specimen 3x3x2 mm^3

3. The specimens were divided by block randomization into 3 groups of 54 specimens each and mounted in plasticine (figure 3).

Figure 3 Mounted specimens in plasticine

3.1) The first group was bleached with 25% H_2O_2 (Zoom2^{TM} bleaching system, Discus Dental, Inc., Culver City, CA, USA) according to manufacturer's instruction. The gel was dispensed into a dappen dish, stirred, applied on the enamel with approximately 1-2 mm thickness by brush, and activated with light for 15 minutes (figure 4 and 5). The treatment was done three times. In between each session, the gel was removed by wiping with cotton pellet and normal saline twice. Finally, each specimen was rinsed once with 10 ml of tap water.

Figure 4 Applied bleaching gel on each specimen

Figure 5 Light activation by Zoom Advance Power™ lamp

3.2) The second group was bleached with 35% H_2O_2 (Beyond™ whitening kit, Beijing, China). The specimens were treated as described for the first group, but light was applied for 8 minutes according to instruction (figure 6).

Figure 6 Light activation by Beyond™ Whitening Accelerator

3.3) The last group was stored in normal saline at room temperature for the same time as the test groups as controls.

Measurement of surface roughness by profilometer

1. All specimens were tested for surface roughness before and after bleaching using contact profilometer (Talyscan 150, England) (figure 7).

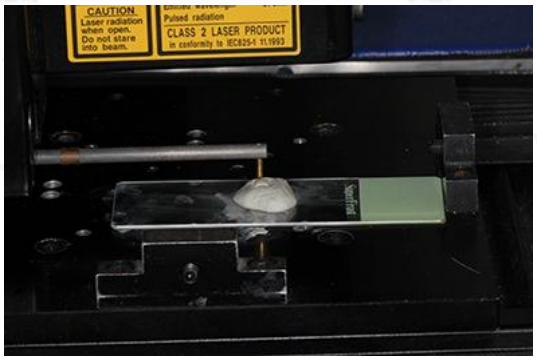

Figure 7 Roughness measurement by contact profilometer

2. Each specimen was traced mesio-distally 3 times at the middle of the sample and 0.5 mm apart on each side in the same directions for the length of 1

mm with gaussian filter at 0.25, spacing at 0.5 μm and speed at 500 μm/s. The average surface roughness (Ra) was determined and recorded.

Bacterial cultures

1. Frozen (-80°C) stocks of *S. mutans* ATCC25175 and *S. sanguinis* ATCC10556 were transferred onto trypticase soy agar and incubated at 37°C with 5% CO_2 for 48 hours.

2. A single colony was inoculated into sterile trypticase soy broth and incubated at 37°C with 5% CO_2 for 16 hours. The bacterial suspension then was diluted to an optical density at 550 nm (OD_{550nm}) of 0.1 and incubated further until reaching an optical density of 0.3 (approximately 3.65×10^8 cells/ml).[46] The optical density was measured by a spectrophotometer (Pharmacia LKB Biotechnology, Inc., Uppsala, Sweden).

Biofilm formation assays

1. The specimens in each treatment group were randomly divided into 3 subgroups, 15 specimens per group as follow;

1.1) Treated with 25% H_2O_2 with *S. mutans*

1.2) Treated with 35% H_2O_2 with *S. mutans*

1.3) Untreated specimens with *S. mutans*

1.4) Treated with 25% H_2O_2 with *S. sanguinis*

1.5) Treated with 35% H_2O_2 with *S. sanguinis*

1.6) Untreated specimens with *S. sanguinis*

1.7) Treated with 25% H_2O_2 without bacteria

1.8) Treated with 35% H_2O_2 without bacteria

1.9) Untreated specimens without bacteria

2. The experiments were performed 3 times (5 samples per group per time). The specimens were mounted into 96 well-plate with putty type silicone (figure 8) and disinfected using Ethylene Oxide gas.[47]

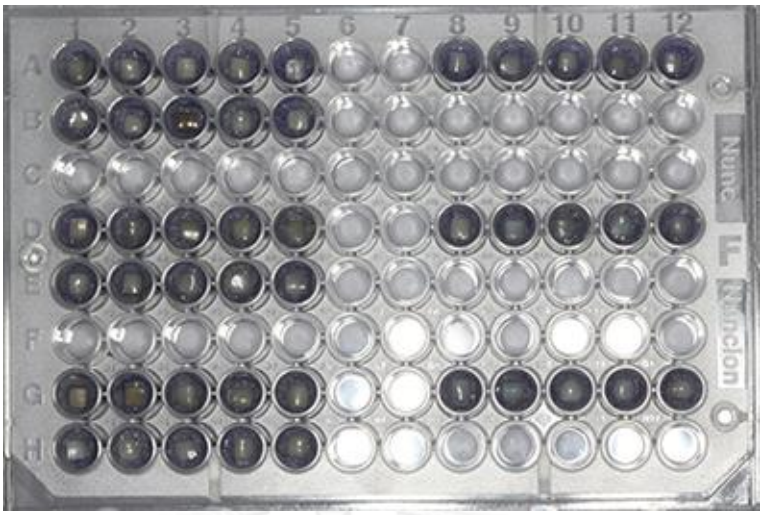

Figure 8 Mounted specimens in 96 well-plate

3. The biofilm formation assay was done as described by Lee and colleagues[42] with some modifications. Artificial saliva was sterilized by 0.2μm pore size filter (Corning, New York, USA) and dispensed 100 μl onto each specimen and incubated at 37°C for 2 hours.

4. Each bacterial suspension (450 μl) was mixed with 40% sucrose (50 μl) in trypticase soy broth to obtain a final concentration of sucrose at 4%. One hundred microliters of bacterial suspension was dispensed onto each specimen and incubated at 37 °C with 5% CO_2 for 24 hours.[48]

5. To obtain the staining background level without bacteria, 100 μl of sterile 4% sucrose in trypticase soy broth was dispensed onto the specimens and incubated at 37 °C with 5% CO_2 for 24 hours.

6. To quantify the total amount of biofilm formation, the crystal violet assay was performed as described.[42] After the incubation period, the specimens were rinsed with 100 μl of sterile Milli Q water for 3 times and stained with 1% crystal violet solution (Sigma-Aldrich, MD, USA) for 10 minutes at room temperature.

7. Then, each specimen was rinsed by sterile Milli Q water to wash away unbound dye and then removed from the silicone. The bound crystal violet was extracted by using 200 μl of destaining solution (80% ethanol, 20% acetone) and transferred to a new 96-well plate (figure 9 and 10).

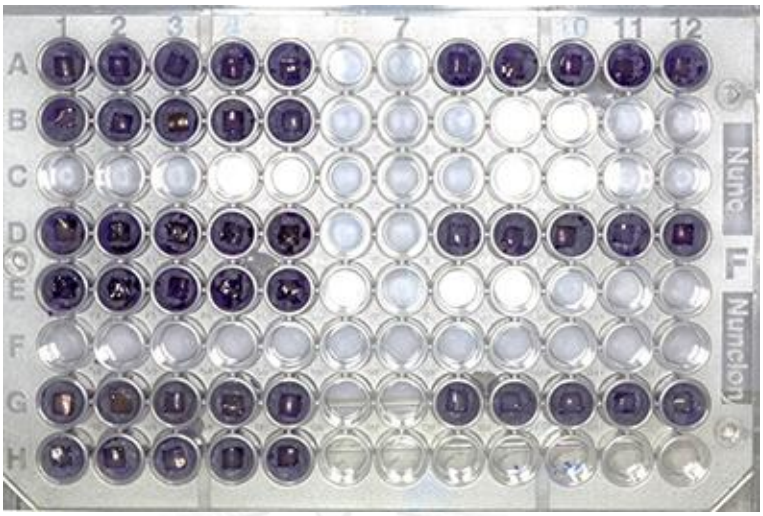

Figure 9 Crystal violet stained on biofilm on enamel

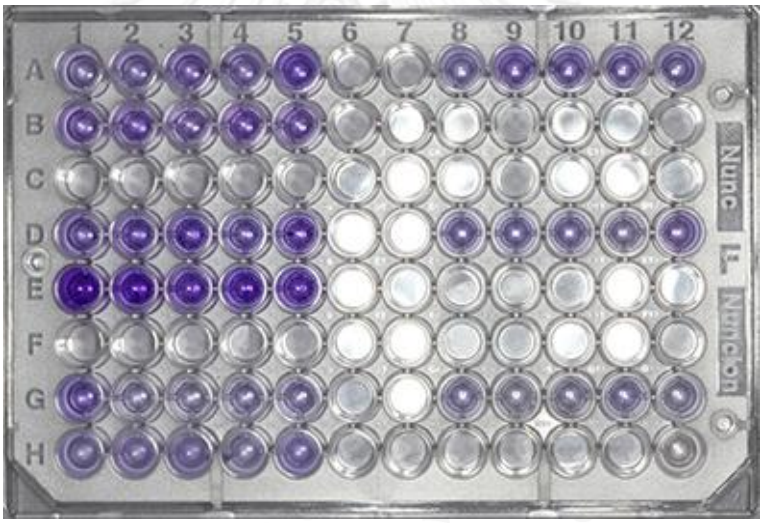

Figure 10 Destaining solution after extracting crystal violet

8. Microplate reader (Biochome Anthos Zenyth 200rt, UK) with ADAP 2.0 Prisma software was used to measure the optical density at 595 nm (OD_{595nm}) of destaining solution samples, which represents the amount of biofilm.

9. Each optical density value of extracted crystal violet of the biofilm groups was subtracted with the mean optical density of the respective background groups.

10. Three specimens of each group were examined under scanning electron microscope (SEM).

10.1) Specimens were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 for 1-2 hours and rinsed twice with 0.1 M phosphate buffer.

10.2) They were then dehydrated successively with 30%, 50%, 70% and 95% ethanol for 10 minutes at each concentration of ethanol, and lastly with 100% ethanol 3 times for 10 minutes each.

10.3) The specimens were dried at critical point by critical point dryer (model CPD 020, Bal-Tec, Balzers, Liechtenstein) and coated by ion sputtering with gold by ion sputter (model SCD 040, Bal-Tec, Balzers, Liechtenstein).

10.4) Specimens were observed by SEM at magnification of 2,000x (model JSM-5410LV, JOEL, Tokyo, Japan).

Outcome measurement

This study measured the roughness of enamel surface by using profilometer. For biofilm formation, the optical density of extracted crystal violet was measured by microplate reader. Each optical density of extracted crystal violet of the biofilm groups was subtracted with the mean optical density of the respective groups without bacteria.

Data collection

Surface roughness

The surface roughness values were recorded automatically by the testing machine.

Biofilm quantification

Biofilm quantity was recorded from optical density of extracted crystal violet using microplate reader.

All data were collected in a Microsoft Excel file for further analysis.

Data analysis

The differences between enamel surface roughness values before and after treatments were evaluated with Wilcoxon Signed Rank test. Kruskal-Wallis test and Mann-Whitney U test with Bonferroni corrections were used to evaluate the levels of

changes in enamel surface roughness and biofilm formation among the test and control groups. The bacterial biofilm data were compared among the same species only. The relationship between enamel surface roughness and biofilm formation was analyzed by linear regression. The level of significance was set at $p<0.05$. SPSS statistical analysis software version 17 (Chicago, IL, USA) was used in this study.

CHAPTER IV
RESULTS

Surface roughness of the samples before and after bleaching was shown in Table 2 as median (interquartile range). When compared with before treatment, enamel surface roughness was decreased significantly after bleaching with both systems ($p<0.001$), while there was no change in the control group ($p=0.233$).

Table 2. Enamel surface roughness before and after bleaching

Group	Median (interquartile range) of roughness value (μm)		
	Before	After	p-value*
Control	0.0854 (0.0693 - 0.1288)	0.0876 (0.0682 - 0.1263)	0.233
25% H_2O_2	0.0916 (0.0657 - 0.1339)	0.0594 (0.0435 - 0.0839)	< 0.001
35% H_2O_2	0.1021 (0.0673 - 0.1256)	0.0706 (0.0531 - 0.0980)	< 0.001

* by Wilcoxon Signed-Rank tests

When comparing the degree of changes in surface roughness between groups, there was no difference between the two bleaching treatments, but significant difference when compared with the control (Figure 11). The results suggest that both bleaching systems reduced enamel surface roughness to a similar degree.

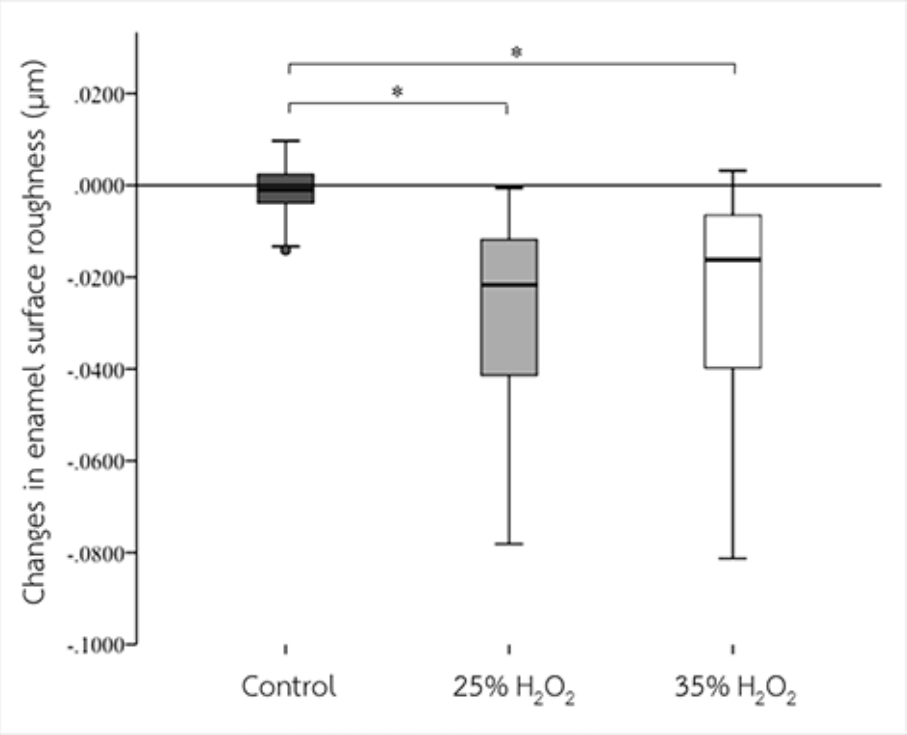

Figure 11 Changes in enamel surface roughness after treatment.

Asterisk (*) represents statistical significant difference ($p<0.001$) by Kruskal-Wallis and Mann-Whitney U tests with Bonferroni corrections. A mild outlier was included in the analysis. Dark grey, light grey and white boxes represent control, 25% H_2O_2 and 35% H_2O_2 treatment groups, respectively.

For the biofilm formation assay, no difference was observed in *S. mutans* biofilm formation among the three treatments ($p=0.139$) (Figure 12). On the other hand, the levels of *S. sanguinis* biofilm formation on enamel specimens were significantly different among the three groups ($p<0.001$) (Figure 13). Interestingly, bleaching with 35% H_2O_2 led to a significantly higher level of *S. sanguinis* biofilm than the control, whereas bleaching with 25% H_2O_2 showed significantly less biofilm than the control group ($p<0.001$).

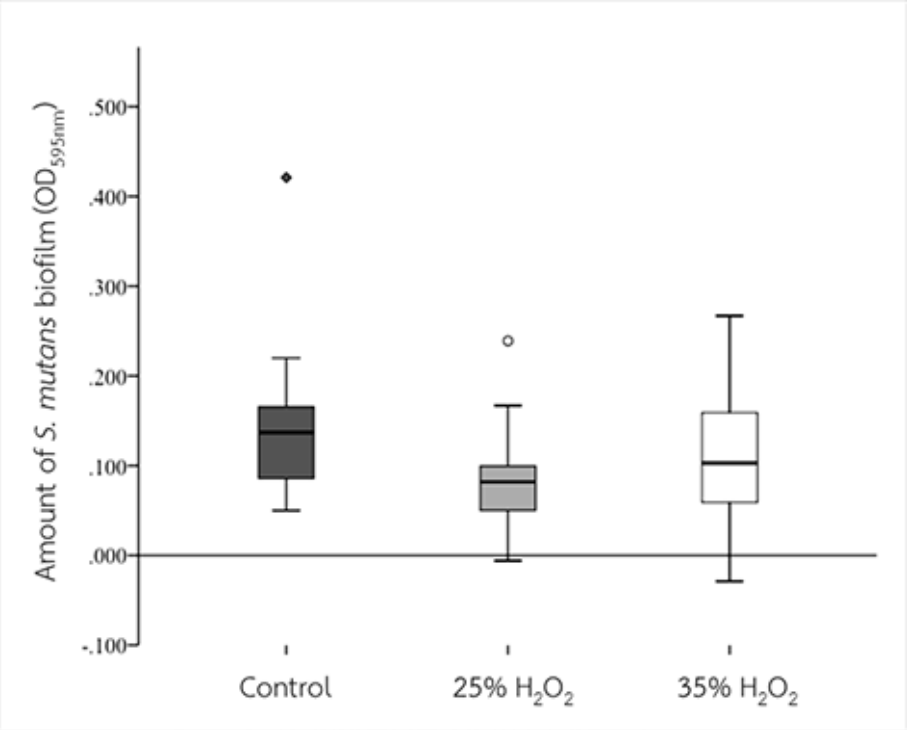

Figure 12 The optical density values at 595 nm (OD595nm) of crystal violet assays represent the amount of biofilm formation of *S. mutans*.

Asterisk (*) represents statistical significant difference ($p<0.001$) by Kruskal-Wallis and Mann-Whitney U tests with Bonferroni corrections. Outliers were included in the analysis. Dark grey, light grey and white boxes represent control, 25% H_2O_2 and 35% H_2O_2 treatment groups, respectively.

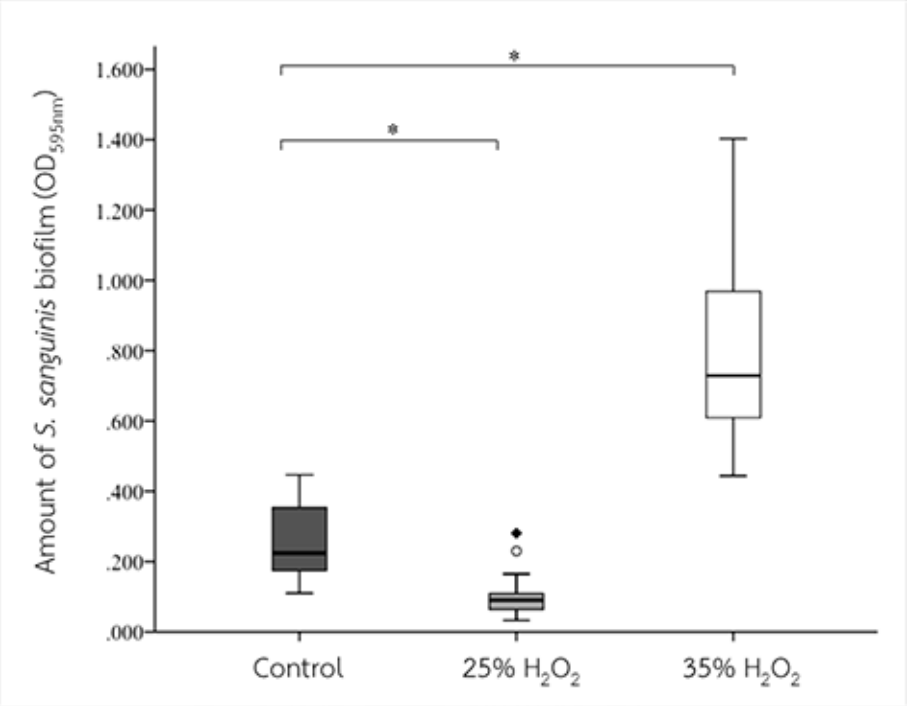

Figure 13 The optical density values at 595 nm (OD595nm) of crystal violet assays represent the amount of biofilm formation of *S. sanguinis*.

Asterisk (*) represents statistical significant difference ($p<0.001$) by Kruskal-Wallis and Mann-Whitney U tests with Bonferroni corrections. Outliers were included in the analysis. Dark grey, light grey and white boxes represent control, 25% H_2O_2 and 35% H_2O_2 treatment groups, respectively.

We also analyzed if there is a correlation between enamel surface roughness and the level of biofilm formation. Interestingly, the result demonstrated that there was a correlation between enamel surface roughness and *S. mutans* biofilm formation ($r=0.105$, $p=0.03$) (figure 14), but this was not observed with *S. sanguinis* ($p=0.93$) (figure 15).

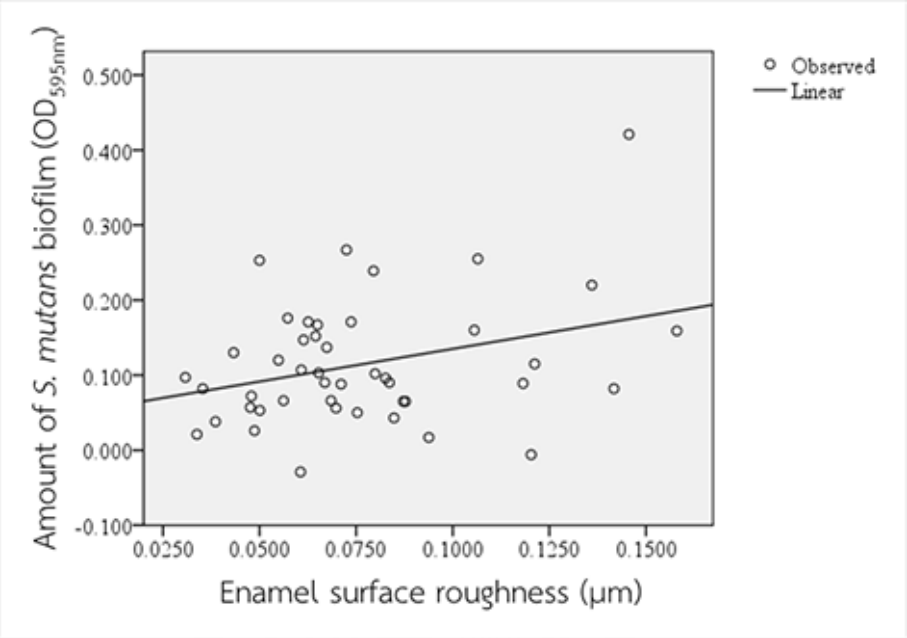

Figure 14 Relationship between enamel surface roughness and *S. mutans* biofilm formation

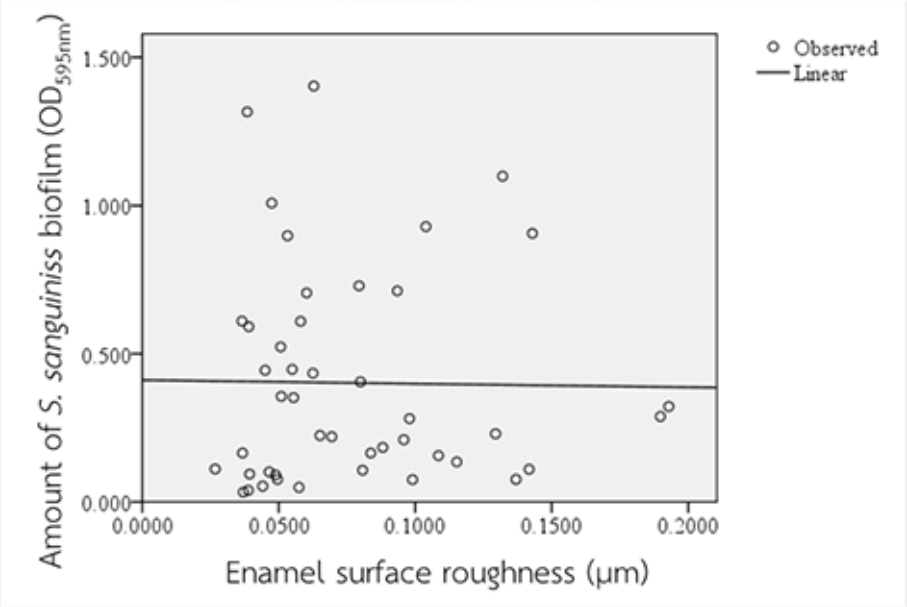

Figure 15 Relationship between enamel surface roughness and *S. sanguinis* biofilm formation

The SEM images illustrated the structure of biofilm in each treatment group (Figure 16). The background group without bacteria did not show any biofilm formation (Figure 16A, 16D, 16G). For *S. mutans* biofilm, similar structure of thin biofilm was observed on the enamel surface in all three treatment groups (Figure 16B, 16E, 16H). Remarkably, *S. sanguinis* formed polysaccharide-encased biofilm that attached to the enamel surface in control and 35% H_2O_2 treated groups (Figure 16C, 16I). However, this was not observed in 25% H_2O_2 treated group (Figure 16F).

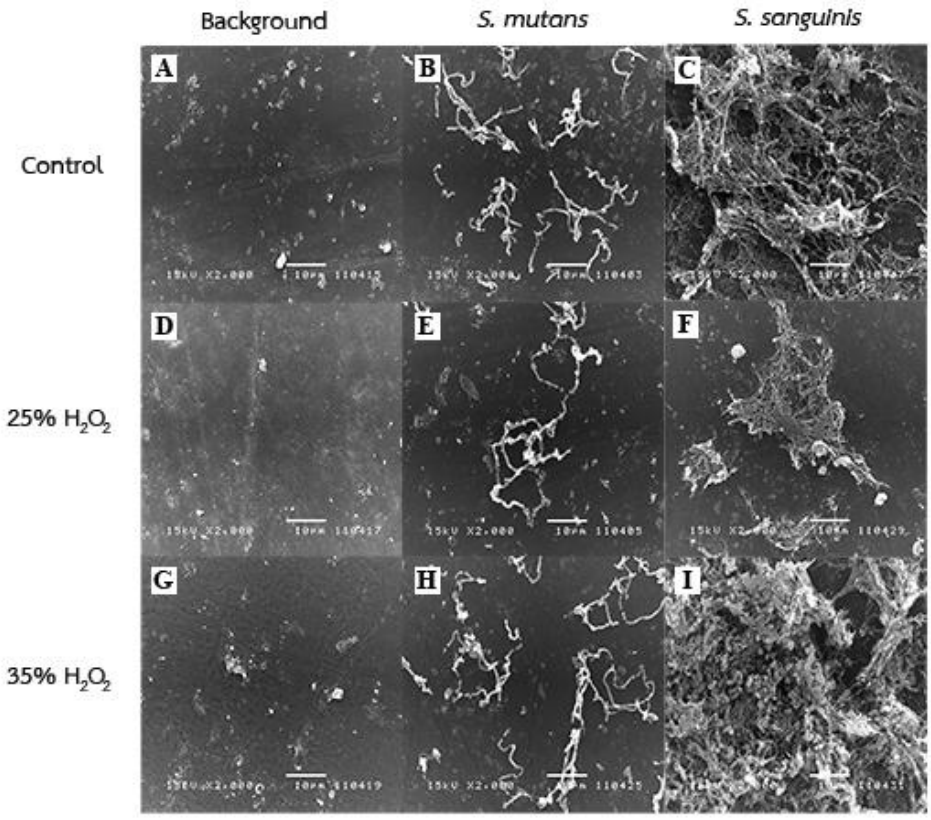

Figure 16 Scanning electron microscope (SEM) images show the structure of biofilm on enamel surface (2,000x).

(A) control group without bacteria, (B) control group with *S. mutans*, (C) control group with *S. sanguinis*, (D) bleaching with 25% H_2O_2 group without bacteria, (E) bleaching with 25% H_2O_2 group with *S. mutans*, (F) bleaching with 25% H_2O_2 group with *S. sanguinis*, (G) bleaching with 35% H_2O_2 group without bacteria, (H) bleaching with 35% H_2O_2 group with *S. mutans*, (I) bleaching with 35% H_2O_2 group with *S. sanguinis.*

CHAPTER V
DISCUSSION

Our results demonstrated that both 25% and 35% H_2O_2 bleaching systems significantly reduced enamel surface roughness comparing to the control group, but there was no difference between the two treatments. Interestingly, a marked increase in *S. sanguinis* biofilm formation was observed on enamel treated with 35% H_2O_2 but not 25% H_2O_2. This finding is the first report of the effect of in-office bleaching on early colonizer biofilm.

Different, and sometimes contradictory, effects of bleaching on enamel surface properties have been reported.[4-14] These could be due to the variations of many conditions among studies, such as tooth substrates (human or bovine), bleaching reagents, pH, treatment time and procedures, and others. Our observation of a reduction in enamel surface roughness after bleaching was similar to the findings reported previously for 20% H_2O_2 (Opalescence PF gel, Ultradent, South Jordan, Utah, USA).[9] In contrast, two *in vivo* studies using non-contact profilometric measurements of surface replica found no significant difference in enamel surface roughness between test and control groups after bleaching with 35% CP or 38% H_2O_2.[10, 11] Moreover, 35% CP was shown to promote alterations of enamel surface *in vitro*; however, CP was applied on enamel surface for 30 minutes without light activation, unlike in clinical situation.[12] Interestingly, bleaching with 35% CP led to enamel demineralization, especially in the area closest to the surface, as observed under a micro-CT scanner.[13] This mineral loss may lead to a decrease in wear resistance of enamel surface. However, it is unclear how these enamel surface changes affect the dental biofilm formation or other clinical parameters after bleaching.

Oral biofilm contains many species of microorganisms that are the causes of major oral diseases.[35] Microbial adhesion and plaque formation occur after saliva coats the tooth surface. Adherence is initiated by a group of oral streptococci called “the early colonizers”, such as *S. gordonii*, *S. mitis*, and *S. sanguinis*.[36] Subsequently, mutans streptococci, such as *S. mutans,* produce glucans from sucrose via the activity of glucosyltransferases to enhance bacterial accumulation, and play an important role in cariogenesis.[39, 40] To investigate both steps of biofilm formation, our study used *S. sanguinis* to represent the early colonizers and *S. mutans* to represent cariogenic bacteria.

Our experiments simulated the clinical procedures as recommended by the manufacturers and tested for streptococcal biofilm formation. Significantly, we observed a marked increase in biofilm formation of *S. sanguinis* on 35% H_2O_2-bleached enamel comparing to control and 25% H_2O_2-bleached enamel. Moreover, SEM demonstrated a large amount of biofilm formation on 35% H_2O_2-bleached enamel and a lesser extent on the control group (Figure 16C and 16I, respectively), corresponding to the biofilm formation assay results. This may help to explain the clinical observation that plaque accumulation scores were higher in the group bleached with 35% H_2O_2 after a 5-day non-brushing period.[15]

On the other hand, neither treatments affects *S. mutans* biofilm formation. SEM analysis that directly visualized the formation of *S. mutans* biofilm on treated-enamel surfaces also showed consistent results (Figure 16B, 16E, 16H). However, this result disagrees with a previous report that showed an increase in biofilm formation of *S. mutans* after bleaching with 35% H_2O_2.[17]

Our study demonstrated that *S. sanguinis* was able to adhere on surfaces better than *S. mutans*. This finding was concordant with several reports on adherence of oral streptococci to hydroxyapatite.[49-52] *S. sanguinis* has been shown to display a high affinity to enamel surfaces and is more effective at adhering on saliva-coated surfaces than *S. mutans*.[51, 52] Moreover, recent study provided genetic information about putative proteins necessary for hydroxyapatite binding ability,[53] yet there are many unknown factors on *S. sanguinis* binding ability remaining to be explored. The formation of *S. sanguinis* biofilms on bleached-enamel surfaces is important since it is the beginning step of dental plaque formation. The colonizing bacteria act as a framework for later colonizers to form multi-species mature biofilms.[53] When bacterial ecology in dental plaque is changed, it will lead to disease development.[35]

Although both H_2O_2 treatments led to similar levels of reduction in surface roughness *S. sanguinis* formed the greatest amount of biofilm on 35% H_2O_2 bleached-enamel, less on the control group and even less on the 25% H_2O_2 treated group. These results indicated that there is no direct correlation between surface roughness and the formation of *S. sanguinis* biofilm. Other factors could also affect biofilm formation. An important factor is the pH of bleaching gels as has been shown that bleaching products with lower pH (4.3-4.9) led to the greatest changes in enamel topography.[22] In this study, the 35% H_2O_2 bleaching system has a pH of 4.4 whereas 25% H_2O_2 bleaching system has a pH of 8, almost equal to the control

group (pH 7). Thus, the acidity may somehow enhance the binding ability of *S. sanguinis* on enamel surfaces. This finding should be further investigated because an increase in early colonizer biofilm formation could have an important clinical implication in choice of in-office bleaching system and post-operative patient care. Furthermore, the effect of bleaching on multi-species biofilm formation, which better reflects *in vivo* situations, should also be carried out.

CONCLUSIONS

Within the limitations of our study, we conclude that bleaching with both 25% and 35% H_2O_2 systems significantly decreased the enamel surface roughness comparing to the control. *S. sanguinis* biofilm formation on enamel bleached with 35% H_2O_2 was markedly higher than the other groups. However, there was no significant difference in *S. mutans* biofilm formation among the three groups.

Implication of the result of this study

Tooth bleaching with 25% or 35% hydrogen peroxide could reduce enamel surface roughness, but 35% hydrogen peroxide may promote more bacterial biofilm formation than 25% hydrogen peroxide. Therefore, this should be further investigated *in vivo,* and efficient plaque control should be emphasized after bleaching with high concentration of hydrogen peroxide.

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APPENDIX

Appendix A Block randomization method

After human permanent anterior teeth and premolars were cut, 162 enamel specimens were randomly divided by block randomization using random number table from http://www.docstoc.com/docs/55841821/Random-Number-Table as shown below.

Random Number Table

13962	70992	65172	28053	02190	83634	66012	70305	66761	88344
43905	46941	72300	11641	43548	30455	07686	31840	03261	89139
00504	48658	38051	59408	16508	82979	92002	63606	41078	86326
61274	57238	47267	35303	29066	02140	60867	39847	50968	96719
43753	21159	16239	50595	62509	61207	86816	29902	23395	72640
83503	51662	21636	68192	84294	38754	84755	34053	94582	29215
36807	71420	35804	44862	23577	79551	42003	58684	09271	68396
19110	55680	18792	41487	16614	83053	00812	16749	45347	88199
82615	86984	93290	87971	60022	35415	20852	02909	99476	45568
05621	26584	36493	63013	68181	57702	49510	75304	38724	15712
06936	37293	55875	71213	83025	46063	74665	12178	10741	58362
84981	60458	16194	92403	80951	80068	47076	23310	74899	87929
66354	88441	96191	04794	14714	64749	43097	83976	83281	72038
49602	94109	36460	62353	00721	66980	82554	90270	12312	56299
78430	72391	96973	70437	97803	78683	04670	70667	58912	21883
33331	51803	15934	75807	46561	80188	78984	29317	27971	16440
62843	84445	56652	91797	45284	25842	96246	73504	21631	81223
19528	15445	77764	33446	41204	70067	33354	70680	66664	75486
16737	01887	50934	43306	75190	86997	56561	79018	34273	25196
99389	06685	45945	62000	76228	60645	87750	46329	46544	95665
36160	38196	77705	28891	12106	56281	86222	66116	39626	06080
05505	45420	44016	79662	92069	27628	50002	32540	19848	27319
85962	19758	92795	00458	71289	05884	37963	23322	73243	98185
28763	04900	54460	22083	89279	43492	00066	40857	86568	49336

The specimens were to be assigned into 9 groups as follow.

Group A = Treated with 25% H_2O_2 with *S. mutans*

Group B = Treated with 35% H_2O_2 with *S. mutans*

Group C = Untreated specimens with *S. mutans*

Group D = Treated with 25% H_2O_2 with *S. sanguinis*

Group E = Treated with 35% H_2O_2 with *S. sanguinis*

Group F = Untreated specimens with *S. sanguinis*

Group G = Treated with 25% H_2O_2 without bacteria

Group H = Treated with 35% H_2O_2 without bacteria

Group I = Untreated specimens without bacteria

The block pattern for each number from the random number table was assigned as follow.

1 = group (A, B, C, D, E, F, G, H, I)

2 = group (B, C, D, E, F, G, H, I, A)

3 = group (C, D, E, F, G, H, I, A, B)

4 = group (D, E, F, G, H, I, A, B, C)

5 = group (E, F, G, H, I, A, B, C, D)

6 = group (F, G, H, I, A, B, C, D, E)

7 = group (G, H, I, A, B, C, D, E, F)

8 = group (H, I, A, B, C, D, E, F, G)

9 = group (I, A, B, C, D, E, F, G, H)

0 was skipped over.

The numbers were read in order from the top of the table from left to right. For each number, the block of specimens was placed into respective 96-well plates according to the indicated group assignments. For example, the first set of numbers (13962) means that the 5 blocks of 9 samples each were placed in the following orders: 1= group (A, B, C, D, E, F, G, H, I); 3 = group (C, D, E, F, G, H, I, A, B); 9 = group (I, A, B, C, D, E, F, G, H); 6 = group (F, G, H, I, A, B, C, D, E); 2 = group (B, C, D, E, F, G, H, I, A).

Appendix B Enamel surface roughness of each specimen before and after treatment

No.	Enamel surface roughness (μm)					
	Control		25% H_2O_2		35% H_2O_2	
	before	after	before	after	before	after
1	0.0694	0.0698	0.1499	0.0826	0.0787	0.0501
2	0.0697	0.0674	0.1448	0.0795	0.1110	0.0626
3	0.1493	0.1360	0.1113	0.0650	0.0990	0.0938
4	0.1654	0.1580	0.0555	0.0549	0.1204	0.0614
5	0.1419	0.1456	0.0701	0.0308	0.0799	0.0711
6	0.0844	0.0881	0.0534	0.0465	0.0958	0.0628
7	0.0539	0.0509	0.0604	0.0393	0.1401	0.1320
8	0.0528	0.0549	0.0699	0.0488	0.0546	0.0365
9	0.0772	0.0694	0.0464	0.0388	0.1540	0.1429
10	0.0835	0.0799	0.0508	0.0267	0.0680	0.0602
11	0.1069	0.1166	0.1054	0.0503	0.0625	0.0657
12	0.0482	0.0525	0.1383	0.0702	0.1193	0.0452
13	0.1266	0.1311	0.1215	0.1032	0.1127	0.0962
14	0.0705	0.0729	0.0918	0.0709	0.0941	0.0753
15	0.0697	0.0712	0.0837	0.0478	0.0886	0.0804
16	0.0574	0.0511	0.1429	0.0735	0.1316	0.1060
17	0.0908	0.0965	0.0675	0.0431	0.1965	0.1567
18	0.1600	0.1692	0.2144	0.1746	0.1145	0.0705
19	0.0653	0.0645	0.0920	0.0476	0.0499	0.0386
20	0.0480	0.0433	0.0475	0.0353	0.0586	0.0608
21	0.0742	0.0685	0.1480	0.0848	0.1489	0.1212
22	0.0525	0.0573	0.1268	0.0487	0.1253	0.0653
23	0.0713	0.0737	0.1448	0.1203	0.0651	0.0606

No.	Enamel surface roughness (μm)					
	Control		25% H_2O_2		35% H_2O_2	
	before	after	before	after	before	after
24	0.1226	0.1085	0.0463	0.0367	0.0421	0.0390
25	0.1914	0.1898	0.1108	0.0990	0.0583	0.0580
26	0.0944	0.0958	0.0902	0.0807	0.1063	0.1039
27	0.1432	0.1417	0.0700	0.0370	0.0404	0.0384
28	0.1090	0.1152	0.0523	0.0441	0.1012	0.0532
29	0.1486	0.1475	0.0403	0.0345	0.1126	0.1073
30	0.0863	0.0911	0.0434	0.0308	0.1734	0.1375
31	0.0843	0.0816	0.1099	0.0927	0.1119	0.1054
32	0.0985	0.0933	0.1053	0.0692	0.1164	0.0706
33	0.1176	0.1049	0.0914	0.0588	0.1416	0.1270
34	0.0868	0.0835	0.1106	0.0449	0.0610	0.0574
35	0.1188	0.1247	0.0735	0.0600	0.1598	0.0849
36	0.0804	0.0765	0.1608	0.1581	0.0785	0.0617
37	0.1398	0.1417	0.0952	0.0562	0.1192	0.1065
38	0.1087	0.1056	0.1497	0.0878	0.0899	0.0872
39	0.1220	0.1182	0.0993	0.0836	0.0735	0.0725
40	0.0722	0.0753	0.0822	0.0799	0.0569	0.0479
41	0.0693	0.0669	0.0489	0.0338	0.1265	0.0500
42	0.0601	0.0625	0.1335	0.1294	0.1616	0.0934
43	0.0539	0.0554	0.0725	0.0574	0.0642	0.0507
44	0.2024	0.1929	0.1393	0.0979	0.0972	0.0794
45	0.0825	0.0837	0.0563	0.0495	0.0746	0.0474
46	0.0635	0.0651	0.1536	0.1369	0.1092	0.0450
47	0.1511	0.1494	0.1352	0.0969	0.1487	0.0775
48	0.1464	0.1462	0.0758	0.0672	0.0636	0.0555

No.	Surface roughness (μm)					
	Control		25% H_2O_2		35% H_2O_2	
	before	after	before	after	before	after
49	0.1357	0.1238	0.0704	0.0429	0.0462	0.0380
50	0.1045	0.0950	0.0593	0.0371	0.1091	0.1035
51	0.1389	0.1352	0.0943	0.0809	0.1029	0.0763
52	0.0665	0.0608	0.0884	0.0436	0.1339	0.0526
53	0.0840	0.0872	0.1934	0.1511	0.2130	0.1838
54	0.0678	0.0697	0.0835	0.0770	0.0941	0.0782
Median	0.0853	0.0876	0.0916	0.0594	0.1020	0.0705
Interquartile range	0.0694 - 0.1288	0.0683 – 0.1263	0.0657 – 0.1339	0.0435 – 0.0839	0.0673 – 0.1256	0.0530 – 0.0980
Min	0.0480	0.0433	0.0403	0.0267	0.0404	0.0365
Max	0.2024	0.1929	0.2144	0.1746	0.2130	0.1838

Appendix C Changes in enamel surface roughness of each specimen after treatment

No.	Changes in enamel surface roughness (μm)		
	Control	25% H_2O_2	35% H_2O_2
1	0.0004	-0.0673	-0.0286
2	-0.0023	-0.0653	-0.0484
3	-0.0133	-0.0463	-0.0052
4	-0.0074	-0.0006	-0.0590
5	0.0037	-0.0393	-0.0088
6	0.0037	-0.0069	-0.0330
7	-0.0030	-0.0211	-0.0081
8	0.0021	-0.0211	-0.0181
9	-0.0078	-0.0076	-0.0111
10	-0.0036	-0.0241	-0.0078
11	0.0097	-0.0551	0.0032
12	0.0043	-0.0681	-0.0741
13	0.0045	-0.0183	-0.0165
14	0.0024	-0.0209	-0.0188
15	0.0015	-0.0359	-0.0082
16	-0.0063	-0.0694	-0.0256
17	0.0057	-0.0244	-0.0398
18	0.0092	-0.0398	-0.0440
19	-0.0008	-0.0444	-0.0113
20	-0.0047	-0.0122	0.0022
21	-0.0057	-0.0632	-0.0277
22	0.0048	-0.0781	-0.0600
23	0.0024	-0.0245	-0.0045
24	-0.0141	-0.0096	-0.0031

No.	Changes in enamel surface roughness (μm)		
	Control	25% H_2O_2	35% H_2O_2
25	-0.0016	-0.0118	-0.0003
26	0.0014	-0.0095	-0.0024
27	-0.0015	-0.0330	-0.0020
28	0.0062	-0.0082	-0.0480
29	-0.0011	-0.0058	-0.0053
30	0.0048	-0.0126	-0.0359
31	-0.0027	-0.0172	-0.0065
32	-0.0052	-0.0361	-0.0458
33	-0.0127	-0.0326	-0.0146
34	-0.0033	-0.0657	-0.0036
35	0.0059	-0.0135	-0.0749
36	-0.0039	-0.0027	-0.0168
37	0.0019	-0.0390	-0.0127
38	-0.0031	-0.0619	-0.0027
39	-0.0038	-0.0157	-0.0010
40	0.0031	-0.0023	-0.0090
41	-0.0024	-0.0151	-0.0765
42	0.0024	-0.0041	-0.0682
43	0.0015	-0.0151	-0.0135
44	-0.0095	-0.0414	-0.0178
45	0.0012	-0.0068	-0.0272
46	0.0016	-0.0167	-0.0642
47	-0.0017	-0.0383	-0.0712
48	-0.0002	-0.0086	-0.0081
49	-0.0119	-0.0275	-0.0082
50	-0.0095	-0.0222	-0.0056

No.	Changes in enamel surface roughness (μm)		
	Control	25% H_2O_2	35% H_2O_2
51	-0.0037	-0.0134	-0.0266
52	-0.0057	-0.0448	-0.0813
53	0.0032	-0.0423	-0.0292
54	0.0019	-0.0065	-0.0159
Median	-0.0010	-0.0217	-0.0162
Interquartile range	-0.0041 – 0.0026	-0.0416 - -0.0113	-0.0409 - -0.0063
Min	-0.0141	-0.0775	-0.0845
Max	0.0097	-0.0006	0.0032

Appendix D Statistical comparison of changes in enamel surface roughness

Comparison	p-value*
Control – 25% H_2O_2	<0.001
Control – 35% H_2O_2	<0.001
25% H_2O_2 – 35% H_2O_2	0.189

* By Kruskal-Wallis and Mann-Whitney U tests with Bonferroni corrections

Appendix E The optical density values at 595 nm of crystal violet assays represent the amount of biofilm formation of each specimen at first experiment

No.	Optical density value at 595 nm.								
	S. mutans			*S. sanguinis*			Background		
	Control	25% H_2O_2	35% H_2O_2	Control	25% H_2O_2	35% H_2O_2	Control	25% H_2O_2	35% H_2O_2
1	0.219	0.238	0.310	0.347	0.243	1.660	0.397	0.166	0.265
2	0.300	0.381	0.428	0.519	0.236	1.356	0.117	0.075	0.259
3	0.383	0.309	0.274	0.610	0.233	0.867	0.122	0.116	0.320
4	0.322	0.262	0.404	0.383	0.181	1.163	0.166	0.187	0.151
5	0.584	0.239	0.345	0.568	0.253	0.962	0.178	0.126	0.288

Appendix F The optical density values at 595 nm of crystal violet assays represent the amount of biofilm formation of each specimen at second experiment

No.	Optical density value at 595 nm.								
	S. mutans			*S. sanguinis*			Background		
	Control	25% H_2O_2	35% H_2O_2	Control	25% H_2O_2	35% H_2O_2	Control	25% H_2O_2	35% H_2O_2
1	0.315	0.199	0.295	0.319	0.307	0.848	0.099	0.124	0.257
2	0.293	0.224	0.364	0.451	0.217	0.866	0.109	0.097	0.234
3	0.229	0.185	0.372	0.373	0.249	1.186	0.076	0.106	0.215
4	0.339	0.168	0.36	0.273	0.175	1.573	0.088	0.091	0.253
5	0.334	0.136	0.228	0.298	0.195	1.155	0.069	0.100	0.264

Appendix G The optical density values at 595 nm of crystal violet assays represent the amount of biofilm formation of each specimen at third experiment

No.	Optical density value at 595 nm.								
	S. mutans			*S. sanguinis*			Background		
	Control	25% H_2O_2	35% H_2O_2	Control	25% H_2O_2	35% H_2O_2	Control	25% H_2O_2	35% H_2O_2
1	0.245	0.208	0.512	0.597	0.372	0.969	0.336	0.162	0.138
2	0.323	0.207	0.322	0.515	0.191	0.78	0.151	0.231	0.231
3	0.252	0.232	0.524	0.485	0.423	0.986	0.143	0.135	0.308
4	0.213	0.244	0.329	0.328	0.218	1.265	0.193	0.229	0.302
5	0.253	0.163	0.51	0.387	0.218	0.701	0.207	0.186	0.367

Appendix H The optical density values at 595 nm of crystal violet assays represent the amount of *S. mutans* biofilm formation after subtracting with mean of optical density value of the background group

No.	Optical density value at 595 nm. of *S. mutans* biofilm formation		
	Control	25% H_2O_2	35% H_2O_2
1	0.056	0.096	0.053
2	0.137	0.239	0.171
3	0.220	0.167	0.017
4	0.159	0.120	0.147
5	0.421	0.097	0.088
6	0.152	0.057	0.038
7	0.130	0.082	0.107
8	0.066	0.043	0.115
9	0.176	0.026	0.103
10	0.171	-0.006	-0.029
11	0.082	0.066	0.255
12	0.160	0.065	0.065
13	0.089	0.090	0.267
14	0.050	0.102	0.072
15	0.090	0.021	0.253
Median	0.137	0.082	0.103
Interquartile range	0.082 – 0.171	0.043 – 0.102	0.053 – 0.171
Min	0.050	-0.006	-0.029
Max	0.421	0.239	0.267

Appendix I The optical density values at 595 nm of crystal violet assays represent the amount of *S. sanguinis* biofilm formation after subtracting with mean of optical density value of the background group

No.	Optical density value at 595 nm. of *S. sanguinis* biofilm formation		
	Control	25% H_2O_2	35% H_2O_2
1	0.184	0.101	1.403
2	0.356	0.094	1.099
3	0.447	0.091	0.610
4	0.220	0.039	0.906
5	0.405	0.111	0.705
6	0.156	0.165	0.591
7	0.288	0.075	0.609
8	0.210	0.107	0.929
9	0.110	0.033	1.316
10	0.135	0.053	0.898
11	0.434	0.230	0.712
12	0.352	0.049	0.523
13	0.322	0.281	0.729
14	0.165	0.076	1.008
15	0.224	0.076	0.444
Median	0.224	0.091	0.729
Interquartile range	0.165 – 0.356	0.053 – 0.111	0.609 – 1.008
Min	0.110	0.033	0.444
Max	0.447	0.281	1.403

Appendix J Statistical comparison of the optical density value at 595 nm. of crystal violet assays of *S. mutans* biofilm formation

Comparison	p-value*
Control – 25% H_2O_2	0.046
Control – 35% H_2O_2	0.340
25% H_2O_2 – 35% H_2O_2	0.325

* By Kruskal-Wallis and Mann-Whitney U tests with Bonferroni corrections

Appendix K Statistical comparison of the optical density value at 595 nm. of crystal violet assays of *S. sanguinis* biofilm formation

Comparison	p-value*
Control – 25% H_2O_2	<0.001
Control – 35% H_2O_2	<0.001
25% H_2O_2 – 35% H_2O_2	<0.001

* By Kruskal-Wallis and Mann-Whitney U tests with Bonferroni corrections

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