The effect of xylitol and erythritol in the presence of sucrose on single and mixed-species biofilm of early colonizer and cariogenic streptococci

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ผลของน้ำตาลไซลิทอลและอิริทริทอลร่วมกับน้ำตาลซูโครสต่อไบโอฟิล์มชนิดเดี่ยวและชนิดผสมของเชื้อกลุ่ม early colonizer และ cariogenic streptococci

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ปนัดดา อยู่อภิบาลรักษ์ : ผลของน้ำตาลไซลิทอลและอิริทริทอลร่วมกับน้ำตาลซูโครสต่อไบโอฟิล์มชนิดเดี่ยวและชนิดผสมของเชื้อกลุ่ม early colonizer และ cariogenic streptococci. (The effect of xylitol and erythritol in the presence of sucrose on single and mixed-species biofilm of early colonizer and cariogenic streptococci) อ.ที่ปรึกษาหลัก : รศ. ทญ. ดร.พนิดา ธัญญศรีสังข์, อ.ที่ปรึกษาร่วม : รศ. ทญ. ดร.อรนาฎ มาตังคสมบัติ

โรคฟันผุเกิดจากหลายปัจจัย ที่สำคัญคือการได้รับซูโครส โรคฟันผุเป็นผลจากการเสียสมดุลระหว่างเชื้อกลุ่มแรก (early colonizers) กับเชื้อก่อโรคฟันผุ (cariogenic bacteria) ในไบโอฟิล์ม น้ำตาลแอลกอฮอล์ไซลิทอล และอิริทริทอลถูกนำมาใช้มากขึ้นเนื่องจากไม่ก่อให้เกิดฟันผุ และยังมีประสิทธิภาพในการป้องกันฟันผุด้วย แต่ในชีวิตประจำวันการบริโภคซูโครสแทบจะหลีกเลี่ยงไม่ได้ การศึกษานี้จึงมีวัตถุประสงค์ที่จะตรวจสอบผลของไซลิทอลและอิริทริทอลร่วมกับซูโครสต่อเชื้อกลุ่ม early colonizers และ cariogenic bacteria ในไบโอฟิล์มทั้งชนิดเดี่ยวและผสม โดยทำการเลี้ยงเชื้อทั้งสองกลุ่มในอาหารเลี้ยงเชื้อชนิดเหลวที่ผสมซูโครสร้อยละ 1 เปรียบเทียบกับเลี้ยงในอาหารเลี้ยงเชื้อที่ผสมไซลิทอลหรืออิริทริทอลแบบที่มีและไม่มีซูโครสผสม เป็นเวลา 36 ชั่วโมง จากนั้นตรวจสอบการผลิตกรดด้วยการวัด pH การสร้างไบโอฟิล์มโดยการวัดมวลไบโอฟิล์ม และจำนวนเชื้อ พบว่าแม้ในสภาวะที่มีซูโครส ไซลิทอลและอิริทริทอลช่วยชะลอการลดลงของค่า pH ทั้งกลุ่ม early colonizers และ cariogenic bacteria ได้ในช่วง 2 ชั่วโมงแรก และพบว่าที่ความเข้มข้นสูงสามารถลดมวลไบโอฟิล์มของ *S. gordonii* และของเชื้อผสมสามชนิดได้อย่างมีนัยสำคัญ และมีแนวโน้มลดมวลไบโอฟิล์มของ *S. mutans* แบบชนิดเดียว และแบบผสมด้วย นอกจากนี้พบว่าจำนวนเชื้อที่มีชีวิตในไบโอฟิล์มของเชื้อทั้ง 2 กลุ่มยกเว้น *S. gordonii* ที่เลี้ยงในน้ำตาลแอลกอฮอล์ทั้งสองชนิดร่วมกับซูโครสมีจำนวนเชื้อมากกว่าที่เลี้ยงในน้ำตาลซูโครสเพียงอย่างเดียว สรุปได้ว่าน้ำตาลแอลกอฮอล์ไซลิทอล และอิริทริทอลที่มีซูโครสมีผลต่อค่า pH มวลไบโอฟิล์ม และจำนวนเชื้อต่อเชื้อทั้งกลุ่ม early colonizers และ cariogenic bacteria

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Panadda Yuapibanrak : The effect of xylitol and erythritol in the presence of sucrose on single and mixed-species biofilm of early colonizer and cariogenic streptococci. Advisor: Assoc. Prof. PANIDA THANYASRISUNG, D.D.S., Ph.D. Co-advisor: Assoc. Prof. ORANART MATANGKASOMBUT, D.D.S., Ph.D.

Dental caries is caused by multiple factors. An essential factor is sucrose. Dental caries is the result of an imbalance between early colonizers and cariogenic bacteria in biofilms. Sugar alcohols, xylitol and erythritol, become more popular because they do not cause cavities and are also effective at preventing caries. In everyday life, however, sucrose consumption is unavoidable. Thus, the aim of this study was to investigate the effect of xylitol and erythritol in the presence of sucrose on single- and mixed-species biofilm of early colonizers and cariogenic bacteria. Bacteria were cultured in broth containing 1% sucrose compared to broth containing xylitol or erythritol with and without sucrose for 36 h. Acid production was then measured using pH meter, biofilm formation using biofilm mass measurement, and colony count. Our result showed that even in the presence of sucrose, xylitol and erythritol slowed pH reduction in early colonizers and cariogenic bacteria during the first 2 h. Moreover, the high concentrations significantly reduced the biofilm mass of *S. gordonii* and the three-mixed species biofilm. The biofilm mass of *S. mutans* single- and mixed-species was also reduced. Furthermore, except for S. *gordonii,* the number of viable cells in the biofilm of both groups of bacteria grown in sugar alcohols with sucrose was greater than that of sucrose alone. In conclusion, sugar alcohols with sucrose influenced the pH, biofilm mass, and cell numbers of early colonizers and cariogenic bacteria.

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Student's Signature .....
Advisor's Signature .....
Co-advisor's Signature .....

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CHAPTER I

INTRODUCTION

Dental caries is among the most common chronic infectious diseases and is an important oral health problem worldwide. There are about 3.5 billion people, and more than 520 million children were affected by dental caries [1]. In Thailand, more than 50% of preschool children and also teenage have dental caries [2]. Although dental caries is not a life-threatening disease, it has an impact on the quality of life, especially in children [3]. The untreated disease causes pains and difficulty chewing which affect the growth and development of children [4]. Dental caries is caused by multiple factors mainly including; a susceptible host, fermentable carbohydrates especially sucrose, microorganisms, and time [5].

Based on the ecological plaque hypothesis, the development of dental caries is a consequence of an imbalance between commensal oral microorganisms and cariogenic pathogens within dental plaque (oral biofilm) [6, 7]. In healthy condition, early colonizers (such as Mitis streptococci) is the first bacterial group that colonizes on the tooth surfaces [8, 9]. They occupy the spaces and have antagonist activities against their opponents through the production of hydrogen peroxide (H_2O_2) and alkali via arginine metabolism [10]. The dysbiosis occurs after the alteration of pH within the biofilm from neutral to acidic conditions which facilitate cariogenic pathogens to grow [11]. Based on the ecological plaque hypothesis, the alteration of

pH biofilm is a result of excessive sugar consumption [12]. In this condition, cariogenic pathogens, such as Mutans streptococci (MS), metabolize sucrose via the glycolytic pathway and further release lactic acids as a by-product [13, 14]. At the same time, they synthesize extracellular polysaccharides (EPS) by converting sucrose to glucans, which are the main component of oral biofilms [15, 16]. The acids then are accumulated within biofilms and eventually develop an acidic environment [17, 18]. Thus, sucrose plays an important role in microbial dysbiosis that finally leads to caries development. Therefore, one of the strategies to prevent dental caries is the use of sugar substitutes, such as sugar alcohols.

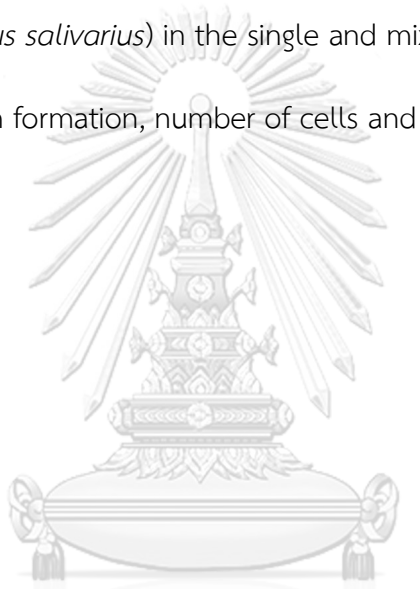
Sugar alcohols (polyols) have been widely used as a sugar substitute for diabetic patients or weight-control individuals since they have a low-glycemic index and lower calories compared to sucrose [19, 20]. Besides the health benefits, the polyols are low- or non-cariogenic [21-23]. Among all polyols, xylitol is the most widely used for caries control with much evidence of its non- and anti-cariogenicity [24-26]. Erythritol is another polyol which is also in the spotlight since it showed higher effectiveness in caries prevention than xylitol [27-29]. With the aim to use xylitol and erythritol for caries prevention, most studies have focused on their effect against cariogenic pathogens, especially *S. mutans.* However, not much is known about the effect on early colonizers which also reside in the biofilms. Moreover, although the utilization of sugar substitutions is increasing, sucrose is still one of the main ingredients in various foods. Therefore, sucrose consumption is hardly

avoidable. With the limited knowledge of the effect of sugar alcohol in the presence of sucrose, this study aims to investigate the impact of xylitol or erythritol in the presence of sucrose on cariogenic streptococci and early colonizers in the biofilm.

CHAPTER II

OBJECTIVE

To investigate the effect of xylitol and erythritol in the presence of sucrose on cariogenic streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*) and early colonizers (*Streptococcus sanguinis*, *Streptococcus gordonii*, *Streptococcus oralis* and *Streptococcus salivarius*) in the single and mixed-species biofilm in term of acid production, biofilm formation, number of cells and biofilm morphology.



CHAPTER III

LITERATURE REVIEW

Dental caries

Dental caries is a common chronic infectious disease and continues to be a global oral health problem. The survey in 204 countries from 1990 to 2019 of the Global Burden of Diseases, Injuries, and Risk Factors Study 2019 (GBD 2019) showed that nearly 3.5 billion people and more than 520 million children were affected by caries [1]. In Thailand, the latest National Oral Health Survey in 2017 revealed that more than 50% of pre-school children have dental caries and more than 40% have a high level of visible plaque which represent the high caries risk. School-age children (12 years old) and teenage (15 years old) also have a caries prevalence of more than 50% [2]. Untreated caries has an impact on the quality of life. The disease causes pain and even systemic infection which results in undernutrition and adverse growth development [30].

Dental caries is a multifactorial disease that is mainly caused by a susceptible host, fermentable carbohydrates, especially sucrose, microorganisms, and time (in terms of frequency) [31]. Based on the ecological plaque hypothesis, the disease begins with the change of pH in biofilm to the acidic environment which leads to the shift of ecology in biofilm (Figure 1) [5]. The environmental low pH promotes the growth of cariogenic pathogens which possesses better acid tolerance and acid

production than commensal microorganism which mainly are early colonizers [31, 32].

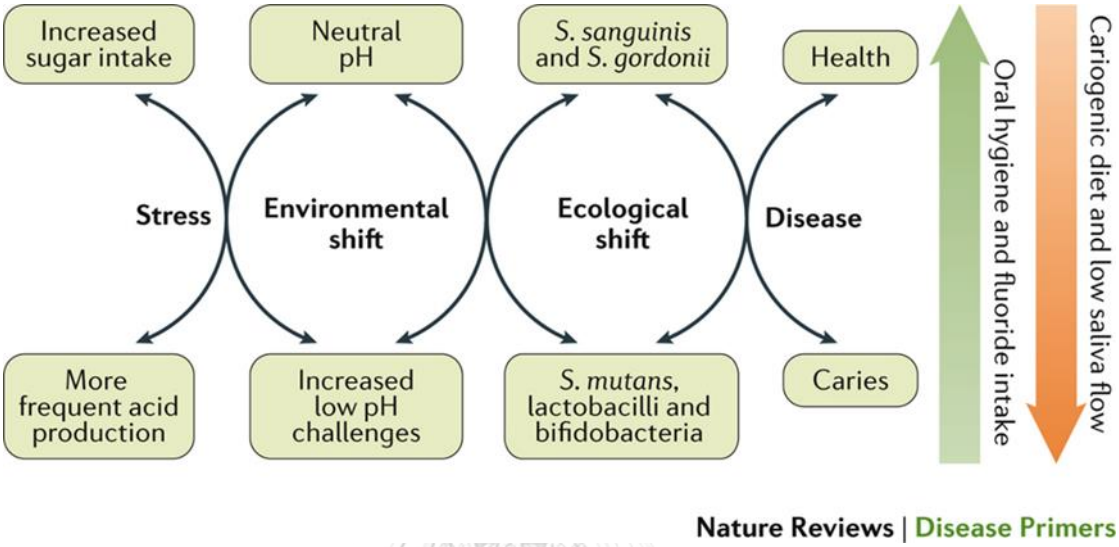


Figure 1 Ecological plaque hypothesis diagram [5]

Cariogenic pathogens

The pathogens, predominantly *Streptococcus mutans,* and *Streptococcus sobrinus*, metabolize "sucrose" via glycolysis pathway and release lactic acids as a by-product (Figure 2). Briefly, "sucrose" enters cells directly or is broken down into monosaccharides (glucose and fructose) before being transported through the major sugar transport mechanism, the phosphoenol pyruvate mediated phosphotransferase transport system (PEP-PTS; EII^{suc}, EII^{man} and EII^{fru}, respectively, Figure 2) [13, 33]. Under sugar excess conditions, internalized sucrose is converted to pyruvate via the glycolysis pathway, then to lactate via the lactate dehydrogenase (LDH) complex.

Lactate is transported across the cell membrane of *S. mutans* as lactic acids [34]. Outside the cells, the remaining "sucrose" (<5%) is converted by the enzyme glucosyltransferase (Gtfs) to glucans (extracellular polysaccharide, EPS), which is the main component of oral biofilm (Figure 2) [13]. *S. mutans* possess 3 types of Gtfs, which are GtfB, GtfC, and GtfD. GtfB produces water-insoluble glucans (WIG), which are important in bacterial cell aggregation within biofilms, whereas GtfC, which produces a mixture of WIG and water-soluble glucans (WSG), is required for adherence to the acquired pellicle on the tooth surface. GtfD synthesizes water-soluble (WSG, called dextran) [13, 35]. The four types of Gtf discovered in *S. sobrinus* are GtfI, GtfS, GtfT, and GtfU, with GtfI being the only WIG [35]. The acid production together with biofilm formation is the cause of caries development. It is noticeable that "sucrose" plays an important role in this incidence.

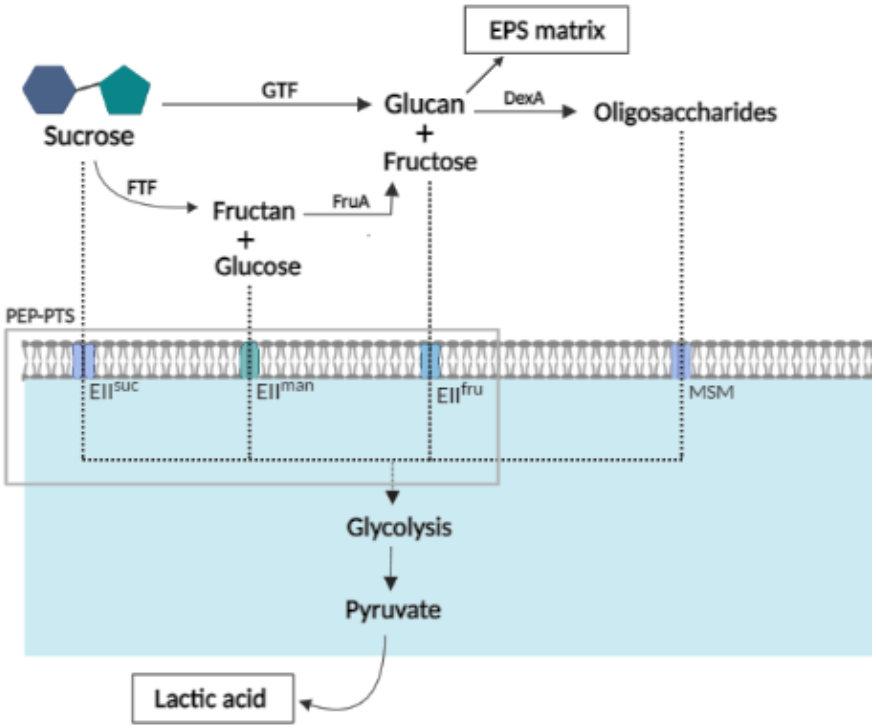


Figure 2 Pathway of sucrose metabolism by *Streptococcus mutans* (modified from [13, 36]). EPS = extracellular polysaccharides; GTF = glucosyltransferases; FTF = fructosyltransferases; DexA = extracellular dextranase; FruA = fructanase enzyme; PEP-PTS = phosphoenolpyruvate-mediated phosphotransferase transport; EII; substrate specific Enzyme II complex, (EII^{suc}= sucrose-PTS, EII^{man} = glucose/mannose-PTS, and EII^{fru} = frutose-PTS); MSM = the multiple-sugar metabolism transport

Early colonizers

The majority of bacteria in healthy plaque, which is mostly new plaque, were early colonizers such as *Streptococcus sanguinis*, *Streptococcus gordonii*, *Streptococcus oralis*, and *Streptococcus mitis* [37]. Early colonizers establish adhesion to the tooth surface through specific interaction between bacterial adhesins (such as the antigen I/II family and multifunctional fibrillar adhesins) and salivary

proteins (including albumin, proline-rich protein, etc.) on the tooth surface [37, 38]. Some early colonizers produce Gtfs, such as *S. sanguinis* GtfP, *S. gordonii* GtfG, *S. oralis* GtfR, and *S. salivarius* GtfJ, GtfK, GtfL, and GtfM, the majority of these are WSG [35].

In terms of acid production, similar to *S. mutans*, early colonizers transport sugars through the PEP-PTS system and metabolize them via the glycolysis pathway, producing acids as a by-product when sugar levels are high [34]. However, early colonizers cannot survive in high acid concentrations (low pH) because the pH homeostasis mechanism is not as effective as in *S. mutans* [39, 40]. *S. mutans* thrives in acidic conditions (acid tolerance) by using the following mechanisms: membrane-bound F_1F_0-ATPase (F-ATPase), which pumps proton (H^+) out of the cells, alteration of membrane fatty acids to block the passive influx of H^+ ions, and production of neutralizing molecules such as ammonia and CO_2 [13]. Furthermore, *S. mutans* encode several DNA/protein repair enzymes which can repair damage caused by acid [40].

Previous study has shown that early colonizers and cariogenic pathogens are incompatible. P W Caufield demonstrated that when *S. sanguinis* colonizes tooth surfaces first, *S. mutans* colonization is delayed [41]. To compete with pathogens, early colonizers used three mechanisms: neutralization of acids, production of H_2O_2, and antimicrobial agents to kill pathogens [37].

Sugar alcohol and caries prevention

Sugar alcohols (polyols) are products from fruits, vegetables, fungi, yeasts, or algae. The polyols are hydrogenated carbohydrates in monosaccharides, disaccharides, and oligosaccharides which are obtained by substituting and aldehyde to change the molecules to polyhydroxy alcohol [20]. The polyols, such as xylitol, are widely used as a sugar substitute for people with diabetes or people who want to control their weight since they do not affect insulin and also slow and incomplete absorption in the intestine [21, 27]. Poor intestinal absorption can cause diarrhea when consuming the excessive amount of xylitol [27].

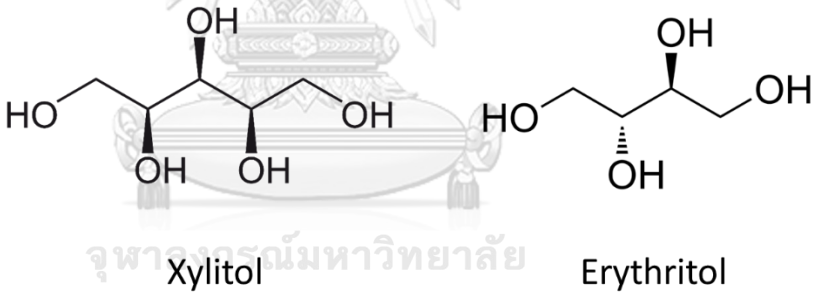


Figure 3 Structure of xylitol and erythritol [42, 43]

Besides the health benefits, there are many evidence showing that polyols, especially xylitol, have an anti-cariogenic property [44, 45]. They are often incorporated into chewing gum, candy, lozenge, mouthwash, or toothpaste [20, 21]. Among all polyols, xylitol has gained the most attention with its superior in non-cariogenicity and caries prevention than other polyols [46]. Xylitol consists of 5

carbon ($C_5H_{12}O_5$) and is soluble in water [47] (Figure 3). Many *in vitro* studies showed that *Streptococcus mutans* cannot metabolize xylitol, thus xylitol can inhibit bacterial growth and acid production [23, 48-50]. Xylitol is transported into *S. mutans* cells via phosphoenolpyruvate phosphotransferase (PEP-PTS). Inside the cell, xylitol is converted to the xylitol-5-.phosphate which inhibits the enzymes in glycolysis [22]. As a result, there is no energy from glycolysis and no acid by-product. In addition, the excess of xylitol 5-phosphate is accumulated and dephosphorylated to xylitol before eliminating from the cells. This futile cycle requires energy, but the cells cannot generate enough energy; this results in the inhibition of growth (Figure 4) [22]. Moreover, there are many *in vitro* evidence showed that xylitol inhibits biofilm formation of *S. mutans* [23, 51]. The inhibition is partly explained by the decrease of viable cells or the polysaccharide synthesis [51]. Xylitol also reduced gene expression of biofilm-producing (Gtf) of *S. mutans* [52].

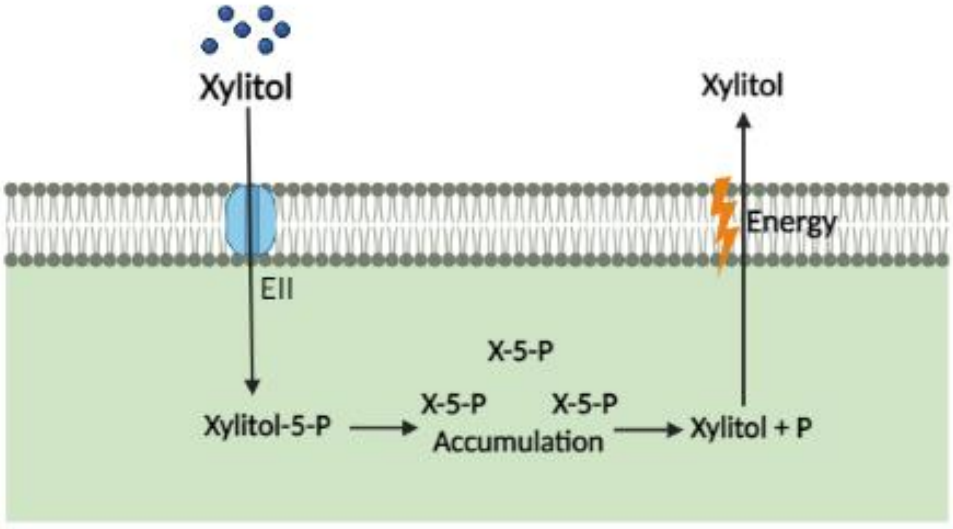


Figure 4 Pathway of xylitol metabolism by *Streptococcus mutans* (Modified from [22]).

Another polyol that has gained increasing attention is erythritol ($C_4H_{10}O_4$) (Figure 3). Erythritol is smaller than xylitol and other polyols which makes it slightly different from other polyols [53]. Erythritol can be absorbed in the small intestine and excreted unchanged in the urine which results in a less laxative effect. Moreover, erythritol is the only polyol that is non-caloric, non-glycemic and non-cariogenic [54]. Similar to xylitol, erythritol cannot be metabolized by *S. mutans*. Erythritol has also inhibited the growth and the biofilm of *S. mutans* and *S. sobrinus* in the *in vitro* study [55, 56], and also inhibited the lactic acid production [57]. A previous study revealed that erythritol has higher effectiveness in the inhibition of growth than xylitol [58]. This phenomenon may be explained by the smaller size of erythritol so it can easily pass through the cell membrane and arrest the growth via several pathways [59]. However, the interaction of erythritol in the *S. mutans* has not quite

been described yet but, there are reports that erythritol was related to the inhibition of expression of enzyme gene encoding such as extracellular proteases that make microorganisms inaccessible amino acids and glucose/fructose transferase are involved in biofilm matrix synthesis, suppress the growth of the bacteria resulting from DNA and RNA depletion, and changing of dipeptide acquisition and amino acid metabolism [22, 59]. Interestingly, the study on the effect of erythritol on the microbial ecology of *in vitro* gingivitis biofilms showed that the microbial ecology shifted from periodontal pathogens to early colonizers in the presence of erythritol [60].

Although the use of sugar substitutes is considered as one of the promising methodologies to prevent dental caries. Nevertheless, in real life, most foods and beverages contain sugars as an ingredient. Even though the use of sugar substitutes is increasing, it is impossible to avoid sugar consumption. So, the property of sugar alcohols in combination with sucrose (the sugar used in daily life), and its effect on microbial ecology especially early colonizers were still limited knowledge. Therefore, this study aims to investigate the effect of xylitol and erythritol in the presence of sucrose on single and mixed-species biofilm between early colonizers and cariogenic streptococci.

CHAPTER IV

MATERIALS AND METHODS

Bacterial strains and growth conditions

Cariogenic streptococci (*Streptococcus mutans* UA159 and *Streptococcus sobrinus* OMZ176) and early colonizers (*Streptococcus sanguinis* ATCC10556, *Streptococcus gordonii* DMST20560, *Streptococcus oralis* ATCC35037, and *Streptococcus salivarius* ATCC7073) were used in this study. Bacterial glycerol stocks were inoculated in Brain-Heart Infusion or BHI agar (HiMedia Laboratories, India) and incubated at 37°C with 5% CO_2 (Thermo Scientific, USA) for 24 h. The isolated colony was inoculated in BHI broth and incubated overnight with continuous shaking at 240 rpm (IKA KS 130 basic Shaker, USA). The optical density at 600 nm (OD_{600nm}) of the overnight culture was measured using a spectrophotometer (Thermo Scientific, USA) and adjusted to 0.1 by dilution in BHI broth. The culture was then incubated at 37°C with 5% CO_2 for approximately 3 h to achieve the log phase (OD_{600nm} = 0.4-0.6, ≈ 10^8 CFU/ml of *S. mutans*, *S. sobrinus*, and 10^7 CFU/ml of *S. sanguinis*, *S. gordonii*, *S. oralis,* and *S. salivarius*) for subsequent experiments.

Preparation of sucrose, xylitol, and erythritol

To prepare BHI broth containing 1% (w/v) sucrose, 10 g of sucrose and 37 g of BHI powder was dissolved in 1 liter of reverse osmosis water (Unipure, USA) and then sterilized by an autoclave (Hirayama, Japan). A 20% (w/v) stock solution of erythritol

(Krungthepchemi, Thailand) and xylitol (Xyliplus, Thailand) was prepared by dissolving 20 g of each sugar alcohol in 100 ml of reverse osmosis water (Unipure, USA) and filter sterilized using a 0.2 μm membrane filter (Sartorius stedim biotech, Germany). To prepare sugar alcohols in the presence of 1% sucrose, a 20% stock solution of erythritol and xylitol was diluted to concentrations of 10%, 5%, 2%, and 1% in BHI containing 1% sucrose. Furthermore, BHI broth, BHI broth supplemented with 10% and 1% of each sugar alcohol, as well as 1% sucrose, was used as a control. Table 1 displays all the media and abbreviations used in this study.

Table 1 List of media used in this study

In the presence of 1% sucrose		In the absence of 1% sucrose	
Media	**Abbreviation**	**Media**	**Abbreviation**
BHI+1% Sucrose	1S	BHI	BHI
BHI+10% Erythritol+1%Sucrose	10E+1S	BHI+10% Erythritol	10E
BHI+10% Xylitol+1%Sucrose	10X+1S	BHI+10% Xylitol	10X
BHI+5% Erythritol+1%Sucrose	5E+1S	BHI+1% Erythritol	1E
BHI+5% Xylitol+1%Sucrose	5X+1S	BHI+1% Xylitol	1X
BHI+2% Erythritol+1%Sucrose	2E+1S		
BHI+2% Xylitol+1%Sucrose	2X+1S		
BHI+1% Erythritol+1%Sucrose	1E+1S		
BHI+1% Xylitol+1%Sucrose	1X+1S		

Formation of biofilm

To form a single-species biofilm, the bacterial cells were collected from 1 ml of log-phase culture (approximately ≈ 10^8 CFU/ml of *S. mutans*, *S. sobrinus*, and 10^7 CFU/ml of *S. sanguinis*, *S. gordonii*, *S. oralis,* and *S. salivarius*) by centrifugation (12,000xg, 4°C, 15 min) and re-suspended in 500 µl of assigned culture media. 500 µl of suspension was placed in a well of a 24-well plate (Corning Costar flat-bottom cell culture plates, Fisher Scientific, USA). In each well, the final bacterial numbers were approximately 10^8 CFU/well of *S. mutans*, *S. sobrinus*, and 10^7 CFU/well of *S. sanguinis*, *S. gordonii*, *S. oralis,* and *S. salivarius*. To observe the dynamic change of biofilm pH, 5 ml of suspension were added into a cell culture dish (SPL cell culture dish 60 x 15 mm, SPL life sciences, Korea), which has tenfold larger surface area than a 24-well plate, providing 10^9 CFU/well of *S. mutans*, and 10^8 CFU/well of *S. sanguinis*. For the Confocal laser scanning microscopy staining assay, the bacterial cells were harvested from 500 µl of log-phase culture and re-suspended in 250 µl of assigned culture media before being transferred into a well of an 8 well cell culture slide (SPL life sciences, Korea), which was about half the size of each well of the 24 well plate, giving $0.5x10^8$ CFU/well of *S. mutans*, and $0.5x10^7$ CFU/well of *S. sanguinis*, *S. gordonii*. The cultures were incubated at 37°C, 5%CO_2 for 36 h to form biofilm, which was used in following experiments.

To form a mixed-species biofilm, *S. mutans* was chosen as a cariogenic pathogen representative since it is commonly found in the biofilm of children with

ECC [61], and *S. sanguinis* and *S. gordonii* were chosen as early colonizers since they are abundant in healthy dental plaque [62, 63]. An equal ratio of the log-phase culture of *S. mutans* and *S. sanguinis* (or *S. gordonii*) (1:1) or a combination of the three species (1:1:1) were mixed [64]. The bacterial cells from the mixture were harvested and further proceeded following the single-species biofilm formation.

Determination of biofilm pH

Based on our preliminary study (data not shown), the pH of media did not differ from the pH of biofilm; thus, the pH of 36-h-old biofilms was determined from the media using a pH meter (Compact pH Meter, Horiba, Japan). The experiment was independently performed three times.

To investigate the dynamic change of pH during biofilm development, the pH was measured at 15, 30, 45, 60, 75, 90, 105, 120 min, and 36 h (the end-point). The experiments were carried out in duplicate and independently three times for *S. mutans* and four times for *S. sanguinis*.

Measurement of biofilm mass by crystal violet or CV staining assay

To determine the total biofilm mass, CV staining assay was performed following O'Toole GA with modifications [65]. Briefly, the 36-h-old biofilms were washed three times with 500 µl of phosphate-buffered saline (PBS, pH 7.4) and stained with 500 µl of 0.1% (w/v) crystal violet solution. After 15 min at room temperature, the plate was washed with tap water until the color of the water became clear. After air drying, the stained biofilm was de-stained with 30% acetic

acid for 15 min at room temperature. The de-stained solution was transferred to a new 24-well plate. The optical density of a de-stained solution at 520 nm (OD_{520nm}) was measured using a microplate reader (EPOCH2 microplate spectrophotometer, BioTek, USA). The 30% of acetic acid was used as a blank. The experiment was independently performed three times.

Investigation of biofilm morphology by scanning electron microscopy

The assay was performed according to the manufacturer's instructions and Zhong Zhang with modifications [66]. Only 1S, 10E+1S, and 10X+1S groups were investigated by SEM since the biofilms were visible. The 36-h-old biofilms were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 at 4°C overnight and rinsed twice with 0.1 M phosphate buffer for 10 min. The specimens were then continually dehydrated with 30%, 50%, 70%, 95% (v/v) ethanol for 10 min of each concentration, and 100% (v/v) ethanol for 10 min three times. The specimens were then dried using a critical point drier (Lecia model EM CPD300, Austria) and coated with gold by a sputter coater (Balzers model SCD 040, Germany). The specimens were thoroughly scanned by the scanning electron microscopy (JSM-6610 Series Scanning Electron Microscope, JEOL Ltd, Japan), and three representative locations were captured at magnifications of 1,000 (1 location) and 20,000 (2 locations).

Determination of the number of cells

Colony count method

The 36-h-old biofilms were washed three times with 500 µl of phosphate-buffered saline (PBS, pH 7.4) to remove unbound cells. One ml of phosphate-buffered saline (PBS, pH 7.4) was added, and the biofilm was scraped off. To break up the cell clumps, the suspension was sonicated twice for 30 sec using an ultrasonic homogenizer with a 25% amplitude [67]. The suspension was then serial diluted and plated on BHI agar for single-species biofilm and on Mitis-Salivarius (MS) agar, which is selective and differential media for oral streptococci, for mixed-species biofilm. The plates were incubated at 37°C, 5%CO_2 48 h, and the colony-forming unit (CFU) was determined. Because some of the first dilution plates had no colony, which could not be determined by log transformation, the number of bacteria was raised by one for each data point and reported as log (CFU+1)/mL [68]. The experiments were carried out in duplicate and independently six times for single species and three times for mixed species.

Staining of biofilm for Confocal Laser Scanning Microscopy analysis

After incubated 36 h, the biofilms were washed three times with 500 µl of 0.9% NaCl and then stained with 100 µl of 0.3% SYTO 9 and Pi [69] (LIVE/DEAD BacLight Bacterial Viability Kits, Invitrogen, USA) solution in 0.9% NaCl and incubated for 15 min at room temperature in a dark room for stained the bacteria live and

dead cells [70] After that, the biofilm was washed three times with 500 µl of 0.9% NaCl and stained with 100 µl of 10 µg/mL calcofluor (Fluorescent Brightener 28, Sigma-Aldrich, USA) solution in 10 mM sodium phosphate (pH 7.5) and incubated for 30 min at room temperature in a dark room to stained extracellular polysaccharides (EPS) and wash three times with 500 µl of 0.9% NaCl [71]. The stained slide was closed with a coverslip and observed in a confocal laser scanning microscopy (ZEISS LSM 900, ZEISS, Germany). Filters were 488/507 nm for detection of SYTO9, 530/615 nm for detection of propidium iodide, and 380/475 nm for detection of calcofluor. The three representative locations were captured at magnifications of 20x (1 location) and 63x (2 locations).

Quantitative PCR (qPCR)

Initially, a standard curve of each bacteria (*S. mutans*, *S. sanguinis*, and *S. gordonii*) was conducted. One ml of a log phase (OD_{600nm} = 0.4-0.6, ≈10^8 CFU/ml of *S. mutans,* and ≈10^7 CFU/ml of *S. sanguinis* and *S. gordonii*) were centrifugated at 12,000 xg, 4°C, 15 min, and culture medium was completely removed. The pelleted cells were harvested for DNA extraction using DNeasy PowerSoil Pro Kit (Qiagen, USA) following the manufacturer's instructions. Briefly, 800 µl of lysis buffer (Buffer CD1) was added to the pelleted cells and transferred to the PowerBead Pro tube. The tube was vortexed on a vortex adapter (Merck, Germany) at maximum speed for 10 min and then centrifuged at 15,000 xg for 1 min. The 600 µl of the supernatant was transferred to a clean 2 ml microcentrifuge tube, mixed with 200 µl of the buffer CD2

to remove inhibitors, and then centrifuged at 15,000 xg for 1 min. The 700 µl of supernatant was transferred to a clean 2 ml microcentrifuge tube and mixed with 600 µl of binding buffer (Buffer CD3) for 5 sec. The mixture was then loaded into an MB spin column and centrifuged at 15,000 xg for 1 min. The MB spin column was washed with 500 µl of wash buffer (EA), discarded the flow-through and followed by 500 µl of another wash buffer (C5). After centrifugation, the column was transferred to a 1.5 ml elution tube. In the elution step, the DNA samples of *S. sanguinis* and *S. gordonii* were eluted with 100 µl sterile distilled water (DW) to prepare a final concentration of 10^8 copies/ml. Importantly, the eluted DNA of *S. mutans* was filled with sterile DW to prepare a final volume of 1 ml and a final concentration of 10^8 copies/ml. Each DNA sample was 10-fold serially diluted with sterilized DW to create a DNA standard containing 10^8 to 10^2 copies/ml. The quantitative PCR (qPCR) was performed using the QuantStudio 5 (Thermo Fisher Scientific, USA) with Luna qPCR Master Mix (New England Biolabs, USA) and 16S rRNA primers, shown in Table 2. The reaction mixtures contained 5 µl of the 2× qPCR master mix, 0.5 µl of each 10 µM forward or reverse primer, 1 µl of sterilized DW, and 3 µl of DNA sample. In the case of *S. gordonii*, 0.4 µl of each 10 µM forward or reverse primer and 1.2 µl of sterilized DW were used. The amplification was carried out following the cycling conditions; one cycle of pre-denaturation at 95°C for 10 min, and 40 cycles of denaturation at 95°C for 15 s, and an extension of 60°C for 1 min. The melting curve analysis was performed among 60 to 95 °C with 0.03 °C/sec increment. The linear equation and

the correlation coefficient (R^2) were calculated from the relationship between DNA concentration and cycle threshold or Ct value.

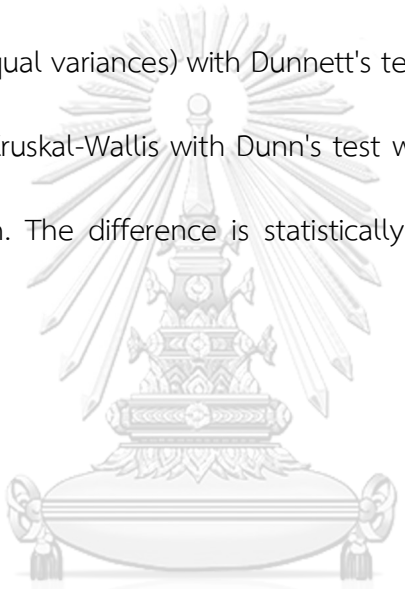
Table 2 Species-specific primers will be used in this study [72]

Species	**Primer names**	**Sequence (5′ → 3′)**
S. mutans	*S. mutans*-F	GATAATTGATTGAAAGATGCAAGC
	S. mutans-R	ATTCCCTACTGCTGCCTCCC
S. sanguinis	*S. sanguinis*-F	AGTTGCCATCATTGAGTTG
	S. sanguinis-R	GTACCAGCCATTGTAACAC
S. gordonii	*S. gordonii*-F	GCTTGCTACACCATAGACT
	S. gordonii-R	CCGTTACCTCACCTACTAG

To quantify the bacteria in each sample, the 36-h biofilms of single and mixed species were washed with 0.5 ml of PBS (pH 7.4) three times to remove unbound cells. Then, the biofilm was scraped using a pipette tip, and the DNA was extracted and amplified as the same protocols with the standard curve. The Ct value of each sample was compared to the standard curve of its own species to calculate DNA copy number (log copies/ml). The experiment was independently performed three times.

Statistical analysis

The statistics were analyzed by the Statistical Package for Social Sciences software (SPSS 28.0, SPSS Inc., IL, USA) and GraphPad Prism version 7.0 and 9.4 software (La Jolla, CA, USA). All results were presented as mean ± standard deviation (SD). A normality test was done by the Shapiro–Wilk test. To compare mean values among groups, the one-way analysis of variance (ANOVA) with Dunnett's test and Welch ANOVA (for unequal variances) with Dunnett's test was performed for a normal distribution data. The Kruskal-Wallis with Dunn's test was used when the data had a non-normal distribution. The difference is statistically significant when a p-value < 0.05.



CHAPTER V

RESULT

5.1 The effect of erythritol and xylitol in the presence of sucrose on acid production

Even though the pH of cariogenic bacteria (*S. mutans* and *S. sobrinus*) biofilm grown in all conditions had no significant difference when compared to 1S (Figure 5A and B), they showed higher level of pH in the sucrose-free media (BHI, 1E, 10E, 1X, and 10X) groups. The pH of early colonizer biofilm, except *S. gordonii*, grown in sucrose-free media was significantly higher than that grown in 1S (Figure 5C, E, and F). Although the *S. gordonii* biofilm grown in sucrose-free media (BHI, 1E, 10E, 1X, and 10X) had a higher pH than those grown in 1S, only 1E, 10E, and 10X showed a significant difference (Figure 5D).

It is noteworthy that when bacteria were cultured in sucrose-containing media, cariogenic pathogens had a pH close to 4, whereas early colonizers had a pH greater than 4.5 (Figure 5). The biofilm of all tested streptococci, except *S. sobrinus*, grown in sucrose-free media had pH values lower than the critical pH of enamel (pH = 5.5).

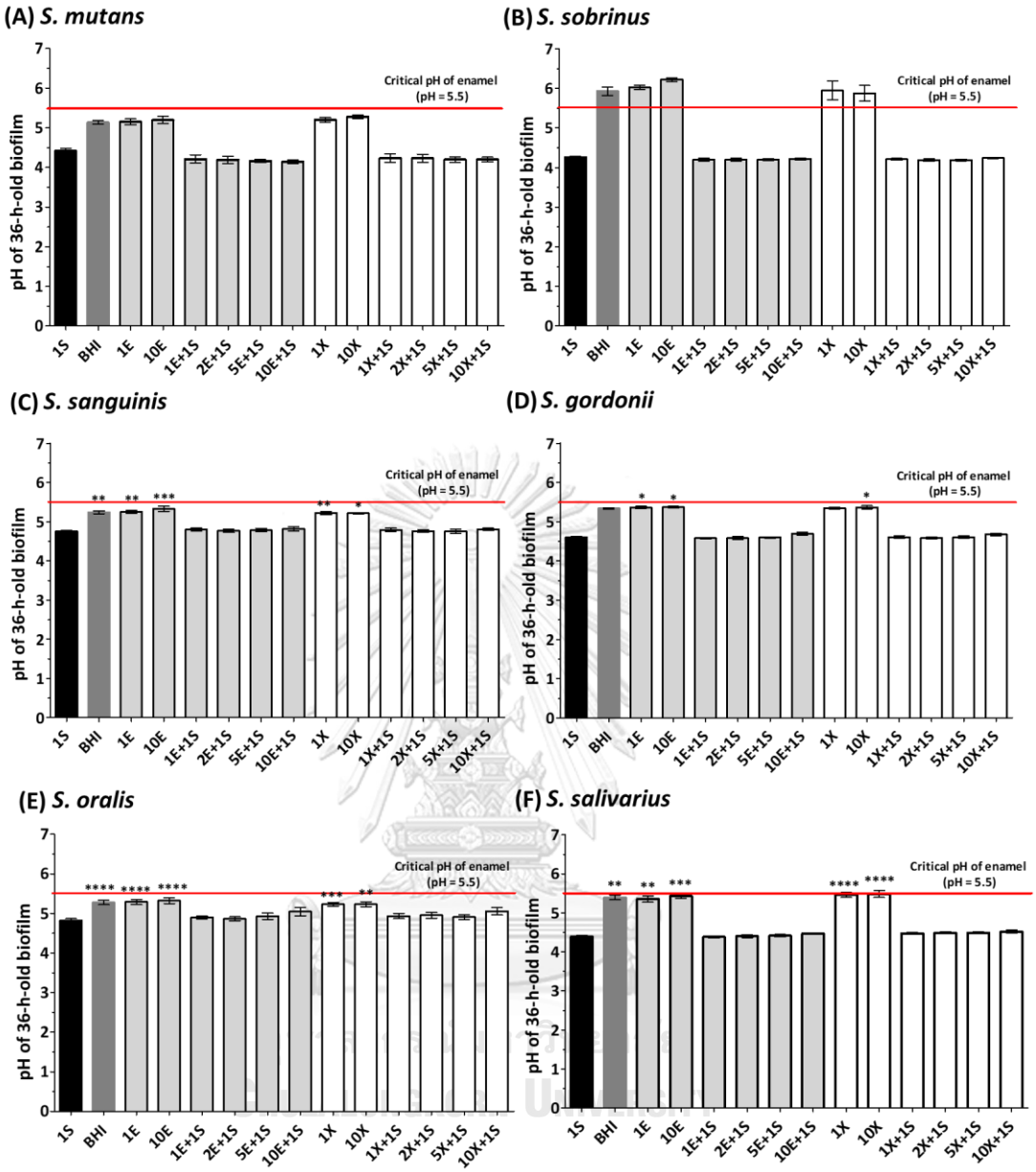


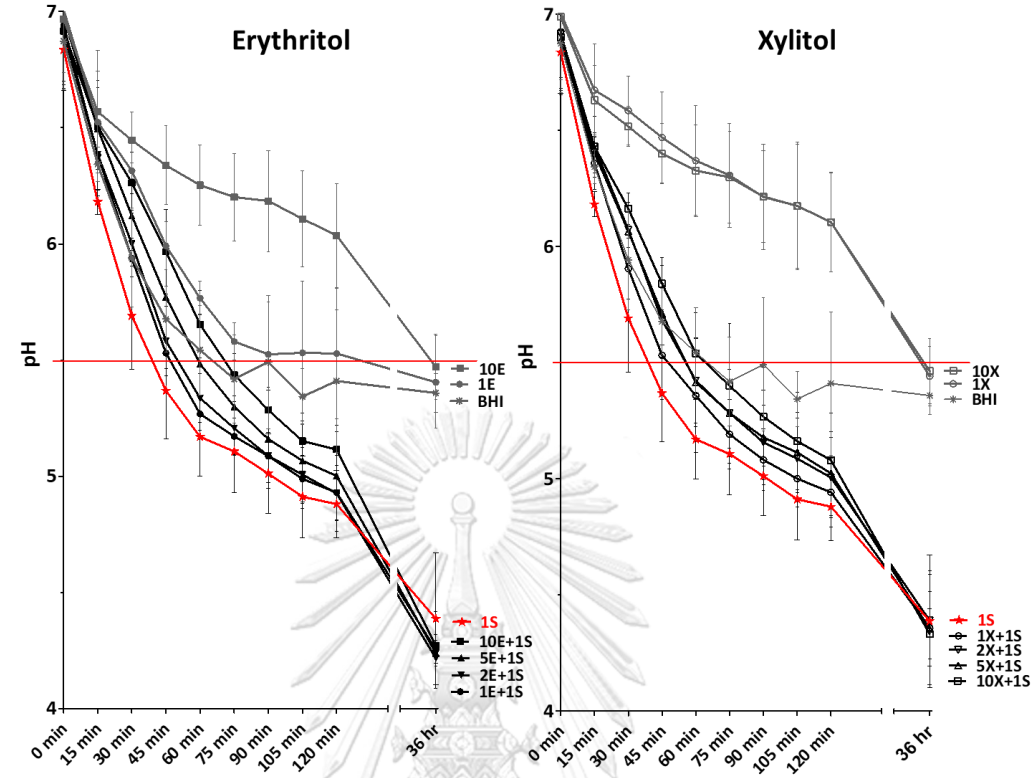
Figure 5 The effect of erythritol (E) and xylitol (X) in the presence of sucrose (S) on the pH of single-species biofilm at 36th hour of cariogenic streptococci (*S. mutans* (A) and *S. sobrinus* (B)) and of early colonizers (*S. sanguinis* (C), *S. gordonii* (D), *S. oralis* (E), and *S. salivarius* (F)). The experiments were performed independently three times, each with a duplicate. The results were presented as mean ± standard deviation (SD). The Kruskal-Wallis with Dunn's test was used for analysis. Statistical significance is marked with asterisks ****p < 0.0001, ***p < 0.001, **p < 0.01, and *p < 0.05 in comparison to 1S.

Because the pH of *S. mutans* biofilm at 36 h was similar to that of early colonizers, the dynamic change in pH biofilm over 36 h was performed to see if there was a difference between cariogenic pathogens and early colonizers. To determine the dynamic change in biofilm pH over 36 h, *S. mutans* and *S. sanguinis* were chosen as representative of cariogenic bacteria and early colonizers, respectively (Figure 6). After 30 min, the pH of *S. mutans* biofilm grown in BHI containing 1% sucrose (1S) was below the critical pH of enamel (pH = 5.5), but the groups grown in erythritol and xylitol-containing 1% sucrose remained above pH = 5.5, particularly, the 10E+1S and 10X+1S groups, which took approximately 60 min to close to pH = 5.5 (Figure 6A). The difference in time that *S. mutans* biofilm had a pH below the critical pH of enamel was more noticeable in the sucrose-free group (BHI, 1E, 10E, 1X, and 10X). At 120 min, the pH of the 10E, 1X, and 10X groups was above pH 6, and the pH of the 1E group had just reached pH 5.5, whereas the pH of the BHI group was slightly below pH 5.5 after 60 min (Figure 6A). However, after 36 h, the pH of all sucrose-free groups was slightly lower than 5.5. Overall, erythritol and xylitol help to delay *S. mutans'* pH decrease, even in the presence of sucrose.

S. sanguinis took longer than *S. mutans* to reach the critical pH of enamel, even in the BHI containing 1% sucrose group (1S) (Figure 6B). At 90 min, only the 1S group was below the critical pH of enamel, and BHI just reached pH 5.5. At 120 min, the 1E+1S and 1X+1S groups had pH levels lower than 5.5, while the (2E, 5E)+1S and (2X, 5X)+1S groups just reached a pH of 5.5. After 36 h, the pH of 10E+1S, 10X+1S,

and all sucrose-free groups except BHI was slightly lower than 5.5 (Figure 6B). As expected, 1S had less effect on the pH of *S. sanguinis* than *S. mutans*, resulting in a slower pH reduction and, at the endpoint (36 h), a higher pH than *S. mutans* for all sucrose-containing groups. Similar to *S. mutans*, erythritol, and xylitol help to delay the pH decrease of *S. sanguinis*, even in the presence of sucrose.

(A) ***S. mutans*** **(n = 3)**



(B) ***S. sanguinis*** **(n = 4)**

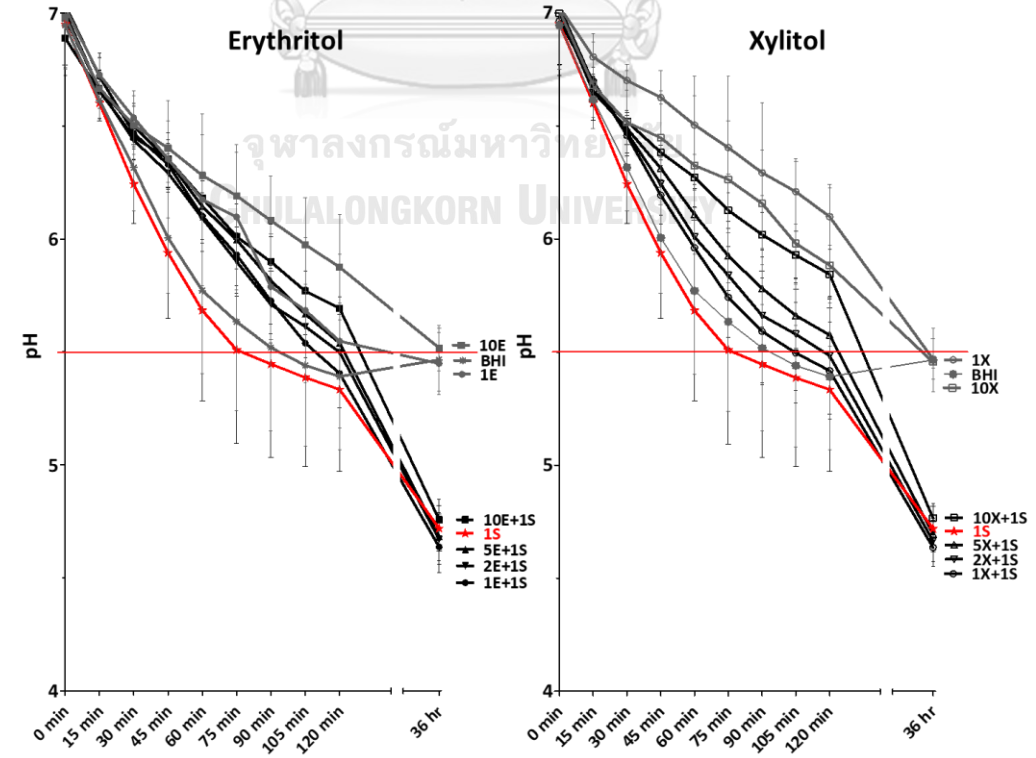


Figure 6 The effect of erythritol and xylitol in the presence of sucrose on the dynamic change of biofilm pH over 36 hours of cariogenic streptococci, *S. mutans* (A), and the early colonizer, *S. sanguinis* (B). The experiments were performed independently at least three times, each with a duplicate. The results were presented as mean ± standard deviation (SD).

The pH of *S. mutans* + *S. gordonii* and of three mixed-species biofilms in all conditions showed no significant difference compared to 1S, but their biofilm pH grown in sucrose-free media was higher (Figure 7B and D). The pH of *S. mutans* + *S. sanguinis* biofilm grown in sugar alcohol without sucrose (1E, 10E, 1X, and 10X) was significantly higher than those grown in 1S (Figure 7A). Early colonizers dual-species biofilm pH was significantly higher in sucrose-free media and 10E+1S than in 1S. (Figure 7C).

Furthermore, in the presence of sucrose, the biofilm containing *S. mutans* had a pH close to 4 (Figure 7A, B, and D), whereas early colonizers dual-species biofilm (*S. sanguinis* + *S. gordonii*) had a pH close to 4.5. (Figure 7C). Despite the fact that the pH of all groups was lower than the critical pH of enamel (pH = 5.5), the pH of the biofilm grown in sucrose-free media was very close to this value (Figure 7, a red line).

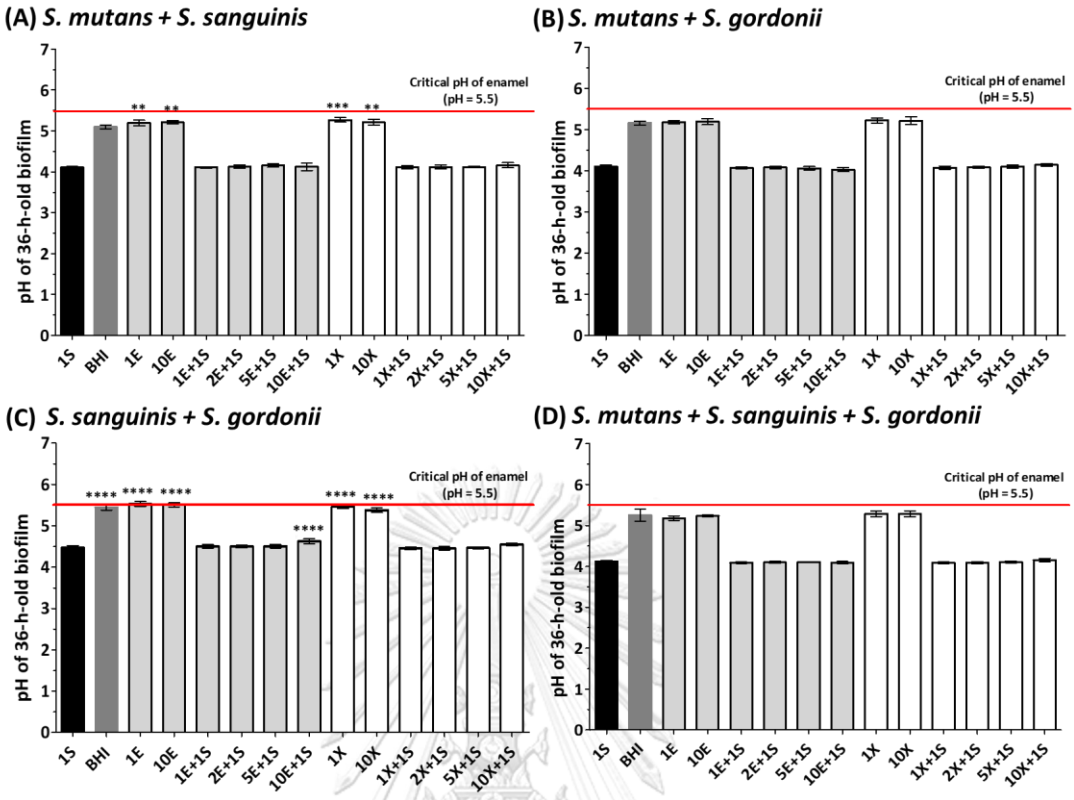


Figure 7 The effect of erythritol (E) and xylitol (X) in the presence of sucrose (S) on the pH of mixed-species biofilm at 36th hour of *S. mutans* + *S. sanguinis* (A), *S. mutans* + *S. gordonii* (B), *S. sanguinis* + *S. gordonii* (C), and mix of the three species (D). The experiments were performed independently three times, each with a duplicate. The results were presented as mean ± standard deviation (SD). Except for *S. sanguinis* + *S. gordonii*, which were analyzed using one-way ANOVA with Dunnett's test, the Kruskal-Wallis with Dunn's test was performed. Statistical significance is marked with asterisks ****p < 0.0001, ***p < 0.001, **p < 0.01, and *p < 0.05 in comparison to 1S.

All of the results showed that the pH at 36 h of cariogenic and commensal streptococci biofilms, despite being grown in the highest concentration of sugar alcohols but containing sucrose, did not differ significantly from those grown in 1S, except the pH of *S. sanguinis* with *S. gordonii* biofilm grown in 10E+1S. The dynamic

change of biofilm pH over 36 hours, on the other hand, revealed that, even in the presence of sucrose, sugar alcohols help to delay the decline of both cariogenic and commensal streptococci biofilm pH in a dose-dependent manner. Moreover, these results showed that the pH of *S. sanguinis* declined slower than that of *S. mutans* in all test conditions.

5.2 The effect of erythritol and xylitol in the presence of sucrose on biofilm mass

When compared to 1S, *S. mutans*, *S. sanguinis*, and *S. salivarius* cultured in sucrose-free media (BHI, 1E, 10E, 1X, and 10X) had significantly lower biofilm mass (Figure 8A, C, and F), whereas *S. sobrinus* showed significantly lower biofilm mass only when grown in 10X (Figure 8B). These findings indicated that when sucrose was present, sugar alcohols had no effect on biofilm formation in these three bacteria. However, despite the presence of sucrose, high concentrations of sugar alcohols (5E+1S, 10E+1S, 5X+1S and 10X+1S) were able to reduce *S. gordonii* biofilm mass, as shown in Figure 8D. Remarkably, sucrose and sugar alcohols appear to have no effect on *S. oralis* biofilm formation (Figure 8E).

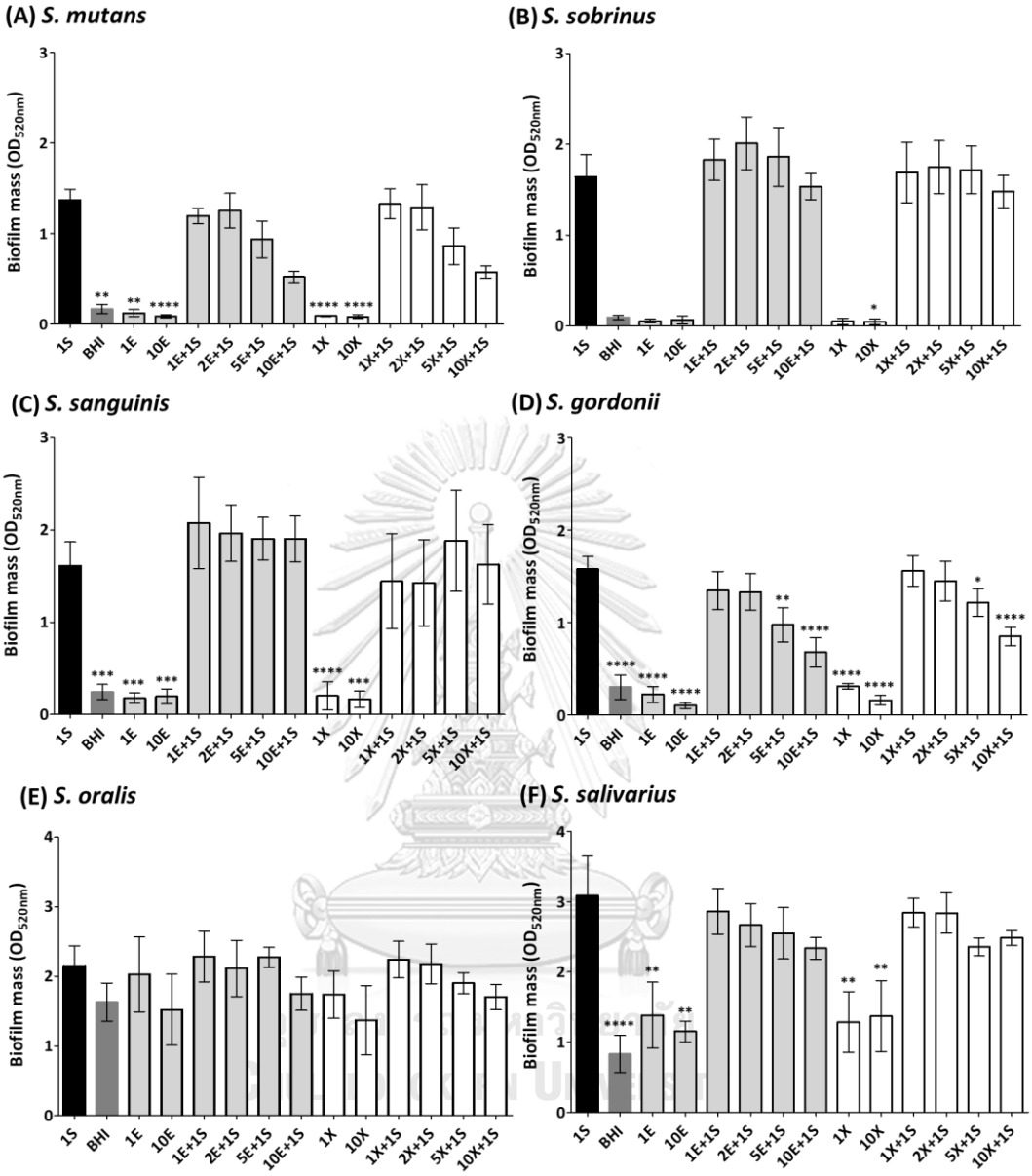


Figure 8 The effect of erythritol (E) and xylitol (X) in the presence of sucrose (S) on the biofilm mass of single-species biofilm at 36th hour of cariogenic streptococci (*S. mutans* (A) and *S. sobrinus* (B)) and of early colonizers (*S. sanguinis* (C), *S. gordonii* (D), *S. oralis* (E), and *S. salivarius* (F)). The experiments were performed independently three times, each with a duplicate. The results were presented as mean ± standard deviation (SD). Except for *S. sanguinis* and *S. gordonii*, which were analyzed using Welch ANOVA with Dunnett's test, the Kruskal-Wallis with Dunn's test was performed.

Statistical significance is marked with asterisks ****p < 0.0001, ***p < 0.001, **p < 0.01, and *p < 0.05 in comparison to 1S.

Dual-species biofilms formed in sucrose-free media (BHI, 1E, 10E, 1X, and 10X) had significantly lower biofilm mass than those formed in 1S (Figure 9A, B and C). Interestingly, 10E+1S and 10X+1S, in addition to sucrose-free media, reduced the biofilm formation of the three-mixed species (Figure 9D).

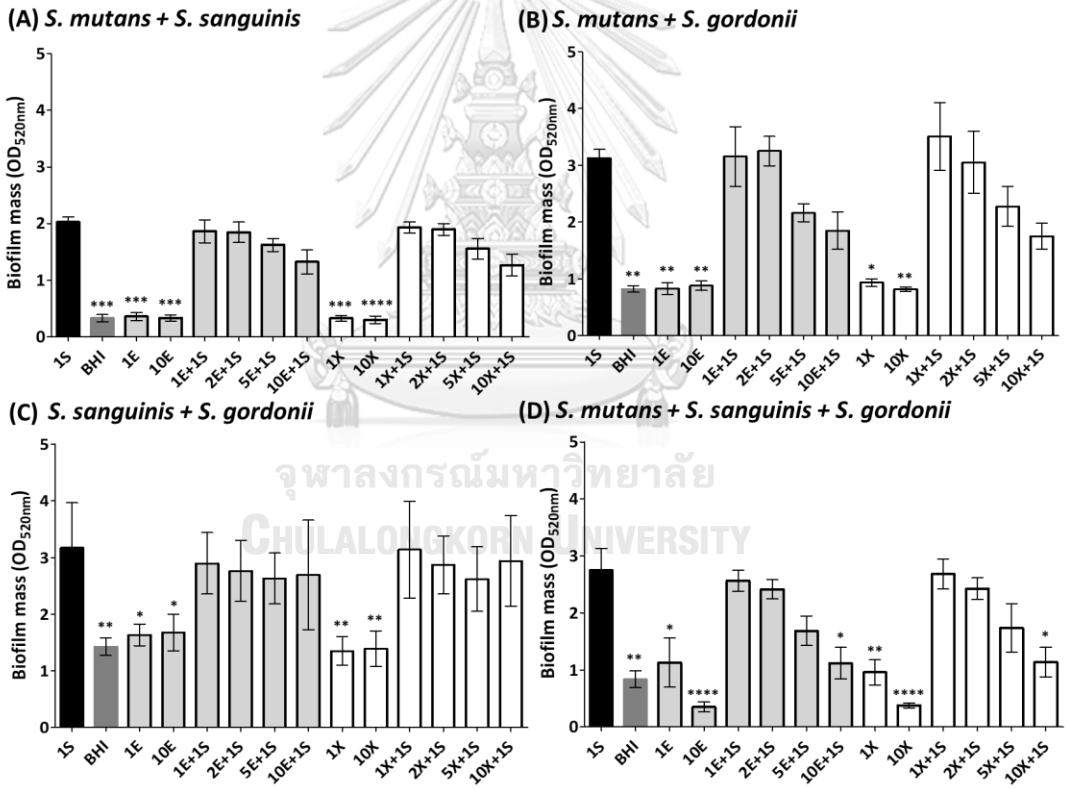


Figure 9 The effect of erythritol and xylitol in the presence of sucrose on the biofilm mass of mixed-species biofilm at 36th hour of *S. mutans* + *S. sanguinis* (A), *S. mutans* + *S. gordonii* (B), *S. sanguinis* + *S. gordonii* (C), and mix of the three species (D). The experiments were performed independently three times, each with a duplicate. The results were presented as mean ± standard deviation (SD). The Kruskal-Wallis with

Dunn's test was used for analysis. Statistical significance is marked with asterisks ****p < 0.0001, ***p < 0.001, **p < 0.01, and *p < 0.05 in comparison to 1S.

All results together indicated that sugar alcohols had an effect on the biofilm formation of both single- and mixed-species in the absence of sucrose, but this effect was not observed in the presence of sucrose, except for three mixed-species that require a high concentration of sugar alcohol.

5.3 The morphology of single-species biofilms formed in sucrose and erythritol and xylitol in the presence of sucrose

The 3 conditions: 1% sucrose (1S), 10% erythritol containing 1% sucrose (10E+1S), and 10% xylitol containing 1% sucrose (10X+1S) were chosen to determine the morphology using a scanning electron microscope since they showed the difference in biofilm mass in all tested streptococci (Figure 10). In 1S, EPS of *S. mutans* and *S. sobrinus* (cariogenic pathogens) was abundant and tightly packed in clumps, whereas early colonizers differed. *S. sanguinis*, similar to *S. gordonii*, had a long, loosely distributed filamentous EPS, whereas *S. oralis* had almost no EPS, but *S. salivarious* EPS was lumpy (Figure 10).

When *S. mutans* and *S. sobrinus* were grown in 10% erythritol or 10% xylitol with 1% sucrose (10E+1S or 10X+1S), their EPS dropped dramatically. Early colonizers, on the other hand, did not affect EPS appearance under the same growth conditions (Figure 10).

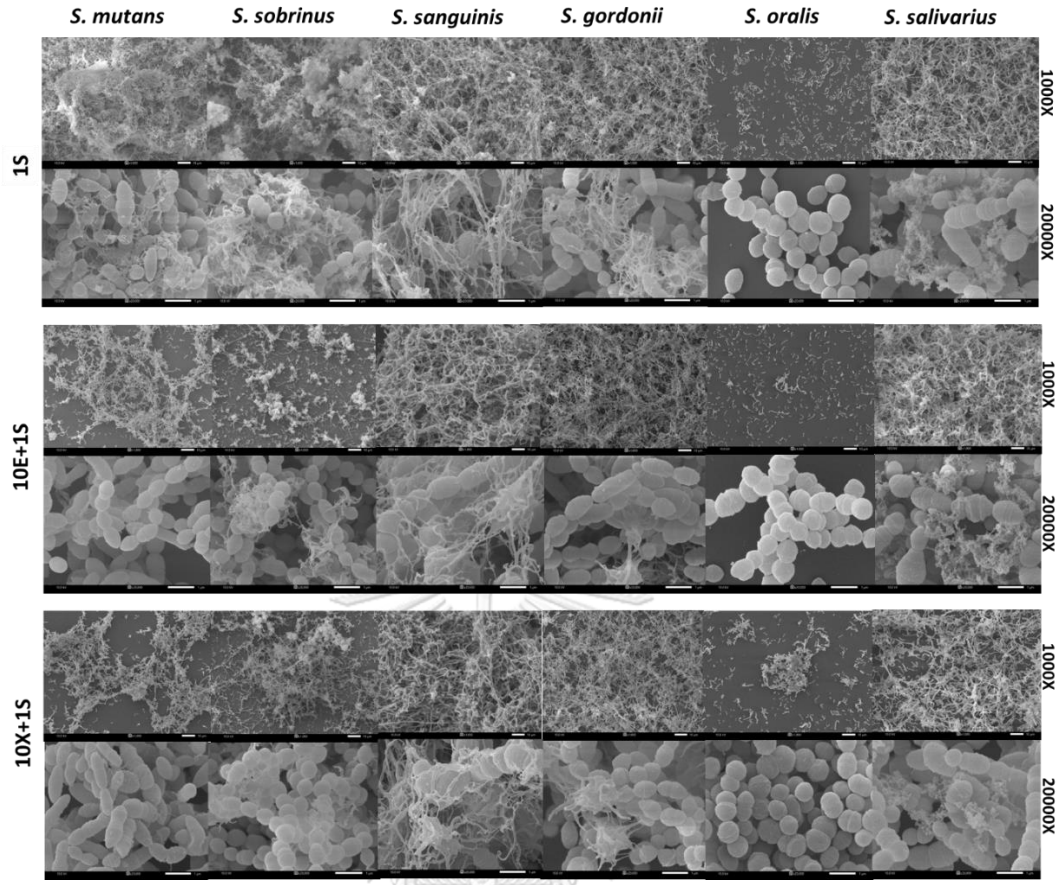


Figure 10 The effect of erythritol (E) and xylitol (X) in the presence of sucrose (S) on biofilm morphology of the single species biofilm at 36th hour of cariogenic streptococci (*S. mutans* and *S. sobrinus*) and of early colonizers (*S. sanguinis*, *S. gordonii, S. oralis* and *S. salivarius*). Magnification at 1,000X and 20,000X.

5.4 The effect of erythritol and xylitol in the presence of sucrose on the viability cells

S. mutans is frequently found in the biofilm of children with ECC [61] and *S. sanguinis* and *S. gordonii* are abundant in healthy dental plaque [62, 63], so they were chosen to study the effect of erythritol or xylitol in the absence and the presence of sucrose on the viability of single-species biofilm. *S. mutans* and *S.*

sanguinis grown in all conditions had significantly greater viable cells than 1S. (Figure 11A and B), whereas only *S. gordonii* grown in sucrose-free media had a significantly higher viable cells number (Figure 11C).

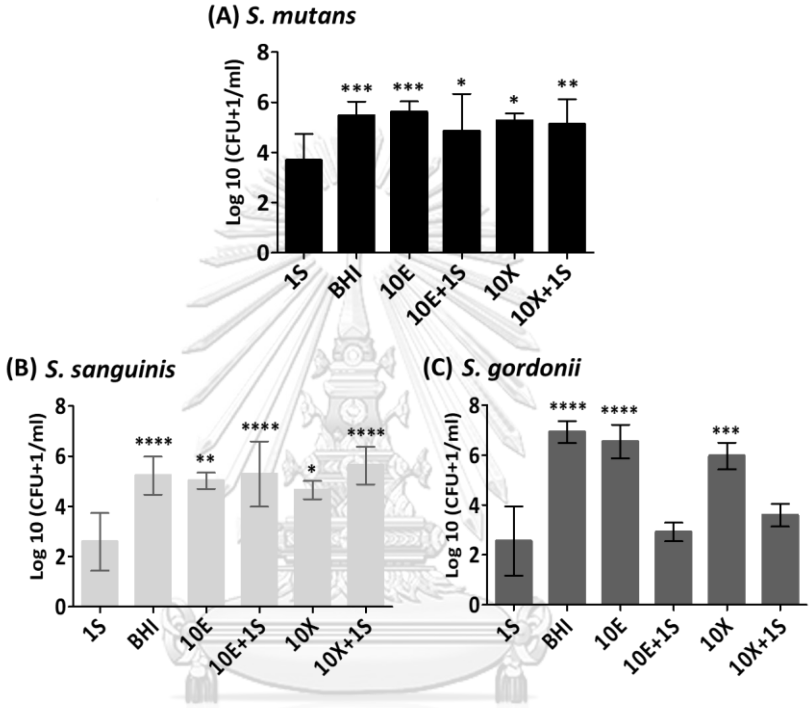


Figure 11 The effect of erythritol and xylitol in the presence of sucrose on the viability cells of single-species biofilm at 36th hour of *S. mutans* (A), *S. sanguinis* (B), and *S. gordonii* (C). The experiments were performed independently six times, each with a duplicate. The results were presented as mean ± standard deviation (SD). The Kruskal-Wallis with Dunn's test was used for analysis. Statistical significance is marked with asterisks ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ in comparison to 1S.

Mixed-species biofilms were created to see how sugar alcohols in the absence and presence of sucrose affected the number of viable cells in each species. However, a limitation in cell count was found in mixed-species biofilm

formation. If one species was counted, another with fewer numbers would fall outside of the countable range. There was no significant difference in the number of *S. mutans* in dual-species biofilms grown in sugar alcohols with and without sucrose compared to those grown in 1S (Figure 12A and B), whereas *S. mutans* in mixed three species biofilms grown in 10X are significantly less than those grown in 1S. (Figure 12D). The numbers of *S. gordonii* grown in sucrose-free media were significantly higher than that grown in 1S in *S. sanguinis* + *S. gordonii* biofilm (Figure 12C).

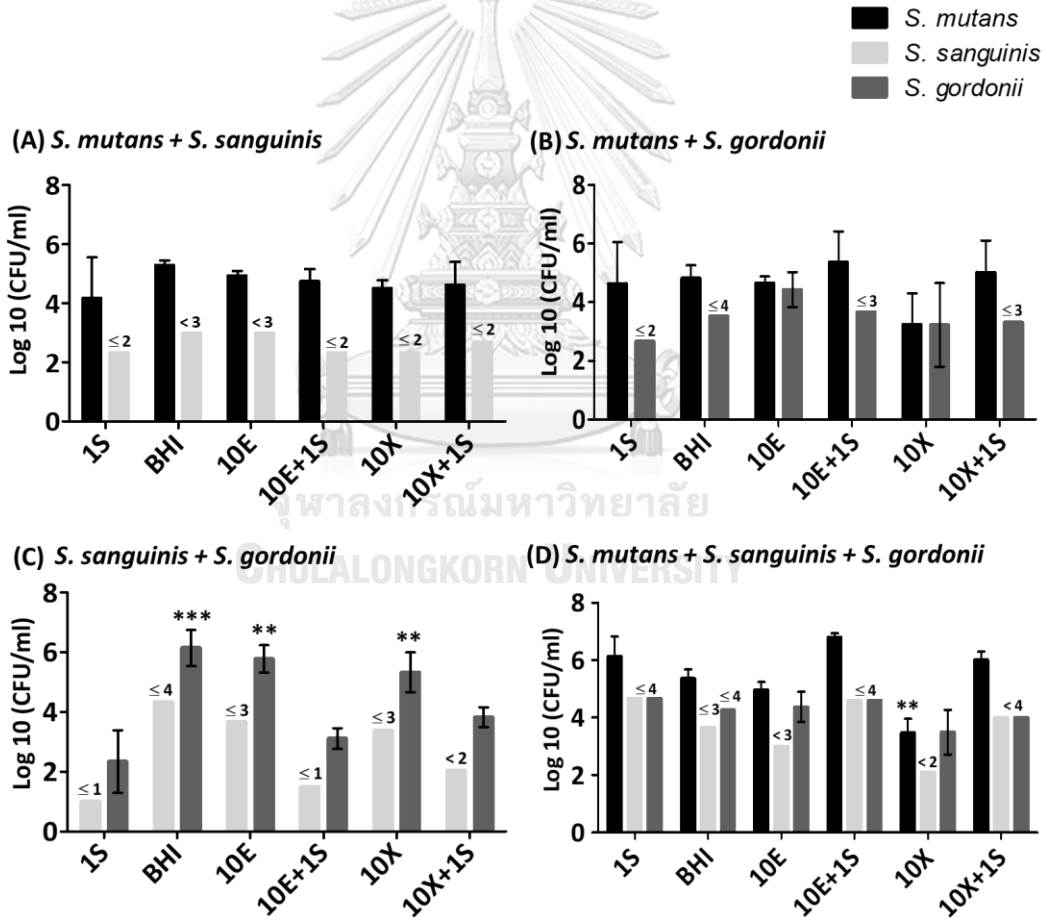


Figure 12 The effect of erythritol and xylitol in the presence of sucrose on the viability of mixed-species biofilm at 36th hour of *S. mutans* + *S. sanguinis* (A), *S. mutans* + *S. gordonii* (B), *S. sanguinis* + *S. gordonii* (C), and mix of the three species

(D). The experiments were performed independently three times, each with a duplicate. The results were presented as mean ± standard deviation (SD). Countable species were analyzed by the Kruskal-Wallis with Dunn's test, except for S. mutans on S. mutans + S. sanguinis, which normally distributed with unequal variance were analyzed using Welch ANOVA with Dunnett's test. Statistical significance is marked with asterisks ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ in comparison to 1S.

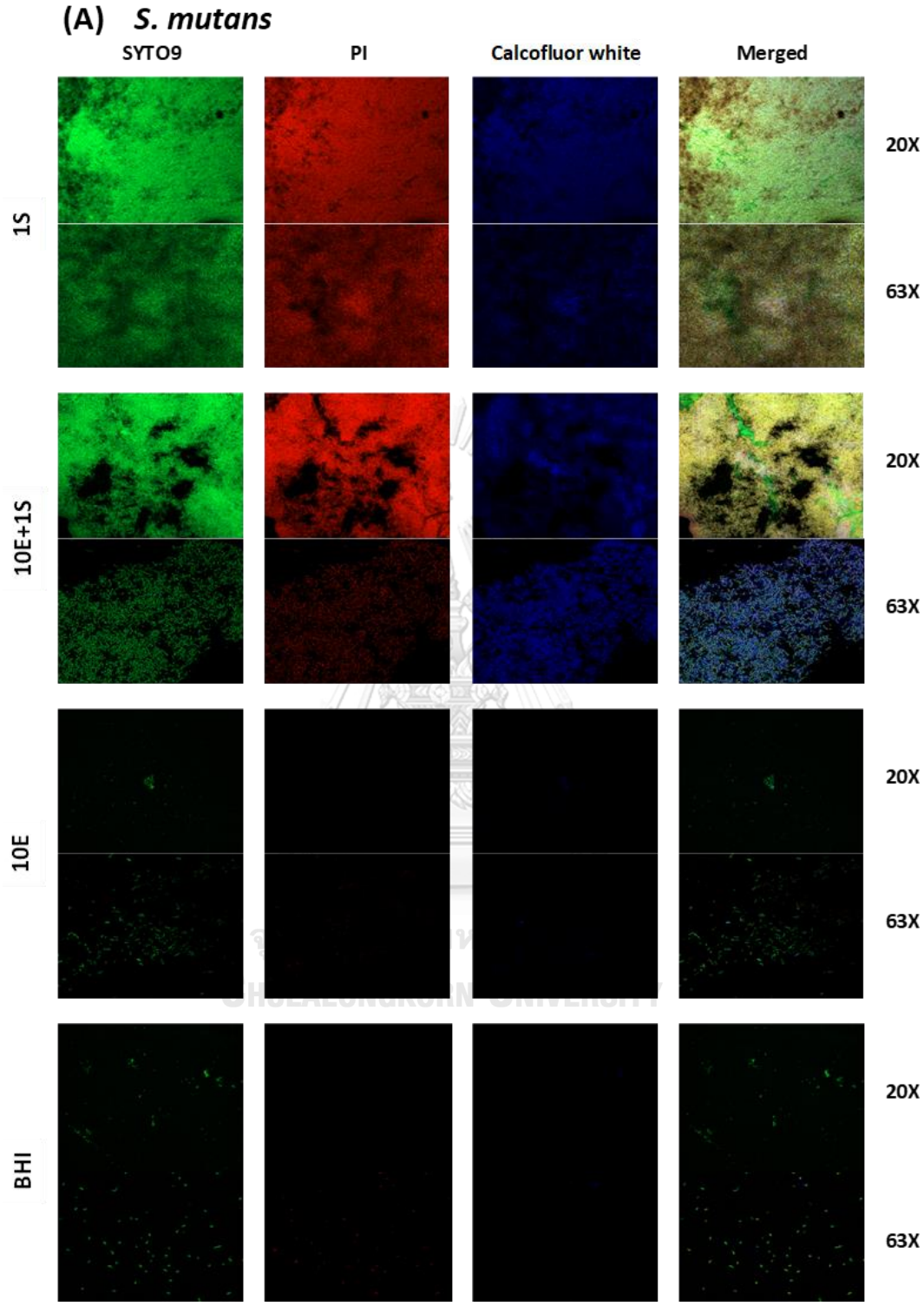
Biofilm grown in sucrose-free media (BHI, 10E, and 10X) had very minimal biofilm mass (Figure 8, and 9) but contained viable cells similar or higher than those grown in sucrose (Figure 11, and 12). Because crystal violet dye stains both viable and dead cells, as well as EPS, groups with a high biofilm mass may contain a high number of dead cells and/or EPS. To prove this assumption, live/dead and EPS staining of the assay was performed.

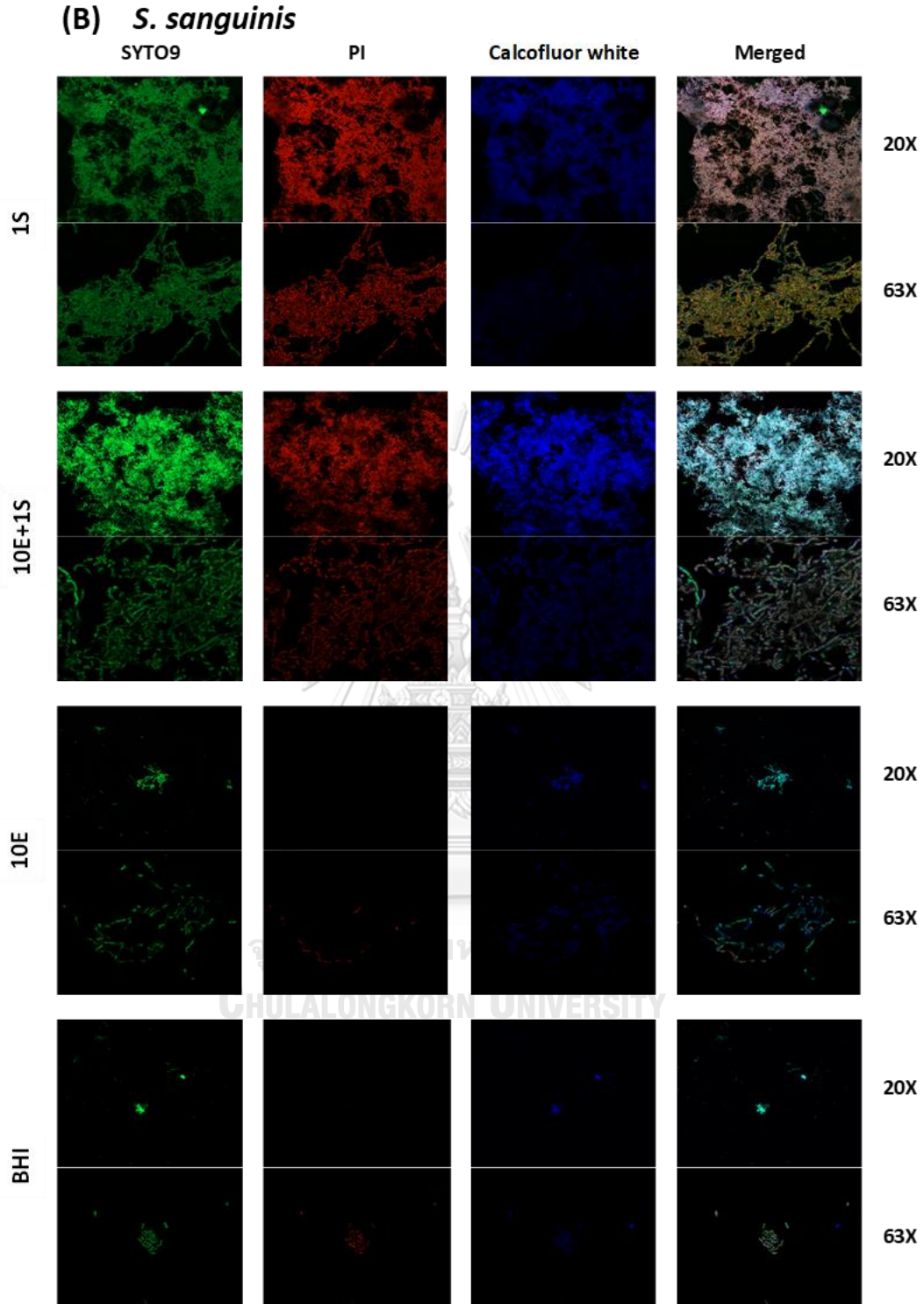
5.5 The live/dead and EPS staining of *S. mutans*, *S. sanguinis*, and *S. gordonii* single and mixed-species biofilms

To stain bacterial cells in biofilm, SYTO9 dye was used, while propidium iodide (PI) dye was used to stain dead cells (the LIVE/DEAD BacLight kit) [69]. At the same time, extracellular polysaccharides (EPS) were stained with calcofluor white [73]. The biofilm of *S. mutans* grown in 1S contained a large number of cells, but most of them were dead. *S. mutans*' EPS was dense, but not as dense as the cell numbers. *S. mutans*' density appeared to decrease when grown in 10E+1S, but other parameters remained unchanged from those found in 1S. When *S. mutans* was grown

in sucrose-free media, the number of cells was very few and most of them are live cells (Figure 13A).

S. sanguinis and *S. gordonii* in 1S were also found to have a large number of cells. They are, however, not as abundant, or dense as *S. mutans*. Furthermore, the majority of the cells died, and EPS was found in the same proportion as the number of cells. The appearance of *S. sanguinis* grown in 10E+1S was similar to that grown in 1S. In contrast, *S. gordonii* appeared to have fewer cells than those grown in 1S, and most of them appeared to be alive (Figure 13B and C). However, *S. gordonii* grown in 10E+1S remained grouped and contained EPS in proportion to the number of cells. When *S. sanguinis* was grown in sucrose-free culture media, it grew similarly to *S. mutans*. mostly the cells are live cells, and some have EPS, which is most likely the cause of the cell separation seen in Figure 13B. On the other hand, in the sucrose-free culture media, *S. gordonii* grown in 10E seemed to have a similar number of cells and EPS as those grown in 10E+1S. Cells grown in 1S and BHI also had the same appearance, but the cells grown in BHI were more dispersed (Figure 13C).


(A) S. mutans
SYTO9
PI
Calcofluor white
Merged
1S
10E+1S
10E
BHI
20X
63X
20X
63X
20X
63X
20X
63X



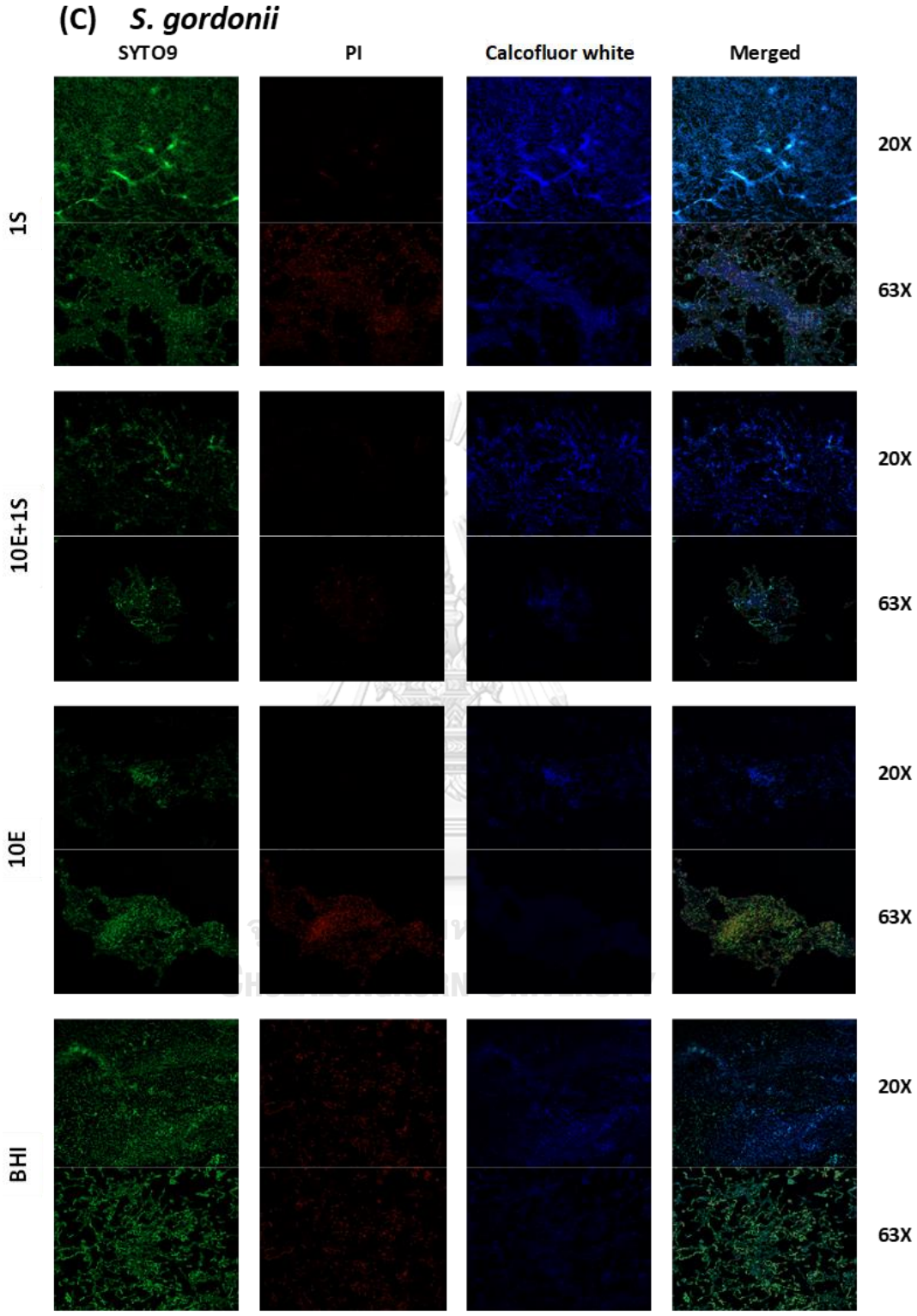
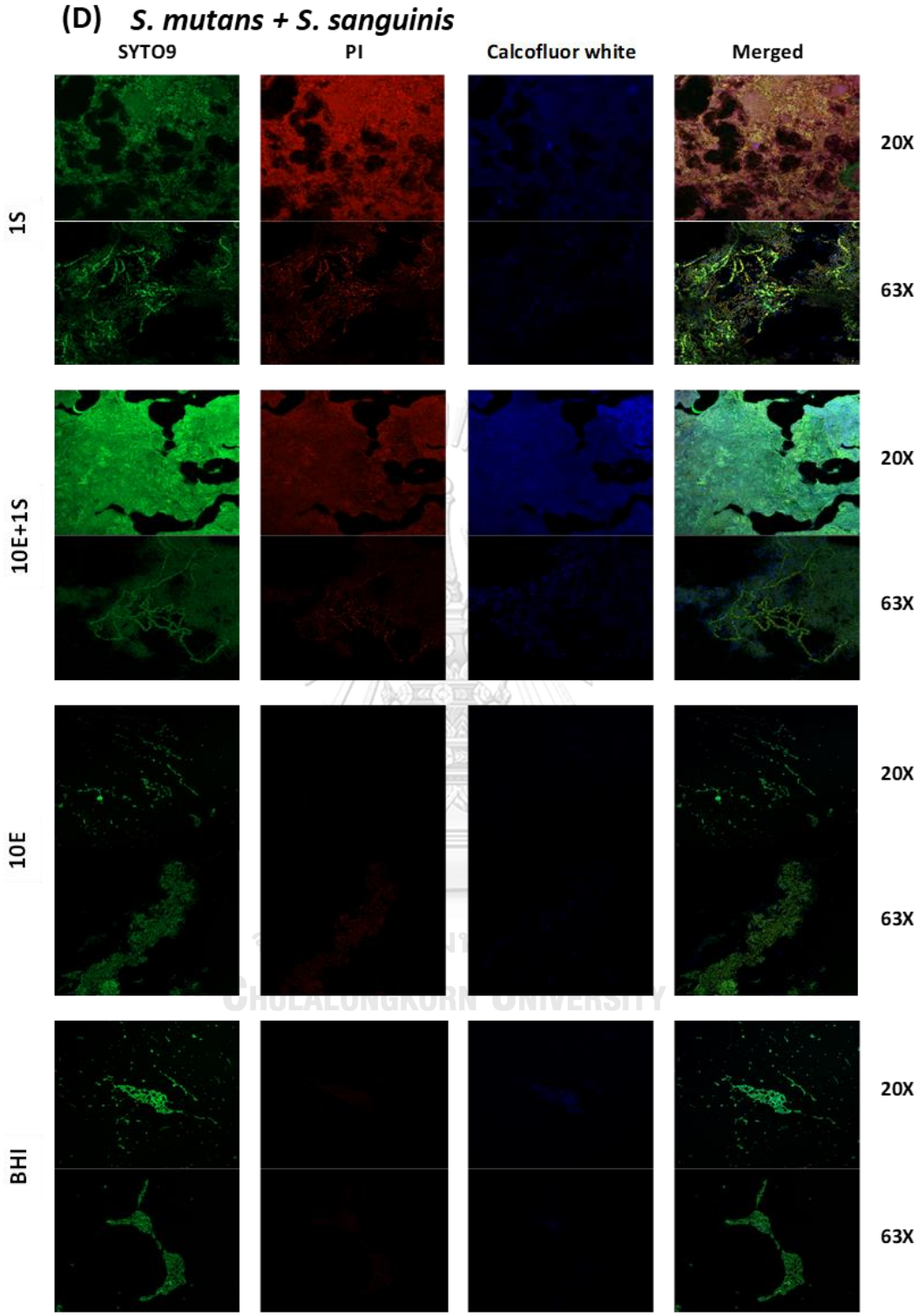
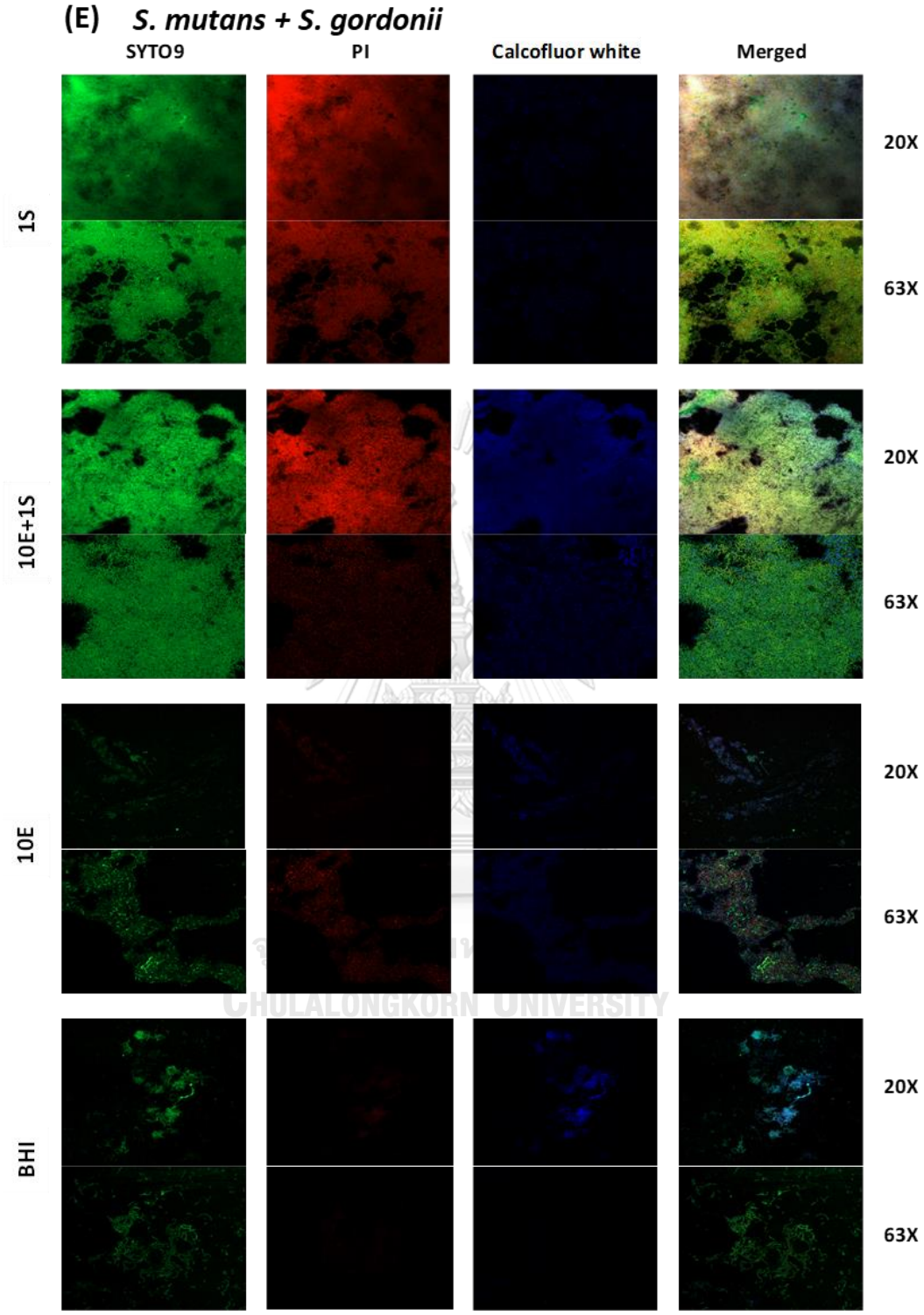
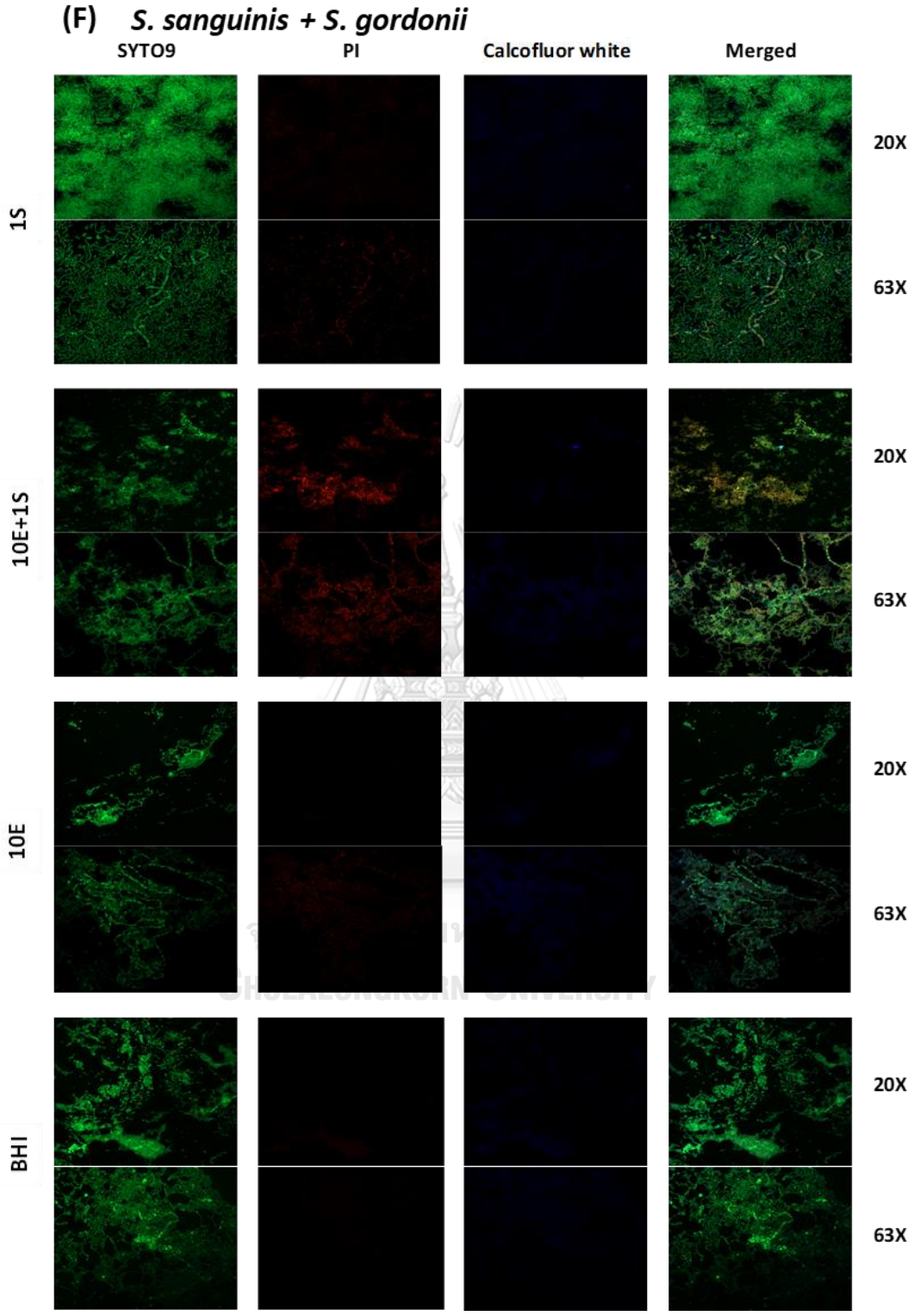

(C) S. gordonii
SYTO9
PI
Calcofluor white
Merged
1S
10E+1S
10E
BHI
20X
63X
20X
63X
20X
63X
20X
63X

Figure 13 Confocal laser scanning microscopy image of single-species biofilm at 36th hour of cariogenic streptococci, *S. mutans* (A), and the early colonizers, *S. sanguinis* (B), and *S. gordonii* (C) grown in 1% sucrose (1S), 10% erythritol with 1% sucrose (10E+1S), 10% erythritol (10E), and BHI. Stained with LIVE/Dead BacLight kit (total cells; green and dead cell; red), and calcofluor white dye (EPS; blue). Magnification at 20X and 63X.

The mixed-species biofilm, which was composed of *S. mutans* and grown in media containing 1S, encapsulated a large number of cells even in the presence of 10% erythritol (10E+1S), but the majority of them appeared to be dead (Figure 14). The mixed-species biofilm of *S. sanguinis* and *S. gordonii* in the presence of sucrose (1S and 10E+1S) contained a large number of cells, the majority of which died, but EPS was almost undetectable (Figure 14F). However, in all groups grown in a sucrose-free environment (10E and BHI), the number of bacteria was drastically reduced, but they were most alive, and a few EPS were observed (Figure 14).


(D) S. mutans + S. sanguinis
SYTO9
PI
Calcofluor white
Merged
1S
10E+1S
10E
BHI
20X
63X
20X
63X
20X
63X
20X
63X




(F) S. sanguinis + S. gordonii
SYTO9
PI
Calcofluor white
Merged
1S
10E+1S
10E
BHI
20X
63X
20X
63X
20X
63X
20X
63X

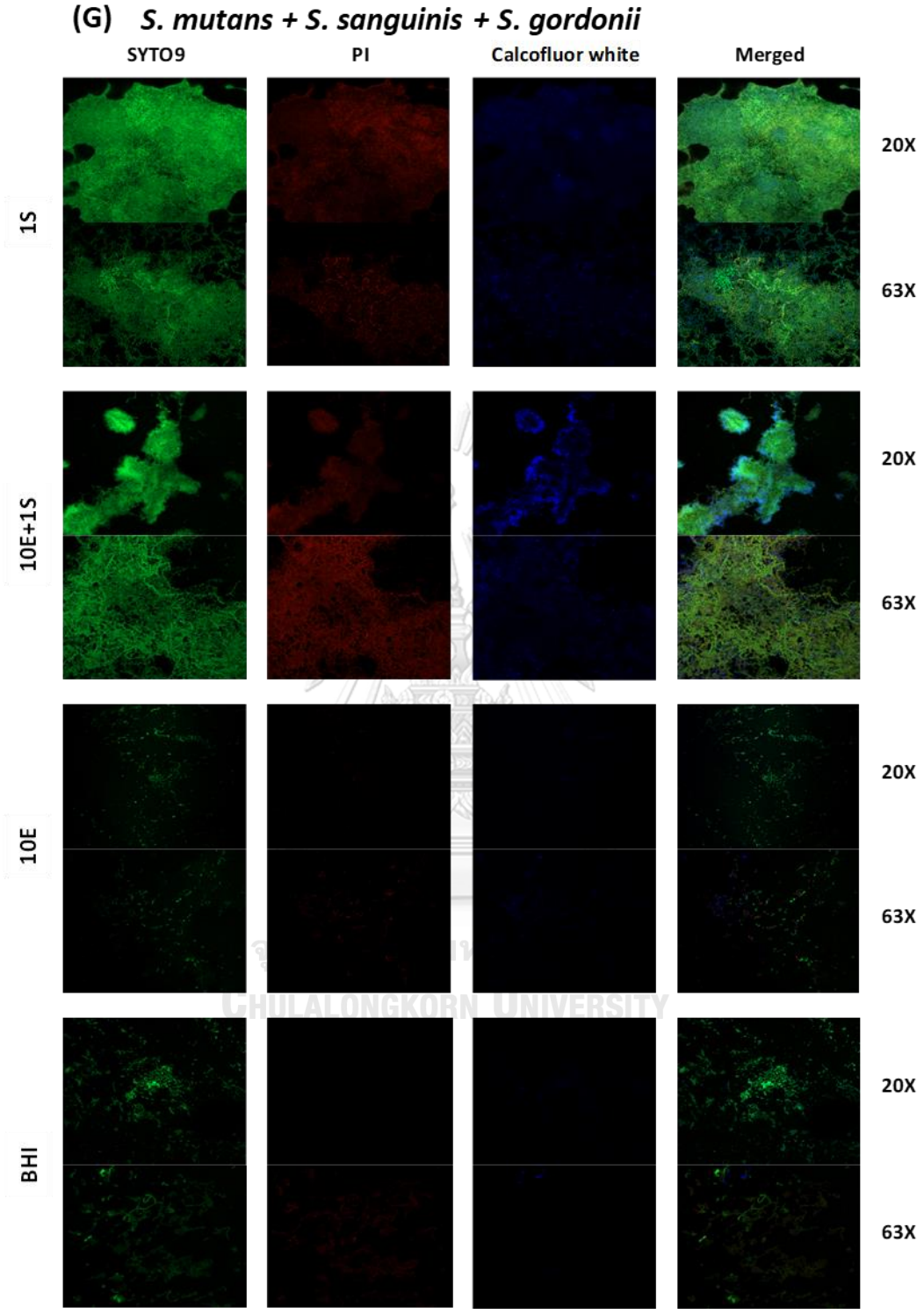


Figure 14 Confocal laser scanning microscopy image of mixed-species biofilm at 36th hour of *S. mutans* + *S. sanguinis* (D), *S. mutans* + *S. gordonii* (E), *S. sanguinis* + *S. gordonii* (F), and mix of the three species (G) grown in 1% sucrose (1S), 10% erythritol with 1% sucrose (10E+1S), 10% erythritol (10E), and BHI. Stained with LIVE/Dead BacLight kit (total cells; green and dead cells; red), and calcofluor white dye (EPS; blue). Magnification at 20X and 63X.

5.6 The effect of erythritol and xylitol in the presence of sucrose on the copy number of cells by using the quantitative PCR

To investigate the effects of erythritol and xylitol in the presence of sucrose on cariogenic streptococci (*S. mutans*) and early colonizers (*S. sanguinis* and *S. gordonii*) in single and mixed-species biofilms, the qPCR was used to quantitate the copy number of 16S rRNA gene (log copies/ml) representing bacterial numbers in the different conditions.

In single-species biofilms, all species grown in sugar alcohols without sucrose (10E and 10X) was significantly lower compared to those grown in 1S (Figures 15A, B and C). Furthermore, *S. mutans* cultured in BHI revealed the same result (Figure 15A).

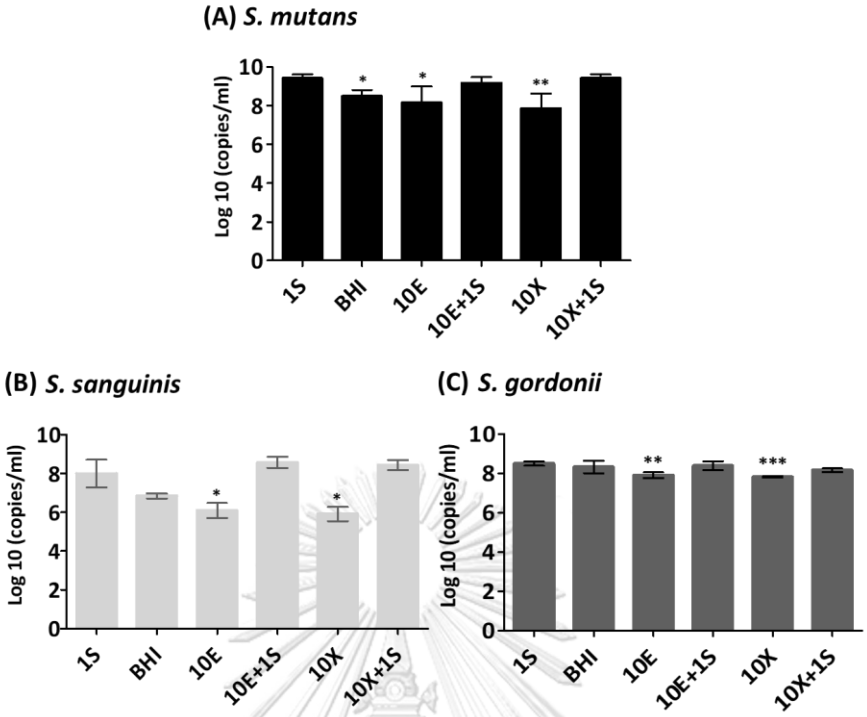


Figure 15 The effect of erythritol and xylitol in the presence of sucrose on the number of cells (log copies/ml) of single-species biofilm at 36th hour of *S. mutans* (A), *S. sanguinis* (B) and *S. gordonii* (C). The experiments were performed independently three times, each with a duplicate. The results were presented as mean ± standard deviation (SD). The Kruskal-Wallis with Dunn's test was used for analysis. Statistical significance is marked with asterisks ****p < 0.0001, ***p < 0.001, **p < 0.01, and *p < 0.05 in comparison to 1S.

All species grown in sugar alcohols without sucrose (10E and 10X) had significantly lower cell numbers in mixed-species biofilm when compared to their own 1S (Figure 16A-D). *S. mutans* + *S. sanguinis* and *S. sanguinis* + *S. gordonii* grown in BHI were also significantly lower (Figure 16A and C).

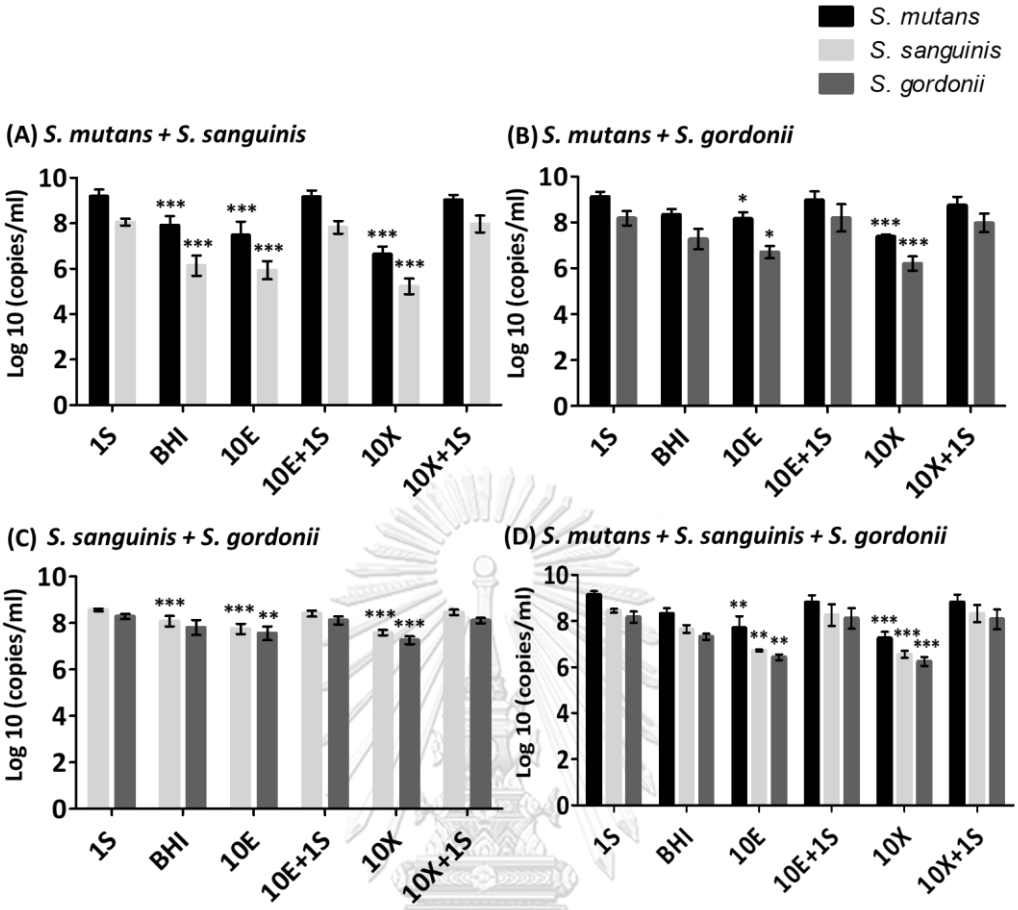


Figure 16 The effect of erythritol and xylitol in the presence of sucrose on the number of cells (log copies/ml) of mixed-species biofilm at 36th hour of *S. mutans* + *S. sanguinis* (A*), S. mutans + S. gordonii* (B), *S. sanguinis* + *S. gordonii* (C), and mix of the three species (D). The experiments were performed independently three times, each with a duplicate. The results were presented as mean ± standard deviation (S.D). The Kruskal-Wallis with Dunn's test was used for analysis, except for *S. mutans* on *S. mutans* + *S. sanguinis*, *S. sanguinis* on *S. mutans* + *S. sanguinis*, and *S. sanguinis* on *S. sanguinis* + *S. gordonii* which normally distributed were analyzed using one-way ANOVA with Dunnett's test. Statistical significance is marked with asterisks ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ in comparison to 1S of their own species**.**

CHAPTER VI

DISCUSSION

Sugar alcohols, particularly xylitol, are known to be non-cariogenic and can help prevent dental caries. However, sucrose consumption is unavoidable in our regular lifestyle. There have been limited studies on the effect of sugar alcohol in the presence of sucrose. Therefore, this study aims to investigate the effect of xylitol and erythritol in the presence of sucrose on single and mixed-species biofilm of early colonizers and cariogenic streptococci.

The pH of single-species biofilms of early colonizers and cariogenic streptococci grown in sugar alcohols (1E, 10E, 1X and 10X) was found to be higher than that of sucrose-grown biofilms (1S). The findings are consistent with previous studies on cariogenic streptococci (*S. mutans*, and *S. sobrinus*) [24, 52]. Because of the limited number of early colonizers studied, only *S. oralis* was reported to have a higher pH in xylitol than in sucrose [24]. After 36 hour, pH of all tested streptococci grown in sucrose-containing sugar alcohols decreased did not differ from those grown in sucrose only (1S). However, when monitoring the pH during the first 2 hours, it was found that the reduction in pH of *S. mutans* and *S. sanguinis* grown in sucrose-containing sugar alcohols was slower than the pH of those grown in sucrose alone. The pH decrease was even slower in strains grown solely in sugar alcohols. These

findings suggested that sugar alcohols help to delay pH reduction, but this ability declined when sucrose was present.

It is notable that the pH measured in all conditions and strains, except for *S. sobrinus*, was lower than the critical pH of enamel (pH=5.5). This could be due to the presence of 0.2% glucose in BHI, which all streptococci can metabolize via glycolysis [74]. For *S. sobrinus*, its glucose PTS activity was not effective at pH 7 [75], which is the pH of BHI. This may result in poor glucose uptake by the bacteria, resulting in a higher pH than other streptococci in conditions absent sucrose. The dynamic change in pH biofilm over 36 h showed that the pH of *S. sanguinis* decreased more slowly than *S. mutans*. This finding may explain by the mutans streptococci are more acidogenic than the early colonizer [76]. When *S. mutans* was present in mixed-species biofilms, the pH was comparable to that of a single species of *S. mutans*. This could be because *S. mutans* are more acid resistant than early colonizers, resulting in higher cell numbers in mixed-species biofilm, as demonstrated by the cell number result. Consequently, they may have had a greater impact on acid production.

Similar to our results, many previous studies have found that xylitol and erythritol can inhibit biofilm formation of cariogenic streptococci (*S. mutans* and *S. sobrinus*) by xylitol inhibiting *gtf* genes expression of *S. mutans* [52] and xylitol and erythritol inhibiting adhesion of *S. sobrinus* to the surface [55, 56]. Although research

on the effects of sugar alcohols on early colonizers is limited, Soderling and Hietala-Lenkker reported that 4% xylitol can inhibit *S. sanguinis* polysaccharide formation, which consistent with our findings. They proposed that xylitol and erythritol reduce early colonizer adhesion via a mechanism other than growth inhibition, which could be the inhibition of water-insoluble glucan production [77]. In the presence of sucrose, high concentrations of sugar alcohols reduce (but not significantly) *S. mutans* biofilm formation, which consistent with Loimaranta *et al.* They discovered that xylitol and erythritol supplements with sucrose can inhibit real-time biofilm formation in the early stages (7-10 h) of *S. mutans*. This maybe because sugar alcohols influence the expression levels of the *gbpB*, *gtfB*, *gtfC*, and *gtfD* genes, all of which are involved in biofilm formation and adhesion [51]. A similar result for *S. mutans* was found in *S. gordonii.* Hashino *et al.* reported that 10% of erythritol and xylitol reduced the biofilm formation of single biofilm *S. gordonii* via several pathways consisting of inhibition of growth induced by DNA and RNA destruction and reduced extracellular matrix production and changed in dipeptide acquisition and amino acid metabolism. They also found that erythritol can reduce the biofilm more than xylitol [59]. However, there is still no explanation for the biofilm mass of other early colonizers or *S. sobrinus* grown in sugar alcohols containing sucrose that were not different from those grown in sucrose alone (1S). Thus, further research is needed to answer these questions.

High concentrations of sugar alcohols with sucrose (5E+1S, 10E+1S, 5X+1S, and 10X+1S) tended to decrease the biofilm mass of mixed-species biofilm containing S*. mutans*, which was similar to that found in single-species *S. mutans* biofilm. This could be explained by the same reason as the pH result: *S. mutans* are more acid resistant than early colonizers, resulting in higher cell numbers in mixed-species biofilm, which could exert influence over early colonizers.

Several methods were used to determine cell numbers, including the colony count method, live/dead and EPS staining, and quantitative PCR (qPCR). The colony count method has a limitation on the mixed-species biofilm formation If one species was counted, another with fewer numbers would fall outside of the countable range. The fluorescence staining was subjective. Furthermore, qPCR with the 16S rRNA gene detects both live and dead cells, which does not represent an accurate count of living cells.

Surprisingly, the colony count method revealed that the number of *S. mutans* grown in sugar alcohols was significantly higher than those grown in sucrose, which corresponded to the results of live/dead and EPS staining. This contradicts previous findings that sugar alcohols can inhibit pathogen growth [22, 24, 52, 78, 79]. However, EM Decker *et al.* demonstrated that Schaedler medium (0.58% endogenous glucose) supplemented with 1% xylitol had no effect on the viability of *S. mutans* biofilms [80].

Our study was conducted *in vitro*, which has several limitations when applied in the real life. Although the study determined the effect of sugar alcohols on the major species found in dental plaque, it cannot represent the composition of dental plaque, which is composed of a variety and number of microorganisms [81]. Moreover, other factors, such as frequency of sugar exposure, were not considered in this study. The recommended sugar alcohol intake for adults is 40-50 g/day and 30 g/day for children to avoid gastrointestinal side effects [82]. The highest concentration of sugar alcohols used in this study was 10% (w/v), which provided superior efficacy over other groups. Therefore, if increasing the concentration of sugar alcohols improves their effect, this maximum sugar alcohol intake level should be considered. We also found that the results of each experiment were inconsistent; for example, confocal laser scanning microscopy revealed fewer cells in sucrose-free groups than other methods. To prepare samples for this method, it requires multiple washing steps, which may cause poorly adherent biofilms such as those grown in sucrose-free media to peel off during washing. Therefore, future studies should consider these limitations and select appropriate testing methods.

CHAPTER VII

CONCLUSIONS

In the presence of sucrose, xylitol and erythritol have no different effect on the biofilm pH of single- and mixed-species at 36 h than sucrose alone. They did, however, help to delay the pH decline of *S. sanguinis* and *S. mutans*. In the presence of sucrose, sugar alcohols had no effect on the biofilm mass of cariogenic streptococci, but a high concentration of them could reduce the single-species biofilm of *S. gordonii*. In mixed-species biofilm, the high concentration of sugar alcohols even with sucrose significantly reduced the biofilm mass of the three-mixed species biofilm (*S. mutans* + *S. sanguinis* + *S. gordonii*) but not others. *S. mutans* grown in sugar alcohols with or without sucrose had significantly higher living cell numbers than those grown in sucrose alone. The same result was found in *S. sanguinis*, but the number of living cells in *S. gordonii* grown in sucrose-containing media was not different.

APPENDIX A

ADDITIONAL RESULTS

Table 3 Raw data of the pH of single-species biofilm at 36 hours. The experiments were performed independently three times, each with a duplicate. The results were presented with mean and standard deviation (SD)

Streptococcus mutans	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	4.17	4.19	4.12	4.08	4.15	4.15	4.143	0.035
5E+1S	4.22	4.21	4.13	4.13	4.15	4.14	4.163	0.037
2E+1S	4.31	4.32	4.13	4.14	4.13	4.14	4.195	0.085
1E+1S	4.33	4.36	4.15	4.17	4.11	4.15	4.212	0.096
10E	5.28	5.24	5.27	5.21	5.1	5.07	5.195	0.081
1E	5.22	5.23	5.1	5.2	5.08	5.07	5.15	0.068
10X+1S	4.28	4.24	4.18	4.11	4.21	4.18	4.2	0.053
5X+1S	4.27	4.29	4.14	4.14	4.17	4.18	4.198	0.06
2X+1S	4.34	4.37	4.13	4.15	4.2	4.18	4.228	0.093
1X+1S	4.35	4.38	4.15	4.16	4.19	4.16	4.232	0.095
10X	5.34	5.32	5.26	5.24	5.27	5.23	5.277	0.04
1X	5.23	5.29	5.18	5.19	5.18	5.12	5.198	0.052
1S	4.5	4.48	4.42	4.39	4.34	4.35	4.413	0.06
BHI	5.18	5.19	5.1	5.15	5.08	5.11	5.135	0.041

Streptococcus sobrinus	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	4.24	4.25	4.22	4.2	4.19	4.22	4.22	0.021
5E+1S	4.21	4.21	4.22	4.23	4.17	4.18	4.203	0.021
2E+1S	4.21	4.21	4.23	4.22	4.12	4.19	4.197	0.036
1E+1S	4.19	4.21	4.23	4.22	4.21	4.12	4.197	0.036
10E	6.22	6.3	6.22	6.2	6.17	6.24	6.225	0.04
1E	5.99	5.96	6.02	6.13	6.01	6.05	6.027	0.054
10X+1S	4.26	4.23	4.23	4.24	4.23	4.26	4.242	0.013
5X+1S	4.22	4.2	4.2	4.19	4.16	4.16	4.188	0.022
2X+1S	4.22	4.21	4.21	4.2	4.16	4.14	4.19	0.029
1X+1S	4.23	4.23	4.21	4.22	4.19	4.18	4.21	0.019
10X	6.22	5.99	5.84	5.79	5.69	5.75	5.88	0.178
1X	6.4	6.01	5.82	5.85	5.77	5.83	5.947	0.216
1S	4.26	4.26	4.29	4.28	4.25	4.27	4.268	0.013
BHI	5.89	5.89	5.98	6.06	5.75	6.01	5.93	0.101

Streptococcus sanguinis	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	4.9	4.87	4.78	4.76	4.83	4.76	4.817	0.054
5E+1S	4.85	4.82	4.75	4.74	4.77	4.78	4.785	0.039
2E+1S	4.82	4.81	4.75	4.74	4.74	4.73	4.765	0.036
1E+1S	4.83	4.85	4.77	4.77	4.78	4.79	4.798	0.031
10E	5.38	5.29	5.26	5.27	5.41	5.36	5.328	0.058
1E	5.27	5.26	5.2	5.25	5.22	5.32	5.253	0.038
10X+1S	4.84	4.82	4.83	4.79	4.79	4.78	4.808	0.023
5X+1S	4.83	4.81	4.68	4.74	4.73	4.77	4.76	0.05
2X+1S	4.8	4.8	4.74	4.71	4.77	4.75	4.762	0.032
1X+1S	4.83	4.83	4.77	4.72	4.82	4.79	4.793	0.039
10X	5.24	5.21	5.21	5.2	5.21	5.21	5.213	0.012
1X	5.2	5.19	5.22	5.18	5.27	5.28	5.223	0.039
1S	4.79	4.76	4.73	4.72	4.77	4.76	4.755	0.024
BHI	5.22	5.26	5.18	5.23	5.25	5.29	5.238	0.034

Streptococcus gordonii	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	4.65	4.68	4.74	4.72	4.71	4.65	4.692	0.034
5E+1S	4.59	4.58	4.61	4.61	4.6	4.59	4.597	0.011
2E+1S	4.55	4.55	4.61	4.64	4.57	4.56	4.58	0.034
1E+1S	4.56	4.56	4.59	4.59	4.61	4.56	4.578	0.02
10E	5.39	5.36	5.4	5.38	5.35	5.35	5.372	0.02
1E	5.34	5.37	5.32	5.39	5.37	5.39	5.363	0.026
10X+1S	4.7	4.67	4.72	4.66	4.67	4.62	4.673	0.031
5X+1S	4.56	4.59	4.65	4.64	4.59	4.59	4.603	0.031
2X+1S	4.56	4.56	4.61	4.61	4.57	4.61	4.587	0.024
1X+1S	4.58	4.58	4.66	4.65	4.59	4.59	4.608	0.033
10X	5.36	5.3	5.42	5.35	5.41	5.35	5.365	0.04
1X	5.33	5.34	5.31	5.38	5.34	5.36	5.343	0.022
1S	4.57	4.59	4.62	4.62	4.63	4.59	4.603	0.021
BHI	5.31	5.36	5.33	5.34	5.32	5.36	5.337	0.019

Streptococcus oralis	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	4.93	4.91	5.07	5.06	5.16	5.14	5.045	0.095
5E+1S	4.84	4.83	4.93	4.96	5.02	5	4.93	0.073
2E+1S	4.81	4.8	4.87	4.87	4.91	4.92	4.863	0.045
1E+1S	4.85	4.85	4.9	4.94	4.92	4.92	4.897	0.035
10E	5.29	5.24	5.28	5.28	5.42	5.41	5.32	0.069
1E	5.21	5.28	5.25	5.27	5.36	5.38	5.292	0.06
10X+1S	4.99	4.94	5.09	5.02	5.2	5.1	5.057	0.085
5X+1S	4.84	4.82	4.93	4.91	4.94	4.98	4.903	0.056
2X+1S	4.86	4.87	4.96	4.98	4.99	5.05	4.952	0.067
1X+1S	4.85	4.88	4.92	4.95	4.97	5.04	4.935	0.062
10X	5.19	5.19	5.19	5.2	5.33	5.29	5.232	0.057
1X	5.19	5.22	5.2	5.2	5.26	5.31	5.23	0.042
1S	4.78	4.76	4.83	4.83	4.89	4.85	4.823	0.043
BHI	5.22	5.31	5.23	5.23	5.33	5.34	5.277	0.051

Streptococcus salivarius	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	4.47	4.44	4.48	4.46	4.49	4.48	4.47	0.016
5E+1S	4.44	4.43	4.38	4.37	4.43	4.43	4.413	0.027
2E+1S	4.42	4.42	4.36	4.35	4.43	4.42	4.4	0.032
1E+1S	4.4	4.4	4.33	4.38	4.39	4.4	4.383	0.025
10E	5.36	5.36	5.45	5.43	5.47	5.5	5.428	0.053
1E	5.28	5.29	5.32	5.37	5.36	5.5	5.353	0.073
10X+1S	4.55	4.5	4.54	4.48	4.57	4.48	4.52	0.035
5X+1S	4.52	4.52	4.47	4.48	4.48	4.46	4.488	0.023
2X+1S	4.5	4.52	4.47	4.48	4.46	4.5	4.488	0.02
1X+1S	4.51	4.48	4.47	4.45	4.45	4.48	4.473	0.021
10X	5.43	5.43	5.43	5.42	5.59	5.6	5.483	0.079
1X	5.37	5.44	5.44	5.45	5.55	5.5	5.458	0.056
1S	4.42	4.4	4.36	4.36	4.42	4.41	4.395	0.026
BHI	5.33	5.41	5.36	5.39	5.36	5.5	5.392	0.055

Table 4 Raw data of the dynamic change of biofilm pH over 36 hours of *S. mutans*. The experiments were performed independently at three times, each with a duplicate. The results were presented with mean and standard deviation (SD)

S. mutans	0 min							
	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	6.58	6.62	6.96	7.14	7.09	7.11	6.917	0.231
5E+1S	6.64	6.59	7.24	7.24	7.08	7.07	6.977	0.265
2E+1S	6.67	6.62	6.98	7.08	7.05	7.05	6.908	0.189
1E+1S	6.63	6.56	7.13	7.14	7.11	7.07	6.94	0.246
10E	6.67	6.68	7.08	7.05	7.15	7.18	6.968	0.212
1E	6.63	6.72	7.24	7.22	7.12	7.13	7.01	0.242
10X+1S	6.61	6.6	7.07	7.05	7.04	7.04	6.902	0.21
5X+1S	6.56	6.62	7.03	6.97	7.1	7.04	6.887	0.214
2X+1S	6.62	6.61	7.08	7.1	7.05	7.03	6.915	0.213
1X+1S	6.63	6.72	7.01	7.03	7.05	7.11	6.925	0.181
10X	6.69	6.6	7.13	7.13	7.19	7.19	6.988	0.245
1X	6.57	6.78	7.19	7.21	7.14	7.14	7.005	0.242
1S	6.63	6.59	6.95	6.97	6.95	6.93	6.837	0.161
BHI	6.58	6.65	6.94	6.94	7.05	7.08	6.873	0.191

S. mutans	15 min							
	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	6.26	6.24	6.66	6.67	6.58	6.62	6.505	0.183
5E+1S	6.32	6.23	6.6	6.63	6.58	6.62	6.497	0.16
2E+1S	6.12	6.23	6.55	6.57	6.42	6.42	6.385	0.162
1E+1S	6.17	6.21	6.5	6.49	6.45	6.48	6.383	0.138
10E	6.24	6.22	6.7	6.76	6.75	6.75	6.57	0.241
1E	6.24	6.27	6.72	6.74	6.6	6.58	6.525	0.2
10X+1S	6.26	6.28	6.46	6.47	6.54	6.57	6.43	0.119
5X+1S	6.32	6.31	6.39	6.4	6.46	6.54	6.403	0.079
2X+1S	6.34	6.38	6.43	6.46	6.48	6.49	6.43	0.054
1X+1S	6.23	6.22	6.39	6.36	6.48	6.47	6.358	0.103
10X	6.45	6.43	6.75	6.75	6.68	6.72	6.63	0.137
1X	6.44	6.4	6.81	6.85	6.77	6.77	6.673	0.182
1S	6.19	6.21	6,.24	6.22	6.12	6.11	6.17	0.046
BHI	6.31	6.34	6.25	6.3	6.41	6.45	6.343	0.068

S. mutans	30 min							
	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	6.12	6.14	6.37	6.46	6.23	6.26	6.263	0.12
5E+1S	6.13	6.13	6.19	6.25	5.99	6.04	6.122	0.087
2E+1S	6	5.99	6.14	6.19	5.83	5.86	6.002	0.132
1E+1S	5.94	5.96	5.99	5.93	5.89	5.92	5.938	0.031
10E	6.28	6.3	6.53	6.52	6.51	6.54	6.447	0.111
1E	6.31	6.33	6.36	6.33	6.26	6.3	6.315	0.031
10X+1S	6.18	6.19	6.06	6.1	6.22	6.23	6.163	0.062
5X+1S	6.17	6.18	5.9	5.92	6.09	6.13	6.065	0.114
2X+1S	6.16	6.14	5.99	6	6.02	6.12	6.072	0.07
1X+1S	6.07	6	5.73	5.77	5.91	5.95	5.905	0.121
10X	6.4	6.43	6.56	6.61	6.51	6.59	6.517	0.079
1X	6.38	6.41	6.7	6.7	6.65	6.67	6.585	0.136
1S	5.94	5.98	5.64	5.7	5.46	5.43	5.692	0.212
BHI	6.14	6.13	5.65	5.7	6.03	6	5.942	0.196

S. mutans	45 min							
	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	5.79	5.78	6.21	6.16	5.92	5.94	5.967	0.166
5E+1S	5.95	5.97	5.82	5.82	5.55	5.52	5.772	0.177
2E+1S	5.69	5.67	5.65	5.7	5.39	5.39	5.582	0.136
1E+1S	5.61	5.6	5.54	5.49	5.49	5.45	5.53	0.059
10E	6.15	6.1	6.44	6.5	6.39	6.45	6.338	0.155
1E	5.89	5.87	6.12	6.1	5.97	6	5.992	0.095
10X+1S	5.94	5.99	5.68	5.78	5.84	5.81	5.84	0.102
5X+1S	5.94	5.98	5.52	5.49	5.65	5.68	5.71	0.189
2X+1S	5.88	5.89	5.55	5.56	5.63	5.59	5.683	0.145
1X+1S	5.76	5.77	5.34	5.37	5.49	5.46	5.532	0.173
10X	6.25	6.23	6.46	6.55	6.44	6.47	6.4	0.118
1X	6.23	6.21	6.57	6.64	6.6	6.57	6.47	0.178
1S	5.6	5.64	5.35	5.29	5.18	5.15	5.368	0.19
BHI	5.84	5.88	5.59	5.57	5.59	5.59	5.677	0.13

S. mutans	60 min							
	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	5.61	5.64	5.79	5.86	5.52	5.5	5.653	0.132
5E+1S	5.71	5.77	5.46	5.41	5.25	5.3	5.483	0.195
2E+1S	5.46	5.45	5.33	5.34	5.24	5.2	5.337	0.097
1E+1S	5.37	5.33	5.24	5.27	5.18	5.22	5.268	0.065
10E	6.07	6	6.38	6.39	6.31	6.37	6.253	0.158
1E	5.71	5.77	5.85	5.86	5.71	5.7	5.767	0.066
10X+1S	5.75	5.77	5.37	5.42	5.45	5.48	5.54	0.159
5X+1S	5.65	5.66	5.23	5.27	5.32	5.36	5.415	0.174
2X+1S	5.68	5.64	5.29	5.32	5.3	5.31	5.423	0.168
1X+1S	5.68	5.66	5.12	5.22	5.23	5.24	5.358	0.224
10X	6.08	6.09	6.48	6.54	6.35	6.42	6.327	0.18
1X	6.09	6.07	6.57	6.62	6.42	6.45	6.37	0.216
1S	5.41	5.32	5.17	5.14	5	4.98	5.17	0.156
BHI	5.65	5.66	5.67	5.69	5.28	5.31	5.543	0.176

S. mutans	75 min							
	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	5.49	5.44	5.52	5.52	5.33	5.32	5.437	0.083
5E+1S	5.55	5.54	5.25	5.23	5.13	5.1	5.3	0.181
2E+1S	5.32	5.33	5.23	5.22	5.04	5.1	5.207	0.106
1E+1S	5.23	5.24	5.13	5.25	5.07	5.11	5.172	0.071
10E	5.97	5.99	6.33	6.44	6.2	6.28	6.202	0.172
1E	5.58	5.55	5.67	5.68	5.52	5.48	5.58	0.074
10X+1S	5.71	5.77	5.27	5.2	5.17	5.28	5.4	0.244
5X+1S	5.56	5.56	5.12	5.12	5.13	5.21	5.283	0.198
2X+1S	5.52	5.53	5.12	5.2	5.15	5.18	5.283	0.173
1X+1S	5.39	5.37	5.12	5.03	5.11	5.13	5.192	0.137
10X	6.07	6.06	6.44	6.54	6.31	6.37	6.298	0.179
1X	6	6.06	6.43	6.56	6.38	6.41	6.307	0.204
1S	5.32	5.31	5.03	5.12	4.95	4.91	5.107	0.161
BHI	5.47	5.44	5.59	5.63	5.18	5.2	5.418	0.174

S. mutans	90 min							
	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	5.34	5.43	5.22	5.33	5.21	5.18	5.285	0.088
5E+1S	5.41	5.44	5.11	5.04	4.98	4.98	5.16	0.193
2E+1S	5.24	5.22	5.07	5.04	4.96	5	5.088	0.106
1E+1S	5.19	5.14	5.08	5.08	5.01	5.02	5.087	0.063
10E	5.96	5.99	6.4	6.49	6.1	6.17	6.185	0.198
1E	5.49	5.43	5.82	5.78	5.34	5.29	5.525	0.205
10X+1S	5.53	5.55	5.1	5.17	5.11	5.14	5.267	0.195
5X+1S	5.43	5.45	5.06	5.02	5.05	5.06	5.178	0.186
2X+1S	5.38	5.33	5.02	5.13	5.05	5.03	5.157	0.145
1X+1S	5.28	5.22	4.97	5.02	5.02	4.98	5.082	0.122
10X	5.99	5.98	6.36	6.47	6.22	6.27	6.215	0.18
1X	5.91	5.96	6.47	6.37	6.28	6.29	6.213	0.207
1S	5.21	5.24	4.94	4.96	4.87	4.85	5.012	0.156
BHI	5.34	5.43	5.82	5.88	5.23	5.25	5.492	0.262

S. mutans	105 min							
	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	5.26	5.24	5.13	5.15	5.04	5.09	5.152	0.078
5E+1S	5.3	5.32	5.03	5.04	4.84	4.87	5.067	0.187
2E+1S	5.16	5.14	4.95	4.98	4.87	4.94	5.007	0.107
1E+1S	5.13	5.11	4.92	4.9	4.92	4.95	4.988	0.094
10E	5.91	5.95	6.28	6.44	6	6.07	6.108	0.19
1E	5.41	5.44	5.91	5.92	5.29	5.22	5.532	0.281
10X+1S	5.44	5.42	5.01	5.02	5.01	5.07	5.162	0.191
5X+1S	5.33	5.34	5.01	4.99	5	5.01	5.113	0.157
2X+1S	5.3	5.31	4.98	5.03	4.99	4.91	5.087	0.158
1X+1S	5.18	5.13	4.91	4.9	4.93	4.95	5	0.112
10X	5.89	5.87	6.44	6.53	6.12	6.2	6.175	0.25
1X	5.88	5.87	6.44	6.5	6.17	6.19	6.175	0.244
1S	5.15	5.12	4.81	4.84	4.78	4.77	4.912	0.16
BHI	5.27	5.24	5.51	5.48	5.28	5.28	5.343	0.108

S. mutans	120 min							
	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	5.22	5.23	5.15	5.1	4.99	5	5.115	0.095
5E+1S	5.23	5.24	4.9	4.99	4.83	4.82	5.002	0.174
2E+1S	5.12	5.14	4.76	4.82	4.84	4.88	4.927	0.148
1E+1S	5.08	5.08	4.88	4.85	4.87	4.82	4.93	0.108
10E	5.86	5.88	6.28	6.36	5.88	5.96	6.037	0.204
1E	5.3	5.34	5.87	5.91	5.37	5.38	5.528	0.257
10X+1S	5.34	5.33	4.98	4.88	5	4.95	5.08	0.184
5X+1S	5.26	5.25	4.89	4.88	4.94	4.92	5.023	0.165
2X+1S	5.21	5.22	5.01	4.89	4.86	4.84	5.005	0.158
1X+1S	5.12	5.14	4.85	4.89	4.84	4.81	4.942	0.135
10X	5.87	5.88	6.37	6.33	6.05	6.13	6.105	0.196
1X	5.86	5.87	6.39	6.28	6.1	6.13	6.105	0.195
1S	5.08	5.04	4.78	4.86	4.77	4.75	4.88	0.132
BHI	5.19	5.13	5.84	5.76	5.28	5.26	5.41	0.281

S. mutans	36 hr.							
	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	4.29	4.3	4.23	4.19	4.32	4.29	4.27	0.045
5E+1S	4.26	4.24	4.21	4.15	4.32	4.25	4.238	0.051
2E+1S	4.24	4.22	4.19	4.2	4.24	4.21	4.217	0.019
1E+1S	4.46	4.44	4.1	4.08	4.26	4.18	4.253	0.151
10E	5.42	5.44	5.3	5.38	5.62	5.66	5.47	0.128
1E	5.24	5.23	5.36	5.29	5.66	5.65	5.405	0.182
10X+1S	4.44	4.43	4.21	4.19	4.38	4.34	4.332	0.099
5X+1S	4.56	4.55	4.32	4.23	4.24	4.22	4.353	0.146
2X+1S	4.64	4.62	4.34	4.35	4.2	4.19	4.39	0.181
1X+1S	4.65	4.66	4.26	4.34	4.1	4.14	4.358	0.224
10X	5.36	5.34	5.35	5.48	5.57	5.68	5.463	0.127
1X	5.35	5.33	5.45	5.33	5.6	5.59	5.442	0.116
1S	4.73	4.74	4.28	4.34	4.13	4.1	4.387	0.26
BHI	5.33	5.31	5.34	5.26	5.42	5.49	5.358	0.076

Table 5 Raw data of the dynamic change of biofilm pH over 36 hours of *S. sanguinis.* The experiments were performed independently at four times, each with a duplicate. The results were presented with mean and standard deviation (SD)

S. sanguinis	0 min									
	1.1	1.2	2.1	2.2	3.1	3.2	4.1	4.2	Average	SD
10E+1S	6.64	6.6	7	6.98	6.97	6.99	6.98	6.95	6.889	0.156
5E+1S	6.63	6.64	7.02	7	7.16	7.11	7.16	7.1	6.978	0.205
2E+1S	6.64	6.6	7.11	7.06	7.17	7.16	7.13	7.13	7	0.222
1E+1S	6.66	6.64	7.17	7.1	7.14	7.13	7.22	7.25	7.039	0.229
10E	6.64	6.63	6.96	6.98	7.15	7.14	7.18	7.18	6.983	0.216
1E	6.63	6.62	7.19	7.13	7.1	7.1	7.22	7.21	7.025	0.235
10X+1S	6.65	6.61	7.16	7.11	7.14	7.11	7.12	7.08	6.998	0.214
5X+1S	6.67	6.62	7.11	7.11	7.12	7.12	7.12	7.16	7.004	0.208
2X+1S	6.69	6.65	7.14	7.1	7.15	7.14	7.17	7.18	7.028	0.208
1X+1S	6.63	6.65	6.85	6.88	7.21	7.17	7.15	7.17	6.964	0.226
10X	6.64	6.6	7.12	7.11	7.11	7.13	7.19	7.18	7.01	0.227
1X	6.65	6.68	7.17	7.15	7.18	7.14	7.12	7.13	7.028	0.21
1S	6.68	6.65	7.02	7	7.09	7.1	7.04	7.02	6.95	0.168
BHI	6.63	6.65	7	7.04	7.13	7.11	7	7	6.945	0.182

S. sanguinis	15 min									
	1.1	1.2	2.1	2.2	3.1	3.2	4.1	4.2	Average	SD
10E+1S	6.6	6.62	6.7	6.69	6.56	6.7	6.67	6.7	6.655	0.051
5E+1S	6.57	6.55	6.64	6.65	6.66	6.69	6.81	6.73	6.663	0.078
2E+1S	6.57	6.52	6.62	6.66	6.73	6.73	6.71	6.76	6.663	0.08
1E+1S	6.61	6.65	6.63	6.63	6.76	6.78	6.86	6.84	6.72	0.095
10E	6.55	6.51	6.58	6.56	6.69	6.76	6.86	6.83	6.668	0.128
1E	6.66	6.67	6.77	6.82	6.63	6.68	6.82	6.76	6.726	0.07
10X+1S	6.55	6.56	6.71	6.67	6.7	6.68	6.65	6.71	6.654	0.06
5X+1S	6.57	6.55	6.64	6.67	6.74	6.66	6.7	6.68	6.651	0.06
2X+1S	6.66	6.65	6.67	6.72	6.73	6.64	6.72	6.73	6.69	0.036
1X+1S	6.67	6.67	6.62	6.63	6.8	6.72	6.73	6.74	6.698	0.057
10X	6.6	6.62	6.41	6.43	6.8	6.87	6.85	6.86	6.68	0.179
1X	6.65	6.64	6.85	6.89	6.88	6.89	6.81	6.83	6.805	0.096
1S	6.69	6.66	6.52	6.5	6.63	6.51	6.65	6.65	6.601	0.073
BHI	6.67	6.65	6.51	6.54	6.52	6.64	6.68	6.71	6.615	0.074

S. sanguinis	30 min									
	1.1	1.2	2.1	2.2	3.1	3.2	4.1	4.2	Average	SD
10E+1S	6.45	6.44	6.37	6.44	6.47	6.52	6.43	6.49	6.451	0.042
5E+1S	6.51	6.53	6.34	6.38	6.48	6.52	6.63	6.61	6.5	0.094
2E+1S	6.38	6.34	6.37	6.35	6.5	6.5	6.53	6.53	6.438	0.079
1E+1S	6.39	6.39	6.35	6.34	6.5	6.51	6.66	6.6	6.468	0.112
10E	6.4	6.44	6.42	6.34	6.57	6.5	6.7	6.66	6.504	0.12
1E	6.62	6.6	6.52	6.54	6.31	6.42	6.65	6.63	6.536	0.111
10X+1S	6.48	6.45	6.5	6.48	6.55	6.59	6.54	6.57	6.52	0.046
5X+1S	6.44	6.43	6.46	6.47	6.53	6.52	6.52	6.54	6.489	0.041
2X+1S	6.57	6.55	6.38	6.36	6.42	6.4	6.51	6.57	6.47	0.083
1X+1S	6.55	6.54	6.32	6.32	6.46	6.5	6.49	6.48	6.458	0.084
10X	6.39	6.34	6.22	6.18	6.71	6.77	6.75	6.76	6.515	0.241
1X	6.65	6.65	6.7	6.74	6.76	6.74	6.68	6.69	6.701	0.039
1S	6.46	6.44	6.04	6.04	6.14	6.13	6.36	6.33	6.243	0.163
BHI	6.53	6.55	6.08	6.09	6.22	6.19	6.43	6.44	6.316	0.181

S. sanguinis	45 min									
	1.1	1.2	2.1	2.2	3.1	3.2	4.1	4.2	Average	SD
10E+1S	6.43	6.44	6.26	6.39	6.35	6.27	6.3	6.39	6.354	0.066
5E+1S	6.41	6.4	6.23	6.21	6.25	6.27	6.5	6.51	6.348	0.114
2E+1S	6.35	6.34	6.17	6.17	6.28	6.28	6.38	6.37	6.293	0.079
1E+1S	6.36	6.34	6.22	6.21	6.25	6.29	6.48	6.49	6.33	0.102
10E	6.37	6.35	6.29	6.26	6.39	6.4	6.57	6.59	6.403	0.112
1E	6.62	6.62	6.39	6.46	5.97	5.95	6.43	6.39	6.354	0.243
10X+1S	6.36	6.34	6.41	6.39	6.34	6.39	6.41	6.42	6.383	0.03
5X+1S	6.34	6.33	6.3	6.36	6.24	6.24	6.33	6.36	6.313	0.045
2X+1S	6.4	6.44	6.15	6.13	6.1	6.11	6.29	6.33	6.244	0.129
1X+1S	6.36	6.35	6.04	6	6.15	6.2	6.25	6.2	6.194	0.122
10X	6.35	6.54	6.01	6	6.6	6.7	6.67	6.71	6.448	0.277
1X	6.62	6.61	6.63	6.67	6.63	6.64	6.57	6.63	6.625	0.026
1S	6.34	6.39	5.76	5.8	5.69	5.61	5.97	5.95	5.939	0.271
BHI	6.34	6.33	5.8	5.82	5.75	5.8	6.09	6.11	6.005	0.229

S. sanguinis	60 min									
	1.1	1.2	2.1	2.2	3.1	3.2	4.1	4.2	Average	SD
10E+1S	6.3	6.32	6.18	6.23	6.09	6.07	6.1	6.16	6.181	0.089
5E+1S	6.22	6.23	5.93	6.03	6.05	6.05	6.29	6.35	6.144	0.138
2E+1S	6.25	6.24	5.97	5.995	5.99	6.04	6.12	6.13	6.092	0.104
1E+1S	6.17	6.15	5.94	5.95	6	5.98	6.34	6.28	6.101	0.146
10E	6.33	6.34	6.07	6.06	6.2	6.21	6.51	6.53	6.281	0.168
1E	6.62	6.61	6.25	6.25	5.62	5.62	6.21	6.21	6.174	0.357
10X+1S	6.26	6.25	6.29	6.29	6.17	6.33	6.27	6.32	6.273	0.047
5X+1S	6.22	6.21	6.11	6.14	5.95	5.94	6.13	6.17	6.109	0.101
2X+1S	6.3	6.34	5.88	5.88	5.74	5.75	6.06	6.12	6.009	0.218
1X+1S	6.22	6.21	5.75	5.78	5.87	5.85	6	6.01	5.961	0.17
10X	6.32	6.33	5.7	5.75	6.6	6.67	6.63	6.6	6.325	0.368
1X	6.3	6.31	6.58	6.59	6.6	6.61	6.48	6.56	6.504	0.121
1S	6.31	6.31	5.48	5.43	5.4	5.31	5.63	5.6	5.684	0.374
BHI	6.31	6.32	5.5	5.57	5.44	5.41	5.81	5.81	5.771	0.344

S. sanguinis	75 min									
	1.1	1.2	2.1	2.2	3.1	3.2	4.1	4.2	Average	SD
10E+1S	6.2	6.21	6.05	6.07	5.86	5.9	5.86	5.93	6.01	0.135
5E+1S	6.19	6.2	5.79	5.81	5.8	5.83	6.17	6.18	5.996	0.189
2E+1S	6.14	6.13	5.77	5.83	5.76	5.79	5.88	5.9	5.9	0.143
1E+1S	6.07	6.09	5.76	5.8	5.76	5.76	6.08	6.12	5.93	0.161
10E	6.3	6.28	5.94	5.96	6.11	6.1	6.41	6.43	6.191	0.179
1E	6.61	6.61	6	6	5.83	5.88	5.94	5.91	6.098	0.301
10X+1S	6.14	6.13	6.21	6.22	6.02	6	6.14	6.15	6.126	0.074
5X+1S	6.08	6.09	5.96	5.98	5.69	5.7	5.93	5.97	5.925	0.143
2X+1S	6.23	6.22	5.7	5.67	5.5	5.6	5.88	5.91	5.839	0.256
1X+1S	6.08	6.09	5.61	5.61	5.53	5.55	5.73	5.74	5.743	0.21
10X	6.28	6.24	5.6	5.54	6.57	6.55	6.67	6.65	6.263	0.426
1X	6.23	6.22	6.41	6.55	6.45	6.44	6.46	6.47	6.404	0.11
1S	6.18	6.16	5.28	5.24	5.21	5.2	5.42	5.38	5.509	0.389
BHI	6.25	6.25	5.41	5.44	5.25	5.34	5.59	5.54	5.634	0.369

S. sanguinis	90 min									
	1.1	1.2	2.1	2.2	3.1	3.2	4.1	4.2	Average	SD
10E+1S	6.13	6.11	5.97	5.92	5.76	5.87	5.71	5.72	5.899	0.155
5E+1S	6.08	6.06	5.69	5.61	5.63	5.66	5.87	5.93	5.816	0.181
2E+1S	5.98	5.99	5.6	5.65	5.58	5.65	5.6	5.63	5.71	0.16
1E+1S	5.89	5.86	5.54	5.58	5.58	5.69	5.82	5.85	5.726	0.136
10E	6.24	6.23	5.8	5.86	5.98	5.99	6.25	6.3	6.081	0.184
1E	6.12	6.11	5.84	5.85	5.53	5.55	5.67	5.64	5.789	0.218
10X+1S	6.03	6	6.19	6.13	5.88	5.98	5.95	5.99	6.019	0.093
5X+1S	6.02	6.03	5.71	5.86	5.57	5.65	5.71	5.7	5.781	0.16
2X+1S	6.13	6.12	5.51	5.49	5.43	5.39	5.61	5.61	5.661	0.277
1X+1S	5.96	5.98	5.46	5.51	5.4	5.45	5.46	5.51	5.591	0.221
10X	6.12	6.14	5.48	5.49	6.54	6.43	6.5	6.55	6.156	0.418
1X	6.13	6.15	6.38	6.4	6.35	6.33	6.3	6.3	6.293	0.094
1S	6.11	6.12	5.23	5.21	5.21	5.22	5.23	5.24	5.446	0.386
BHI	6.11	6.1	5.35	5.37	5.22	5.23	5.37	5.4	5.519	0.344

S. sanguinis	105 min									
	1.1	1.2	2.1	2.2	3.1	3.2	4.1	4.2	Average	SD
10E+1S	6.03	6	5.83	5.88	5.59	5.68	5.58	5.56	5.769	0.179
5E+1S	5.97	5.96	5.53	5.52	5.53	5.55	5.59	5.69	5.668	0.179
2E+1S	5.89	5.88	5.56	5.59	5.46	5.54	5.48	5.5	5.613	0.162
1E+1S	5.73	5.78	5.41	5.42	5.4	5.45	5.53	5.59	5.539	0.139
10E	6.15	6.16	5.69	5.76	5.83	5.87	6.17	6.17	5.975	0.194
1E	6.18	6.17	5.74	5.6	5.43	5.4	5.48	5.47	5.684	0.301
10X+1S	5.92	5.97	6.09	6.07	5.84	5.88	5.81	5.85	5.929	0.099
5X+1S	5.88	5.87	5.68	5.67	5.5	5.58	5.54	5.57	5.661	0.136
2X+1S	5.98	5.97	5.52	5.44	5.41	5.43	5.41	5.47	5.579	0.231
1X+1S	5.84	5.86	5.46	5.39	5.33	5.4	5.33	5.36	5.496	0.208
10X	6.13	6.11	5.39	5.41	6.15	6.3	6.14	6.21	5.98	0.34
1X	6.02	6.01	6.32	6.37	6.32	6.33	6.15	6.15	6.209	0.136
1S	6.02	6.02	5.23	5.15	5.14	5.13	5.21	5.19	5.386	0.367
BHI	6.01	6.02	5.28	5.3	5.16	5.2	5.32	5.23	5.44	0.336

S. sanguinis	120 min									
	1.1	1.2	2.1	2.2	3.1	3.2	4.1	4.2	Average	SD
10E+1S	5.95	5.97	5.74	5.73	5.58	5.57	5.5	5.5	5.693	0.176
5E+1S	5.85	5.88	5.43	5.42	5.49	5.45	5.39	5.4	5.539	0.191
2E+1S	5.75	5.77	5.44	5.47	5.42	5.44	5.32	5.38	5.499	0.157
1E+1S	5.63	5.66	5.32	5.29	5.36	5.39	5.29	5.3	5.405	0.143
10E	6.16	6.13	5.56	5.58	5.79	5.77	6.02	5.99	5.875	0.219
1E	6.16	6.17	5.42	5.41	5.25	5.32	5.33	5.32	5.548	0.36
10X+1S	5.85	5.86	6	6.01	5.78	5.77	5.75	5.73	5.844	0.102
5X+1S	5.81	5.84	5.61	5.6	5.41	5.44	5.4	5.49	5.575	0.162
2X+1S	5.9	5.88	5.44	5.42	5.27	5.32	5.33	5.31	5.484	0.24
1X+1S	5.73	5.77	5.34	5.42	5.3	5.32	5.24	5.22	5.418	0.201
10X	6.12	6.1	5.32	5.31	6	6.2	6	6.02	5.884	0.335
1X	5.97	5.98	6.19	6.22	6.24	6.22	5.98	5.99	6.099	0.12
1S	5.91	5.93	5.16	5.11	5.15	5.14	5.14	5.13	5.334	0.339
BHI	5.9	5.93	5.25	5.22	5.19	5.22	5.22	5.2	5.391	0.303

S. sanguinis	36 h									
	1.1	1.2	2.1	2.2	3.1	3.2	4.1	4.2	Average	SD
10E+1S	4.88	4.89	4.72	4.81	4.67	4.73	4.7	4.66	4.758	0.085
5E+1S	4.78	4.75	4.8	4.75	4.66	4.65	4.55	4.52	4.683	0.099
2E+1S	4.8	4.87	4.7	4.65	4.58	4.59	4.57	4.61	4.671	0.104
1E+1S	4.75	4.77	4.74	4.7	4.54	4.53	4.52	4.54	4.636	0.105
10E	5.4	5.44	5.61	5.7	5.49	5.57	5.45	5.48	5.518	0.094
1E	5.22	5.24	5.54	5.55	5.52	5.53	5.54	5.45	5.449	0.13
10X+1S	4.85	4.86	4.79	4.77	4.73	4.7	4.73	4.7	4.766	0.059
5X+1S	4.78	4.76	4.74	4.76	4.63	4.63	4.61	4.65	4.695	0.067
2X+1S	4.76	4.79	4.72	4.72	4.6	4.6	4.56	4.57	4.665	0.086
1X+1S	4.71	4.75	4.68	4.64	4.52	4.51	4.64	4.64	4.636	0.079
10X	5.41	5.44	5.45	5.47	5.47	5.48	5.49	5.44	5.456	0.024
1X	5.33	5.36	5.57	5.59	5.47	5.49	5.46	5.48	5.469	0.084
1S	4.88	4.85	4.73	4.64	4.67	4.61	4.73	4.64	4.719	0.094
BHI	5.2	5.29	5.53	5.56	5.52	5.57	5.51	5.54	5.465	0.13

Table 6 Raw data of the pH of mixed-species biofilm at 36 hour of S. *mutans + S. sanguinis, S. mutans + S. gordonii, S. sanguinis + S. gordonii*, and mix of the three species. The experiments were performed independently three times, each with a duplicate. The results were presented with mean and standard deviation (SD)

S. mutans + S. sanguinis	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	4.12	4.14	3.96	4.09	4.2	4.22	4.122	0.085
5E+1S	4.15	4.21	4.13	4.1	4.19	4.17	4.158	0.037
2E+1S	4.17	4.15	4.08	4.07	4.15	4.14	4.127	0.038
1E+1S	4.11	4.12	4.11	4.08	4.12	4.12	4.11	0.014
10E	5.22	5.24	5.19	5.26	5.18	5.15	5.207	0.037
1E	5.1	5.19	5.24	5.29	5.18	5.17	5.195	0.059
10X+1S	4.09	4.11	4.18	4.13	4.25	4.21	4.162	0.057
5X+1S	4.11	4.1	4.09	4.13	4.14	4.13	4.117	0.018
2X+1S	4.19	4.16	4.08	4.08	4.11	4.09	4.118	0.042
1X+1S	4.14	4.16	4.08	4.09	4.12	4.08	4.112	0.031
10X	5.2	5.22	5.21	5.35	5.15	5.15	5.213	0.067
1X	5.28	5.22	5.24	5.37	5.25	5.24	5.267	0.05
1S	4.12	4.09	4.1	4.08	4.14	4.11	4.107	0.02
BHI	5.02	5.06	5.12	5.11	5.13	5.15	5.098	0.045

S. mutans + *S. gordonii*	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	4.04	4.01	3.95	4.01	4.05	4.1	4.027	0.046
5E+1S	4.11	4.06	4.07	4.06	3.99	4.08	4.062	0.036
2E+1S	4.11	4.13	4.06	4.05	4.08	4.06	4.082	0.029
1E+1S	4.1	4.11	4.06	4.05	4.06	4.05	4.072	0.024
10E	5.17	5.18	5.21	5.32	5.1	5.17	5.192	0.066
1E	5.12	5.17	5.21	5.24	5.18	5.16	5.18	0.038
10X+1S	4.14	4.11	4.16	4.1	4.19	4.16	4.143	0.031
5X+1S	4.11	4.11	4.05	4.05	4.17	4.12	4.102	0.042
2X+1S	4.09	4.1	4.06	4.06	4.11	4.12	4.09	0.023
1X+1S	4.09	4.14	4.03	4.05	4.08	4.06	4.075	0.035
10X	5.14	5.12	5.2	5.38	5.21	5.22	5.212	0.084
1X	5.16	5.14	5.29	5.28	5.23	5.21	5.218	0.056
1S	4.08	4.11	4.17	4.09	4.1	4.09	4.107	0.03
BHI	5.12	5.22	5.1	5.16	5.15	5.2	5.158	0.042

S. sanguinis + *S. gordonii*	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	4.57	4.58	4.73	4.59	4.63	4.65	4.625	0.055
5E+1S	4.52	4.55	4.55	4.48	4.5	4.44	4.507	0.039
2E+1S	4.51	4.55	4.53	4.48	4.48	4.46	4.502	0.031
1E+1S	4.55	4.56	4.49	4.46	4.47	4.47	4.5	0.04
10E	5.49	5.55	5.53	5.55	5.42	5.51	5.508	0.045
1E	5.51	5.53	5.58	5.62	5.48	5.48	5.533	0.052
10X+1S	4.52	4.51	4.59	4.58	4.54	4.53	4.545	0.03
5X+1S	4.45	4.44	4.45	4.49	4.47	4.49	4.465	0.02
2X+1S	4.4	4.41	4.48	4.46	4.51	4.49	4.458	0.041
1X+1S	4.44	4.41	4.45	4.47	4.47	4.5	4.457	0.028
10X	5.38	5.33	5.31	5.39	5.41	5.47	5.382	0.052
1X	5.47	5.44	5.41	5.44	5.5	5.51	5.462	0.035
1S	4.45	4.48	4.53	4.51	4.38	4.49	4.473	0.049
BHI	5.44	5.41	5.35	5.46	5.45	5.53	5.44	0.054

Mixed of 3 species	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	4.14	4.1	4.08	4.06	4.1	4.09	4.095	0.024
5E+1S	4.11	4.1	4.1	4.11	4.12	4.11	4.108	0.007
2E+1S	4.09	4.06	4.12	4.11	4.09	4.12	4.098	0.021
1E+1S	4.07	4.07	4.1	4.09	4.12	4.09	4.09	0.017
10E	5.24	5.27	5.21	5.22	5.22	5.24	5.233	0.02
1E	5.22	5.2	5.1	5.12	5.22	5.21	5.178	0.049
10X+1S	4.14	4.11	4.2	4.21	4.12	4.12	4.15	0.04
5X+1S	4.09	4.09	4.15	4.12	4.09	4.11	4.108	0.022
2X+1S	4.06	4.06	4.12	4.11	4.08	4.1	4.088	0.023
1X+1S	4.07	4.07	4.09	4.1	4.12	4.09	4.09	0.017
10X	5.21	5.42	5.27	5.28	5.25	5.28	5.285	0.065
1X	5.35	5.38	5.23	5.22	5.24	5.3	5.287	0.062
1S	4.16	4.11	4.12	4.13	4.13	4.11	4.127	0.017
BHI	5.07	5.22	5.44	5.41	5.14	5.23	5.252	0.134

Table 7 Raw data of the biofilm mass of single-species biofilm at 36 hours. The experiments were performed independently three times, each with a duplicate. The results were presented with mean and standard deviation (SD)

S. mutans	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	0.622	0.548	0.511	0.49	0.524	0.435	0.522	0.057
5E+1S	0.774	0.675	1.243	1.072	0.909	0.944	0.936	0.186
2E+1S	1.14	1.04	1.55	1.11	1.34	1.34	1.253	0.174
1E+1S	1.24	1.06	1.17	1.15	1.27	1.28	1.195	0.077
10E	0.071	0.072	0.101	0.102	0.067	0.111	0.087	0.018
1E	0.084	0.108	0.136	0.186	0.114	0.1	0.121	0.033
10X+1S	0.514	0.675	0.591	0.502	0.622	0.551	0.576	0.061
5X+1S	0.568	0.651	0.979	1.081	0.949	0.936	0.861	0.185
2X+1S	1.1	1.03	1.69	1.35	1.43	1.15	1.292	0.226
1X+1S	1.24	1.12	1.49	1.55	1.33	1.23	1.327	0.151
10X	0.053	0.069	0.112	0.074	0.091	0.099	0.083	0.02
1X	0.09	0.078	0.098	0.091	0.087	0.089	0.089	0.006
1S	1.29	1.26	1.41	1.57	1.29	1.41	1.372	0.107
BHI	0.122	0.171	0.242	0.22	0.14	0.106	0.167	0.05

S. sobrinus	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	1.66	1.68	1.58	1.56	1.33	1.39	1.533	0.131
5E+1S	1.91	2.41	1.7	2	1.52	1.63	1.862	0.294
2E+1S	2.31	2.34	1.9	2.1	1.81	1.61	2.012	0.264
1E+1S	1.91	2.21	1.8	1.84	1.6	1.61	1.828	0.205
10E	0.102	0.138	0.035	0.039	0.035	0.031	0.063	0.041
1E	0.046	0.055	0.052	0.085	0.034	0.038	0.052	0.017
10X+1S	1.59	1.5	1.76	1.4	1.27	1.36	1.48	0.161
5X+1S	1.91	2.11	1.46	1.65	1.74	1.43	1.717	0.24
2X+1S	2.06	2.15	1.58	1.72	1.52	1.46	1.748	0.265
1X+1S	2.11	2.03	1.79	1.47	1.35	1.39	1.69	0.304
10X	0.058	0.051	0.09	0.04	0.009	0.009	0.043	0.028
1X	0.053	0.059	0.09	0.077	0.009	0.01	0.05	0.031
1S	1.71	2.11	1.58	1.5	1.48	1.46	1.64	0.226
BHI	0.099	0.1	0.056	0.066	0.127	0.087	0.089	0.023

S. sanguinis	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	1.87	1.69	1.73	1.9	2.38	1.85	1.903	0.226
5E+1S	2.03	1.74	1.84	1.75	1.76	2.32	1.907	0.21
2E+1S	1.99	1.75	1.82	1.7	2.54	1.99	1.965	0.28
1E+1S	2.06	1.63	1.77	1.8	2.99	2.22	2.078	0.452
10E	0.127	0.171	0.182	0.114	0.29	0.295	0.197	0.072
1E	0.168	0.18	0.127	0.112	0.215	0.26	0.177	0.05
10X+1S	1.15	1.06	1.74	1.86	2.14	1.82	1.628	0.391
5X+1S	1.07	1.39	2.22	1.93	2.18	2.51	1.883	0.5
2X+1S	0.826	0.885	1.55	1.59	1.99	1.71	1.425	0.427
1X+1S	0.811	0.921	1.41	1.52	2.1	1.9	1.444	0.469
10X	0.086	0.079	0.127	0.145	0.296	0.247	0.163	0.081
1X	0.079	0.073	0.127	0.145	0.375	0.412	0.202	0.138
1S	1.89	1.74	1.25	1.34	1.65	1.8	1.612	0.236
BHI	0.253	0.164	0.242	0.22	0.398	0.189	0.244	0.075

S. gordonii	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	0.715	0.737	0.656	0.928	0.524	0.507	0.678	0.142
5E+1S	1.13	1.01	1.15	0.91	1.03	0.64	0.978	0.171
2E+1S	1.47	1.24	1.6	1.4	1.24	1.04	1.332	0.182
1E+1S	1.59	1.15	1.56	1.43	1.25	1.11	1.348	0.19
10E	0.117	0.158	0.069	0.076	0.095	0.096	0.102	0.029
1E	0.327	0.299	0.14	0.116	0.263	0.191	0.223	0.079
10X+1S	0.814	0.9	0.826	0.694	0.972	0.906	0.852	0.088
5X+1S	1.47	1.32	1.09	1.14	1.11	1.18	1.218	0.135
2X+1S	1.81	1.57	1.48	1.28	1.28	1.29	1.452	0.195
1X+1S	1.82	1.56	1.46	1.47	1.69	1.37	1.562	0.152
10X	0.143	0.112	0.138	0.17	0.148	0.261	0.162	0.047
1X	0.296	0.355	0.318	0.309	0.291	0.279	0.308	0.024
1S	1.61	1.6	1.59	1.76	1.33	1.59	1.58	0.127
BHI	0.457	0.448	0.165	0.162	0.318	0.258	0.301	0.12

S. oralis	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	1.57	1.5	1.85	2.04	1.55	2	1.752	0.22
5E+1S	2.3	2.46	2.15	2.13	2.42	2.18	2.273	0.13
2E+1S	1.75	1.55	2.24	2.13	2.63	2.4	2.117	0.368
1E+1S	2.01	1.7	2.52	2.31	2.67	2.51	2.287	0.335
10E	1.11	1.11	2.19	2.15	1.33	1.23	1.52	0.466
1E	1.57	1.42	2.65	2.7	1.87	1.96	2.028	0.491
10X+1S	1.57	1.71	1.74	1.65	2.03	1.52	1.703	0.164
5X+1S	2.06	2.09	1.94	1.81	1.72	1.79	1.902	0.139
2X+1S	1.89	1.8	2.57	2.3	2.24	2.28	2.18	0.261
1X+1S	1.98	1.97	2.59	2.2	2.52	2.19	2.242	0.24
10X	0.72	0.82	1.94	1.76	1.42	1.56	1.37	0.455
1X	1.43	1.23	2.08	2.02	1.93	1.74	1.738	0.313
1S	1.8	1.8	2.22	2.44	2.32	2.35	2.155	0.259
BHI	1.32	1.27	1.8	1.89	1.65	1.85	1.63	0.249

S. salivarius	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	2.27	2.13	2.46	2.21	2.45	2.5	2.337	0.14
5E+1S	2.23	2.34	2.42	2.34	3.18	2.8	2.552	0.333
2E+1S	2.39	2.28	2.93	2.83	3.02	2.56	2.668	0.276
1E+1S	2.6	2.56	3.25	2.92	2.61	3.25	2.865	0.297
10E	1.14	1.04	1.13	0.96	1.34	1.31	1.153	0.136
1E	1.18	1	1.27	1.37	1.17	2.31	1.383	0.429
10X+1S	2.43	2.58	2.48	2.56	2.31	2.56	2.487	0.095
5X+1S	2.16	2.46	2.27	2.45	2.49	2.33	2.36	0.118
2X+1S	2.76	2.36	2.84	2.91	3.25	2.93	2.842	0.264
1X+1S	2.69	2.69	3.2	2.92	2.68	2.88	2.843	0.186
10X	1.21	1.38	2.36	1.24	1.03	1	1.37	0.461
1X	1.84	1.47	1.56	1.29	0.69	0.87	1.287	0.397
1S	2.09	2.79	3.53	3.21	3.41	3.51	3.09	0.513
BHI	0.654	0.512	1.27	0.87	0.715	0.954	0.829	0.244

Table 8 Raw data of the biofilm mass of mixed-species biofilm at 36 hours of *S. mutans + S. sanguinis, S. mutans + S. gordonii, S. sanguinis + S. gordonii,* and mix of the three species. The experiments were performed independently three times, each with a duplicate. The results were presented with mean and standard deviation (SD)

S. mutans* + *S. sanguinis	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	1.59	1.3	1.12	1.05	1.4	1.49	1.325	0.192
5E+1S	1.64	1.46	1.78	1.71	1.56	1.59	1.623	0.103
2E+1S	2.063	2.066	1.83	1.66	1.7	1.76	1.847	0.163
1E+1S	2.01	1.78	1.68	2.21	1.73	1.784	1.866	0.185
10E	0.364	0.396	0.375	0.34	0.293	0.251	0.337	0.05
1E	0.412	0.215	0.391	0.365	0.39	0.379	0.359	0.066
10X+1S	1.12	1.29	1.09	1.108	1.48	1.52	1.268	0.177
5X+1S	1.34	1.41	1.57	1.49	1.72	1.81	1.557	0.165
2X+1S	1.91	2.054	1.9	1.93	1.73	1.86	1.897	0.096
1X+1S	1.86	2.04	1.78	2.01	1.945	1.96	1.933	0.088
10X	0.31	0.265	0.361	0.389	0.208	0.282	0.302	0.06
1X	0.266	0.286	0.371	0.414	0.29	0.34	0.328	0.052
1S	2.07	2.1	2.08	1.97	1.86	2.067	2.025	0.084
BHI	0.328	0.378	0.278	0.233	0.4	0.357	0.329	0.058

S. mutans + *S. gordonii*	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	1.87	2.4	1.5	1.52	1.98	1.82	1.848	0.303
5E+1S	1.97	2.28	2.22	1.97	2.35	2.18	2.162	0.145
2E+1S	3.42	3.5	3.35	3.37	2.88	2.97	3.248	0.235
1E+1S	3.45	3.72	3.21	3.51	2.61	2.41	3.152	0.481
10E	0.854	0.96	0.99	0.809	0.875	0.778	0.878	0.076
1E	0.745	0.731	0.833	0.754	0.997	0.902	0.827	0.096
10X+1S	1.8	1.84	1.54	1.41	1.98	1.93	1.75	0.206
5X+1S	1.95	2.71	1.98	2.66	1.986	2.34	2.271	0.321
2X+1S	3.7	3.53	2.95	3.26	2.49	2.37	3.05	0.497
1X+1S	3.67	4.12	3.84	3.87	2.83	2.69	3.503	0.543
10X	0.816	0.767	0.86	0.815	0.848	0.765	0.812	0.036
1X	0.804	0.957	0.948	0.926	0.994	0.96	0.932	0.06
1S	3.29	3.19	3.21	3.19	2.96	2.89	3.122	0.145
BHI	0.862	0.856	0.767	0.73	0.852	0.848	0.819	0.051

S. sanguinis + *S. gordonii*	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	3.77	3.67	2.92	2.64	1.56	1.59	2.692	0.882
5E+1S	3.15	3.24	2.44	2.46	2.31	2.18	2.63	0.411
2E+1S	3.36	3.5	2.62	2.45	2.46	2.18	2.762	0.492
1E+1S	3.28	3.58	2.85	3.13	2.36	2.18	2.897	0.495
10E	1.86	2.12	1.592	1.85	1.275	1.374	1.679	0.294
1E	1.825	1.906	1.579	1.543	1.52	1.425	1.633	0.172
10X+1S	3.67	3.81	3	3.18	1.98	2	2.94	0.725
5X+1S	3.16	3.48	2.51	2.3	2.09	2.17	2.618	0.521
2X+1S	3.26	3.66	2.73	2.73	2.2	2.63	2.868	0.469
1X+1S	3.38	3.7	4.45	2.61	2.18	2.51	3.138	0.784
10X	1.611	1.648	1.505	1.594	0.98	0.998	1.389	0.286
1X	1.53	1.661	1.418	1.388	1.07	1.025	1.349	0.231
1S	3.86	4.27	2.7	3.4	2.4	2.4	3.172	0.724
BHI	1.476	1.56	1.316	1.417	1.185	1.604	1.426	0.143

Mixed of 3 species	1.1	1.2	2.1	2.2	3.1	3.2	Average	SD
10E+1S	1.48	1.43	0.8	0.92	1.05	1.04	1.12	0.251
5E+1S	2.02	1.95	1.64	1.32	1.57	1.62	1.687	0.236
2E+1S	2.63	2.29	2.39	2.26	2.3	2.62	2.415	0.154
1E+1S	2.47	2.7	2.46	2.39	2.5	2.87	2.565	0.166
10E	0.333	0.316	0.213	0.357	0.429	0.45	0.35	0.078
1E	1.14	0.967	1.513	1.75	0.628	0.789	1.131	0.393
10X+1S	0.95	1.06	1.2	0.82	1.55	1.25	1.138	0.234
5X+1S	1.52	2.04	1.43	1.17	2.26	2	1.737	0.387
2X+1S	2.3	2.38	2.19	2.39	2.59	2.71	2.427	0.174
1X+1S	2.76	3.1	2.29	2.59	2.61	2.76	2.685	0.243
10X	0.441	0.321	0.34	0.388	0.372	0.405	0.378	0.04
1X	0.788	0.776	1.27	1.21	0.847	0.853	0.957	0.203
1S	3.27	3.19	2.38	2.6	2.42	2.64	2.75	0.352
BHI	0.932	1.076	0.767	0.778	0.669	0.782	0.834	0.133

Table 9 Raw data of the viability cells of single-species biofilm at 36 hours of *S. mutans, S. sanguinis*, and *S. gordonii*. The experiments were performed independently six times, each with a duplicate.

CFU/ml												
S. mutans	**1.1**	**1.2**	**2.1**	**2.2**	**3.1**	**3.2**	**4.1**	**4.2**	**5.1**	**5.2**	**6.1**	**6.2**
10E+1S	8.60E+04	9.10E+04	1.40E+06	1.40E+06	1.70E+06	2.40E+06	2.60E+02	1.30E+02	9.10E+03	5.20E+03	4.60E+05	4.00E+05
10E	1.10E+05	1.20E+05	1.60E+05	2.30E+05	1.50E+06	2.00E+06	5.60E+05	5.80E+05	contaminate	9.20E+05	3.40E+05	3.70E+05
10X+1S	2.80E+05	3.50E+05	9.60E+05	8.80E+05	2.90E+05	3.00E+05	2.80E+03	1.50E+03	2.10E+04	contaminate	4.70E+05	7.00E+05
10X	9.50E+04	9.00E+04	5.70E+04	6.80E+04	2.30E+05	2.50E+05	2.60E+05	1.80E+05	3.60E+05	3.40E+05	3.80E+05	3.40E+05
1S	4.00E+02	2.70E+02	1.80E+04	1.40E+04	1.10E+05	1.00E+05	2.00E+03	2.10E+03	2.20E+02	2.60E+02	5.10E+04	4.30E+04
BHI	1.50E+04	5.30E+04	2.00E+05	2.10E+05	4.90E+05	4.80E+05	9.20E+05	1.10E+06	2.90E+05	1.60E+05	1.00E+06	9.40E+05
S. sanguinis	**1.1**	**1.2**	**2.1**	**2.2**	**3.1**	**3.2**	**4.1**	**4.2**	**5.1**	**5.2**	**6.1**	**6.2**
10E+1S	4.20E+06	4.40E+06	6.40E+05	6.80E+05	8.90E+05	9.10E+05	1.30E+03	1.30E+03	1.30E+06	1.30E+06	1.50E+04	1.20E+04
10E	1.70E+05	1.50E+05	4.00E+05	2.30E+05	7.00E+04	9.00E+04	7.90E+04	9.70E+04	1.30E+05	1.20E+05	3.00E+04	3.10E+04
10X+1S	5.00E+06	5.10E+06	3.80E+05	4.60E+05	2.20E+05	1.80E+05	2.00E+04	2.40E+04	1.10E+06	9.80E+05	5.80E+05	6.20E+05
10X	4.70E+04	3.40E+04	1.20E+05	1.70E+05	1.90E+04	2.40E+04	6.30E+04	7.20E+04	8.00E+04	7.10E+04	1.20E+04	1.50E+04
1S	7.00E+02	<10	6.30E+02	5.80E+02	2.10E+03	2.20E+03	2.00E+01	2.00E+01	4.90E+03	9.90E+03	6.80E+02	7.00E+02
BHI	4.40E+04	4.60E+04	9.80E+05	1.00E+06	1.00E+06	8.90E+05	1.60E+05	1.40E+05	4.40E+05	3.50E+05	1.00E+04	9.30E+03
S. gordonii	**1.1**	**1.2**	**2.1**	**2.2**	**3.1**	**3.2**	**4.1**	**4.2**	**5.1**	**5.2**	**6.1**	**6.2**
10E+1S	3.20E+02	2.30E+02	6.60E+02	7.00E+02	3.30E+03	3.50E+03	4.40E+02	5.20E+02	7.50E+02	7.00E+02	1.60E+03	1.40E+03
10E	5.20E+05	4.20E+05	4.70E+06	4.20E+06	1.00E+07	1.00E+07	8.60E+05	8.80E+05	3.00E+06	2.40E+06	3.60E+07	4.40E+07
10X+1S	9.90E+02	9.20E+02	1.10E+04	8.50E+03	8.50E+03	1.80E+04	1.60E+03	9.10E+02	3.90E+03	5.00E+03	7.10E+03	5.10E+03
10X	1.00E+06	1.10E+06	2.30E+05	3.30E+05	1.10E+06	1.00E+06	2.20E+05	contaminate	1.10E+06	1.20E+06	1.60E+07	contaminate
1S	6.80E+04	8.10E+04	1.20E+03	8.00E+02	2.00E+01	<10	2.30E+02	2.30E+02	5.00E+01	3.00E+01	7.40E+02	6.50E+02
BHI	2.50E+06	2.80E+06	4.00E+06	5.00E+06	3.10E+07	3.00E+07	3.90E+06	4.70E+06	1.20E+07	contaminate	2.30E+07	2.40E+07

Table 10 Raw data of the viability cells of mixed-species biofilm at 36 hours of *S. mutans + S. sanguinis, S. mutans + S. gordonii, S. sanguinis + S. gordonii,* and mix of the three species The experiments were performed independently three times, each with a duplicate. SM; *S. mutans*, SS; *S. sanguinis*, SG; *S. gordonii*

CFU/ml								
SM+SS		**1.1**	**1.2**	**2.1**	**2.2**	**3.1**	**3.2**	
10E+1S	**SM**	8.90E+04	8.90E+04	1.90E+04	1.40E+04	1.40E+05	1.00E+05	
	SS	1.00E+02	1.00E+02	1.00E+02	1.00E+02	1.00E+03	1.00E+03	$\leq 10^2$
10E	**SM**	9.30E+04	1.00E+05	4.80E+04	7.20E+04	7.80E+04	1.50E+05	
	SS	1.00E+03	1.00E+03	1.00E+03	1.00E+03	1.00E+03	1.00E+03	$<10^3$
10X+1S	**SM**	1.70E+04	3.40E+04	9.20E+03	7.00E+03	4.50E+05	3.30E+05	
	SS	1.00E+03	1.00E+03	1.00E+02	1.00E+02	1.00E+03	1.00E+03	$\leq 10^2$
10X	**SM**	1.80E+04	1.20E+04	5.30E+04	5.10E+04	3.90E+04	5.20E+04	
	SS	1.00E+02	1.00E+02	1.00E+03	1.00E+03	1.00E+02	1.00E+02	$\leq 10^2$
1S	**SM**	6.00E+02	6.00E+02	1.00E+04	7.20E+03	5.00E+05	8.80E+05	
	SS	1.00E+02	1.00E+02	1.00E+02	1.00E+02	1.00E+03	1.00E+03	$\leq 10^2$
BHI	**SM**	2.90E+05	2.80E+05	1.40E+05	1.20E+05	1.90E+05	2.30E+05	
	SS	1.00E+03	1.00E+03	1.00E+03	1.00E+03	1.00E+03	1.00E+03	$<10^3$

SM+SG		**1.1**	**1.2**	**2.1**	**2.2**	**3.1**	**3.2**	
10E+1S	**SM**	1.10E+06	7.10E+05	1.00E+04	1.10E+04	1.60E+06	1.10E+06	
	SG	1.00E+04	1.00E+04	1.00E+03	1.00E+03	1.00E+04	1.00E+04	$\leq 10^3$
10E	**SM**	5.90E+04	1.60E+04	6.40E+04	6.00E+04	4.60E+04	4.70E+04	
	SG	8.00E+03	3.00E+03	5.00E+04	4.70E+04	7.60E+04	9.10E+04	
10X+1S	**SM**	5.10E+04	3.10E+04	1.20E+04	1.10E+04	2.10E+06	2.80E+06	
	SG	1.00E+03	1.00E+03	9.00E+02	9.00E+02	1.00E+04	1.00E+04	$\leq 10^3$
10X	**SM**	8.00E+01	9.00E+01	2.00E+04	1.80E+04	3.20E+03	3.00E+03	
	SG	3.00E+01	2.00E+01	1.70E+04	1.70E+04	1.00E+04	1.30E+04	
1S	**SM**	3.00E+03	2.00E+03	1.60E+04	1.20E+04	2.40E+06	2.70E+06	
	SG	1.00E+03	1.00E+03	1.00E+02	1.00E+02	1.00E+03	1.00E+03	$\leq 10^2$
BHI	**SM**	4.50E+04	3.10E+04	3.00E+04	3.70E+04	1.70E+05	3.20E+05	
	SG	1.00E+03	1.00E+03	2.60E+04	6.00E+04	1.00E+03	1.00E+03	$\leq 10^4$

SS+SG		1.1	1.2	2.1	2.2	3.1	3.2	
10E+1S	SS	1.00E+01	1.00E+01	1.00E+02	1.00E+02	5.00E+01	2.00E+01	$\leq 10^1$
	SG	7.50E+02	8.80E+02	3.20E+03	4.20E+03	6.90E+02	7.70E+02	
10E	SS	1.00E+03	1.00E+03	1.00E+04	1.00E+04	1.00E+04	1.00E+04	$\leq 10^3$
	SG	1.80E+05	1.60E+05	8.30E+05	7.80E+05	1.50E+06	1.90E+06	
10X+1S	SS	1.00E+02	1.00E+02	2.00E+02	1.00E+02	1.00E+02	1.00E+02	$<10^2$
	SG	3.50E+03	2.30E+03	1.70E+04	1.40E+04	7.10E+03	7.30E+03	
10X	SS	1.00E+03	2.00E+03	1.00E+03	1.00E+03	1.00E+04	1.00E+04	$\leq 10^3$
	SG	4.00E+04	4.50E+04	2.00E+05	1.80E+05	1.10E+06	1.40E+06	
1S	SS	1.00E+01	1.00E+01	1.00E+01	1.00E+01	1.00E+01	1.00E+01	$<10^1$
	SG	1.00E+01	1.00E+01	7.00E+02	1.10E+03	1.30E+03	1.20E+03	
BHI	SS	1.00E+04	1.00E+04	1.00E+04	1.00E+04	1.00E+05	1.00E+05	$\leq 10^4$
	SG	5.60E+05	8.90E+05	4.00E+05	5.60E+05	7.50E+06	9.00E+06	

Mixed of 3		1.1	1.2	2.1	2.2	3.1	3.2	
10E+1S	SM	5.60E+06	8.50E+06	6.90E+06	3.80E+06		7.80E+06	
	SS	1.00E+04	1.00E+04	1.00E+05	1.00E+05	เพสลแตก	1.00E+05	$\leq 10^4$
	SG	1.00E+04	1.00E+04	1.00E+05	1.00E+05		1.00E+05	$\leq 10^4$
10E	SM	7.20E+04	4.90E+04	8.80E+04	8.10E+04		2.60E+05	
	SS	1.00E+03	1.00E+03	1.00E+03	1.00E+03	เพสลแตก	1.00E+03	$<10^3$
	SG	5.00E+03	9.00E+03	8.20E+04	4.00E+04		5.00E+04	
10X+1S	SM	2.90E+06	9.70E+05	7.30E+05	5.30E+05	1.80E+06	5.70E+05	
	SS	1.00E+04	1.00E+04	1.00E+04	1.00E+04	1.00E+04	1.00E+04	$<10^4$
	SG	1.00E+04	1.00E+04	1.00E+04	1.00E+04	1.00E+04	1.00E+04	$<10^4$
10X	SM	7.00E+02	1.00E+03	2.70E+03	3.50E+03	1.00E+04	1.00E+04	
	SS	1.00E+02	1.00E+02	1.00E+02	1.00E+02	2.00E+02	2.00E+02	$<10^2$
	SG	4.00E+02	3.00E+02	3.40E+03	9.30E+03	1.30E+04	1.90E+04	
1S	SM	1.80E+06	5.00E+06	2.20E+05	1.70E+05	4.30E+06	4.70E+06	
	SS	1.00E+05	1.00E+05	1.00E+04	1.00E+04	1.00E+05	1.00E+05	$\leq 10^4$
	SG	1.00E+05	1.00E+05	1.00E+04	1.00E+04	1.00E+05	1.00E+05	$\leq 10^4$
BHI	SM	3.10E+05	3.40E+05	8.10E+04	1.00E+05	4.10E+05	4.30E+05	
	SS	1.00E+04	1.00E+04	1.00E+03	1.00E+03	1.00E+04	1.00E+04	$\leq 10^3$
	SG	1.00E+04	1.00E+04	7.40E+04	5.40E+04	1.00E+04	1.00E+04	$\leq 10^4$

Table 11 Raw data of the copy number of single-species biofilm at 36 hours of *S. mutans, S. sanguinis*, and *S. gordonii*. The experiments were performed independently three times, each with a duplicate. SM; *S. mutans*, SS; *S. sanguinis*, SG; *S. gordonii*, CT; cycle threshold, Tm; melting temperature, and N-C; negative control.

SM	1				2				3			
		CT	Tm	Tm		CT	Tm	Tm		CT	Tm	Tm
10E1S	1	7.675	86.179		1	7.526	85.892		1	9.976	86.187	
	2	7.617	86.179		2	7.554	85.892		2	10.054	86.187	
10E	1	10.005	85.886		1	16.819	85.745		1	10.655	86.041	
	2	9.612	86.179		2	16.967	85.745		2	11.188	86.041	
10X1S	1	7.681	86.032		1	8.382	85.892		1	9.132	86.041	
	2	7.645	86.032		2	8.619	85.745		2	9.32	85.894	
10X	1	11.776	84.716		1	17.222	85.745		1	11.235	85.894	
	2	11.926	85.886		2	16.89	85.745		2	11.263	85.894	
1S	1	7.325	85.886		1	8.113	85.892		1	6.567	86.041	
	2	7.259	85.886		2	8.607	85.599		2	6.67	85.894	
BHI	1	9.418	85.886		1	11.531	85.745		1	12.293	85.894	
	2	9.65	85.886		2	11.658	85.745		2	12.579	85.894	
N-C	1	29.377	85.74	75.941	1	31.322	85.307	74.921	1	30.397	76.532	83.261
	2	26.685	85.594	75.795	2	31.726	76.237		2	28.984	85.602	76.532

SS	1											
		CT	Tm	Tm		CT	Tm	Tm		CT	Tm	Tm
10E1S	1	10.757	83.836		1	8.397	83.987		1	8.222	83.992	
	2	10.856	85.005		2	8.114	83.841		2	7.738	83.992	
10E	1	16.812	83.689		1	18.374	83.695		1	20.595	83.699	
	2	17.008	85.005		2	18.697	83.695		2	20.543	83.553	
10X1S	1	10.54	83.836		1	8.531	83.841		1	8.226	84.87	
	2	10.281	83.689		2	8.524	83.695		2	8.068	85.016	
10X	1	19.884	83.543		1	21.451	83.695		1	18.076	83.699	
	2	19.821	83.543		2	21.447	83.695		2	17.932	83.699	
1S	1	14.867	83.689		1	9.585	85.011		1	8.763	84.431	
	2	14.808	83.689		2	9.564	83.695		2	8.648	84.431	
BHI	1	16.569	83.689		1	15.314	83.841		1	15.737	83.846	
	2	16.553	83.689		2	15.636	83.841		2	15.492	83.699	
N-C	1	33.774	83.982	76.524	1	27.82	83.987		1	31.16	85.016	
	2	Und.	90.708	82.958	2	Und	91.739	84.426	2	33.029	85.016	

SG	1											
		CT	Tm	Tm		CT	Tm	Tm		CT	Tm	Tm
10E1S	1	8.423	83.249		1	6.651	82.943		1	8.008	83.007	
	2	8.712	83.249		2	6.737	82.943		2	7.847	83.154	
10E	1	9.344	82.956		1	10.166	82.651		1	9.014	82.859	
	2	9.575	82.956		2	10.348	82.651		2	8.971	83.007	
10X1S	1	8.966	82.956		1	8.04	82.797		1	8.508	83.007	
	2	8.997	82.81		2	8.347	82.651		2	8.789	83.007	
10X	1	9.885	82.956		1	9.995	82.651		1	9.973	82.859	
	2	9.971	82.81		2	10.008	82.651		2	9.609	82.859	
1S	1	7.41	83.103		1	7.691	82.797		1	6.859	83.007	
	2	7.499	82.956		2	7.74	82.797		2	6.795	83.007	
BHI	1	8.714	82.956		1	8.615	82.797		1	6.46	83.007	
	2	8.94	82.81		2	8.755	82.797		2	6.468	83.007	
N-C	1	Und	81.933	92.168	1	Und	60.001		1	33.333	82.417	
	2	Und	92.461	88.659	2	Und	87.035	83.382	2	35.21	72.536	

Table 12 Raw data of the copy number of cells of mixed-species biofilm at 36 hour of *S. mutans* + *S. sanguinis*, *S. mutans* + *S. gordonii*, *S. sanguinis* + *S. gordonii*, and mix of the three species The experiments were performed independently three times, each with a duplicate. SM; S. mutans, SS; *S. sanguinis*, SG; *S. gordonii*, CT; cycle threshold, and Tm; melting temperature.

SM_SM+SS	1				2				3			
		CT	Tm	Tm		CT	Tm	Tm		CT	Tm	Tm
10E1S	1	7.888	85.865		1	7.554	85.579		1	9.982	85.727	
	2	8.086	85.865		2	7.38	85.579		2	9.698	85.727	
10E	1	12.96	85.865		1	17.456	85.287		1	14.824	85.727	
	2	12.727	86.012		2	18.772	85.433		2	14.821	85.727	
10X1S	1	8.112	85.573		1	8.744	85.579		1	9.835	85.581	
	2	8.093	85.719		2	8.776	85.579		2	10.18	85.581	
10X	1	16.998	85.135		1	20.319	85.433		1	19.174	85.435	
	2	17.172	85.427		2	19.804	85.287		2	18.408	85.435	
1S	1	7.374	85.719		1	10.061	85.433		1	7.514	85.581	
	2	7.886	85.573		2	9.744	85.579		2	7.482	85.581	
BHI	1	11.529	85.573		1	15.095	85.14		1	13.923	85.581	
	2	11.593	85.573		2	15.299	85.433		2	13.622	85.581	
SS_SM+SS	1				2				3			
		CT	Tm	Tm		CT	Tm	Tm		CT	Tm	Tm
10E1S	1	11.928	84.973		1	10.535	83.815		1	12.642	83.82	
	2	12.32	83.659		2	10.502	83.815		2	12.904	83.674	
10E	1	17.879	83.659		1	21.675	83.523		1	19.605	83.674	
	2	18.07	83.659		2	21.612	83.523		2	19.106	83.674	
10X1S	1	11.483	83.513		1	9.198	83.669		1	12.902	83.381	
	2	11.906	83.367		2	9.333	83.523		2	12.278	83.527	
10X	1	20.863	83.367		1	24.034	83.523		1	22.849	83.381	
	2	20.987	83.367		2	24.214	83.523		2	22.9	83.527	
1S	1	11.622	83.513		1	10.605	83.523		1	10.524	83.527	
	2	11.602	83.513		2	10.527	83.669		2	10.102	84.843	
BHI	1	16.875	83.513		1	20.889	83.523		1	17.955	83.527	
	2	17.729	83.367		2	21.37	83.669		2	18.109	83.527	

SM_SM+SG	1				2				3			
		CT	Tm	Tm		CT	Tm	Tm		CT	Tm	Tm
10E1S	1	8.05	85.86		1	8.46	85.725		1	11.143	86.016	
	2	7.951	86.152		2	8.521	85.871		2	11.208	86.016	
10E	1	12.93	85.714		1	13.517	85.579		1	11.064	85.87	
	2	13.056	86.006		2	13.514	85.725		2	11.066	86.016	
10X1S	1	8.287	85.714		1	10.723	85.725		1	11.532	85.724	
	2	8.214	85.86		2	10.757	85.725		2	11.517	85.724	
10X	1	15.508	85.714		1	15.896	85.433		1	15.394	85.724	
	2	15.592	85.568		2	15.31	85.579		2	16.375	85.724	
1S	1	8.35	85.568		1	9.444	85.433		1	7.721	85.724	
	2	8.861	85.568		2	9.834	85.579		2	7.71	85.724	
BHI	1	13.615	84.107		1	11.196	85.579		1	11.524	85.724	
	2	13.614	85.568		2	11.23	85.725		2	11.494	85.724	
SG_SM+SG	1				2				3			
		CT	Tm	Tm		CT	Tm	Tm		CT	Tm	Tm
10E1S	1	7.94	82.932		1	6.391	82.904		1	11.309	82.831	
	2	7.486	83.078		2	6.493	82.904		2	11.307	82.831	
10E	1	14.766	82.493		1	12.505	82.321		1	14.183	82.537	
	2	14.843	82.493		2	13.333	82.466		2	15.198	82.537	
10X1S	1	8.291	82.639		1	8.5	82.612		1	11.333	82.537	
	2	7.906	82.786		2	8.653	82.612		2	11.206	82.537	
10X	1	16.356	82.347		1	14.776	82.175		1	17.118	82.389	
	2	16.38	82.201		2	14.401	82.321		2	17.169	82.389	
1S	1	8.068	82.786		1	10.138	82.466		1	7.524	82.831	
	2	7.887	82.932		2	9.912	82.466		2	7.607	82.831	
BHI	1	13.791	82.347		1	9.966	82.466		1	12.553	82.537	
	2	13.558	82.493		2	10.022	82.466		2	12.05	82.537	

SS_SS+SG	1				2				3			
		CT	Tm	Tm		CT	Tm	Tm		CT	Tm	Tm
10E1S	1	9.868	84.266		1	9.575	83.962		1	8.918	84.477	
	2	9.857	84.704		2	9.328	84.254		2	8.496	84.624	
10E	1	11.084	83.535		1	12.842	83.67		1	12.101	83.887	
	2	10.831	83.827		2	13.012	83.67		2	12.586	83.74	
10X1S	1	9.627	84.412		1	8.491	84.108		1	8.952	84.624	
	2	9.736	84.412		2	9.184	83.962		2	8.727	84.624	
10X	1	12.588	83.681		1	12.258	83.816		1	13.299	83.887	
	2	12.876	83.681		2	12.247	83.816		2	13.291	83.887	
1S	1	8.828	84.119		1	8.296	84.108		1	8.614	84.624	
	2	8.804	84.412		2	8.983	84.108		2	8.548	84.33	
BHI	1	9.922	83.973		1	11.968	83.962		1	10.402	84.182	
	2	9.992	83.827		2	11.807	83.816		2	10.136	84.182	
SG_SS+SG	1				2				3			
		CT	Tm	Tm		CT	Tm	Tm		CT	Tm	Tm
10E1S	1	9.283	83.073		1	8.032	82.904		1	9.527	82.973	
	2	9.163	83.073		2	8.097	82.904		2	8.438	82.973	
10E	1	9.539	82.927		1	11.818	82.758		1	11.223	82.678	
	2	9.492	82.927		2	12.045	82.758		2	11.249	82.826	
10X1S	1	9.25	82.927		1	8.26	82.758		1	8.856	82.973	
	2	9.225	82.781		2	8.245	82.904		2	8.802	82.973	
10X	1	11.525	82.781		1	11.65	82.613		1	12.976	82.678	
	2	11.567	82.635		2	11.658	82.758		2	12.674	82.826	
1S	1	8.025	82.781		1	8.707	82.758		1	7.785	82.973	
	2	7.889	82.781		2	8.634	82.758		2	7.761	82.973	
BHI	1	9.033	82.781		1	11.521	82.613		1	9.206	82.973	
	2	8.955	82.781		2	11.495	82.613		2	9.441	82.973	

SM_SM+SS+SG	1				2				3			
		CT	Tm	Tm		CT	Tm	Tm		CT	Tm	Tm
10E1S	1	8.15	83.793		1	9.687	85.557		1	10.942	85.85	
	2	7.959	85.837		2	9.733	85.557		2	10.95	85.85	
10E	1	13.261	85.691		1	16.242	85.411		1	12.483	85.704	
	2	14.235	85.691		2	17.51	85.557		2	12.56	85.704	
10X1S	1	8.106	85.691		1	10.269	85.557		1	10.958	85.558	
	2	8.152	85.691		2	10.452	85.411		2	10.965	85.704	
10X	1	14.974	85.545		1	17.462	85.411		1	16.159	85.558	
	2	15.046	85.545		2	17.496	85.265		2	15.93	85.558	
1S	1	8.228	84.377		1	9.158	85.411		1	7.916	85.558	
	2	8.208	85.399		2	9.166	85.411		2	8.067	85.412	
BHI	1	12.487	82.917		1	12.683	85.411		1	10.672	85.558	
	2	12.279	85.399		2	12.764	85.557		2	10.924	85.558	
SS_SM+SS+SG	1				2				3			
		CT	Tm	Tm		CT	Tm	Tm		CT	Tm	Tm
10E1S	1	9.874	84.233		1	7.908	84.195		1	11.936	83.701	
	2	10.542	81.605		2	8.172	84.195		2	11.947	83.701	
10E	1	16.537	83.649		1	16.113	83.612		1	16.321	83.554	
	2	16.574	83.649		2	16.349	83.612		2	16.563	83.554	
10X1S	1	9.128	84.087		1	8.111	83.903		1	11.525	83.554	
	2	9.354	84.525		2	8.375	84.049		2	11.672	83.701	
10X	1	16.366	83.649		1	17.03	83.466		1	17.731	83.554	
	2	16.356	83.503		2	17.235	83.466		2	17.873	83.554	
1S	1	9.14	84.379		1	9.532	83.903		1	8.912	84.437	
	2	9.154	84.087		2	9.629	83.757		2	8.76	84.731	
BHI	1	13.223	83.503		1	11.827	83.757		1	12.071	83.701	
	2	13.418	83.503		2	12.012	83.757		2	12.269	83.554	
N-C	1	32.165	83.649		1	29.544	83.757		1	33.106	75.31	
	2	29.668	83.649		2	33.243	75.012		2	31.865	77.077	
SG_SM+SS+SG	1				2				3			
		CT	Tm	Tm		CT	Tm	Tm		CT	Tm	Tm
10E1S	1	8.119	83.051		1	7.128	82.874		1	10.93	82.801	
	2	8.448	83.197		2	7.203	82.874		2	10.764	82.801	
10E	1	15.356	82.468		1	14.56	82.437		1	15.41	82.507	
	2	15.45	82.614		2	14.568	82.437		2	15.639	82.36	
10X1S	1	8.183	82.76		1	7.615	82.728		1	11.156	82.654	
	2	8.098	82.905		2	7.626	82.728		2	10.876	82.654	
10X	1	15.203	82.322		1	15.686	82.291		1	16.618	82.36	
	2	15.182	82.176		2	15.68	82.291		2	16.898	82.36	
1S	1	7.606	82.76		1	10.086	82.582		1	8.073	82.654	
	2	8.316	82.76		2	9.297	82.437		2	8.075	82.801	
BHI	1	12.278	82.468		1	11.872	82.437		1	11.357	82.507	
	2	12.39	82.468		2	11.774	82.437		2	11.096	82.36	

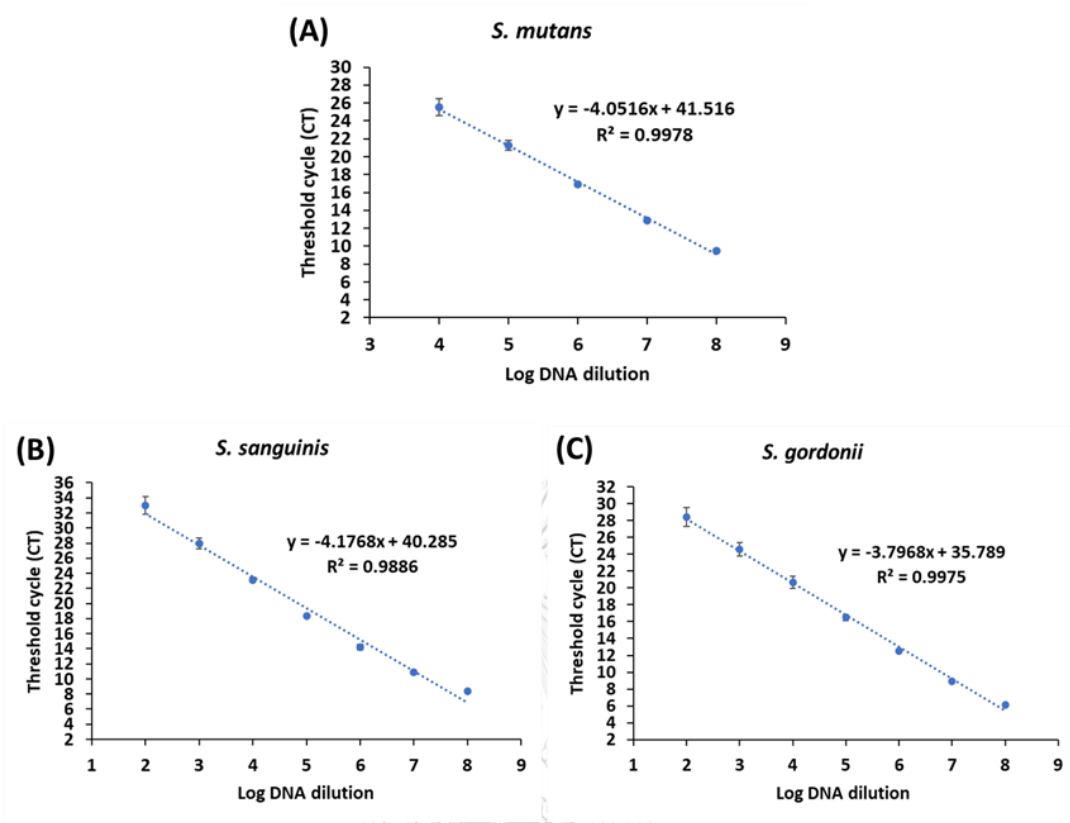


Figure 17 Serial dilution standard curve from 16S rRNA gene of *S. mutans* (A), *S. sanguinis* (B) and *S. gordonii* (C).

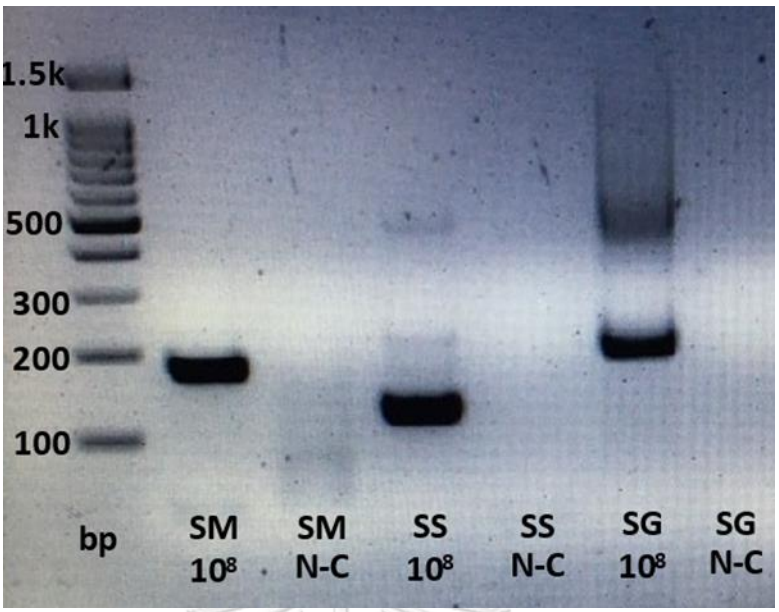


Figure 18 Gel electrophoresis from qPCR product of *S. mutans* (SM), *S. sanguinis* (SS) and *S. gordonii* (SG) and the negative control (N-C) of each species. The product size of primer; *S. mutans* is 175 bp, *S. sanguinis* is 126 bp, and *S gordonii* is 196 bp.

APPENDIX B

MATERIALS AND EQUIPMENT

1.	0.2 µm sterilize membrane filter	(Biotech, Germany)
2.	8-well cell culture slide	(SPL life sciences, Korea)
3.	24-well flat-bottom plate	(Thermo Fisher Scientific, USA)
4.	60 x 15 mm cell culture dish	(SPL life sciences, Korea)
5.	Autoclave	(Hirayama, Japan)
6.	Confocal laser scanning microscopy	(ZEISS, Germany)
7.	Coverslip	(Thermo Fisher Scientific, USA)
8.	Critical point drier	(Lecia, Austria)
9.	Incubator	(Thermo Fisher Scientific, USA)
10.	Micro Centrifuge	(Tomy Digital Biology, Japan)
11.	Microplate reader	(BioTek, USA)
12.	pH meter	(Horiba, Japan)
13.	Real-Time PCR System (QuantStudio 5)	(Thermo Fisher Scientific, USA)
14.	Scanning electron microscopy	(JEOL, Japan)
15.	Shaker	(IKA, USA)
16.	Spectrophotometer	(Thermo Fisher Scientific, USA)
17.	Sputter Coater	(Balzers, Germany)
18.	Ultrasonic homogenizer	(Thermo Fisher Scientific, USA)
19.	Vortex	(Scientific Industries, USA)

APPENDIX C

CHEMICAL AND REAGENTS

1.	16S rRNA forward and reverse primers	(Macrogen, Korean)
2.	Acetic acid	(Merck, Germany)
3.	Agarose gel	(Vivantis, Malaysia)
4.	Brain-Heart Infusion (BHI) agar	(HiMedia Laboratories, India)
5.	Brain-Heart Infusion (BHI) broth	(HiMedia Laboratories, India)
6.	Calcofluor-white	(Sigma-Aldrich, USA)
7.	Crystal violet	(PanReac AppliChem, USA)
8.	Disodium hydrogen phosphate	(Univar Solutions, USA)
9.	DNeasy PowerSoil Pro Kit	(Qiagen, USA)
10.	Erythritol	(Krungthepchemi, Thailand)
11.	Ethanol	(Merck, Germany)
12.	Glutaraldehyde	(Merck, Germany)
13.	Hydrochloric acid	(Univar Solutions, USA)
14.	Monosodium phosphate	(Sigma-Aldrich, USA)
15.	Mitis-Salivarius (MS) agar	(BD, USA)
16.	Potassium chloride	(Univar Solutions, USA)
17.	Potassium dihydrogen phosphate	(Univar Solutions, USA)
18.	Potassium tellurite	(Sigma-Aldrich, USA)
19.	LIVE/DEAD BacLight Bacterial Viability Kits	(Invitrogen, USA)

20. Sodium azide	(Sigma-Aldrich, USA)
21. Sodium chloride	(Univar Solutions, USA)
22. Sodium hydroxide	(Univar Solutions, USA)
23. Sucrose	(Univar Solutions, USA)
24. SYBR Green PCR Luna Master Mix	(New England Biolabs, USA)
25. Xylitol	(Xyliplus, Thailand)

APPENDIX D

MEDIA AND REAGENTS PREPARATION

1. 0.1% (w/v) crystal violet

Crystal violet	0.1	g
Sterile water	100	ml

2. 0.1 M phosphate buffer, pH 7.2

Stock solution (0.5 M phosphate buffer)

Monosodium phosphate	6	g
Distilled Water	100	ml

Working solution (0.1 M phosphate buffer)

Stock solution (0.5 M)	20	ml
Distilled Water	80	ml

Adjust to pH 7.2 with HCl or NaOH

3. 0.3% SYTO 9 and Pi (LIVE/DEAD BacLight Bacterial Viability Kits)

SYTO 9	1.5	µl
Pi	1.5	µl
0.9% NaCl	1000	µl

4. 0.9% NaCl

NaCl	9	g
Sterile water	1000	ml

5. 2.5% glutaraldehyde

25% glutaraldehyde	10	ml
0.1 M phosphate buffer, pH 7.2	60	ml

6. 10 µg/ml Calcofluor-white

Calcofluor-white	100	µg
10 mM sodium phosphate, pH 7.5	10	ml

7. 10 mM sodium phosphate, pH 7.5

H_4NaO_5P	0.386	g
NaCl	8.766	g
Na_2HPO_4	1	g
NaN_3	0.5	g
Distilled Water	1000	ml

Adjust to pH 7.5 with HCl or NaOH

8. 30% acetic acid

100% acetic acid	30	ml
Sterile water	70	ml

9. 30% ethanol

100% ethanol	30	ml
Sterile water	70	ml

10. 50% ethanol

100% ethanol	50	ml

Sterile water	50	ml

11. 70% ethanol

100% ethanol	70	ml
Sterile water	30	ml

12. 95% ethanol

100% ethanol	95	ml
Sterile water	5	ml

13. Brain-Heart Infusion (BHI) agar

BHI agar powder	52	g
Sterile water	1000	ml

14. Brain-Heart Infusion (BHI) broth

BHI broth powder	37	g
Sterile water	1000	ml

15. Mitis-Salivarius (MS) agar

MS agar powder	90	g
Sterile water	1000	ml

After autoclave, add 1 ml of 1% tellurite in the 50-55°C

16. Phosphate Buffer Saline (PBS), pH 7.4

Stock solution (10X PBS)

NaCl	80	g
KCL	2	g

Na_2HPO_4	11.5	g
KH_2PO_4	2	g
Distilled Water	1000	ml

Working solution (1X PBS)

Stock solution (10X PBS)	100	ml
Distilled Water	900	ml

Adjust to pH 7.4 with HCl or NaOH

17. Quantitative Real-Time Polymerase Chain Reaction (qPCR) master mix

Working solution (10 µl/reaction)

For *S. mutans* and *S. sanguinis*

2× qPCR master mix	5	µl
10 µM forward or reverse primer	0.5	µl
Distilled Water	1	µl
DNA sample	3	µl

For *S. gordonii*

2× qPCR master mix	5	µl
10 µM forward or reverse primer	0.4	µl
Distilled Water	1.2	µl
DNA sample	3	µl

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