THE POTENCY OF CAJUPUTS CANDY IN MAINTAINING THE COMPETITIVE CAPACITY OF STREPTOCOCCUS SANGUINIS UPON STREPTOCOCCUS MUTANS

Christofora Hanny Wijaya13
Bernadeta R.E. Sari1
Boy M. Bachtiar2

1Department of Food Science and Technology, Bogor Agricultural University, Bogor, Indonesia
2Department of Oral Biology and Oral Science Research Center, University of Indonesia, Jakarta, Indonesia.
3Tropical Biopharmaca Research Center, Bogor Agricultural University, Bogor, Indonesia

ABSTRACT

Streptococcus mutans were competing Streptococcus sanguinis in biofilm formation. As pioneer colonizer, S. sanguinis were able to control S. mutans growth. This study was aimed to explore the ability of sucrose and non-sucrose cajuputs candies (SCC and NSCC) in maintaining the antagonistic relationship between the indigenous oral flora when they grew as dual-species biofilms (S. sanguinis and S. mutans). SCC and NSCC contained cajuput and peppermint oils as the flavor which the volatile compounds had been identified. Unflavored sucrose candy and unflavored non-sucrose candy were prepared similarly to the SCC and NSCC, respectively, except the flavor addition. SCC, NSCC, unflavored sucrose candy, unflavored non-sucrose candy, and the control were exposed in vitro to the biofilms. The biofilm was examined for biofilm inhibition capacity, DNA amount, and the expression level of spxB mRNA. The biofilm inhibition by SCC and NSCC were higher than the unflavored ones and were significantly different compared to the control. The SCC and NSCC managed to decrease the total DNA amount in the biofilm, but unflavored candies did not. The qPCR assays showed that the exposure of candies did not alter the proportion of S. sanguinis DNA to S. mutans DNA in the biofilms. Meanwhile, spxB mRNA expression indicated the ability of S. sanguinis to control S. mutans growth.

Keywords: Biofilm; cajuputs candy; Melaleuca cajuputi; spxB gene; Streptococcus mutans; Streptococcus sanguinis.

ABSTRAK

Streptococcus mutans bersaing dengan Streptococcus sanguinis dalam pembentukan biofilm. Sebagai pionir kolonisasi, S. sanguinis mampu mengendalikan pertumbuhan S. mutans. Penelitian ini bertujuan untuk mengeksporlasi kemampuan permen cajuputs sukrosa dan non-sukrosa (SCC dan NSCC) dalam menjaga hubungan antagonistik pada flora di mulut saat tumbuh sebagai dual-species (S. sanguinis dan S. mutans). Permen beraroma (SCC dan NSCC) mengandung minyak cajuput dan pepermint sebagai rasa yang telah teridentifikasi sebagai senyawa volatil. Permen tanpa rasa dibuat mirip dengan permen dengan rasa tetapi tanpa penggunaan rasa. Permen rasa, permen tanpa rasa, dan kontrol diekspos secara in vitro ke biofilm. Biofilm dianalisa untuk kapasitas penghambatan biofilm, jumlah DNA, dan tingkat ekspresi mRNA spxB. Penghambatan biofilm oleh permen dengan rasa lebih tinggi daripada yang tidak diberi rasa dan secara signifikan berbeda dibandingkan dengan kontrol. Permen rasa berhasil mengurangi jumlah DNA total dalam biofilm, tetapi sampel tanpa rasa tidak. Tes qPCR menunjukkan bahwa paparan permen tidak mengubah proporsi DNA S. sanguinis ke DNA S. mutans dalam biofilm. Sementara itu, ekspresi mRNA spxB menunjukkan kemampuan S. sanguinis untuk mengendalikan pertumbuhan S. mutans.

Kata kunci: Biofilm; Melaleuca cajuputi; permen cajuputs; spxB gene; Streptococcus mutans; Streptococcus sanguinis.
INTRODUCTION

Dental caries is one of the most common diseases in oral cavity (Somaraj et al., 2017; Eslami et al., 2016). Eco-systemic factors such as saliva, dietary habit, and microbial composition in biofilm contribute to its formation (Fejerskov, 2004; Becker et al., 2002). In addition, interaction among polymicrobial could lead to cases of dental caries (Becker et al., 2002; Kreth et al., 2005). *Streptococcus sanguinis* is a health-associated species (Percival et al., 2006; Kreth et al., 2005) and is considered as beneficial bacterium in regards to dental caries (Percival et al., 2006; Kreth et al., 2005; Magalhaes et al., 2016). On the contrary, *Streptococcus mutans* is the most commonly found species on dental caries (Oda et al., 2015) because it manages to grow in acidic environment and produces acidic compounds by fermenting carbohydrates (Percival et al., 2006; Becker et al., 2002; Kreth et al., 2005). Interestingly, *S. sanguinis* is able to antagonize *S. mutans* activity and protects the host from the negative effect of this opportunistic pathogen (Kreth et al., 2009).

*S. sanguinis* pioneers the development of oral biofilm, which have a role for the healthy dental plaque (Kreth et al., 2009). This bacterium is able to produce hydrogen peroxide (H$_2$O$_2$) that inhibits *S. mutans* growth under aerobic condition (Zheng et al., 2011a). *S. mutans* is not able to tolerate H$_2$O$_2$ (Zheng et al., 2011b). The H$_2$O$_2$ production in *S. sanguinis* was generated by pyruvate oxidase (SpxB). SpxB activity was controlled by SpxB gene (Zheng et al., 2011a). Expression of *spxB* mRNA represented the activity of *spxB* gene that correlated with the production of H$_2$O$_2$ by *S. sanguinis* (Magalhaes et al., 2016). Therefore, the physiological activity of *S. sanguinis* to inhibit *S. mutans* can be evaluated by the expression level of *spxB* mRNA (Zheng et al., 2011a).

Cajuputs candy is an Indonesian herbal-based candy that has been developed since 1997 as a functional food. Cajuputs candy was invented to increase the economic value of cajuput oil from *Melaleuca cajuputi* plant and to create a novel and distinctive Indonesian food product. Cajuputs candy has been patented (ID 0000385 S) (Wijaya et al., 2002) and developed into several variants including Sucrose Cajuputs Candy (SCC) (Wijaya et al., 2011) and Non-Sucrose Cajuputs Candy (NSCC) (Iftari et al., 2013). Both SCC and NSCC have been produced and marketed in certain area in Indonesia with good consumer acceptance.

Based on the previous studies, it is known that SCC and NSCC could suppress the growth of several pathogenic microbes in the oral system such as *S. mutans*, *S. sobrinus* (Wijaya et al., 2011) and *Candida albicans* (Wijaya et al., 2014). Iftari et al. (2013) reported that NSCC showed inhibition on biofilm formation and *gfpC* expression of *S. mutans* serotype c. Cajuputs candy activity could be caused by a synergistic effect between cajuput oil and peppermint oil, which were utilized as the main flavoring components. Cajuput oil contains α-terpineol and terpien-4-ol which had been reported having antimicrobial activity against *Streptococcus spp.* and *C. albicans* (Jedlickova et al., 1994). Peppermint oil, which contains menthol as the major component, had been also reported for its antimicrobial activity against cariogenic bacteria (Dwivedi et al., 2012; Galvao et al., 2012).

Antibiofilm potency of cajuputs candy formulas toward single type of pathogenic bacteria that related to dental caries has been proven (Wijaya et al., 2011; Iftari et al., 2013). However, its effect on polymicrobial biofilms, especially *S. sanguinis* and *S. mutans*, has not been reported. The aim of this study was to determine the ability of SCC and NSCC to maintain the competitive capacity of *S. sanguinis* toward *S. mutans*. The effect of cajuput oil and peppermint oil as flavor and the effect of sucrose and non-sucrose as the raw material were validated in vitro by biofilm assay, quantification of *spxB* mRNA expression level and total bacterial DNA using qPCR technique.

MATERIALS AND METHODS

Materials

Food grade cajuput oil distilled from *M. cajuputi* was obtained from Pulau Buru Maluku and sweeteners such as sucrose, liquid glucose, isomalt and acesulfame-K were obtained from local supplier while peppermint oil and honeysuckle flavor were from flavor houses. Analytical grade
Preparation of candy formulas

Four candy formulas in this experiment are: (1) Unflavored sucrose candy, (2) Sucrose Cajuputs Candy (SCC), (3) Unflavored non-sucrose candy, and (4) Non-Sucrose Cajuputs Candy (NSCC). SCC and NSCC were prepared based on the procedures conducted by Wijaya et al. (2002) and Iftari et al. (2013), respectively. Similar procedures were conducted by removing flavor (cajuput and peppermint oil) to prepare unflavored sucrose and non-sucrose candy. For in vitro assay, all candy formulas were diluted 1:1 (w/v) aseptically in sterile BHI broth. They will be used in biofilm inhibition assay and qPCR analysis. BHI broth without candy formula was used as a negative control.

Volatile compounds identification

SCC or NSCC (50 grams) were diluted with 20 mL distilled water and 5 mL hexane. They were put on a shaker (160 rpm) for 15 hours until all candies were dissolved. The supernatant was pipetted then added with sodium sulphate anhydrous. This water-free flavor extract was then pipetted into a new vial and added with 0.5 mL of 1,4-dichlorobenzene 1% (diluted with hexane) as an internal standard. The final extract solution was flushed with N2.

Each extract (0.5 mL) was injected into the GC-MS (Agilent) on split mode (50:1 for SCC and 5:1 for NSCC) that was equipped with a DB-5 capillary column (60m length; 0.25mm i.d.; 0.25µm film thickness; helium carrier gas). The injector temperature was 250°C, and detector was 280°C. The initial oven temperature of the column was 60°C (held for 5 min), increased to 250°C at 10°C/min and held constantly for 2 min. Alkane standard C8-C20 (Fluka) was used as an external standard.

Qualitative identification of the constituents was performed by comparison of their linear retention indices (LRI) with the literature and their mass spectral data (NIST library) (Muchtaridi et al., 2010; Adams, 2009). The volatile profiles of SCC and NSCC were compared to volatile profile of cajuput and peppermint oil from Iftari et al. (2013).

Bacterial strains, media, and culture conditions

S. sanguinis ATCC 10556 and S. mutans XC from -70°C culture stocks were grown in BHI agar in an anaerobic jar under microaerophilic condition (CO2 10%, N2 80%, H2 10%) and incubated for 24 hours at 37°C. For in vitro assay, the bacteria were harvested and adjusted to achieve 0.477 optical density in 490 nm (OD490) for S. sanguinis and 0.061 in OD650 for S. mutans, which equaled to 1×10^6 colony forming units (CFU) mL^-1.

Preparation of dual-species biofilm

Preparation of dual-species biofilm for in vitro assay was conducted based on the method of Kreth et al. (2008) with modification. Two types of microplates were used, 96-well microtitre plate for biofilm inhibition assay and 6-well microtitre plate (Takara, Tokyo, Japan) for qPCR analysis (mRNA and DNA quantification). Briefly, 100 µL of S. sanguinis suspension (1×10^6 CFUmL^-1) was inoculated into 96-well microplate and 350 µL (1×10^6 CFUmL^-1) of the same culture was inoculated into 6-well microplate. They were incubated for two hours in anaerobic jars under a microaerophilic condition (CO2 10%, N2 80%, H2 10%) at 37°C. In order to prepare dual-species biofilms, 100 µL (1×10^6 CFUmL^-1) and 350 µL (1×10^6 CFUmL^-1) of S. mutans suspensions were respectively added into the 96 and 6-well microplate containing S. sanguinis biofilm, and re-incubated for 18 hours in a similar condition.

In regards to the inhibition assay, dual-species biofilm on 96-well microplate was added with 200 µL of diluted candies in BHI broth (1:1 v/v). For qPCR analysis, 6-well microplate biofilm was added with 700 µL of diluted candies. Biofilm
added with only BHI broth was used as the negative control. The microplates were incubated for 18 hours, after which, the medium in 96 and 6-well plates were decanted and the remaining planktonic cells were removed by rinsing the wells with PBS pH 7.2) three times.

**Biofilm inhibition assay**

The inhibition effect of candy formulas toward dual-species biofilm was analyzed using method described by Yamanaka et al. (2004). After PBS rinsing, the plates were air dried and the adhered bacteria was stained with 200 μL of 0.5% crystal violet (CV, Sigma Aldrich) for 15 minutes at 37°C. After rinsing twice with 200 μL of PBS, bound dye was extracted from the stained cells using 200 μL of 95% ethanol. The OD_{490} of the extracted CV was measured with a microplate reader (Bio-Rad Laboratories, Hercules, CA). The absorbance value OD samples compared to OD control. Each experiment was performed in triplicate and repeated two times in separated occasions.

**Bacterial DNA quantification**

Microbial DNA was extracted using Trizol® reagent, following the instruction provided by the company. The DNA concentration was determined by spectrophotometer and standardized prior to qPCR analysis. The qPCR mixture for DNA quantification (10 μL) included 5 μL SYBR Green 1x Universal (KAPA Biosystem), 1 μL DNA (100 μg/mL), 3.2 μL DEPC water, and 0.3 μL of 5 mM forward and reverse real-time PCR primers that was Ss 16S rRNA, Sm 16S rRNA, and universal primers of 16S rRNA gene (Table 1). The qPCR protocol included one cycle of 95°C for 3 minutes, followed by 40 cycles of 95°C for 3 second and 60 minutes for 30 second. The bacterial load was determined based on the proportion of each species compared with total bacteria. It was determined by using the ΔCt method (Yoshida et al., 2003).

**Analysis of the expression of spxB mRNA**

RNA extraction was performed in similar procedures with DNA extraction. It was reversed to cDNA using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Moreover, reactions mixture (10 μL) which contained 1xKAPA SYBR Green Master Mix (KAPA Biosystems), 1 μL cDNA, forward and reverse primers (0.5 μM) of spxB were centrifuged at 2000 rpm for 1 minutes. The house keeping gene (16S rRNA) was used as internal control. All primers used in this study are shown in Table 1. The real-time PCR cycle was carried out under the similar conditions as previous DNA quantification. The relative change in spxB mRNA expression was analyzed using 2-ΔΔCt (Suzuki et al., 2005).

**Statistical analysis**

Student’s t test (SPSS Inc 17.0 software) was carried out to analyze the data significance on a p-value of <0.05 among the biofilms OD, spxB mRNA expression, and bacterial proportion of experimental samples to the control groups. One way analysis of variance (ANOVA) by SPSS Inc 17.0 was used in biofilm inhibition analysis with significance value p<0.05. ANOVA analysis was carried out to support the assumption/discussion about significance biofilm inhibition between samples.
The potency of cajuputs candy in maintaining the competitive capacity of *Streptococcus sanguinis* upon *Streptococcus mutans*

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**Table 1. Primers used for qPCR**

| Primer                | Sequence (5’-3’)                  | Purpose                     | Ref.                  |
|----------------------|-----------------------------------|-----------------------------|-----------------------|
| Sm 16S rRNA forward  | CCTACGGGGAGCAGCAGTAG              | *S. mutans* quantification  | (Shemesh et al., 2007) |
| Sm 16S rRNA reverse  | CAACAGAGCTTTACGATCCGAAA           | *S. mutans* quantification  | (Shemesh et al., 2007) |
| Ss 16S rRNA forward  | CCGCCTAAGGTGGGAATGATGGTAG        | spxB mRNA and *S. sanguinis* quantification | (Zheng et al., 2011a) |
| Ss 16S rRNA reverse  | ACCTTCCGATACGCCTACCTTGTTAC       | spxB mRNA and *S. sanguinis* quantification | (Zheng et al., 2011a) |
| spxB Ss forward      | AATTCGGCGGCTCAATCG                | spxB mRNA quantification    | (Zheng et al., 2011a)  |
| spxB Ss reverse      | AAGGATAGCAAGGAAATGAGTG           | spxB mRNA quantification    | (Zheng et al., 2011a)  |
| Universal forward    | TCTACGGAGGGACAGCTATG             | Total bacteria quantification | (Suzuki et al., 2003) |
| Universal reverse    | GGACTACCAGGGGTATCTAATCCTGTT      | Total bacteria quantification | (Suzuki et al., 2003) |

**RESULTS**

**Volatile compounds contents**

Volatile compounds of SCC and NSCC are presented in Table 2. The major compounds in SCC were 1,8-cineole and α-terpineol, while in NSCC were menthol and α-terpineol. Both SCC and NSCC contained 1,8-cineole, α-terpineol, β-caryophyllene, terpen-4-ol, menthol, menthone, limonene, β-pinene, α-terpinene, γ-terpinene, and viridiflorol. Monoterpene groups, especially oxygenated monoterpenes, dominated these bioactive components.

**Inhibition of dual species biofilm by Cajuputs candy**

SCC, unflavored sucrose candy, NSCC, and unflavored non-sucrose candy were evaluated for their effect on the dual-species biofilm formation. Figure 1 shows that after incubation, both sucrose and non-sucrose candy showed inhibition activity. The inhibition of all candy samples were significantly different from control (p<0.05). Moreover, the biofilm inhibition between samples were observed. OD values with different subset (a, b, ab, and c) mean they were significantly different with each other. As could be seen from Figure 1, the inhibition of SCC and NSCC were significantly higher compared with unflavored sucrose candy indicated the lower growth of the tested bacteria in biofilm mass of SCC and NSCC. The addition of cajuput and peppermint oils as flavoring ingredients in SCC and NSCC significantly reduced the biofilm formation by more than 50% compared to the control (growth medium cultured with bacteria without any formula addition). Meanwhile, the inhibition of SCC was not significantly different from unflavored non-sucrose candy (p>0.05). Biofilm inhibition was also observed in unflavored sucrose candy. Biofilm inhibition of unflavored sucrose candy was significantly lower compared to the control.
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Table 2. Chemical composition of volatile compounds of Sucrose Cajuputs Candy (SCC) and Non-Sucrose Cajuputs Candy (NSCC)

| No. | Compounds          | LRI Refa | SCC     | NSCC     | References for antimicrobial activities                                      |
|-----|--------------------|----------|---------|----------|--------------------------------------------------------------------------------|
|     |                    | LRI expb | %       | LRI expb | %                                 |
| 1   | α-pinene           | 939      | 940     | 0.98     | 986                                | 0.11                           | (Maggi et al., 2009) |
| 2   | β-pinene           | 979      | 984     | 0.86     | 986                                | 0.11                           | (Inouye et al., 2001) |
| 3   | p-cymene           | 1026     | 1031    | 1.63     | 1031                               | 0.41                           | (Maggi et al., 2009; Hamoud et al., 2012) |
| 4   | D-limonene         | 1029     | -       | -        | 1039                               | 1.92                           | (Inouye et al., 2001) |
| 5   | 1,8-cineole        | 1031     | 1043    | 23.67    | 1044                               | 8.48                           | (Maggi et al., 2009; Hamoud et al., 2012) |
| 6   | γ-terpinene        | 1059     | 1064    | 2.78     | 1065                               | 0.51                           | (Inouye et al., 2001) |
| 7   | Terpinolene        | 1088     | 1094    | 1.9      | 1095                               | 0.66                           | (Dwivedi et al., 2012; Maggi et al., 2009) |
| 8   | Linalool           | 1096     | 1098    | 0.36     | 1100                               | 0.34                           | (Maggi et al., 2009) |
| 9   | Isopulegol         | 1145     | 1156    | 0.32     | 1158                               | 0.63                           | (Inouye et al., 2001) |
| 10  | Menthone           | 1152     | 1165    | 3.3      | 1168                               | 4.43                           | (Jedlickova et al., 1994; Inouye et al., 2001) |
| 11  | Isomenthone        | 1162     | 1175    | 2.91     | 1178                               | 4.34                           | (Jedlickova et al., 1994; Maggi et al., 2009) |
| 12  | Menthol            | 1171     | 1185    | 7.29     | 1191                               | 13.32                          | (Jedlickova et al., 1994; Inouye et al., 2001) |
| 13  | Terpinen-4-ol      | 1177     | 1189    | 1.86     | 1194                               | 1.28                           | (Dwivedi et al., 2012; Inouye et al., 2001) |
| 14  | Neoisomenthol      | 1186     | 1195    | 0.26     | 1199                               | 0.51                           | (Dwivedi et al., 2012; Inouye et al., 2001) |
| 15  | α-terpineol        | 1188     | 1203    | 9.17     | 1209                               | 9.97                           | (Dwivedi et al., 2012; Inouye et al., 2001) |
| 16  | Pulegone           | 1237     | 1252    | 0.47     | 1256                               | 0.92                           | (Maggi et al., 2009) |
| 17  | Piperitone         | 1252     | 1267    | 0.29     | 1271                               | 0.73                           | (Jedlickova et al., 1994; Hamoud et al., 2012) |
| 18  | Menthy acetate     | 1295     | 1299    | 1.37     | 1302                               | 2.56                           | (Jedlickova et al., 1994; Hamoud et al., 2012) |
| 19  | α-terpinyl acetate | 1354     | 1359    | 3.66     | 1362                               | 3.38                           | (Heleno et al., 2011) |
| 20  | α-copaene          | 1376     | 1398    | 0.34     | 1400                               | 0.39                           | (Heleno et al., 2011) |
| 21  | β-elemene          | 1390     | 1409    | 0.32     | 1412                               | 53                             | (Heleno et al., 2011) |
| 22  | β-caryophyllene    | 1419     | 1452    | 6.78     | 1456                               | 7.64                           | (Heleno et al., 2011) |
| 23  | α-humulene         | 1454     | 1475    | 4.2      | 1489                               | 4.67                           | (Heleno et al., 2011) |
| 24  | Viridiflorol       | 1592     | 1631    | 0.57     | 1635                               | 0.77                           | (Iscan et al., 2002) |

Note:
a: LRI reference (Adams, 2009) with DB-5 column; b: LRI experiment with DB5-MS column.

Figure 1. Inhibition of candies exposures on dual-species (S. sanguinis and S. mutans) biofilms
**Bacterial DNA quantification: effect of candy exposure to the relative amount and the proportion of S. sanguinis and S. mutans in dual-species biofilm**

Table 3 showed the quantification of relative amount of total DNA in the four formulas compared to the total DNA in the control group (defined as 100% growth). Our data showed that the exposures of SCC and NSCC decreased the amount of total bacterial DNA significantly, indicating the involvement of the flavor. In contrast, when the unflavored candies were exposed into the bacterial biofilm, the amount of bacterial DNA were significantly increased compared to those of control. Nevertheless, the DNA proportion of S. sanguinis and S. mutans in the biofilm did not show any significant difference between samples and control (Table 3). This study showed that the exposure of the candies did not alter the antagonistic interaction between S. sanguinis and S. mutans, *in vitro*.

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**Tables 3. Effect of candies exposures to the relative amount of total DNA bacteria and DNA proportion of S. sanguinis and S. mutans in dual-species (S. sanguinis and S. mutans) biofilm using qPCR**

| Formula                  | Relative amount of total DNA bacteria (%) | Proportion |
|--------------------------|-----------------------------------------|------------|
|                          |                                         | S. sanguinis (%) | S. mutans (%) |
| Control                  | 100.00                                  | 41.47      | 58.53        |
| Unflavored sucrose candy| 184.77                                  | 35.58      | 64.42        |
| SCC                      | 1.20                                    | 35.39      | 64.61        |
| Unflavored non-sucrose candy| 221.29                           | 36.35      | 63.65        |
| NSCC                     | 1.04                                    | 38.95      | 61.05        |

SCC: Sucrose Cajuputs Candy, NSCC: Non-Sucrose Cajuputs Candy

No significance difference in proportion was observed between sample and control in the analysis of student’s t test with p>0.05

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**Effect of candy exposure on the expression level of spxB mRNA in the biofilms**

Figure 2 showed that the exposure to all sample formulas did not eliminate the expression of spxB gene. No statistical difference was found between the expression of samples and the control group, which meant that the ability of S. sanguinis in producing H₂O₂ as its competing agent could be maintained. The spxB expression of NSCC and unflavored non-sucrose candy were higher than SCC.
DISCUSSION

Volatile profile

The volatile profiles of SCC and NSCC showed a similarity but vary in percentage (Table 2). Both candies were using cajuput and peppermint oil as the flavor. The flavor concentration in these candies were about 0.9%, that consisted of cajuput oil (0.7-0.8%) and peppermint oil (0.1-0.2%) based on their original formulations. Different heating temperature during production of SCC and NSCC may caused variation in their volatile percentage (Iftari et al., 2013). Table 2 showed that oxygenated monoterpenes (1,8-cineole, α-terpineol, menthol, menthone, and terpinen-4-ol) were dominated the bioactive volatiles in SCC and NSCC. Functional groups of bioactive compounds might contribute to their antimicrobial activities. As reported by Inouye et al. (2001), the antimicrobial activity of terpene alcohols (menthol, α-terpineol, and terpinen-4-ol) were higher than terpene ketone (menthone) as well as terpene ether (1,8-cineole), and terpene hydrocarbons (limonene, β-pinene, and γ-terpinene) were lowest in activity.

Biofilm inhibition

This study showed cajuputs candy exposures suppressed the formation of dual-species (S. sanguinis + S. mutans) biofilms. In comparison to the control, cajuputs candy (SCC and NSCC) were more effective in inhibiting biofilm formation than the unflavored candies (Figure 1). Meanwhile, biofilm inhibition of unflavored sucrose candy was significantly lower compared to the control, despite of the missing flavor as anti-bacterial agent. The potency of cajuput and peppermint oils as antibiofilm agent was due to their bioactive volatiles. Cajuput and peppermint oils contain highly hydrophobic substances, such as 1,8-cineole, menthol, and menthone (Table 2). These volatile components have been reported for their antimicrobial capacity (Inouye et al., 2001; Maggi et al., 2009; Hamoud et al., 2012; Iscan et al., 2002; Jedlickova et al., 1994; and Dwivedi et al., 2012). Direct contact of these oils to the biofilm at long period of incubation (over 18 hours) could inhibit biofilm growth. The inhibition mechanism can be vary by disturbing membrane function, inhibition of cells respiration, and alteration of ion transport processes which lead to the death of microbial cell (Hamoud et al., 2012), thus can reduce biofilm formation. The biofilm inhibition of NSCC was higher compared to SCC, which meant that the combination of flavor and non-sucrose carbohydrate in NSCC may play a role. However, further study is needed to elucidate the exact mechanism of SCC and NSCC in inhibiting biofilm formation.
Not only NSCC and SCC, the unflavoured non-sucrose candy in comparison with the control also exhibited a significant inhibition in biofilm formation. As shown in Figure 1, the inhibition of unflavored non-sucrose candy was not significantly different from SCC. Lower biofilm density produced when biofilms were exposed to non-sucrose candies than that were exposed to sucrose candies. It showed that the usage of isomalt to substitute sucrose in the candy formulation exhibited biofilm inhibition. In the present study, different kind of carbohydrate were used, and this may promote different biomass density. Therefore, the usage of isomalt in non-sucrose candy formulations will produce lower biomass density in comparison with sucrose or glucose containing candies (Mayo and Ritchie, 2009). Sucrose is naturally more available to be fermented by microorganism than isomalt (Childers et al., 2011).

The unflavored candies were also reported to have lower biofilm density than control (Figure 1). In this study, the unflavored sucrose candy contained high amount of sucrose and glucose, while the unflavored non-sucrose candy contained high amount of isomalt. The sucrose and isomalt concentration were approximately 50% of the candy formulations (data not shown), meanwhile the growth medium (BHI broth) itself already contained sucrose and glucose. The high amount of such carbohydrates (e.g. sucrose and isomalt formulations) in the biofilm may cause environmental disturbance to bacterial growth. High content of external carbohydrates exposed to the biofilm will modify the growth environment, increases osmotic pressure, that will disturb bacterial growth (Touger-decker and Loveren, 2003). These disturbances were able to reduce the ability of bacteria to form biofilm.

In this study, the result of biofilm inhibition was presented by optical density (OD) value. OD value sometimes represents number of bacterial cell, but in term of biofilm, it does not merely indicating bacterial cell, but biofilm mass. Biofilm is a complex mixture of several materials consisting of bacterial cells, proteins, carbohydrates, water, lipids, including non-viable cell of bacteria that form a biofilm mass (Kreth et al., 2008). It means that OD value also represented biofilm density. Biofilm can have low OD value, eventhough its bacterial number (DNA) was high. As it can be shown, a contradictive result was found between OD value (Figure 1) and DNA amount (Table 3) of unflavored candies compared to the control.

DNA quantification

Both unflavored sucrose candy and unflavored non-sucrose candy were able to decrease biofilm formation compared with control (Figure 1). Meanwhile, higher bacterial DNA was detected in those unflavored candies (Table 3). It seemed that OD values from the biofilm inhibition measurement could not be used to distinguish the amount of bacterial load. Since the crystal violet assay did not only determine the number of bacterial cells but also measure the extracellular matrix within biofilm, low OD value did not mean low bacterial number. OD value is the determination of turbidity, which represents the biomass density of the biofilm (Bakke et al., 2001). Therefore, in contrast with crystal violet assay, qPCR is a sensitive method to quantify total and/or individual bacterial DNA in clumping cells, including the presence of viable and non-viable cells (Childers et al., 2011). Moreover, it can also be used to quantify specific bacterial species (Fortin et al., 2001).

In this study, unflavored non-sucrose candy had the higher number of bacterial DNA and even higher than that in unflavored sucrose candy (Table 3). This result indicated that isomalt was used in biofilm metabolism. However, isomalt can still be used in bacterial metabolism, thus can support bacterial growth. Mayo and Ritchie (2009) reported that the incubation time of 18-24 hours implied isomalt degradation by Streptococci isolates used. Frequent and prolonged contact of isomalt (an equimolar mixture of D-glucopyranosyl-1,6-sorbitol and α-D-glucopyranosyl-1,6-mannitol) used in “sugar-free” cough drop syrup could be degraded to produce glucose, sorbitol, and mannitol.

This study showed that NSCC had the greatest antibacterial activity and inhibition toward biofilm formation (Figure 1), which was significantly different from the control. NSCC inhibited biofilm formation by approximately 68.2% compared to
control and decreased bacterial DNA to 1% of the control (Table 3). These phenomena were due to antibacterial volatiles provided from the flavor, supported by the limitations of the substrate. Essential oils tend to inhibit more powerfully on Gram-positive bacteria than the growth of Gram-negative bacteria, which could be attributed to the different structure and cell membrane compositions (Güiterrez et al., 2008). S. mutans and S. sanguinis, which are Gram-positive bacteria, have single membrane structure with thick peptidoglycan layer that are sensitive to essential oil (Trombetta et al., 2005).

The DNA proportion was measured to determine the effect of cajuputs candy formulas (SCC and NSCC) on the ability of S. sanguinis to maintain its antagonistic competency upon S. mutans growth. Both SCC and NSCC were able to decreased bacterial load in the biofilm (Table 3) and inhibited biofilm growth (Figure 1), but the result of DNA proportion (Table 3) indicated that they did not show any capacity to interfere the natural interaction among S. mutans and S. sanguinis. The absence of cajuputs candy increased the cells number of both bacterial in biofilm. On the contrary, their presence simultaneously inhibited (Table 3). Kreth et al. (2005) reported that S. sanguinis and S. mutans have a competitive exclusion in the biofilm system caused by the production of diffusible substances that inhibited the other growth. S. sanguinis produces peroxidase, while S. mutans produces bacteriocin. This study showed that, the cajuputs candy exposures did not interfere such competitiveness effect (Table 3). Thus, the exposures may have a capability in maintaining the antagonistic competency of S. sanguinis upon S. mutans in the biofilm.

**Gene Expression of spxB mRNA**

We have demonstrated that the presence of cajuputs candy in biofilm development resulted in up-regulating of spxB mRNA expression, which implied the transcription activity of spxB gene of viable S. sanguinis. The spxB gene produces an enzyme responsible for production of H2O2 by S. sanguinis to inhibit the growth of S. mutans (Zheng et al., 2011a). The detectable expression of spxB mRNA in all of the tested formula (Figure 2) indicated the competitive capacity of S. sanguinis against S. mutans and showed that viable bacteria remained within the biofilms. No negative effect was found in the expression of spxB mRNA compared to control since there were no statistically difference between samples and control (Figure 2). This indicated that the bacteria still has capacity in controlling S. mutans as naturally occurred *in vivo* (Kreth et al., 2008).

The exposure of unflavored sucrose candy showed the lowest expression level of spxB mRNA compared to others formulas (Figure 2), whereas the amount of total DNA bacteria was very high (Table 3). Kreth et al. (2005) mentioned that in high sucrose concentration, S. sanguinis preferred to use the carbon source for growth rather than spend a lot of energy to produce H2O2 to suppress the growth of the S. mutans.

The spxB mRNA expression of that in NSCC exposure was higher than SCC exposure (Figure 2). The use of isomalt on NSCC might create a stress condition due to the lack of carbon source that could be metabolized (Lemos et al., 2005). Therefore, under these conditions, S. sanguinis would focused on producing H2O2 to maintain the bacteria coexistence (Kreth et al., 2005), and increase the bacterium competitive capacity in the biofilm (Zhu and Kreth, 2012).

It is known that the essential oil is a mixture of various components that are mostly hydrophobic compounds and have antibacterial properties. The mechanism essential oil biofilm inhibition were very diverse but mostly related to disruption of membrane function by penetration of hydrophobic compounds (Nazaro et al., 2013). Other proposed mechanisms include the alteration of the membrane fatty acids, degradation of cell wall, alteration of the proton motive force, increase of permeability of the membrane, and membrane protein damage (Nazaro et al., 2013). Even though SCC and NSCC exposure effectively inhibited the dual-species biofilm formation (Figure 1), the inhibition did not eliminate the ability of S. sanguinis to produce H2O2, as proven by the up-regulation of spxB mRNA expression because S. sanguinis is less susceptible to bioactive compounds in essential oil than S. mutans (Magalhaes et al., 2016). Therefore,
the survival *S. sanguinis* still has the ability to express the *spxB* gene. However, more studies are needed to fully understand the inhibition mechanism of cajuputs candy in the dual-species biofilm formation.

**CONCLUSIONS**

The data showed that the absence of flavor in the candy triggered greater biofilm growth than the control. Flavored candy exposures effectively inhibited the bacterial growth within the biofilms. Each tested candy showed the ability to maintain the antagonistic competency of *S. sanguinis* toward *S. mutans* in dual-species biofilms. The combination of active volatiles and non-sucrose material made NSCC exposures as the most effective formula to inhibit the biofilm growth without eliminating its competitive capacity of *S. sanguinis* toward *S. mutans* in dual-species biofilm. However, more studies are needed to fully understand the inhibition mechanism of cajuputs candy in polymicrobial biofilm formation. The data in this study can be used for further exploration of the SCC and NSCC potency in preventing dental caries in vivo.

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