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

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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 mengeksplorasi 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.*

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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_2O_2) that inhibits *S. mutans* growth under aerobic condition (Zheng et al., 2011a). *S. mutans* is not able to tolerate H_2O_2 (Zheng et al., 2011b). The H_2O_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 corelated with the production of H_2O_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 *gtfC* 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 distillated 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 honeydew flavor were from flavor houses. Analytical grade

chemicals were used: Brain Heart Infusion (BHI) agar and liquid (Acumedia), distilled water, milli-Q water, hexane (JT Baker), alkane standard (C_8-C_{20}), Nitrogen, carbon dioxide, violet crystal 1%, ethanol 95% (Merck), ethanol p.a. (Merck), phosphate buffer saline (PBS), trizol reagent (Invitrogen), isopropanol (Merck), chloroform, SYBR green (Kapa Biosystems), ultrapure distilled water (Invitrogen) and RT-PCR primer (1st BASE Custom Oligos, Singapore).

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 flashed with N_2.

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 C_8-C_{20} (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 (CO_2 10%, N_2 80%, H_2 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 (OD_{490}) for *S.sanguinis* and 0.061 in OD_{450} 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 (CO_2 10%, N_2 80%, H_2 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 µLof 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 ΔC_t 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 1×KAPA SYBR Green Master Mix (KAPA Biosystems), 1 µL cDNA, forward and reverse primers (0.5 µM) of spxBSs was 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.

Table 1. Primers used for qPCR

Primer	Sequence (5′-3′)	Purpose	Ref.
Sm 16S rRNA forward	CCTACGGGAGGCAGC AGTAG	*S. mutans* quantification	(Shemesh et al., 2007)
Sm 16S rRNA reverse	CAACAGAGCTTTACG ATCCGAAA	*S. mutans* quantification	(Shemesh et al., 2007)
Ss 16S rRNA forward	CCGCCTAAGGTGGGA TAGATGATTG	*spxB* mRNA and *S. sanguinis* quantification	(Zheng et al., 2011a)
Ss 16S rRNA reverse	ACCTTCCGATACGGC TACCTTGTTAC	*spxB* mRNA and *S. sanguinis* quantification	(Zheng et al., 2011a)
spxB Ss forward	AATTCGGCGGCTCAA TCG	*spxB* mRNA quantification	(Zheng et al., 2011a)
spxB Ss reverse	AAGGATAGCAAGGAA TGGAGTG	*spxB* mRNA quantification	(Zheng et al., 2011a)
Universal forward	TCCTACGGGAGGCAG CAGT	Total bacteria quantification	(Suzuki et al., 2003)
Universal reverse	GGACTACCAGGGTAT CTAATCCTGTT	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, terpinen-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.

Table 2. Chemical composition of volatile compounds of Sucrose Cajuputs Candy (SCC) and Non-Sucrose Cajuputs Candy (NSCC)

No.	Compounds	LRI Ref[a]	SCC		NSCC		References for antimicrobial activities
			LRI exp[b]	%	LRI exp[b]	%	
1	α-pinene	939	940	0.98	-	-	
2	β-pinene	979	984	0.86	986	0.11	(Maggi et al., 2009)
3	p-cymene	1026	1031	1.63	1031	0.41	
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	
8	Linalool	1096	1098	0.36	1100	0.34	(Dwivedi et al., 2012 ; Maggi et al., 2009)
9	Isopulegol	1145	1156	0.32	1158	0.63	
10	Menthone	1152	1165	3.3	1168	4.43	(Inouye et al., 2001)
11	Isomenthone	1162	1175	2.91	1178	4.34	
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	(Jedlickova et al., 1994 ; Maggi et al., 2009)
14	Neoisomenthol	1186	1195	0.26	1199	0.51	
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	
17	Piperitone	1252	1267	0.29	1271	0.73	
18	Menthyl acetate	1295	1299	1.37	1302	2.56	
19	α-terpinyl acetate	1354	1359	3.66	1362	3.38	
20	α-copaene	1376	1398	0.34	1400	0.39	
21	β-elemene	1390	1409	0.32	1412	53	
22	β-caryophyllene	1419	1452	6.78	1456	7.64	(Heleno et al., 2011)
23	α-humulene	1454	1475	4.2	1489	4.67	
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.

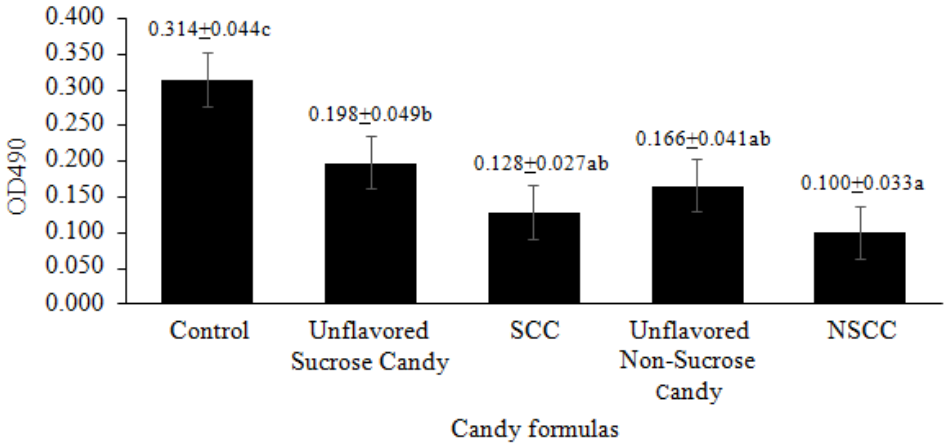


Figure 1. Inhibition of candies exposures on dual-species (*S. sanguinis* and *S. mutans*) biofilms

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.*

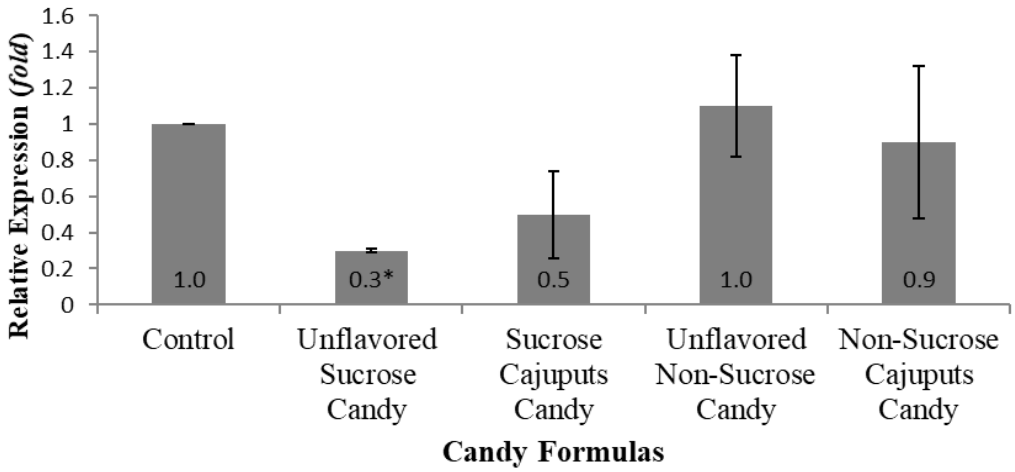
Tables 3. Effect of candies exposures to the relative amount of total DNA bacteria and DNA proportion of *S. sanguinis* and *S. mutans* in dual-species (*S. sanguinis* and *S. mutans*) biofilm using qPCR

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 $p>0.05$

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 H_2O_2 as its competing agent could be maintained. The *spxB* expression of NSCC and unflavored non-sucrose candy were higher than SCC.



*p<(0.05) indicated statistically significant difference between sample and the control group.

Figure 2. *spxB* mRNA expression level affected by candy formulas in dual-species (*S. sanguinis* and *S. mutans*) biofilm

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 ammount 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 (Guiterrez 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. sangiunis* 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 H_2O_2 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 bacterium 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* prefered to use the carbon source for growth rather than spend a lot of energy to produce H_2O_2 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 H_2O_2 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 H_2O_2, 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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REFFERENCES

Adams RP. Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry. Carol Stream, IL: Allured Publ. Corp., 2009.

Bakke R, Kommedal R, Kalvenes S. Quantification of biofilm accumulation by an optical approach. J Microbiol Methods, 2001; 44: pp.13-26.

Becker MR, Paster BJ, Leys EJ, Moeschberger ML, Kenyon SG, Galvin JL, Boches SK, Dewhirst FE, Griffen AL. Molecular analysis of bacterial species associated with childhood caries. J Clin Microbiol, 2002; 40: pp.1001–1009.

Childers NK, Osgood RC, Hsu KL, Manmontri C, Momeni SS, Mahtani HK, Cutter GR, Ruby JD. Real-time quantitative polymerase chain reaction for enumeration of *Streptococcus mutans* from oral samples. Eur J Oral Sci, 2011; pp.119:447-454.

Dwivedi D, Khandelwal G, Patidar RK, Singh V. Antimicrobial activity of *Menthaarvensis* against clinical isolates of human cariogenic pathogens- an *in vitro* study. Int J Pharm Sci Res, 2012;3: pp.1355-1360.

Eslami H, Zarandi A, Pouralibaba F, Fakhrzadeh V, Falsafi P, Sepas RR. Assesment of relationship between. *Streptococcus mutans*, dental caries, and TGF-β. Asian J Biomed Pharmaceutic Sci, 2016; 6 (56): pp.20-22.

Fejerskov O. Changing paradigms in concepts on dental caries: consequences for oral health care. Caries Res, 2004;38 pp.182–191.

Fortin NY, Mulchandani A, Chen W. Use of real-time polymerasechain reaction and molecular beacons for the detection of *Escherichia coli* O157:H7.Anal Biochem, 2001; pp.289:281–288.

Galvao LC, Furletti VF, Bersan SM, da Cunha MG, Ruiz AL, de Carvalho JE, Sartoratto A, Rheder VL, Fiqueira GM, Duarte MC, Ikegaki M, de Alencar SM, Rosalen PL. Antimicrobial activity of essential oils against *Streptococcus mutans* and their antiprolifrative effects. Evid based Complement Alternat Med, 2012;doi: http://dx.doi.org/10.1155/2012/751435.

Gutierrez J, Barry-Ryan C, Bourke P. The anti-microbial efficacy of plant essential oil combinations and interactions with food ingredients. Int J Food Microbiol, 2008; pp.124:91–97.

Hamoud R, Sporer F, Reichling J, Wink M. Antimicrobial activity of a traditionally used complex essential oil distillate (Olbas[R]

Tropfen) in comparison to its individual essential oil ingredients. Phytomedicine, 2012; 19: pp.969-975.

Heleno VC, Martins CG, Morais GO, da Silva EH, Wakabayashi K, Carvalho C, Critti AM. Antimicrobial activity of the essential oils and non-polar extracts from leaves and flowers of *Tithonia diversifolia* against cariogenic bacteria. Planta Med, 2011; 12: pp.77-PE47.

Iftari W, Fardiaz D, Bachtiar BM, Wijaya CH. The potency of non-sucrose Cajuputs candy in inhibiting the formation of biofilm by *Streptococcus mutans* serotype c. XIII Asean Food Conference, Singapore; 2013.

Inouye S, Takizawa T, Yamaguchi H. Antibacterial activity of essential oils and their major constituents against respiratory tract pathogens by gaseous contact. J AntimicrobChemother, 2001; 47: pp.565-573.

Işcan G, Kirimer N, Kürkcüoğlu M, Başer KHC, Demirci F. Antimicrobial screening of *Mentha piperita* essential oils. J Agric Food Chem, 2002;50: pp.3943-3946.

Jedlickova Z, Ery V, Mott O, Nguyen DC. Antibacterial properties of cajuput oil. Medicaments et Aliments: L'Approche Ethnopharmacologique, 1994; 2: pp.293.

Kreth J, Merritt J, Wenyuan S, Fengxia Q. Competition and coexistence between *Streptococcus mutans* and *Streptococcus sanguinis* in the dental biofilm. J Bacteriol, 2005;187: pp.7193-7203.

Kreth J, Zhang Y, Herzberg MC. Streptococcal antagonism in oral biofilms: *Streptococcus sanguinis* and *Streptococcus gordonii* interference with *Streptococcus mutans*.J Bacteriol, 2008, 190: pp.4632-4640.

Lemos JA, Abranches J, Burne RA. Responses of cariogenic *Streptococci* to environmental stresses.*Curr Issues Mol Biol*, 2005; pp.7:95-107.

Magalhães CF, de Siqueira EP, de Oliveira EA, Zani CL, Peres RL, dos SantosKV, Andrade AA. Antimicrobial activity of *Piper aduncumleaf* extracts against the dental plaque bacteria *Streptococcus mutans* and *Streptococcus sanguinis*. J Med Plants Res, 2016; 10: pp.331-337.

Maggi F, Bramucci M, Cecchini C, Coman MM, Cresi A, Cristalli G, Lupidi G, Papa F, Quassinti L, Sagratini G, Vittori S. Composition and biological activity of essential oil of *Achillea ligustica All. (Asteraceae)* naturalized in central Italy: ideal candidate for anti-cariogenic formulations. Fitoterapia, 2009; 80: pp.313-319.

Mayo JA, Ritchie JR. Acidogenic Potential of "Sugar-Free" Cough Drops. Open Dent. J, 2009; 3: pp.26-30.

Muchtaridi, Subarnas A, Apriyantono A, Mustarichie R. Identification of compounds in the essential oil of nutmeg seeds (*Myristicafragrans Houtt.*) that inhibit locomotor activity in mice. Int J Mol Sci, 2010;11: pp.4771–4781.

Nazzaro F, Fratianni F, de Martino L, Coppola R, de Feo V. Effect of Essential Oils on Pathogenic Bacteria. Pharmaceuticals, 2013;6: pp.1451-1474.

Oda Y, Hayashi F, Okada M. Longitudinal study of dental caries incidence associated with *Streptococcus mutans* and *Streptococcus sobrinus* in patient with intellectual disabilities. BMC Oral Health, 2015; 15: pp.102.

Percival RS, Devine DA, Duggal MS, Chartronand S, Marsh PD. The effect of cocoa polyphenols on the growth, metabolism, and biofilm formation by *Streptococcus mutans* and *Streptococcus sanguinis*. Eur J Oral Sci,2006;114: pp.343-348.

Saharkhiz MJ, Motamedi M, Zomorodian K, Pakshir K, Miriand R, Hemyari K. Chemical composition, antifungal and antibiofilm activities of essential oil of *Mentha piperita L.*

ISRN Pharm, 2012; doi: http://dx.doi.org/10.5402/2012/718645.

Shemesh M, Ashyalom T, Steinberg D. Expression of biofilm-associated genes of *Streptococcus mutans* in response to glucose and sucrose. J Med Micobiol, 2007;56: pp.1528-1535.

Somaraj V, Shenoy RP, Panchmal GS, Kumar V, Jodalli PS, Sonde L. Effect of herbal and fluoride mouth rinse on *Streptococcus mutans* and dental caries among 12-15-year-old school children: a randomized controlled trial. Int J Dent, 2017; doi: http://doi.org/10.1155/2017/5654373.

Suzuki N, Yoshida A, Nakano Y. Quantitative analysis of multi-species oral biofilms by taqMan Real-Time PCR. Clin Med Res, 2005; 3: pp.176-185.

Touger-Decker R, Loveren C. Sugar and dental caries (suppl). Am J Clin Nutr, 2003; 78: pp.881S-92S.

Trombetta D, Castelli F, Sarpietro MG, Venuti V, Cristani M, Daniele C, Saija A, Mazzanti G, Bisignano G. Mechanisms of antibacterial action of three monoterpenes. Antimicrob Agents Chemother, 2005; 49: pp.2474–2478.

Wijaya CH, Halimah, Kindly, Taqi FM.(2002). The composition of Cajuput candy as throat lozenges (Komposisi permen cajuput untuk pelega tenggorokan). Indonesia Patent No. 0000385S. The Ministry of Justice and Human Rights, Republic of Indonesia.

Wijaya CH, Nurramdhan IF, Bachtiar BM. Ability of Cajuput candy in maintaining oral health: *in vitro* inhibition of cajuput candy flavor formula against *Streptococcus mutans* and *Streptococcus sobrinus*. International Food Conference. Surabaya, Indonesia; 2011.

Wijaya CH, Rachmatillah AR, Bachtiar BM.Inhibition of Cajuputs candy toward the viability of *Candida albicans* by using *in vitro* assay. *J Teknologi dan Industri Pangan*, 2014; 25: pp.158-167.

Yamanaka A, Kimizuka R, Kato T, Okuda K. Inhibitory effects of cranberry juice on attachment of oral streptococci and biofilm formation. Oral Microbiol Immunol, 2004; 19: pp.150-154.

Yoshida A, Suzuki N, Nakano Y, Oho T, Kawada M, Koga T. Development of a 5′ fluorogenic nuclease-based real-time PCR assay for quantitative detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis.* J Clin Microbiol, 2003; 41: pp.863-866.

Zheng L, Chen Z, Itzek A, Ashby M, Kreth J. Catabolite control protein A controls hydrogen peroxide production and cell death in *Streptococcus sanguinis*. *J Bacteriol*, 2011;193: pp.516-526.

Zheng L, Itzek A, Chen Z, Kreth J. Oxygen dependent pyruvate oxidase expression and production in *Streptococcus sanguinis*. Int J Oral Sci, 2011;3; pp.82-89.

Zhu L, Kreth J. The role of hydrogen peroxide in environmental adaptation of oral microbial communities. Oxic Med Cell, 2012; pp.1-10.