Current Applied Science and Technology Vol. 21 No. 3 (July-September 2021)

# Effects of Natural Sugar on Acidogenic Potential, Biofilm Biomass, and Antiseptic Resistance of Oral Streptococci

Pimpikar Kanchanadumkerng[1] and Karn Wongsariya[2]*

[1]Department of Food Chemistry, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand
[2]Department of Biology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand

Received: 8 August 2020, Revised: 13 January 2021, Accepted: 3 February 2021

## Abstract

Natural sugar is deliberated as ordinary, non-chemical, and healthy alternatives. However, a diet rich in sugar is well documented as a causative agent for dental caries. The purpose of this study was to investigate the effects of natural sugars, including raw cane, palmyra palm and coconut sugar on the acidogenic profiles, biofilm formation and antiseptic treatment efficacy compared with refined sugar. The study was based on single-species and dual-species of *Streptococcus mutans* ATCC 25175 and *Streptococcus sobrinus* ATCC 33402. Our results showed that sucrose was a major component of all samples, with percentages of relative content higher than 89.0. Palmyra sugar gave the least pH change at 180 min of 4.84-4.93, which indicated it was the least acidogenic. Coconut sugar formed the lowest level of biofilm biomass compared to refined sugar ($p < 0.05$) and other samples. Antiseptic treatment was performed to study the level of percent eradication of bacterial plaque using MTT assay to determine cell viability under biofilm in each sugar medium. Biofilm derived from coconut sugar had a susceptibility to antiseptic treatment with 56.2-61.99% eradication which was higher than the biofilm from palmyra and raw cane sugar. As a result, this study points out the effects of various natural sugars (especially different sources of plant material) on cariogenic potential. However, further experiment should be done to confirm the results *in vivo* and further study the cariogenic effects of diet supplementation of these fermentable sugars.

**Keywords:** natural sugar; sweetener; dental caries; biofilm
DOI 10.14456/cast.2021.41

## 1. Introduction

Dental caries is widespread condition affecting about one-third of the global population at all ages and is considered to be the most common noncommunicable disease (NCD) worldwide [1]. This multifactorial disease is a biofilm-mediated, sugar-driven, and dynamic circumstance resulting in the phasic demineralization and remineralization of tooth surface. Acidogenicity is a consequence

*Corresponding author: Tel.: (+66)2-329-8400 Fax: (+66)2-329-8412
E-mail: karn.wo@kmitl.ac.th

of microbial sugar metabolism which accelerates demineralization and results in caries lesions [2]. Mutans streptococci (MS) has long been connected with dental caries in humans. Certain species, including *Streptococcus mutans* and *Streptococcus sobrinus*, have been shown to be related to the incidences and activities of dental caries [3]. These bacteria are able to efficiently utilize free sugars and thus promote the formation of cariogenic biofilm [4]. Biofilm is a matrix of polysaccharides synthesized by bacterial glucosyltransferase which produces extracellular polysaccharides (EPS) from dietary sugar. Biofilm further promotes bacterial adhesion, and generates a microenvironment which enhances the growth of acidogenic and cariogenic bacteria [5]. Recent research has established that a single specific cause of dental caries is free sugars [6, 7]. WHO recommends avoiding the excessive consumption of added sugars or "free sugars" to prevent dental caries. Free sugars can be defined as all the monosaccharides and disaccharides added to, and naturally present in, foodstuff [8, 9]. These sugars serve as the building block of EPS which is the core of biofilm or dental plaque. It is now commonly accepted that sucrose, or table sugar, is the most cariogenic sugar because it is readily metabolized by mutans streptococci yielding glucose and fructose, from which EPS is made up and acid is produced [5, 10, 11]. Recently, natural sugar has attracted interest as an alternative sweetener due to it being natural and unrefined, full-flavored and sweet-scented. It is generally used as sweetener for beverages and desserts, especially traditional dishes. These natural sugars are produced from several tropical plants including sugarcane (*Saccharum officinarum* L.), and several species of palms such as coconut palm (*Cocos nucifera* L.), palmyra palm (*Borassus flabellifer* L.), nipa palm (*Nypa fruticans* Wurmb.) [12, 13]. Sugarcane is primarily used to produce table sugar, also known as white sugar, which is a refined product containing up to 99.9% sucrose. Non-refined sugar alternatives have also attracted the interest of a range of people due to their nutritional and antioxidant properties [12-14]. Raw and brown sugar from sugarcane showed antioxidant activity which was directly related to the degree of refining, and was due to the retention of phenols and flavonoids [13]. Many studies have reported on palm sap sugar characteristics such as proximate analysis, aroma profile, reducing sugars content and antioxidant activity [12]. Moreover, recent work indicated that due to its antioxidant and nutritional properties, coconut sap, the material from which coconut sugar is derived, was a potential healthier sugar source which had a low glycemic index [15].

Therefore, natural sugar has become a popular non-refined sugarcane alternative to refined white sugar and a possible healthier candidate to be used instead of it. As for our knowledge, there is no report of these alternatives on their cariogenic potential. It remains unclear whether natural sugars have different cariogenic potentials to refined sugar. In this study, two palm sap-based sugars and two sugarcane-derived sugars were evaluated for their cariogenicity. Acidogenicity or glycolytic pH drop from sugar metabolization by cariogenic bacteria was evaluated. Biofilm biomass were determined using crystal violet staining. Moreover, antiseptic treatment of biofilm-induced from different sugars was studied to evaluate the robustness of the microbial matrix and metabolic activity was assessed using MTT assay. Our aim was to evaluate the cariogenic potential of natural sugars compared to that of refined sugar. Our research may well offer a base for further *in vivo* and dietary studies of these sugars versus refined sugar as causative agents of dental caries.

## 2. Materials and Methods

### 2.1 Microorganisms and sample preparation

*Streptococcus mutans* ATCC 25175 and *Streptococcus sobrinus* ATCC 33402 were cultivated on Brain Heart Infusion agar (BHI agar) in candle extinction jar (5% $CO_2$) for 48 h at 37°C. Pure refined, raw cane, coconut (*Cocos nucifera* L.), and palmyra palm (*Borassus flabellifer* L.) sugar

were purchased from local stores and supermarkets in Bangkok, Thailand. All samples were dissolved in deionized water and prepared as stock solution at concentration of 20% w/v.

## 2.2 HPLC analysis of monosaccharide and disaccharide in natural sugar

The analysis was performed using a Chromaster HPLC system (Hitachi High-Tech Corporation, Japan) and refractive index detector (RID) (Chromaster 5450 model). Samples were injected at the volume of 20 µl and separated using Benson polymeric (BP-800 H+) column at 35ºC with deionized water as mobile phase. The flowrate applied was 0.5 ml/min with a run time of 30 min. Standard sucrose solutions were injected to obtain the retention time. Sucrose content was determined based on peak area and calculated as % relative content.

## 2.3 Analysis of Acidogenic potential

Acidogenic potential was determined according to the method of Stegues *et al.* [16] with some modifications. A purified single colony of tested bacteria was inoculated into BHI broth (5 ml) and incubated in candle extinction jar at 37°C for 18 h. The overnight culture was centrifuged at 8,000 rpm for 10 min, and cell pellets were resuspended in a mixture solution of 50 mM KCl and 1 mM $MgCl_2$ (pH 7.4) in order to maintain pH value of the mixture in neutral range, which correlated with physiological pH in oral cavity. The cell suspension was required with the optical density at 600 nm of 1.0 (approximately $10^9$ cfu/ml). For co-culture, the mixture of *S. mutans* ATCC 25175 and *S. sobrinus* ATCC 33402 was prepared in the ratio of 1:1. Then 2 ml of bacterial suspension was transferred to 50 ml conical centrifuge tube that contained 18 ml of mixture solution supplemented with 2% w/v of the tested sugar. The pH value was monitored at different time points for 180 min (0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 90, 120, 150, and 180 min) using a polycarbonate electrode (Ionix instruments, Singapore). Time duration of 180 min was based on the duration that microorganism used to decrease the pH value from neutral pH to critical pH. The mixture solution of 50 mM KCl and 1 mM $MgCl_2$ (pH 7.4) was used as a negative control.

## 2.4 Biofilm formation of oral streptococci

The biofilm of oral streptococci was grown in 96-well polystyrene plate. *Streptococcus mutans* ATCC 25175 and *S. sobrinus* ATCC 33402 were cultured in BHI broth (5 ml) at 37°C for 18 h, and after that the turbidity of overnight cultures was adjusted to 0.1 at optical density 600 nm (approximately $10^7$ cfu/ml). Twenty microliters of bacterial suspension were added into 180 µl of BHI broth supplemented with tested sugar at concentration of 2% w/v. For dual-species biofilm, the mixture of *S. mutans* ATCC 25175 and *S. sobrinus* ATCC 33402 was prepared in the ratio of 1:1. The plates were then incubated and biofilm was allowed to form at 37°C for 24 h. Time duration for 24 h was based on the time needed for biofilm to mature and become well-attached.

## 2.5 Quantitation of biofilm biomass

To assess biofilm biomass, crystal violet (CV) assay as previously described method of He *et al.* [17] with some modifications was used. First, the growth medium was softly removed. Then the 96-well polystyrene microplates were washed three times with sterile phosphate-buffered saline (PBS), pH 7.4. The biofilm was fixed with 200 µl of absolute ethanol for 15 min, and after that the fixed biofilm was stained by adding 200 µl of CV solution (0.01% w/v). In order to remove the excess dye, the wells were rinsed three times with sterile PBS and 200 µl of DMSO was then added to solubilize the bound CV. The solubilized solution was transferred to a new 96-well polystyrene

microplates and measured the absorbance at a wavelength of 590 nm by microplate reader (Biochrom EZ Read 2000, UK).

### 2.6 Antiseptic treatment of biofilm-induced by different sugars

Biofilms of *S. mutans* ATCC 25175, *S. sobrinus* ATCC 33402, and dual-species was performed in 96-well polystyrene microplates using BHI broth supplemented with 2% w/v of tested sugars along with the negative control (BHI broth). Then, each biofilm was subjected to treatment with 0.12% w/v of chlorhexidine gluconate according to the following procedure. After washing with PBS, chlorhexidine gluconate solution was added and incubated for 1 min. Then, the antiseptic solution was removed and gently rinsed twice with sterile PBS (pH 7.4) to remove the residual antiseptics and dead cells. The remaining biofilm was assessed for metabolic activity of viable cells under the biofilm using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the method of Zhong *et al*. [18] with some modifications. Briefly, 200 µl of MTT solution (1 mg/ml, MTT in PBS (pH 7.4)) was added to biofilm and incubated under light protecting condition at 37°C for 3 h to allow the metabolically active cells to reduce the MTT into formazan crystals. After the designated time, excess MTT solution was removed, and the formazan crystals were solubilized by adding 150 µl of DMSO. The solubilized solution was transferred to a new 96-well polystyrene microplate and the absorbance at 560 nm was determined using microplate reader (Biochrom EZ Read 2000, UK). The percentage of eradication was calculated using the equation as described below:

$$\%\ Eradication = \frac{A-B}{A} x\ 100 \quad (1)$$

A was defined as the average absorbance of untreated biofilm, B was defined as the average absorbance of treated biofilm.

### 2.7 Statistical analysis

All experiments were carried out independently in triplicate. Data were represented as mean value ± standard deviation. For statistical analysis, IBM SPSS statistics 21 was used to perform the one-way analysis of variance (ANOVA) followed by Dunnett test to compare the test groups with the control group. Level of significance at 5% ($p < 0.05$) was considered to indicate statistically significant difference.

## 3. Results and Discussion

### 3.1 Composition of monosaccharide and disaccharide in natural sugar

The type of monosaccharide or disaccharide which was the major component in selected natural sugars was determined by using HPLC. The retention times and chromatograms of samples compared with standard sucrose were presented in Figure 1. The major peaks of all samples and standard sucrose were detected after 10 min with retention time in the range of 10.60-10.610 min. The results showed that that sucrose was the main component of refined sugar, palmyra sugar, coconut sugar, and raw cane sugar with percentages of relative content at 99.85, 99.73, 93.08, and 89.03, respectively (Table 1). In addition, glucose and fructose were presented in small amount in coconut sugar and palmyra sugar, with approximate retention time at 12.1 and 13.1 min (data not shown). The high level of sucrose in natural sugar might be one of the crucial factors related to the cariogenic potential of oral pathogens.

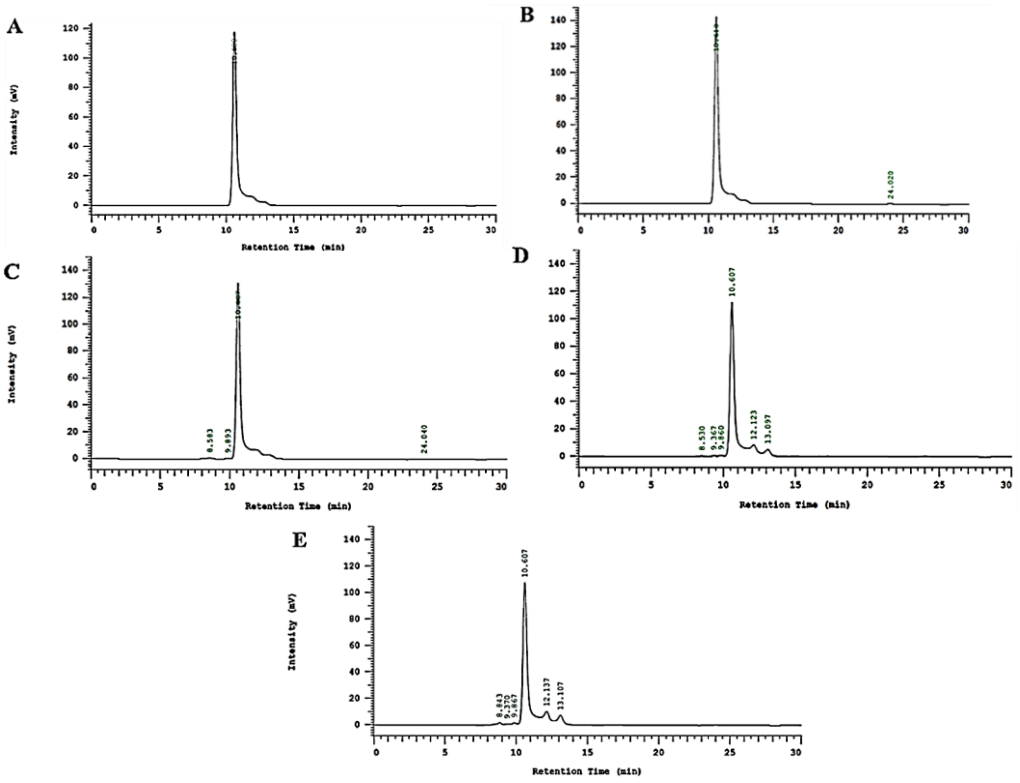


**Figure 1.** HPLC chromatographic profile of standard sucrose (A), refined sugar (B), raw cane sugar (C), coconut sugar (D), and palmyra sugar (E). The major peaks of standard sucrose, refined sugar, raw cane sugar, coconut sugar, and palmyra sugar were detected after 10 min.

**Table 1.** The HPLC analytical results of natural sugars

| Samples | Retention time (min) | % Relative content of sucrose |
|---|---|---|
| Standard sucrose | 10.600 | 100.00 |
| Refined sugar | 10.610 | 99.85 |
| Raw cane sugar | 10.607 | 99.73 |
| Coconut sugar | 10.607 | 93.08 |
| Palmyra sugar | 10.607 | 89.23 |

## 3.2 Acidogenicity

Glycolytic pH drop from sugar fermentation by cariogenic bacteria was shown in Figure 2. A similar pattern in pH fall curve was observed among various sugars in *S. mutans* ATCC 25175, *S. sobrinus* ATCC 33402 and co-culture. Significant difference of pH value for the four types of sugar was distinguished at 5 min ($p < 0.05$). Refined sugar showed significantly higher level of acidogenicity

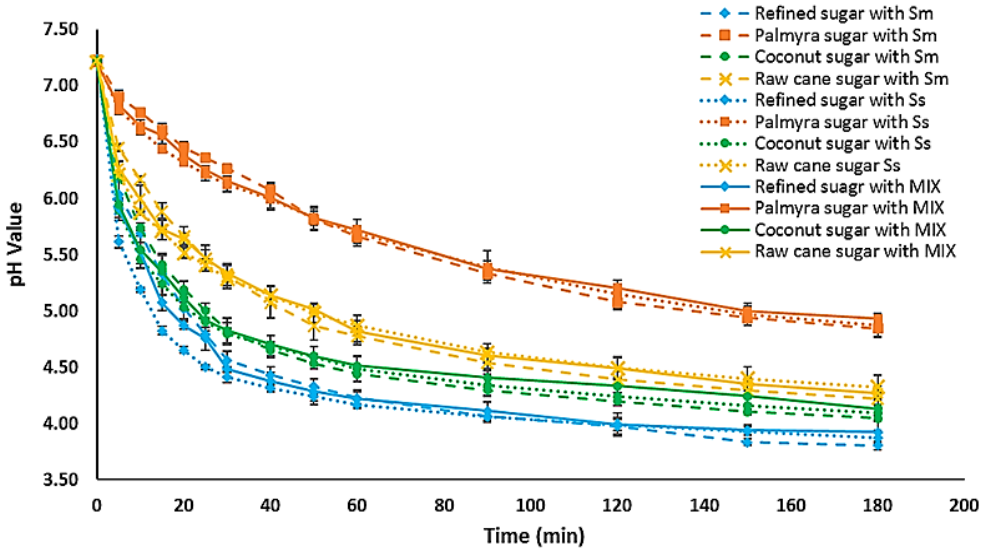


**Figure 2.** Acidogenic potential of *S. mutans* ATCC 25175 (Sm), *S. sorbrinus* ATCC 33402 (Ss), and co-culture (MIX) against refined sugar, raw cane sugar, coconut sugar, and palmyra sugar were presented as blue line, yellow line, green line, and brown line, respectively.

compared to other natural sugars in *S. mutans* ATCC 25175, *S. sobrinus* ATCC 33402 and co-culture at 5 min ($p < 0.05$), except that the variance between refined and coconut sugar in co-culture was observed for statistically difference at 15 min.

The presence of refined sugar influenced the pH fall the most, with the final pH values at 180 min of 3.80±0.04, 3.87±0.06 and 3.92±0.10 in *S. mutans* ATCC 25175, *S. sobrinus* ATCC 33402 and co-culture, respectively. Palmyra sugar resulted in the least pH change with the final pH values at 180 min of 4.84±0.07, 4.87±0.10 and 4.93±0.04 in *S. mutans* ATCC 25175, *S. sobrinus* ATCC 33402 and co-culture, respectively. Time to reach critical pH, which is acidic pH at 4.5-5.5 that affects the dental enamel of refined sugar fermentation faster than that of natural sugar fermentation. The slowest fermentation rate was found in palmyra sugar medium. It needed 90 min to reach a pH value of 5.3 whereas refined sugar took between 10 to 15 min. Hence, approximately about half of acidic end products were produced from glucose and sucrose [19]. According to the HPLC results, palmyra sugar showed the lowest sucrose content among the natural sugars resulting in the smallest pH dropping effect.

Even though sucrose is the major component of those natural sugars, brown color is from caramelization that occurs during production process from heat resulting in sucrose content reduction [12, 13]. As the same plant is the source of refined and raw cane sugar, it may well be that it is the degree of refining that results in different caloric nutrient or fermentable carbohydrate content in the sugar products. As reported by Seguí *et al*. [13], the sucrose contents in brown or non-refined sugarcane products ranged from 0.83 to 0.94 g per g product with low amount of glucose and fructose [13]. Moreover, retained components such as molasses and phenolic compounds in brown or non-refined sugarcane have been reported for their anti-mutans streptococci activities [20]. Recent study determined the sugar profile and total phenolic content of starting materials used for the production of natural sugars including sugarcane juice, coconut sap, and palm juice. Coconut sap had the highest monosaccharide and phenolic content [15]. As a result of this current study, sugar allocation in cariogenic bacteria may be one reason to explain the different effects of white and brown cane sugar on acidogenicity. In addition, it may primarily imply that impurities and

processing may affect some virulence factors of cariogenic microorganisms and more research is needed to clarify this.

### 3.3 Effect on biofilm biomass

The effect of sugar types on biofilm biomass based on crystal violet assay was shown in Figure 3 and Table 2. Compared to refined sugar, coconut sugar produced the least biofilm formation with statistically significance among all cariogenic biofilms ($p < 0.05$ in *S. mutans* ATCC 25175 and *S. sobrinus* ATCC 33402 biofilm and $p < 0.001$ in dual-species biofilm) whereas raw cane and palmyra sugar showed no significant difference from refined sugar. As describe above, the mono- and di-saccharide profile of tested sugar showed different sucrose content [15]. Sucrose influences pH change and promotes ecological and biofilm structural shift [11]. It is biologically described that external sucrose is used to produce extracellular polysaccharide (EPS), whereas internal sugars such as glucose or fructose are utilized for glycolysis and other mediated processes [21]. However, monosaccharide fructose plus glucose in combination can also cause caries [6]. On a dry basis, biofilm is mainly composed of cariogenic bacteria and EPS matrix which is created directly from sucrose substrate [22-24]. Previous study clearly demonstrated that sucrose formed cariogenic dental plaque to a greater degree than glucose plus fructose [10]. Duarte *et al.* [25] also reported that the polysaccharide matrix in sucrose supplementation was higher than in sucrose plus glucose supplementation. This suggests that the impurities in natural sugar provided less available substrate for EPS production, and this caused a decrease in the biofilm biomass which is consistent with the results of the current study.

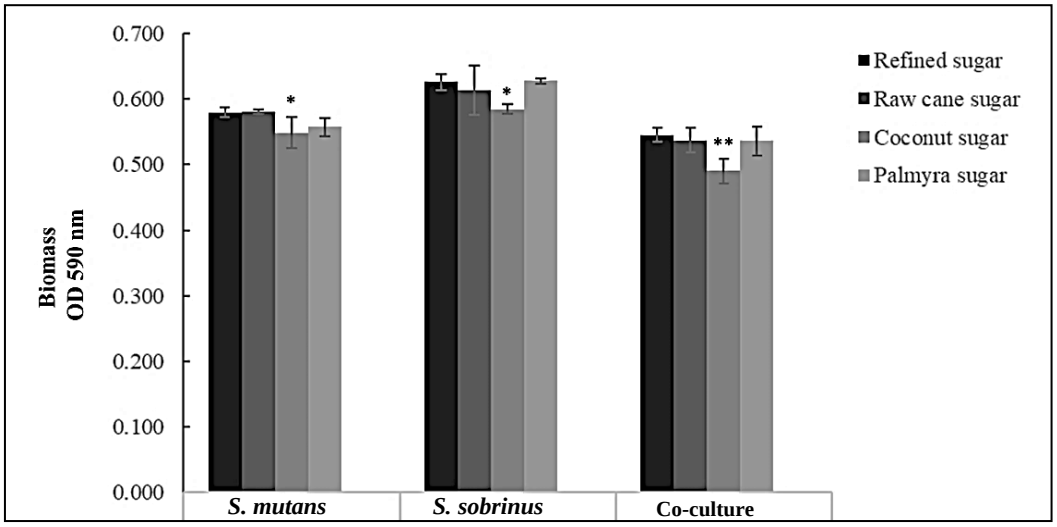


**Figure 3.** The effect of selected sugars (2% w/v) on biofilm formation of *S. mutans* ATCC 25175, *S. sobrinus* ATCC 33402, and co-culture. Statistical analysis was performed by one-way ANOVA followed by Dunnett t-tests by using refined sugar as control. Mean difference is significant at the level of significance: $p < 0.05$ (*) and $p < 0.001$ (**).

**Table 2.** Biofilm biomass based on crystal violet assay. Different superscript letters in the same column indicate significant differences at $p < 0.05$. Different capital letter in the same row indicates significant difference at $p < 0.05$ between bacterial individual and/or co-culture.

| **Biofilm formation (OD590 nm)** | ***S. mutans* ATCC 25175** | ***S. sobrinus* ATCC 33402** | **Co-culture** |
|---|---|---|---|
| Refined sugar | $0.580\pm0.007^{aA}$ | $0.626\pm0.012^{aB}$ | $0.545\pm0.010^{aC}$ |
| Raw cane sugar | $0.581\pm0.003^{aA}$ | $0.602\pm0.041^{aB}$ | $0.537\pm0.019^{aA}$ |
| Coconut sugar | $0.549\pm0.023^{bA}$ | $0.584\pm0.007^{bB}$ | $0.490\pm0.019^{bC}$ |
| Palmyra sugar | $0.557\pm0.014^{aA}$ | $0.628\pm0.004^{aB}$ | $0.536\pm0.018^{aA}$ |

Furthermore, the biofilm formation in the presence of coconut sugar yielded the lowest biomass level. This demonstrated that coconut sugar is not a suitable resource for biofilm formation of *S. mutans* ATCC 25175, *S. sobrinus* ATCC 33402, and co-culture when compared with refined sugar. This is preliminary data and human studies should be performed to confirm the effect of sugars on cariogenic plaque formation. In addition, the biofilm from *S. sobrinus* ATCC 33402 culture gave higher biomass than *S. mutans* ATCC 25175 and co-culture in all sugars. Acid by-products cause the micro-environment of dental plaque to be suitable for the growth of acid tolerant microbes. *Streptococcus sobrinus* strains are usually acidophilic and aciduric microbes [26, 27]. In addition, *S. sobrinus* can utilize glucose more efficiently than *S. mutans* [28]. In agreement with previous studies, *S. sobrinus* can produce more plentiful polysaccharide and may be more cariogenic than *S. mutans* [4].

### 3.4 Antiseptic treatment of biofilm

Palm-derived sugars gave less metabolically active microorganisms than refined sugar ($p < 0.05$ in co-culture biofilm in the presence of palm-derived sugars) (Table 3). The percentage of eradication was shown in Figure 4. After 1-min contact with 0.12% chlorhexidine, biofilm in the refined sugar medium was the most susceptible to antiseptic with 71.7% eradication of *S. sobrinus* ATCC 33402 biofilm while raw cane sugar dependent biofilm was the most resistant to antiseptic with 36.5% eradication of *S. mutans* ATCC 25175 biofilm.

Previous reports have mentioned that crude cane and molasses contained a protective agent that had an anticariogenic effect. In brown sugar, an anti-caries mechanism involved an enzyme inhibitor and its mineral content that reduced demineralization [29, 30]. This was contrary to our result in which raw cane sugar had more viability biofilm cells, and this may be due to the response to antiseptic of planktonic cells and microbes in biofilm being different. Usually, bacteria incorporated into biofilms are more resistant to antimicrobial agents than planktonic cells [31]. In addition, the different kinds of mono- or di-saccharide and other components in various natural sugars may affect the susceptible to antiseptics of cariogenic bacteria within biofilm. A different medium can have different effects on the bacterial growth, cell amount, glucosyltransferase (GTF) activity, and insoluble glucan yield [32].

Previous studies demonstrated that biofilm formed in sucrose media was significantly thicker than that in high fructose corn syrup media [33]. Moreover, the quality of unrefined sugars had variation based on source materials and non-standard processing technologies [34]. The analysis of compounds that responsible for the effect of microbial biofilm should be accomplished.

In addition, *S. sobrinus* can utilize glucose efficiently when compared to *S. mutans* [28]. In agreement with previous studies, *S. sobrinus* produced more plentiful polysaccharides and may be more cariogenic than *S. mutans* [4].

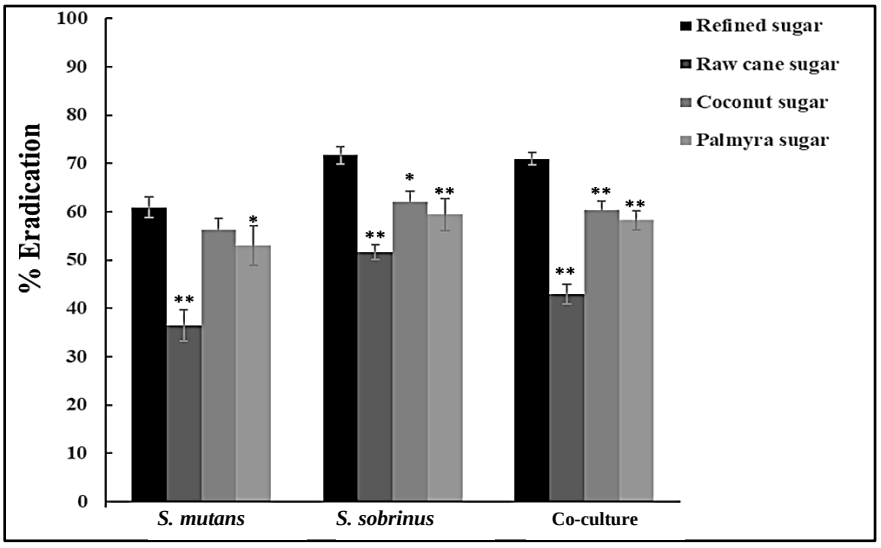


**Figure 4.** Percent eradication of viable cells under the biofilm after treated with antiseptic. Statistical analysis was performed by one-way ANOVA followed by Dunnett t-tests using refined sugar as control. Mean difference is significant at the level of significance: $p < 0.05$ (*) and $p < 0.001$ (**).

**Table 3.** Viable cell analysis of 24-h biofilm (before and after antiseptic treatment) by MTT assay. Different superscript letters in the same column indicate significant differences at $p < 0.05$.

| **Biofilm viable cell (OD560)** | ***S. mutans* ATCC 25175** | | ***S. sobrinus* ATCC 33402** | | **Co-culture** | |
|---|---|---|---|---|---|---|
| | Untreated | Treated | Untreated | Treated | Untreated | Treated |
| Refined sugar | $1.233\pm0.025^{a}$ | $0.482\pm0.017^{a}$ | $1.490\pm0.062^{a}$ | $0.422\pm0.010^{a}$ | $1.210\pm0.031^{a}$ | $0.351\pm0.009^{a}$ |
| Raw cane sugar | $1.189\pm0.010^{a}$ | $0.756\pm0.043^{b}$ | $1.414\pm0.034^{a}$ | $0.685\pm0.014^{b}$ | $1.200\pm0.030^{a}$ | $0.685\pm0.007^{b}$ |
| Coconut sugar | $1.210\pm0.032^{a}$ | $0.530\pm0.043^{a}$ | $1.433\pm0.091^{a}$ | $0.545\pm0.013^{b}$ | $1.130\pm0.041^{b}$ | $0.448\pm0.008^{b}$ |
| Palmyra sugar | $1.218\pm0.019^{a}$ | $0.573\pm0.048^{a}$ | $1.377\pm0.043^{a}$ | $0.559\pm0.036^{b}$ | $1.139\pm0.030^{b}$ | $0.476\pm0.017^{b}$ |

## 4. Conclusions

Natural sugar is commonly used in cultural dishes and desserts in Asia and South East Asia. However, fermentable carbohydrate, especially sugar, plays a pivotal role in the development of dental caries. This study aimed to clarify the cariogenic potential of various alternative natural sugars. The results of this study suggest that the palm-derived sugars, coconut and palmyra sugar, may have less acidogenic potential than refined sugars, and furthermore biofilm from palm-derived

sugars contained less metabolically active microorganisms than biofilm from refined sugar. Due to the variation in traditional processing and sources, sugar profile and presence of other compounds found in natural sugars need to be individually analyzed. Assuredly, analyses of virulence-related factors and biofilm such as confocal laser scanning microscopy and fluorescent staining should be conducted. Further studies included in *vivo*, and *in situ* investigations and studies involving human volunteers need to be performed to further discover and clarify the effects of sugar type on dental caries.

## 5. Acknowledgements

This research was kindly supported by Department of Biology, Faculty of Science, KMITL, Thailand and Department of Food Chemistry, Faculty of Pharmacy, Mahidol University, Thailand.

## References

[1] Kassebaum, N.J., Smith, A.G.C., Bernabé, E., Fleming, T.D., Reynolds, A.E., Vos, T., Murray, C.J.L., Marcenes, W. and GBD 2015 Oral Health Collaborators, 2017. Global, regional, and national prevalence, incidence, and disability-adjusted life years for oral conditions for 195 countries, 1990-2015: a systematic analysis for the global burden of diseases, injuries, and risk factors. *Journal of Dental Research*, 96(4), 380-387.

[2] Pitts, N.B., Zero, D.T., Marsh, P.D., Ekstrand, K., Weintraub, J.A., Ramos-Gomez F, Tagami J, Twetman, S, Tsakos, G. and Ismail, A., 2017. Dental caries. *Nature Reviews Disease Primers*, 3(1), 1-16.

[3] Okada, M., Kawamura, M., Oda, Y., Yasuda, R.I.E., Kojima, T. and Kurihara, H., 2012. Caries prevalence associated with *Streptococcus mutans* and *Streptococcus sobrinus* in Japanese schoolchildren. *International Journal of Paediatric Dentistry*, 22(5), 342-348.

[4] Zhan, L., 2018. Rebalancing the caries microbiome dysbiosis: targeted treatment and sugar alcohols. *Advances in Dental Research*, 29(1), 110-116.

[5] Bowen, W.H., Burne, R.A., Wu, H. and Koo, H., 2018. Oral biofilms: pathogens, matrix, and polymicrobial interactions in microenvironments. *Trends in Microbiology*, 26(3), 229-242.

[6] Sheiham, A. and James, W.P.T., 2014. A reappraisal of the quantitative relationship between sugar intake and dental caries: the need for new criteria for developing goals for sugar intake. *BMC Public Health*, 14(1), 863.

[7] Giacaman, R.A., 2018. Sugars and beyond. The role of sugars and the other nutrients and their potential impact on caries. *Oral Diseases*, 24(7), 1185-1197.

[8] WHO, 2015. *Guideline: Sugars Intake for Adults and Children*. Geneva: World Health Organization.

[9] Moynihan, P., 2016. Sugars and dental caries: evidence for setting a recommended threshold for intake. *Advances in Nutrition*, 7(1), 149-156.

[10] Cury, J.A., Rebelo, M.A.B., Cury, A.D.B., Derbyshire, M.T.V.C. and Tabchoury, C.P.M., 2000. Biochemical composition and cariogenicity of dental plaque formed in the presence of sucrose or glucose and fructose. *Caries Research*, 34(6), 491-497.

[11] Leme, A.P., Koo, H., Bellato, C.M., Bedi, G., and Cury, J.A., 2006. The role of sucrose in cariogenic dental biofilm formation-new insight. *Journal of Dental Research*, 85(10), 878-887.

[12] Saputro, A.D., Van de Walle, D. and Dewettinck, K., 2019. Palm sap sugar: A review. *Sugar Tech*, 21(6), 862-867.

[13] Seguí, L., Calabuig-Jiménez, L., Betoret, N. and Fito, P., 2015. Physicochemical and antioxidant properties of non-refined sugarcane alternatives to white sugar. *International Journal of Food Science & Technology*, 50(12), 2579-2588.
[14] Phillips, K.M., Carlsen, M.H. and Blomhoff, R., 2009. Total antioxidant content of alternatives to refined sugar. *Journal of the American Dietetic Association*, 109(1), 64-71.
[15] Asghar, M.T., Yusof, Y.A., Mokhtar, M. N., Ya'acob, M.E., Mohd Ghazali, H., Chang, L. S. and Manaf, Y.N., 2020. Coconut (*Cocos nucifera* L.) sap as a potential source of sugar: Antioxidant and nutritional properties. *Food Science & Nutrition*, 8(4), 1777-1787.
[16] Stegues, C. G., Arthur, R. A. and Hashizume, L. N., 2016. Effect of the association of maltodextrin and sucrose on the acidogenicity and adherence of cariogenic bacteria. *Archives of Oral Biology*, 65, 72-76.
[17] He, Z., Huang, Z., Jiang, W. and Zhou, W., 2019. Antimicrobial activity of cinnamaldehyde on *Streptococcus mutans* biofilms. *Frontiers in Microbiology*, 10, 2241.
[18] Zhong, H., Xie, Z., Wei, H., Song, Y. and Wang, M., 2019. Antibacterial and Antibiofilm Activity of Temporin-GHc and Temporin-GHd Against Cariogenic Bacteria, *Streptococcus mutans*. *Frontiers in Microbiology*, 10, 2854.
[19] Aizawa, S., Miyasawa-Hori, H., Nakajo, K., Washio, J., Mayanagi, H., Fukumoto, S. and Takahashi, N., 2009. Effects of α-amylase and its inhibitors on acid production from cooked starch by oral streptococci. *Caries Research*, 43(1), 17-24.
[20] Takara, K., Ushijima, K., Wada, K., Iwasaki, H. and Yamashita, M., 2007. Phenolic compounds from sugarcane molasses possessing antibacterial activity against cariogenic bacteria. *Journal of Oleo Science*, 56(11), 611-614.
[21] Kawada-Matsuo, M., Oogai, Y. and Komatsuzawa, H., 2017. Sugar allocation to metabolic pathways is tightly regulated and affects the virulence of *Streptococcus mutans*. *Genes*, 8(1), 11.
[22] Razak, F.A., Baharuddin, B.A., Akbar, E.F.M., Norizan, A.H., Ibrahim, N.F. and Musa, M.Y., 2017. Alternative sweeteners influence the biomass of oral biofilm. *Archives of Oral Biology*, 80, 180-184.
[22] Koo, H., Xiao, J., Klein, M.I. and Jeon, J.G., 2010. Exopolysaccharides produced by *Streptococcus mutans* glucosyltransferases modulate the establishment of microcolonies within multispecies biofilms. *Journal of Bacteriology*, 192(12), 3024-3032.
[24] Gupta, P., Gupta, N., Pawar, A.P., Birajdar, S.S., Natt, A.S. and Singh, H.P., 2013. Role of sugar and sugar substitutes in dental caries: a review. *International Scholarly Research Notices*, 2013.
[25] Duarte, S., Klein, M.I., Aires, C.P., Cury, J.A., Bowen, W.H. and Koo, H., 2008. Influences of starch and sucrose on *Streptococcus mutans* biofilms. *Oral Microbiology and Immunology*, 23(3), 206-212.
[26] Coulter, J., Jakubovics, N.S., Preshaw, P.M. and German, M.J., 2019. An in vitro model to assess effects of a desensitising agent on bacterial biofilm formation. *Acta Biomaterialia Odontologica Scandinavica*, 5(1), 1-8.
[27] Conrads, G., de Soet, J.J., Song, L., Henne, K., Sztajer, H., Wagner-Döbler, I. and Zeng, A.P., 2014. Comparing the cariogenic species *Streptococcus sobrinus* and *S. mutans* on whole genome level. *Journal of Oral Microbiology*, 6(1), 26189.
[28] Homer, K.A., Patel, R.U.P.A.L. and Beighton, D.A.V.I.D., 1993. Effects of N-acetylglucosamine on carbohydrate fermentation by *Streptococcus mutans* NCTC 10449 and *Streptococcus sobrinus* SL-1. *Infection and immunity*, 61(1), 295-302.
[29] Jaffé, W.R., 2015. Nutritional and functional components of non centrifugal cane sugar: A compilation of the data from the analytical literature. *Journal of Food Composition and Analysis*, 43, 194-202.
[30] Jaffé, W.R., 2012. Health effects of non-centrifugal sugar (NCS): a review. *Sugar Tech*, 14(2), 87-94.

[31] Liu, C., Worthington, R. J., Melander, C. and Wu, H., 2011. A new small molecule specifically inhibits the cariogenic bacterium *Streptococcus mutans* in multispecies biofilms. *Antimicrobial Agents and Chemotherapy*, 55(6), 2679-2687.

[32] Wiater, A., Choma, A. and Szczodrak, J., 1999. Insoluble glucans synthesized by cariogenic streptococci: a structural study. *Journal of Basic Microbiology: An International Journal on Biochemistry, Physiology, Genetics, Morphology, and Ecology of Microorganisms*, 39(4), 265-273.

[33] Ma, R., Sun, M., Wang, S., Kang, Q., Huang, L., Li, T. and Xia, W.W., 2013. Effect of high-fructose corn syrup on the acidogenicity, adherence and biofilm formation of *Streptococcus mutans*. *Australian Dental Journal*, 58(2), 213-218.

[34] Lee, J.S., Ramalingam, S., Jo, I.G., Kwon, Y.S., Bahuguna, A., Oh, Y.S., Kwon, O.J. and Kim, M., 2018. Comparative study of the physicochemical, nutritional, and antioxidant properties of some commercial refined and non-centrifugal sugars. *Food Research International*, 109, 614-625.