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COMPARATIVE EVALUATION OF CARIOGENIC POTENTIAL OF NATURAL SWEETENERS ON STREPTOCOCCUS MUTANS BIOFILM FORMATION AND ENAMEL DEMINERALIZATION: IN VITRO STUDY

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ABSTRACT

Objectives: The purpose of this study is to examine cariogenicity of commercial sweeteners, including Stevia sweetener, coconut sugar, monk fruit sugar, and date molasses, and their influence on *Streptococcus mutans (S. mutans)* biofilms, in comparison to sucrose.

Methods: Bacterial optical density was measured after culturing *Streptococcus mutans* with different sugar solutions using colony forming unit (CFU) assay. Moreover, twenty-five human caries-free premolars were incubated in sugar solutions with *Streptococcus mutans* and brain heart infusion growth medium for 21 days.

Results: We found that monk fruit and date molasses showed significant differences in bacterial optical density when compared to sucrose ($p<0.0001$). On the other hand, a mild noticeable reduction was associated with coconut sugar. Furthermore, microhardness tests of sucrose recorded significantly the lowest levels, while monk fruit showed the highest levels, with statistical significance ($p<0.001$).

Conclusion: According to our findings, all the experimental natural sweeteners evaluated in the present study have reduced cariogenicity and demineralizing effect, when compared to sucrose. However, they should be taken cautiously and in small doses because they have not been demonstrated to be completely cariogenic.

KEYWORDS: Sucrose, Natural sweeteners, Microhardness, Enamel demineralization, Dental caries

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INTRODUCTION

Dental caries is a prevalent disorder that affects approximately one-third of the world's population and is regarded as the most frequent non-communicable disease in the world [1]. This disease is multifactorial, biofilm-mediated, sugar-driven, and dynamic. It causes demineralization and remineralization of the tooth enamel. Moreover, acidogenicity is caused by the bacterial metabolism of sugar, which hastens demineralization and leads to caries lesions. *Streptococcus mutans* has long been linked to human dental caries [2]. These bacteria can efficiently use free sugars and increase the production of cariogenic biofilms [3]. The biofilm is a polysaccharide matrix produced by microbial glucosyltransferase that converts dietary sugar into extracellular polysaccharides (EPS). Additionally, biofilms stimulate bacterial adherence and create a milieu that enhances acidogenic and cariogenic bacterial development [4].

Several studies have found that free sugars are a single unique cause of dental caries [5,6]. Therefore, to prevent dental caries, the World Health Organization suggests reducing the quantity of added sweets or "free sugars" consumed. All monosaccharides and disaccharides, introduced to or naturally present in foods, are considered free sugars [7,8]. They provide the foundation of EPS, which is the basic component of biofilms or dental plaque. In addition, sucrose is often considered the highest cariogenic sugar since it is easily metabolized by mutans streptococci, generating both fructose and glucose, from which EPS is formed and acid is created [4,9].

Natural sugar has recently gained popularity as a substitute for sweeteners due to its natural and unprocessed nature, as well as its rich flavor and pleasant fragrance. These natural sugars are derived from various tropical plants and palm species, such as coconut palms [10]. It is commonly used to sweeten drinks and pastries. Consequently, sugar alternatives are being examined to avoid the influence of sucrose on the virulence of S. mutans. These sugar alternatives cannot be metabolized by cariogenic microbes, which results in decreased or no acid production. Moreover, they will not be substrates for glucan or fructan synthesis, and will not cause a reduction in the pH of the biofilm, lowering the pathogenic potential of dental plaque [11].

Stevia sugar extracts produced from the leaves of stevia include glycosides, especially stevioside, and rebaudioside-A, which makes them one of the most popular sucrose alternatives. These chemicals have a sweetness intensity that is 300 times greater than that of sucrose. As a result, they are utilized as sweeteners in foods and beverages [12]. In addition, there has been an increase in utilizing coconut sugar as a sweetening agent in foodstuffs. It is produced using the sap of the coconut tree. It is comparable to brown granulated sugar with regard to its physical characteristics and flavor. However, despite the benefits of coconut sugar, there are still no sufficient studies on its behavior in relation to dental caries [13]

Another sucrose substitute that has appeared recently is monk fruit sugar. Monk fruit is only found in China and Indonesia. It contains diverse bioactive compounds, such as essential oils, saccharides, proteins, vitamins, and flavones. This fruit has traditionally been used as a home treatment for curing sunstroke, severe thirst, sore throat, cough, and the cold [14]. Moreover, it is high in glycosides, such as mogroside. Hence, it might be an effective sugar alternative for people suffering from diabetes and obesity because of its high degree of sweetness (>300 times more than sucrose) and low calories [15]. Obviously, the growing demand for non-nutritive sweeteners derived from natural sources has expanded the appeal for monk fruit in the international market, including the nutraceutical, food, and beverage sectors [16]. Thus, long-term studies on the effects of mogrosides and the extract of monk fruit might be a unique topic for further research.

While glucose and fructose are a main form of carbohydrates, dates are also an adequate source of

it [17]. Dates fruit syrup is utilized in culinary goods to replace sugar. Studies have shown the significant impact dates have on human health [18]. Nonetheless, almost no studies have been performed on its cariogenic potential. Furthermore, information on the cariogenic potential of minimally processed sugars obtained from natural sources other than sugarcane is still unclear and limited. There have been very few researches conducted on it, particularly on date molasses and monk fruit. Natural healthier alternatives to sucrose are becoming increasingly popular worldwide. Therefore, this study aims to compare between sucrose and four commercial natural sweeteners (Stevia sweetener, coconut sugar, monk fruit sugar, and date molasses) concerning their cariogenic potential and effect on *Streptococcus mutans* biofilms. In order to do this, we presented and tested two alternate hypotheses: The researched natural sweeteners can have lower cariogenic potential than that of sucrose based on that (i) they will show less biofilm thickness, (ii) they will show higher degree of microhardness.

MATERIALS AND METHODS

This observational study included performing biofilm formation assessment of each sugar type using CFU assay. Moreover, their demineralization capacity was evaluated by measuring teeth microhardness.

Biofilm formation assessment

The standard *Streptococcus mutans'* strain (Sigma Aldrich, USA) was inoculated into a sterile tube containing brain heart infusion broth (BHI) growth medium. Then, it was incubated overnight at 37°C and 5% CO_2. On the next day, cells were harvested by centrifugation at 900g, 25 °C for 5 minutes under aerobic conditions, followed by Dulbecco's Phosphate buffer saline (PBS) (Gibco, Thermosientific, USA). Finally, the bacterial pellet was resuspended in 500μL of PBS.

Biofilm formation in 96-well microtiter plate

The cultured *Streptococcus mutans* were diluted to an optical density (OD) of 0.1 at 600 nm using a sterile growth medium. This corresponds to approximately 1 × 10^7 CFU/mL.100 µL of the diluted culture that was dispensed into each well of a sterile 96-well microtiter plate. Additionally, positive and negative controls are included. After that, the plate was incubated at 37°C for the desired biofilm formation period for 48 hours.

Bacterial count using optical density:

After the incubation of *Streptococcus mutants* with different sweeteners for a period of 48 hours at 37° C, the sample in each well was diluted 1:10 by transferring 100 µL of bacterial suspension into 900 µL of sterile, distilled water. Moreover, the OD was measured for each sample at 600nm using the spectrophotometer (***DIALAB Diagnostics DTN-405***, ***Neudorf, Austria)*** [19]***.*** Finally, the bacterial count was determined by using the standard curve, and multiplied by the dilution factor.

$OD_{final} = (OD_{test} - OD_{control})$ for each sweetener [20]

A standard curve of OD was plotted against the bacterial count (CFU/mL), by preparing a cell suspension of the *Streptococcus mutans*' strain, followed by adjustment of the spectrophotometer on OD 600nm for the bacteria and on Zero for the culture media (**Fig. 1**).

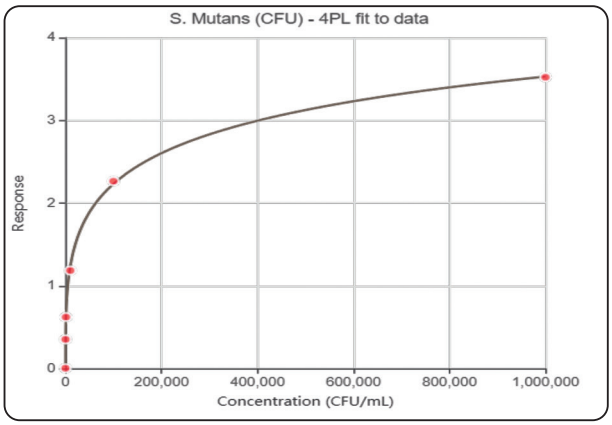

Fig. (1). XY plot illustrating the standard curve for S. mutans CFU/mL; the data was plotted as the O.D.600 of each standard dilution (Y) VS. the respective CFU/mL in each dilution (X).

Preparing the biofilm for CFU assay

Human enamel specimens were collected, sterilized, and cut into uniform pieces of approximately 5 mm × 5 mm. A single colony of *Streptococcus mutants* strain (Sigma Aldrich, USA) was inoculated into 5 mL of brain heart infusion (BHI) broth, and then incubated overnight at 37°C with shaking at 180 rpm. After that, the overnight culture was diluted to an OD 600 of approximately 0.1 in fresh LB, corresponding to roughly 10^7 CFU/mL.

Moreover, sterile 96-well flat-bottom polystyrene plates were used for biofilm formation. Each well received an enamel specimen with 200 μL of the diluted bacterial culture and the tested sugar. Furthermore, control wells containing sucrose instead of tested sugar were included to account for background absorbance. The plates were incubated at 37°C for 48 hours under static conditions to allow biofilm formation on the enamel specimens.

After incubation, planktonic cells were carefully aspirated from the wells without disturbing the biofilm on the enamel. Each well was gently washed three times with 200 μL of sterile phosphate-buffered saline (PBS) to remove non-adherent cells. Then, 200 μL of sterile PBS was added to each one. The biofilm was disrupted by sonication (5 minutes at 40 kHz) to release the cells into suspension.

Bacterial count adopting CFU assay:

Streptococcus mutants strain (Sigma Aldrich, USA) from batch culture, co-cultured with different types of sweaters, was diluted 1:10 by transferring 100 µL of bacterial suspension into 900 µL of sterile, distilled water. Besides, 100 μL of the diluted bacteria were pipetted and spread onto agar plates of brain heart infusion (BHI) agar supplemented, using a cell spreader. The plates were labeled with the Dilution Factor (1:10) and incubated for 48 hours at 37°C. At the end of incubation, the number of colonies was counted on each plate [20]. The number of colonies was calculated using the following equation:

Volume plated (mL) x "DF of the plate" = Total Dilution Factor

Number of colonies counted (CFU) / Total Dilution Factor = Total CFU/mL

It is noteworthy that the standard unit of measure for CFU is the number of culturable microorganisms present per 1 mL of culture (CFU/mL) [21]. In addition, inter-group Comparative analysis was done between the CFU/mL of S. mutants after being cultured with each of the five tested sweeteners for 48 hours. Every sample was performed in triplicates (n=3), and the mean±SD as well as the range was calculated.

Enamel microhardness assessment

Sample size for microhardness testing

To calculate the sample size for a particular group, G * power analysis adopted the mean and SD of the depth of demineralization in micrometers for the Stevia group and the sucrose group, based on a previous study [11]. Since there are five groups, the Type I error probability associated with this test is 0.05. Minimally, the study needed 3 subjects in each group, and the total sample size increased to 4 subjects in each group to compensate for 20% drop out. Therefore, we increased the sample size to 5 x 5 = 25. Additionally, the inclusion criteria included caries-free human premolars, without any development defects, cracks, white spots, or restorations.

Twenty-five human premolars that were extracted for orthodontic treatment were stored in 10% formalin solution for disinfection and to prevent bacterial growth. Moreover, a non-fluoridated pumice with prophylactic rubber cups was used to clean and polish the teeth. A nail polish was also used to cover all the surfaces, leaving only the buccal surface. This was followed by root

resection and blocking of the root end with wax. Besides, preparation and sterilization of the BHI solution were performed.

Subsequently, the *Streptococcus mutans* bacterial suspension was prepared. Each tooth was incubated at 37 °C with 3 ml of BHI broth for 24 hours. Then, contamination assessment was performed through the evaluation of the turbidity of the samples. After that, the tubes were randomly divided into 5 groups (n=5). Each group was placed into new flasks containing 100 ml of one of the different solutions.

Preparation of sample sweeteners

Each sample was prepared by adding 10ml distilled water and 90ml sterilized BHI broth to10g of one of the used sweeteners according to the group: Group 1: Sucrose; Group 2: Stevia; Group 3: Coconut sugar; Group 4: Monk-fruit sugar; and Group 5: Date molasses. Furthermore, each flask received 1.5 x 108 *Streptococcus mutans* ATCC 2517 cells (equal to 0.5 McFarland units). Daily, 2 ml of the old solution was withdrawn and replaced with a fresh 2 ml. The removed solution was grown in agar medium to ensure that it was free of contamination. The teeth were removed after 21 days and were mounted in acrylic resin and sectioned mesiodistally using a microtome.

Moreover, microhardness testing was performed for every sample before exposure to sugar solutions (baseline) and following demineralization. The microhardness was also evaluated by means of a digital display Vickers microhardness tester equipped with a Vickers diamond indenter and a 20X lens. Each specimen was exposed to 25 g stress for 5 seconds [22]. For each sample, three measurements were recorded, and the mean value was used to calculate the hardness number. Additionally, three microhardness measurements were taken for each specimen.

Statistical analysis

Statistical analysis was performed using SPSS 16® (Statistical Package for Scientific Studies), Graph pad prism, and windows excel. It has been presented in 2 tables and 1 graph. Exploration of the given data was performed using Shapiro-Wilk test and Kolmogorov-Smirnov test for normality, which revealed that the data was originated from normal data. In addition, One-way ANOVA was used. Upon observation of the significance of the p-value (i.e., P value < 0.05), a multiple comparison test (Tukey's test) was used to determine which pair of groups show a significant difference.

RESULTS

Comparative analysis has been done between the optical density of *Streptococcus mutans* after being cultured with each of the five tested sweeteners for 24 hours. *Streptococcus mutans* colonization was observed in all samples.

Assessment of the colonization of *Streptococcus mutans* in all groups was performed whereas, the number of colonies (CFU/mL) was measured. A high significant difference was detected among the CFU/ mL of the five tested solutions ($F=1.53$, $p<0.0001$). The data obtained are displayed in **Table 1, Table 2 and Fig. 2**.

Compared to microbial growth in sucrose sugar, a highly significant reduction in *Streptococcus mutans* growth was detected in date molasses ($p<0.0001$), monk ($p<0.0001$), and Stevia ($p=0.003$). However, a mild noticeable reduction was associated with coconut sugar ($p=0.035$). Moreover, as detected by Tukey's multiple comparisons test, no significant difference was identified for the CFU in monk fruit, when compared to data molasses ($p= 0.474$). In contrast, high remarkable difference was found among the other groups ($p<0.001$). The data are shown in **Table 2 and Fig. 3.**

TABLE (1) Number of S. mutans colonies calculated in different sugars

	OD at 600nm	CFU/mL	CFU/mL mean±SD	Range
S. mutans (untreated)	0.701	146,098.50	143,15±3,05	140,06 – 146,0
S. mutans (untreated)	0.697	143,367.70		
S. mutans (untreated)	0.692	140,006.30		
S. mutans + Sucrose	0.401	23,789.10	22,97±8,41	22,109 – 23,78
S. mutans + Sucrose	0.397	23,032.10		
S. mutans + Sucrose	0.392	22,109.40		
S. mutans + Stevia sugar	0.328	12,434.10	14,28±1,74	12,43 – 15,91
S. mutans + Stevia sugar	0.354	15,910.10		
S. mutans + Stevia sugar	0.344	14,503.60		
S. mutans + Coconut sugar	0.369	18,190.80	18,65±2,71	16,20 – 21,56
S. mutans + Coconut sugar	0.389	21,568.20		
S. mutans + Coconut sugar	0.356	16,202.10		
S. mutans + Monk fruit	0.242	4,621.80	4,38±0.27	4,08 – 4,62
S. mutans + Monk fruit	0.233	4,080.50		
S. mutans + Monk fruit	0.239	4,436.30		
S. mutans + Date molasses	0.186	1,929.00	2,28±0.46	1,93 – 2,803
S. mutans + Date molasses	0.191	2,108.00		
S. mutans + Date molasses	0.208	2,803.00		

OD: optic density; CFU: colony forming unit; S.mutans: Streptococcus mutans

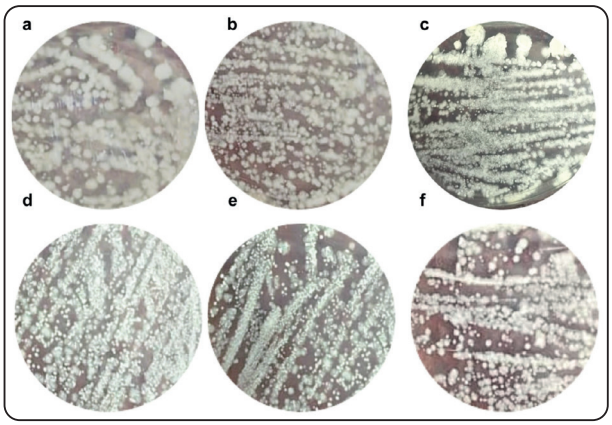

Fig. 2. Colony forming unit assay: (a) *S. mutans* grown in DMEM medium; (b) *S. mutans* grown in sucrose supplemented medium; (c) *S. mutans* grown in **Stevia sugar** supplemented medium; (d) *S. mutans* grown in **coconut sugar** supplemented medium; (e) *S. mutans* grown in **monk fruit** supplemented medium; and (f) *S. mutans* grown in **date molasses** supplemented medium. The magnification is 20X, and the scale bar is 20μm.

TABLE (2) Tukey's multiple comparisons analysis between CFU/mL for S. mutans cultured with five different sweeteners (one-way ANOVA test)

Tukey's multiple comparisons test	Mean diff.	95.00% CI of diff.	Adjusted P value
Sucrose VS. Stevia sugar	8694	4634 to 12754	0.0003[HS]
Sucrose VS. Coconut sugar	4323	263.4 to 8383	0.0359[S]
Sucrose VS. Monk fruit	18598	14538 to 22658	<0.0001[HS]
Sucrose VS. Date molasses	20697	16637 to 24757	<0.0001[HS]
Stevia sugar VS. Coconut sugar	-4371	-8431 to -311.1	0.0338[S]
Stevia sugar VS. Monk fruit	9903	5843 to 13963	<0.0001[HS]
Stevia sugar VS. Date molasses	12002	7942 to 16062	<0.0001[HS]
Coconut sugar VS. Monk Fruit	14274	10214 to 18334	<0.0001[HS]
Coconut sugar VS. Date molasses	16373	12313 to 20433	<0.0001[HS]
Monk fruit VS. Date molasses	2099	-1961 to 6159	0.4742[NS]

HS: high statistical significance (p<0.01); S: significant difference between compared groups (p<0.05); CI: confidence interval; ANOVA: Analysis of Variances

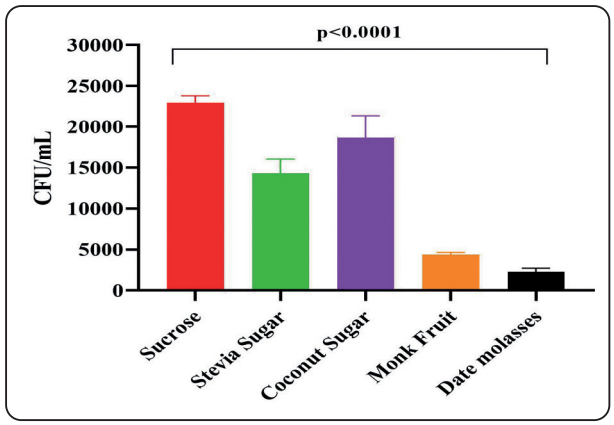

Fig. (3). Bar chart presenting a significant difference in CFU/mL for *S. mutans* growth after culture with five different tested sweeteners. Data are presented in mean±SD values.

Microhardness analysis

Microhardness analysis for the five groups showed that in post measurements, there was a significant difference between them (P=0.005). Obviously, sucrose (256.38±23.28) showed the least microhardness. On the other hand, date molasses (369.95±46.17) and monk fruit (339.9±49.28) manifested the highest microhardness, with insignificant difference between them. However, Stevia (314.65±32.34) and coconut sugar (291.8±21.9) revealed insignificant difference with all the other groups. Based on comparing between pre- and post-microhardness, the sucrose group (-121.4±20.87) showed the highest decrease in microhardness. In contrast, date molasses (-16.3±2.45) and monk fruit (14.85±2.86) reflected the least decrease in microhardness. Regarding percentage of change, the sucrose group (-32.15±5.46) showed the highest decrease in microhardness, but date molasses (-4.51±1.1) and monk fruit (-4.38±0.61) revealed the least decrease in microhardness (**Table 3** and **Fig. 4**).

TABLE (3) Mean and standard deviation of all groups pre- and post-microhardness, the difference between them, and percentage of change

	Sucrose		Stevia		Coconut sugar		Date molasses		Monk fruit		P value
	M	SD	M	SD	M	SD	M	SD	M	SD	
Pre	377.78 ^{a}	15.58	368.75 ^{a}	30.37	380.78 ^{a}	8.11	386.25 ^{a}	43.85	354.75 ^{a}	51.09	0.72
Post	256.38 ^{a}	23.28	314.65 ^{ab}	32.34	291.80 ^{ab}	21.90	369.95 ^{b}	46.17	339.90 ^{b}	49.28	0.005*
Diff	-121.40 ^{a}	20.87	-54.10 ^{b}	6.54	-88.99 ^{c}	17.13	-16.30 ^{d}	2.45	-14.85 ^{d}	2.86	0.0001*
% of change	-32.15 ^{a}	5.46	-14.77 ^{b}	2.29	-23.41 ^{c}	4.69	-4.51 ^{d}	1.10	-4.38 ^{d}	0.61	0.0001*

Significant difference as P<0.05. Means with different superscript letters were significantly different as P<0.05. Means with the same superscript letters were insignificantly different as P>0.05.

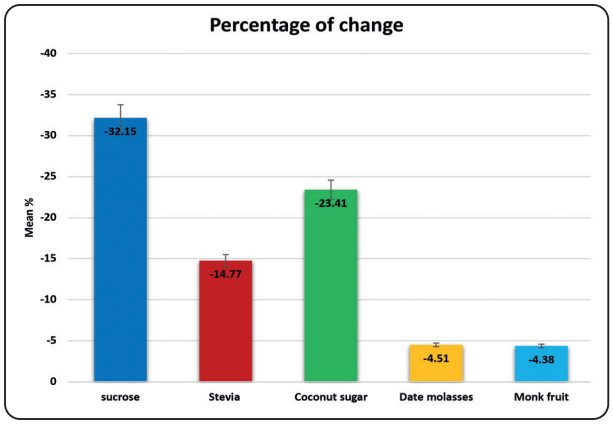

Fig. (4) Bar chart clarifying percentage of change in microhardness in all groups.

DISCUSSION

The detrimental role of sugar, especially in tooth decay, remains a subject of research [23]. For oral biofilms, sucrose is known to be a highly cariogenic substrate. Sweeteners are becoming more popular across the world. However, the cariogenicity of commercially available sweeteners, both nutritious and nonnutritive, has not been determined [24]. Numerous studies have linked caries to increased numbers of bacteria that create the biofilm since the amount of *S. mutans* isolated from plaque was assumed to have a key role in defining the caries risk of individuals [5].

Our results revealed that biofilms treated with Stevia, monk fruit, and date molasses had less biomass and viable cells than sucrose. However, they remained equivalent to it regarding the negative control. This implies that natural sweeteners are unlikely to be bactericidal since they do not encourage cell proliferation like sucrose does.

It was found that CFU in case of date molasses was the least among the five tested sugars, followed by monk fruit, and then stevia. On the other hand, it was found that coconut sugar recorded the highest bacterial count, which was comparable to the bacterial count in case of sucrose. These results agree with those found by Giacaman et al. who reported that stevia showed significantly less viable cells when compared with sucrose [24].

In addition, these results indicate that natural sweeteners, except coconut sugar that showed numbers so close to those of sucrose, reflect a consistent decrease in polysaccharide production, which is responsible for 40% of the composition of the biofilm [25]. Polysaccharides are one of the main virulence factors of the bacteria since they allow the adhesion of bacteria to the acquired pellicle, act as scaffolds for biofilm maturation, and increase structural porosity, which allows the diffusion of sugar within the biofilm [25].

In contrast, coconut sugar showed results regarding biofilm formation as well as enamel demineralization almost compatible with sucrose. This is in accordance with Marques et al. who found that *S. mutans* has a similar acidogenic potential and adhesion in solutions containing sucrose and coconut sugar [26]. In fact, the composition of coconut sugar is essentially sucrose (approximately 70%), with glucose, mannose, and fructose [27]. These components are readily metabolized by saccharolytic bacteria, which often results in exposure of tooth biofilms to low pH. This condition can cause an imbalance in the microenvironment, and, consequently, lead to the development of caries lesions [28].

Another clarification was proposed by recent research using messenger ribonucleic acid (mRNA) sequencing methods. It pointed out that the presence of sucrose and fructose significantly alters the expression of the *S. mutans* gene, affecting its energy metabolism, production of acid, tolerance of stress, and cell-to-cell communication. Fructose modifies the expression of a great number of genes related to virulence [29]. Thus, the presence of fructose in coconut sugar could lead to different cariogenic traits in S. mutans; nonetheless, its presence in coconut sugar did not reduce the acidogenicity and adhesion of *S. mutans* [26].

As expected, natural sugar substitutes induced enamel demineralization lower than sucrose. Obviously, no substitute could demineralize the enamel at levels similar to sucrose, except for coconut sugar. These data demonstrate that sucrose has a cariogenic potential higher than any other carbohydrate. These results were consistent with previous studies performed on sucrose, which showed that sucrose is highly cariogenic, and resulted in high demineralization [9,30]. Additionally, the concentrations of soluble and insoluble extracellular polysaccharides (EPS), and the proportion of insoluble EPS, were higher in the biofilm formed in the presence of sucrose.

Additionally, the current investigation found that the degree of demineralization with Stevia was lower than that generated by sucrose. These findings agree with those of a recent study that compared Stevia to glucose and fructose. The enamel caries depth of stevia extracts was less than conventional sweeteners such as glucose and fructose [31]. Furthermore, earlier in vitro and in vivo investigations have shown that Stevia is helpful in lowering cariogenic bacteria counts and enamel demineralization evaluated by polarized light. Stevia was also compared with xylitol, which proved to show the least in-vitro biofilm formation and depth of enamel demineralization when compared to sucrose and other natural sweeteners [11]. Stevia has many effects that can explain such results since it diminishes bacterial acid production, formation of extracellular polysaccharides, and triggering cell aggregation [31].

Regarding date molasses, despite its high viscosity, it showed low biofilm mass and demineralization[32]. Previous study suggested that sugar that was minimally processed preserved its mineral content; and during the metabolism of sugar by bacteria from the biofilm, this mineral content might interact in the demineralization/ remineralization process and lead to a different cariogenic response. Furthermore, it was earlier mentioned that molasses protects against decalcification due to the presence of calcium and potassium, with a significantly lower incidence of caries and minimally processed sugar; this is in comparison to refined sugar in in vitro and animal model studies [33].

The very low cariogenic potential of monk fruit sugar was explained by studies performed on its chemical and physical properties that stated monk fruit is noncariogenic with a zero glycemic index. Besides, monk fruit has antibacterial effects against not only *S. mutans* but also Porphyromonas gingivalis and Candida albicans [34]. Lastly, the low demineralization potential of some of the commercial natural sugars tested could be due to the lack of metabolism by *S. mutans* to other components added to the commercial forms, such as sugar alcohols, which are fermented poorly or not at all by oral bacteria, and consequently, have negligible cariogenicity [35].

Conclusion and Recommendations

According to the current study, it is possible to infer that all of the experimental natural sweeteners evaluated here are less cariogenic and have less demineralizing potential than sucrose. The amount and frequency of consumption of these tested sugars should be controlled even if they are marketed as "healthy products". Moreover, it is still critical to adopt a new policy for natural sugar substitution for sucrose as a means of controlling dental caries.

Furthermore, *S. mutans* metabolizes coconut sugar, presenting biofilm formation and demineralization almost comparable to sucrose. Accordingly, more studies evaluating the transcription of microorganisms in biofilms might explain whether there is any influence of glucose and fructose in the composition of coconut sugar on bacterial metabolism. Conclusively, the results of this study indicated that some caution should be taken regarding its cariogenicity.

Finally, since this study has not assessed the degree of enamel demineralization, we urge additional research with a bigger sample size that utilizes polarized light microscopy to reach more thorough results on demineralization caused by these natural sweeteners.

List of abbreviations:

S. mutans: *Streptococcus mutans*

OD: optical density

CFU: colony forming unit

EPS: extracellular polysaccharides

BHI: brain heart infusion

SDS-HCL: sodium dodecyl sulfate with hydrochloric acid

mRNA: messenger ribonucleic acid

Declarations:

Ethics approval:

This study has been accepted by the Ethics Committee of Faculty of Oral and Dental Medicine, Ahram Canadian University (IRB00012891#72).

Consent for publication

Not Applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All the authors contributed to all the steps in the practical part, including writing and reviewing the manuscript.

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