Alternative sweeteners influence the biomass of oral biofilm

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1. Introduction

Sugar is a common household ingredient that adds sweetness to enhance the taste of foods. The sugar consumed in our diet may come from fruits, vegetables, grains and dairy products. Simple sugars like glucose serves as an instant substrate for the generation of energy in the form of sweets and drinks, and often taken by athletes as instant energy booster prior to sport events. Sucrose is a disaccharide sugar that is easily available in the granulated form, and is the most commonly consumed sugar in human diet (Gupta et al., 2013). Today, a world population of seven billion people consumes roughly 165 million tonnes of sugar, that is 23 kg per capita on average and four billion of this consumers are concentrated in Asia (Arif, 2014). Despite its importance stance in the food industry, excess consumption of sucrose has been implicated with several health conditions including diabetes and dental caries.

In relation to oral health, stability and health of the oral cavity is influenced by the biomass of oral biofilm. The bacterial microflora characterizes the oral environment, and their responses to dietary factors in caries formation (Colby & Russel, 1997). Metabolism of fermentable sugars such as sucrose, fructose and glucose by oral streptococci produces acidic by-products in oral biofilm which in thin plaque, are continuously cleansed by oral fluids to neutralize and maintain pH within the biofilm (Marsh, 1994; Samaranayake, 2002). The type of organic acids produced may vary from one strain of streptococci to another as it is determined by the availability and type of the sugar substrate. When the availability of sugar is high, its utilisation by S. mutans is more efficient comparative to other streptococci. Under such condition, lactate dehydrogenase is activated which then accelerates the production of lactic acid (Banas, 2004). When lactate becomes a major component of plaque acids, the enamel structure becomes vulnerable as demineralization of the enamel begins at a critical pH of 5.5 and less (Ferguson, 2006; Leme, Koo, Bellato, ...
Bedi, & Cury, 2006; Marsh & Martin, 1999). When the provision of substrate is low, pyruvate-formate lyase instead becomes activated to produce organic acids of lower acidity such as formate (Marsh & Martin, 1999; Washio & Takahashi, 2016).

Apart from efficiently carrying out acidogenic activities, streptococci possess extracellular glycosyltransferase (GTF) and/or fructosyltransferase (FTF) that catalyse the formation of extracellular polysaccharides (ECP) such as glucans and/or fructans when the supply of sucrose is in excess (Schonfeld, 1992; Weiger, Netuschil, von Ohle, Schlagenhaus, & Brecc, 1995). ECP forms the backbone of the biofilm matrix and contribute to 50–95% of its dry weight (Gupta et al., 2013). If a less soluble ECP such as the branched mutant that is synthesised by S. mutans is produced, a voluminous sticky biofilm matrix is formed (Bradhaw, Marsh, Watson, & Allison, 1997; Marsh & Bradshaw, 1999).

The non-porous biofilm retains acid metabolites close to the tooth surface which upon long exposure, makes the enamel structure unstable and susceptible to demineralisation (Dawes, 2003; Ferguson, 2006). ECP also serves as a continuous source of substrate for saccharolytic resident bacteria. Due to these reasons, sucrose and other fermentable carbohydrates are categorised as cariogenic or caries-promoting sugars.

Sugar substitutes or alternative sweeteners have been shown to be effective in reducing the prevalence of dental caries as many of them are not metabolised to acid by plaque bacteria (ElSalhy, Sayed Zahid, & Honkala, 2012; Gupta et al., 2013; Matsukubo & Takazo, 2006). Sweeteners can be categorised as carbohydrate or non-carbohydrate origin. Carbohydrate sweeteners include sugar alcohols such as sorbitol and xylitol while non-carbohydrate sweeteners are chemically synthesised such as saccharin, aspartame and sucralose (Gupta et al., 2013; Maguire & Rugg-Gunn, 2003; Matsukubo & Takazo, 2006). Sorbitol is the most frequently used non-sugar sweetener. Although sorbitol could be adapted by the oral microbiota as a substrate, studies showed no increase in caries upon its frequent use (Hogg & Rugg-Gun, 1991; Marsh & Bradshaw, 1999). Non-carbohydrate sweeteners are usually calorie-free high-intensity sweeteners which accounts for their popular usage in slimming and health care products. Plant derived sweeteners such as stevioside and thaumatins are also calorie free (Matsukubo & Takazo, 2006). A lot of previous works had shown the inability of alternative sweeteners to produce acids but not many had focus on their effects on the ecology and structure of dental plaque.

This study aimed to assess the effect of several alternative sweeteners which include Equal Stevia®, Tropicana Slim® Pal Sweet® and xylitol on the matrix-forming activity of plaque bacteria, at both early and established stages of biofilm formation. The microbial component of the plaque biofilm includes S. mutans, S. mitis and S. sanguinis which constitute the dominant species in early dental biofilm. The cariogenic potential of the sweeteners was comparatively evaluated and analysed with reference to sucrose.

2. Materials and methods

2.1. Preparation of bacterial suspension

Stock cultures of S. mutans, S. sanguinis and S. mitis were separately revived in brain heart infusion (BHI) broth and incubated at 37 °C for 18 h. The bacteria cells were then washed in PBS and harvested by centrifugation at 3000 rpm, 4 °C, for 15 min. Bacterial suspension of each species was prepared in nutrient broth and the turbidity was standardised at an optical density (OD) of 0.144 at 550 nm. At this absorbance, the concentration of cells is standardised to about 10⁶ cells/ml, an equivalent of McFarland Standard # 0.5 (BioMerieux, France) (Fathilah & Rahim, 2003).

2.2. Collection of saliva and preparation of saliva-coated glass beads

3 ml of whole stimulated saliva was collected from a healthy subject. The saliva was first clarified by low speed centrifugation to remove debris and then passed through a filter of 0.2 μm pore size to sterilize before it was poured into a Petri dish. Filtration is the most common method used for saliva sterilization although the process may reduce the amount of total salivary protein (Ruhl et al., 2011). Sterile glass beads of 3 mm diameter were introduced and the dish was gently swirled on a rocker for 2 min to allow coating of the beads by the saliva. The saliva-coated glass beads (sGB) represent the pellicle-coated substratum for plaque formation.

2.3. Preparation of sucrose and alternative sweeteners

Equal Stevia®, Pal Sweet®, Tropicana Slim® and xylitol were used as test sweeteners and sucrose was included as the positive control. Treatment with distilled water was also performed to represent a negative control. Details on the composition and manufacturer’s information of the respective sweeteners are presented in Table 1. All test sweeteners were prepared to a standardised concentration of 10%. The test sweeteners were filter-sterilised and stored at 4 °C in sterile centrifuge tubes prior to use.

2.4. Preparation of dental plaque models

6 ml of mixed-bacterial suspension consisting of equal ratio (1:1:1) of S. mutans, S. sanguinis and S. mitis were pipetted into sterile Petri dishes. sGBs were aseptically introduced and immersed into the suspension. The Petri dishes were then incubated in a shaking incubator at 37 °C. After 3 h the Petri dishes were removed and the beads with biofilm formed on the surfaces were used to represent a 3 h-plaque model. Similar procedure was repeated with an extension of the incubation period to 24 h for the 24 h-plaque model. The former represented biofilm at the early stage, while the later at the established stage of formation. The population of adherent bacteria in the 3 h-plaque and 24 h-plaque models was determined and compared.

2.5. Determination of effect of test sweeteners on 3 h- and 24 h-plaque models

The 3 h- and 24 h-plaque models were separately placed in Petri dishes containing basic Nutrient broth as growth media. Three doses of test sweeteners (10%) were introduced at three intervals to simulate the

| Table 1 |
| Composition list and manufacturer’s information of each of the test sweeteners used in the study. |

<table>
<thead>
<tr>
<th>Test sweeteners</th>
<th>Presentation</th>
<th>Constituents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>Powder</td>
<td>Pure sucrose</td>
</tr>
<tr>
<td>Xylitol</td>
<td>Powder</td>
<td>Pure xylitol</td>
</tr>
<tr>
<td>Tropicana Slim®</td>
<td>Powder</td>
<td>Aspartame</td>
</tr>
<tr>
<td>Pal Sweet®</td>
<td>Powder</td>
<td>Sorbitol</td>
</tr>
<tr>
<td>Pal Sweet®</td>
<td>Powder</td>
<td>Corn powder</td>
</tr>
<tr>
<td>Pal Sweet®</td>
<td>Powder</td>
<td>Aspartame</td>
</tr>
<tr>
<td>Pal Sweet®</td>
<td>Powder</td>
<td>Acesulfame</td>
</tr>
<tr>
<td>Pal Sweet®</td>
<td>Powder</td>
<td>Lactose</td>
</tr>
<tr>
<td>Equal Stevia®</td>
<td>Powder</td>
<td>Stevia</td>
</tr>
<tr>
<td>Equal Stevia®</td>
<td>Powder</td>
<td>Erythritol</td>
</tr>
<tr>
<td>Equal Stevia®</td>
<td>Powder</td>
<td>Cellulose powder</td>
</tr>
</tbody>
</table>

| Table 2 |
| Adherent streptococci to saliva-coated glass beads indicated the colonization of bacteria at the early 3 h and established 24 h phases of biofilm formation. |

<table>
<thead>
<tr>
<th>Plaque age (h)</th>
<th>Adherent cells (×10⁶ cells/ml)</th>
<th>Colonization rate (×10⁶ adhering cells/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>1.4 ± 0.6</td>
<td>467</td>
</tr>
<tr>
<td>24</td>
<td>3.5 ± 0.4</td>
<td>146</td>
</tr>
</tbody>
</table>
exposure of dental plaque to sugar during three consecutive food intakes. The first dose which consisted of 0.6 ml of 10% preparation of the test sweetener was added into the Petri dish at zero-h. A 30 min exposure period was allowed before the medium was removed and replaced with 6 ml of fresh mixed bacterial suspension. The Petri dishes were further incubated for 3 h at 37 °C in a shaking incubator, after which a second dose of the sweetener was introduced. The procedure was repeated to receive the third dose of the sweetener. This regime was designed to mimic the three exposures of dental plaque to sugary food intakes at breakfast, lunch and tea. As a positive control, sucrose was similarly tested. The 10% sugar doses were replaced with distilled water as a negative control. Once the cycle was completed, the treated plaque models were removed and placed in microfuge vials containing 1000 μl of PBS. The adherent plaque mass was dislodged following a 10 s sonication (Fathilah & Rahim, 2003). Turbidity of the suspension due to the presence of plaque mass was measured using a spectrophotometer at a wave length of 550 nm, blanked with the negative control.

2.6. SEM examination

Upon completion of the treatment cycles, two sGB from each of the treatment on the 3 h and 24 h-plaque models were removed from incubation and fixed overnight in separate vials containing 1 ml of 4% glutaraldehyde (v/v). Following removal of the fixative and a gentle wash, 1 ml of sodium cacodylate buffer (0.1 M) was added. The beads were again fixed in 1% aqueous osmium tetro-oxide (w/v) overnight at 4 °C. The osmium tetro-oxide was then displaced with sodium cacodylate buffer and washed three times with fresh buffer each time. The sGBs were then treated with ascending percentage (10% to 100%) of ethanol (15 min each) to dehydrate the biofilms. Finally each sGBs were mounted onto aluminium stubs and coated with gold before examination under the SEM (JOEL JFC1100, Tokyo).

2.7. Determination of cariogenic potential of test sweeteners

The cariogenic strength of a sweetener was inferred by the reduced plaque mass produced in its presence in comparison to sucrose. It was used to indicate the inhibitory effect of the sweetener on the synthesis and production of extracellular plaque matrix. These values were derived using the following formula:

\[
\text{% matrix inhibition} = \frac{\text{OD}_{\text{positive control}} - \text{OD}_{\text{test sweetener}}}{\text{OD}_{\text{positive control}}} \times 100
\]

2.8. Statistical analysis

Data obtained was comparatively analysed with ANOVA and Tukey’s test using the Microsoft Excel 2010 statistical software.

3. Results

Higher colonization activity of streptococci to sGB was observed in the first three hours of biofilm formation compared to the subsequent period. This was indicated by the high adherence rate of bacterial cells at 467 × 10^3 cells/h reaching an adherent cell population of 1.4 × 10^6 cell/mL at 3 h of formation. The adherence activity in the subsequent hours was 3-fold slower at a rate of 146 × 10^3 cells/h, reaching a population of 3.5 × 10^6 cell/mL at the 24-h (Table 2).

Upon receiving the three doses of sucrose, a significant increase in turbidity was observed in both the 3 h- and 24 h-plaque models. A corresponding 70% higher level of plaque mass was recorded in the 24 h-plaque with a higher bacterial population (Fig. 1). A significant decline in the level of plaque mass in the 24 h-plaque

Table 3

<table>
<thead>
<tr>
<th>Test sweeteners</th>
<th>Reduction of plaque mass (%)</th>
<th>p value</th>
<th>Cariogenic potential with reference to sucrose equivalence (Tukey’s test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pal Sweet®</td>
<td>57</td>
<td>0.0034</td>
<td>Stevia &gt; Trop Slim = Pal</td>
</tr>
<tr>
<td>TropicanaSlim®</td>
<td>55</td>
<td>0.0076</td>
<td>Sweet &gt; Xylitol</td>
</tr>
<tr>
<td>Equal Stevia®</td>
<td>42</td>
<td>0.0193</td>
<td></td>
</tr>
<tr>
<td>Xylitol</td>
<td>68</td>
<td>0.0002</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Levels of plaque-mass produced in 3 h-plaque and 24 h-plaque models following three doses of Pal Sweet®, Tropicana Slim®, Equal Stevia® and xylitol in comparison to sucrose. Data produced were the mean of three trials performed in triplicates (n = 9).
was observed upon replacement of sucrose by the test sweeteners. Xylitol exhibited a 68% drop in the level of plaque mass (p < 0.05). Compared to sucrose, Pal Sweet®, Tropicana Slim® and Equal Stevia® exhibited a lesser degree but still significant reduction of plaque mass of between 42 and 57% (p < 0.05) (Table 3). Although not significantly different, the production of plaque mass with Equal Stevia® was higher compared to Pal Sweet® and Tropicana Slim® (Fig. 1). In contrast to the 24 h-plaque, production of plaque mass in the 3 h-plaque was not detected with Pal Sweet®, Tropicana Slim®, Equal Stevia® or xylitol indicating total inhibition of matrix synthesis.

Results obtained from the turbidity study were in accordance to that examined in the SEM micrographs (Fig. 2). The 3h-plaque models treated with Pal Sweet®, Tropicana Slim®, Equal Stevia® and xylitol showed minimal bacterial adherence as isolated single or short chain units. Over a longer period, bacterial adhesion in the 24 h-plaque remained low but bacterial aggregation forming small isolated colonies was observed with Pal Sweet®, Tropicana Slim® and xylitol. Heavy bacterial aggregation with significant presence of plaque matrix was however observed following treatment with Equal Stevia®. Sucrose, representing a positive control had caused heavy bacterial aggregation and produced dense plaque mass in the 24 h-plaque (Fig. 2). Distilled water did not support the adhesion of streptococci to the sGB. The degree to which the respective sweeteners were able to reduce the mass of formed plaque gives an indication as to its effectiveness as an anticariogenic agent. Table 3 summarizes the cariogenic potential of the test sweeteners with respect to sucrose.

4. Discussion

During the early phase of plaque formation, streptococci successfully settled on the tooth surface by adhering to the various binding receptors provided by components in the salivary-pellicle coating the tooth surface. These early plaque colonisers constitute predominantly of the streptococci, hence the selection of S. mitis, S. sanguinis and S. mutans for the 3 h- and 24 h-plaque models in this study. Adherence of bacteria to the saliva-coated tooth surface happens in a selective manner and thus occurs in sequential phases with time. Once anchored, these bacteria survive by utilising endogenous carbon compounds present in the pellicle for energy generation to ensure smooth running of biosynthetic processes for their maintenance and reproduction (Cole & Eastoe, 1988; Marsh, 1994). Due to the limited availability of endogenous substrates, microbial generation during the initial stage of plaque formation is generally slow.

In this study, the 3 h-plaque model represented plaque at the early stage of its formation while the 24 h-plaque model represented a more established biofilm. Within the first 3 h of its formation, approximately $1.4 \times 10^6$ cell/mL of streptococci were attached to the glass beads forming the 3 h-plaque. As the biofilm was allowed more time to establish, the microbial population was significantly increased by 1.5-fold to $3.5 \times 10^6$ cell/mL in the 24 h-plaque. The colonization of sGB at the early phase was faster compared to subsequent hours (Table 2). In addition to bacterial adherence, the increased cell population in the established plaque may also be due to the multiplication of pre-existing streptococci, made possible using energy generated from glycolysis. Such activity is common and has been reported in established 24 h dental plaques (Weiger et al., 1995). Bradshaw et al. (1997) had also reported that microorganisms may synthesize a wide range of molecules with potential for involvement in biofilm formation. Among factors identified to be involved include various receptors and signalling molecules (Sambanthamoorthy et al., 2014).

In the present study, sucrose was found to be highly utilised by S. mitis, S. sanguinis and S. mutans to produce ECP as indicated by the high turbidity (Fig. 1) and matrix presence (Fig. 2) both in the early and established plaques. Lower ECP yield in the early plaque was because sucrose was mainly utilised for the generation of energy required to support active colonization activity (Table 2). Aspartame, acesulfame, erythritol and stevia that are respectively present in Tropicana Slim®, Pal Sweet® and Equal Stevia® are however, non-fermentable sweeteners. And like sorbitol or xylitol, cannot be utilised for energy generation (ElSalhy et al., 2012; Runnel et al., 2013). These alternative sweeteners also did not support the formation of ECP (Fig. 1).

In the established biofilm however, alternative sweeteners seem to have caused an increase in plaque turbidity (Fig. 1). It is suggested that some of the constituents added to the sweeteners such as corn and cellulose powder (Table 1) may have contributed to this effect. When bacteria is forced to grow under an unfavourable growth condition, which in this case the absence of sucrose, they may adapt by producing...
enzymes that allows them to oxidize environmental energy sources to survive (Fathilah, Aishah, & Zarina, 2007). These enzymes include amylase which degrades starch, protease for proteins, lipase for lipids, and DNase which degrades DNA. This may explain the presence of some ECP in the micrograph of Equal Stevia-treated 24 h-plaque (Fig. 2). Erythritol and cellulose present in Equal Stevia. Although erythritol is an uncommon substrate for bacterial energy generation, it has been reported that a minimal amount can be digested by intestinal bacteria to generate short fatty acids and other organic acids (Hiele, Ghoos, Rutgeerts, & Vantrappen, 1993; Munro et al., 1998). It has also been shown that S. mutans-biofilm formed in the presence of erythritol tended to be less adherent and easily removed upon rinsing (Söderling & Hietala-Lenkkeri, 2010). A broad array of carbohydrates like cellulose has also been reported metabolizable by certain enterococci, which gives them robust advantage in adapting to a competitive growth environment (Ramsey, Hartke, & Huycke, 2014). However, utilisation of cellulose by streptococci needs further investigations. In the 3 h-plaque, such adaptations are unlikely as the process requires time and extension of the lag phase of bacterial growth (Fathilah et al., 2007; Gerhardt et al., 1981).

5. Conclusion

As a conclusion, Pal Sweet®, Tropicana Slim® and Equal Stevia® were equally effective as xylitol in reducing the presence of extracellular matrix in streptococci biofilms. Substituting sucrose with these alternative sweeteners does not support formation of biofilm matrix, hence improves the porosity of biofilm leading to the formation of thin, porous and healthier plaque.

Conflict of interests

All authors declare no conflict of interests.

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