PJN
ISSN 1680-5194
ansinet.com/pjn

PAKISTAN JOURNAL OF NUTRITION

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OPEN ACCESS Pakistan Journal of Nutrition

ISSN 1680-5194
DOI: 10.3923/pjn.2021.101.107

Research Article
Natural Sweetness of Fruits May Lead to Cariogenic Oral Biofilm: An *in vitro* Study

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Abstract

Background and Objective: Fruits are rich in essential vitamins and minerals and develop protection against many common ailments when taken adequately. The continuous presence of sugars in the mouth causes a microbial imbalance in the oral ecosystem and may lead to dental caries and gum disease if oral hygiene is compromised. This study aimed to determine whether regular consumption of mango, banana and pineapple juices have any effect on oral biofilm and to associate their sweetness properties to cariogenic potential. **Materials and Methods:** Mixed-bacterial suspension consisting of *Streptococcus mutans*, *Streptococcus sanguinis* and *Streptococcus mitis* was used to form early biofilm models of 3 h and established at 24 h. The biofilms were exposed to doses of fruit juices at three intervals to mimic three daily intakes. The treated biofilms were collected and determined for biofilm mass. The cariogenic potential was calculated with reference to sucrose. The sweetness properties of juices were evaluated based on Brix index, thin layer chromatography profile and reactions to Benedict's test. **Results:** The treated biofilms showed an increase in biofilm mass of approximately 11.3-, 5.7- and 4.6-fold for pineapple, banana and mango, respectively. The cariogenic potential of pineapple was almost equal to sucrose, followed by banana and mango. Brix index indicated mango and banana as the sweetest in the form of fruit chunks compared to pineapple. Whereas, in the form of fruit juices, banana exhibited the highest value of sweetness followed by mango and pineapple. Pineapple juice (pH 3.82) was the most acidic, while mango and banana were weak acids (pH 4.35 and 4.42). Reducing sugar glucose was higher in mango and pineapple than that of banana. All three juices have a high content of disaccharide sucrose. **Conclusion:** Banana, mango and pineapple juices have a high content of sucrose and glucose and are categorized as high sugar-containing fruits. All fruits have high cariogenic potential especially pineapple that is almost equal to sucrose.

Key words: Cariogenic potential, banana, mango, pineapple, biofilm mass, Brix index

Citation: Fathilah A. Razak, Syarifah Nur S.A. Rahman, Nur Sariyah M. Rosli, Azrin A.A. Rahim, Adyani A.A. Halim and Muhammad Luthfi, 2021. Natural sweetness of fruits may lead to cariogenic oral biofilm: An *in vitro* study. Pak. J. Nutr., 20: 101-107.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Fruits contain many healthful vitamins and minerals. They provide essential nutrients for the human diet. Many fruit juices are consumed on a daily basis as it is a convenient way to receive the benefits of fruits. Some fruits however, are relatively high in sugar compared to other whole foods. All fruits contain fructose, glucose, sucrose and sorbitol, however, different fruits have varying levels of each of these sugars[1]. During fruit ripening process, the level of glucose and fructose decreases while sucrose level increases[2]. In obese and diabetic people, it was suggested to replace sucrose or glucose with fructose as it decreases the blood glucose and insulin levels. This effect was however, to a lesser degree in people with normal glucose levels[3] because liver converts fructose into glucose before it can affect blood glucose concentrations. Thus, if sugar consumption cannot be avoided then it is probably better to choose fructose than other types of sugar. Fructose is richly present in fruits, over other types of sugar.

Concern of oral health professionals is more on the effect of fruit sugars which are often associated with sweetness, on dental health. These sugars, especially sucrose when consumed in excess, has been highlighted as one of the four etiological factors in caries formation[4]. Metabolism of fermentable sugars (sucrose, fructose and glucose) by oral streptococci residing in the oral biofilm produces acidic by-products that often gets accumulated within the biofilm when its structure is thick and compact. Acidity of the biofilm may cause demineralization of the tooth enamel at pH 5.5 or below[5]. Such incidence however, seldom happens when the biofilm structure is thin and porous, as the unrestricted in and out flow of saliva helps neutralize and maintain pH of the biofilm to about neutral[6,7].

Structure of the biofilm matrix is also greatly influenced by sugars. Besides performing efficient acidogenic activities, the oral streptococci also possess extracellular matrix-forming enzymes glycosyltransferase (GTF) and fructosyltransferase (FTF) that catalyze the formation of extracellular polysaccharides (ECP) when there is surplus supply of sugars[8,9]. ECP is the main component of biofilm matrix whether the biofilm is thin and porous, or thick and sticky, depends on the type of sugars forming it. Due to these properties, sucrose and other fermentable carbohydrates are categorized as cariogenic or caries-promoting sugars.

Based on the popular trend of drinking fruit juices, this study thus aimed to assess whether regular consumption of fruit juices of banana, mango and pineapple have any influence on the oral biofilm. The effect of the selected fruit juices on biofilm mass was measured by the change in the density of the biofilm suspension upon consecutive exposures to the juices.

MATERIALS AND METHODS

Effect of fruit juices on early and established oral biofilms

Preparation of bacterial suspension: Stock cultures of *S. mutans*, *S. sanguinis* and *S. mitis* were separately revived in brain heart infusion (BHI, Oxoid) broth and incubated at 37°C for 18 h. The bacteria cells were then washed in phosphate-buffered saline (PBS) and harvested by centrifugation at 5800 g, 4°C, for 10 min. Bacterial suspension of each species was prepared in nutrient broth (NA, Oxoid) and the turbidity was standardized at an optical density (OD) of 0.144 at 550 nm wavelength. At this absorbance, the concentration of cells is standardized to about 10^6 cells mL^{-1}, an equivalent of McFarland Standard No. 0.5[10].

Collection of saliva and preparation of saliva-coated glass beads: A volume of 20 mL of stimulated saliva was collected from a healthy subject. The subject was asked to chew sterile rubber for 15 min to stimulate saliva secretion. Following which the saliva was aseptically collected into a sterile flask. The saliva was centrifuged at 1000 rpm at 4°C, for 5 min to remove debris and then poured into a Petri dish. Sterile glass beads of 3 mm diameter were introduced and the dish was gently swirled in a shaking incubator for 2 min. After 2 min the saliva was removed and the sGBs were used to represent pellicle-coated substratum for biofilm formation. sGBs were kept sealed in the freezer (-20°C) prior to use.

Preparation of fruit juices, sucrose, glucose and xylitol: Three types of fruits (banana, mango and pineapple) were purchased from local market. The fruits were cleaned, peeled and cut into chunks. Concentrated juices (±100%) were prepared by mixing 50 g of each fruit chunks with 50 mL distilled water using a mortar. Once homogenized, the juices were left to set for 15 min prior to centrifugation at 2100 g, at 4°C for 10 min. The clear supernatant was collected while the pellet was re-suspended in distilled water and then centrifuged. The supernatant was again collected and the pellet washed. The procedure was repeated until the pooled supernatant make a stock of 50 mL. Each juice stocks were dispensed in smaller volume in separate centrifuge tubes for use in experiments. Stocks of sucrose, glucose and xylitol were each prepared at 10% concentration to be used as control in the experiments by mixing 5 g of sugar/xylitol with 50 mL of distilled water. All stock samples were kept in the freezer prior to use.

Preparation of experimental biofilm models: A 6 mL volume 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 and the Petri dishes were placed in a shaking incubator at 37°C. The Petri dishes were removed after 3 h and the formed biofilm on sGBs were used as a 3 h-biofilm model. Similar procedure was repeated with an extension of the incubation period to 24 h for the preparation of 24 h-biofilm model. The former was represented as the early stage, while the later was represented as established stage of biofilm. The population of adherent bacteria in the 3 h-biofilm and 24 h-biofilm models was determined and compared.

Determination of the influence of fruit juices on 3 h- and 24 h-biofilm models: The 3 and 24 h-biofilm models were separately placed in Petri dishes containing basic nutrient broth (Oxoid) as growth media. The experimental biofilm models were treated with three doses of concentrated fruit juices (±100%) at three subsequent intervals to simulate the exposure of oral biofilm during three consecutive daily intakes. The first dose of 3 mL of concentrated juice was added to the Petri dish at 0 h. A 30 min exposure period was allowed before the medium was removed and replaced with 6 mL of fresh nutrient broth. The biofilms were further incubated in a shaking incubator for 3 h at 37°C. The medium was removed and the biofilm coated glass beads were washed using PBS before a second dose of juices was given. Similar cycle of procedure was repeated to receive the third dose of fruit juices. This regime was designed to mimic the three exposures of oral biofilm to food intakes at breakfast, lunch and tea. In this experiment, sucrose (10%) was regarded as the positive control, whilst xylitol was used as the negative control. Once the cycle was completed, the treated 3 and 24 h-biofilm models were removed and placed in microcentrifuge vials containing 1 mL of PBS. The adherent biofilm mass was dislodged off the glass beads through sonication for 10 s[10]. Turbidity of the suspension which was due to the presence of biofilm mass was measured using ELISA reader at a wavelength of 550 nm.

Determination of cariogenic potential of fruit juices: The procedure of Razak *et al.*[11] was adopted to measure the cariogenic potential of the respective fruit juices. The cariogenic strength of the fruit juices was inferred by production of less plaque mass as compared to sucrose. It was used to indicate the inhibitory effect of the fruit juices on the production of extracellular biofilm matrix. These values were derived using the following formula:

$$\text{Matrix inhibition } (\%) = \frac{OD_{\text{Positive control}} - OD_{\text{Sample}}}{OD_{\text{Positive control}}} \times 100$$

Assessment on sweetness properties of fruit juices

Determination of sweetness index: The sweetness index of banana, mango and pineapple [% soluble sugar content (SSC) or Brix] was measured using a handheld refractometer (Atago, Tokyo). The % SSC or Brix reading was evaluated based on soluble (dissolved) sugar (or solid) content of the fruit. In this experiment, two sweetness measurements were recorded; (1) The solid content and (2) Juices of the fruits. For the former, the fruits specimen was cut into small pieces, crushed using a mortar and then filtered through cheesecloth to obtain a cleared, fruit filtrate. Whilst, for the later, stocks of the respective fruit juices prepared at ±100% were used. The refractometer was calibrated using distilled water and cleaned with disinfectant prior usage. The calibration adjuster was adjusted to zero. Few drops of sample were dropped on the glass measuring surface using plastic pipette and the flip was covered. The eyepiece was focused under a light ray and the % SSC or Brix value was recorded once the reading becomes stable. After each reading, the glass measuring surface was cleaned using distilled water.

Determination of sugar constituents in fruit juices using TLC: Thin Layer Chromatography (TLC) technique was used to determine sugar constituents of the fruits. Analytical TLC was performed on glass plates pre-coated with silica gel 60 F_{254} (Merck of 20.25 mm). A small amount of the respective fruit juices was spotted at 1 cm apart, about 1 cm from the bottom of the TLC plate. Spots of sucrose and glucose were included as reference. The glass plate was then placed in a covered developing tank containing solvent mixture of 1-butanol-2-propanol-water (1:3:1)[12]. The chromatography was run to allow separation of sugar molecules contained in the spots. The system was stopped when the solvent front is about 0.5 cm from the glass edge. The plate was removed, dried and sprayed with vanillin ethanol solution (1 g vanillin, 100 mL 95% ethanol and 10 mL of 95% sulphuric [VI] acid) for detection of separated sugar spots. The plate was then heated to 100°C until the color spots became visible and stable. The spots were marked and the retention factor (R_f) value was determined. R_f is used to quantify the movement of sugar molecules along the plate. These values were derived using the following formula:

$$R_f \text{ of component A} = \frac{d_A}{d_S}$$

d_A : Distance spot moved
d_S : Distance solvent moved

Determination of reducing sugar presence in fruit juices: A conventional Benedict's test was performed to determine the presence of reducing sugar in the fruit juices. A volume of 2 mL of Benedict's reagent was added to a test tube containing 1 mL of the respective fruit juices. The test tubes were then heated in a beaker of boiling water for a few minutes. Using a test tube holder, the test tubes were gently agitated until a color change from green to red precipitation was observed. The presence of reducing sugars was indicated by the appearance of brick-red precipitation.

pH measurement of fruit juices: The pH of each fruit juice samples was determined using a pH meter (Hanna Instruments). The pH meter was standardized and calibrated by dipping the electrode into standard solution with pH value of 4 and 7. The electrode was rinsed with distilled water prior to use and dipped into a volume of 2 mL of each juice samples. The pH reading for each samples was recorded.

RESULTS

The effect of fruit juices on early and established oral biofilm: Oral biofilm develops and mature over time. At the early phase of its formation, direct adhesion of bacteria to sGB resulted in colonization of saliva-coated surface. With time the biofilm becomes established as more bacteria are able to adhere and accumulate on the sGB. This was clearly shown in this study where turbidity of adherent bacteria was increased by 37% from the 3 h-biofilm to the established 24 h-biofilm.

Comparative to the 3 h-biofilm, juices of banana, mango and pineapple seem to have greater impact on the 24 h-biofilm. Profound increase in biofilm turbidity was seen in the 24 h-biofilm model. Pineapple juice exhibited the greatest increase in turbidity of the 24 h-biofilm (~11.3-fold), almost equivalent to the reading of the control, sucrose (Fig. 1). Slightly at a lesser degree, banana and mango each exhibited a 5.7-fold and 4.6-fold increase in biofilm turbidity following the three consecutive exposures. The effect of fruit juice exposures on the 3 h-biofilm was very low on both banana (OD of 0.007) and mango (OD of 0.008) and minimal (OD of 0.003) on pineapple. Upon receiving the three doses of sugar controls, sucrose was found to greatly support turbidity increase of the 3 h-biofilm. The effect of glucose and xylitol exposures was very low. In ascending order, the juices and sugars that supported 24 h-biofilm development was xylitol <glucose<mango<banana<pineapple<sucrose (Fig. 1).

The cariogenic potential was calculated with reference to sucrose equivalence based on the reduction in biofilm mass. The cariogenic potential of pineapple was the highest followed by banana and the least by mango. Pineapple exhibited the highest cariogenic potential almost at par to sucrose as it showed the lowest percentage of biofilm mass reduction. Glucose and xylitol had lower cariogenic potential, at 53 and 79% respectively (Table 1).

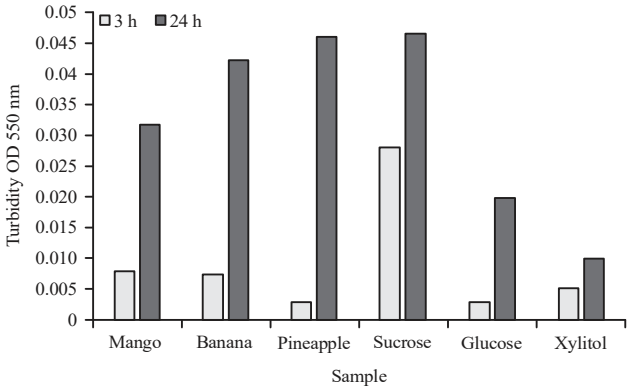

Fig. 1: Biofilm mass produced by 3 h- and 24 h-biofilms upon exposures to fruit juices and sugars
Levels of biofilm mass produced in 3 h- and 24 h-biofilm models following three doses of mango, banana and pineapple in comparison to control sugars glucose, xylitol and sucrose. Data produced were the mean of three trials performed in triplicates (n = 9)

Table 1: Cariogenic potential of banana, mango and pineapple juices

Test samples	Reduced biofilm mass (%)	Cariogenic potential with reference to sucrose equivalence
Sucrose	0	
Glucose	53	
Xylitol	79	Pineapple>banana>mango>glucose>xylitol
Mango	47	
Banana	11	
Pineapple	2	

The cariogenic potential of banana, mango and pineapple juices as indicated by their ability in causing a reduction in biofilm mass. The percentage of reduced mass was determined in comparison to sucrose

The sweetness properties of fruit juices: As observed in Table 2, the tabulated indexes represented sweetness as compared to sucrose which has a relative sweetness value of 100% wherein, 1-degree Brix (written °Bx) means 1 g of sucrose per 100 g of aqueous solution. Result showed that all three fruits were categorized as very sweet with >10°Bx, which is higher than the Brix index for the control sugars, sucrose, glucose and xylitol. Mango and banana showed the highest sweetness level in fruit chunks compared to pineapple. Whilst, in the form of fruit juices, banana exhibited the highest value of sweetness followed by mango and pineapple. Table 2 shows the pH values of each samples. The pH values 1-3, 4-6 and 7 represented strong acid, weak acid and neutral. In the present study, pineapple was the most acidic at the pH of 3.82. Mango and banana were categorized as weak acid with pH values of 4.35 and 4.42, respectively.

Based on Benedict's test, high presence of reducing sugar glucose (>2.0%G) was determined in mango and pineapple and slightly lower (1.5%G) in banana. Sucrose and xylitol remained blue (unchanged) as both are non-reducing sugars (Table 3). In addition to glucose, juices of mango, pineapple and banana were also shown to have high content of the disaccharide sucrose. This was displayed by the color spots on the TLC plates which was represented as the R_f values in Table 3.

DISCUSSION

According to the Dietary Guidelines for Americans, consumption of fruit juice has been positively associated with the intake of key nutrients such as vitamin C, folate, magnesium and potassium which have been identified as under-consumed nutrients in the year 2010[13,14]. Fruit juices are also sources of flavonoids and polyphenolic compounds that may confer health benefits[15]. The choice of Malaysian people regarding fruits is more multifarious than those of the Southeast Asian. Banana, pineapple and mango fruits were selected in this study because they are cheap and regularly consumed by the Malaysian. Although consumption of fruit juices has no adverse effects on health conditions[16], banana, mango and pineapple are categorized as high sugar-containing fruits and this is of great concern to the health professionals.

Sugar is a great nuisance to oral healthcare practitioners as it impacts the health of oral biofilm. Oral biofilm, which is defined as structured microbial communities on tooth surfaces, play an important role in most bacterial diseases such as dental caries and periodontitis[17]. *Streptococcus mitis*, *S. mutans* and *S. sanguinis* that constituted the experimental biofilm models in this study are predominant components of supragingival biofilm[18]. Streptococci species being saccharolytic bacteria utilize sugars via glycolysis to produce acidic by-products such as lactate and acetate that may lead to lowering the pH of the biofilm that is much associated with the initiation of dental caries[19,20]. It has been described that the critical pH for enamel demineralization is 5.5 and the teeth will erode in the pH range of 2.0-4.0. The chemical erosive potential of beverages can be segregated into 3 zones:

Extremely erosive : pH lower than 3.0
Erosive : pH 3.0 to 3.99
Minimally erosive : pH more than or equal to 4.0[5]

Table 2: The sweetness index (% SSC or °Bx) and acidity of the fruit juices

	SSC (%) or °Bx		
Test samples	Solid form	Liquid form	pH
Mango	16.0	13.9	4.35±0.07
Banana	16.0	15.4	4.42±0.04
Pineapple	13.3	10.3	3.82±0.06
Sucrose	-	10.0	6.53±0.07
Glucose	-	10.6	6.54±0.05
Xylitol	-	10.6	6.48±0.09

The table shows the sweetness index (% SSC or °Bx) and acidity of the fruit juices comparative to sucrose, glucose and xylitol sugar controls. The pH readings were the average of three measurements (n = 3)

Table 3: Sugar content of banana, mango and pineapple juices

	Benedict's test		Thin layer chromatography
Test samples	Precipitation (colour)	Reducing sugar (%)	Retention factor (R_f)
Glucose	Brick-red	>2.0 G%	0.65±0.01
Sucrose	Absent	0 G%	0.64±0.02
Xylitol	Absent	0 G%	0.65±0.02
Mango	Brick-red	>2.0 G%	0.66±0.03
Banana	Yellow	1.5 G%	0.66±0.01
Pineapple	Brick-red	>2.0 G%	0.67±0.02

Verification of sugar presence and reducing sugar content (G%) of banana, mango and pineapple juices based on thin layer chromatography technique and Benedict's test, respectively. The migration distance of each separated sugar molecules that appeared as dark blue spots on the TLC plate, were recorded as the retention factor (R_f)

However, another fact that is often missed is the ability of these species to synthesize polymers of saccharides known as extracellular polysaccharide (EPS) that contributes to increase the thickness of the oral biofilm[21,22]. However, this ability is influenced by the type of sugars that are used as substrate in the process, as well as the presence of GTF and/or FTF extracellular to the bacterial cell.

In this study, consecutive exposures of saliva-coated surface to juices of mango, banana and pineapple influenced the mass of the experimental biofilms, both in the early (3 h) and established (24 h) models (Fig. 1). In other words, mango, banana and pineapple can be utilized by oral streptococci for the production of extracellular matrix resulting in high density readings after treatments of biofilm models with the juices. This was to be expected as the three fruits (mango, banana and pineapple) contain high levels of sucrose and glucose (Table 2 and 3) which can be used as substrates for EPS synthesis. However, an increase in biomass density (turbidity) was seen in the established (24 h biofilm) models compared to the early (3 h biofilm) models (Fig. 1). Razak *et al.*[11], reported that the low count of bacteria at the early colonization phase (3 h) would explain the low synthesis of extracellular matrix captured by the density reading. In other words, less biofilm mass was produced in the early biofilm as the substrates were used more for energy generation to enable active colonization process rather than for the formation of ECP.

With regards to the control sugars, sucrose was shown to be better utilized by oral streptococci for the production of extracellular matrix (EPS) compared to glucose as the increase in biofilm mass density was recorded >2-folds higher for sucrose (Fig. 1). Xylitol supported very little production of biofilm mass. This is because, although, xylitol has a sweetness index equivalent to sucrose, its utilization as substrates for streptococci is limited[11].

Based on the ability of the fruit juices in reducing biofilm mass production with reference to sucrose, the cariogenic potential of each was evaluated (Table 1). Pineapple was found to be the most cariogenic as its cariogenic potential was almost at par to sucrose, followed by banana and the least by mango. Interestingly, all three fruits juice have cariogenic potential higher than glucose.

This study discovered that although fruit juices may have many beneficial nutrients but their frequent consumptions and prolonged exposures to the oral cavity may pose a risk to dental health as it supports EPS formation. Hence, leading to thick and compact-structured oral biofilm.

CONCLUSION

Pineapple, mango and banana fruit juices were found to influence the structure of oral biofilm. Frequent exposures to these high sugar-containing juices tend to increase the mass of oral biofilm especially when it is in the matured and established state. These juices have high content of sucrose and glucose and exhibited high cariogenic potential, especially pineapple that is almost equal to sucrose.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to the staff at the Balai Ungku Aziz Research Laboratory, Faculty of Dentistry and the Faculty of Science, University of Malaya for their efforts during the conduct of this experiment. The study was financially supported by the Faculty of Dentistry, University of Malaya through research grant No. GPF004E-2018.

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