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Influences of starch and sucrose on *Streptococcus mutans* biofilms

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Introduction: The combination of starch and sucrose has been shown to be potentially more cariogenic than either alone. The aim of this study was to examine the influence of starch and sucrose, alone or in combinations, on formation, polysaccharide composition, gene expression, and acidogenicity of *Streptococcus mutans* biofilms.
Methods: *S. mutans* UA159 biofilms were formed on saliva-coated hydroxyapatite (sHA) discs in batch culture for 5 days in the presence of 1% (weight/volume) starch, 1% sucrose, 1% starch plus 1% sucrose, 1% starch plus 0.5% fructose plus 0.5% glucose, or 1% sucrose plus 1% glucose.
Results: Amylase activity from sHA disks was detected up to 48 h, thereby increasing the availability of reducing sugars and acidogenicity in the early stages of biofilm development. *S. mutans* grown in the presence of sucrose alone or in combinations formed well-defined and tightly adherent biofilms comprised of mostly water-insoluble polysaccharides (INS); in contrast, the presence of starch or starch + glucose + fructose resulted in little biofilm formation with minimal amounts of INS. However, the combination of starch + sucrose produced biofilms with more biomass and acidogenicity, and a higher content of INS than those grown in sucrose or sucrose + glucose ($P < 0.05$). The INS extracted from biofilms formed in the presence of starch + sucrose displayed a higher percentage of 3-linked branching (3,4-, 3,6-, and 3,4,6-linked glucose) compared to those from biofilms grown in sucrose or sucrose + glucose. Furthermore, biofilms grown in starch + sucrose expressed significantly higher levels of *gtfB* messenger RNA than sucrose-grown or sucrose + glucose-grown biofilms ($P < 0.05$).
Conclusion: The combination of starch and sucrose has profound effects not only on the composition and structure of the polysaccharide matrix but also on gene expression of *S. mutans* within biofilms, which may enhance the cariogenic potential of dental biofilms.

Key words: amylase; biofilms; starch; *Streptococcus mutans*

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Dental caries results from the interaction of specific bacteria with constituents of the diet within dental biofilms known as plaque. Sucrose is considered to be the 'arch criminal' from the dietary perspective, because it is fermentable, and serves as a substrate for the synthesis of polysaccharides in dental biofilms (3, 27). In addition, starches are also an important source of fermentable carbohydrates, and are usually consumed simultaneously or interspersed with sucrose (24); starch is considered non-cariogenic or slightly cariogenic when used as the sole source of carbohydrate in the diet (24). However, combinations of starch and sucrose are potentially more cariogenic than either carbohydrate alone (2, 13, 29).

Streptococcus mutans is regarded as the primary microbial culprit of dental caries; this bacterium synthesizes extracellular polysaccharides, mostly glucans, from sucrose (and may also use starch hydrolysates as acceptors); it is acidogenic and acid-tolerant, which are critical virulence properties involved in the pathogenesis of dental caries in animals and humans (25, 28, 33, 37). Glucans promote the accumulation of microorganisms on the tooth surface, and contribute to the

establishment of the extracellular polysaccharide (EPS) matrix, which provides bulk and structural integrity for dental biofilms, and serve as a reserve source of energy (3). The formation of EPS matrix by *S. mutans* involves the interaction of at least three glucosyltransferases (GTFs) and an endodextranase, which participate in the synthesis and degradation of glucans; these enzymes are products of the *gtfB*, *gtfC*, *gtfD*, and *dexA* genes (15, 22). *S. mutans* synthesizes glucans directly from sucrose, but not from undigested starch. However, starches can be digested by salivary α-amylases to maltose, maltodextrins, and other oligosaccharides, some of which can be acceptors during glucan synthesis (14, 36).

Enzymatically active α-amylase and GTFs have been identified in salivary pellicles formed *in vitro* and *in vivo* (1, 23, 32, 35). Furthermore, starch hydrolysates produced by salivary α-amylase bound to saliva-coated hydroxyapatite (sHA) increased the synthesis of glucans from sucrose by surface-adsorbed GTF B; the hydrolysates also affected the structure and bacterial binding sites of the glucans (36). Moreover, maltose and maltodextrins from starch hydrolysis can be metabolized into acids by mutans streptococci (6). Clearly, starch could enhance the cariogenic potential of sucrose, as indicated by previous *in vivo* and *in situ* studies (2, 14, 29); the interaction of sucrose and starch through GTF enzymes and amylase adsorbed on the tooth surface may modulate *in situ* the development of cariogenic biofilms by influencing the synthesis of the EPS at structural and molecular levels, and the availability of fermentable carbohydrates for acid production.

Thus, the explanation for the greater cariogenicity of the dietary combination of starch and sucrose may be associated with biochemical and structural changes in the biofilms. In this study, we investigated whether combinations of starch and sucrose in the presence of surface-adsorbed salivary amylase and *S. mutans*, influence biofilm formation by affecting the synthesis and structure of EPS, and expression of the *gtfB*, *gtfC*, *gtfD*, and *dexA* genes using our sHA disc biofilm model (19).

Materials and methods

Amylase activity of salivary pellicle

Hydroxyapatite discs (Clarkson Chromatography Products, Inc., South Williamsport, PA; surface area 2.7 ± 2 cm^2) were coated with filter-sterilized, GTF-free, clarified human whole saliva (10, 19). The levels of amylase in saliva were unaffected by filtration, as determined experimentally by immunodetection and direct enzyme assay as described elsewhere (35). The sHA disc was incubated in ultrafiltered (Amicon 10 kDa molecular weight cut-off membrane; Millipore Co., Billerica, MA) buffered tryptone yeast-extract broth (pH 7.0) containing 1% starch (soluble starch −80% amylopectin and 20% amylose; Sigma Chemical Company, St Louis, MO) at 37°C and 5% CO_2 for 5 days; neither bacteria nor saliva was added to this solution. The 1% starch solution was replaced daily until the fifth day of the experimental period (120 h). Amylase activity was determined by measuring the amount of reducing sugars (4) released into the solution at different time-points to determine whether the surface-adsorbed amylase remain active on the HA surface over time.

Biofilm preparation and analysis

Biofilms of *S. mutans* UA159 (ATCC 700610) were formed on sHA discs placed in a vertical position using a disc holder (see Fig. 1) in batch cultures at 37°C in 5% CO_2 for 5 days (19). The biofilms were grown in buffered tryptone yeast-extract broth containing: (i) 1% starch, (ii) 1% sucrose; (iii) 1% starch + 1% sucrose; (iv) 1% starch + 0.5% glucose + 0.5% fructose; or (v) 1% sucrose + 1% glucose. The culture medium was replaced daily; pH values and amounts of reducing sugars and total carbohydrates in the medium were measured daily after the first 24 h of incubation. At the end of the experimental period (120-h-old biofilms), the biofilms were dip-washed three times, and then gently swirled in physiological saline to remove loosely adherent material. The biofilms were placed in 5 ml sterile saline solution, and the hydroxyapatite surfaces were gently scraped with a sterile spatula to harvest adherent cells. The removed biofilms were subjected to sonication using three 30-s pulses at an output of 7 W (Branson Sonifier 150; Branson Ultrasonics, Danbury, CT). The homogenized suspension was used for dry weight, total protein, and polysaccharide analyses. For the dry weight determination, three volumes of cold ethanol (−20°C) were added to 1 ml biofilm suspension, and the resulting precipitate was collected (10,000 ***g*** for 10 min at 4°C). The supernatant was discarded, and the pellet was washed twice with cold ethanol, and then lyophilized and weighed. Total protein in the biofilm suspension was determined by acid digestion followed by ninhydrin assay (26). The polysaccharide composition (extracellular water-soluble and insoluble, and intracellular polysaccharides) was determined by colorimetric assays as detailed by Koo et al. (18); the polysaccharide content was expressed per mg of dry weight or protein. Briefly, an aliquot (4 ml) of the suspension was centrifuged at 10,000 ***g*** for 10 min at 4°C. The supernatant was collected and the biofilm pellet was resuspended and washed in the same volume of water; this procedure was repeated twice. All the supernatants were pooled and three volumes of cold ethanol were added, and the resulting precipitate was collected. The precipitate, or water-soluble polysaccharides, were collected by centrifugation and washed three times with cold ethanol and resuspended in 1 ml MilliQ H_2O; the total amount of carbohydrate was determined

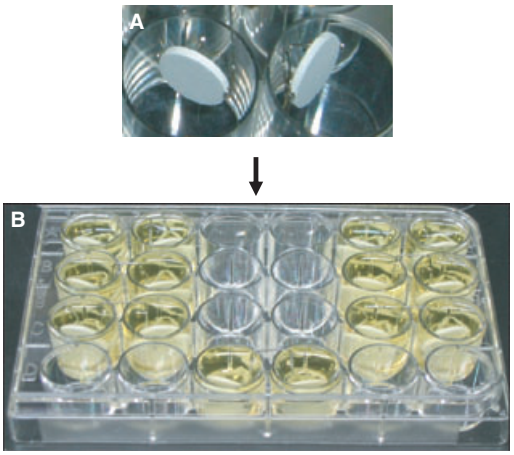

Fig. 1. Saliva-coated hydroxyapatite (sHA) biofilm model. (A) sHA discs placed in a vertical position; (B) biofilms forming in a 24-well plate.

by the phenol–sulfuric acid method (11). The biofilm pellet was dried in a Speed Vac concentrator and used for determination of: (i) extracellular insoluble polysaccharides; and (ii) intracellular iodophilic polysaccharides. The insoluble polysaccharides were extracted using 1 M NaOH (1 mg biofilm dry weight/0.3 ml of 1 M NaOH) under agitation for 2 h at 37°C. The supernatant was collected by centrifugation, and precipitated with three volumes of cold ethanol. The precipitate was washed three times with cold ethanol and resuspended in 1 ml 1 M NaOH; the total amount of carbohydrate was determined by the phenol–sulfuric acid method (11). The intracellular iodophilic polysaccharides were extracted with hot 5.3 M KOH (0.8 mg of biofilm dry weight/ml KOH) and quantified using 0.2% I_2/2% KI solution as described by DiPersio et al. (9).

Glycosyl linkage analysis

The extracellular water-soluble and insoluble polysaccharides were extracted as described above, and dissolved in dimethyl sulfoxide (21). For glycosyl linkage analysis, the polysaccharide extracts were methylated by a modification of the method of Ciucanu & Kerek (5) followed by combined gas chromatography/mass spectrometry (GC/MS) analysis as described by York et al. (38). The partially methylated aldital acetates were analyzed on a 30-m Supelco 2330 bonded phase fused silica capillary column by GC/MS using a Hewlett Packard 5890 GC interfaced to a 5970 MSD (mass selective detector, electron impact) as detailed elsewhere (21).

Extraction of RNA and real-time polymerase chain reaction

The RNA extraction and purification, and reverse transcriptase polymerase chain reaction (PCR) conditions and specific primers (for *gtfB*, *gtfC*, *gtfD*, and *dexA*) were similar to those described previously (7, 20). Complementary DNAs (cDNAs) were synthesized using a BioRad iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA). To check for DNA contamination, purified total RNA without reverse transcriptase served as the negative control. The resulting cDNA and negative control were amplified by a MyiQ real-time PCR detection system with iQ SYBR Green supermix (Bio-Rad Laboratories, Inc.) and specific primers. The critical threshold cycle (C_t) was defined as the cycle at which the fluorescence becomes detectable above the background and is inversely proportional to the logarithm of the initial number of template molecules. A standard curve was plotted for each primer set as detailed elsewhere (20). The standard curves were used to transform the C_t values to the relative number of cDNA molecules. Relative expression was calculated by normalizing each gene of interest of the biofilms grown in the presence of various carbohydrates to the 16SrRNA gene (internal control). These values were then compared to those from sucrose-grown biofilms to determine the change in gene expression.

Statistical analyses

An exploratory data analysis was performed to determine the most appropriate statistical test; the assumptions of equality of variances and normal distribution of errors were also checked. The data were then analyzed using analysis of variance, and the *F*-test was used to test any difference among the groups. When significant differences were detected, pairwise comparisons were made between all the groups using Tukey's method to adjust for multiple comparisons. Triplicates from at least three separate experiments were conducted in each of the assays. Statistical software JMP version 3.1 (30) was used to perform the analyses. The level of significance was set at 5%.

Results

The amylase activity of sHA discs in bacteria-free culture medium containing 1% starch was measured daily, and the results are shown in Fig. 2. The salivary amylase adsorbed on the hydroxyapatite surface was active 48 h after salivary pellicle formation, although the enzyme activity declined between 48 and 72 h.

The presence of sucrose alone or in combinations resulted in biofilms displaying four to seven times more biomass, seven to 15 times more total protein, and 10 to 15 times more total EPS than starch or starch + glucose + fructose-grown biofilms (Table 1). However, biofilms of *S. mutans* grown with starch in combination with sucrose exhibited significantly more biomass and total amount of EPS than the biofilms formed in the presence of sucrose, either alone or in combination with glucose ($P < 0.05$).

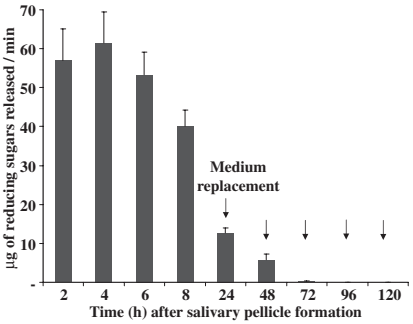

Fig. 2. Amylase activity indicated by the amount of reducing sugars released into the medium during incubation of sHA in 1% starch. The amylase activities at 2, 4, and 6 h after salivary pellicle formation were not significantly different from each other ($n = 12$; $P > 0.05$, ANOVA, comparison for all pairs using Tukey test).

The total amount (in µg/total biofilm dry weight) and content (in µg/mg protein) of extracellular insoluble (INS) and water-soluble (WSP) polysaccharides, and of intracellular polysaccharides (IPS) in biofilms are shown in Fig. 3. Biofilms formed in the presence of sucrose alone or in combinations displayed a significantly higher content of INS than starch or starch + glucose + fructose-grown biofilms ($P < 0.05$). The INS content in starch + sucrose-grown biofilms was significantly higher than that of the sucrose-grown and sucrose + glucose-grown biofilms ($P < 0.05$). On the other hand, starch- or starch + glucose + fructose-grown biofilms were comprised of mostly WSP, and showed higher content of the soluble polysaccharides than biofilms grown in sucrose alone or in combinations ($P < 0.05$). The amount (and content) of

Table 1. Biomass (dry-weight), total amount of protein, and EPS in *Streptococcus mutans* UA159 biofilms formed in the presence of starch and sucrose, alone or in combinations

Experimental groups	Dry-weight (mg)	Total amount of protein (mg)	Total amount of EPS (µg)
Starch	0.75 (0.27)[1]	0.1 (0.08)[1]	88.98 (17.84)[1]
Starch + sucrose	6.25 (0.69)[2]	1.3 (0.12)[2]	1747.99 (146.62)[2]
Sucrose	5.50 (0.45)[3]	1.5 (0.32)[3]	1411.28 (256.45)[3]
Starch + glucose + fructose	1.25 (0.42)[1]	0.2 (0.12)[1]	126.37 (16.58)[4]
Sucrose + glucose	3.92 (0.92)[4]	1.4 (0.21)[2]	850.31 (190.79)[5]

Values (SD, $n = 12$) in the same column followed by the same superscript numbers are not significantly different from each other ($P > 0.05$, ANOVA, comparison for all pairs using Tukey test).

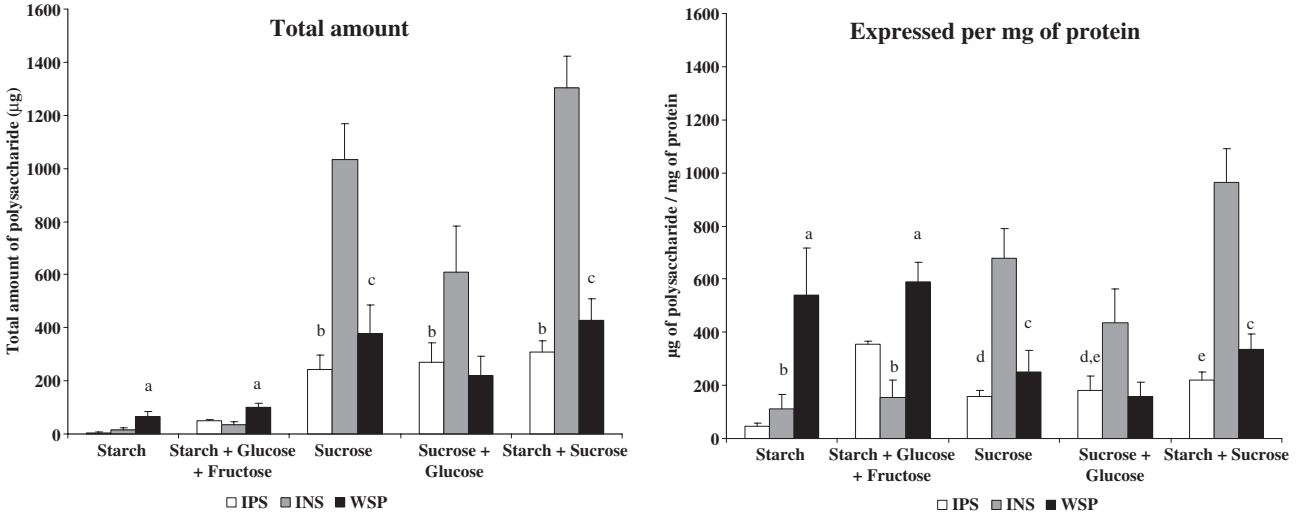

Fig. 3. Total amount (in µg/total biofilm dry-weight) and content (expressed per mg of protein) of intracellular (IPS) and extracellular water-soluble (WSP) and insoluble (INS) polysaccharides in *Streptococcus mutans* UA159 biofilms formed in the presence of starch and sucrose, alone or in combinations. Values (SD, $n = 12$) for each type of polysaccharides marked by the same letters are not significantly different from each other ($P > 0.05$, ANOVA, comparison for all pairs using Tukey test).

IPS in biofilms formed with sucrose alone or in combinations was significantly higher than in starch-grown biofilms ($P < 0.05$).

The activity of surface-adsorbed amylase on starch could increase the levels of fermentable carbohydrates in the culture medium, and thereby enhance the acid production by biofilms. Therefore, the reducing sugars and total carbohydrate levels, and the pH of the culture medium surrounding the biofilms were determined daily.

The pH of the culture medium was measured at various time-points during each 24 h incubation period, and the pH-drop curves are illustrated in Fig. 4. Biofilms grown in sucrose, alone or in combinations, rapidly lowered the culture pH to values below 4.5 each day of growth. Biofilms formed in the presence of starch + sucrose displayed the highest rate of acid production during the first 8 h of incubation of early-formed biofilms (between 24 and 48 h) showing significantly lower pH values than starch (t_{28h}, t_{32h}, t_{36h}, and t_{48h}), starch + glucose + fructose (t_{28h}, t_{32h}, and t_{36h}), sucrose (t_{28h} and t_{32h}), and sucrose + glucose (t_{28h}) -grown biofilms ($P < 0.05$). Biofilms formed in starch also lowered the culture pH to 5.3 in the first 48 h. The rate of acid production was slowed after 72 h even though fresh medium was added daily, which is consistent with a decline of amylase activity after 48 h of incubation. Furthermore, biofilms of *S. mutans* grown in starch, either alone or in combinations, displayed elevated levels of reducing sugars in the earlier stages (24–48 h) of biofilm formation (data not shown), which agrees well with the amylase activity data (Fig. 2) and the pH drop curves (Fig. 4).

In an approach to determine whether polysaccharide matrices formed in the presence of different carbohydrates had distinctive structures, the type of glycosyl linkages in WSP and INS extracted from the biofilms were determined (Table 2). Major structural differences were observed in WSP and INS from biofilms grown with starch or sucrose, alone or in combinations. Soluble polysaccharides from starch + sucrose-grown biofilms displayed higher percentages of 4-linked glucose and

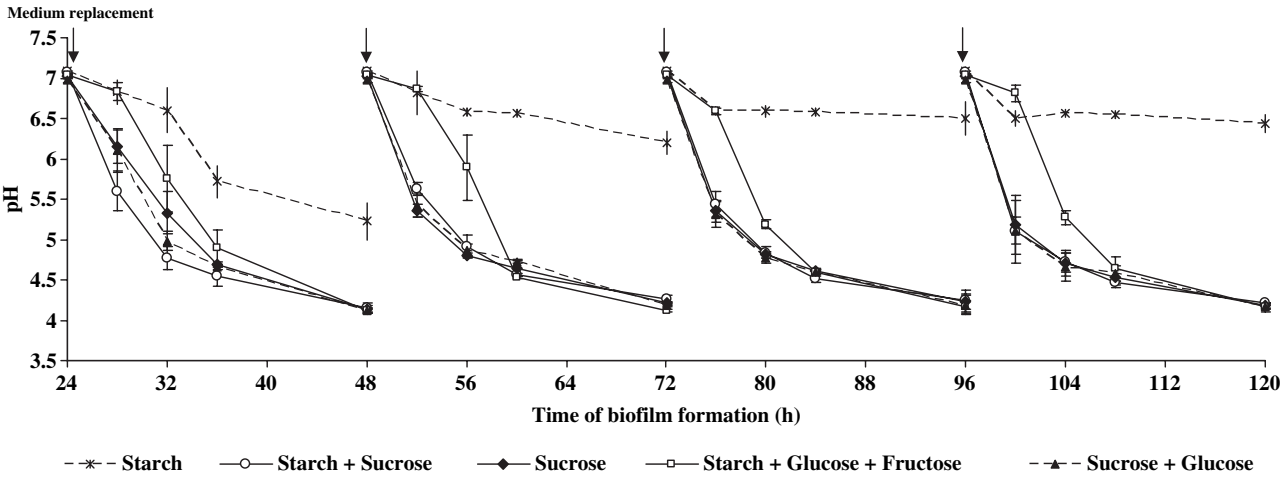

Fig. 4. pH measurements in the culture medium during *Streptococcus mutans* biofilm formation. The medium was replaced daily with fresh medium. The pH values ($n = 12$) were determined after 4, 8, 12, and 24 h of incubation; the reducing sugars and total carbohydrate levels ($n = 12$) were measured after a 24-h period of incubation for each day.

Table 2. Percentage of glycosyl linkages of water-soluble (WSP) and insoluble (INS) polysaccharides extracted from *Streptococcus mutans* biofilms grown in the presence of starch and sucrose, alone or in combinations

	Starch		Starch + sucrose		Sucrose		Starch + glucose + fructose		Sucrose + glucose	
Glycosyl residue	WSP	INS	WSP	INS	WSP	INS	WSP	INS	WSP	INS
3-linked glucose	−	n/d	±	+++	++	+++	−	n/d	++	+++
4-linked glucose	+++++	n/d	+++++	+	+++	+++	+++++	n/d	++	++++
6-linked glucose	−	n/d	+	+++	+++	++	−	n/d	++	++
3,4-linked glucose	−	n/d	−	±	−	−	−	n/d	−	−
3,6-linked glucose	−	n/d	±	++	++	+	−	n/d	+	+
3,4,6-linked glucose	−	n/d	−	±	−	−	−	n/d	−	−

n/d, not determined.
−, 0–1%; ±, 1–4%; +, 5–9%; ++, 10–19%; +++, 20–29%; ++++, 30–59%; +++++, ≥ 60% or more.

less 3-, 6-, and 3,6-linked glucose than those from biofilms grown in sucrose or sucrose + glucose. In contrast, the INS from starch + sucrose biofilms showed higher levels of 3-linked branching (3,4-, 3,6-, and 3,4,6-linked glucose) and considerably less 4-linked glucose than the sucrose-based biofilms. The WSP of starch-grown and starch + glucose + fructose-grown biofilms were comprised predominantly of 4-linked glucose.

Lastly, the expression of *gtfB*, *gtfC*, *gtfD*, and *dexA* in *S. mutans* biofilms grown in starch or sucrose, alone or in combinations, was determined by real-time reverse transcription PCR. Overall, the expression of *gtfB* messenger RNA (mRNA) in biofilms formed with starch + sucrose was significantly increased (25–40%) whereas *gtfD* mRNA levels were decreased (20–30%) when compared with sucrose and sucrose + glucose-grown biofilms ($P < 0.05$); *gtfC* and *dexA* expression was also decreased in starch + sucrose-grown biofilms but the differences were not statistically significant ($P > 0.05$). The gene expression of starch- and starch + glucose + fructose-grown biofilms was not determined because of minimal biofilm formation (and poor RNA yield).

Discussion

The results of this study showed that the combination of starch and sucrose exposed to surface-adsorbed salivary amylase and *S. mutans* clearly influenced the formation and acidogenicity of biofilms by at least four routes: (i) enhanced the total biomass and the content of extracellular insoluble polysaccharides, (ii) synthesized a structurally distinctive EPS matrix, (iii) enhanced acid production in the early stages of biofilm formation, and (iv) affected the expression of specific genes involved in EPS matrix formation (e.g. *gtfB*). Our monospecies biofilm model is advantageous in examining specific actions of carbohydrates on *S. mutans* physiology and genetics, especially on the glucan-mediated processes involved in the formation of the polysaccharide matrix in biofilm, although it does not mimic the complex microbial community found in dental plaque.

S. mutans growing in the presence of sucrose alone or in combinations formed a well-defined, firmly adherent, and highly acidogenic biofilm on the surface of sHA comprised mostly of insoluble polysaccharides containing 3-, 4-, and 6-linked glucose; which agrees well with the glycosyl linkage profile of insoluble glucans synthesized by surface-adsorbed streptococcal glucosyltransferases (17, 21). In contrast, the presence of starch alone or in combination with glucose and fructose resulted in little (and loosely attached) biofilm formation displaying predominantly soluble polysaccharides with 1,4-linked glucose, which suggests that starch and its hydrolysates might be incorporated on to the sHA surface (36). The inability of *S. mutans* to form adherent and established biofilms on the surface of sHA in the presence of starch or starch + glucose + fructose may be related to a lack of insoluble polysaccharide synthesis because insoluble glucans are essential in providing structural integrity and bulk to biofilms (3). However, the combination of starch and sucrose enhanced the acidogenicity of early-formed biofilms (up to 48 h, when salivary amylase is active), and more importantly increased the production of insoluble EPS by *S. mutans* within biofilms when compared to sucrose alone or sucrose with glucose. The starch hydrolysates released by the action of surface-adsorbed amylase combined with sucrose in the medium enhanced the extracellular and intracellular sugar metabolism by *S. mutans* at the pellicle–biofilm interface by providing oligosaccharides to serve as acceptors in glucan synthesis by glucosyltransferases (14, 36), and fermentable carbohydrates for acid production (6). These effects would certainly increase the biofilm accumulation on the tooth surface and accelerate the breakdown of microbial homeostasis in dental plaque (3, 27). Interestingly, the addition of excess glucose in the sucrose medium resulted in less biomass and EPS content than biofilms formed with sucrose alone; an observation consistent with previous studies showing that *S. mutans* growing with glucose in excess diminished the synthesis of extracellular polysaccharides and repressed the sugar uptake by the phosphotransferase system (12, 16). The intracellular polysaccharide accumulation was not markedly affected whether biofilms were grown in the presence of sucrose alone or in combinations, although the IPS content in starch + sucrose-grown and sucrose + glucose-grown biofilms was slightly higher than in sucrose-grown biofilms.

Furthermore, biofilms grown in the presence of starch + sucrose resulted in a structurally distinct EPS matrix when compared to those formed in sucrose or sucrose + glucose. It is noteworthy that the presence of starch + sucrose resulted in insoluble polysaccharides comprised predominantly of $1 \rightarrow 3$ and $1 \rightarrow 6$ linkages, and higher percentages of branch points from 3,4-, 3,6-, and 3,4,6-linked glucose than those from sucrose- or sucrose + glucose-grown biofilms. It is apparent that the presence of oligosaccharides from starch hydrolysis is contributing to the insolubilization of exopolysaccharide matrix in starch + sucrose-grown biofilms by (i) enhancing the content of insoluble polysaccharides containing (ii) a higher percentage of insoluble 3-linked branching. The higher content of insoluble polysaccharides in starch + sucrose-grown biofilm matrix can be explained by previous observations that starch hydrolysates in combination with sucrose increased the synthesis of insoluble glucans, and also affected the structure of glucans synthesized by surface-adsorbed

GTF B, resulting in enhanced *S. mutans* binding compared to those formed with sucrose alone (21, 36); this enhancement may be associated with changes in the binding sites of the modified glucans (21, 36). Clearly, the influence of the oligosaccharides on the GTF B activity plays a critical role in changing the physical and biochemical properties of the biofilms matrix, and thereby influencing its cariogenic properties. However, in biofilms, the formation and maturation of the EPS matrix is a result of a dynamic interaction of all of the three GTFs acting in concert and is influenced by an endodextranase produced simultaneously by *S. mutans* (15, 17, 22). The presence of starch in combination with sucrose may be modulating all of the enzymes responsible for synthesis and degradation concomitantly, resulting in a structurally distinct matrix. Further studies shall elucidate how starch and sucrose influence the synthesis and degradation of glucans concomitantly during the EPS matrix development at molecular and structural levels.

A recent *in situ* study showed that a combination of 2% starch + 10% sucrose was potentially more cariogenic than 10% sucrose alone, despite the total amounts of EPS in the matrices of the biofilms being similar to each other (29). Although higher levels of acidogenic and aciduric bacteria, such as *Lactobacilli*, were found in the biofilms, the enhanced cariogenicity of starch + sucrose may be also explained by the structural differences of the EPS matrix between starch + sucrose-grown biofilms and those formed in sucrose alone. The structural changes in the matrix may affect the diffusion properties, bacterial binding sites, physical integrity, and architecture of the biofilms (8, 21, 34, 36, 39). It is therefore feasible that such changes in the EPS matrix of biofilms could modulate the pathogenesis of dental caries, substantiating the concept that there is a starch hydrolysate contribution to the formation of cariogenic dental plaque. The exact mechanisms by which the structural changes enhance the virulence of the biofilms need further elucidation.

Lastly, we examined the expression profile of the genes encoding the synthesis (*gtfB*, *gtfC*, *gtfD*) and degradation (*dexA*) of glucans by *S. mutans* within biofilms, in an attempt to explain the structural differences of the EPS matrix observed between starch + sucrose-grown and sucrose-grown biofilms. Our data indicate that biofilms formed in starch + sucrose expressed significantly higher levels of *gtfB* mRNA and less *gtfD* mRNA than those formed in sucrose. It is noteworthy that expression of *gtfC* and *dexA* was decreased in starch + sucrose biofilms (although the differences were not statistically significant), indicating an overall effect of induction of *gtfB*. This observation could explain, in part, the differences observed in the structure of the EPS matrix, e.g. higher percentage of 3-linked branching in insoluble polysaccharide and less 6-linked glucose in the soluble polysaccharide matrix of starch + sucrose biofilms. Furthermore, *gtfB* is a critical virulence gene associated with the pathogenesis of dental caries (37); *S. mutans* treated with therapeutic agents that repress the expression of *gtfB*, or mutant strain of this organism defective in *gtfB*, are far less cariogenic than untreated or parent strains *in vivo* (19, 20, 37). Thus, the presence of a combination of starch and sucrose would result in a more virulent (cariogenic) biofilm. We are currently pursuing detailed gene expression profiling at different stages of biofilm formation to better understand the molecular mechanisms involved in the EPS synthesis in the presence of starch and sucrose.

Our data offer, in part at least, an explanation for why starch and sucrose combinations are potentially more cariogenic than either alone; and furthermore illustrate that composition of the diet can influence the virulence traits of the oral pathogen *S. mutans*. Clearly, surface-adsorbed α-amylase may have an additional role in dental biofilm formation other than promoting specific bacterial adhesion (31) by contributing directly with the synthesis of a structurally distinct extracellular polysaccharide matrix and by enhancing the expression of *gtfB*. Further studies using additional microorganisms that bind amylase in a multispecies biofilm model shall elucidate even further the role of starch and sucrose in the virulence of cariogenic biofilms.

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