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Effect of starch on the cariogenic potential of sucrose

Cecilia C. C. Ribeiro, Cínthia P. M. Tabchoury, Altair A. Del Bel Cury, Livia M. A. Tenuta, Pedro L. Rosalen and Jaime A. Cury*

Faculty of Dentistry of Piracicaba, University of Campinas, Av. Limeira 901, CEP 13414-903, Piracicaba, São Paulo, Brazil

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Since *in vitro* and animal studies suggest that the combination of starch with sucrose may be more cariogenic than sucrose alone, the study assessed *in situ* the effects of this association applied *in vitro* on the acidogenicity, biochemical and microbiological composition of dental biofilm, as well as on enamel demineralization. During two phases of 14 d each, fifteen volunteers wore palatal appliances containing blocks of human deciduous enamel, which were extra-orally submitted to four groups of treatments: water (negative control, T1); 2 % starch (T2); 10 % sucrose (T3); and 2 % starch + 10 % sucrose (T4). The solutions were dripped onto the blocks eight times per day. The biofilm formed on the blocks was analysed with regard to amylase activity, acidogenicity, and biochemical and microbiological composition. Demineralization was determined on enamel by cross-sectional microhardness. The greatest mineral loss was observed for the association starch + sucrose ($P<0{\cdot}05$). Also, this association resulted in the highest lactobacillus count in the biofilm formed ($P<0{\cdot}05$). In conclusion, the findings suggest that a small amount of added starch increases the cariogenic potential of sucrose.

Starch: Sucrose: Dental biofilm: Demineralization: Enamel

Among dietary carbohydrates, starch has been pointed out as non-cariogenic or slightly cariogenic when used as the sole source of carbohydrate in the diet. This observation has been supported by experiments on dental biofilm acidogenicity (Stephan, 1940; Imfeld, 1977; Lingström *et al.* 1989), experimental studies with animals (König & Grenby, 1965; Green & Hartles, 1967; Hefti & Schmid, 1979; Bowen *et al.* 1980), controlled studies in man (Gustaffson *et al.* 1954), epidemiological data (Marthaler & Froesch, 1967; Fisher, 1968; Newbrun *et al.* 1980) and *in situ* experiments (Lingström *et al.* 1994), which demonstrated that starch is less cariogenic than sucrose.

However, while in primitive diets starch was consumed as the main energy source, in contemporary ones it is consumed simultaneously or interspersed with sucrose (Lingström *et al.* 2000). This combination, which is consumed by both adults and children, may influence dental biofilm composition and consequently dental caries. Thus, a greater prevalence of caries lesions was found in children who consume milk supplemented with a combination of cereal and sucrose (Mattos-Graner *et al.* 1998). Such observation in human subjects is supported by the results of experimental caries studies in animals (Firestone *et al.* 1982; Mundorff-Shrestha *et al.* 1994), suggesting that starch would enhance the cariogenic potential of sucrose.

The explanation for the greater cariogenicity of the association of dietary starch with sucrose may reside in the dental biofilm formed. It is well known that the biofilm formed in the presence of sucrose is more cariogenic due to its high concentration of extracellular insoluble polysaccharides (IP), which alter the matrix of the biofilm, making it more porous (Dibdin & Shellis, 1988). These polysaccharides are produced from sucrose by bacterial enzymes named glucosyltransferases. Thus it has been shown *in vitro* that, in the presence of starch, not only the synthesis of insoluble glucans by glucosyltransferase-B increases, but also these glucans present a biochemical (Vacca-Smith *et al.* 1996) and physical structure (Kopec *et al.* 1997) different from those formed in the presence of sucrose alone. In addition, the presence of these 'new polysaccharides' increases the adherence of strains of *Actinomyces naeslundi* and mutans streptococci to hydroxyapatite (Vacca-Smith *et al.* 1996). This greater adhesion observed *in vitro* may possibly contribute to *in vivo* adherence and accumulation of biofilm on dental surfaces.

Considering that the dietary combination of starch and sucrose seems to affect the structure of the biofilm formed, which would consequently influence caries development, studies closer to *in vivo* conditions in man are necessary in order to assess this hypothesis. Therefore, the present experimental research aimed to study *in situ* the effect of the combination of starch and sucrose on amylase activity, acidogenicity, biochemical and microbiological composition of the dental biofilm formed, and its relationship with demineralization of deciduous dental enamel. Deciduous enamel was chosen because neither the cariogenic effect of starch alone nor that of starch + sucrose on this dental substrate is known.

Abbreviations: %AC, percentage of *Actinomyces naeslundi* in relation to total micro-organisms; CFU, colony-forming units; cH^+, hydrogen ionic concentration; CSMH, cross-sectional microhardness; IP, inorganic polysaccharides; %LB, percentage of lactobacilli in relation to total micro-organisms; MSB, mitis salivarius agar plus bacitracin; $pH_{5\,min}$, pH at 5 min; P_i, inorganic P; %SM, percentage of mutans streptococci group in relation to total micro-organisms; ΔZ, mineral loss.

* **Corresponding author:** Professor Jaime A. Cury, fax +55 19 3412 5218, email jcury@fop.unicamp.br

Materials and methods

Experimental design

The study was approved by the Research and Ethics Committee of the Faculty of Dentistry of Piracicaba (Protocol No. 132/2002). It was carried out in two phases of 14 d each, during which fifteen volunteers, 18–33 years old, wore acrylic palatal appliances, containing two sets of four blocks of human deciduous dental enamel. In each phase, each set of four blocks was submitted outside the mouth to one of the following treatments: T1, distilled and deionized water (negative control); T2, 2 % starch solution (soluble starch S-9765; Sigma, St. Louis, MO, USA); T3, 10 % sucrose solution; and T4, 2 % starch + 10 % sucrose solution. The volunteers were randomly assigned to the different treatments; those who dripped T1 and T3 in the first phase dripped T2 and T4 in the second phase, and vice versa (Fig. 1). The use of two treatments (split-mouth) in the same intra-oral palatal appliance (Fig. 2(A)) was supported by the absence of a cross-effect in previous studies (Cury *et al.* 2001; Hara *et al.* 2003; Paes Leme *et al.* 2004; Pecharki *et al.* 2005). On the day 13 of the experiment, the biofilm formed on the most posterior blocks of each treatment (Fig. 2(B)) was collected for determination of amylase activity; on this day the acidogenicity of the dental biofilm formed was also determined (Fig. 2(C)). On the day 14 of each phase, the biofilms were collected for biochemical and microbiological analyses (Fig. 2(D)); the variation of mineral content was determined in the enamel (Fig. 2(E)). For statistical analysis, the volunteer was considered as an experimental block. This study was blind only with respect to the examiner, since the volunteers were able to identify the treatments by the flavour and consistency of the solutions.

Enamel blocks and palatal appliance preparation

Two hundred and forty dental enamel blocks (3 mm × 3 mm × 2 mm) were obtained from the middle third of the buccal face of sound human deciduous incisors, and were randomly distributed to the different treatments. An acrylic resin intra-oral palatal appliance, containing two lateral cavities measuring 13 mm × 4 mm × 3 mm, in which four blocks of enamel were placed on each side, was made for each volunteer. The three anterior blocks were kept together and the fourth was separated from them by resin (Fig. 1). Plastic meshes were fixed over the cavities to protect the enamel block surfaces from mechanical attrition, leaving a 1 mm space for accumulation of dental biofilm. Colourless or red acrylic resin was used to fix the meshes, indicating where each treatment should be made (Cury *et al.* 2001; Paes Leme *et al.* 2004; Pecharki *et al.* 2005). Further details of appliance preparation are given in previous publications (Cury *et al.* 1997, 2000; Hara *et al.* 2003).

The concentration of 10 % sucrose used in the present experiment is usually found in soft drinks. Also, it simulates the addition of 1 soupspoon of sugar to a 150 ml baby bottle, and is close to the concentration found in some Brazilian infant formulas (Bebelac 2®, Sustain® and Soya Diet®). The 2 % starch concentration was adopted in the experiment because it is the concentration frequently found in infant formulas available on the Brazilian market and is the maximum solubility of the starch used (soluble starch S-9765; Sigma).

Treatments

Throughout the entire experiment, volunteers used a dentifrice containing 1100 μg F/g (NaF) and silica as abrasive, consumed water optimally fluoridated (0·67 mg F/l) and received instructions as previously described (Cury *et al.* 2000). During the 14 d of each experimental phase, eight times per day (08.00, 09.30, 11.00, 14.00, 15.30, 17.00, 19.00 and 21.00 hours) each volunteer removed the appliance from the oral cavity, dripped the treatment solutions on the dental blocks, and after 5 min the appliance was replaced in the mouth. The labels of the dropping bottles containing treatments T3 and T4 were marked in red, indicating to the volunteers that these solutions should be dripped on the side of the appliance where the mesh was fixed with red resin (Figs. 1 and 2). The excess fluid was removed with gauze in an attempt to avoid carry-across effect of the treatments. A wash-out interval of 14 d was established between the experimental phases. Considering the crossover design of this study, no restriction was made

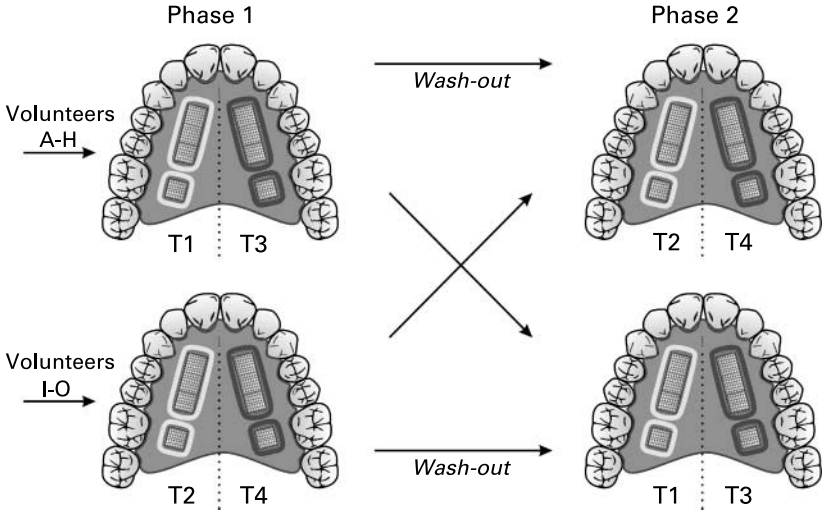

Fig. 1. Schematic illustration of the experimental design. T1, Distilled and deionized water (negative control); T2, 2% starch solution; T3, 10% sucrose solution; T4, 2% starch + 10% sucrose solution.

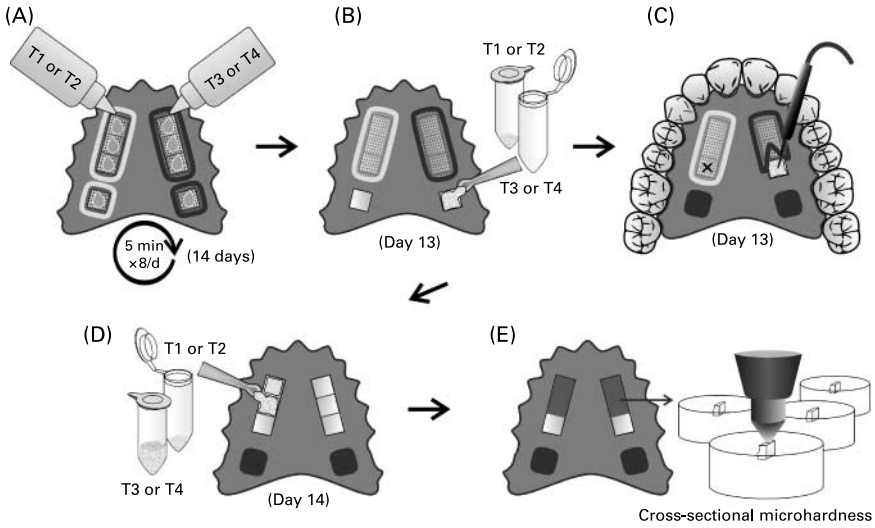

Fig. 2. Schematic illustration of the treatments and analyses made. (A) Treatments; (B) amylase activity analysis; (C) pH measurement *in situ*; (D) microbiological and biochemical analyses; (E) enamel demineralization analysis. T1, Distilled and deionized water (control); T2 2% starch solution; T3, 10% sucrose solution; T4, 2% starch + 10% sucrose solution.

with regard to the volunteers' diet, but they were instructed to remove the appliances during their meals (Cury *et al.* 1997, 2000).

Dental biofilm analysis

Determination of amylase activity in dental biofilm. On day 13 of each experimental phase, approximately 10 h after the last exposure to treatments, with the volunteers in fasting condition and without having brushed their teeth, the biofilm formed on the most posterior enamel block on each side of the appliance (Fig. 2(B)) was collected. For each 1 mg biofilm, 20 μl of 1·5 % NaCl solution, containing 2·0 mg F/ml, was added (Dodds & Edgar, 1986) and homogenized using a tissue micro-grinder. This suspension was incubated with 1 % starch solution (soluble starch F1000552.139; Merck, Darmstadt, Germany) buffered with 0·02 M-phosphate (pH 6·9) for 15 min. Amylase activity was determined by means of the Bernfeld (1955) colorimetric method using maltose as standard, with absorbance determined in a spectrophotometer (DU-70®; Beckman, Fullerton, CA, USA) at 540 nm. The results are expressed in U/g per min, where 1 U is defined as the amount of enzyme able to release 1 μmol maltose from starch substrate.

Dental biofilm acidogenicity assessment. On the same day 13 of the experiment, after biofilm collection for amylase activity analysis and with the intra-oral appliance positioned in the volunteer's oral cavity (Fig. 2(C)), the pH of the biofilm was determined after overnight fasting and 5 min after treatment with the respective solutions. A contact micro-electrode (Beetrode® MEPH-3L; WPI, Sarasota, FL, USA) connected to a pH meter (720-A; Orion, Boston, MA, USA) in combination with a reference electrode (9002; Orion) was used. For this analysis, the plastic mesh that covered the third dental block on each side of the appliance was dislocated to facilitate positioning of the electrode in the biofilm. A salt bridge was created in a 3 M-KCl solution between the reference electrode and the volunteer's finger (Lingström *et al.* 1994). pH was measured at time 0 (baseline), the intra-oral appliance was removed from the oral cavity and the respective treatment solutions dripped onto the blocks. After 1 min, the appliance was replaced in the oral cavity and, after 4 min had elapsed, the pH was determined again ($pH_{5\,min}$). After these procedures, the plastic mesh was replaced and the volunteers continued carrying out the treatments until the following day. By means of a computer program (Larsen & Pearce, 1997), the pH data were converted into hydrogen ionic concentration (cH^+) and the cH^+ area between the times of 0 and 5 min was calculated. This determination at 5 min was chosen as a parameter of plaque acidogenicity, rather than the curve of pH *v.* time, on the basis of the results of a pilot study conducted with three volunteers, which showed that it was suitable for comparing the difference of fermentation between starch and sucrose. Furthermore, this procedure allowed the analysis of acidogenicity in up to eight volunteers in the same morning period.

Dental biofilm composition analysis

On the day 14 of each experimental phase, under the same conditions as described for the previous day, the dental biofilm from the three remaining blocks was collected (Fig. 2(D)); a homogeneous aliquot was used for the microbiological analyses and the rest of the biofilm was dehydrated for the biochemical analyses.

For the microbiological analyses, the dental biofilm was weighed (± 0·01 mg) in sterile microcentrifuge tubes, suspended in 0·9 % NaCl solution (1 ml/mg wet weight) and sonicated using Sonicador Vibra Cell (Sonics and Materials, Danbury, CT, USA) at 40 W, amplitude 5 %, six pulses of 9·9 s each (Bowen *et al.* 1986). The suspensions were diluted in 0·9 % NaCl in series up to $1{:}10^5$ and automatically inoculated in duplicate (Whitley Automatic Spiral Plater®; Don Whitley Scientific Ltd, Shipley, UK) in the following culture media: blood agar, for total microbiota; mitis salivarius agar plus 0·2 units of bacitracin/ml (MSB), for mutans streptococci group (Gold *et al.* 1973); Rogosa SL agar (Difco 248020; Becton Dickinson, Sparks, MD, USA), for lactobacillus; and CFAT medium (Zylber & Jordan, 1982), for *A. naeslundi*. The plates were incubated in 10 % CO_2

at 37°C for 48 h (blood agar, MSB, Rogosa) or 72 h (CFAT). The blood agar plates were additionally incubated for 24 h at 37°C in aerobiosis. The colony-forming units (CFU) were counted and the results expressed in CFU/mg dental biofilm (wet weight) and in percentage of mutans streptococci group (%SM), lactobacilli (%LB) and *A. naeslundi* (%AC) in relation to total micro-organisms.

After removing an aliquot for the microbiological analysis, the rest of the collected biofilm was dried over P_2O_5 for 24 h and treated as described previously (Cury *et al.* 1997, 2000), but with a modification in the proportions of solutions used in extraction of the inorganic components (100 µl HCl/mg biofilm (dry weight)) and extraction of the IP (200 µl NaOH/mg biofilm (dry weight)). F^-, Ca^{2+}, inorganic P (P_i) and IP analyses were done as previously described (Cury *et al.* 1997, 2000).

Dental enamel analysis

Two blocks from each treatment, which were placed in the most anterior position (Fig. 2(E)), were sectioned longitudinally through the centre for enamel cross-sectional microhardness (CSMH) determination. The CSMH was determined according to Cury *et al.* (2000), and the impressions were made at the distances of 10, 20, 30, 40, 50, 60, 80, 100, 120, 140, 160, 180 and 200 µm from the outer enamel surface. CSMH values were converted to mineral content (vol%) according to Featherstone *et al.* (1983), and the area of mineral loss (ΔZ) for each treatment was calculated (White & Featherstone, 1987). The microhardness tester Future-Tech FM, coupled to software FM-ARS (Future-Tech, Tokyo, Japan), was used with a 25 g load for 5 s.

Statistical analyses

For all analyses, the experimental unit considered was the volunteer. The assumptions of equality of variances and normal distribution of errors were checked, and data that violated these statistical principles were transformed (Box *et al.* 1978), with the exception of the variable %SM, since it was not possible to normalize these data. The data of biofilm biomass, cH^+ area, counts of total micro-organisms, lactobacilli and *A. naeslundi*, Ca^{2+} and ΔZ were transformed into $\log_{10}$. The values of %LB, %AC and IP were transformed by the power of $-0{\cdot}5$; amylase activity by the power of 2·5; F^- by the power of 0,1; initial pH by the power of 3; $pH_{5\,min}$ by the power of -1; P_i by the power of $-0{\cdot}3$; and counts of mutans streptococci group by the power of $-0{\cdot}1$. ANOVA followed by the Tukey test was used for all variables, with the exception of the variable %SM, for which the Friedman test followed by the *t* test was used. The software SAS (version 8.02, 1999; SAS Institute Inc., Cary, NC, USA) was used and the significance level fixed at $P < 0.05$.

Results

ANOVA showed a significant effect of treatments for the majority of the variables studied ($P<0{\cdot}001$), except for amylase activity in the biofilm ($P=0{\cdot}231$).

With regard to the baseline pH of the biofilm (Table 1), no statistical difference was observed ($P>0{\cdot}05$) between sucrose and starch + sucrose treatments, but both presented pH values 11 % lower ($P<0{\cdot}05$) than the negative control and starch treatments, which did not differ from each other ($P>0{\cdot}05$). For $pH_{5\,min}$ and cH^+ area (Table 1), no difference was also found ($P>0{\cdot}05$) between sucrose and starch + sucrose treatments, which presented $pH_{5\,min}$ values 18 % lower and cH^+ area values ten times higher ($P<0{\cdot}05$) than the treatment with starch; the negative control differed significantly ($P<0{\cdot}05$) from all the other treatments.

The total micro-organism count (Table 2) in the biofilm of the negative control group was 3·2 times higher ($P<0{\cdot}05$) than that of the sucrose and starch + sucrose groups, which did not differ from each other ($P>0{\cdot}05$). The starch treatment did not differ from the other treatment groups ($P>0{\cdot}05$).

For the populations of mutans streptococci (Table 2), the highest values were found for the sucrose and starch + sucrose treatments, which differed from the other groups ($P<0{\cdot}05$), but did not differ from each other ($P>0{\cdot}05$). No significant difference was observed between the control group and the group treated with starch ($P>0{\cdot}05$). The same observations were found for %SM in relation to total micro-organisms, which showed an average of 300 times more mutans streptococci in the biofilm exposed to sucrose or starch + sucrose than in that exposed to starch or the negative control group.

In relation to the lactobacilli (Table 2), the treatment with starch + sucrose presented respectively 2·2, 16·4 and 4525 more CFU than sucrose, starch and the negative control ($P<0{\cdot}05$). Sucrose and starch treatments did not differ from each other ($P>0{\cdot}05$), but presented higher counts ($P<0{\cdot}05$) than the negative control group. With respect to %LB in relation to total micro-organisms, starch + sucrose treatment presented the highest values ($P<0{\cdot}05$), which did not differ significantly from only the sucrose treatment (Table 2). Starch treatment did not differ ($P>0{\cdot}05$) from the control or sucrose treatments; however,

Table 1. Analysis of dental biofilm pH according to treatment

(Mean values with their standard deviation and number of determinations)

	Treatment*											
	T1			T2			T3			T4		
	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*
Baseline pH	7·5[a]	0·4	14	7·4[a]	0·5	15	6·6[b]	0·6	14	6·7[b]	0·8	15
$pH_{5\,min}$	7·2[a]	0·4	14	6·4[b]	0·7	13	5·3[c]	0·3	14	5·2[c]	0·3	14
cH^+ area (µmol/l × min)	0·1[a]	0·2	14	2·0[b]	2·0	13	18·3[c]	11·7	14	22·7[c]	13·3	14

$pH_{5\,min}$, pH at 5 min; cH^+ area, hydrogen ionic concentration between the times of 0 and 5 min (see text, p. 49 for details of procedures).

*T1, H_2O; T2, 2 % starch; T3, 10 % sucrose; T4, 2 % starch + 10 % sucrose.

[a,b,c] Mean values with unlike superscript letters were significantly different ($P<0{\cdot}05$).

Table 2. Microbiological analysis of dental biofilm according to treatment
(Mean values with their standard deviation and number of determinations)

	Treatment*											
	T1			T2			T3			T4		
	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*
Total micro-organisms (CFU/mg × 10^7)	3·2[a]	3·1	14	1·9[ab]	1·2	15	0·9[b]	0·6	14	1·1[b]	0·8	15
Mutans streptococci (CFU/mg × 10^3)	0·02[a]	0·04	14	0·07[a]	0·12	15	8·51[b]	16·37	14	7·98[b]	18·36	15
%SM	0·0001[a]	0·0000	14	0·0012[a]	0·002	15	0·22[b]	0·46	14	0·17[b]	0·40	15
Lactobacilli (CFU/mg × 10^5)	0·004[a]	0·006	14	1·1[b]	3	15	8·2[b]	18·1	14	18·1[c]	3	15
%LB	0·017[a]	0·00	14	1·8[ab]	5·6	15	12·6[bc]	22·9	14	23·1[c]	29·6	15
Actinomyces naeslundi (CFU/mg × 10^5)	2·3[a]	7·7	14	3·1[a]	8·1	15	7·0[b]	10·1	14	8·4[b]	10·8	15
%AC	2·8[a]	7·2	14	2·8[a]	7·6	15	10·4[b]	15·7	14	7·3[b]	7·0	15

CFU, colony-forming units; %SM, percentage of mutans streptococci group in relation to total micro-organisms; %LB, percentage of lactobacilli in relation to total micro-organisms; %AC, percentage of *A. naeslundi* in relation to total micro-organisms.
* T1, H_2O; T2, 2 % starch; T3, 10 % sucrose; T4, 2 % starch + 10 % sucrose.
[a,b,c] Mean values with unlike superscript letters were significantly different ($P<0·05$).

the %LB was 741 times higher in the biofilm exposed to sucrose compared with the negative control ($P<0·05$).

For the *A. naeslundi* count (Table 2), the highest values were presented by sucrose and starch + sucrose treatments, which did not show difference from each other ($P>0·05$), but differed from the other groups ($P<0·05$). Nor was a significant difference found between the control group and the treatment with starch ($P>0·05$). The same pattern was observed for %AC in relation to the total micro-organisms.

The biomass formed on the dental blocks in the presence of sucrose and starch + sucrose treatments (Table 3) was 112 % greater than that of the other groups ($P<0·05$), but did not differ from each other ($P>0·05$). No significant difference was found between the negative control and the treatment with starch ($P>0·05$) either.

For F^- concentration in the dental biofilm (Table 3), the lowest values were found with sucrose and starch + sucrose treatments, which did not differ from each other ($P>0·05$), but differed significantly from the other groups ($P<0·05$). F^- concentration in the biofilm of the negative control group was respectively 2·0, 3·9 and 8·4 times higher than in that exposed to starch, starch + sucrose and sucrose ($P<0·05$). The same pattern was observed for the variables Ca^{2+} and P_i concentration in the biofilm (Table 3).

IP concentration (Table 3) found in the biofilms treated with sucrose and starch + sucrose did not differ from each other ($P>0·05$), but was about 4 times greater ($P<0·05$) than that of the negative control and starch groups, which did not differ from each other ($P>0·05$).

In ΔZ analysis, the negative control and starch treatment did not differ from each other (Table 4), but presented 2·7 times less mineral loss ($P<0·05$) than the other groups. The greatest mineral loss was found for starch + sucrose treatment ($P<0·05$), which was 33 % higher than for sucrose exposure and 69 % higher than for starch and the negative control groups.

Discussion

The results of mineral loss (Table 4) show that starch at 2 %, used at the frequency of eight times a day, did not cause demineralization on deciduous human dental enamel, confirming results observed with permanent teeth (Lingström *et al.* 1994). These findings also confirm studies conducted with animals (König & Grenby, 1965; Green & Hartles, 1967; Hefti & Schmid, 1979; Bowen *et al.* 1980) and epidemiological studies (Fisher, 1968; Newbrun *et al.* 1980), which also found little or no caries in enamel when starch is used as the only carbohydrate source in the diet.

The absence of demineralization in the dental blocks exposed to the treatment with starch (T2) cannot be attributed to a cross-effect during the study, since sucrose (T3) caused significant more enamel demineralization than T1, which was dripped on the opposite side of the same oral appliance (Table 4). The

Table 3. Biochemical analysis of dental biofilm according to treatment
(Mean values with their standard deviation and number of determinations)

	Treatment*											
	T1			T2			T3			T4		
	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*
Amylase activity (U/g per min)	24·2[a]	3·5	14	22·4[a]	4·5	15	25·3[a]	3·1	14	24·1[a]	4·1	15
Biomass (mg dry weight)	2·2[a]	1·2	14	2·6[a]	1·6	15	5·0[b]	2·0	14	5·2[b]	3·8	15
F^- (μg/g)	468·4[a]	401·8	10	239·8[b]	251·3	15	119·9[c]	179·3	14	55·7[c]	144·3	15
Ca^{2+}(μg/mg)	45·9[a]	50·9	10	17·4[b]	25·4	15	5·1[c]	7·9	14	4·9[c]	10·5	15
P_i (μg/mg)	27·1[a]	29·5	10	11·6[b]	14·6	15	4·1[c]	4·6	14	3·6[c]	5·0	15
IP (μg/mg)	47·5[a]	22·8	10	49·8[a]	13·5	15	181·6[b]	115·8	14	201·6[b]	137·6	15

P_i, inorganic P; IP, insoluble polysaccharides.
* T1, H_2O; T2, 2 % starch; T3, 10 % sucrose; T4, 2 % starch + 10 % sucrose.
[a,b,c] Mean values with unlike superscript letters were significantly different ($P<0·05$).

Table 4. Analysis of dental enamel according to treatment
(Mean values with their standard deviation and number of determinations)

	Treatment*											
	T1			T2			T3			T4		
	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*
Mineral loss	447·9[a]	169·0	14	420·0[a]	160·1	15	955·6[b]	543·6	14	1421·8[c]	653·8	14

*T1, H_2O; T2, 2 % starch; T3, 10 % sucrose; T4, 2 % starch + 10 % sucrose.
[a,b,c] Mean values with unlike superscript letters were significantly different ($P<0·05$).

non-cariogenicity of starch may be explained by the biofilm acidogenicity parameters (Table 1), as the baseline pH of this group was similar to that of the negative control (T1). Thus, although starch presented higher cH^+ area value than the negative control and caused a significant pH drop after 5 min (Table 1), these values were still above the critical pH for enamel dissolution, which is around 5·5. In addition, the IP concentration in the matrix of the biofilm formed in the presence of starch (T2) did not differ from that of the negative control (Table 3). Considering the role of these IP in dental biofilm cariogenicity (Rölla *et al.* 1985; Dibdin & Shellis, 1988; Cury *et al.* 2000; Mattos-Graner *et al.* 2000; Nobre dos Santos *et al.* 2002), this observation would give greater support to the absence of demineralization observed in the presence of starch (Table 4).

The mineral content data of the dental blocks treated with the starch + sucrose association (T4) presented the greatest value of mineral loss. These *in situ* data are consistent with the results found in observational studies in human subjects (Mattos-Graner *et al.* 1998) and in experiments with animals (Firestone *et al.* 1982; Mundorff-Shrestha *et al.* 1994), in which the association starch + sucrose was more cariogenic than sucrose alone. Such findings, showing the highest cariogenic potential for the combination of starch + sucrose, are relevant since starchy foods in modern diets may not be considered safe for the teeth (Lingström *et al.* 2000), especially dentine.

With regard to IP in the biofilm formed, our *in situ* findings (Table 3) did not confirm *in vitro* data showing higher IP concentration formed in the presence of starch + sucrose than sucrose alone (Vacca-Smith *et al.* 1996). However, in the present study the total amount of alkali-soluble polysaccharides (Cury *et al.* 1997, 2000) was determined, without differentiating the IP synthesized by the different glucosyltransferases. Also, the methodology used did not allow confirmation of whether the IP formed *in situ* by treatment T4 is structurally different from that formed in the exclusive presence of T3, as shown *in vitro* (Kopec *et al.* 1997). Further studies should be conducted to evaluate these possibilities *in situ*. Moreover, another explanation could be the source of starch used, although it was the same as used in the cited papers.

Thus, the most evident effect of the combination starch + sucrose in the biofilm formed seems to be related to bacterial ecology, since in the presence of this treatment there was a greater ($P<0·05$) absolute *Lactobacillus* count than in presence of sucrose alone (Table 2). Our results showed that, although starch (T2) did not result in enamel demineralization (Table 4), it caused an increase in the populations of lactobacilli in the biofilm compared with the negative control (Table 2). Thus, although the pH drop during starch fermentation (Table 1) did not reach the critical pH for enamel solubility, it was sufficient for bacterial selection (Marsh, 1994). Such effect on the dental biofilm – selection of more aciduric bacteria both by starch and by sucrose – may help explain the question posed by Green & Hartles (1967), i.e. whether the consumption of a mixture of highly cariogenic sucrose with a virtually non-cariogenic starch would lead to more dental caries in rats than when sucrose is mixed with moderately cariogenic roll-dried starch.

With regard to *A. naeslundi* populations in the biofilm formed, the results of the present *in situ* study (Table 2) did not confirm those obtained *in vitro*, which showed greater adherence of these micro-organisms in the presence of glucans synthesized from starch and sucrose (Vacca-Smith *et al.* 1996), since the difference between T4 and T3 was not statistically significant ($P>0·05$).

In conclusion, the results of the present *in situ* study indicate that a small amount of added starch accentuates the cariogenic potential of sucrose solution. Since these findings were obtained by exposure of the dental biofilm to 2 % soluble starch and 10 % sucrose, further studies should be conducted using different concentrations and combinations with other carbohydrates.

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