

Raisin effects on in vitro demineralization of teeth

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California Raisin Marketing Board**

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Abstract:

A bacterially modulated caries model system was used to evaluate the demineralizing potential of raisins and other foods on human dental enamel. This system mimics several of the conditions of the oral environment including the cyclic nature of caries attacks followed by a period of remineralization, continuous salivary flow, and *S. mutans* as the source of the acid attacks. The demineralizing effects of a 10 % (w/w) puree of raisins (raisin juice) was compared to that of whole milk, orange juice, remineralizing saliva-like solution (SLS, system control) and Brain-Heart Infusion broth (BHI, system control). The change in mineral was determined by x-ray microradiography which determines both the surface loss of the tooth as well as reduction of mineral density within the enamel. It was found that there was no mineral loss in the controls (SLS and BHI) and Milk, and mineral loss due to raisin juice and orange juice. The orange juice was the most aggressive and caused the greatest mineral loss.

The original proposal submitted by Dr. Nicola Richards in July 2006 to the California Raisin Marketing Board for this research is attached to this report as Appendix A. It was a major loss to all of us when Dr. Richards fell ill in October 2006 and died in July of 2007. Her obituary is attached as Appendix B.

Introduction:

There is evidence that food particles retained on the teeth will lead to demineralization and cavitation of tooth surfaces (caries) (Kashket et al, 1996). The process for the development of caries from food particles, is that the bacteria in the plaque mass metabolize the sugars in the retained food particles generating acids that attack the tooth structure. Thus foods that are retained in the dentition are suspects for the development of dental caries. The general logic is that foods that are perceived to be “sticky” will be more cariogenic than non-sticky snack foods. Raisins have been perceived (by the general public) as the ninth stickiest food out of a list of twenty-one popular snacks (Kashket et al, 1991), however there is no evidence that raisins contribute to the demineralization of teeth. Researchers measured the weight of food retained just after swallowing compared to the weight remaining 5 minutes after swallowing and found that foods that are less soluble in oral fluids are retained for longer times. Although raisins are perceived to be sticky, they are easily cleared from the oral cavity. The fast clearance argues that raisins may not contribute to tooth demineralization significantly because the sugars are removed from the dentition before the plaque mass has the opportunity generate sufficient acid to lower the pH below 5.5 (Linke *et al.*, 1997). There is also research that shows that raisins contain compounds that inhibit the *in vitro* growth of *S. mutans*, thus making raisins less cariogenic than other foods with high sugar content (Wu et al, 2003). Thus the purpose of this study was to determine the effects of raisins on demineralization of teeth using an *in vitro* model that mimics the oral environment. An additional purpose was to compare the cariogenic potential of raisins to that of other foods.

Our *in vitro* caries model was developed to determine the potential of raisins and other foods to stimulate intraoral bacteria to demineralize human teeth in a system that mimics the oral environment as much as possible. We developed our *in vitro* caries model by building on the concepts described by Fontana *et al.* (1996) in their bacterially moderated system to assess the efficacy of potential therapies for the prevention of secondary caries. Our caries model continues to use *S. mutans* as the cariogenic vector, and we have developed methods to simulate the oral environment where the samples are cycled between periods of demineralization and remineralization as occurs in the mouth. The demineralizing cycle is due to the metabolic acids produced by the *S. mutans* when exposed to sugars. The remineralizing cycle is a continuous flow across the samples of saliva-like solution (SLS) that is supersaturated with respect to tooth enamel.

Test specimens of caries free human enamel were subjected to repeated exposures of “raisin juice” within this *in vitro* caries model and compared to exposures of milk, orange juice, brain-heart infusion broth (system control) and saliva-like solution (negative control). The resultant demineralization of tooth structure was evaluated using x-ray microradiography (Chow *et al.*, 1990; Carey *et al.*, 2007; Schmuck *et al.*, 2007). With x-ray microradiography, the change in enamel surface loss (or gain) and mineral density of enamel in cross-section was measured as a function of time.

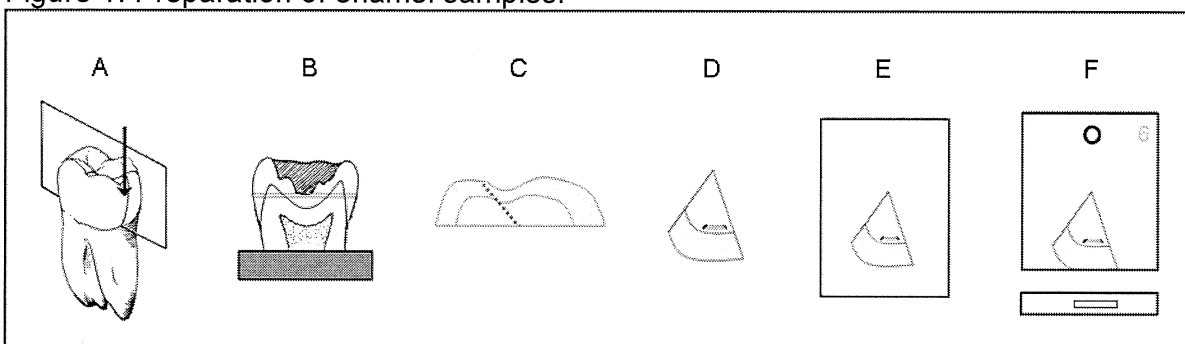
Our hypotheses were 1. the high sucrose content of raisin (raisin juice) will feed the *S. mutans* and cause enamel demineralization; 2. the amount of raisin caused demineralization will be significantly less than other acidic foods (orange juice) and greater than neutralized foods (whole milk).

Our long term goals are to demonstrate the validity of the new *in vitro* caries model with other methods, and to put into perspective the results of the cariogenic potential of raisins with other foods.

Methods:

Detailed descriptions of the methods are in Appendix C. Caries-free human molars were sliced and embedded in epoxy, then sanded to expose a small enamel window on one edge (see Fig. 1).

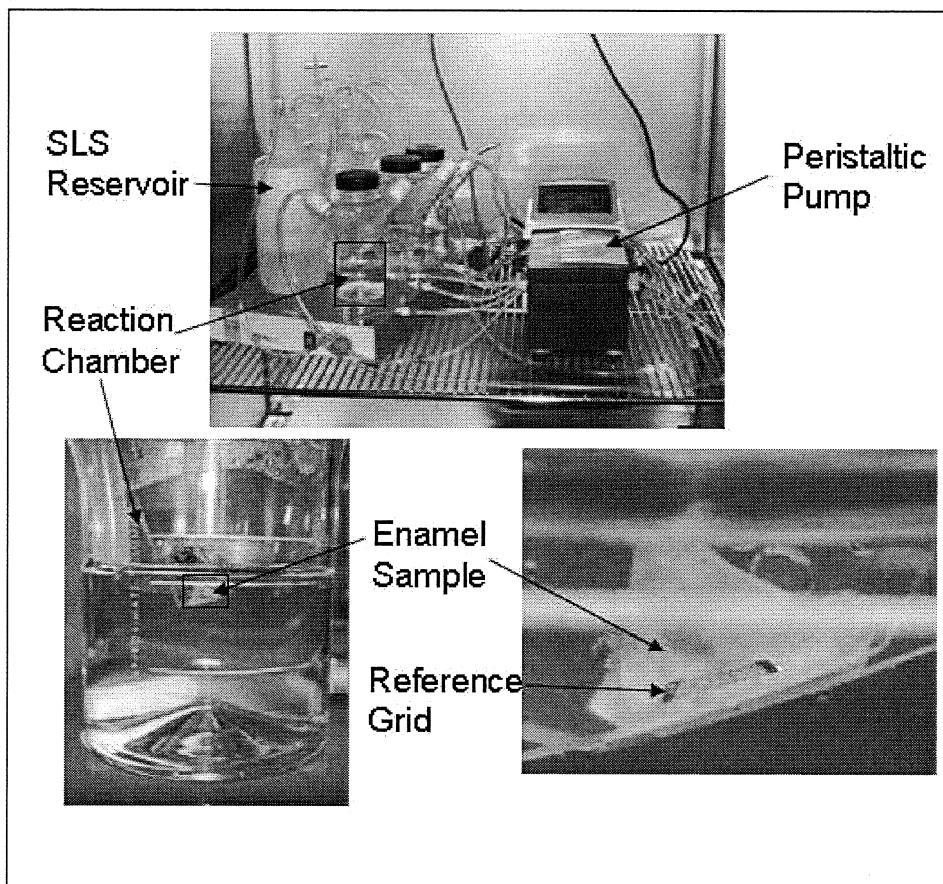
Figure 1: Preparation of enamel samples.



Specimen preparation. A sound, human molar was sliced along the sagittal plane (A), then each half was cross sectioned along the buccal and lingual faces (B). Smaller pieces of the cross sections were isolated based on analysis of sound enamel (C), then each piece was sanded to 100 µm thickness. A copper microgrid was mounted on each sample parallel to the desired enamel edge (D). Each sample was embedded in epoxy, which was sanded and polished to reach the desired size (E). The bottom edge was sanded and polished to expose an enamel window, followed by labeling and drilling of a small hole for hanging in the reaction vessel (F).

On treatment day 1, the samples were incubated in an inoculum of *Streptococcus mutans* for two hours to allow bacterial attachment onto the exposed enamel surface. Inoculation was followed with a 2 h incubation period in Brain-Heart Infusion media broth with 5 % dextrose to allow the bacteria to feed and attach. The samples were then exposed to the experimental solutions for 15 min and placed in SLS for the remineralization cycle. During treatment day 1, the samples were given two 15-min exposures to the experimental solution. A continuous flow of SLS through the reaction chambers clears the bacterially produced acids and unmetabolized sugars to mimic natural salivary oral clearance (Fig. 2).

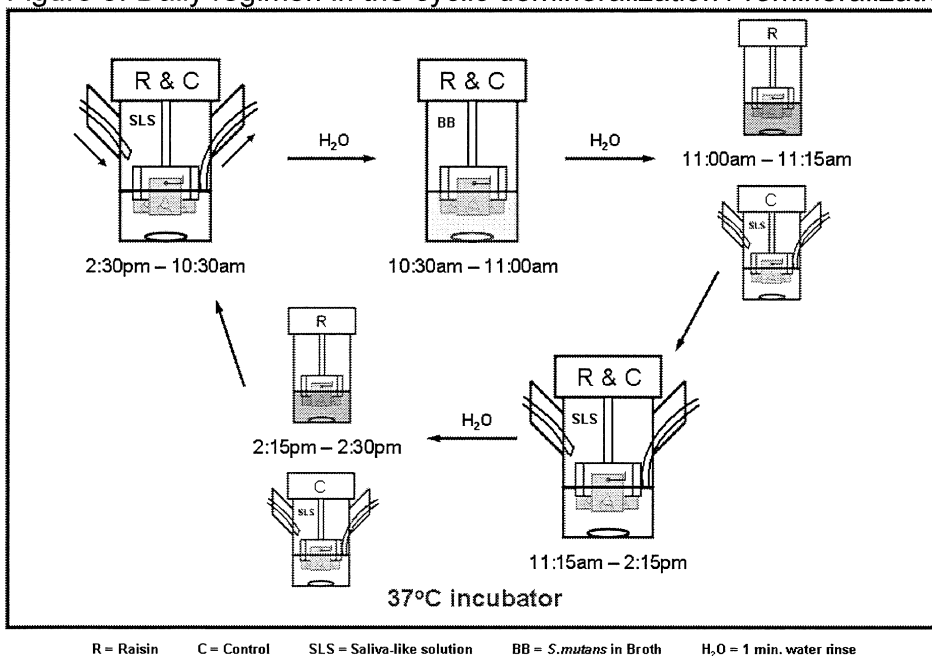
Figure 2: Experimental Setup in the Incubator



Subsequent daily regimen for treatment days 2, 3, and 5, (see Fig. 3) consisted of a 30 min re-inoculation of *S. mutans*, two 15 min exposures to the experimental solution, and exposure to circulating SLS washing the enamel samples between treatments. The process we are modeling is two exposures to experimental solutions daily with intervening remineralization cycles. On weekend days (non-treatment days) were experimental days 6 and 7. The samples remained in the reaction chambers with circulating SLS and received no treatments.

On treatment day 4, the samples were removed from the reaction chambers rinsed with dH_2O , sterilized with UV light and a sodium azide solution rinse and x-ray micrographs were taken. Following x-ray microradiography, the samples were treated as on treatment day 1. Results are given in terms of the experimental day (either day 4 or day 7) when the x-ray micrographs were taken.

Figure 3: Daily regimen in the cyclic demineralization / remineralization experiments.



The samples underwent one of the five following treatments during the seven day experiments: continuous saliva-like solution (negative control), or two 15 minute exposures per day of 10 % (w/w) raisin juice, 100 % orange juice, whole milk, or brain-heart infusion with 5% dextrose (system control).

X-ray microradiography: Evaluation of the enamel samples for mineral loss.

X-ray microradiographs of each sample were evaluated prior to experiments and after 4 d and 7 d of treatments (Chow *et al.*, 1990; Carey *et al.*, 2007; Schmuck *et al.*, 2007). These microradiographs were compared for changes as a function of time to determine the amount of enamel surface loss, and change in mineral density (lesion development). The change in the amount of mineralization at 80 % mineral density (Δ_{80}) was used as a measure of the mineral loss for each sample. This mineral loss includes both surface loss as well as a reduction in the mineral density (demineralization).

pH Measurement:

The pH was determined for each experimental solution, using an Orion pH electrode calibrated with NIST traceable pH standards.

Data Analysis:

The 4- and 7-day Δ_{80} measurements obtained for each specimen were averaged to give a mean Δ_{80} value for each treatment. This data was analyzed using ANOVA, Duncan's Rank Multiple Comparison, and paired t-Test. We have assumed that the population variances will be equal, and normal distribution to allow these statistical comparisons. Our number of samples from each group is too small to indicate otherwise.

Results:

Table 1 shows the pH and change in mineral (Δ_{80}) for the different treatments. Tables 2 and 3 gives the ANOVA statistics for Day 4 and Day 7, and find that there are significant differences between the groups.

Table 1. The pH of the exposure solutions and their descriptive statistics for the loss of mineral Δ_{80} .

Treatment	pH	4 day Mean \pm S.D. (n) μm	7 day Mean \pm S.D. (n) μm
Brain-Heart Infusion	7.2	-2.1 \pm 1.7 (2)	-2.7 \pm 0.2 (2)
Whole Milk	6.8	0.5 \pm 1.3 (3)	-0.1 \pm 1.8 (3)
Saliva-like Solution	6.8	1.1 \pm 3.5 (6)	3.6 \pm 3.8 (6)
Raisin Juice	3.6 – 3.9	8.9 \pm 4.2 (9)	15.0 \pm 7.3 (9)
Orange Juice	3.5	11.3 \pm 5.6 (6)	22.4 \pm 6.2 (6)

Table 2: ANOVA comparisons between the five exposure solutions at Day 4.

ANOVA 4-Day					
Source of Variation	SS	df	MS	F	P-value
Between Groups	617.015156	4	154.2538	6.154355	0.001933
Within Groups	526.3475556	21	25.06417		
Total	1143.362712	25			

Table 3: ANOVA comparisons between the five exposure solutions at Day 7.

ANOVA 7-Day					
Source of Variation	SS	df	MS	F	P-value
Between Groups	2038.301978	4	509.5755	14.30451	8.75E-06
Within Groups	748.0918833	21	35.62342		
Total	2786.393862	25			

Paired difference t-test of the Δ_{80} between Day 4 and Day 7 found no significant differences for Brain-Heart Infusion ($p = 0.32$) or Milk ($p = 0.18$) and significant differences for SLS ($p = 0.02$), Raisin Juice ($p = 0.002$) and Orange Juice ($p < 0.001$).

Duncan's Ranked Multiple Comparison at $p \leq 0.05$ finds significant differences in the amount of mineral loss (Δ_{80}) between the different treatments.

Day 4: Brain-Heart Infusion = Milk = SLS < Raisin Juice = Orange Juice

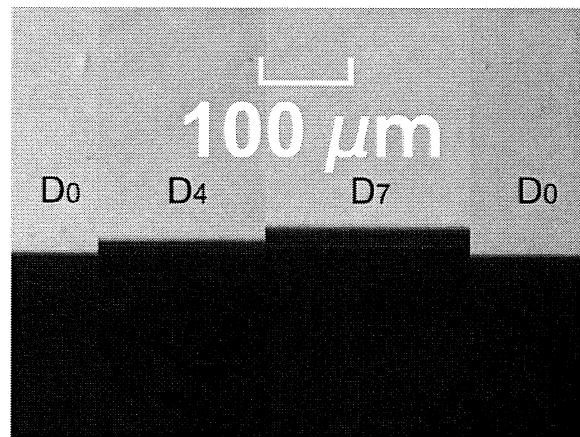
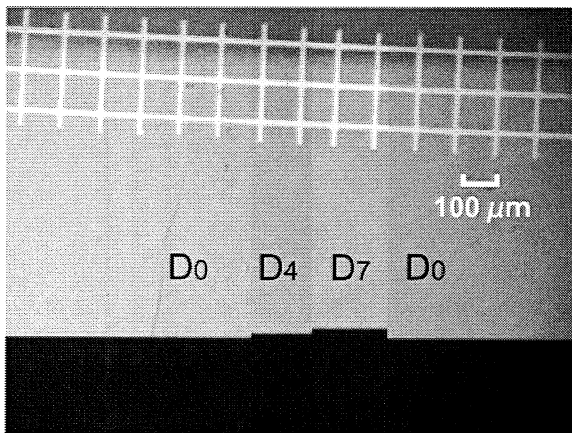
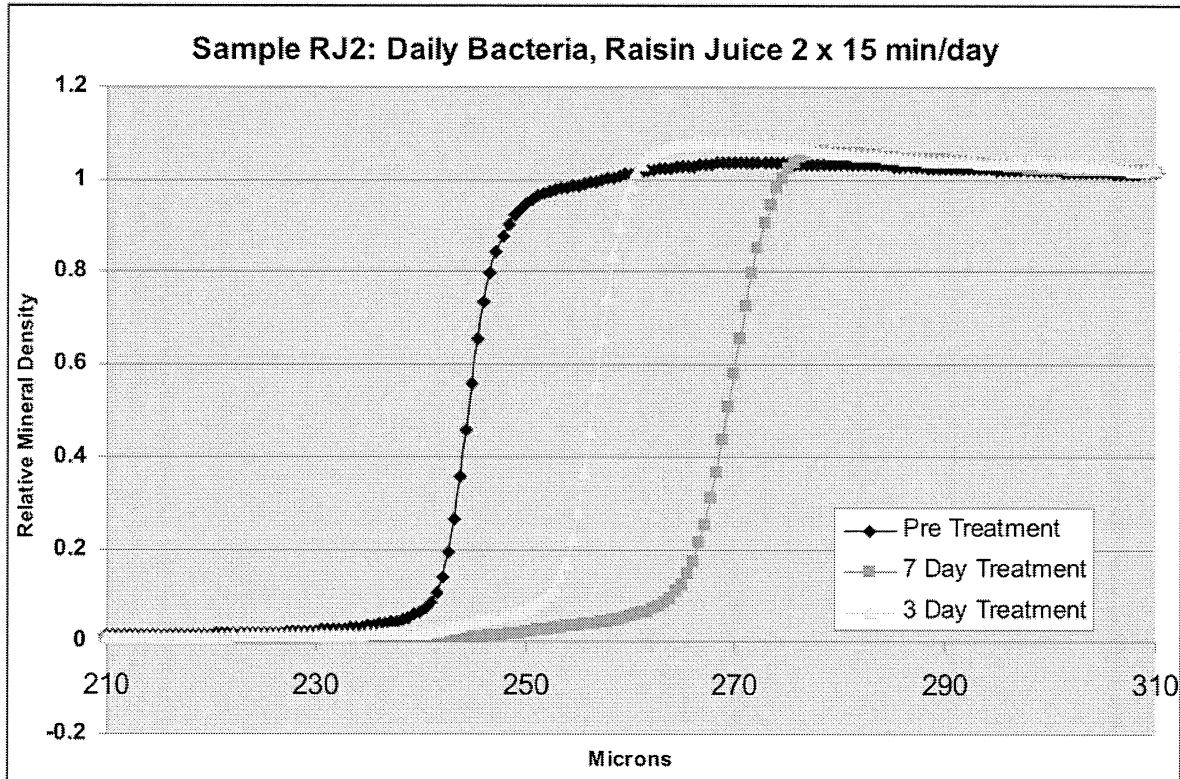
Day 7: Brain-Heart Infusion = Milk = SLS < Raisin Juice < Orange Juice

Loss of Mineral: The change in the amount of mineralization at 80 % mineral density (Δ_{80}) is reported in Table 1. Figures 4 through 8 present the photomicrographs and x-ray micrographs for representative samples of each experimental solution. The x-ray micrographs are overlaid to show the extent of surface loss due to the experiments. The graphs of the mineral density show the reduction in mineral density from the enamel. These graphs can be interpreted as a cross-section of the enamel slice.

Figure 4: Composite x-ray micrographs for Raisin Juice treatment from Day 0, Day 4 and Day 7 of the experiments along with the digital profile of the sample.

Raisin Solution

4 Day Δ_{80} : 12.17 μm 7 Day Δ_{80} : 23.23 μm

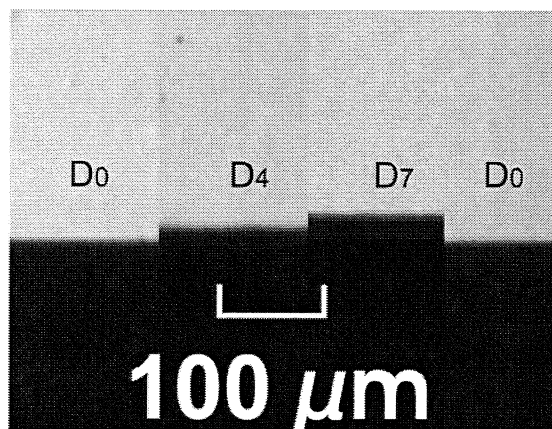
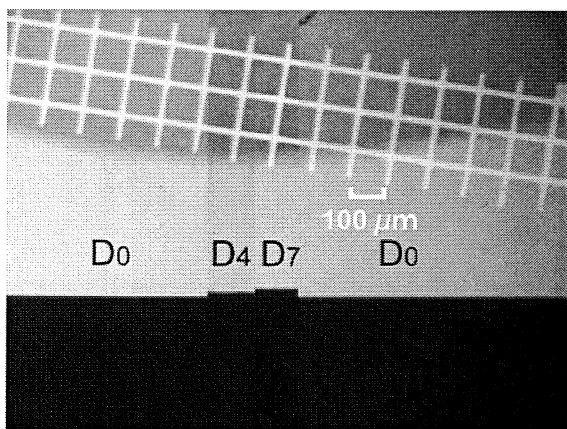
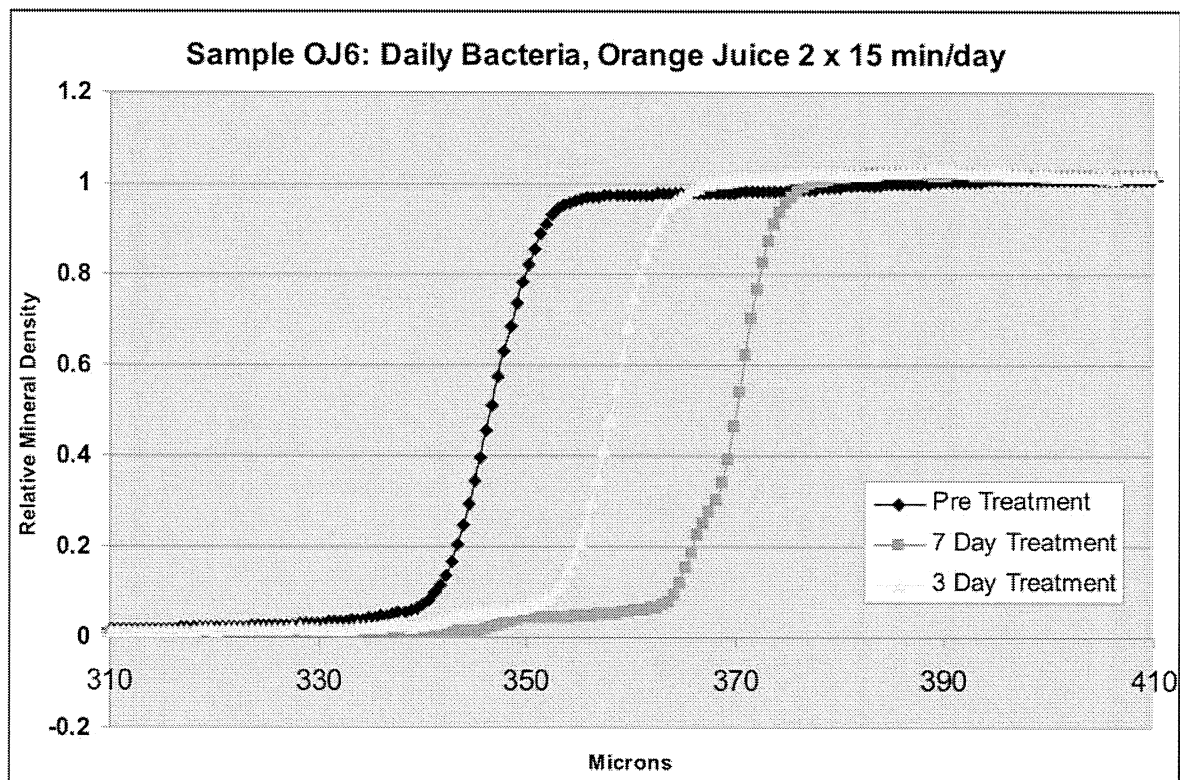


The x-ray micrographs are overlaid in alignment with the reference grid to show the relative mineral loss (Δ_{80}) as a function of time. The digital profile shows the position of the enamel surface and mineral density as a function of time.

Figure 5: Composite x-ray micrographs for Orange Juice treatment from Day 0, Day 4 and Day 7 of the experiments along with the digital profile of the sample.

Orange Juice

4 Day Δ_{80} : 11.43 μm 7 Day Δ_{80} : 21.22 μm

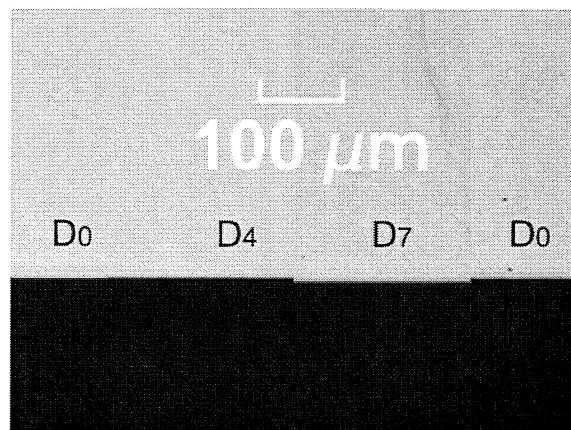
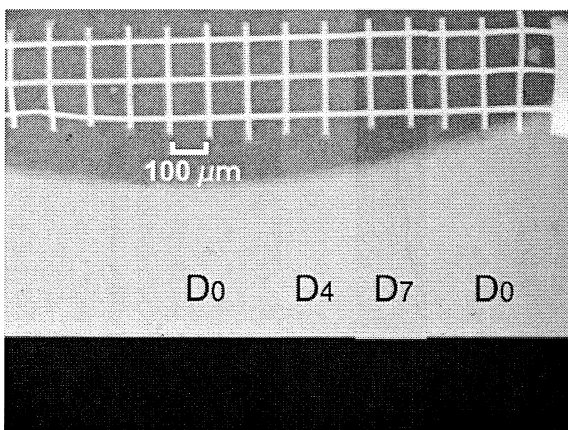
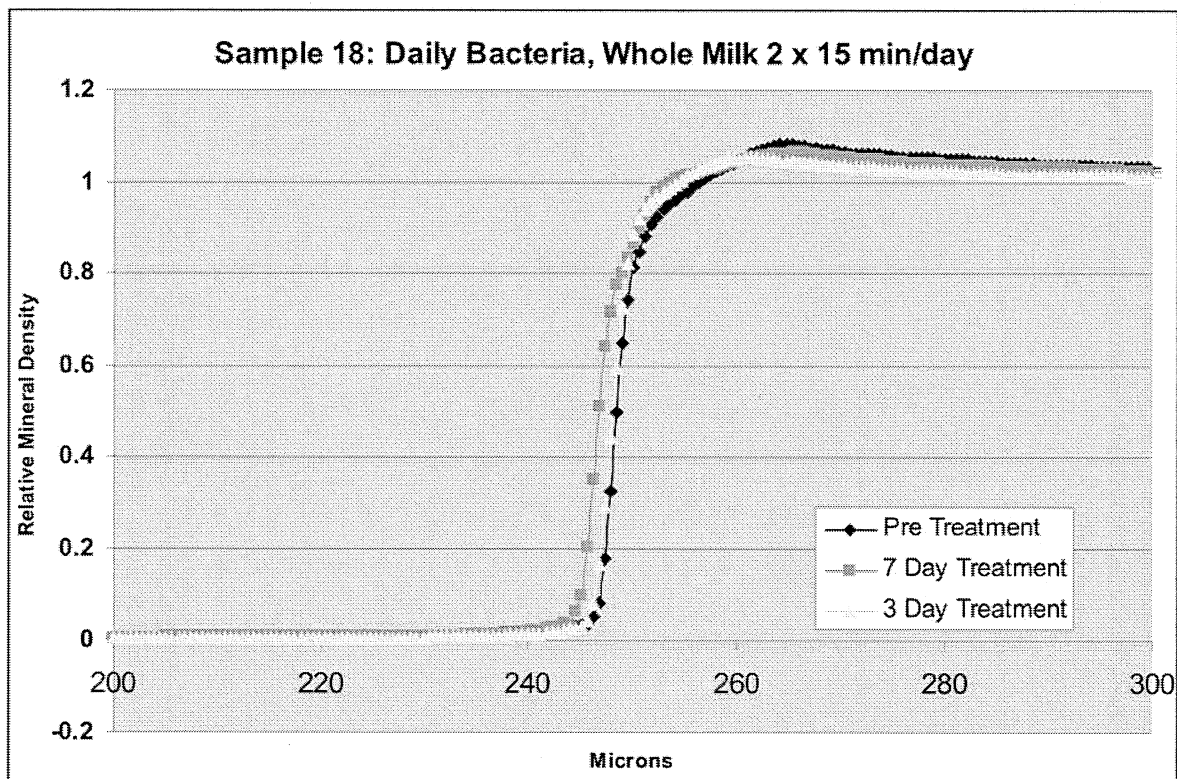


The x-ray micrographs are overlaid in alignment with the reference grid to show the relative mineral loss (Δ_{80}) as a function of time. The digital profile shows the position of the enamel surface and mineral density as a function of time.

Figure 6: Composite x-ray micrographs for Whole Milk treatment from Day 0, Day 4 and Day 7 of the experiments along with the digital profile of the sample.

Whole Milk

4 Day Δ_{80} : 0.62 μm 7 Day Δ_{80} : 0.85 μm

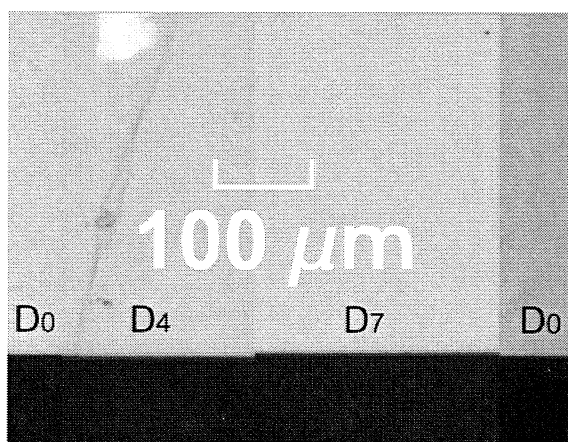
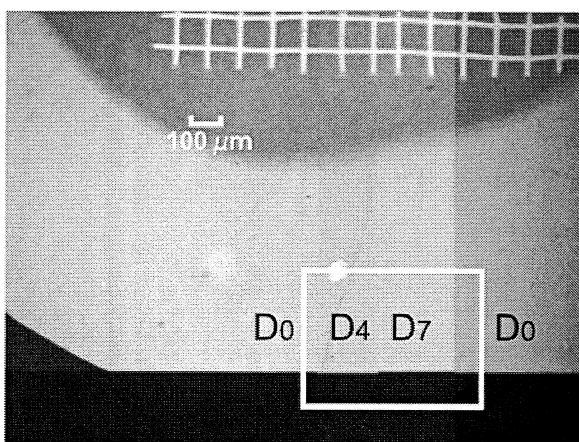
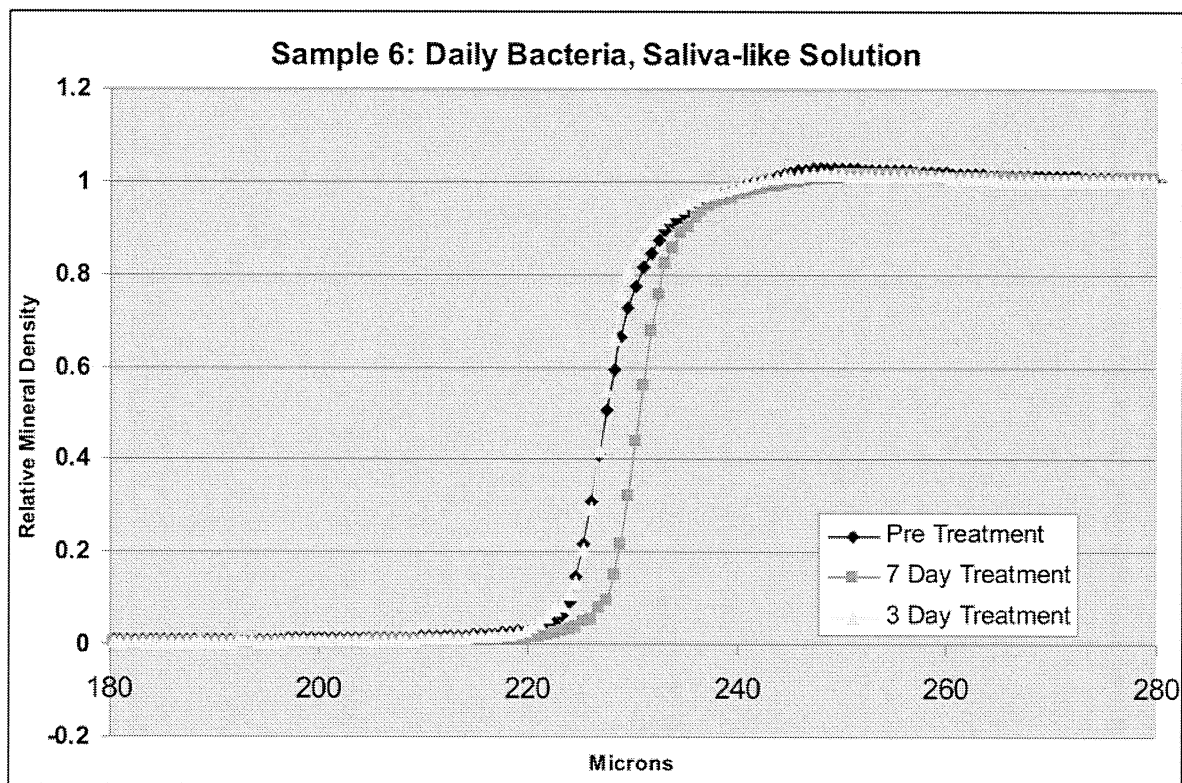


The x-ray micrographs are overlaid in alignment with the reference grid to show the relative mineral loss (Δ_{80}) as a function of time. The digital profile shows the position of the enamel surface and mineral density as a function of time.

Figure 7: Composite x-ray micrographs for Saliva-like Solution treatment from Day 0, Day 4 and Day 7 of the experiments along with the digital profile of the sample.

Saliva-like Solution

4 Day Δ_{80} : $-1.15 \mu\text{m}$ 7 Day Δ_{80} : $2.24 \mu\text{m}$

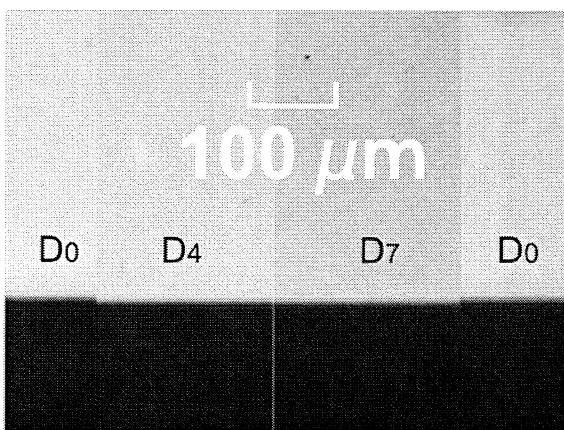
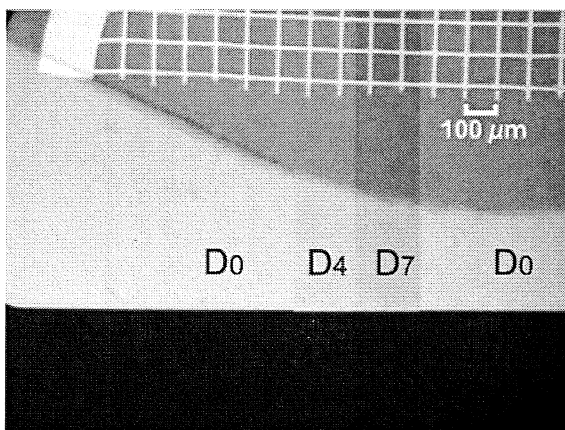
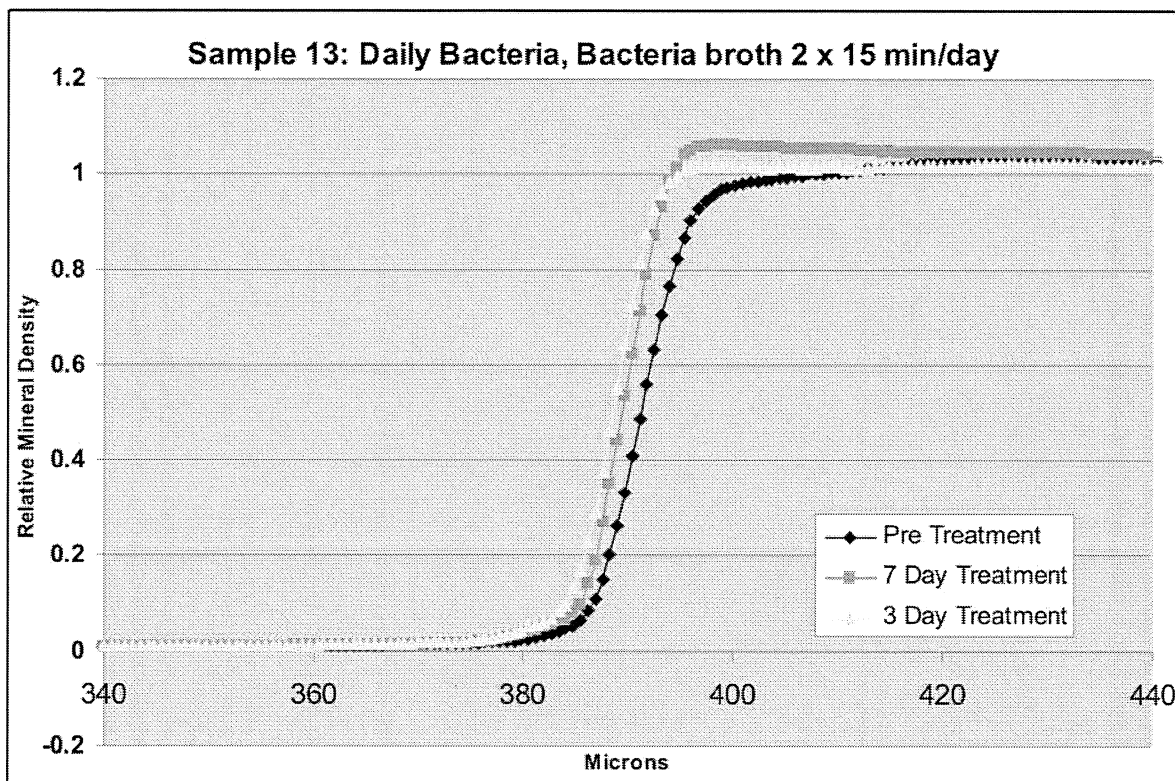


The x-ray micrographs are overlaid in alignment with the reference grid to show the relative mineral loss (Δ_{80}) as a function of time. The digital profile shows the position of the enamel surface and mineral density as a function of time.

Figure 8: Composite x-ray micrographs for Brain-Heart Infusion Broth treatment from Day 0, Day 4 and Day 7 of the experiments along with the digital profile of the sample.

Brain-Heart Infusion Broth

4 Day Δ_{80} : $-3.25 \mu\text{m}$ 7 Day Δ_{80} : $-2.87 \mu\text{m}$



The x-ray micrographs are overlaid in alignment with the reference grid to show the relative mineral loss (Δ_{80}) as a function of time. The digital profile shows the position of the enamel surface and mineral density as a function of time.

Discussion:

This *in vitro* caries model is designed to mimic the conditions in the mouth by mimicking several important conditions of the oral environment, including the cyclic nature of caries attacks followed by a period of remineralization, continuous salivary flow, and *S. mutans* as the source of the acid attacks. However, the model does not include the effects of increased salivary flow during eating, tooth pellicle, plaque inhibition by salivary components, and fluoride. Thus, the apparent severity of a demineralizing attack on tooth enamel as measured in microns of mineral loss determined by this *in vitro* caries model is much greater than what is observed *in vivo*. Relative rankings of food solutions would be indicative of their potential to cause demineralization.

The *in vitro* caries model developed for this project allows one to determine the relative demineralization potential of different foods in solution. The three food solutions tested were whole milk, raisin juice (10 % w/w), and orange juice. The amount of mineral loss was significantly more than Saliva-like solutions for raisin juice and orange juice and less for whole milk. The lack of demineralization caused by milk is because it is supersaturated with respect to tooth mineral and does not contain large amounts of readily metabolized sucrose. Raisin juice and orange juice are acidic, undersaturated with respect to tooth mineral and contain large amounts of sucrose that the *S. mutans* metabolizes into acids. The relative increase in mineral loss due to the higher sucrose containing food solutions is consistent with other reports (Pollard *et al.*, 1996). It is noteworthy to observe that although the pH of the raisin juice and the orange juice are similar, the amount of mineral loss was less in the raisin juice experiments. This may be due to the phytochemicals identified by Wu and coworkers (Wu *et al.*, 2003; La Monte *et al.*, 2004) or could be due to the difference in the acids that predominate the solutions. Orange juice contains large amounts of citric acid which is a particularly strong calcium kelator, and thus is able to dissolve tooth mineral more aggressively. Complementary experiments are under way to help determine which of these two factors, or what combination of them is making the difference.

The pattern of mineral loss is mostly from the surface of the samples and less from the body of the enamel samples. This observation is similar to findings in demineralization experiments where there is no fluoride present (Carey *et al.*, 2004). The presence of fluoride tends to enhance remineralization of the surface, resulting in stabilization of the enamel surface, effectively halting 'erosion-like' mineral loss (Carey *et al.*, 2007). Another feature of this caries model is that the acidic attack is severe causing rapid mineral loss only from the surface of the enamel. The relative intensity of the attack is such that the surface is lost, and thus the remineralization cycle has nothing to remineralize. Additionally, subsurface lesions do not form under these conditions. The addition of very small amounts of 1 ppm (1 µg/mL) fluoride to the SLS has been shown to be sufficient to stabilize the surface of enamel under these conditions. Because fluoride is typically present in the oral cavity the caries model should include experiments that have fluoride in the SLS. Further studies using this *in vitro* caries model including fluoride in the SLS are planned.

Planned presentations and publications:

Raisin Effects on *in vitro* Demineralization of Human Teeth, Rose, J.L., Hoffman, K.M., Schmuck, B.D., Dickens, S.H., and Carey, C.M. Submitted for presentation at the American Association of Dental Research meeting to be held April 2008.

Demineralization of tooth enamel involves a sensitive balance of several factors, including oral bacteria, dietary components, salivary flow and clearance. Previous studies using rats inoculated with oral bacteria have indicated a high cariogenic potential for raisins. The bacterial action is believed to be an etiological agent of human dental caries. **Objective:** To evaluate the cariogenic potential of raisins with respect to the factors listed above; this study utilized an *in vitro* model to determine the affect of raisins upon the demineralization of human enamel by intraoral bacteria under a continuous flow of saliva-like solution (SLS). **Methods:** The crowns of extracted caries-free human molars were sliced laterally to produce 180µm thick sections of buccal or lingual enamel. The enamel slices were embedded in epoxy, and then sanded to expose a 300µm treatment window on the edge. The epoxy samples were incubated (37°C) in an inoculum of *Streptococcus mutans* for 2h to allow bacterial attachment onto the exposed enamel. For 7d, the control group was treated with SLS, and the experimental group with SLS plus two 15-min exposures/day of 10% (w/w) raisin juice. Microradiographs of each sample were evaluated prior to, during, and following treatments for change in lesion depth plus surface loss at 80% mineral density (Δ_{80}). **Results:** At 3d, the raisin samples showed a Δ_{80} of $5.7 \pm 4.1 \mu\text{m}$, relative to $1.1 \pm 3.5 \mu\text{m}$ in the SLS. After 7d, the raisin samples showed a Δ_{80} of $11.2 \pm 4.7 \mu\text{m}$, relative to $3.6 \pm 3.8 \mu\text{m}$ in the SLS ($p < 0.06$, $n = 6$, t-test). **Conclusion:** Although not statistically significant, the 7d results indicate that 10% raisin juice may stimulate intraoral bacteria to demineralize human teeth despite the neutralizing capabilities of salivary flow. More study is needed to determine the relative intensity of the cariogenic attack compared to other common food stuffs. Support: California Raisin Marketing Board, Great West Life Insurance, ADAF, and NIST.

Papers in progress:

A Microbial *in vitro* Caries Model to Assess the Effects of Food Solutions on Human Teeth. This paper will focus on the caries model and use raisin juice, orange juice, and milk as examples. To be submitted to the Journal of Dental Research.

Effects of Food Solutions on Human Teeth. This paper will discuss the differences between food solutions on the demineralization of teeth as found by this caries model. To be submitted to the Journal of the American Dental Association.

Comparison of Erosion to Demineralization by Food Solutions on Human Teeth. This paper will present erosion data measured by a non-bacterial model and mineral loss data determined by the microbial *in vitro* caries model. The differentiation between the causes of erosion and bacterially mediated mineral loss will be discussed. To be submitted to the Journal of Dental Research.

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