

Physiological Effects of Sucrose Substitutes and Artificial Sweeteners on Growth Pattern and Acid Production of Glucose-Grown *Streptococcus mutans* Strains *in vitro*

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Streptococcus mutans, Sweeteners, Caries, Polysorbate, Saccharin

The synergistic effects of four sucrose substitutes, polysorbate and five artificial sweeteners were studied *in vitro* on growth pattern and acid production of seven glucose-grown *Streptococcus mutans* strains, representing the five serological groups after Bratthall. Four distinct growth patterns during glucose fermentation were observed: high rate of growth with low acid production, moderate growth rate with moderate acid production, moderate growth rate with high acid production, and slow rate of growth with moderate acid production. Depending on the strain used, the final OD at 546 nm ranged from 0.55 to 0.99 and the final pH of the medium varied between 4.65 and 4.15. While added sucrose substitutes, with exceptions, usually enhanced growth rate, most artificial sweeteners suppressed or, at higher concentrations, even inhibited growth of *S. mutans*; addition of polysorbate to the medium always increased growth rate of *S. mutans* significantly. The presence of sucrose substitutes during glucose fermentation had no effect on final pH of the medium, but addition of artificial sweeteners, especially sodium saccharin, elevated final pH up to 1.8 units. The observed physiological patterns and differences within the several strains of *S. mutans* during glucose fermentation *in vitro* do not necessarily relate to the five serological groups of the species.

Introduction

Currently, there is much interest in the cariogenic properties of sucrose substitutes^{1, 2} and the development of new non-nutritive artificial sweeteners³. Sucrose substitutes and artificial sweeteners are widely used to sweeten a variety of food preparations and health aids such as "sugarless" candy, chewing gums, low-calorie beverages and tooth paste. Little knowledge is available as to how sucrose substitutes or artificial sweeteners interfere with the physiology of *Streptococcus mutans*, the etiological agent of dental caries, growing in a sucrose and/or glucose containing environment *in vitro* or *in vivo*.

The present study aimed to examine the physiological effects of several food additives – four sucrose substitutes, polysorbate and five artificial sweeteners – on growth pattern and acid production of 7 glucose-grown *S. mutans* strains, representing the five serological groups after Bratthall, *in vitro*.

Materials and Methods

Bacterial strains

Seven strains of *S. mutans*, representing the five Bratthall serological groups⁴, were used in this study

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(Table I). Five of these strains are isolates from humans, and the other two strains are isolates from hamster and rat.

Table I. Origin and Bratthall serological groups of the seven studied *Streptococcus mutans* strains.

| Strain number | Sero-logical group | Origin | Source |
|---------------|--------------------|---------|---|
| HS-6 | a | hamster | Dr. R. J. Fitzgerald, Miami, Florida, USA |
| AHT | a | human | Dr. H. D. Slade, Chicago, Illinois, USA |
| FA-1 | b | rat | |
| NCTC 10449 | c | human | |
| OMZ-176 | d | human | |
| B-2 | e/E | human | Dr. S. Edwardsson, Malmö, Sweden |
| P-4 | e/E | human | |

Compounds

The following chemicals were purchased: D-(–)-fructose (J. T. Baker Chemical Co., Phillipsburg, N. J.), D-sorbitol, D-mannitol and tween 80 (Sigma Chemical Co., St. Louis, Mo.), and D-xylitol (ICN Pharmaceuticals, Inc., Plainview, N. Y.). The dihydrochalcones of naringin and neohesperidin were synthesized in this laboratory⁵. The following artificial sweeteners were obtained as a gift: sodium saccharin (Sherwin Williams Co., Cleveland, Ohio), sodium cyclamate (Abbott Laboratories, North Chicago, Illinois) and aspartyl-phenylalanine methyl ester (G. D. Searle Co., Arlington Heights, Illinois).



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Media

The *S. mutans* strains were maintained in a medium composed of 2% glucose (Bacto-Dextrose, Difco) and 2% yeast extract (Difco) at pH 6.5 in screw cap test tubes containing 9 ml, sterilized in an autoclave for 10 min at 20 lb./sq. in. and 126 °C. The tubes were supplemented with 10% inoculum and were incubated at 28 or 36 °C. The cultures were transferred every 4 to 5 days. The strains were preserved for a longer time period in Bacto Transport Medium Stuart (Difco) by inoculating 1 ml of a cell suspension to 5 ml of the transport medium in screw cap test tubes, mixing well, and freezing at -20 °C. To reactivate these cultures, 1 ml of the thawed suspensions was added to 9 ml of yeast extract-glucose medium and incubated at 28 °C until growth appeared.

Experimental approach

Stock solutions of the four sucrose substitutes, polysorbate and the five artificial sweeteners, containing 2.8 g of compound/20 ml of distilled water, were made up and sterilized by ultrafiltration; solutions of aspartyl-phenylalanine methyl ester and the dihydrochalcones were sterilized by tyndallization. The solutions were then diluted to yield the desired sweetener concentrations. For each test, 1.0 ml of the appropriate sweetener dilutions and 0.5 ml of sterile water were added to 5.0 ml of a 2% glucose, 2% yeast extract medium at pH 6.5 in screw cap test tubes. The final concentrations of the sweeteners in the medium were 2% (20 mg/ml), and 1/10, 1/20, 1/100 and 1/1000 of the 2% concentration.

The tubes were then inoculated with 0.5 ml of a 48 hour culture of each of the seven *S. mutans* strains to yield a total volume of 7.0 ml. Inoculated tubes with 1.5 ml of sterile water added to the glucose-yeast extract medium served as a control. The test tubes were incubated in a gyrotory water bath shaker (New Brunswick Scientific, Model G76) at 36 °C. After 24 hours the optical density, as a measure of growth, was determined at 546 nm using a Bausch & Lomb Spectronic 20 Spectrophotometer, and the final pH was read using a Fisher Accumet Model 230 pH/ion meter.

All tests were carried out in duplicate and the obtained data were averaged to unify the experiment.

Results

I. Growth and fermentation patterns of *S. mutans* in the presence of glucose

To study the growth and fermentation patterns of *S. mutans* in the presence of glucose, the control

data of all utilized strains were compared. Four distinct growth patterns could be observed (Fig. 1):

- Pattern 1: high rate of growth with low acid production (Strain OMZ-176).
- Pattern 2: moderate growth rate with moderate acid production (Strains FA-1, AHT, HS-6).
- Pattern 3: moderate growth rate with high acid production (Strains 10449, P-4).
- Pattern 4: slow rate of growth with moderate acid production (Strain B-2).

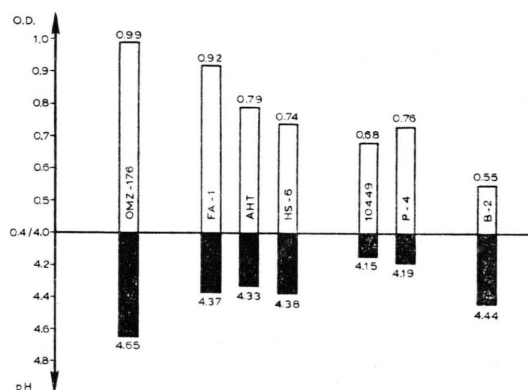


Fig. 1. Growth pattern (OD at 546 nm) and acid production (pH) of *Streptococcus mutans* strains during glucose fermentation *in vitro*.

Pattern 1 represents an extreme deviation from the expected pattern of growth. The atypically high pH of 4.65 and the high optical density of 0.99 suggest that the glucose is primarily incorporated into the cell for growth, while a relatively smaller amount of the glucose is converted to acidic end products, accounting for the higher pH value.

Pattern 2 agrees with the normally expected growth and fermentation. While all three strains yield moderate pH values in the 4.33 to 4.38 range, the differences in the optical density, ranging from 0.74 to 0.92, can be accounted for by the differences in their rate of growth. Strain FA-1 possesses the highest rate of growth of the three strains in this group. This suggests that more glucose is being utilized for accumulation of cell material as compared to the strains AHT and HS-6.

Pattern 3 deviates from the expected norm in that the pH of 4.15 and 4.19 is much lower than the norm. Since growth is moderate- OD = 0.68 and 0.76, the low pH indicates that relatively more glucose is utilized for the acid yielding fermentation

process as compared to glucose-C incorporated for cell growth.

Pattern 4 represents the other extreme in its pattern of growth. Strain B-2 is a slow grower, OD = 0.55, and the produced pH of 4.44 is in the medium range. This observation may give indications as to the way glucose is utilized or may reflect a lower tolerance level of this strain to produced acids, thereby inhibiting its growth.

With the many strains of *S. mutans* that have been identified, this study demonstrates that there are several physiological patterns and differences within the species during glucose fermentation. No conclusions can be drawn with respect to trends within the five Bratthall serological groups, because of the limited number of strains tested.

II. The effects of sucrose substitutes, polysorbate, and artificial sweeteners on the growth of glucose-grown *S. mutans*

A. Sucrose substitutes

1. Fructose

Fructose promotes the growth of glucose-grown *S. mutans* (Fig. 2). This trend is most apparent in the more prolific strains (AHT, FA-1, OMZ-176, HS-6) and seems to be present in the other three strains as well. The growth enhancement effect is greatest at the low and intermediate fructose concentrations, while in some cases the 2% concentration seems to slow down growth.

2. Mannitol

Under test conditions, mannitol concentrations ranged from 0.2 mg/ml, where mannitol may function as a cofactor, to 20 mg/ml, where mannitol can be utilized as a carbon source. The resulting data (Fig. 2) indicate that the intermediate concentrations produce an enhancement of growth of most strains. On the other hand, mannitol at the 2% concentration tends to inhibit the majority of the strains tested.

3. Sorbitol

Sorbitol in the presence of glucose causes an overall inhibition of *S. mutans* growth; however, intermediate concentration ranges, in most cases, appear to have a slight growth depressing effect while the 2% concentration is clearly inhibitory (Fig. 2). This observed inhibition may be attributed

to a competitive effect with the glucose present in the system.

4. Xylitol

We found that in the presence of glucose, xylitol promotes growth of most *S. mutans* strains in the low and intermediate concentration ranges. The results are most obvious in the fast growing strains while the slower growing strains 10449, B-2 and P-4 produce only slight, if any enhancement of growth (Fig. 2). Xylitol at the 2% concentration seems to have an inhibitory effect on all strains under test conditions.

B. Polysorbate (Tween 80)

We assumed that polysorbate as a nonionic surfactant would exert a growth inhibitory effect on *S. mutans*, but this study demonstrated that addition of polysorbate to the medium caused an increased growth activity of the glucose-grown *S. mutans* strains at all concentrations; the optimum concentration for enhancement of growth was found to be at 0.1% (Fig. 2).

C. Artificial sweeteners

1. Sodium cyclamate

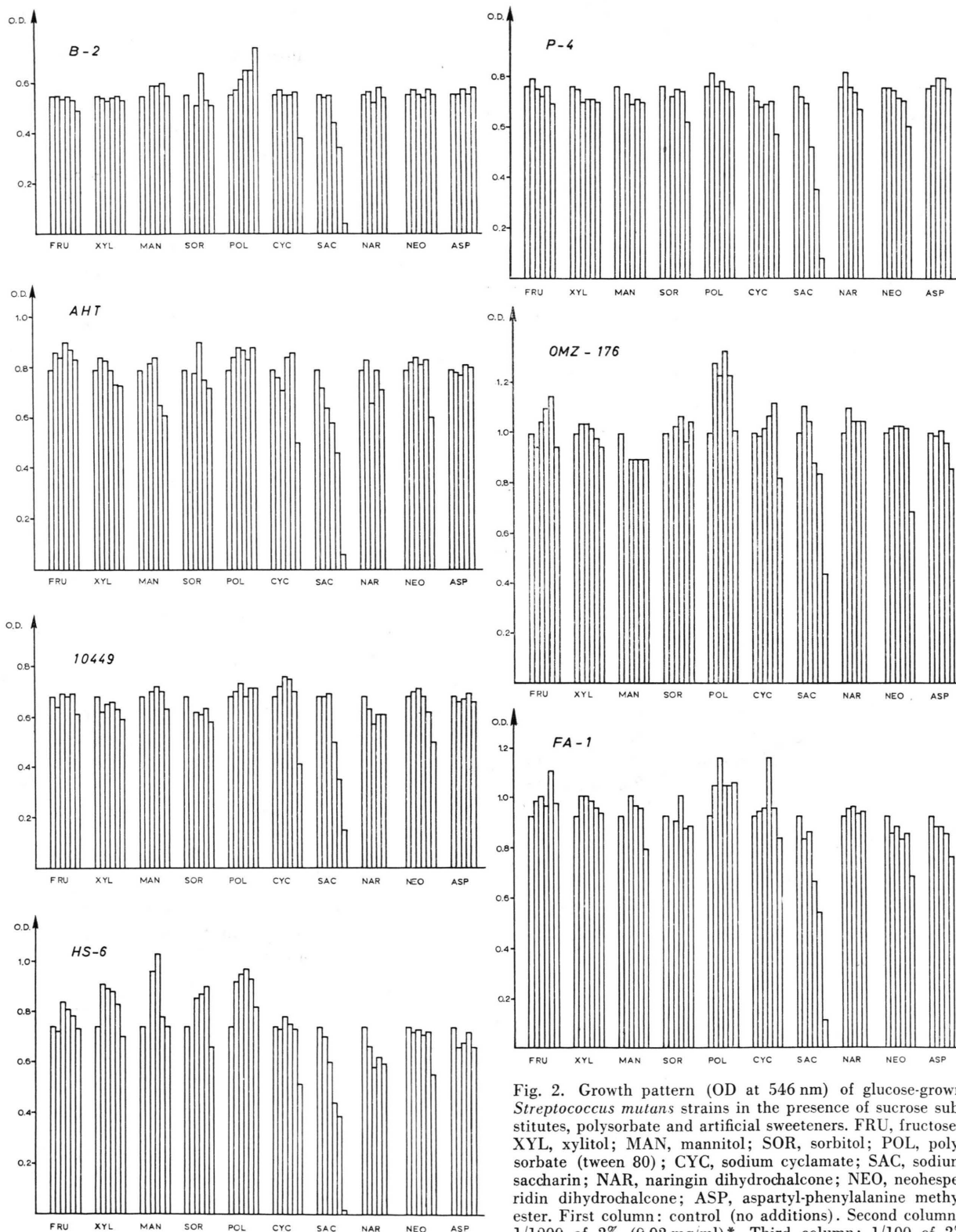
This artificial sweetener exhibits a dual effect on glucose-grown *S. mutans*. In examining optical density, the trend indicates a promotion of growth up to the 1/10 concentration of 2% (2 mg/ml), but the 2% concentrations clearly induce reduction of growth (Fig. 2).

2. Sodium saccharin

Sodium saccharine definitely exerts a growth inhibiting effect on glucose-grown *S. mutans* throughout the studied concentration range from 0.02 to 20 mg/ml (Fig. 2). The magnitude of the growth inhibition is proportional to the saccharin concentrations and affects all seven strains of *S. mutans* tested.

3. Naringin dihydrochalcone (NDHC)

In the presence of NDHC no particular trends are evident (Fig. 2); each strain is affected differently. At the 1/10 concentration of 2%, NDHC produces a slight inhibitory or no effect on growth. In some cases (OMZ-176, FA-1, AHT, P-4 and B-2), a slight increase of growth could be observed at the lower concentration (0.02 mg/ml). Data at



* No data for MAN and SOR at this concentration.

** No data for NAR and ASP at this concentration.

Fig. 2. Growth pattern (OD at 546 nm) of glucose-grown *Streptococcus mutans* strains in the presence of sucrose substitutes, polysorbate and artificial sweeteners. FRU, fructose; XYL, xylitol; MAN, mannitol; SOR, sorbitol; POL, polysorbate (tween 80); CYC, sodium cyclamate; SAC, sodium saccharin; NAR, naringin dihydrochalcone; NEO, neohesperidin dihydrochalcone; ASP, aspartyl-phenylalanine methyl ester. First column: control (no additions). Second column: 1/1000 of 2% (0.02 mg/ml)*. Third column: 1/100 of 2% (0.2 mg/ml). Fourth column: 1/20 of 2% (1 mg/ml). Fifth column: 1/10 of 2% (2 mg/ml). Sixth column: 2% (20 mg/ml)**.

the 2% concentration could not be obtained since 2% exceeds the solubility of NDHC in an aqueous system at room temperature.

4. Neohesperidin dihydrochalcone (NHDHC)

Though NDHC and NHDHC are structurally similar compounds, their effects on *S. mutans* seem to be unrelated. NHDHC in most cases suppresses growth throughout the studied concentration range (Fig. 2), especially at the higher concentrations. In case of the strains OMZ-176, AHT, 10449 and B-2 a slight growth enhancement at the lower concentration (0.02 mg/ml) of NHDHC could be observed.

5. Aspartyl-phenylalanine methyl ester

This dipeptide sweetener has unexpectedly little or no effect on the growth of glucose-grown *S. mutans* (Fig. 2), and in a parallel study, it was found that this compound, given as sole carbon source, could not be utilized by the strains tested.

III. The effect of sucrose substitutes, polysorbate and artificial sweeteners on the acid production (pH) of glucose-grown *S. mutans*

A. Sucrose substitutes and polysorbate

The addition of fructose, mannitol, sorbitol, xylitol and polysorbate to *S. mutans* growing in a glucose containing medium caused no significant changes in acid production. At all concentrations tested the final pH of the medium remained, with slight variations, at the level as indicated for the seven strains in Fig. 1.

B. Artificial sweeteners

Aspartyl-phenylalanine methyl ester had no significant effect on the acid production of *S. mutans* during glucose fermentation at all concentrations tested; the pH of the medium remained at the levels as stated in Fig. 1. On the contrary, a dramatic elevation of the final pH was observed during the growth of *S. mutans* in the presence of the other artificial sweeteners. In comparison to the control, the pH increased, especially at the higher concentrations (2 and 20 mg/ml), in the order NDHC (+0.1 unit) < sodium cyclamate (+0.2 units) < NHDHC (+0.6 units) < sodium saccharin (+1.8 units). From examining the produced pH of the

various strains, the trend is a steadily increasing pH with increasing concentrations. This would indicate that these artificial sweeteners negatively affect the production of acids from glucose, yet in most cases allow glucose-C to be assimilated for growth (Fig. 2), with the exception of sodium saccharin, where the pH rises concomitantly with higher concentrations (Fig. 3). This could indicate

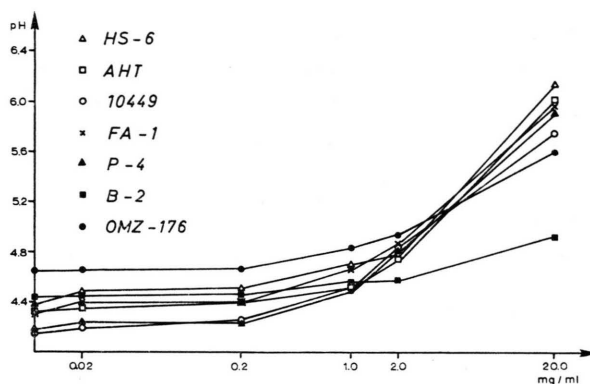


Fig. 3. Effect of sodium saccharin on acid production (pH) of *Streptococcus mutans* strains during glucose fermentation *in vitro*.

that the glycolytic sequence is not fully operational and that the assimilation of glucose-C for growth is strongly reduced (Fig. 2).

Discussion

We are living in a society where sucrose and glucose contribute substantially to our daily diet, and it is of interest to find out more about the possible synergistic effects of sucrose substitutes and artificial sweeteners on this specific diet in relation to dental caries formation by *S. mutans*. One would expect that the addition of certain concentrations of these sweeteners to this environment may result in either an increased or decreased growth rate of *S. mutans*; these sweeteners may even cause complete growth inhibition due to possible toxic effects of these compounds.

Recent rat feeding studies indicated that animals fed a sucrose diet had a higher incidence of caries development compared to animals that were fasted or intermittently fed diets of various carbohydrates⁶. In another study involving human volunteers, it was found that ingestion of a mixture of sucrose substitutes reduced plaque formation when compared to sole intake of a sucrose diet⁷. The uptake

of foods sweetened with fructose or xylitol in the total absence of sucrose led to plaque inhibition² and significant reduction of dental caries⁸. Microbiological data of the latter study indicated a lower occurrence of *S. mutans* in the plaque samples of the test persons after one year of xylitol consumption in contrast to the sucrose and fructose groups⁹. From these studies, one might assume that a population on a sucrose diet supplemented with additional sucrose substitutes for the purpose of "diluting" the sucrose intake, would develop caries at a rate significantly lower than a population solely on a sucrose diet. Certainly, this would depend on the *in vivo* effects of this diet on *S. mutans*. Since the *in vivo* effects are much too complex to be foreseen, this simplified *in vitro* physiological study was conducted. For the growth of *S. mutans* we chose a glucose system rather than a sucrose system to avoid excessive glucan formation and clumping together of cells. The results indicate that all *S. mutans* strains tested are high acid producers. The final pH, ranging from 4.65 to 4.15, is far below the critical pH of 5.50¹⁰, necessary for demineralization of the teeth and caries formation¹¹. The final pH of the glucose medium was not or only slightly influenced by addition of the four sucrose substitutes, polysorbate and aspartyl-phenylalanine methyl ester. We noted, however, that addition of the other four artificial sweeteners to the glucose medium elevated the final pH significantly. Most effective was sodium saccharin, which increased the pH up to 1.8 units at the highest concentration. Therefore, sodium saccharin may have some potential in reducing the incidence of dental caries. In a study involving 24 student volunteers receiving a diet enriched with a low-calorie sweetener, composed of saccharin and up to 98% of glucose (including short chains of glucose units linked by α -1,4 bonds), the amount of plaque formation and caries experience was significantly lower than in the control group¹². It is not clear from this study whether the plaque reduction was due to the glucose or saccharin content in the diet; unfortunately, the author reported no data on the exact saccharin content of the used low-calorie sweetener. Since the intake

was 42 g of sweetener per day per test person, the plaque reduction could well have been due to the presence of saccharin. Our study would confirm these observations. Saccharin at all tested concentrations in the presence of glucose inhibited or reduced the growth rate of all seven *S. mutans* strains significantly. Saccharin is the only compound of all 10 tested food additives which exerts such a toxic effect on *S. mutans* with increasing concentrations.

In previous studies it was observed that *S. mutans* 10449, in comparison to other streptococci, is not able to utilize xylitol for acid production¹, even after an adaptation period of up to 10 months¹³. It was suggested that xylitol behaved as an inert compound toward *S. mutans*⁹. In an *in vivo* study, involving human volunteers, a xylitol diet produced up to 90% less caries than a sucrose diet⁸, indicating that *S. mutans* is probably not affected *in vivo* by this sucrose substitute. Our study indicates that lower and intermediate concentrations of xylitol promote growth of four out of the seven *S. mutans* strains, most striking in the case of *S. mutans* HS-6. Higher concentrations (up to 2%) of xylitol always produced a reduction of the growth rate of the *S. mutans* strains, with the exception of *S. mutans* FA-1. Our study also confirms the former observation¹, that *S. mutans* 10449 is not able to utilize xylitol for growth and acid production. The present study also is in agreement with former findings that *S. mutans* is able to utilize fructose¹, mannitol¹, and sorbitol^{1,14} for growth and acid production; all three sucrose substitutes increased the growth rate of the seven *S. mutans* strains when added to the glucose medium, with very few exceptions.

In summary, one may conclude from this study that there are many physiological patterns and differences present within the several strains of *S. mutans*. These physiological differences must be taken into consideration for further *in vitro* and *in vivo* studies utilizing *S. mutans*.

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