Turku sugar studies X

Occurrence of polysaccharide-forming streptococci and ability of the mixed plaque microbiota to ferment various carbohydrates

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Dental plaque samples collected from the subjects during the last 20 months of the 2 year trial were subjected to quantitative and qualitative analysis of the occurrence of *S. mutans*, *S. sanguis*, *S. salivarius* and the total growth on phenol red agar. Lyophilized plaque samples were homogenized and incubated on a sucrose containing medium under anaerobic conditions. In addition, the pH-values were measured after incubation of the mixed plaque flora in media containing 1 % respectively xylitol (X), sorbitol, sucrose (S), fructose (F) or no carbohydrates. The results show a significantly lower incidence of *S. mutans* in the X-group relative to the S- and F-groups. The corresponding difference could not be observed between the S- and F-groups. The logarithmic means and standard deviations of the colony counts of *S. sanguis*, *S. salivarius* and total bacteria yielded no significant differences between the 3 sugar groups during the test period. Repeated pH-measurements, carried out at the 4, 12, 18 and 24 month phases, showed that, except in the presence of X, the mean values all fell below the pH-limit of 5.5. In the course of the study, no evidence was obtained of adaption or mutation enabling acidogenic decomposition of X. These findings emphasize the importance of low acidogenic potential in dental plaque, generally parallelled by a low incidence of dental caries.

Key-words: Streptococcus mutans, Streptococcus sanguis, Streptococcus salivarius, dental plaque, xylitol; sucrose; fructose; sorbitol

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The present study reports additional and final information regarding the occurrence of *Streptococcus sanguis, S. mutans, S. salivarius* and total bacteria counts in plaque samples obtained from the subjects in the Turku sugar studies. The basic experimental conditions have been described by *Gehring et al.*, 1974.

S. sanguis was included in these investigations because this species produces extracellular polysaccharides through the metabolism of sucrose and represents the dominating streptococci in the anaerobic system of dental plaque (*Carlsson*, 1965). *S. Salivarius* is the dominating bacterium in the aerobic system of the saliva and was also included in these experiments along with total microflora counts. The cariogenic properties of *S. sanguis* and *S. salivarius* do not seem to be comparable with that of *S. mutans*. A negative association of *S. sanguis* with caries has been noted by *de Stoppelar et al.* (1969) in man, and by *Bowen* (1965) in monkeys. Yet, certain

S. sanguis strains have been shown to cause caries in rats (*Guggenheim*, 1968; *Ranke & Ranke*, 1971; *Mikx et al.*, 1972). *S. salivarius* does not multiply to any appreciable degree on the tooth surface and is probably a contaminant from the tongue, its usual habitat (*Carlsson*, 1965 a, *Krasse*, 1968).

The proportion of *S. mutans* in the plaque samples was considered important, as this streptococcus plays an etiologic role in dental caries. *S. mutans* and its role in human dental caries has been the subject of recent reviews e.g. *Gibbons & van Houte* (1975) and *Hardie & Bowden* (1975). However, the taxonomic position of these potential caries-inducing streptococci is still uncertain. In the latest edition of *Bergey's Manual of Determinative Bacteriology* (1974) *S. mutans* has not been described as falling exactly within the genus STREPTOCOCCUS. It has only been mentioned in connection with the description of *S. salivarius* with comment as follows: »*Carlsson* (1965) has suggested a relation between dental caries and *S. mutans* (*Clarke*, 1924), an organism quite similar to *S. salivarius*. *S. mutans* has not yet been extensively studied and compared with *S. salivarius*.»

In addition to measuring the quantitative occurrence of *S. mutans* in the plaque of test persons, a main purpose of the present study was to determine whether the observation (*Gehring et al.* 1974) from the first year of the study would hold true for the second year as well, i.e. that under chronic xylitol diet no adaptation or mutation of the plaque flora occurred which would enable them to degrade xylitol into acids.

MATERIAL AND METHODS

Subjects and plaque sampling. The subjects, divided into sucrose (S), fructose (F) and xylitol (X) groups were the same as reported previously (*Mäkinen & Scheinin*, 1975). For the present studies, all plaque samples were obtained as lyophilized preparations. The third microbiological examination was carried out at the 20-month phase and the fourth at the end of the study. Table I shows the number of plaque samples examined in the four determinations. The variations in the numbers of subjects were due to conditions described separately (*Mäkinen & Scheinin*, 1975) and to the fact that some samples were rejected due to incorrect handling of the lyophilized plaque material.

Table I. *Number of plaque samples in the four examinations*

Examination Phase	Xylitol	Fructose	Sucrose
I 4 months	51	38	35
II 12 »	51	37	35
III 20 »	48	36	35
IV 24 »	47	35	33

Culturing of plaque samples and identification of isolated streptococci strains. As described previously (*Gehring et al.*, 1974), a Potter S¹) homogenizer was used (10 sec) to disperse the freeze-dried plaque samples in a solution containing 1.0 ml Bacto-NIH-Thioglycollate broth²) and 2.0 ml saline. Ten-fold serial dilutions (10^{-1}—10^{-4}) were made and 0.1 ml was spread on phenol red agar base plates supplemented with 5 % sucrose. In the first set of registrations the colony counts were generally ascertained three times. No significant differences between the colony counts were found. Thus, in subsequent examinations the colony counts were

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²) Difco Laboratories, Detroit, Michigan, U.S.A.

determined with only one serial dilution. All cultures were incubated in a 95 % N_2 5 % CO_2 atmosphere (BBL GasPak-jars)3) at 37°C for 48 hrs. Plates showing single located colonies on the surface of the culture medium were counted using a stereomicroscope and the percentages of *S. mutans* and *S. sanguis* determined.

The identification and enumeration of specific streptococci species in human oral samples is usually based on the distinctive appearance of the colonies on a 5 % sucrose containing culture medium. The colonial characteristics on the specific sucrose-containing medium (*Gehring*, 1972, 1976) were used to determine the presence of these extracellular polysaccharide-producing streptococci. From each sample typical *S. mutans* colonies were selected and the isolates were differentiated as described in the first report (*Gehring et al.*, 1974).

Shklair & Keene (1974) have developed a biochemical scheme for the separation of *S. mutans* into 5 biotypes, »a»—»e», which correlate with the recognized serotypes a—e (E) (*Brathall*, 1970, 1972). This biotype identification of *S. mutans* is based on a number of tests: fermentation of mannitol (with and without bacitracin), sorbitol, raffinose, melibiose and the production of ammonia from arginine. This biochemical scheme was applied to the plaque samples in the 3rd and 4th series of the present experiments. A 0.1 ml portion of each plaque suspension was placed in a test medium (TM) of the following composition (*Shklair & Keene*, 1974): thioglycollate medium (no carbohydrate or indicator) 2.4 % (w/v), lactoalbumin 0.25 %, mannitol 0.5 %, thallium acetate 0.025 %, crystal violet 0.0001 % and brom cresol purple 0.0015 %. Inoculated screw-cap test tubes filled with 5 ml of this medium were incubated for 7 days at 37°C. A color change to yellow indicated degradation of mannitol through acid production and, thus, growth of *S. mutans*. A loopful of the culture was then streaked onto phenol red medium containing 5 % sucrose. After incubation under anaerobic conditions for 2 days at 37°C, typical *S. mutans* colonies were selected and identified as described above.

S. salivarius colonies were detected on the same culture medium, but after aerobic incubation (1—2 days at 37°C). Identification of the adherent, rubber-like colonies of *S. sanguis* and the large mucoid forms of *S. salivarius* was carried out randomly. The methods used have been described elsewhere (*Hardie & Bowden*, 1974). All bacteria counts are given in units per mg plaque material.

pH-measurements. Changes in pH-values of culture media containing 1 % respectively of xylitol, sorbitol, sucrose or fructose or no carbohydrate addition were measured. Phenol red broth base with the above mentioned sugars served as culture medium. Screw cap test tubes, in duplicate, filled with 2.5 ml of the test media were inoculated with 0.05 ml of plaque suspension. These were incubated 7 days at 37°C. At days 1 and 7 the pH-values were measured directly in the test tubes with a Digi 510 1) pH-meter using combined microelectrodes of type 405/M3 2).

RESULTS

S. mutans. The growth of *S. mutans* in the 3 sugar groups is shown in Fig. 1. Using the χ^2-test the observed differences between the X and the two other sugar groups proved to be significant at the 5 %

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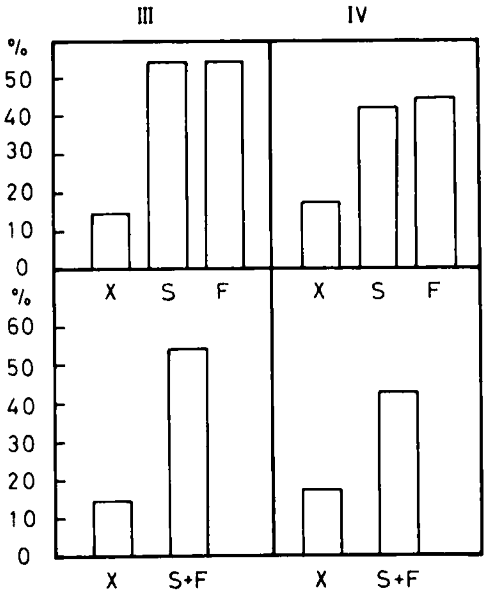

Fig. 1. Occurrence of anaerobic *S. mutans* in the three sugar groups at the 3d and 4th examinations. The findings appear also for the xylitol-, and the pooled sucrose- and fructose-groups.

level in the 3rd ($\chi^2 = 18.37$, $\alpha = 0.0001$) and 4th examinations ($\chi^2 = 8.15$, $\alpha = 0.017$). As there was no difference between the S- and the F-groups in these series the frequencies are combined in the lower part of Fig. 1.

The relative distribution of cases harbouring qualitative *S. mutans* in the three sugar groups during the diet period, is shown in Figs. 2 and 3. In these figures, all cases in which *S. mutans* was either not present or found in the 4 sets of determinations are noted. *S. mutans* was absent from the X-group in the majority of cases. In the other 2 sugar groups the cases with positive findings of *S. mutans* were predominant. Table II shows the

Table II. *Distribution of cases harbouring anaerobic S. mutans in the three sugar groups during the two year study*

Examination						
1	2	3	4	Xylitol	Fructose	Sucrose
—	—	—	—	30	9	6
—	—	—	+	1	1	2
—	—	+	—	0	3	6
—	—	+	+	1	4	0
—	+	—	—	2	1	1
—	+	—	+	1	1	1
—	+	+	—	0	0	0
—	+	+	+	0	1	1
+	—	—	—	1	3	2
+	—	—	+	1	0	0
+	—	+	—	3	1	1
+	—	+	+	1	3	5
+	+	—	—	2	0	1
+	+	—	+	1	0	1
+	+	+	—	0	1	1
+	+	+	+	2	5	3
Total				47	33	33

+ : S. mutans found
— : S. mutans not found

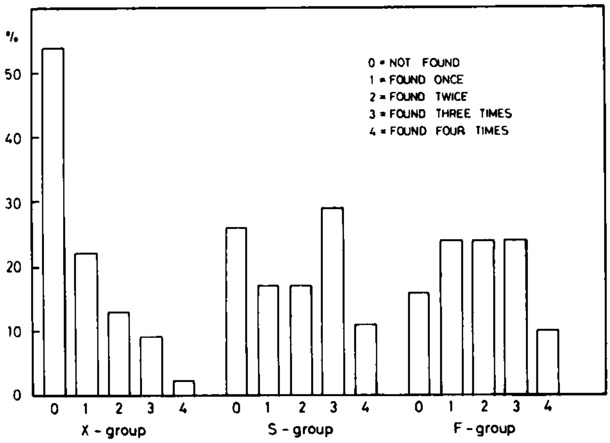

Fig. 2. Distribution of cases harbouring anaerobic *S. mutans* in the three sugar groups during the actual diet period. Summary of the four microbiological examinations.

distribution of cases harbouring *S. mutans* during the study. The X-group had 35.8 % positive and 64.2 % negative findings of anaerobic *S. mutans* as compared with 76.6 % positive and 23.4 % negative findings in the pooled S- and F-group ($\alpha = 0.0001$).

As mentioned in the Material and methods section, in the 3rd examination, *S. mutans* was identified with a new selective test medium. This medium is a highly sensitive indicator, theoretically, one single cell of *S. mutans* per plaque sample. A color change from purple to yellow indicates a positive test. It allows, however, no conclusions about the quantitative occurrence of *S. mutans* in the individual plaque samples. Data obtained in this manner are shown in Figs. 4 and 5. In the 3rd examination the differences in the positive findings of anaerobic *S. mutans* in the persons of the 3 sugar groups were not clearly significant. When analyzed using the Fisher test, the X-group and the pooled S- and F-group differences resulted in the value $\alpha = 0.0675$, which slightly exceeds the accepted 0.05-level of significance. In the 4th examination, however, significant differences were found between the 3 sugar groups ($\chi^2 = 13.7$; $\alpha = 0.0011$). The same was true of the pooled S- and F-group where the Fisher test gave $\alpha = 0.0005$. The use of the new test medium resulted in a higher number of cases with positive findings of *S. mutans* in all sugar groups.

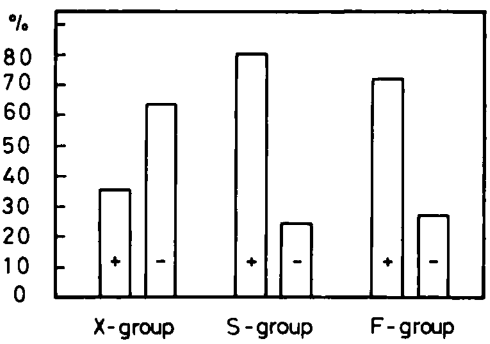

Fig. 3. Occurrence of cases harbouring anaerobic *S. mutans* in the three sugar groups during the diet period. Summary of the four microbiological examinations.

+ = positive findings in at least one examination

— = negative findings in all examinations.

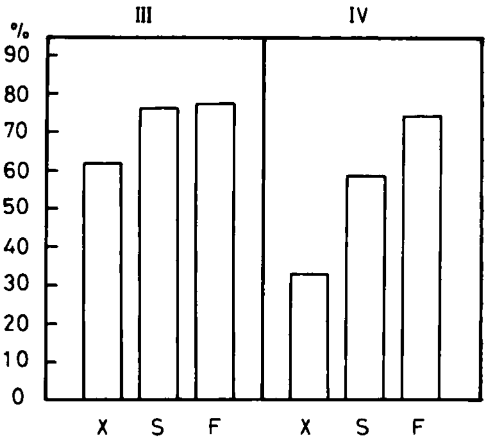

Fig. 4. Occurrence of cases harbouring anaerobic *S. mutans* identified with a liquid test medium in the three sugar groups at the 3d and 4th examinations.

Some of the colonial variants of *S. mutans* obtained on phenol red agar base containing 5 % sucrose are shown in Fig. 6. There were rough, smooth and mucoid types. Many strains formed a puddle-like or drop-shaped extracellular exudate. The proportions of different polysaccharides synthesized by *S. mutans* may determine the morphology of the colonies on sucrose-containing media (*Edwardsson*, 1970).

One *S. mutans* strain was isolated from each plaque sample obtained in the 3rd and 4th examination. The typical colony form of *S. mutans* was diagnosed stereomicroscopically. In addition, the microscopical identification was confirmed in each case on the basis of the biochemical test scheme as specified in the Material and methods

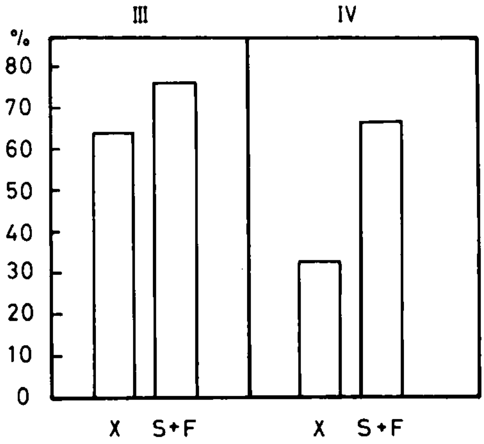

Fig. 5. Occurrence of anaerobic *S. mutans* identified with a liquid test medium in the X-group and the pooled S- and F-groups at the 3d and 4th examinations.

section. Table III shows the scheme for the differentation of *S. mutans* biotypes together with the reactions of those strains which did not conform to any of the classes. The positive fermentation of mannitol and the production of extracellular polysaccharides from sucrose indicate that those strains which were non-typeable using these criteria did, however, belong to the species *S. mutans*. Table IV presents all the data on the prevalence of *S. mutans* biotypes in the plaque samples from the subjects in the 3 sugar groups from the last examinations. The biotype »c» was clearly dominant and was followed in order of decreasing frequency by the biotypes »b» and »e». The biotype »a» was found only once and the biotype »d» was not identified at all. The proportional prevalence of all isolated *S. mutans* biotypes within the 3 sugar groups in examinations 3 and 4 may summarized as follows. From a total of 149 isolated *S. mutans* strains, 87.2 % were of biotype »c»; 4.0 % »b»; 3.4 % »e»; 0.7 % »a». Some 4.7 % were not classifiable into any of these biotypes.

S. sanguis, S. salivarius and total counts. In addition to the investigations of *S. mutans*, quantitative analysis of the occurrence of *S. sanguis*, *S. salivarius* and total bacteria in the dental plaque material was also carried out. Table V shows the frequencies of occurrence of the two glucan-, respectively fructan-producing *Streptococcus* species investigated.

Under anaerobic incubation, *S. sanguis* occurred in practically all subjects in relatively high numbers. The logarithmic means and standard deviations of the anaerobic colony counts in the 3 sugar groups and all 4 examinations showed no significant differences.

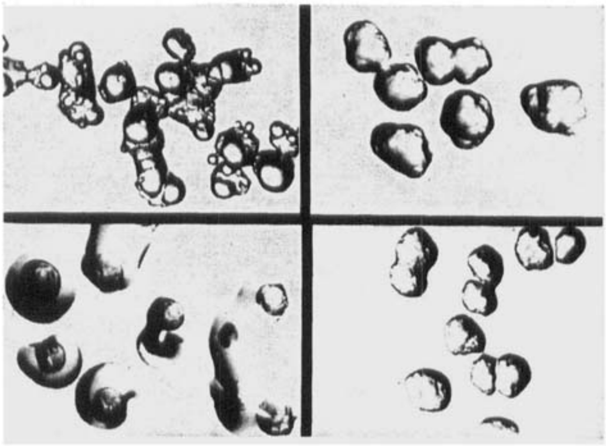

Fig. 6. Variants of *S. mutans* on phenol red agar containing 5 % sucrose.

Table III. *Biochemical scheme for the separation of S. mutans biotypes (»serotypes a — e») and additional strains which did not conform to any of these classes*

	Biotypes (»Serotypes»)					Nonclassifiable strains Number of isolated strains						
Biochemical test	»a»	»b»	»c»	»d»	»e»	1	1	1	1	1	1	1
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	+	+	—	—	+	+	+
Raffinose	+	+	+	—	+	+	—	+	—	—	—	+
Melibiose	+	+	+	—	—	—	—	—	—	—	—	—
NH_3 from L(+)-arginine	—	+	—	—	—	—	—	+	—	+	+	+
Mannitol + 2 units/ml bacitracin	—	+	+	+	+	—	—	—	+	—	+	+

Table IV. *Prevalence of S. mutans biotypes in dental plaque in examinations III and IV*

		Biotypes													
	Exami-	»a»		»b»		»c»		»d»		»e»				Total	
Groups	nation	No.	%	No.	%	No.	%	No.	%	No.	%	Φ*)	%	No.	%
Xylitol	III	0	0	4	13.3	24	80.0	0	0	0	0	2	6.7	30	100
	IV	0	0	0	0	15	83.3	0	0	1	5.6	2	11.1	18	100
Sucrose	III	0		0	0	24	85.7	0	0	3	10.7	1	3.6	28	100
	IV	0	0	1	5.0	18	90.0	0	0	0	0	1	5.0	20	100
Fructose	III	1	3.7	1	3.7	24	88.9	0	0	1	3.7	0	0	27	100
	IV	0	0	0	0	25	96.2	0	0	0	0	1	3.8	26	100

*) nonclassifiable strains.

S. mutans dominated only in a very few cases (Table VI). These cases occurred more frequently in the pooled S- and F-group than in the X-group. During the diet period, the greatest differences in *S. mutans* counts were detected in the second examination. As mentioned in the intermediate report (*Gehring et al.*, 1974) this was probably due to a difference in the method of plaque sampling. Samples were then obtained without regard to the location of the plaque material. Likewise there were no significant divergencies with *S. salivarius* and total counts (Table V), all observations checked by the Kruskal-Wallis test. Since *S. salivarius* occurs primarily in the aerobic system of saliva the colony counts for these streptococci were determined aerobically. All the remaining colony counts were made after anaerobic incubation.

The ability of the mixed plaque microbiota to ferment various carbohydrates. As in the first two examinations, the possibility of adaptation or mutation of the plaque flora enabling their acidogenic fermentation of xylitol was also assessed after 20 and 24 months of the sugar diets. The pH-values measured in culture tubes containing the various sugars and sugar-free controls are shown in Tables VII & VIII. The alterations of the pH-values occurring between day 1 and 7 for all sugar groups and the control were exam-

Table V. *Logarithmic means, standard deviations, $\min_{\log}$ and $\max_{\log}$ of the anaerobic S. mutans and S. sanguis, as well as aerobic S. salivarius and total colony counts in the three sugar groups during the diet period*

								S. mutans							
			X					S					F		
Examination	n	$\bar{x}_{\log}$	$SD_{\log}$	$\min_{\log}$	$\max_{\log}$	n	$\bar{x}_{\log}$	$SD_{\log}$	$\min_{\log}$	$\max_{\log}$	n	$\bar{x}_{\log}$	$SD_{\log}$	$\min_{\log}$	$\max_{\log}$
I	51	1.23	1.88	2.46	5.61	35	2.35	2.60	2.46	6.57	38	2.52	2.30	2.34	6.18
II	52	0.97	1.87	2.48	6.18	35	1.38	2.03	2.48	6.08	37	1.02	1.88	3.30	6.42
III	49	0.51	1.40	3.28	5.08	35	2.40	2.22	2.48	6.11	36	2.73	2.31	2.87	6.15
IV	47	0.68	1.50	2.30	4.67	33	2.14	2.22	2.48	5.98	35	2.13	2.17	2.60	5.66
								S. sanguis							
			X					S					F		
I	51	5.80	0.80	3.71	6.76	35	5.64	1.03	3.26	7.49	38	5.62	0.87	3.58	6.66
II	51	5.90	0.71	4.30	7.04	35	5.51	0.84	4.00	7.08	37	5.59	0.93	3.18	7.26
III	48	5.71	0.65	3.18	7.08	35	5.61	0.72	3.60	6.48	36	5.61	1.16	4.23	6.89
IV	47	5.01	0.83	3.48	7.04	33	5.10	0.90	2.90	6.66	35	5.29	0.74	3.00	6.54

								S. salivarius							
			X					S					F		
	n	$\bar{x}_{\log}$	$SD_{\log}$	$\min_{\log}$	$\max_{\log}$	n	$\bar{x}_{\log}$	$SD_{\log}$	$\min_{\log}$	$\max_{\log}$	n	$\bar{x}_{\log}$	$SD_{\log}$	$\min_{\log}$	$\max_{\log}$
I	51	0.34	1.05	2.52	4.30	35	0.64	1.33	2.30	3.74	38	1.08	1.63	2.88	4.18
II	51	0.85	1.45	2.30	4.00	35	0.42	1.06	2.30	3.51	37	0.76	1.43	2.30	5.48
III	48	1.59	1.65	2.30	3.95	35	0.93	1.51	2.30	3.85	36	1.76	1.73	2.30	5.08
IV	47	1.34	1.56	2.00	3.96	33	1.48	1.78	2.30	4.59	35	1.60	1.65	2.00	4.72
								Total counts							
			X					S					F		
I	51	6.08	1.41	3.93	7.61	35	6.22	0.84	4.04	7.89	38	6.19	0.68	4.40	7.18
II	52	6.00	1.62	4.85	7.53	35	5.98	0.75	4.30	7.08	37	6.00	1.31	3.38	7.20
III	49	5.75	1.76	4.48	7.28	35	6.20	0.47	5.48	7.43	36	6.18	1.19	4.93	7.58
IV	47	4.99	1.80	4.00	7.08	33	5.52	1.18	3.15	6.83	35	5.74	0.64	3.95	6.91

Table VI. *Case numbers in which the counts of S. mutans in the respective plaque samples from the xylitol group and the pooled sucrose & fructose groups were higher than the counts of S. sanguis in all four examinations*

Examination	Sugar groups	
	Xylitol	Sucrose & Fructose
I	No. 94*)	No. 1, 11, 119*), 120, 125
II	No. 94*)	—
III	—	No. 4, 5, 14, 34, 38, 64
IV	No. 105	No. 17, 80, 98, 119*), 126
Total	3	16

*) Found twice in same subject.

Table VII. *Alterations of pH-values after 1 and 7 days incubation at the 20-months phase of the diets*

Decomposition of substrate (1%)	Day	X-group (n = 47) x̄	S.D.	F-group (n = 35) x̄	S.D.	S-group (n = 33) x̄	S.D.
Xylitol	1	6.59	0.19	6.66	0.21	6.66	0.21
	7	6.34	0.50	6.32	0.48	6.01	0.37
Sorbitol	1	5.97	0.61	5.76	0.68	6.06	0.54
	7	4.86	0.50	4.69	0.38	4.78	0.54
Sucrose	1	4.19	0.10	4.17	0.12	4.20	0.11
	7	4.19	0.22	4.20	0.22	4.20	0.19
Fructose	1	4.35	0.33	4.23	0.35	4.27	0.38
	7	4.32	0.21	4.34	0.39	4.19	0.26
Control	1	6.44	0.32	6.36	0.45	6.37	0.44
	7	6.44	0.45	6.57	0.48	6.30	0.24

Table VIII. *Alterations of pH-values after 1 and 7 days incubation at the end of the study*

Decomposition of substrate 1%	Day	X-group (n = 47) x̄	S.D.	F-group (n = 35) x̄	S.D.	S-group (n = 33) x̄	S.D.
Xylitol	1	6.65	0.27	6.71	0.20	6.63	0.32
	7	6.45	0.56	6.37	0.54	6.41	0.58
Sorbit	1	6.43	0.47	6.36	0.51	6.39	0.61
	7	5.23	0.84	4.79	0.70	5.05	0.87
Sucrose	1	4.50	0.86	4.34	0.60	4.54	1.00
	7	4.44	0.80	4.21	0.24	4.40	0.76
Fructose	1	4.48	0.67	4.34	0.56	4.42	0.75
	7	4.44	0.61	4.22	0.19	4.33	0.58
Control	1	6.56	0.28	6.51	0.44	6.55	0.29
	7	6.59	0.46	6.44	0.38	6.45	0.44

ined by means of Wilcoxon's test for paired differences. The changes in the 2 final sets of registrations after 1 and 7 days incubation show that the pH-values remained above 6 in the presence of xylitol and in the control. However, in the presence of either sorbitol (after 7 days), sucrose or fructose (after 1 and 7 days) the pH was considerably decreased. Similar results were obtained in all 4 examinations. The degradation of sucrose and fructose resulted in rapid and intensive acid production. In the presence of xylitol, the pH remained rather constant between days 1 and 7. A summary of all cases examined appears in Table IX. The

Table IX. *Number of subjects whose plaque samples were able to reduce the pH-values below 5,5 after 7 days incubation on xylitol as a carbon source for all sugar groups (Summary of the 4 examinations)*

Groups	Examination											
	I			II			III			IV		
		%	n		%	n		%	n		%	n
Xylitol	3	5.9	51	2	3.9	51	0	0	48	0	0	47
Sucrose	3	8.5	35	0	0	35	3	8.6	35	0	0	33
Fructose	0	0	38	1	2.7	37	2	5.5	36	1	2.8	35

number of subjects harbouring xylitol-decomposing microorganisms in their plaque material did not increase during the course of the 2 year study. This suggests that the plaque flora were neither able to decompose xylitol into acid nor to adapt doing so.

DISCUSSION

Generally the bacterial counts appear low. Therefore, some remarks on this problem are indicated. Only one culture medium was used for the determination of total counts in the plaque material. Moreover the incubation of the inoculated culture-plates occurred under anaerobic conditions, except for the aerobic determination of *S. salivarius*. Furthermore the incubations occurred exclusively at 37°C for 2 days. Therefore it cannot be excluded that more fastidious cells were not detected and it seems safe to assume then that the total counts were actually higher. On the other hand, one may assume that the viability of all the streptococci species investigated were not injured by freeze-drying. It is well known (*Heckly*, 1961) that the grampositive cocci are perhaps the most resistant of the nonspore formers to the process of lyophilization. Yet, there are many other micro-organisms more sensitive to freeze-drying. In case of *S. mutans*, this bacterium was not detected in all subjects with the technique described. In each plaque sample the boundary for demonstration of specified bacteria species, e.g. *S. mutans*, was less than 30 cells. These zero-values are included in the calculations of all bacteria counts (Table V). For this reason the *S. mutans* counts appear comparatively low. The method used does, however, conform to the common practice in microbiology for determination of bacterial counts. The diagnosis of the isolated *S. mutans* variants (Table III) was made using the scheme devised by *Shklair* & *Keene* (1974). Certain methodological deficiencies of the biochemical identification tests did, however, complicate the reproducibility of this classification. For example, the production of NH_3 from L-arginine was not always clearly positive or negative. Therefore, the stated differences between »c» and »b» biotypes are sometimes uncertain. *Facklam* (1974) reported in a recent paper that the problem of clearly defining serotypes *c*, *d*, and *e* has not been completely resolved. Troublesome crossreactions between the serotypes *a* and *d* (*Bratthall*, 1972) remain to be resolved. *Grenier et al.* (1973) found a significant discrepancy between the serological identification and the cultural isolation of *S. mutans* and suggested that nonspecific reactions may be one of the possible reasons. *Perch et al.* (1974) have established 2 new *S. mutans* serotypes, namely, type *f* and *g*, and have recommended a subdivision of *S. mutans* into 3 biotypes. Further *Facklam* (1974) divided the numerous known *S. mutans* stock strains according to their physiological reactions into 8 biotypes. In all the plaque samples tested in the present study *S. mutans* strains were found which could not be classified according to the biochemical scheme which was employed (Table III). The differences in the biochemical and serological behaviour of *S. mutans* are well known. They indicate the great variability of the streptococci in general. In spite of these restrictions, there is no doubt that in all the plaque samples investigated in the Turku sugar study the »serotype c» is predominant. Because of the considerable amount of material and time required for the isolation and differentiation of the various *S. mutans* strains from each plaque sample, only one isolation per subject was carried out. Had several representative colonies per subject been examined, combinations of the different biotypes as reported by *Shklair & Keene* (1974), might possibly have been found in numerous other plaque samples. Nevertheless, the present findings support the results of *Bratthall* (1972 a) who has studied the worldwide distribution of the various streptococcus serotypes by aid of serological methods. His observations indicate that different serotypes predominate in different parts of the world. In Europe, the serotype *c* predominates, whereas, strains of *a* and *b* are rarely found. In contrast to these data, serotypes *a* and *b* predominated, with only a limited number of type *c* strains in a small number of plaque samples from Cairo, Egypt (*Bratthall,* 1972 a). The reasons for these discrepancies are still unknown. In the U.S.A., *Shklair & Keene* (1974) and *Bratthall* (1972 a) found all 5 serotypes with the *c* strains being predominant. A high frequency of the type »*c*» was also confirmed in an area of the Federal Republic of Germany (*Gehring et al.,* 1976). The incidence of different *S. mutans* types in the oral cavities of rats and hamsters, which are the primary model systems for experiments on dental caries, is of interest as well. *Gehring et al.,* (1976) have shown that biotype »c» dominates in the oral cavities of rats, while in the plaques of hamsters the biotypes »e» and »d» occur most frequently.

Previous papers of this series have shown (*Scheinin et al.,* 1974, 1975; *Gehring et al.,* 1974) that the occurrence of *S. mutans* was correlated with the incidence of dental caries. In the 3rd and 4th examinations, *S. mutans* still occurs significantly less frequently in the X-group than in subjects on sucrose and fructose diets. The mean values of quantitative counts of *S. mutans* show the same trend, but due to large individual variations, as in the preceding studies, significant differences cannot be established. It is generally known (*Krasse,* 1968) that changes in the diet influence the composition of the oral flora and, that, if the amount of carbohydrate consumed is increased or decreased a corresponding variation in the number of specific micro-organisms is also obtained. Thus *Larmas et al.* (1974) have shown in the Turku studies that the number of candida colonies in Sabourand agar was strongly reduced in the X-group compared with the F- and S-groups. In contrast, it seemed very difficult to displace *S. mutans* from its ecological niche in the oral cavity of man. Yet it should not be forgotten that dental caries in man is a multifactorial process and probably the more or less individualistic manner of development of the disease is due to varying concurrence of the different variables. It should be borne in mind that other oral bacteria can also become involved depending on circumstances. In an extensive bacteriological study of areas of deep carious dentine, *Edwardsson* (1974) concluded that the observed incidence of gram-positive facultative anaerobic cocci, including the polysaccharide-producing species *S. sanguis* and *S. mutans,* was low. Additional epidemiologic and ecologic investigations in this field might help to clarify these correlations. It is certain that *S. mutans* is the primary cause of initial lesions of smooth surface caries. Also important in this connection are the observations of *Theilade et al.* (1973) that the central fissures seem to represent an ecosystem differing from that of smooth surfaces of the teeth.

In none of the examinations of the 2 year study did bacteriological results show any significant differences in the occurrence of *S. mutans* between the F- and S-groups. Because the presence of *S. mutans* biotypes is known to be fostered by sucrose, a higher frequency of this bacterium was expected in the S-group. Through the metabolism of sucrose *S. mutans* strains are able to synthesize extracellular glucans and fructans which in turn may account, in part, for the caries-promoting potential of this sugar (*Gibbons*, 1975). Such adhesive polymers can not be produced from fructose. The present observations emphasize the predominant role of microbial acid production in the etiology of caries in general.

During the diet period *S. mutans* dominated quantitatively compared with the *S. sanguis* counts (Table VI) in only a few cases in the S- and F-group and in just 2 plaque samples in the X-group. *DeStoppelaar et al.* (1970) found that during a carbohydrate-free period of 17 days the occurrence of *S. mutans* in human dental plaque decreased to a very low or undedectable level while the counts of *S. sanguis* increased. In the present case, the percentage of *S. sanguis*, *S. salivarius*, and the total counts fluctuated without any significant correlation to the different sugar sources in the 3 test groups. During the 2 year test period *S. salivarius* occurred in all 3 test groups. *Carlsson* (1965) found that the number of *S. salivarius* both in plaque and saliva increased markedly when a basic diet was supplemented with a frequent intake of sugar. However, this author suggested that *S. salivarius* does not grow to any appreciable degree on the tooth surface and thus does not seem likely to play any major role in plaque formations. The present results confirm this.

As in the 1st and 2nd examinations, the last 2 examinations reveal that only in the xylitol-containing media did the mean values of pH not fall below 5.5 after the 1 and 7 days incubation. The xylitol medium behaves much as the control medium without sugar. The results indicate that the plaque flora even after 2 years of exposure, had not adapted to utilize xylitol. Extensive *in vitro* studies (*Knuuttila & Mäkinen*, 1975 a,b), concerning the adaptation of *S. mutans* to xylitol substrate support these observations. With xylitol as the sole carbohydrate *S. mutans* cells preferentially utilized protein as a source of carbon. Table IX shows that extremely few plaque samples from any of the sugar groups were able to reduce the pH-values to below 5.5 on the 7th day in the presence of xylitol. In addition, the number of subjects harbouring xylitol-decomposing organisms in their plaques did not increase in any of the 4 examinations. Hence, there is no evidence for a selective development of micro-organisms capable of decomposing xylitol during the 2 year xylitol diet.

From the plaque samples listed (Table IX) some streptococcal strains were isolated which neither group serologically into known types nor produce extracellular polysaccharides, but, which do fermentate xylitol very slowly (*Gehring*, 1974). In experiments with gnotobiotic rats the cariogenicity of such streptococci has been tested, and in comparison with a *S. mutans* strain, they have practically no cariogenic capabilities (*Karle & Gehring*, 1976).

Furthermore, a number of experiments with conventionally fed laboratory animals have shown that, relative to sucrose, xylitol is not cariogenic (*Mühlemann et al.*, 1970; *Karle & Büttner*, 1971; *Grunberg et al.*, 1973; *Karle & Gehring*, 1974; *Gehring*

& *Karle*, 1975). In a recently published paper *Mikx et al.* (1975), on the basis of animal experiments, concluded that the concentration of sucrose in the diet and the microbial composition, rather than the gross amount of plaque are the important factors in the caries process in SPF-rats.

In the present study the decomposition of sorbitol by the mixed plaque flora was also studied. In all 4 examinations the decrease of pH below the critical limit of 5.5 after 7 days incubation was definitely clear. However, after 1 day incubation the measured values exceeded pH 6. In comparison with sucrose or fructose, sorbitol was degradated more slowly and is therefore presumed to be less cariogenic. Nearly all caries-inducing *S. mutans* strains readily ferment this carbohydrate. Some enterococci and micrococci strains and perhaps still other bacteria in the mouth are also able to degrade sorbitol with consequent weak acid production (*Gehring & Patz*, 1974).

Frostell (1965) reported that substitution of sucrose in sweets by sorbitol resulted in less acids being produced *in vivo*. However, it has been suggested that the long term use may lead to an increase in the sorbitol utilizing organisms in the oral flora. All things considered, the status of sorbitol regarding cariogenicity remains unclear (*Gülzow*, 1971).

The literature concerning the role of sugar and sugar substitutes in the development of dental caries is extensive and many aspects have been reviewed in recent years by various authors (*Mäkinen*, 1972, 1974; *Newbrun*, 1973; *Scheinin & Mäkinen*, 1972, *Scheinin*, 1973; *Winter*, 1968). The clinical results of the present long-term dietary studies were a substantial reduction in the caries incidence in the X-group compared with the S- and F-groups. (*Scheinin et al.*, 1974, 1975). The present microbiological studies suggest that this might be due the fact that very little if any adaptation occurred regarding the ability of plaque microbiota, including *S. mutans*, to ferment xylitol even after chronic exposure for 2 years. As a result the plaque flora in general were unable to produce enamel decomposing acids from this substance. On the basis of the present results it is not possible to ascribe this effect to any specific bacterium. Rather a generalized phenomenon regarding the total plaque flora is indicated.

In summary, the microbiological results of the Turku sugar study also support the conclusion that, among all sugar substitutes known to date, xylitol is the most advantageous in caries prophylaxis.

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