Effect of culture medium on acid production from sorbitol by oral bacteria

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The fermentation of sorbitol or glucose and the acid production by strains belonging to the genera *Actinomyces, Lactobacillus,* and *Streptococcus* isolated from the predominant sorbitol-fermenting human dental plaque flora were studied in cultures in complex or defined bacteriologic broths and in saliva-based broth. The growth yields of *Lactobacillus* and *Streptococcus* in the saliva-based media and of *Actinomyces* in the defined broth were poor. Addition of fermentable carbohydrate to the saliva-based broth favored the growth of *Streptococcus* and *Lactobacillus* but not that of *Actinomyces*. The results showed obvious differences in the capacity of oral bacteria to ferment sorbitol between cultures in saliva-based and bacteriologic broths. *Lactobacillus* failed to utilize sorbitol when saliva was the only source of nutrients. Lower proportions of lactic and formic acids were formed from sorbitol by *Actinomyces* and *Lactobacillus* in the saliva-based than in the bacteriologic media. The findings illustrate some mechanisms possibly involved in the interactions between sorbitol and dental plaque flora. ☐ *Carbohydrate; fermentation; saliva*

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In a recent study (1) the acid production activities from sorbitol and glucose in resting cell suspensions of oral bacteria were examined. It was found, among other things, that the growth conditions of the cultures and the treatment of the resting cells before and during the experiments could affect the catabolic activity and the acid production rate of the cell suspensions. These observations are in line with previous knowledge of the effect of environmental conditions on bacterial physiology (2).

It has earlier been shown that the predominant microflora of the supragingival dental plaque can utilize saliva as a complete source of nutrients (3, 4). Saliva is considered the main microbial substrate in the oral cavity, since it is continuously provided at a rate of about 800 ml/day (3). Compared with bacteriologic culture media, saliva contains about 10–1000 times lower concentrations of several essential nutrients, such as minerals, amino acids, and vitamins (5–7). It might therefore be expected that the metabolic activities of oral bacteria, grown in saliva-based media, differ from those obtained with cultures in complex microbiologic substrates.

To elucidate the fermentation of sorbitol to acids by oral bacteria, the present study was conducted to compare the growth patterns and the formation of metabolic end products from sorbitol or glucose in growing cells of microorganisms from the predominant sorbitol-fermenting plaque flora (8) in saliva-based and bacteriologic culture broths.

Materials and methods

Culture media

Three media with different base compositions were prepared and sterilized in accordance with previous descriptions. The media used were peptone-yeast extract without (PY) (9) and with sorbitol (PYS) or glucose (PYG), a defined broth (FMC) (10) with sorbitol (FMCS) or glucose (FMCG), and a saliva-based broth without (SB) (3) and with sorbitol (SBS) or glucose (SBG).

The concentration of the sugars added to the media was 0.5% (w/v). The sorbitol product used (Merck, quality puriss) contained approximately 0.5% glucose. As negative controls non-sugar-supplemented media were used with the exception of FMC, which did not support the growth of the bacteria studied without the addition of a fermentable carbohydrate. The sterilized media were kept pre-reduced in screw-capped tubes. The storage time of the media never exceeded 1 week.

Bacterial cultures

Isolates from human dental plaque, capable of fermenting sorbitol and belonging to the species (number of strains within square brackets): *Actinomyces naeslundii* [3], *A. viscosus* [1], *Lactobacillus casei* var. *casei* [1], *L. casei* var. *rhamnosus* [2], *L. plantarum* [1], *Streptococcus mutans* [2], *S. sanguis* I [1], and *S. sanguis* II/*S. mitis* [1] were examined. All strains had previously been identified (8).

The fermentation of sorbitol or glucose was examined with medium-adapted and non-adapted cultures. Tubes with 10 ml of each broth were inoculated with 0.1 ml bacterial cell suspension (equivalent to 10^8 viable cells) in phosphate saline buffer (3 mM, pH 6.8). Adaptation was performed with a five-time transfer of a 24-h culture to a new tube with fresh broth. All cultures were incubated anaerobically (5% CO_2 + 9% H_2 in N_2) at 37°C until they reached the late stationary growth phase (incubation time, 2–5 days). The cultures were thereafter centrifuged at 10,000 *g* for 10 min. The pH of the cell-free supernatants was measured, and an aliquot of each was distributed in small cryo tubes (A/S Nunc Inter Med, Roskilde, Denmark) and stored at −20°C, until analyzed for the presence of fermentation end products.

The pelleted cells were washed twice with 10 ml distilled water and finally resuspended in 1 ml water. A 0.5-ml volume of the suspension was pipetted into a pre-weighed cup, dried at 90°C for 18 h and weighed to the nearest 0.1 mg. As negative controls, non-inoculated media incubated under similar conditions were used.

Analyses of fermentation end products

The amount of ethanol in the supernatants was assayed with an enzymatic method by using a commercial kit (Boehringer Mannheim GmbH Biochemica, Mannheim, FRG, cat. no. 17690). Gas–liquid chromatography and isotachophoresis were used for determination of the acids in the same samples.

For chromatographic analyses a 2-m glass column (inside diameter, 2 mm), packed with SP 1220-impregnated Chromosorb® (Supelco Inc., Bellefonte, USA, cat. no. 1-2144), was used. The temperature of the injector and the column was 160°C, and that of the flame-ionization detector 190°C. Nitrogen was used as carrier gas, with a flow rate of about 20 ml/min. This system detects lactic, pyruvic, and succinic acids in non-derivatized samples, besides acetic, butyric, caproic, isobutyric, isocaproic, isovaleric, propionic, and valeric acids. Each sample was acidified with metaphosphoric acid, and 0.5 µl of it was injected into the column.

A tachophor δ (Itaba AB, Vällingby, Sweden) was used for isotachophoretic analyses. The leading electrolyte was a 10-mM HCl solution in re-distilled water containing 0.25% (w/v) hydroxypropylmethylcellulose and 10% (v/v) ethanol. The pH of the solution was adjusted to 4.3 with ε-aminocaproic acid. The terminating electrolyte contained 10 mM caproic acid in 10% (v/v) ethanol in re-distilled water. The detection current was set at 15 µA. Calibration curves were obtained with standard solutions of acetic, formic, lactic, orthophosphoric, propionic, pyruvic, and succinic acids within the concentration range 1 to 10 mM. The volume of the sample injected was either 2 or 5 µl from appropriate dilutions of the supernatants.

The results obtained with both methods were comparable. However, the methods were supplementary to each other, since some compounds could only be detected either by chromatography or electrophoresis. The reproducibility of the latter method

Table 1. Mean dry weight (in mg) of cellular mass from 10 ml medium-adapted cultures. *n* = number of strains showing visible growth in each broth. Within each genus, four strains were examined. Dry weights of non-inoculated broths, ≤0.2 mg

Culture* broth	*Streptococcus*		*Lactobacillus*		*Actinomyces*	
	n	mg	*n*	mg	*n*	mg
PY	4	1.5	4	1.2	4	1.2
PYS	4	2.9	4	2.0	4	1.9
PYG	4	3.8	4	3.3	4	2.8
FMCS	4	3.4	3	2.2	1	0.6
FMCG	4	3.7	4	2.5	1	0.9
SB	1	0.4	1	0.3	4	1.1
SBS	3	1.1	4	0.4	3	0.9
SBG	4	1.2	4	1.1	2	1.4

* For abbreviations see Culture media under Materials and methods.

(methodologic error, about 5% or less) was considered more satisfactory than that of the former. Moreover, none of the samples contained end products from bacterial carbohydrate fermentation which were not detected by isotachophoresis. The quantitative data presented below are therefore calculated from the isotachopherogram of each sample.

Results

Non-medium-adapted cultures grew in all three media both with and without sorbitol or glucose. After the adaptation differences were observed between cultures in PY broths and those in FMC or SB broths (Table 1). The strains *L. plantarum*, two *A. naeslundii*, and *A. viscosus* failed to grow in FMCS. The same *Actinomyces* strains did not grow in FMCG. None of the strains included could grow in the base medium FMC without addition of a fermentable carbohydrate. The base medium with saliva (SB) supported the growth of all *Actinomyces*, one *L. casei* var. *rhamnosus* and one *S. mutans*. When SB was supplemented with sorbitol, all *Lactobacillus* and three strains from each of the other genera showed visible growth. In SBG, all *Streptococcus* and *Lactobacillus* and two *A. naeslundii* strains grew.

The patterns of growth, expressed as the cellular dry mass of the culture, and of acid production from the carbohydrates observed with the non-medium-adapted cultures deviated slightly from those found in the medium-adapted cultures. In brief, all non-medium-adapted cultures showed growth of variable intensity. In addition, adaptation to the culture broth resulted in increased cellular dry mass only of the cultures of streptococci and lactobacilli in the bacteriologic media. The main difference in the acid production was the increased concentration of lactate (about 50% of the metabolic end products) in the adapted cultures of streptococci in the sorbitol-containing bacteriologic broths PYS and FMCS. In the following, the results obtained with the medium-adapted cultures are presented and discussed.

The growth yields are presented in Table 1. Greater cellular dry weights were found in the PY- and FMC-growing cultures than in the saliva broth (SB). In all bacteriologic media the yield of growth became greater when the base was supplemented with carbohydrates. The saliva base broth supported the growth of *Actinomyces* but not of *Streptococcus* and *Lactobacillus*. The dry weights, observed for *Lactobacillus* in SBS, were slightly higher than that of the blank.

The mean lowest final pH values from each carbohydrate were observed in the PY-based broth, for each genus (Table 2). The corresponding values for the cultures in SB were comparable with those in the negative

Table 2. Mean final pH (FpH) and pH decrease (DpH) of the cultures in the different broths. DpH is calculated as the difference in FpH of the cultures in non-carbohydrate-supplemented and the carbohydrate-containing broth

Culture*	*Streptococcus*		*Lactobacillus*		*Actinomyces*	
broth	FpH	DpH	FpH	DpH	FpH	DpH
PY	6.3	—	5.4	—	6.5	—
PYS	4.6	1.7	4.3	1.1	5.3	1.2
PYG	4.3	2.0	4.0	1.3	4.7	1.8
FMCS	5.5	1.4	5.6	1.3§	6.5	0.4†
FMCG	5.2	1.7	5.2	1.7	6.2	0.7†
SB	7.3†	—	7.4†	—	7.2	—
SBS	6.0	1.3	7.2	0.2	5.4	1.5§
SBG	4.7	2.6	5.1	2.3	4.5	2.7‡

* For abbreviations see Culture media under Materials and methods.
Values from one†, two‡, or three§ strains.

controls. The final pH found in SBG cultures was about 1 unit or more lower than the corresponding pH in SBS. On the other hand, the differences observed in the final pH values between the cultures in glucose- and sorbitol-supplemented bacteriologic broths were less than 0.6 units. The final pH for the *Lactobacillus* cultures in SBS remained around 7.2.

The pH decreases—that is, the differences in final pH values between the cultures in non-carbohydrate-supplemented and sugar-containing broths—varied from 0.4 to 2.7 units, with a mean value around 1.5 (Table 2). Exceptions are the results with *Lactobacillus* in SBS. In general, the differences were greater in the media containing glucose than in those with sorbitol. The greatest decreases were recorded in SBG for all cultures, ranging between 2.1 and 2.7 pH units.

The concentrations of the metabolic end products from sorbitol or glucose found in the supernatants of the cultures are presented in Fig. 1 as means for each genus. They have been calculated as the differences in the amounts of the products between the carbohydrate-containing and the non-inoculated broths. With the exceptions of *Streptococcus* and *Lactobacillus* in FMC-based broths, the total amount of end products was somewhat greater in glucose-supplemented broths than in the sorbitol-containing media. Furthermore, this amount in saliva-based media was less than one-third of the corresponding figures in the bacteriologic media. In general, the total concentrations of acidic end products were in line with the patterns of pH decreases in the different cultures.

Sorbitol was mainly fermented by the strains of *Streptococcus* and *Lactobacillus* to ethanol, formate, and lactate in all broths. *Actinomyces* formed also acetate and succinate. The major end product from glucose was lactate. *Streptococcus* and *Lactobacillus* also formed various amounts of acetate, ethanol, and formate in PYG and FMCG, whereas only traces of these products could be found in the SBG cultures of the same strains. *Actinomyces* formed all five products in PYG. In FMCG and SBG they produced ethanol, lactate, and succinate and, in addition, traces of pyruvate in SBG.

When formed, acetate was found in low concentrations. All non-inoculated PY- and FMC-based media contained about 20 mM and 60 mM acetate, respectively. The concentrations of this acid appeared decreasing in all sugar-containing broths and particularly in those with sorbitol.

Discussion

Compared with the bacteriologic broths and in agreement with earlier findings (3, 4), all

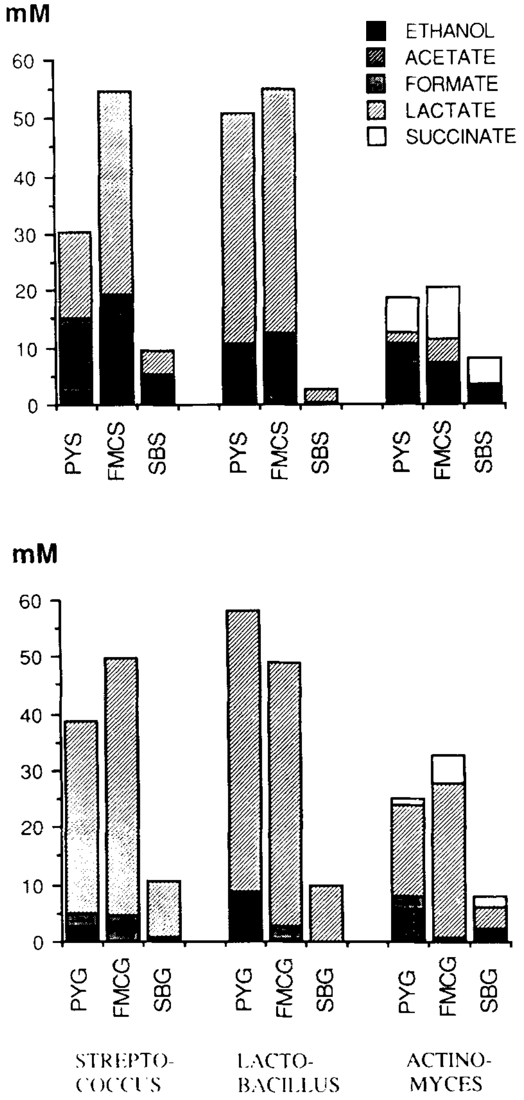

Fig. 1. Concentrations of metabolic end products from sorbitol or glucose in the supernatants of cultures in different broths. Mean values for each bacterial genus. (For abbreviations see Culture media under Materials and methods.)

strains grew less in the saliva-based media. Re-confirming previous observations (11–13), addition of glucose appeared to be necessary to support the growth of *Streptococcus* and *Lactobacillus*. In contrast, all *Actinomyces* strains were capable of growing in SB, but only two of them became stimulated by the increased glucose concentration in SBG. Supplementing SB with sorbitol led to different growth patterns for each of the genera. The growth yield of *Streptococcus* increased, whereas that of *Actinomyces* and *Lactobacillus* was not stimulated. Thus, the growth patterns of the genera tested are considerably changed when the nutritional environment reflects more closely the in vivo conditions.

Irrespective of the culture medium, the pH decreases and the final pH values of the cultures were lower in the glucose- than in the sorbitol-containing broths. Similar results have previously been reported (14–16). The various final pH and pH-decrease values for the different broths are influenced by at least three factors: the buffering capacity, the initial pH, and the content of other fermentable carbon sources in the non-inoculated medium. The pH decreases in glucose- and sorbitol-containing bacteriologic broths differed slightly, being about 0.5 pH units or less. The corresponding figures recorded in the salivary environment were much greater and ranged from 1.3 to 2.1 units. The greatest difference was found with the cultures of *Lactobacillus*, indicating that these strains failed to ferment the polyol to acids and to utilize it as a carbon source, as was revealed by the scanty growth of these strains in SBS. The fact that the growth of *Lactobacillus* was not maintained in SB but in SBS, although sorbitol was not utilized as a carbon source in the latter broth, might be explained by the contaminating amount of glucose added to the medium with the sorbitol preparation. Thus, the results of the pH measurements illustrate that the acidogenic capacities of sorbitol-challenged cultures of *Streptococcus and Lactobacillus* are diminished when saliva is the only source of nutrients.

Two certain patterns could be distinguished from the analyses of the fermentation products. Irrespective of the strain and the carbohydrate, the relative proportions of the end products were influenced by the culture medium. Furthermore, the composition of them was dependent on the fermentable carbohydrate in the medium. In agreement with earlier results (1, 3, 9) lactate was always the main end product from glucose. Moreover, the ratio low pK_A/high pK_A organic acids was lower in cultures in SBS than in SBG. These findings are in line with those reported for dental plaque suspensions (17, 18) and resting cell suspensions (1).

To sum up, saliva-based medium with sorbitol supports the growth of oral strains of *Streptococcus*, but it neither stimulates the growth of and the acid production by oral *Lactobacillus* nor encourages the growth of *Actinomyces* to a greater extent than that obtained in the non-sorbitol-containing base broth. These findings elucidate some methodologic differences that are important in microbiologic studies of carbohydrate fermentation.

Assuming that the culture conditions in the saliva-based media are more similar to those existing in the oral cavity than are conditions in bacteriologic media, the present results might explain earlier findings (8, 18) showing that the proportions of sorbitol-fermenting strains of *Lactobacillus* and *Actinomyces* did not increase in dental plaque of people given frequent mouth rinses with sorbitol solution. Furthermore, the results support our previous concept (1) that sorbitol-fermenting strains of *Streptococcus* mainly account for the acid production in dental plaque in vivo when sorbitol is supplied to the oral cavity.

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