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Development of multi-species consortia biofilms of oral bacteria as an enamel and root caries model system

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Abstract

The aim was to establish defined-species consortium plaque biofilms to investigate enamel and root caries in an artificial mouth. Strains of the putative enamel and root caries pathogens, *Streptococcus mutans, Strep. sobrinus, Actinomyces naeslundii* and *Lactobacillus rhamnosus*, were screened in batch culture for potential cariogenic properties: a low terminal pH, ability to aggregate, and catabolic diversity. The strains selected were grown as monoculture biofilms and as consortium plaque biofilms in a multiplaque artificial mouth. The biofilms were supplied with a constant flow of a simulated oral fluid and were given periodic sucrose (and in some instances glucose) to simulate meals. All the bacteria except *L. rhamnosus* formed large, monospecies biofilms with resting pH in the range 5.3–5.8. The consortia biofilms were larger and had a resting pH of 4.9–5.3. The consortia biofilms supplied with 8-hourly carbohydrate comprised mainly 'mutans' streptococci (58, SD 5.5%) and *L. rhamnosus* (42, SD 5.7%). *A. naeslundii* characteristically was absent or present in a low percentage (up to 4% colony-forming units). All biofilms demineralized polished bovine enamel and dentine blocks, as assessed by microradiography and enamel-surface microhardness measurement. The consortia also demineralized intact enamel and tooth roots; they were more cariogenic to enamel than any of the monoculture biofilms, as measured by enamel-surface softening, but variation in lesion depth was proportional to biofilm wet weight irrespective of acidogen composition ($r = 0.93$, $p < 0.05$). Enamel lesions had a well-mineralized intact surface and a zone of subsurface demineralization, typical of early natural lesions. Dentine and root lesions showed extensive demineralization but lacked a pronounced surface mineralized zone. Substitution of glucose for sucrose had no effect on the cariogenicity of the consortium to bovine enamel or human roots and had no major effect on the plaque composition. Continuously supplied fluoride (19 parts/10^6) resulted in a substantially reduced enamel surface softening and subsurface demineralization of intact roots. It was concluded that consortia biofilms of selected caries pathogens generate realistic caries lesions in all tooth hard tissues under controlled growth conditions in the artificial mouth. This in vitro caries experimental model may prove useful for the study of interrelations between the plaque biofilm, tooth tissues and the oral environment, and for the development of procedures to modify the course of caries development.

Keywords: Dental plaque; Biofilm; Bacterial consortia; Caries; Enamel; Root; Model system

Abbreviations: BHY, brain–heart infusion yeast extract agar; c.f.u., colony-forming unit.

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1. Introduction

Bacteria in plaque biofilms metabolize carbohydrates to the acids that cause dental caries. Laboratory models of this process are potentially valuable in understanding the mechanisms involved and in developing and testing procedures to combat caries (Marsh, 1995; Bowden, 1995). Chemical induction of caries by organic acids is one of the principal approaches to clarifying the mechanisms involved in de- and re-mineralization of enamel and dentine, but direct acid exposure lacks the biofilm interactions that characterize caries in vivo. In vivo caries studies have the advantage of including host factors involved in the natural caries process but have some fundamental limitations. The oral environment is difficult to control and varies greatly with intraoral location over time and between different persons (Scheie et al., 1992). Experimental studies in both humans and animals are further restricted by ethical issues and expense (Zero, 1995; Sissons, 1997). Accordingly, simple monobacterial biofilm models have been developed, for example, using *Streptococcus mutans* (Noorda et al., 1986a,b). However, because the in vivo plaque microbiota is highly diverse and complex (Moore and Moore, 1994), plaque biofilms containing multiple species of appropriate bacteria should be more relevant for studying both enamel and root caries.

The same organisms probably cause caries in both enamel and dentine (Bowden, 1990; van Houte et al., 1994; Schüpbach et al., 1995, 1996; Aamdal-Scheie et al., 1996). Highly acidogenic and aciduric bacteria such as *Strep. mutans* and lactobacilli are major odontopathogens involved in the initiation and progression of caries (van Houte, 1994). However, caries results when a range of different bacteria create a cariogenic environment in the plaque biofilm following repeated carbohydrate exposure (Bowden, 1990; van Houte, 1994; Bradshaw and Marsh, 1998). Studies of the predominant cultivable microflora of sound and carious root surfaces has suggested that root caries development might involve organisms in addition to mutans streptococci and lactobacilli, but the role, for example, of *Actinomyces* sp. or *Strep. mitis* has proved difficult to establish (van Houte et al., 1994; Schüpbach et al., 1995; Brailsford et al., 1998). In individuals with root surface caries, Aamdal-Scheie et al. (1996) found no obvious difference in metabolic activity assessed by pH measurement, or in microbial composition and detection frequency, between plaque overlying sound and carious root surfaces. Association of particular species or groups of species unique to the development of root rather than enamel caries is still uncertain.

We have developed a multistation artificial-mouth experimental system for the simultaneous culture of five plaque biofilms under controlled experimental conditions (Sissons et al., 1991, 1992, 1995; Wong et al., 1994; Sissons,1997). Our studies to date have mainly involved validating the use of microcosm dental plaque biofilms, i.e., those evolved in vitro from a mixed oral flora (Wimpenny, 1988; Tatevossian, 1988; Sissons, 1997). We have examined their growth, metabolism, pH, mineralization (Sissons et al., 1991, 1992, 1994, 1995, 1998; Wong et al., 1994), and their attack on tooth tissue substrates (Cutress et al., 1995). Microcosm plaques are similar in composition, growth, pH behaviour, biochemical properties and probably in complexity to natural supragingival plaque, but they allow more detailed and controlled study than plaque in vivo. Defined-species biofilm consortia, although simpler than plaque microcosms, have the advantage of allowing detailed control and study of the properties of the individual bacterial species present (Marsh, 1995; Sissons, 1997). Even in batch culture, oral multispecies consortia develop complex biofilms on enamel that can induce carious lesions similar to those in vivo (Yue et al., 1992).

Our objective now was to establish an enamel and root caries experimental system in a multistation artifi-

Table 1
Bacteria

Species	Strain	ATCC no.[a]	Source
Streptococcus mutans	Type strain	25175	DRG[b]
	KSPK (JC2)		DRG
	13 M		J. Tagg[d]
	UTB1		J. Tagg
Streptococcus sobrinus	O1H1		DRG
	K1R	27351	DRG
Actinomyces naeslundii	WVU 626		G. Bowden[e]
	B 236		G. Bowden
	B 120		G. Bowden
	Be 32		G. Bowden
	Type strain	12104	G. Bowden
	16D		DRG
	33A		DRG
Lactobacillus casei	10302		D. Hartley[f]
	Type strain	393	D. Hartley
Lactobacillus rhamnosus	Type strain	7469	NZRM[c]
	B 259		J. Tagg

[a] ATCC: American Type Culture Collection.
[b] DRG: Culture collection, Dental Research Group, Department of Pathology, Wellington School of Medicine, New Zealand.
[c] NZRM: New Zealand Reference Culture Collection, Medical Section.
[d] J. Tagg, Department of Microbiology, University of Otago, New Zealand.
[e] G.H. Bowden, Department of Oral Biology, University of Manitoba, Canada.
[f] D. Hartley, Institute for Dental Research, University of Sydney, Australia.

cial mouth based on defined-species consortia. The approach taken was to screen four putative root-caries pathogens, *Strep. mutans, Strep. sobrinus, Actinomyces naeslundii* and *Lactobacillus rhamnosus*, for likely cariogenic properties and to study the strains selected in monoculture and together as a mixed-culture consortium. The demineralization of enamel and dentine under the plaque biofilms was then measured by microhardness measurement and microradiography. Plaque formation, species composition, resting pH, pH responses to 5% sucrose and the effect of continuously applied fluoride (19 parts/10^6) on caries formation were assessed.

2. Materials and methods

2.1. Bacterial strains

The origin and strains of *Strep. mutans, Strep. sobrinus, A. naeslundii, L. rhamnosus/casei* are described in Table 1.

2.2. Media

Bacteria were maintained on BHY comprising 3.7% Bacto brain–heart infusion supplemented with 0.5% yeast extract (both from Difco Laboratories, Detroit, MI), 1.5% agar, 5 mg/l haemin, and 0.5 mg/l menadione. To screen for cariogenic properties, basal medium (BM) broth was supplemented with 0.2% or 1% glucose, or 1% sucrose to give, respectively, BMG (0.2%), BMG (1%), and BMS (1%). BM broth contained 0.5% trypticase (BBL®, Becton Dickinson Microbiological Systems, MD), 1.0% proteose peptone (Oxoid, Unipath Ltd, Basingstoke, UK), 0.5% yeast extract, 0.25% KCl, 2.5 mg/l haemin, and 1 mg/l menadione, adjusted to pH 7 before autoclaving. For inoculation of the artificial mouth, bacteria were cultured in BMG (0.5%). Basal medium mucin (BMM) comprising BM broth supplemented with 0.25% partially purified pig gastric mucin (type III; Sigma Chemical Co., St Louis, MO) was continuously supplied as a simulated oral fluid during biofilm growth (Sissons et al., 1991). Selective media used for cultural analysis of the biofilms included mitis salivarius, Rogosa agars (both from Difco, and CFAT agar, selective for *Actinomyces* (Zylber and Jordan, 1982).

2.3. Selection of putative root-caries pathogens

Features used to indicate a potentially high cariogenic activity of the bacteria in Table 1 were assessed after batch culture in BMG (0.2%), BMG (1.0%) and BMS (1.0%). They included growth (cell protein), acidogenicity (terminal pH), and the degree of aggregation during growth as a measure of their potential to form a biofilm. The bacteria were inoculated from BHY plates to 10 ml of BMG (0.2%), BMG (1.0%) and BMS (1.0%) in 16 × 160 mm screw-capped culture tubes and were grown shaken at 200 rev/min at 35°C (Model G24 Incubator; New Brunswick Scientific, New Brunswick, NJ) for 24 h. Portions of each culture (100 μl) were inoculated into a fresh set of media in triplicate and also grown for 24 h. Aggregation was assessed using a specially developed, composite scoring system: clumping in culture (graded from − to +++), bacterial adherence to the wall of the culture tube after shaking, and wall growth at the liquid/gas interface of the medium. The culture tubes were then centrifuged at 3260 ***g*** at room temperature for 15 min. Pellet biomass was quantified as total protein (Sissons et al., 1985, 1995) and the terminal pH measured in the supernatant.

The ability of the different strains to utilize nutrient sources, important for establishing metabolically versatile collaborations in consortia, was determined after growth on BHY plates using the Minitek™ system (BBL Microbiology System, Cockeysville, MD), and a panel of enzymes was measured semiquantitatively by API-ZYM™ techniques (Bio Merieux SA, Marcy-l'Etoile, France), both according to the manufacturers' instructions. A primary requisite for this study was the ability to discriminate species on selective media to facilitate analysis. This was established by examining growth, colony size, colour, and morphology on BHY, mitis salivarius, Rogosa, and CFAT agar plates, after 72 h culture and confirmed by Gram stain and catalase test. Mitis salivarius plates were incubated at 35°C in an anaerobic hood (Coy Laboratories Products Inc, Ann Arbor, MI) for 48 h and then left on the bench at room temperature (20°C) for 24 h (Gold et al., 1973). Rogosa and CFAT plates were incubated at 35°C in the anaerobic hood and a 10% CO_2 incubator, respectively.

2.4. General biofilm growth conditions in the artificial mouth

Details of the five-plaque artificial mouth have been described previously (Sissons et al., 1991, 1992; Wong et al., 1994). In brief, the plaque biofilm culture chamber consists of a horizontal, 110-mm diam. glass cylinder with detachable end-plates; five plaque-growth stations 80 mm apart; gas and thermometer ports and a port in the bottom to waste and gas traps (Sissons et al., 1991). Each plaque-growth station includes a port for insertion of a removable plaque holder (Sissons et al., 1992, 1995), and other ports for the fluid-head assembly, which has three lines for supply of nutrients and experimental treatments (Sissons et al., 1992), for inoculation, and for the insertion of pH and reference

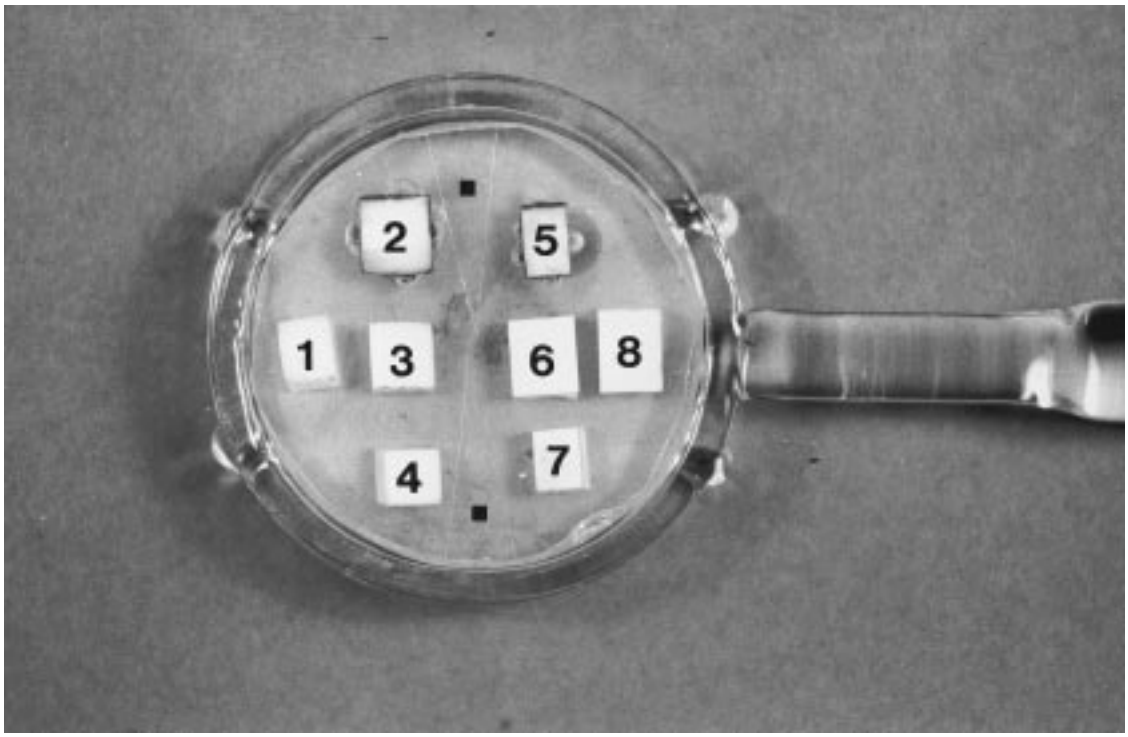

Fig. 1. Arrangement of tooth tissues exposed to plaque biofilms in experiment II. Enamel blocks (1–4) and dentine blocks (5–8), 1 and 8 with their hardness measured. Two wedge-shaped shallow gouges in the polyester resin in which Thermanox™ wedges were placed are labelled ■.

electrodes (Wong et al., 1994). The plaque holders support 25-mm Thermanox™ coverslips (Nunc, Inc, Naperville, IL) or resin-embedded tooth samples (Fig. 1). The culture chamber is housed in a custom-built perspex air incubator at 35°C. Humidified gas (5% CO_2 in N_2) is supplied regularly into the chamber for 30 min every 2 h. A computer with LabVIEW software (National Instruments, Austin, TX) controls both the supply of nutrients to the plaques and pH data-acquisition (Wong et al., 1994).

Thirty minutes after inoculating the plaque stations with approx. 4×10^8 c.f.u. of bacteria, a continuous flow of BMM was delivered at 3.6 ml/h to each plaque through a peristaltic pump (Watson-Marlow, Model 503 U, Falmouth, England). Carbohydrates, 1.5 ml of 5% w/v sucrose (or glucose), were supplied for 6 min every 6 or 8 h according to the experimental protocol described for each experiment. BMM addition was stopped during the 6-min carbohydrate addition. Three separate 3-week experimental 'runs' in the five-plaque artificial mouth were carried out and designated experiments I, II, and III.

2.5. Inoculation procedure

To prepare the inoculum, each bacterial species was grown on BHY plates for 2 days, and single colonies inoculated into 10 ml BMG (0.5%) broth in triplicate. *L. rhamnosus* was incubated in the anaerobic hood. The bacteria were grown (24 h, 35°C, shaken at 200 rev/min), and 100 μl of each culture inoculated into a fresh set of media and grown for a further 24 h. For each species, the culture tube with the highest turbidity was centrifuged (3260 ***g***, 20°C, 15 min), the pellet resuspended by vigorous vortexing in 1 ml sterile H_2O and 1 ml 1% Proteose peptone–0.25% KCl solution and transferred to a sterile Universal bottle. Suspensions (600 μl) of each species were combined into a further Universal bottle. The inocula composition was analysed culturally. Bacterial suspension (1 ml) from each of these five Universal bottles containing the single and the mixture of four species was inoculated onto the coverslips or embedded tooth tissues.

2.6. Experiment I

The aim of experiment I was to establish monoculture and consortium plaque biofilms in the artificial mouth on Thermanox™ coverslips. After 12 days of growth with 5% sucrose supplied 6-hourly, plaque wet weight and depth were measured (Sissons et al., 1995).

A quarter of each plaque was aseptically removed into 10 ml H_2O, dispersed by vortexing followed by 60-s sonication with a Microson 50 sonicator at 50% power (Heat Systems-Ultrasonics Inc, Farmingdale, NY). Protein content was measured by a modification of the Lowry reaction on a pellet centrifuged from 1 ml of the suspension (Sissons et al., 1985). Dilutions in 1% Proteose peptone–0.25% KCl were spiral-plated on to BHY, mitis salivarius, Rogosa, and CFAT plates for bacterial composition analysis (CV approx. 15%; Spiral Systems, 1991). The remainder of the plaque was spread evenly around the coverslip, growth was allowed to resume and pH measurements were made during the following 10 days.

2.7. Experiment II

The aim of experiment II was to examine the cariogenicity of the monoculture and consortium plaque biofilms to blocks of polished bovine enamel and dentine. Enamel blocks underlaid with dentine ($5 \times 4 \times 3$ mm) were cut from the crowns of bovine permanent incisors having a smooth labial surface. Bovine dentine blocks were prepared with the pulpal side uppermost. The surface of the blocks to be exposed to the biofilms was ground flat with 600-grade carborundum paper parallel to the natural surface. Red nail varnish and hard yellow wax were then applied to the cut surfaces to prevent resin entry into dentinal tubules. Four enamel and four dentine blocks were embedded together in polyester resin (Epiglass 552p, polyester encapsulating resin; Epiglass NZ Ltd, Auckland, New Zealand) to fit a single, 25-mm dia. plaque holder (Fig. 1). The exposed tooth surfaces were polished to a high gloss with 6-μm and 1-μm diamond abrasives (Perlos Oy Olozon® Division, SF-01900 Nurmijärvi, Finland). Their surface hardness was measured (as described below). To give a control area on each block, red nail varnish was applied at one edge to cover about 20% of the exposed surface. The embedded tooth samples were weighed and sterilized with ethylene oxide.

The artificial mouth was inoculated as for experiment I and then reinoculated similarly on days 5 and 8 of growth to facilitate acquisition of all species into the consortium. Sucrose (5% w/v) was supplied 8 hourly instead of 6 hourly to allow more time to reach a resting pH (Sissons et al., 1998). After 12 days of growth, plaque wet weight and depth were measured. About 5% of the total plaque (50% for the *L. rhamnosus* monoculture biofilm) was removed for cultural analysis as for experiment I. Growth was allowed to resume. pH electrodes were installed on day 13 and pH responses to the 8-hourly, 6-min sucrose applications were recorded between day 14 and day 16 of growth. On day 22, the caries attack on enamel blocks was analysed (see below) by microradiography and microhardness measurement. The association between plaque size and cariogenicity was assessed by linear regression and the significance of the regression coefficient analysed by two-tailed *t*-test. The dentine blocks were too uneven for microhardness measurement and were analysed by microradiography only. One of the three dentine blocks under each plaque was demineralized and stained for light microscopy (see below).

2.8. Experiment III

The aim of experiment III was to examine the effect of variation in carbohydrate supply, inoculation procedure and continuously applied 1 mmol/l (19 parts/10^6) fluoride on the composition and cariogenicity of the consortia biofilms on polished bovine enamel and human root tissue with unpolished natural surfaces. Human teeth extracted at Wellington Hospital Dental Department were obtained with patient consent and examined for defects and caries under a stereomicroscope. The Greater Wellington area water supply is fluoridated to 1 part/10^6. Cervical portions of human premolars with intact enamel and root surfaces were prepared. Each plaque station included one polished bovine enamel block and two blocks prepared from the cervical region of human teeth to include both the intact enamel and adjacent root. Three plaque stations were supplied with 5% sucrose 8 hourly (Table 3; nos 11, 14, 15), one with 5% glucose (Table 3; no. 12), and one with 2.5% sucrose and 2.5% glucose (Table 3; no. 13). Plaque no. 14, was first inoculated with *A. naeslundii*, a biofilm grown for 3 days and then *Strep. mutans, Strep. sobrinus* and *L. rhamnosus* were also inoculated, a procedure aimed at increasing the percentage of *A. naeslundii* in the consortium. The other plaques were inoculated with all bacteria on both days. All plaques were reinoculated with all bacteria on day 4 of growth. The BMM supply to plaque no. 15 (also supplied with 5% sucrose) was supplemented with 19 parts/10^6 NaF. After 12 days of growth, plaque size and species composition were measured and the plaque was sampled for electron microscopy (see below). The plaques were reshaped to prevent loss of medium through the channel formed by removal of part of the plaque, growth allowed to resume, and then electrodes installed and the pH response to 5% sucrose measured. On day 22 the plaque was removed and the hardness of the polished bovine enamel block remeasured, one human tooth block was sectioned for microradiography and the other human tooth block demineralized and Gram stained.

The fluoride-treated plaque (Table 3; no. 15) was terminated after 12 days of growth because the BMM supply had become blocked. A fresh plaque station and set of tissues was reinoculated from the 'control'

sucrose-grown plaque on day 12, and cultured with 19 parts/10^6 fluoride in the BMM for 22 days before end-point analysis as plaque no. 15, exposing the tissues to plaque metabolism for the same period as those under the other plaques.

For the day-12 analyses, a quarter of each plaque was taken for protein and cultural analysis as in experiment I. The 200-fold dilutions used in this experiment were too large to allow accurate differential counting of the bacteria. No *A. naeslundii* were detectable on either the CFAT or BHY plates so the *L. rhamnosus* % was calculated from the Rogosa agar counts of *L. rhamnosus* related to the BHY counts of total bacteria, and an aggregated 'mutans streptococci %' from the difference between total BHY and Rogosa counts. A 10-mg sample of intact plaque was taken for electron microscopy, fixed in half-strength Karnovsky's fixative (Karnovsky, 1965), postfixed in 1% osmium tetroxide, dehydrated in ethanol, embedded in Epon and sections stained with saturated uranyl acetate/lead citrate and examined in an electron microscope (Siemens 102, Germany).

2.9. Tooth tissue analysis

For microradiography, sections through the demineralized area of tooth blocks were cut with a water-cooled diamond blade and ground to a thickness of 100 μm. Microradiographs were taken on spectroscopic film (Kodak 649-0; Eastman Kodak Co., Rochester, NY) with soft X-rays (λ 2.5 Å) using a Softex CMR X-ray machine (Hosoda and Co., Tokyo, Japan). Approximate lesion depth was measured for a representative area of the lesion on calibrated photos of the microradiographs. Due to desiccation and consequent shrinkage of the dentine blocks (e.g. during microradiography), the depth of the dentine lesion could not be determined as accurately as that for enamel and, therefore, the absolute depths derived relate only to other plaques in the same experiment.

Hardness measurements were made with a miniload tester (Leitz Wetzlar Model 6980, Germany) with a Knoop diamond indentor using a 50-g load. The length of the long diagonal of the indentations in μm was recorded. Six indentations were made on the surface of each block in a set pattern. Following exposure to plaque in the artificial mouth, the tooth samples were brushed with a soft toothbrush and another six indentations were made just below and to the right of the previous set. The change in average length of the six indentations in μm was used as a measure of softening or hardening. Statistical significance was tested by a paired Student *t*-test.

Infiltration of micro-organisms into dentinal tubules was examined by light microscopy after demineralization and staining. The dentine blocks were fixed in half-strength Karnovsky's fixative for 48 hs at 4°C, washed in 0.185 mol/l sodium cacodylate buffer (pH 7.4), followed by a 2-h 1.33% osmium tetroxide postfixation in s-collidine buffer (0.2 mol/l, pH 7.6). After fixation, dentine blocks together with embedding resin were demineralized in 0.25 mol/l EDTA and 4% glutaraldehyde for 3 months, dehydrated in alcohol (Schüpbach et al., 1989), and embedded in glycol methacrylate resin (Kulzer Technovit resin; Kulzer & C GmbH, D-6393 Wehrheim/Ts). Thin sections (2 μm) were stained with Gram stain.

2.10. Plaque pH measurement

After removal of the plaque samples for microbiota analysis, the remaining plaque was mechanically flattened with a sterile spatula to cover the whole of the coverslip or tooth sample, returned to the artificial mouth and the BMM and carbohydrate supply resumed. After 24 h further growth, a micro-oesophageal glass pH electrode (1.4 mm dia.; Model MI-508; Microelectrodes, Inc, Bedford, NH) mounted in stainless-steel tubing, and a microreference electrode (Model 401; Diamond General Corp., Ann Arbor, MI) were inserted into each plaque through the inoculation and sampling ports (respectively) for computer-based pH measurement (Sissons et al., 1992; Wong et al., 1994). The reference electrode was positioned near the edge of the plaque and the pH electrode placed deep into the plaque (Sissons et al.,1992). The electrodes were calibrated with pH 7 and pH 4 buffer before placement in the artificial mouth and again at the conclusion of the experiment. The resting pH in this study was defined as the pH to which the plaque returns 8 h after a carbohydrate application (Sissons et al., 1998). Several pH responses to 5% sucrose of each plaque were recorded sequentially, and both the resting and minimum pH values were averaged as described in Table 3.

3. Results

3.1. Bacterial selection for a four-species cariogenic biofilm consortium

The bacteria shown in Table 1 were screened using the criteria described in the Methods. The properties of the four *Strep. mutans* strains examined were similar. The type strain *Strep. mutans* ATCC 25175 was selected. *Strep. sobrinus* 01H1 was selected because it had a lower terminal pH and greater aggregation in culture than strain KIR. *A. naeslundii* WVU 626 was selected from the *Actinomyces* spp. because it had the lowest terminal pH in 1% sucrose, was catalase-positive (aiding detection) and could metabolize lactose.

Table 2
Terminal pH, aggregation during growth, and metabolic properties of bacterial species selected to construct consortia biofilms

Variables			*Strep. mutans* ATCC 25175	*Strep. sobrinus* 01H1	*A. naeslundii* WVU 626	*L. rhamnosus* ATCC 7469
Terminal pH[a] in:	0.2% glucose		4.52 (0.10)	4.29 (0.01)	5.16 (0.06)	4.66 (0.12)
	1% glucose		4.59 (0.03)	4.20 (0.02)	5.09 (0.02)	4.11 (0.02)
	1% sucrose		4.20 (0.00)	4.11 (0.04)	4.90 (0.02)	5.70 (0.03)
Aggregation after growth in:	1% glucose	clump	++	++	+	–
		wall	–	–	+	–
		ring	+	+	+	–
	1% sucrose	clump	+++	+++	++	–
		wall	+	+	+	–
		ring	+	+	+	–
Metabolism of:[b]	Lactose		+	–	+	+
	Glycerol		–	–	+	+
	Raffinose		+	–	+	–
	Inulin		+	–	+	–
	Salicin		+	–	–	+
	Adonitol		+	–	–	–
	Mellibiose		+	–	+	–
Enzymes present:[c]	Catalase		–	–	+	–
	Urease		–	–	+	–
	Alkaline phosphatase		–	–	–	+
	Esterase(C4)		+	–	+	+
	Chymotrypsin		+	–	–	–
	Valine arylamidase		–	–	+	+
	β-glucosidase		+	–	+	+
	α-galactosidase		–	–	+	–
	β-galactosidase		–	–	+	+
	β-glucuronidase		–	+	–	+

[a] Mean and SD of triplicates.
[b] All four species were able to metabolize glucose, sucrose, galactose, maltose, trehalose, inositol, fructose, and esculin; none metabolized xylose, starch, or produced NH_3 from arginine.
[c] All four species had acid phosphatase, esterase(C8), phosphoamidase, α-glucosidase, and leu arylamidise activities, but lacked nitrate reductase, lipase, cystine arylamidase, trypsin, *N*-acetyl β-glucosaminidase, and mannosidase.

The *L. rhamnosus* strains were similar but strain ATCC 7469 was selected because strain B 259 was negative for sucrose metabolism by the Minitek procedure. Table 2 shows the putative cariogenic and metabolic properties of the selected strains. Choice of these particular strains also maximized the potential metabolic diversity of the consortia, and the species could be distinguished on BHY plates by colony morphology as well as on selective growth plates.

3.2. Growth of monoculture and consortium biofilms

After 11–13 days of growth in the artificial mouth, most biofilms reached 1–2 g wet wt (Table 3), had a maximum thickness of 4–6 mm, and ranged from approx. 1 to 5×10^{10} c.f.u. *L. rhamnosus* formed a smaller biofilm virtually invisible to the naked eye in experiment I, somewhat larger in experiment II (0.24 g), and formed a substantial biofilm after infecting the neighbouring *A. naeslundii*-inoculated biofilm. This cross-contamination probably occurred during inoculation. The consortium biofilms were generally larger than the monoculture biofilms.

The consortia in experiments I and II comprised mainly streptococci with *Strep. mutans* tending to predominate over *Strep. sobrinus*, and *L. rhamnosus* contributing most of the balance (Table 3). The composition varied between experiments. *Strep. mutans* was particularly high (76%) and *A. naeslundii* was not detected in the consortium of experiment I where the sucrose was delivered every 6 h. The sucrose applications were subsequently reduced to 8-hourly with lower amounts of *Strep. mutans*. *A. naeslundii* reached 4% in experiment II. When the carbohydrates and inoculation procedures were changed in experiment III, the consortia had a similar composition with slightly more mutans streptococci than *L. rhamnosus* but no detectable *A. naeslundii*. Overall, the consortia in experiments II and III, which were receiving 8-hourly carbohydrate, contained 58 ± 6 (SD) c.f.u.% mutans

Table 3
Biofilm size, composition and pH at day 12, and caries activity at 22 days of growth

Experiment no.	Plaque no.	Inoculum	Carbohydrate[a] (%)	Wet wt day 12 of growth (g)	Composition: day 12 of growth (%c.f.u.)		Resting pH[b]	Sucrose-induced minimum pH[b]	Enamel softening[c] (Δμm from control)	Lesion Depth[d] (μm)	
										Enamel	Dentine
I	1	*Strep. mutans*	sucrose (5)	1.01	*Strep. mutans*	100					
	2	*Strep. sobrinus*	sucrose (5)	0.70	*Strep. sobrinus*	100					
	3	*A. naeslundii*	sucrose (5)	0.99	*A. naeslundii*	100					
	4	*L. rhamnosus*	sucrose (5)	0.017	*L. rhamnosus*	100					
	5	Consortium	sucrose (5)	1.35	*Strep. mutans*	76					
					Strep. sobrinus	0.7					
					A. naeslundii	0					
					L. rhamnosus	23					
II	6	*Strep. mutans*	sucrose (5)	1.71	*Strep. mutans*	100	5.77 (0.09)	4.17 (0.14)	64.1 (5.7)	200	200
	7	*Strep. sobrinus*	sucrose (5)	1.21	*Strep. sobrinus*	100	5.53 (0.06)	4.26 (0.19)	64.2 (10.7)	165	200
	8	*A. naeslundii*	sucrose (5)	0.87	*L. rhamnosus*	98[e]	5.34 (0.02)	4.30 (0.09)	68.3 (7.9)	115	150
					A. naeslandii	2					
	9	*L. rhamnosus*	sucrose (5)	0.24	*L. rhamnosus*	100	5.38 (0.07)	3.90 (0.08)	96.0 (11.3)	100	190
	10	Consortium		1.66	*Strep. mutans*	30	5.29 (0.00)	3.66 (0.10)	126.4 (4.7)	225	175
					Strep. sobrinus	24					
					A. naeslundii	4					
					L. rhamnosus	42					
III	11	Consortium	sucrose (5)	1.69	mutans strep.[f]	60	5.01 (0.09)	4.07 (0.09)	114.0 (25.7)	–	–
					A. naeslundii	0					
					L. rhamnosus	40					
	12	Consortium	glucose (5)	1.08	mutans strep.	65	5.19 (0.16)	3.86 (0.02)	123.3 (5.5)	–	–
					A. naeslundii	0					
					L. rhamnosus	35					
	13	Consortium	sucrose (2.5) glucose (2.5)	1.49	mutans strep.	53	4.93 (0.12)	3.81 (0.09)	125.3 (6.5)	–	–
					A. naeslundii	0					
					L. rhamnosus	47					
	14	Consortium Sequential Inoculation	sucrose (5)	1.25	mutans strep.	53	5.20 (0.15)	3.83 (0.12)	93.5 (16.2)	–	–
					A. naeslundii	0					
					L. rhamnosus	47					
	15	Consortium 19 parts/10^6 F	sucrose (5) 19 parts/10^6 F	1.94	mutans strep.	57	5.58 (0.05)	4.41 (0.02)	38.3 (3.7)	–	–
					A. naeslundii	0					
					L. rhamnosus	43					

[a] Applied for 15 min every 6 h in experiment I, every 8 h in experiments II and III.
[b] Mean and SD of six successive determinations following 8-hourly sucrose applications in experiment II, and three successive determinations in experiment III (see Fig. 2).
[c] Δμm: Mean (SD) of the difference between Knoop diamond indentations before (47.9, SD 1.16) and after exposure of polished bovine enamel and dentine blocks to biofilms (six repeated indentations in a predetermined pairing pattern on one enamel block).
[d] Approximate lesion depth from microradiographs.
[e] Dominated by *L. rhamnosus*, which probably infected the plaque station during inoculation.
[f] mutans strep. is the total of *Strep. mutans* and *Strep. sobrinus* (see Methods and Results).

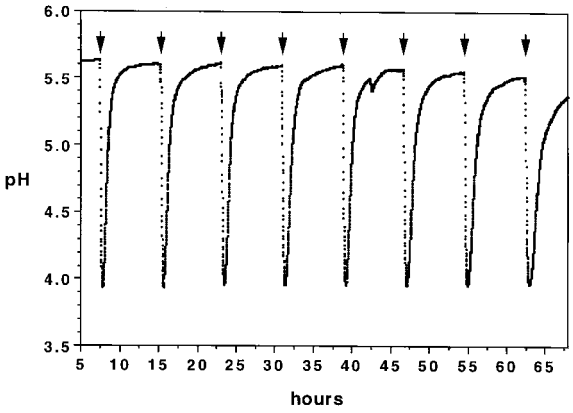

Fig. 2. pH response to 5% sucrose of a consortium plaque biofilm (experiment II; Table 3; no. 10). pH measurements were taken between day 14 and day 16 of growth. Arrows show the addition of 5% sucrose over 6 min.

streptococci and 42 ± 6 c.f.u.% *L. rhamnosus*. This composition was different from the original colony-forming unit proportions of the inoculating species, which comprised about 50% *L. rhamnosus* and equal units (17%) of the other species. Examination by electron microscopy indicated that the consortia biofilms grew predominantly as defined microcolonies (data not shown). The resting pH of the consortia plaques ranged from 4.9 to 5.3 (Table 3). Sucrose (5%) caused a rapid pH drop to a minimum pH below 4 (Fig. 2 and Table 3).

3.3. Formation of caries in tooth tissues

In experiment II, bovine enamel blocks exposed to plaques for 3 weeks (Table 3; nos 6–10) changed from translucent white to an opaque, chalky white. The decrease in enamel surface hardness under all biofilms,

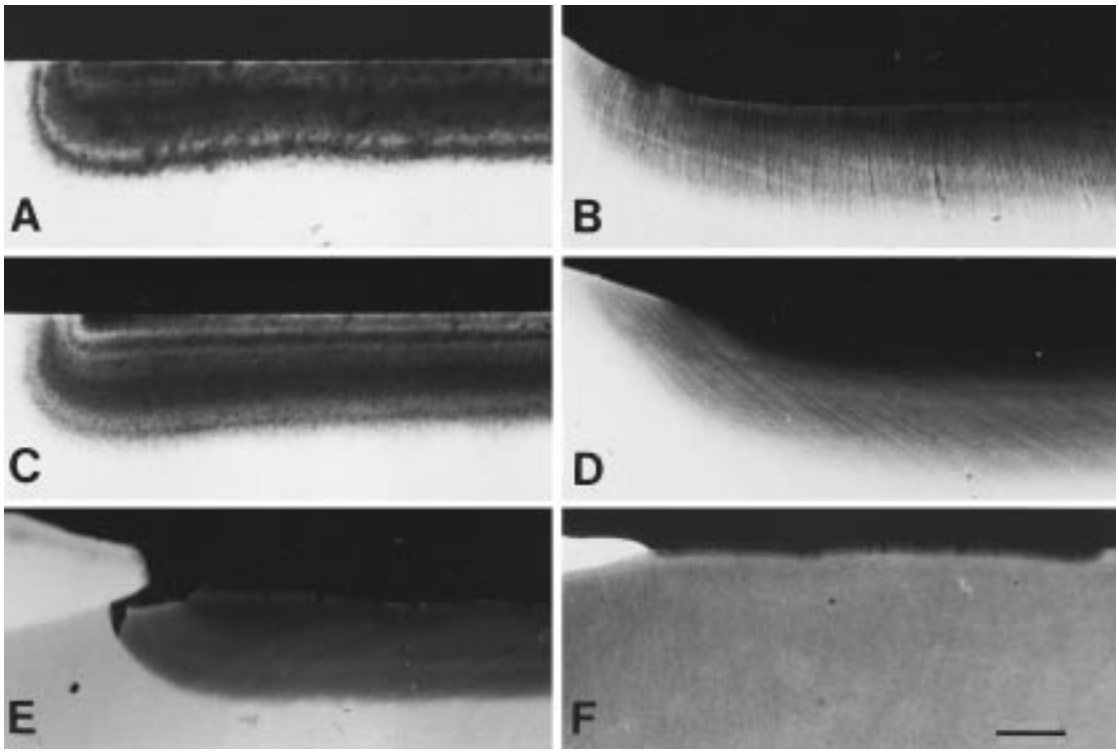

Fig. 3. Microradiographs of lesions induced in bovine enamel and dentine and in intact human roots. Microradiographs (A) and (B) are of bovine enamel and dentine blocks, respectively, which had been exposed to a *Strep. mutans* biofilm (experiment II; Table 3; no. 6) for 22 days; (C) and (D) are of bovine enamel and dentine blocks exposed to a consortium plaque biofilm (experiment II; Table 3; no. 10). The left corner of microradiographs (A–D) shows the undemineralized control areas that were covered by nail varnish to protect them from caries attack. Microradiograph (E) is a human tooth cervical region optimized for root demineralization exposed to a consortium plaque supplied with 5% sucrose (experiment III; Table 3; no. 11) for 22 days. Microradiograph (F) is a similar tooth block with 19 parts/10^6 fluoride supplied in the BMM during 22 days of growth (experiment III; Table 3; no. 15). A control area of the root that had been covered with varnish was on the right hand side. Bar = 100 μm.

including the *L. rhamnosus* biofilm, was highly significant ($p < 0.0001$). The enamel under the consortium plaque in experiment II was significantly softer than the enamel under the monoculture (and the *A. naeslundii*/*L. rhamnosus* mixed culture; Table 3; no. 8) biofilms ($p < 0.003$). The polished dentine blocks turned yellow–brown, and 0.2–0.3 mm of the original surface layer was lost. Hardness measurements could not be made due to the resulting uneven surface. In experiment III, which included human cervical region enamel-root blocks (Table 3; nos 11–13), similar enamel surface softening occurred under the consortia biofilms irrespective of substitution of sucrose by glucose or prior formation of an *A. naeslundii* biofilm (Table 3; no. 14).

Microradiography of enamel blocks in experiment II showed the development of demineralized subsurface lesions but virtually no erosion of the enamel surface (Fig. 3). The lesion was slightly deeper under the consortium (225 μm) than under the other biofilms (Table 3). It had a pronounced surface hypermineralized zone and a thick radiolucent area containing five layers of laminations (Fig. 3C). The lesion produced by the *Strep. mutans* biofilm (Fig. 3A) was similar in size (depth 200 μm) and appearance. The others were smaller (100–160 μm deep, not shown). Apart from the lesions formed under the small *L. rhamnosus* biofilm (Table 3; no. 9) and with 19 parts/10^6 fluoride present (Table 3; no. 15), enamel lesion depth was mainly explained by the biofilm wet weight ($r^2 = 0.68$, $p < 0.05$) (Table 3). Despite this relationship, lesions in the dentine blocks produced by both monocultures and consortia were of similar size (Table 3) and appearance, with a radiolucent carious area, surface erosion and no radiopaque zone at the lesion surface (Fig. 3B,D). All had concave outer surfaces due to shrinkage. Bacteria penetrated the tubules of bovine dentine blocks under all five biofilms in experiment II, with *Strep. mutans* having the deepest maximum penetration (approx. 280 μm) (Fig. 4).

In intact human roots, the consortia in the absence of fluoride (experiment III; Table 3; nos 11–14) induced saucer-shaped artificial lesions on the roots, which were similar to those in bovine dentine blocks (Fig. 3E). At the coronal and apical extremities of the root-surface lesion, the bands curved toward the enamel and also toward the apical surface of the root, with some contraction at the cervical part of the root. The thickness of the dentine demineralization zones of the four lesions were very similar, and there was no surface radiopaque zone. Hence, for the tooth samples with intact surfaces, the different carbohydrate supplies still resulted in lesions having a similar microradiographic appearance, possibly explained by the similar microbiota composition of the plaques. Bacteria penetrated all the intact human root samples but only as far as the cementum, despite the presence of substantial (up to 170 μm), subcementum, dentinal demineralization (Fig. 3E). Subsurface lesions were detectable in the enamel.

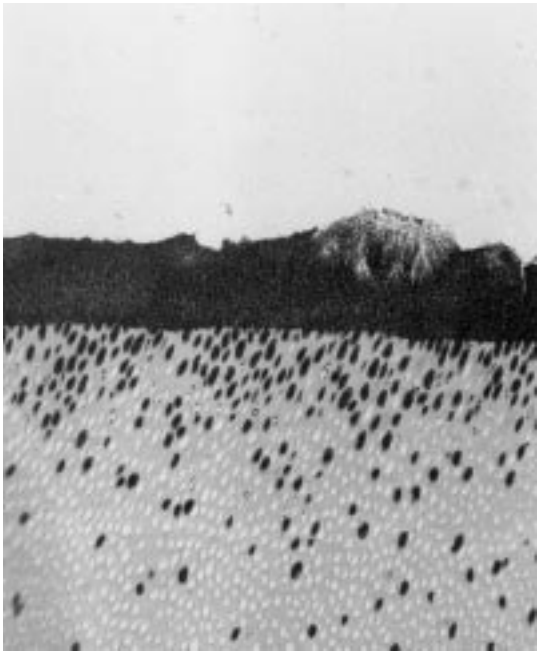

Fig. 4. A Gram-stained *Strep. mutans* biofilm overlaying bacteria penetrating a demineralized dentine block that had been exposed in the artificial mouth for 22 days (experiment II; Table 3; no. 6) (×290). The microradiograph in Fig. 3(B) is another equivalent dentine block (that had not been demineralized) under the same *Strep. mutans* biofilm.

In the presence of 19 parts/10^6 fluoride, softening of polished enamel was reduced by two-thirds (Table 3; no. 15). In the intact human tooth-root surfaces, some erosion of the cementum surface may have occurred with only a slight indication of a subsurface lesion (Fig. 3F).

4. Discussion

4.1. Selection of strains to form defined-species consortia biofilms

In constructing laboratory caries models based on consortia biofilms of selected species of bacteria, the properties of the particular strain of each species selected will determine the activities of the biofilm and hence the outcome of experiments. Random selection of available strains is a common approach improved somewhat by use of type strains, but this approach yields bacteria with arbitrary properties, possibly no direct biological link to the induction of caries and

hence uncertainty in the applicability of findings to natural caries. In our study, we screened several strains for appropriate cariogenic properties and selected the ones that performed best. This method probably offers the best strategy for obtaining highly cariogenic biofilms, although the degree of collaboration between the bacteria may be less than occurs in vivo. The use of bacteria from a natural ecosystem (e.g. a single root-caries lesion) is an alternative and has the advantage of an increased probability of ecosystem compatibility and involvement in lesion formation. Such isolates were not available in the early stages of this study.

4.2. Caries induction by the consortia biofilms

Strep. mutans, Strep. sobrinus, A. naeslundii, and *L. rhamnosus* are all potential root-caries pathogens (Ellen et al., 1985; Bowden, 1990; van Houte, 1994; Schüpbach et al., 1995, 1996). Their monoculture biofilms, and the consortia biofilms, were highly cariogenic however, overall, the consortia plaque biofilms grew better and induced more surface enamel softening. They produced lesions in polished bovine enamel having a surface mineralized layer and a subsurface demineralized zone, usually with a laminated appearance on microradiography. Lesions in dentine blocks involved bacterial penetration of the tubules and lacked a radiopaque surface zone. Except for this latter feature, both dentine and enamel lesions were similar to those that occur naturally in vivo (Nyvad and Fejerskov, 1982; Pearce, 1983; Schüpbach et al., 1989). Following exposure of human roots with an intact cementum to the consortia for 3 weeks, the bacteria were confined to the cementum, the thickness of which was only about 10% of the total demineralized region. The subcementum demineralization observed (Fig. 3E) indicates that acid produced by bacterial metabolism diffused into the dentine and demineralized the inorganic components. The substantial enamel softening induced by the *L. rhamnosus* plaque (experiment II; Table 3; no. 9), which did not visibly cover the particular enamel block used for hardness measurement (Table 3), is also an indication that the ability of bacteria to produce an acidic liquid environment may be more important in lesion generation than close juxtaposition of the bacteria to the surface of the mineralized tissue. Despite large numbers of mutans streptococci present in experiment II, replacing the sucrose with glucose, which should lower glucan production, had little effect on the biofilm pH or cariogenicity (Table 3). These findings reinforce the suggestion that sucrose-induced glucans may not be of great importance in modulating cariogenicity (van Houte, 1994).

Fluoride in the low part/10^6 range of concentration modulates calcium phosphate de- and remineralization (Wong et al., 1987; Fejerskov et al., 1996) and may also affect bacterial metabolism (Guha-Chowdhury et al., 1997). We have previously shown that 5 parts/10^6 fluoride in the sucrose supply significantly inhibits caries induced by microcosm plaques that had been cultured in the artificial mouth under the same regimen as the consortia plaques (Cutress et al., 1995). The present findings show that continuously supplied 19 parts/10^6 fluoride strongly inhibits lesions induced by highly acidic cariogenic consortia biofilms in both enamel and the roots of teeth. In terms of the hard-tissue attack, caries induced by these defined species consortia has proved to be a good model of caries in vivo, with potential for dissecting the processes involved and, for example, determining the relative role of different potential effects of fluoride.

4.3. Composition and nature of the defined-species consortia biofilms

The dominant bacteria of the consortia biofilms formed in these experiments were the mutans streptococci and *L. rhamnosus*, all known to be highly acidogenic and aciduric caries pathogens (van Houte, 1994). The presence of these cariogenic properties was reinforced by the present screening programme (Table 2). The bacterial composition of the consortia biofilms varied between experiments compared to growth in chemostat planktonic culture, which is more reproducible (Marsh, 1995; Kinniment et al., 1996). Such variation is intrinsic to biofilms and their associated stochastic growth leading to spatial differentiation (Wimpenny and Colasanti, 1997). As might be expected for very simple biofilm consortia, the bacteria grew mainly in microcolonies, which might allow microscale predominance of a particular acidogenic species. It has been suggested that this mode of biofilm growth may facilitate its cariogenicity (Nyvad and Kilian, 1990).

Despite the range of biofilm composition in experiment II, caries induction measured as lesion depth was proportional to biofilm biomass irrespective of composition, suggesting that for these highly acidogenic bacteria, lesion depth may relate more to the environment, the substrate supply (van Palenstein Helderman et al., 1996) and biofilm rates of acid/base metabolism than to the overall composition of the biofilms. Ecosystems tend to show stability of function despite major changes in species composition (Steneck and Dethier, 1994); for example, in plaque microcosms we have previously reported a reproducible resting pH despite a complex changing flora (Sissons et al., 1998). The possibility that the caries attack may be insensitive to changing proportions of 'mutans' streptococci and lactobacilli could be another example of this. If so, it would reinforce the robustness of biofilm consortia-

based caries model systems. To further validate this possibility, an analysis of variance of the caries attack within enamel blocks at different positions under the plaques, and between stations, would be desirable. A related but difficult study of great interest would be an analysis of the development of temporal and spatial heterogenity in composition and structure using simple consortia.

A. naeslundii proved difficult to colonize and maintain in the consortia biofilms, even if a preformed 3-day *A. naeslundii* monoculture biofilm was established, or sucrose was replaced with glucose. Similar findings in vitro using defined species consortia biofilms have been reported by others (Ahmed and Russell, 1978; Donoghue and Perrons, 1988). The in vivo growth rate of *A. naeslundii* (viscosus) is considerably slower than that of *Strep. mutans* (Beckers and van der Hoeven, 1984); however, the most likely reason for the failure of *A. naeslundii* to be maintained was that the consortia biofilms were too acidic at pH 5.3 and below (Table 3) to allow its growth (Bradshaw and Marsh, 1998). Direct bacterial interference by bacteriocins did not appear to be involved (deferred antagonism assay; unpublished observation). These results suggest that *Actinomyces* spp. are unlikely to be prominent in simple, highly acidogenic, biofilm caries model systems, although *Actinomyces* has been maintained for 20 h in a consortium also containing *Strep. sanguis* and low concentrations of *Strep. mutans* (Simmonds et al., 1995). It might prove possible to counter the sensitivity to extreme acid conditions of a ureolytic *A. naeslundii* by increasing the supply of urea to raise the resting pH (Sissons and Cutress, 1988; Sissons et al., 1998; Morou-Bermudez and Burne, 1999).

4.4. The multiplaque artificial mouth as a caries model experimental system

The term 'artificial mouth' coined by Pigman et al. (1952) is applied usually to dental plaque biofilm experimental systems that have a continuous, open-surface film fluid flow, and excludes closed-flow cells (Sissons, 1997; Sissons et al., 1999). Artificial mouths have a long history of use in caries investigations (Miller, 1890; reviewed by Tatevossian, 1988; Marsh, 1995; Sissons, 1997). Early systems examined caries in extracted teeth but tended to immerse plaque and tooth fully in nutrients and had problems with contamination (Pigman et al., 1952; Sidaway et al., 1964; Tatevossian, 1988). Russell and Coulter (1975) described continuous monitoring of pH and redox potential of developing plaque on a tooth surface, and Dibdin et al. (1976) described an artificial mouth for growing several plaques simultaneously. The technique used here was a further development of these systems (Sissons et al., 1991, 1992, 1999; Wong et al., 1994; Sissons, 1997). However, the early studies and those subsequently of Donoghue and co-workers (Hudson et al., 1986; Donoghue and Perrons, 1988; reviewed by Sissons, 1997) and Simmonds et al. (1995) focused on the properties of the microbial biofilm and did not examine cariogenicity. Noorda et al. (1986a,b) induced enamel caries by *Strep. mutans* monoculture biofilms in a rotating-platter artificial mouth, and this system, also with *Strep. mutans* biofilms, was further modified by Zampatti et al. (1994) to allow periodic application of slurries (e.g. toothpaste) to tooth sections. Batch culture of a mixture of *Strep. mutans, A. naeslundii, L. rhamnosus* plus *Strep. salivarius* and *Strep. sanguis* with saliva-coated enamel blocks results in mixed-species biofilms overlying caries-like lesions (Yue et al., 1992). Consortia of nine oral species have been established in a constant-depth film fermenter (Kinniment et al., 1996), but installation of tooth tissues in such a system is likely to prove difficult.

The data presented here indicate that plaque biofilm consortia of putative caries pathogens established in an appropriate biofilm culture system are valid and useful models for the study of enamel and root caries. The consortia plaques had sucrose-induced pH curves that were similar to those in natural plaque, induced realistic caries lesions, and were more cariogenic than monocultures. Artificial-mouth technology allowed a range of tooth tissues to be exposed to experimentally flexible biofilm growth, metabolism and treatment protocols under controlled conditions. In particular, use of cervical sections of teeth with intact surfaces allowed the investigation of enamel and root caries in the same tooth, together with a comparison of carious attack on standardized, polished enamel blocks. We conclude that defined-species consortia caries models represent a significant improvement over monoculture biofilm systems. Their use opens new possibilities for detailed studies of enamel and root caries and the effects of interactions between plaque biofilms, the oral environment and factors such as fluoride.

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