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Establishment of Defined Mixed Bacterial Plaques on Teeth in a Laboratory Microcosm (Model Mouth)

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Actinomyces viscosus WVU627, '*Streptococcus mitior*' LPA-1 and *Veillonella alcalescens* OMZ193 were grown in pure or mixed culture on teeth in a model mouth which was supplied with synthetic saliva and an intermittent nutrient supplement containing 1 per cent (w/v) glucose. All organisms became established in pure culture although *V. alcalescens* colonised very poorly unless 1 per cent (w/v) lactate was supplied in place of the glucose. Mixed cultures of all three organisms were readily established. In general, numbers and proportions of species varied widely in mixed culture, but were comparable to those observed in human dental plaque studies. When grown in association with *A. viscosus* and '*S. mitior*', *V. alcalescens* achieved similar numbers to pure cultures supplied with lactate, thus demonstrating that a food chain existed. Higher viable counts of '*S. mitior*' were obtained from mixed plaques, compared with pure cultures. However, less *A. viscosus* could be isolated when co-cultured with streptococci and veillonellae. Comparison of differential viable counts at 45 h, 66 h and 90 h after inoculation with these three organisms showed that both veillonellae and actinomycetes increased in numbers and proportions with incubation time. *Streptococcus mutans* C67-1 became established when inoculated together with the other organisms, although it attained lower numbers compared with pure cultures. It appeared that *S. mutans* antagonised *A. viscosus* and partially displaced this organism in mixed plaques which contained all four species.

KEY WORDS—*Actinomyces viscosus*; '*Streptococcus mitior*'; *Streptococcus mutans*; *Veillonella alcalescens*; Model mouth; Microbial interactions.

INTRODUCTION

The microbial film on teeth, known as dental plaque, consists of a complex community of many different microorganisms and their extracellular products. The acidic end-products of these organisms are implicated in dental caries and there has been much debate on whether specific, highly acidogenic and aciduric bacteria such as *Streptococcus mutans* initiate caries.[15,25] An alternative view is that caries results from 'pathogenic synergy', where a group of different bacteria under appropriate conditions creates a cariogenic environment.[7]

Microbial interactions are fundamental to the concept of pathogenic synergy. However, interactions must also be of prime importance if caries is indeed initiated by a specific organism. For example, *S. mutans* is not one of the initial colonisers of the tooth surface,[9,28] and it is not yet understood how this species can become established within a pre-existing plaque that contains organisms known to produce potentially inhibitory products.[11,13] Work with animal models for caries has shown *S. mutans* induced caries which can be enhanced[32] or reduced[29] by the presence of other plaque bacteria.

There is disagreement about the potential effect on caries of the interaction between *S. mutans* and the lactate-utilising organism *Veillonella alcalescens*. Early reports[20,29] suggested that veillonellae reduce experimental caries by metabolising the lactate produced by *S. mutans*. More recent work, however, has found that veillonellae can stimulate the growth and glycolytic activity of streptococci by the continual removal of lactate,[17] and it has been suggested[27] that the role of veillonellae in plaque may have to be re-assessed.

The purpose of the present study, therefore, was to establish populations of plaque bacteria on teeth in a controlled environment, in order to investigate the stability of the flora and the interactions occurring within it. Bacteria in dental plaque are

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growing on a surface and are subjected to a fluctuating environment caused by the salivary flow and intermittent nutrient supplements derived from the host diet. Under such conditions, organisms are known to differ in their phenotype and metabolism from bacteria in batch or continuous cultures.[41] The laboratory microcosm[42] known as the artificial or model mouth was therefore used, in order to study plaque bacteria under controlled conditions but analogous to those *in vivo*.

Three organisms which are abundant in human supragingival plaque,[18] *Actinomyces viscosus*, '*Streptococcus mitior*' and *Veillonella alcalescens*, were used to produce a simple bacterial plaque, analogous to those formed in gnotobiotic animal models where defined microbial ecosystems are established.[29] The ability of these organisms to become established on teeth was compared in pure and mixed culture, and the effect of incubation time or the presence of *Streptococcus mutans* was determined.

MATERIALS AND METHODS

Microorganisms

All organisms were originally isolated from the human oral cavity but have been maintained in the laboratory for many years.

Actinomyces viscosus WVU627 is a human serotype 2 strain, isolated by Gerencser, West Virginia University, USA, and was obtained from Dr P. S. Handley, University of Manchester, England.

'*Streptococcus mitior*' LPA-1, Carlsson's strain 44,[8] has been described previously.[14] This strain produces extracellular polysaccharide and was received as *Streptococcus sanguis* from Professor H. Luoma, University of Kuopio, Finland. Although the taxonomy of this group is still under discussion, the name '*S. mitior*' is used in this study, because of its familiarity in the dental literature.

Streptococcus mutans C67-1 is a serotype c strain which closely resembles the type strain NCTC 10449 and is cariogenic in rats.[37] It was obtained from Prof. J. D. de Stoppelaar, University of Utrecht, The Netherlands.

Veillonella alcalescens OMZ193 was supplied by Dr J. S. van der Hoeven, University of Nijmegen, The Netherlands. This organism is a subculture of strain V5, originally isolated by Dr R. J. Gibbons, Forsyth Dental Center, Boston, Mass., USA.

Cultural conditions

The model mouth described by Hudson *et al.*[22] was used, and methods were generally similar to those described previously.[14,22] Briefly, bacteria were grown on sound, human upper premolar crowns, that had been sliced into halves and mounted back to back to simulate a stagnation site in the mouth. Each culture flask contained one such tooth unit. Generally, six replicate culture flasks were used per experiment. The flasks, tubing and teeth were steam-sterilised (134°C for 6 min). Fluids were filtered into the reservoirs using Durapore membranes (Millipore [UK] Ltd, Harrow, Middlesex, England).

Synthetic saliva based on bovine submaxillary glycoprotein[36] was provided continuously at 0·5 ml/h. At this flow rate saliva was delivered to the teeth as separate drops, which were retained there until displaced by the next drop. An additional nutrient supplement, streptococcal basal medium (SBM) broth was supplied intermittently, for one hour every 6 h, also at 0·5 ml/h. SBM broth contained (g/l): Bacto-casitone (Difco) 20·0; yeast extract (Oxoid) 5·0; NaCl 2·0; K_2HPO_4 4·0; KH_2PO_4 1·0; pH 7·0. After sterilisation (15 min at 121°C), glucose was added from a separately sterilised (10 min at 110°C) stock solution to give a final concentration of 1 per cent (w/v). In one experiment the glucose was replaced by a 1 per cent (w/v) sodium lactate solution. The apparatus was swept with 5 per cent (v/v) CO_2 in nitrogen at 7–14 kPa (1–2 psi), which reduces the oxygen tension but does not create strictly anaerobic conditions because fluids in reservoirs were not deoxygenated and the tubing permits seepage of atmospheric gases into the system. Culture flasks were maintained at 35°C via their waterjackets.

Inoculation

Organisms were maintained by weekly subculture on brain heart infusion (Oxoid) agar and selective agar plates (see below). For inocula, organisms were grown in an appropriate broth for 24 h at 35°C. From these, rapidly-growing (2–5 h) broth cultures were prepared and diluted to give similar numbers of organisms. Streptococci were grown in brain heart infusion (BHI) broth. *A. viscosus* was cultured in the basal medium described by Beighton and Colman[3] but without agar or serum. Rogosa's medium V17[35] was used to culture *V. alcalescens*. Inocula were always counted (see below) and contained between 10^7–10^9 CFU/ml. A single

inoculum (1·5 ml) of a pure culture or a freshly-prepared mixture of equal volumes of three or four strains, was delivered rapidly (30 s) to each tooth via the vaccine cap, using a hypodermic syringe.

Sampling and counting procedure

Tooth units were removed, generally 66 h after inoculation, and placed in 10 ml 1/4 strength Ringer's solution (Oxoid). Bacteria were released from the surface by placing the bottles containing the teeth in an ultrasonic cleaning bath for 2 min and the resulting suspension serially diluted in 1/4 strength Ringer's solution. Direct cell and CFU counts were peformed on the appropriate dilution (usually 10^{-1}) using a counting chamber. Viable counts were performed in triplicate on BHI agar and various selective agars. Diluted bacterial suspensions (usually 10^{-1}–10^{-7}) were spotted on to each well-dried plate, using a 1/30 ml dropper.

A. viscosus was cultured on the enrichment medium described by Beighton and Colman,[3] made selective with 5 µg/ml colistin sulphate solution, but with the sodium fluoride replaced by 300 µg/ml cadmium sulphate.[43]

Streptococci were cultured on GSTB agar, a version of glucose-sucrose-potassium tellurite-bacitracin agar[38] that included 5 µg/ml colistin sulphate. '*S. mitior*' was selected on GSTB agar containing 7·5 µg/ml potassium tellurite but no bacitracin. Although *S. mutans* grew well on this medium in pure culture, it was almost totally excluded when '*S. mitior*' was present, although the reason for this inhibition is not known. Any colonies of *S. mutans* were easily distinguishable as they were small and dark, whereas '*S. mitior*' colonies were raised and translucent, with a dark rim. *S. mutans* was cultured on GSTB agar without tellurite but containing 0·3 units/ml bacitracin.

V. alcalescens was grown on Rogosa's medium V15[35] that contained 7·5 µg/ml vancomycin (RVM).

Plates were incubated at 35°C for 72 h. BHI and RVM plates were cultured in an atmosphere of 10 per cent (v/v) CO_2 and 90 per cent (v/v) hydrogen. GSTB and *Actinomyces* selective agar (ASA) plates were incubated in 10 per cent (v/v) CO_2 and 90 per cent (v/v) air. The most appropriate dilutions were chosen, and the number of CFU per tooth unit was found by calculation. Differential counts of each organism were obtained from the BHI plates, using a Kyowa low-power zoom microscope with transmitted and incident light.

Calculations and statistical analysis

Selective media were used whenever possible for differential counts as interactions apparently occurred on the BHI plates. Although the four organisms used in the study could be distinguished readily on this medium, many colonies were mixed. Counts of veillonellae were consistently higher when grown in association with the other organisms. However, *A. viscosus* grew less well on the mixed BHI plates, especially when *S. mutans* was present. Proportions of the organisms in mixtures were therefore obtained from the sum of the separate selective counts.

Individual bacterial counts were converted to $\log_{10}$ values before any calculations or analysis, to ensure normal distribution of the data. The Student's t test was used to compare bacterial counts and $p<0{\cdot}05$ was deemed to be significant. The percentage composition of the mixed plaques was calculated for each tooth and the median plus the range determined.

RESULTS

A. viscosus, '*S. mitior*' and *S. mutans* were readily grown in pure culture on teeth (Fig. 1). *V. alcalescens* colonised poorly with synthetic saliva and the intermittent nutrient supplement of SBM + 1 per cent (w/v) glucose, and could not be detected on 3/12 teeth. However, when the glucose was replaced by sodium lactate, high numbers were obtained in pure culture after 66 h (Fig. 1).

Viable counts in pure culture were next compared with selective counts obtained when mixed plaques of *A. viscosus*, '*S. mitior*' and *V. alcalescens* were produced. Lower numbers of actinomycetes were recovered ($p<0{\cdot}001$), but '*S. mitior*' attained a higher viable population in the mixture ($p<0{\cdot}001$). When grown in mixed cultures supplied with SBM + 1 per cent glucose, *V. alcalescens* was isolated consistently and with far higher counts than when grown in pure culture ($p<0{\cdot}001$). Indeed, numbers of veillonellae in a mixed culture with glucose were similar to the pure culture supplied with lactate.

Mixed plaques of these three organisms were then compared at 45, 66 or 90 h after incubation (Fig. 2). There was an increase in total direct count at 90 h compared with the count at 45 h ($p<0{\cdot}025$) and 66 h ($p<0{\cdot}05$). The total viable count varied between replicate teeth, although there was a significant rise ($p<0{\cdot}02$) between 45 and 66 h (Fig. 2). '*S. mitior*' counts were similar at all three

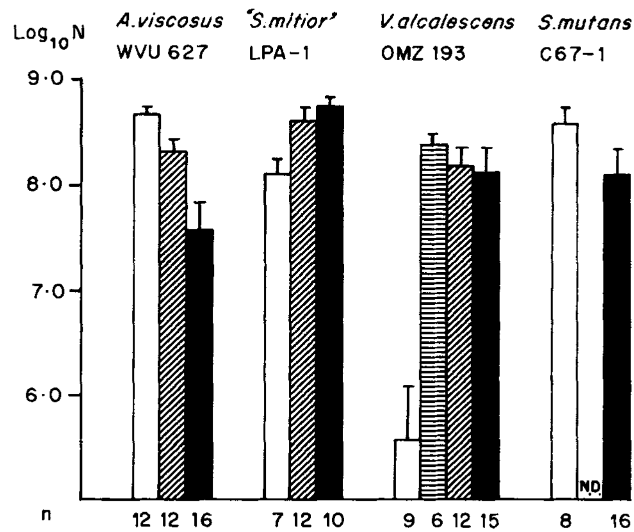

Figure 1. Mean $\log_{10}$ selective viable counts (+SEM) of bacteria cultured for 66 hours in pure or mixed culture on teeth in a model mouth
□ Pure culture
▤ Pure culture with 1 per cent glucose replaced by 1 per cent lactate
▨ 3-species mixed culture of *Actinomyces viscosus* WVU627, '*Streptococcus mitior*' LPA-1 and *Veillonella alcalescens* OMZ193
■ 4-species mixed culture including *Streptococcus mutans* C67-1
N.D. Not Done
n = number of replicate teeth

sampling times. However, both *A. viscosus* and *V. alcalescens* increased in numbers significantly between 45 and 90 h ($p<0{\cdot}005$). Proportions of each species varied considerably between teeth (Table 1) although the same trend was seen, of an increase in the proportion of actinomycetes and veillonellae with time, and a declining proportion of '*S. mitior*'.

S. mutans consistently colonised teeth when inoculated simultaneously with the three other species (Fig. 1), although lower numbers were recovered after 66 h compared with the pure culture ($p<0{\cdot}001$). Direct counts (Table 1) were greater in the four-species plaques ($p<0{\cdot}005$) but total viable counts and selective counts of '*S. mitior*' and *V. alcalescens* were similar to those obtained with the three-organism mixture. However, fewer viable actinomycetes were recovered from mixed plaques containing *S. mutans* ($p<0{\cdot}001$), and there was a dramatic drop in the proportion of *A. viscosus* in the four-species mixture with a concomitant rise in the proportion of streptococci (Table 2).

DISCUSSION

Laboratory bacterial strains were used in the present study because this enabled comparisons to be made with other studies in which they were used, and knowledge of their characteristics aided interpretation of the results. However, any conclusions need to be tested at some stage, using fresh isolates or mixed plaque, to ensure their general validity.

The pure culture experiments demonstrated that all four organisms were able to colonise the tooth segments, when provided with sufficient nutrients. *Veillonella alcalescens* is unable to utilise glucose, and did not colonise consistently unless a supplement of 1 per cent lactate was provided at regular intervals. Both *V. alcalescens* and *Streptococcus mutans* adhere poorly to salivary pellicle[19,24] and it is assumed that their accumulation in the model mouth was encouraged by the provision of a suitable retention site.

Comparison of the populations of individual species obtained in pure and mixed cultures enables the effect of any microbial interactions to be assessed. In the first instance, the establishment of a simple mixed plaque of three species was examined.

Work with chemostat cultures of a mixed oral flora has shown that *Actinomyces viscosus* requires mucin to maintain its population at slow growth rates.[16] In the present study, mucin was supplied

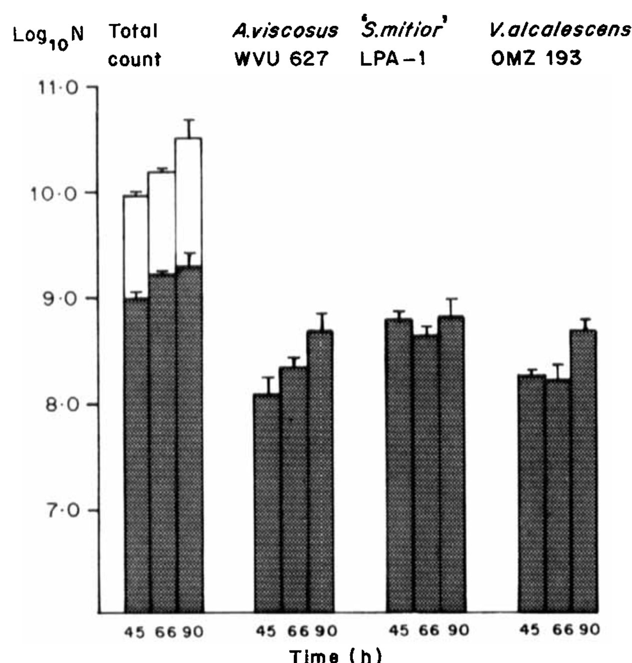

Figure 2. Mean $\log_{10}$ direct count, total viable count and selective viable count (+SEM) of *Actinomyces viscosus* WVU627, '*Streptococcus mitior*' LPA-1 and *Veillonella alcalescens* OMZ193, co-cultured for 45, 66 or 90 h on teeth in model mouth. Number of replicates is shown in Table 1

□ Direct CFU count

■ Viable count

Table 1. Mean $\log_{10}$ total direct and viable count (±SEM) of *Actinomyces viscosus* WVU627, '*Streptococcus mitior*' LPA-1 and *Veillonella alcalescens* OMZ193 grown in mixed culture alone (3-species mixture) or with *Streptoccocus mutans* C67-1 (4-species mixture), on teeth in a model mouth for 66 h

	3-Species mixture	4-Species mixture
Log_{10} direct count (±SEM)	10·17±0·06 *n=12	10·29±0·03 n=16
Log_{10} total viable count (±SEM)	9·21±0·05 n=6	8·99±0·18 n=10

*n = number of replicate teeth.

continuously, and the organisms were grown in a surface-associated microbial film, which has been shown to enhance bacterial metabolic rate[4] and may also thus encourage *A. viscosus* growth.[5] It was, therefore, not surprising that *A. viscosus* was readily recovered from all mixed plaques. The numbers and proportion of actinomycetes increased with incubation time, a finding which is reflected by studies *in vivo*.[2,33] The slow accumulation of actinomycetes *in vivo* appears to be due to their relatively slow doubling time compared with streptococci.[2]

'*S. mitior*' attained higher populations in mixed cultures, although its proportion declined with time. This agrees with *in vivo* observations,[33,39] although due to the complexity of the microbial flora and the oral environment, direct comparisons need to be made with caution. '*S. mitior*' produces neuraminidase and is positively selected by growth in saliva.[23] Therefore, the nutrient regime in the present study, consisting of 5 h intervals with saliva only, would be expected to encourage the growth of this organism. A consortium of oral organisms was more effective than pure '*S. mitior*' in utilising saliva,[23] which may be one of the reasons for the higher numbers of '*S. mitior*' obtained when it was co-cultured with actinomycetes and veillonellae in the present study.

Table 2. Median per cent (range) of *Actinomyces viscosus* WVU627, '*Streptoccocus mitior*' LPA-1 and *Veillonella alcalescens* OMZ193 co-cultured with or without *Streptoccocus mutans* C67-1 on teeth in a model mouth

	Incubation time (hours)			
	3-Species mixture			4-Species mixture
Bacterial species	45 *n = 5	66 n = 12	90 n = 6	66 n = 15
A. viscosus	11·9 (6·8–27·4)	22·9 (17·3–40·2)	34·3 (17·3–37·0)	3·9 (0·16–22·1)
'*S. mitior*'	62·5 (54·0–74·9)	46·1 (21·2–77·6)	41·1 (26·1–50·4)	46·9 (3·9–92·1)
V. alcalescens	21·8 (6·7–30·7)	26·9 (4·8–55·8)	34·3 (16·5–40·0)	15·0 (1·5–88·6)
S. mutans	—	—	—	16·2 (1·2–56·8)

*n = number of replicate teeth.

V. alcalescens grew as well in mixed cultures supplied with 1 per cent glucose as in pure culture with 1 per cent lactate, thus supporting the existence of a food chain.[12,30] Counts of veillonellae in mixed plaques were particularly variable, which was possibly due to their dependence on the prior growth and production of metabolites by the co-cultivated plaque species, as there was no added lactate. The establishment of a food chain would account for the observed increase in numbers and proportions of veillonellae in 90 h plaque. This phenomenon would also explain the prevalence of mixed colonies containing veillonellae recovered from teeth on BHI plates, and the higher *Veillonella* counts which were obtained from teeth when BHI agar rather than selective agar was used.

It has been suggested[27] that the ready utilisation of lactate by veillonellae enhances streptococcal growth by stimulating their glycolytic rate,[17] so this may be another reason why '*S. mitior*' grew better in mixed culture in the model mouth.

There have been several reports of co-aggregation occurring between streptococci and actinomycetes[6,10] or veillonellae[26]. However, there is no evidence to suggest that this phenomenon was of any long-term significance in the present investigation, as indicated by the comparable or greater counts of veillonellae and actinomycetes obtained after 66 h in pure culture, as opposed to the mixed cultures with streptococci.

An incubation time of 66 h was chosen for the majority of experiments with mixed plaques, as the total viable count showed no significant increase with further incubation to 90 h.

S. mutans colonised the teeth when inoculated together with the three other species in the model mouth. Present work is investigating the inhibition of *S. mutans* by a pre-existing plaque flora that has had sufficient time to become well-established, a phenomenon demonstrated originally in an animal model.[21,31]

It has been noted previously, using a different model mouth, that *A. viscosus* could not tolerate conditions in plaque when *S. mutans* was present,[1] although this was with a nutrient-rich regime that included sucrose. In the present study, comparison of the proportions of organisms in the 3- and 4-species mixtures indicates that *S. mutans* partially displaces *A. viscosus* in the plaque. A pilot experiment showed that when a broth culture of *S. mutans* C67-1 was spotted on to a lawn of *A. viscosus* WVU627, a large inhibition zone was produced (data not shown). This observation, together with the direct inhibition of *A. viscosus* by *S. mutans* seen on the BHI viable count plates in the present study, also suggests that such antagonism occurred on the teeth in the model mouth.

There are several possible explanations for the inhibition of *A. viscosus* in mixed culture, including: hydrogen peroxide produced by '*S.*

mitior'; inhibitory low pH due to streptococcal acid production; competition for substrates; bacteriocin production; or a combination of one or more of these factors.[13,40] *S. mutans* C67-1 is bacteriocinogenic and prevented the establishment of *A. viscosus* in the dental plaque of di-associated gnotobiotic rats although a non-bacteriocinogenic mutant was unable to do so.[34] *S. mutans* C67-1 was also able to retard the actinomycete growth rate on teeth in gnotobiotic rats.[2] Therefore, although it remains to be proved, this evidence, together with the observations from the present study, suggests strongly that bacteriocin production by this strain of *S. mutans* was a significant factor in the inhibition of *A. viscosus* on teeth in the model mouth.

The numbers and proportions of the different organisms from mixed plaques were much more variable when all four species were present, suggesting that the populations were less stable in the presence of *S. mutans*. Such variability has been observed in clinical studies,[18,39] suggesting that these relatively simple plaques formed in the model mouth may provide an ecosystem of sufficient complexity to provide an experimental analogue of dental plaque. This could be of use, not only in studies of plaque ecology, but also in preliminary investigations on possible anti-plaque or antibacterial agents.

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