INVESTIGATION OF CHANGES IN THE ORAL MICROFLORA OF CHILDREN WITH TIME AND THEIR POSSIBLE RELATIONSHIP TO CARIES

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Past study of the microbial aetiology of dental caries was often focussed on particular species. Most studies have been cross-sectional, either confined to saliva or plaque samples. In the latter, fissure plaque has been studied less than other types although fissures are the most common site for caries.

A study of fissure plaque and salivary microflora was conducted over a 12 month period in a group of 58 children aged 6-8 years. No statistically significant differences were seen in the micro-flora in relation to caries but some trends were apparent.

There was a consistent trend for the anaerobic count to be higher in plaque from carious sites and for *Actinomyces* to be detected in higher proportions from sites which remained caries-free.

The ratio of aerobic to anaerobic counts was higher in plaque from caries-free sites. In contrast, the ratio was less variable and showed little evidence of relationship to caries when saliva was studied.

A higher ratio of *S. mutans* to *Actinomyces* was found in plaque from carious sampling sites. The ratio was also higher in saliva samples of children who developed caries but on only two of the three sampling occasions.

The oral microflora is complex and includes a large number of interacting bacterial species. The two relationships studied in this investigation showed as much relationship to caries as did any one species or group. Results from saliva samples proved more difficult to interpret than did those from plaque and may have been influenced more by the nature of saliva.

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1. INTRODUCTION

It is accepted that oral micro-organisms are essential for the development of dental caries. However, no one species has been shown to be solely responsible for the disease. The oral microflora is complex and it seems likely that the inter-relationships and interactions of bacterial species may be at least as important as the presence and numbers of any one species or group.

Whilst the micro-organisms contributing to caries must be a part of the oral flora as a whole it would be anticipated that the caries process is most intimately related to the plaque immediately overlying it. Caries is site-specific and in permanent teeth the most common site of caries in children is the occlusal fissures of first molars. Despite this, fissure plaque would appear to have been studied less than that found on smooth tooth surfaces, both in relation to the differing species present and their inter-relationships. This may partly be a result of difficulty of sampling fissure plaque. The salivary flora is known to be contributed to by various oral compartments and although it could not directly reflect the changes occurring at a particular site, it may provide an overview of changes occurring in the oral cavity in relation to caries. However, relatively few studies have considered both plaque and saliva samples in the same subject making it difficult to discover the relationship between the two aspects of the oral ecosystem.

The aims of the present study were therefore:-

1. To investigate the bacterial ecology of fissure plaque and that of saliva in a group of children. In particular to consider inter-relationships occurring between species in these samples.

2. To relate plaque flora to caries developing at the site sampled during the 12 month period and to relate salivary flora to caries developing in the mouth as a whole during the study period.

3. To investigate the inter-relationships between plaque microflora and that of saliva.

2. REVIEW OF THE LITERATURE

2.1 THE ORAL FLORA AND CARIES

Current belief holds dental caries to be a disease caused by acid attack on the hard tissues of the tooth. The acid responsible is produced through fermentation of dietary carbohydrates by oral bacteria (Miller, 1890). Miller believed that dental caries was not caused by a single species of micro-organisms but was related to the activity of many micro-organisms in a process involving both acid and protein degradation. Studies in experimental animals have since confirmed the essential role of bacteria in dental caries (Orland *et al.*, 1954; 1955). In a classical series of experiments it was found that germ-free rats of a susceptible strain did not develop caries even when they were fed on a diet rich in fermentable carbohydrates (Orland *et al.*, 1954). However, if these same animals were infected with enterococci (*Streptococcus faecalis*) together with a proteolytic anaerobic rod or an anaerobic pleomorphic rod caries developed (Orland *et al.*, 1955). Studies with experimental animals have shown the transmissible nature of dental caries (Keyes, 1960; Fitzgerald and Keyes, 1960). Keyes (1960) reported that caries-resistant Syrian albino hamsters (where penicillin-sensitive flora had been depressed prior to feeding the test diet) failed to develop caries even when maintained on a high sucrose diet. Caries developed in these animals if they were caged with caries-active hamsters or if they were orally inoculated with the faecal flora from this second group of animals.

2.1.1 Micro-organisms thought to be related to caries

The species of bacteria thought to be related to caries have been studied in both experimental animals and in man. Experimental caries studies with conventional hamsters and primates have shown that a wide

variety of bacterial species are able to induce caries in these animals. Species include lactobacilli, including *Lactobacillus fermentum*, *Lactobacillus salivarius* and streptococci, including *S. mutans*, *S. salivarius*, *S. sanguis* and *Actinomyces viscosus* (Thylstrup and Fejerskov, 1986).

Species found to induce caries in primates include *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus fermenti* and differing species of streptococci especially *S. mutans* (Bowen, 1965; 1969; Caldwell *et al.*, 1977; Colman and Hayday, 1980).

In a study of the relative caries-inducing ability of streptococci, Krasse and Carlsson (1970) tested a total of 24 strains of streptococci. It was reported that all strains of *S. mutans* produced highly significant amounts of caries whilst none of the other species investigated (*S. sanguis*, *S. mitior* and *S. bovis*) produced caries at all. However, it is of note that these latter three species were not successfully implanted in the mouth during this experiment.

In at least one study, strains of *S. milleri* have been reported to be cariogenic in gnotobiotic rats (Drucker and Green, 1978; 1979).

In human caries, the capacity of a large proportion of the organisms present in the oral cavity to produce acid from dietary carbohydrates is believed to be the single most important factor in the pathogenesis of dental caries. Acidogenic bacteria are those bacteria in plaque which can metabolise sugars and carbohydrates to acids. It has been thought that the acidogenic bacteria are responsible for caries as a whole rather than as a single group or species (Thylstrup and Fejerskov, 1986). These bacteria are found to be present in plaque of

caries-active, caries-inactive and caries-free individuals. In human studies, the numbers of acidogenic organisms were found to be larger in dental plaque from persons with high caries activity when compared with dental plaque from persons with low caries activity (Handelman *et al.*, 1968).

The production of acid will alter the bacterial environment and reduce pH. At the critical pH value at which significant amounts of enamel are considered to dissolve (pH 5.4-4.4), only a few bacterial species are still able to survive and to continue to produce acids. These bacteria are known as aciduric bacteria and it is these species which are believed to be especially important in the pathogenesis of caries. The acidogenic bacteria which are also aciduric include both lactobacilli and streptococci. However, the precise role which these species play in human caries is still not clear (Burnett and Scherp, 1968; Scherp, 1971).

In relation to lactobacilli for example, it has also been found that these contribute a minor fraction of the plaque flora (Gibbons, 1964) and that the amount of acid formed by this small number of lactobacilli present in plaque was insignificant compared to the amount produced by other acidogenic organisms (Stralfors, 1950). It is now thought that lactobacilli may play a more extensive role in the progression than in the initiation of the lesion (Duchin and Van Houte, 1978; Van Houte *et al.*, 1982).

Streptococci are the predominant organisms found in dental plaque from all tooth surfaces. Species of streptococci of human origin have been reported to produce caries in gnotobiotic animals (Zinner *et al.*,

1965; 1966; Gibbons *et al.*, 1966; Krasse, 1966; Jablon and Zinner, 1966; Edwardsson, 1968; Guggenheim, 1968). Among the streptococcal species, *S. mutans* is thought to be the most important organism related to caries aetiology in humans (Klock and Krasse, 1977; 1978; Togelius and Brathall, 1982; Carlsson *et al.*, 1985). However, despite a great amount of investigation the relationship between caries and *S. mutans* is not wholly understood. This organism is not invariably isolated from individual surfaces prior to caries initiation and caries has occurred in some subjects in whom *S. mutans* could not be detected (Ikeda, Sandham and Bradley, 1973; Mikkelsen and Poulsen, 1976; Swenson *et al.*, 1976).

In one study in humans (Shiera *et al.*, 1951) a relationship of *S. salivarius* to caries was demonstrated and more recently there has been further interest in this species. It has been thought that *S. salivarius* is unable to colonise tooth surfaces but Drucker *et al.* (1984) reported that *S. salivarius* resembled *S. mutans* both in its potential ability to form plaque *in vivo* in rats and to induce fissure caries. Acid production by *S. salivarius* under specific conditions was said to be greater than that of *S. mutans* (Marsh *et al.*, 1984). This finding was in contrast to a general belief that acid accumulation by *S. mutans* is greater than any other oral streptococci. Despite this apparent potential, in most studies *S. salivarius* has been found to constitute only a minor fraction (some 0.4-0.77 per cent) of the mature microbiota of dental plaque (Bowden *et al.*, 1976; Thomson *et al.*, 1980). Few epidemiological studies have been carried out into the prevalence and numbers of *S. salivarius* so that the possibility of this species having a significant role cannot yet be conclusively rejected.

Actinomyces species have been reported to be found in plaque over carious lesions and in necrotic carious dentine (Loesche and Syed,

1973). Although they are numerically dominant among the plaque micro-organisms, the association of *Actinomyces* with enamel caries has not yet been proved. Studies by Hardie *et al.* (1977) and Minah *et al.* (1977) did not support the suggestion that these organisms play a major role in the initiation of caries. Species found in the human mouth are *A. israeli*, *A. naeslundii*, *A. viscosus* and *A. odontolyticus*. Among these, *A. viscosus* has been shown to be related to root caries as well as periodontal disease in laboratory rodents (Jordan, 1976; Jordan and Hammond, 1972), and to be numerically associated with established root lesions in humans both *in vivo* (Syed *et al.*, 1975) and in extracted teeth *in vitro* (Sumney and Jordan, 1974). Despite the vast numbers of studies done in experimental animals as well as in humans, it could not be concluded that a particular group of micro-organisms is solely responsible for dental caries. Most of the studies carried out have been cross-sectional in nature and as Silverstone *et al.* (1981) have pointed out, such studies may show an association between the disease and a particular micro-organism but are not capable of demonstrating a cause and effect relation. Longitudinal investigations are therefore essential in this context.

2.1.2 The normal oral flora

Over 325 species of micro-organisms have been isolated from the oral cavity (Moore, 1987). The principal groups of micro-organisms found in studies of plaque and salivary flora include Gram-positive cocci, Gram-positive rods and filaments, Gram-negative cocci, Gram-negative rods and other micro-organisms. A brief summary of the major groups of organisms is given in Table 1.

Table 1 Principal groups of micro-organisms found in the mouth

G (+) cocci	G (-) cocci	G (+) rods and filaments	G (-) rods and filaments	Others
Streptococci	Veillonella	Actinomyces	Pseudomonas	Spirochates
Staphylococci	Neisseria	Lactobacilli	Haemophilus	Yeasts
		Diphtheroids	Fusobacterium	Viruses
			Bacteroides	

(from Silverstone et al., 1981)

Gram-positive cocci

Streptococci are the predominant Gram-positive cocci found in dental plaque from all tooth surfaces. The prevalent species of streptococci isolated from the mouth include *S. mutans*, *S. sanguis*, *S. mitior*, *S. milleri* and *S. salivarius*.

Staphylococci and micrococci are not oral commensals and they are not commonly isolated in large numbers from the oral cavity.

Gram-negative cocci

Veillonella and *Neisseria* species are included in this second major category. *Veillonella* are strict anaerobes and can be separated into two species, *V. parvula* and *V. alcalescens* which differ in the ability of *V. alcalescens* to break down hydrogen peroxide.

Neisseria are isolated in low numbers from most sites in the oral cavity. Together with *S. sanguis*, they are among the earliest colonisers of clean tooth surfaces (Marsh and Martin, 1984).

Gram-positive rods and filaments

Lactobacilli are Gram-positive facultatively anaerobic rods which are both acidogenic and aciduric (acid-tolerant). They are usually recovered in low numbers in dental plaque (0-4.2 per cent of the total viable count) (Hardie and Bowden, 1974). On the other hand, these organisms are present as the predominant microflora in the depths of advancing dentinal lesions (Fitzgerald *et al.*, 1980).

The bacterial flora of the mouth also include large numbers of pleomorphic Gram-positive rods and filaments. Many of the Gram-positive rods isolated from the mouth were found to be *Actinomyces* species.

Gram-negative rods and filaments

Haemophilus (aerobic and facultatively anaerobic Gram-negative rods) are frequently present in saliva and dental plaque. *Pseudomonas* species, mainly *Ps. aeruginosa* have also been found to be present in saliva. Anaerobes such as *Fusobacterium* and *Bacteroides* species could also be isolated from dental plaque especially from the gingival crevice region.

Other micro-organisms

Other organisms such as yeasts (especially *Candida albicans*), Mycoplasmas (pleuropneumonia-like organisms) and Spirochaetes are also common oral inhabitants.

Apart from *Herpes simplex* virus, most viruses are regarded as transient members of the normal oral flora.

2.2 ECOLOGICAL SITUATIONS IN THE ORAL CAVITY

The many bacterial species found in the oral cavity differ in their growth and nutritional requirements and are suited to differing environments. The oral cavity presents a series of different environments, each of which may be suitable for colonisation by different micro-organisms (Hardie and Bowden, 1974). The study of relationships of organisms to their environment and to each other is commonly termed ecology (Brock, 1966). In the case of the oral cavity, interaction between the oral flora and its host constitutes an ecosystem. Thus the bacteria of the human oral cavity and the tissues on which they grow are inter-related and have been defined as constituting the essential components of the oral ecosystem (Kleinberg, 1977).

2.2.1 The oral ecosystem

The components of the oral ecosystem, as described by Kleinberg, are shown in Figure 1. Bacteria from the external environment after entering the mouth become part of the salivary compartment. These bacteria may either attach to the oral surfaces (soft, hard or oral appliance compartments) or leave the oral cavity with the saliva that is swallowed. In the scheme, the soft, hard or oral appliance compartments are termed the peripheral compartments. The soft tissue compartment includes organs and tissues such as the tongue, cheek, palate and gingiva which have in common a renewable surface consisting of epithelial cells that are continually replaced and shed.

In the case of the other two peripheral compartments (hard tissue and oral appliance compartments) bacteria grow on non-renewable surfaces. The appliance compartment is also distinct from the hard tissue compartment because the devices involved (such as dentures or orthodontic bands) not only provide surfaces different in composition from that of the enamel but are also present in the mouth only on an interrupted basis.

The gastro-intestinal tract may be regarded as a system sink into which salivary bacteria can drain. A constant interchange occurs between peripheral compartments and the salivary compartment. Most of the bacteria in saliva come from the microbial masses that continually grow on the hard and soft tissues of the mouth and any intra-oral appliances that may be present. Only a small proportion are newly arrived from the external environment.

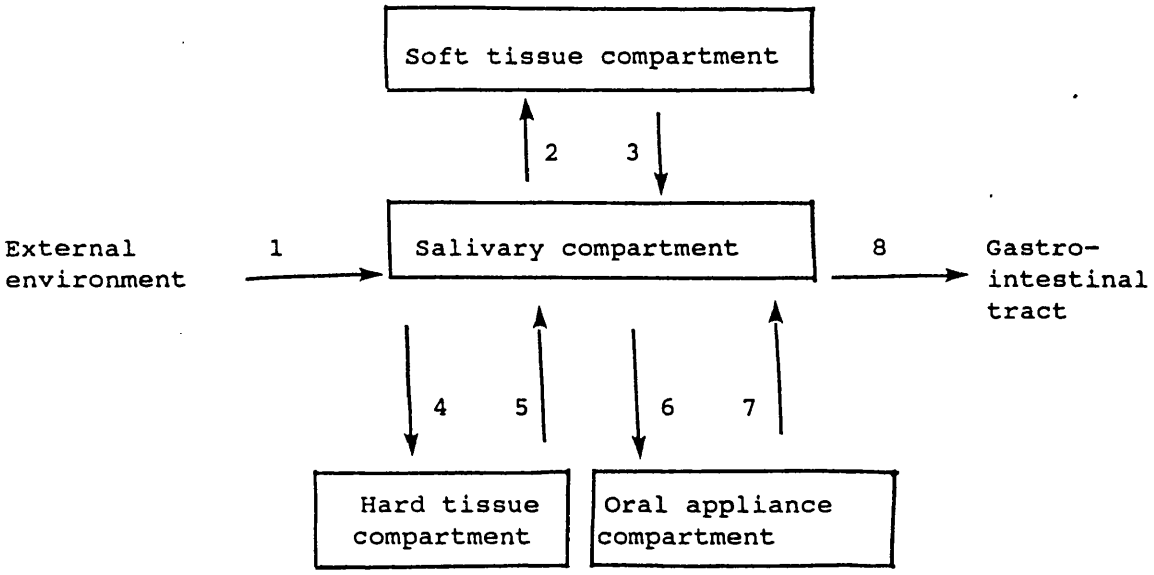


Figure 1 Compartments of the oral ecosystem.

Steps 2, 4 and 6 involve attachment of salivary bacteria to the teeth, the soft tissues and dental appliances respectively. Steps 3, 5 and 7 involve dislodgement or desquamation of bacteria from the three peripheral compartments. Except during the establishment of the resident flora following birth, the number of bacteria that attach to the tissues by steps 3, 5 and 7 is less than those lost by steps 2, 4 and 6. The differences are made up by the growth of bacteria on the peripheral compartment surfaces. The nutrients essential for this growth are derived mainly from the diet, the oral fluids (saliva and gingival crevicular fluid) and cells and cellular debris of mammalian and bacterial origin.

(from Kleinberg, 1977).

This description of the oral ecosystem may represent an oversimplification since each of Kleinberg's "compartments" may contain a wide variety of smaller environments and ecosystems. In the case of hard tooth surfaces, for example, systems and flora differ between approximal, buccal, lingual and occlusal surfaces. Even this may be an imprecise definition of differing ecosystems. For example, in relation to fissured surfaces, large individual variations in the microbiota of different fissures have been shown from studies of fissure plaque. Even samples from fissures implanted in the same tooth in the same subject showed considerable variations in the relative composition of the cultivable microbiota (Theilade *et al.*, 1974). In this study, five subjects had two consecutive fissures implanted in the same tooth. It was shown that flora in the pairs of fissures differed very widely as did the flora of the five study subjects.

2.2.2 Development of the oral ecosystem

The way in which the oral ecosystem develops and the oral flora becomes established may be seen in studies of the oral microflora in young children and also in the short-term in studies of plaque formation. The first micro-organisms to colonise are termed pioneer species and they collectively make up the pioneer microbial community (Marsh and Martin, 1984). One genus of species is usually dominant during the development of the pioneer community (Hobson, 1969).

Development of the oral microflora in young children

An illustration of the developing flora in infants was seen in the study reported by McCarthy *et al.* (1965) who investigated the development of oral flora in 51 newborn and 44 one month old infants.

Further specimens were collected from the one month old infants at eight and 12 months. Streptococci were found to be numerically dominant throughout the study period (although the percentage fell from 98 to 70 per cent of the cultivable flora by the end of the first year of life), perhaps suggesting their role as pioneer species. *S. salivarius* was especially common in the mouths of newborns but became less so with increasing age.

It was suggested that the metabolic activities of the initial bacterial population alter their environment and influence the subsequent flora (Hobson, 1969). The importance of streptococci may again be recognised since many streptococcal species including *S. salivarius* are capable of metabolising carbohydrate with the formation of lactic acid. This in turn could favour the emergence of such species as *Veillonella* which could utilise lactic acid as a carbon source but are unable to use glucose (Mikx *et al.*, 1972). *Veillonella* was reported in the study of McCarthy *et al.* (1965) in all subjects by the age of 12 months but in only one out of 51 newborns studied. Pioneer species continue to grow and colonise until environmental resistance is encountered. Both chemical and physical factors may act as limiting barriers. In the oral cavity, physical factors include the shedding of epithelial cells and shear forces from chewing and saliva flow. All of these will act to remove bacteria that are not firmly adherent to tooth or soft tissue surfaces. Nutrient requirements, Eh (Redox potential), pH and antibacterial properties of saliva can all act as chemical barriers limiting growth (Marsh and Martin, 1984). All of these barriers may be especially effective in the newborn child since only a few of the oral micro-organisms and even fewer of the large

numbers of bacteria found in the environment are able to establish successfully in these young infants (Socransky and Manganiello, 1971).

Although the eruption of teeth was seen to have a marked effect on indigenous flora, it was not necessary for the establishment of some species. Thus the presence of teeth did not appear to be essential for the establishment of filamentous bacteria since examples of *Fusobacterium*, *Leptotrichia*, *Actinomyces* and *Nocardia* (Rothia) species were all isolated occasionally from predentate infants. However, the frequency of isolation of *Fusobacterium* and *Actinomyces* species increased after the eruption of teeth (Hardie and Bowden, 1974).

The presence of teeth has been shown to be more crucial for the establishment of *S. mutans* and *S. sanguis* in the mouths of infants (Carlsson *et al.*, 1970). Carlsson *et al.* reported that in their study (which involved 27 infants during their first years of life) *S. sanguis* became established in all these infants only some three months after eruption of teeth.

The increase in the number of anaerobes once teeth are present is an example of autogenic succession in which community development is influenced by microbial factors. The metabolism of the aerobic and facultatively anaerobic pioneer species lowers the Eh (redox potential) in plaque and creates conditions suitable for colonisation by strict anaerobes such as *Fusobacterium*, *Veillonella*, *Actinomyces* and *Bacteroides*. Once teeth have erupted, the climax community in children is similar to that in adults except that numbers of *Bacteroides melaninogenicus* and oral spirochaetes increase during adolescence (Marsh and Martin, 1984).

Development of the oral ecosystem during plaque formation

The development of the oral flora with establishment of pioneer species and subsequent colonisation is not only observable in the long term in the development of the oral flora from birth onwards but also in the short term in relation to plaque formation on a previously clean tooth surface. Thus, in a developing coronal plaque over a nine day period there is broadly a progressive shift from mainly aerobic and facultatively anaerobic species in the early stages to a situation where facultatively anaerobic and anaerobic organisms become dominant (Ritz, 1967). Streptococci are again an important pioneer species with *Neisseria* and *Nocardia* also having an important role in the early stages. After nine days, streptococci, *Actinomyces*, *Veillonella* and *Corynebacterium* were predominant. Results of this study indicated that growth of anaerobic organisms such as *Veillonella* and fusobacteria was dependent upon prior growth of aerobic and facultative organisms. As the plaque thickness increased with age, conditions became more suitable for anaerobic organisms. Thus, autogenic succession is seen in both plaque development and in development of the oral flora in infants.

In the case of plaque development, bacterial changes (growth) reduce the availability of oxygen at the plaque/enamel interface and thus alter the predominant flora at this point. From the studies of the developing oral flora, it may be concluded that growth and activity of some bacterial species are influenced by the presence and activities of other species. In this context it should be remembered that in the series of experiments which confirmed the importance of bacteria in caries aetiology, Orland *et al.* (1955) had utilised more than one species at once to induce caries. Inter-relationships between species

have been less widely studied than the presence/absence and numbers of selected species. Nevertheless, a few inter-relationships have been investigated in relation to caries aetiology.

2.2.3 Bacterial inter-relationships and caries

The ability of micro-organisms to induce carious lesions could be altered by numerous microbial interactions which take place within the complex bacterial community on the tooth. Theilade *et al.* (1974) for example found that the relative proportions of different micro-organisms in occlusal fissures seemed to be more crucial than their absolute numbers in caries aetiology. In this study, crowns of extracted third molars were held in the oral cavity by an intra-oral appliance and exposed to the oral environment for one to 21 days (nine plaque growth periods). Changes in the fissure plaque flora were found as the plaque increases in age. There was a decrease in the recovery of Gram-positive cocci from 47 per cent to 13 per cent while Gram-negative cocci remained relatively constant over time (20 per cent). The Gram-positive rods and the pleomorphic rods increased from 30 per cent to 54 per cent with plaque age while the Gram-negative rods stayed much the same (4 per cent) over the study period. A statistically significant correlation ($p<0.005$) was found between decreasing and increasing percentages of Gram-positive cocci and Gram-positive rods respectively with increasing plaque age. On the other hand, the recovery of *S. mutans* was largely variable between subjects from sample to sample and no statistically significant correlation could be observed between the total number of streptococci recovered and the percentage distribution of *S. mutans* with increasing plaque age (Theilade *et al.*, 1974).

Although there have been fewer studies of such relationships than of the numbers of individual species, some work has been carried out into this subject in both animals and man. These studies can be broadly considered in terms of:-

Relationships between *Veillonella* and *S. mutans*.

The effect of the presence of *S. mutans* and *S. sobrinus* together.

Possible interactions between *Actinomyces viscosus*, *S. mutans* and *S. sanguis*.

Influence of *S. salivarius* on *S. mutans*.

Relationship of *S. sanguis* to *S. mutans*.

Relationships between *Veillonella* and *S. mutans*

It has been postulated that the cariogenic potential of *S. mutans* may be modified by the presence of *Veillonella* species (Mikx *et al.*, 1972). At least six studies have investigated this relationship in animals and in man. In one study it was shown that caries activity (as expressed by the number of fissure lesions) in rats with *Veillonella* and streptococci was lower than in rats mono-infected with streptococci. It was thought that *Veillonella* could have exerted an inhibitory effect on the metabolism of streptococci, mediated through its ability to convert the lactic acid (produced by *S. mutans*) into weaker acids such as acetic acid and propionic acid. As some support for the concept of a relationship between *Veillonella* and lactic acid, it had already been observed that *Veillonella* strains increased in numbers in dental plaque after lactic acid-producing organisms have first colonised (Ritz, 1967).

In a third study (Mikx *et al.*, 1976), it was noted that there was a significant reduction in dentinal tissue lesions in rats di-associated with *S. mutans* and *V. alcalescens* on a highly cariogenic diet when compared with rats mono-associated with *S. mutans* and fed in the same way (Van der Hoeven *et al.*, 1978).

The symbiotic relationship of *Veillonella alcalescens* and *S. mutans* in dental plaque in gnotobiotic rats formed the basis of a fourth study. In this study, two strains of *S. mutans* were used; strain C67-1 described by de Stoppelaar *et al.* (1971) and FIL, described by Yamada *et al.* (1976). It was found that the proportion of *Veillonella* was higher in combination with FIL than with C67-1. *S. mutans* FIL was also found to be capable of greater lactic acid production than was C67-1. Furthermore, a significant caries reduction was observed in the rats harbouring *S. mutans* FIL and *Veillonella* at the same time compared to those with *S. mutans* C67-1 and *Veillonella*.

All of these studies in experimental animals, therefore suggest that the presence of *Veillonella* in combination with *S. mutans* could reduce the cariogenicity of *S. mutans* by utilising lactic acid.

In man, in sequential samples taken from distal surfaces of upper first premolars in nine 13-14 year old children, low levels of *Veillonella* were detected before carious lesions were seen. On the other hand, the mean percentage levels of *S. mutans* were found to be higher in lesion sites than the opposing caries-free sites in these children (Bowden *et al.*, 1976). It was suggested that this low level of *Veillonella* may have indicated a reduced ability of the plaque to cope with lactic acid produced by other species such as *S. mutans*. However,

this postulation may not be borne out by results of all studies in humans.

Milnes and Bowden (1985) carried out longitudinal microbiological examinations of dental plaque from children who developed nursing caries. Nine children were included in the study and five of these developed carious lesions while four remained caries-free. The results from this study showed that there were significant differences between the levels of *S. mutans*, lactobacilli and *Veillonella* at the sites which developed caries and at the control sites in the same mouths of caries-active children. Increases in the levels of *S. mutans* and lactobacilli could be seen associated with the white spot lesions and cavities. In addition, significantly higher levels of *Veillonella* were found on the surfaces believed to be susceptible to nursing caries when compared to the sites believed to be resistant. However, it was also found that there was no significant difference in the number of *Veillonella* at susceptible sites which developed lesions or susceptible sites that remained caries-free in the group of caries-active children. In other words, although *Veillonella* were found in significantly higher amounts on the susceptible surfaces when compared to the control sites, this finding was independent of the development of a lesion. *S. mutans* levels were also found to be high at these susceptible sites which developed a lesion but the same high levels were increasingly recovered in the absence of lesion formation at susceptible sites. These findings provide little evidence to support the idea that *Veillonella* modified the caries attack in these children. It was concluded that high levels of *Veillonella* may simply reflect an environment high in lactic acid rather than consuming lactate (Milnes and Bowden, 1985). However, it

should be noted that although the study was longitudinal in nature, only nine children were studied. Both this investigation and the one reported by Bowden *et al.* (1976) placed more emphasis on the individual species rather than to the relative proportions of organisms and to how these changed with time making it difficult to draw conclusions about the exact influence of the inter-relationships.

The effect of the presence of *S. mutans* and *S. sobrinus* together

In a recent study in a group of Icelandic children (aged 11 to 12 years) it was reported that 60 children who harboured both *S. mutans* serotypes c/e/f and *S. sobrinus* (previously known as *S. mutans* serotype d/g) had a significantly higher caries prevalence and higher Lactobacillus count counts than did 139 children who harboured *S. mutans* (serotypes c/e/f) alone.

Both groups of strains are prevalent in humans and have differing properties which may be important in human caries. Of the two however, *S. mutans* strains are able to continue fermentation of carbohydrates at a lower pH than *S. sobrinus* and is also able to synthesise intracellular polysaccharides. It is thought that both of these properties are crucial in caries aetiology (Kohler and Bjarnason, 1987). The authors pointed out that the presence of *S. sobrinus* could possibly be a consequence of high sucrose consumption in these children rather than their acting directly to potentiate the effect of *S. mutans*. This finding has also been noted in another human study (Thomson *et al.*, 1980) as well as in rats (Huisin't Veld *et al.*, 1982).

Possible interactions between *Actinomyces viscosus*, *S. mutans* and *S. sanguis*

In animal experiments it was found that extensive plaque formation and a higher caries rate resulted when gnotobiotic rats were inoculated with *A. viscosus* and *S. mutans* together than when they were inoculated separately (Edwardsson, 1986). The balance between species may be complex since, when *A. viscosus* and *S. sanguis* were introduced in the oral cavity of specific pathogen-free (SPF) rats and allowed to form stable communities on the teeth it was difficult for *S. mutans* to establish itself (Mikx *et al.*, 1976). It was also noted that pre-inoculation of *A. viscosus* and *S. mutans* also reduced the subsequent proportions of *S. sanguis*. Thus, it may be concluded that the establishment and proportions of specific micro-organisms in the microbial communities on the teeth are affected by the sequence of introduction of micro-organisms into the oral cavity. The effect was apparently complex since it had been demonstrated in a previous study that pre-inoculation of either of these two species alone failed to have an inhibitory effect on the establishment of *S. mutans* in SPF rats (Mikx *et al.*, 1975) or in germ-free rats (Van der Hoeven *et al.*, 1974).

Influence of *S. salivarius* on *S. mutans*

The influence of *S. salivarius* on *S. mutans* in caries aetiology formed the subject of three consecutive studies on the inhibition of caries by means of bacterial competition within the plaque of rats (Tanzer *et al.*, 1982; 1985a and b).

In the initial study on the competition between a rough non-cariogenic *S. salivarius*-like strain (subsequently known as *S. salivarius* TOVE-R) and *S. mutans* in plaque, it was found that

S. salivarius TOVE-R was able to pre-empt initial colonisation of teeth by *S. mutans*. In a subsequent study, the same authors demonstrated the ability of *S. salivarius* TOVE-R to compete with *S. mutans* for tooth surface sites resulting in a concurrent decline in *S. mutans* on teeth. There was, in addition, an associated caries inhibitory effect in the experimental animals.

In the third study (Tanzer *et al.*, 1985b) it was confirmed that initial oral colonisation of rats by *S. salivarius* TOVE-R inhibited the ecological emergence of *S. mutans* which again was found to be associated with a reduction in caries. Again, this demonstrates the importance of initial colonisation in determining the final indigenous oral flora.

Relationship of *S. sanguis* to *S. mutans*

In in vitro investigations it has been reported that a strain of *S. sanguis* was able to grow in a medium free from p-aminobenzoic acid (B48) while a strain of *S. mutans* was unable to do so (Carlsson, 1971). In this study it was found that on incubation of a mixture of *S. mutans* and *S. sanguis*, *S. mutans* became the predominant organism in p-aminobenzoic acid-free medium. This was reported to occur as a result of the growth of *S. mutans* being supported by the presence of *S. sanguis*, but in the following year Holmberg and Hollander (1972) reported that *S. sanguis* inhibited rather than supported the growth of *S. mutans*. In a series of assays of inhibitory actions, both solid media (modified NAYS agar medium) and liquid media (modified NAYS broth or Trypticase soy broth, TSB) were utilised to culture the organisms. This was in contrast to the study of Carlsson (1971) where a single growth medium (B48, a p-aminobenzoic-free medium) was used.

In experimental animal work when *S. mutans* and *S. sanguis* were inoculated into rats with an intact oral flora it was noted that the final content of *S. sanguis* in plaque tended to be inversely proportional to content of *S. mutans* (Huxley, 1973).

An inverse relationship between the occurrence of *S. mutans* and *S. sanguis* has also been reported in a small study in humans (De Stoppelaar *et al.*, 1970). During a carbohydrate-free period of 17 days, the percentage of *S. mutans* in total cultivable flora decreased to a very low or undetectable level while simultaneously the percentage of *S. sanguis* increased.

In human studies, the salivary concentration of *S. mutans* and *S. sanguis* were found to be crucial for their establishment in artificial fissures. Svanberg and Loesche (1977) discovered that experimental reduction of salivary concentrations of *S. mutans* around the time of insertion of artificial tissues prevented the colonisation of *S. mutans* in these fissures even though the salivary concentrations were allowed to increase subsequently. Once *S. mutans* was established however, experimental reduction in saliva did not influence the proportional distribution of *S. mutans* in the fissures. The same broad pattern was observed for *S. sanguis*. Pre-inoculation with *Actinomyces viscosus* and *S. mutans* reduced the subsequent proportions of *S. sanguis* again (Mikx *et al.*, 1976).

These findings indicate that just as in the studies of *Actinomyces viscosus* discussed earlier, the initial inoculum may be the main determinant for fissure colonisation in man. From the results of the studies carried out in experimental animals and in man it has been

anticipated that numerous interactions could take place within the plaque ecology which could modify caries activity. However, in relation to caries, relationships between bacterial species and groups are likely to be at least as complex and variable as the presence and number of any one species. A majority of the studies carried out in the past have investigated the role of particular groups of micro-organisms thought to be related to caries rather than to the changes in proportions of these organisms that could have occurred with the development of caries. Perhaps partly because longitudinal studies may be time-consuming, administratively difficult and more expensive, cross-sectional studies have been carried out more often. Nevertheless, longitudinal studies would seem essential if changes in plaque are to be related to caries occurrence.

2.3 SITE SPECIFICITY OF CARIES

Caries does not occur randomly on the tooth surface but has been well recognised to have a predilection for specific sites (Kidd and Joyston-Bechal, 1987). The most common site for caries to occur is in the pits and fissures of the tooth surface (Dummer *et al.*, 1988; Rock and Kidd, 1988). The next most commonly affected areas are the approximal surfaces (where carious attack occurs cervical to the contact area). Smooth tooth surfaces, i.e. buccal, labial and lingual surfaces, are the least commonly affected. When they do become carious, lesions most often affect that part of the surface adjacent to the gingival margin (Shafer, 1983; Kidd and Joyston-Bechal, 1987).

It is believed to be no coincidence that these sites are the ones thought to favour the undisturbed growth and accumulation of micro-

organisms in the form of plaque. Plaque normally collects most rapidly and extensively in inaccessible areas of the mouth, particularly interproximally and in the pits and fissures. These areas have been referred to as stagnation areas or uncleanable areas (Silverstone *et al.*, 1981).

2.3.1 Caries in pits and fissures

Different authors have different opinions in considering whether fissures are normal areas, developmental faults or the space between abutting enamel coverings (Kronfield, 1935; Brucker, 1944; Gillings and Buonocore, 1961). Fissures can be extremely diverse in shape and have been described as broad or narrow funnels, constricted hourglasses, multiple invaginations with inverted Y-shaped divisions and irregularly shaped (Newbrun, 1989).

Based on their morphology, the fissures of human teeth have been categorised into five different types (Nagano, 1961). These are illustrated in Figure 2. V-types are described as those which were wider at the top and gradually narrowed towards the bottom forming 34 per cent of the fissures studied by Nagano. IK type fissures, which have an extremely narrow slit associated with a larger space at the bottom formed 26 per cent of those investigated. Nineteen per cent of the fissures had an extremely narrow slit and were categorised as I type. Those fissures which had almost the same width from top to bottom (14 per cent of the fissures) were classified as U type. Other more rarely seen types constituted 6 per cent of the fissures studied.

The high caries susceptibility of this area was said to be directly related to the morphology of the pits and fissures (Newbrun,

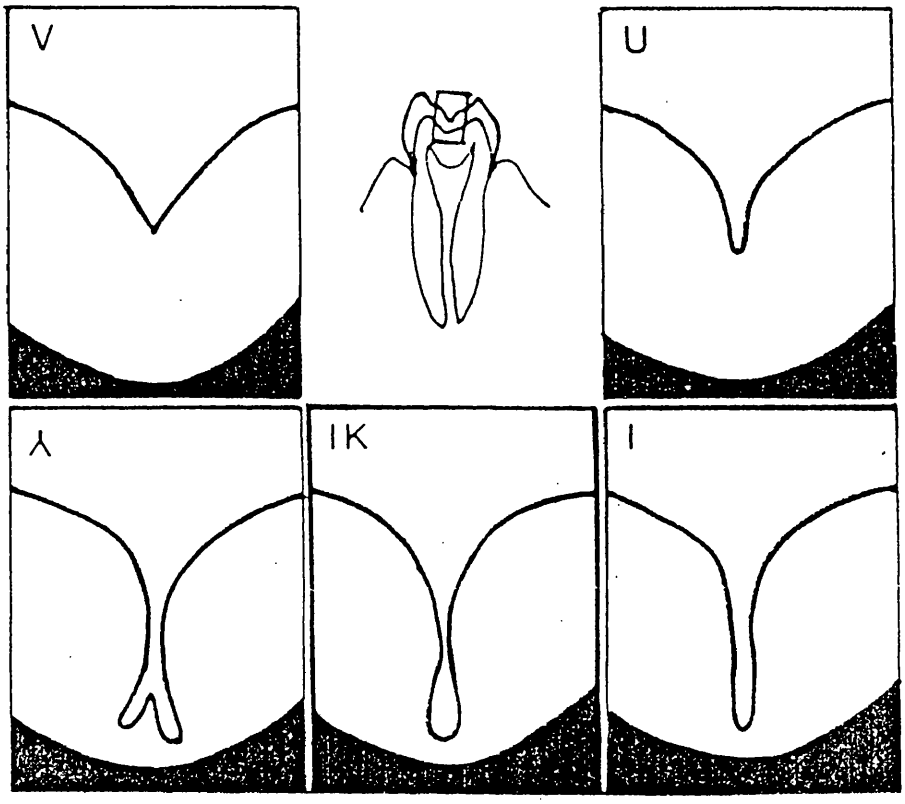


Figure 2 Diagram of various morphological types of occlusal fissures. V, wide at top and gradually narrowing toward the bottom; U, almost same width from top to bottom; ⅄, inverted Y, bifurcating at bottom; IK, hour-glass, extremely narrow slit associated with a large space at the bottom; I, extremely narrow slit.
(from Newbrun, 1989)

1989; Shafer, 1983). The relationship between fissure morphology and sites of early caries development has been studied by a number of authors (Bossert, 1933; Nagano, 1961; Konig, 1963; Mortimer, 1964; Awazawa, 1969; Zuhrt and Vierus, 1967; Juhl, 1983).

Bossert (1933) reported that the steeper the sides of the valley, the greater the likelihood of caries in the pit. Konig (1963) too described a higher susceptibility to caries in deep and narrow fissures (teeth with grooves formed by angles smaller than approximately 70^{o}). In contrast to these authors, Zuhrt and Vierus (1967) found that wide and U-shaped fissures were more susceptible to caries. It was Mortimer (1964) who reported an equal susceptibility to caries in wide and narrow fissures.

There has been disagreement concerning the position of carious lesions in relation to fissure morphology. Nagano (1961) and Konig (1963) reported that the first site of early caries is not the same in wide and narrow fissures. In wide fissures, early carious lesions were said to occur in the base of the fissures whereas in the narrow fissures, the walls were said to be the first site of carious attack. Two other authors (Mortimer, 1964; Gustafson, 1957) have similarly shown that the walls of the fissures were the first sites to become carious. Fissure caries began bilaterally on the fissure walls giving rise to an appearance of two small smooth surface lesions on histological examination. As the lesion increased in size, they came to coalesce at the base of the fissure (Silverstone *et al.*, 1981). This process is shown diagrammatically in Figure 3.

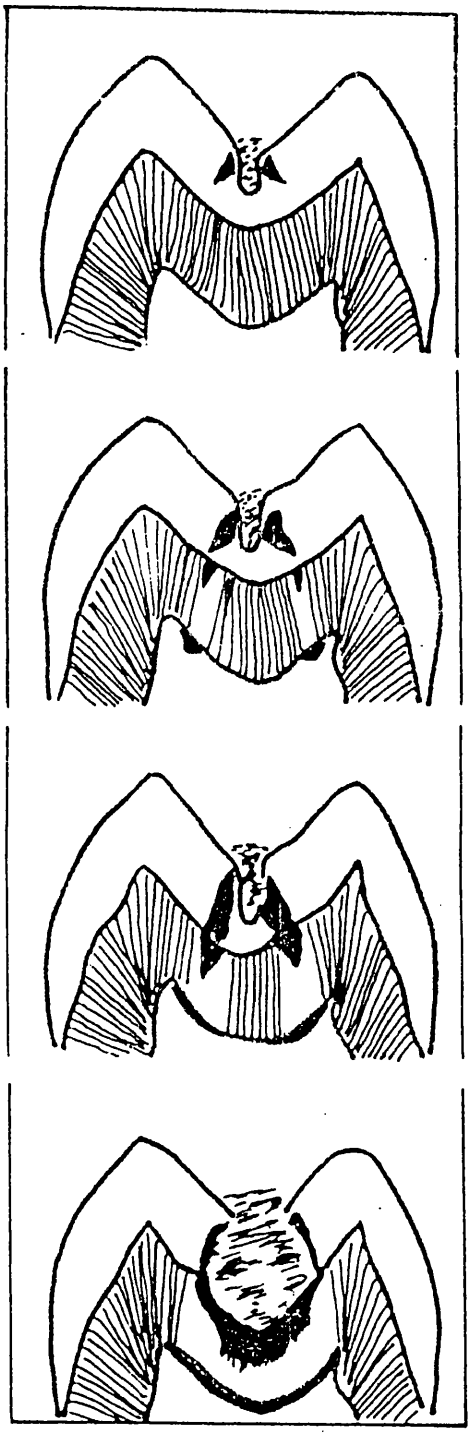


Figure 3 Schematic illustration of the various stages of fissure caries development. (from Thylstrup and Fejerskov, 1986)

2.3.2 The diagnosis of occlusal caries

It is now accepted that the diagnosis of caries may be difficult, especially in the early stages of the disease (Kidd, 1984). Achieving a valid, reproducible diagnostic method is important both in research into the aetiology and prevention of caries and in clinical practice.

Diagnosis of occlusal caries may present a particular problem and a number of methods have been used.

Use of a mirror and a sharp probe

Fissures may be examined using a mirror for visual inspection and a sharp probe for tactile investigation. Caries has been said to be present when the point of the probe sticks without doubt when applied with a little pressure and a definite pull is required for its removal (Jackson, 1950). For many years, this was the most common method of diagnosing caries in pits and fissures (Parfitt, 1954; Miller and Hobson, 1956; Slack *et al.*, 1958; Andlaw and Tucker, 1975; Anderson *et al.*, 1976; King and Shaw, 1979; Anderson, 1981; Beighton *et al.*, 1987) but its use has been found to be unreliable (Kidd, 1984; Rock, 1987).

Histologically, the white spot lesion forms bilaterally on the walls of the fissure rather than the base of the fissure. Thus, a fissure which looks clinically caries-free and has a base hard to probe may histologically show signs of early lesion formation. In addition, the fissure that is sticky to the sharp probe may not be carious histologically (Kidd, 1984) since the retention of an explorer in the fissure is dependent not only on the presence of caries but also on other factors such as the physical characteristics of the explorer

point, the pressure applied during its use and the morphology of the fissure examined (Downer, 1989).

The use of a probe for diagnosis is also now considered questionable by some authors, as it has been reported that forceful probing with a sharp probe can damage the initial lesion and may also serve to transfer cariogenic organisms to non-infected fissures (Bergman and Linden, 1969; Ekstrand *et al.*, 1987; Kay *et al.*, 1988).

Use of a mirror and a blunt probe

Blunt probes have been used in more recent studies instead of sharp probes (Rugg-Gunn *et al.*, 1973; Todd, 1975; Downer and O'Mullane, 1975; Todd and Dodd, 1985; Dummer *et al.*, 1988). In these studies, the probe was mainly used for removing food debris (e.g. Rugg-Gunn *et al.*, 1973; Dummer *et al.*, 1988) or to investigate surfaces where doubt existed after visual inspection (Todd, 1975; Todd and Dodd, 1985). The catching of a probe in a pit or fissure alone was not enough for a diagnosis of caries (Todd and Dodd, 1985).

Visual method

With this method, diagnosis of caries is based on visual assessment alone. It has been used in the dental surveys carried out in the United Kingdom (Winter *et al.*, 1966; Holt *et al.*, 1982; 1988; Kay *et al.*, 1988; Pelkwijk *et al.*, 1990) and has been reported to be efficient for diagnosis of occlusal caries in clinical trials (Howat *et al.*, 1981; Howat, 1981).

Use of radiographs

Examiner reproducibility of diagnosis has been shown to be consistently higher for radiographic examinations than for clinical caries assessment when approximal lesions were investigated (Shaw and Murray, 1975) but this may not be the case with occlusal caries. At least two studies have considered this problem. Miller and Hobson (1956) studied 173 lower molars. Radiographs were taken and caries of pits and fissures diagnosed from these and independently at clinical examination with a mirror and probe. In this study, examiner reproducibility was higher for clinical examination. A similar study design was used by King and Shaw (1979). These authors evaluated the use of bitewing radiographs in the detection of occlusal caries in first permanent molars. Standardised bitewing radiographs taken for 1,172 schoolchildren (aged 11-13 years) were assessed for occlusal caries under uniform magnification and illumination. The radiographic scores were then compared with records of a clinical examination carried out independently for the same children. Of the 4,688 first permanent molars selected for study, a total of 834 teeth were diagnosed as having occlusal caries on either clinical or radiographic examination. Occlusal caries was diagnosed clinically in 804 teeth (96.4 per cent of 834 teeth). Of these 804 teeth, 247 teeth (29.6 per cent of the total) were also recorded as carious on radiographic examination. Reproducibility was assessed in a random sample from 150 of the radiographs of these children. It was concluded from the results that an examiner trained in the use of well defined criteria for the diagnosis of occlusal caries from bitewing radiographs can achieve an acceptable standard of reproducibility (82.6 per cent). However, although the detection of occlusal caries from bitewing radiographs was

more reproducible than clinical examination, it was found to be less sensitive. Only 30 occlusal surfaces (3.6 per cent of the total carious) were diagnosed from radiographs alone.

Very recently Sawle and Andlaw (1988) reported that the appearance of occlusal caries has changed in recent years and as a result, the number of lesions not detected has significantly increased. In these cases, the teeth appear sound but a radiolucent area under the enamel may be detected radiographically. Caries of underlying dentine becomes apparent when the overlying enamel is removed and this might be extensive. This has been explained as a possible consequence of greater exposure of children's teeth to fluoride toothpaste masking the presence of underlying caries to examination by mirror and probe. Thus the authors suggested that bitewing radiographs should be used as an aid to the diagnosis of occlusal caries in addition to clinical examination.

Electronic caries detection

As early as 1951, Pincus suggested the detection of occlusal caries by measuring the electrical resistance at the tooth surface. Mayuzami *et al.* (1964) reported that teeth with a resistance greater than 600,000 ohms had very low caries susceptibility and that a resistance of less than 250,000 ohms indicated actual dental caries.

Electronic caries detectors such as Vanguard (Massachusetts Manufacturing Corporation, Massachusetts) have been said to be particularly useful in the detection and monitoring of early occlusal caries but results with this method have been equivocal (Flaitz *et al.*, 1986; Rock and Kidd, 1988). Other methods such as the use of fibre optic transillumination, use of fluorescent dyes, ultrasound or visible

laser have also been studied for the diagnosis of caries but most have related to caries of other tooth surfaces and no widely acceptable substitute has yet emerged for clinical and/or radiographic methods for detection of occlusal caries.

Without knowledge of the validity of the different diagnostic methods used, it is difficult to evaluate the results of these studies but determination of validity may be problematic (Downer, 1989).

Microscopic examination is most often considered the absolute diagnostic method.

2.3.3 Microbial composition of plaque at a specific site in relation to caries at the same site

It has already been pointed out that the microbial composition of plaque differs in samples from different sites in the same mouth and even between different areas on a single tooth (Hardie and Bowden, 1974). The importance of these differences in caries aetiology has been investigated. Hardie and Bowden reported the composition of plaque collected from adjacent sites on freshly extracted teeth. The sites studied included the contact area, the gingival crevice area (2 mm below the contact area) and the buccal surface. It was reported that streptococci were more highly recovered from the gingival crevice areas than were *Bacteroides* and *Fusobacterium*. *Veillonella* made up a greater proportion of the viable count from buccal surfaces than in samples from either of the other sites in two of the three subjects. They were not recovered at all from the gingival crevice on these two occasions (Hardie and Bowden, 1974).

The microbial composition of plaque may vary as a consequence of the presence or absence of caries. For example, the proportion of *S. mutans* has been reported to be generally higher in plaque overlying carious surfaces. This effect has been demonstrated in at least four studies (Littleton *et al.*, 1970; Rogers, 1973; Shklair *et al.*, 1972; Duchin and van Houte, 1978). *S. mutans* was present in samples from all of 26 carious lesions examined and accounted for 40 per cent of the total streptococcal flora in one investigation (Littleton *et al.*, 1970). In contrast, the organism was recovered in only six of the 26 sound tooth surfaces sampled. Rogers (1973) also found *S. mutans* in 23 of the samples from 25 carious occlusal surfaces in contrast to only six out of 23 samples from sound lingual and buccal surfaces.

Plaque samples were collected from three surfaces in 25 subjects in a third study (Shklair *et al.*, 1972). The sites sampled were carious lesions, sound enamel immediately adjacent to these lesions and sound enamel more distant to the cavities. The isolation frequency of *S. mutans* at these sites was 22, 10 and four respectively.

In the fourth study, a significantly higher proportion of *S. mutans* was found to be present in plaque associated with the development of smooth surface incipient lesions and cavities compared to plaque covering clinically sound enamel immediately adjacent to the lesion (Duchin and van Houte, 1978). *S. mutans* in plaque overlying a carious lesion may be present as a result of conditions created by the lesion itself but it has been suggested that sound surfaces with a greater disposition to caries may also show greater levels of *s. mutans* in their overlying plaque than surfaces which more often remained sound. Shklair and Keene (1974) analysed pooled plaque samples from all four

quadrants in the oral cavity for specific types of anatomic sites. Approximal and occlusal sites which have a higher susceptibility to caries yielded the highest isolation frequency of *S. mutans*. In a study of differing sites, Kohler *et al.* (1981) also found that occlusal and approximal surfaces of caries-susceptible teeth showed the greatest amount (expressed as a percentage of the total direct microscopic count) of *S. mutans*.

Although the exact relationship of specific organisms to specific sites is not known, different organisms have some selectivity to which tooth surface they attack (Newbrun, 1989). Studies with rat fed on a diet rich in carbohydrates have suggested that a number of bacterial species have a cariogenic potential on different tooth surfaces (van Houte, 1980). Findings are summarised in Table 2.

Strains of *S. mutans*, whether from animal or human origin, were nearly always cariogenic in animals fed on a high sucrose diet and often induced high caries activity in fissures. *S. mutans* strains may also be related to caries on approximal and smooth tooth surfaces. Other types of streptococci, lactobacilli and *Actinomyces* were found to be much more variable in their cariogenicity and in general caries induced by these bacteria were usually restricted to the fissures (van Houte, 1980).

These studies have demonstrated that the microbial composition of plaque varies from site to site and that this variation may relate to the presence or absence of caries. This illustrates that collecting plaque samples from specific sites may be especially important if the events at that point are to be better understood. Thus, for example, study of plaque from fissures seems likely to yield especially useful

Table 2 Cariogenicity of different bacteria in gnotobiotic, relatively gnotobiotic or conventional rats (van Houte, 1980)

Organism	Caries		
	Fissure	Smooth enamel	Root surface
Streptococcus			
mutans	+	- and +	- and +
sanguis	- and +	- and +	-
salivarius	- and +	-	- and +
mitis	- and +	-	-
milleri	+*	-	-
Enterococcus	- and +	-	-
Lactobacillus			
acidophilus	- and +	-	-
casei	+	-	-
fermenti	-*	-	-
Actinomyces			
viscosus	- and +	+	+
naeslundii	+	-	-
israelii	+*	-	-
Streptococcus lactis, *diphtheroids* *Leptotrichia*, yeasts	-	-	-

information in relation to the development of caries at this site. Nevertheless, the salivary flora may provide a better indicator of the overall oral flora. Both types of sample have been used to investigate the relationships of oral microflora and caries.

2.4 METHODS OF STUDY OF ORAL FLORA IN RELATION TO CARIES

2.4.1 Types of bacterial sample

Use of saliva samples

Salivary bacterial samples have been widely used in the study of oral organisms that have been related to caries. Some studies (e.g. Woods, 1971; Edwardsson *et al.*, 1972; Klock and Krasse, 1977; 1978; Kohler *et al.*, 1981; 1984; Togelius and Bratthall, 1982; Stecksen-Blicks, 1985; Carlsson, 1985) have utilised stimulated saliva samples whereas others (e.g. Lehner *et al.*, 1978; Van Houte *et al.*, 1982) used unstimulated saliva samples for investigation. Stimulated or unstimulated saliva may be collected by expectorating saliva into a sterile container but this method may not be appropriate for young children. In this age group, the method described by Kohler and Bratthall (1979) may be more suitable since less co-operation is required. A wooden spatula is rotated in the mouth of the child and withdrawn through the pressed lips to remove excess saliva. Both sides of the spatula must be immediately pressed against the surface of a suitable medium.

The chewing of paraffin wax before saliva sampling could increase the shedding of bacteria from the teeth which is the known habitat of *S. mutans* (Carlsson, 1967). Salivary organisms have been thought to be dislodged from plaque and from gingival debris through the washing

action of saliva (Gibbons *et al.*, 1964). However, reviewing studies of predominant salivary flora, Krasse (1953; 1954a and b) has suggested that the tongue is the principal source of salivary bacteria.

In relating salivary flora to caries, the two types of organisms most commonly studied have been streptococci, especially *S. mutans*, and lactobacilli.

Streptococci

Amongst the studies of salivary micro-organisms, *S. mutans* has been most widely investigated because of its association with caries (Hamada and Slade, 1980; Loesche, 1986; Burt *et al.*, 1983; Beighton *et al.*, 1987). In reviewing a total of 33 separate studies, Chong (1986) reported that those studies employing stimulated saliva samples (e.g. Klock and Krasse, 1977; 1978; Zickert *et al.*, 1982; Stecksen-Blicks, 1985) had more often shown a relationship between caries activity and *S. mutans* than those in which unstimulated samples were used. However, it was pointed out that interpretation of results may be more complex than was first apparent because whilst the latter method is suitable for young children, the first is not. Thus the disparity in ages of subjects included in these studies could have contributed to some differences in caries activity and oral flora (Chong, 1986).

It has been reported that the number of colony-forming units (cfu's) of *S. mutans* per ml saliva showed a significant correlation with caries activity (Klock, 1978; Klock and Krasse, 1979; Togelius and Bratthall, 1982).

An investigation of caries-predictive ability of salivary *S. mutans* (Chong, 1986) found a trend for *S. mutans* to be isolated more often from unstimulated saliva samples taken from 15 2-5 year old children with rampant caries, than in those from an equivalent number of caries-free children of the same age group. Samples were taken using Kohler and Bratthall's method and using a Calgiswab attached to a broach.

Van Houte *et al.* (1982) studied the oral flora of six 1.5-3.5 year old children with rampant caries. As in Chong's study, unstimulated saliva samples were obtained by saturating Calgiswabs in saliva from the floor of the mouth. The authors reported that *S. mutans* was present in all samples. *S. salivarius* was also investigated in this study and the proportion of this organism in saliva was found to be very low and it was not detected in the saliva of four out of the six children included in the study. Chong (1986) too reported on this organism. She found a trend for children with rampant caries to have lower levels of *S. salivarius* in their saliva samples taken in this way than did those who were caries-free.

Lactobacilli

Stimulated saliva samples have been widely used for culture and counts of lactobacilli or to determine the acid production of this species on a selective medium.

For cultivation of lactobacilli, Rogosa selective Lactobacilli (SL) agar (Rogosa *et al.*, 1951) has been widely used in a number of studies (Klock and Krasse, 1977; 1978; 1979; Zickert *et al.*, 1982; Kohler and Bjarnason, 1987; Bentley *et al.*, 1988). Alternatively, a dip

slide test (Dentocult, Orion Diagnostica, Helsinki) has been used for estimating salivary lactobacilli. This method also used wax-stimulated saliva (Crossner and Hagberg, 1977; Crossner, 1981; Stecksen-Blicks, 1985; Crossner and Unell, 1986; Pienihakkinen, 1987; Wilson and Ashley, 1989). Snyder medium (pH 4.8-5.1) is an acidic medium which inhibits the growth of all the acid-producing organisms except lactobacilli. Therefore this medium has been used to measure the acid production of lactobacilli (Snyder, 1940). Perhaps not surprisingly, it has been reported that there was an excellent agreement between a zero count or negative Snyder reading and the absence of caries activity (Sims, 1970). Both techniques have been thought to provide an indication of caries activity and have been widely used, especially in Scandinavia (e.g. Klock and Krasse, 1978; Crossner, 1981; Zickert *et al.*, 1982). However, the main criticism of the diagnostic use of Lactobacillus counts has been their failure to predict future caries activity, especially on an individual basis (Green and Weisenstein, 1959; Snyder *et al.*, 1963; Klock and Krasse, 1979). It has been reported that the best prediction of caries activity could be obtained by combining Lactobacillus counts with other factors such as the number of incipient smooth surface lesions and *S. mutans* counts (Klock and Krasse, 1979), salivary *S. mutans* counts (Stecksen-Blicks, 1985), clinical examination (Crossner and Unell, 1986) or with a combined use of incipient carious lesions, salivary buffering capacity and numbers of yeasts in salivary samples (Pienihakkinen, 1987).

Recently, Bentley *et al.* (1988) has reported that salivary microbial counts have a day-to-day variability and that quantitation of salivary micro-organisms on a single day could not adequately define the oral microflora.

The salivary flora may represent the source for plaque organisms and will also include organisms dislodged from the tooth surface where caries occur. It is also thought to reflect the ecology of other oral tissues, especially the tongue, perhaps providing a broader indication of the overall flora. Saliva has a further practical advantage in being easy to collect, even in mouths with minimal plaque levels.

Use of plaque samples

A summary of 21 studies utilising plaque samples to investigate the relationship of oral microflora to caries is illustrated in Table 3. Three basic types of samples may be recognised in these studies.

First, plaque samples may be taken from all available surfaces and combined to form pooled plaque samples. This technique was used in studies (nos. 4, 9, 13 and 21 in Table 3).

However, as described earlier (Shklair and Keene, 1974; Kohler *et al.*, 1981) colonisation of some species, for example *S. mutans* is believed highly localised and it has been thought to be important to obtain plaque samples from the precise area or areas likely to be affected by the disease. Thus some studies have used samples drawn from very specific sites. Studies nos. 1, 2, 3, 5, 6, 7, 8, 10, 11, 12, 15, 18 and 19 in Table 3 utilised samples drawn from a specific site. Drawing samples from specific sites for separate analysis may be very difficult. Sites may be inaccessible, as in the case of approximal areas, of the samples derived may be too small to allow culture.

In a third type of study, samples may be pooled after collection from a similar type of tooth site. Studies illustrated in Table 3 (nos. 14, 16, 17, 19 and 20) used this type of sampling technique.

Table 3 Epidemiological studies on dental plaque and caries

Study no. and Authors	Type of study	Sample size	Age of subjects	Type of plaque sample	Findings
Study no. 1 Bowden et al. (1976)	L (12 mths)	9	13-14 yrs	S Distal surfaces of 4/4; abrasive strip used.	The microflora of all sites was similar at the generic level with Streptococcus, Actinomyces and Veillonella comprising a high proportion of the counts. Qualitative and quantitative differences were detected at the species level and showed that each site could be considered as a distinct ecological niche. Comparison of the carious to caries-free sites suggested that: (a) domination of S. mutans in plaque on a tooth surface can be associated with the development of a lesion. (b) domination by S. mutans and lactobacilli could produce a lesion. (c) the flora of plaque above a lesion after formation may not be obviously different from that which initiated it.
Study no. 2 Boyar and Bowden (1985)	L (12 mths)	22	4-9 yrs	S Incipient approximal lesions; abrasive strip method.	Significant positive associations were detected between S. mutans, lactobacilli, Veillonella and Actinomyces odontolyticus and progressive lesions. On the other hand, significant negative associations were detected with S. mitior, A. naeslundii and A. viscosus.
Study no. 3 Boyar et al. (1989)	L (1-14 days)	12 children (34 caries-free premolars)	14-16 yrs	S Accumulated plaque under preformed orthodontic band collected after extraction of tooth.	The numbers of streptococci and Veillonella were significantly higher at day 1 than at day 14 ($p<0.05$). S. mutans and A. viscos were isolated more frequently at day 14 than day 1 ($p<0.01$). Ratios of viable counts of bacteria showed no significant differences between day 1 and day 14. Lactobacilli were isolated from 2 out of 8 samples at day 4 and 4 out of 8 samples at day 1

Table 3 Epidemiological studies on dental plaque and caries (contd)

Study no. and Authors	Type of study	Sample size	Age of subjects	Type of plaque sample	Findings
Study no. 4 Bratthall (1972)	C	275	5-45 yrs	P From buccal, lingual and occlusal tooth surfaces.	Results of this study suggested to consider s. mutans as a possible important factor for the occurrence of dental caries.
Study no. 5 Burt et al. (1983)	L (2 yrs)	279	6-7 yrs	S Collected from occlusal fissure of lower first permanent molar.	Teeth remained sound throughout the study period irrespective o S. mutans levels (low, not detectable or above median levels). Nevertheless, there was a significant relationship between S. mutans levels and the development of caries. No association was found between S. sanguis and caries.
Study no. 6 De Stoppelaar et al. (1969)	L	63	≈13 yrs	S From buccal smooth surface of 7 , except 11 children from 6.	An inverse relationship was found between the occurrence of S. mutans and S. sanguis. A tendency towards a negative correlation was found between the presence of S. sanguis and dental caries.
Study no. 7 De Stoppelaar et al. (1970)	L	6	19-34	S Obtained from 3 sites and treated separately 1.Interproximal area between 76 . 2.The buccal smooth surface of 7. 3.Buccal surface of 3\.	An inverse relationship between S. mutans and S. sanguis was observed in this study. During a carbohydrate-free period (17 d the percentage of S. mutans on the total cultivable flora decreased to a very low or undetectable level while simultaneously the percentage of S. sanguis increased.

Table 3 Epidemiological studies on dental plaque and caries (contd)

Study no. and Authors	Type of study	Sample size	Age of subjects	Type of plaque sample	Findings
Study no. 8 Hardie et al. (1977)	L (2 yrs)	19	Average= 12.5 yrs	S From distal surface of 4/4; abrasive	No marked increase in either S. mutans or lactobacilli before c detection. The microbial composition of plaque samples from car free sites and carious sites before and after radiographic detection of lesions was broadly similar.
Study no. 9 Hayes et al. (1983)	C L	109 8	3-23 yrs -	P Collected from all available non-carious surfaces and pooled.	A high acidogenic ratio was found in individuals with active lesions or recent restorations. Individuals who were caries-fre had no recent lesions had a lower ratio although total organism were similar. Streptococci, Actinomyces and S. mutans were isolated from all samples but no consistent relationship was found between either their isolation frequency or proportions and carrier status.
Study no. 10 Ikeda and Sandham (1971)	C	12	7-9 yrs	S Collected from pits and fissures of first molar, mesial approximal area (contact point) and buccal surface.	Occlusal pits and fissures constituted the major reservoir of S. mutans (39 percent of the total streptococci). Fewer S. muta were found on the buccal surface (2.9 percent) and this organis was absent in samples from approximal tooth surfaces.
Study no. 11 Ikeda, Sandham and Bradley (1973)	L	12	7-9 yrs	S From 3 sites:- 1.Occlusal pits and fissures of 6. 2.Buccal smooth surface of 6. 3.Contact point between 6 and E.	1. The mean number of S. mutans increased with time on all 3 surfaces but it remained much higher in the pits and fissure than on other surfaces. 2. Initiation of a carious lesion on a tooth surface was closel associated with a history of previous S. mutans infection of the surface. 3. No similar association was observed between lactobacilli and initial caries.

Table 3 Epidemiological studies on dental plaque and caries (contd)

Study no. and Authors	Type of study	Sample size	Age of subjects	Type of plaque sample	Findings
Study no. 12 Kohler et al. (1981)	L 2.5 yrs	10	13-16 yrs	S Collected from 10 selected surfaces with different degrees of caries susceptibility.	A relationship between S. mutans and the development of initial carious lesions was observed in this study. Nine of the 16 surf with higher proportions of S. mutans showed caries or progressiv caries. In contrast, surfaces with low proportions of S. mutans remained caries-free.
Study no. 13 Krasse et al. (1968)	L	235	4-27 yrs	P From buccal and lingual surfaces of all teeth.	1. Caries-inducing streptococci constituted 80-90 percent of the cultivable streptococci in plaque samples of some persons from various population groups in Sweden whereas they were consistently absent in others. No correlation was found between the frequency of caries and the occurrence of these streptococci. 2. In a selected group of school children who developed smooth surface lesions during the study period (1 year), a high incidence of the specific streptococci was isolated. On the other hand, the majority of children with no new buccal or lingual lesions had a low frequency of these organisms.
Study no. 14 Lehner et al. (1978)	C	27	2.5-5.5 yrs	SP From approximal surfaces of deciduous molars and cervical aspects of lower incisors	S. mutans was cultured in more children from rampant caries grou (9 out of 15 plaque samples) than in children with non-rampant caries (4 out of 12 plaque samples) although this finding was no significant statistically.
Study no. 15 Littleton et al. (1970)	C	26	13-14 yrs	S From fissures or selective smooth surfaces	Caries-inducing streptococci accounted for more than 50 percent of the total streptococcal count in 12 of the 26 samples from carious lesions.

Table 3 Epidemiological studies on dental plaque and caries (contd)

Study no. and Authors	Type of study	Sample size	Age of subjects	Type of plaque sample	Findings
Study no. 16 Marsh et al. (1989)	C	29	Mean= 13.5 ± 1.4 yrs	SP Collected from multiple sites around the contact areas of premolars.	The isolation frequencies as well as the mean percentages of viable counts of mutans streptococci and Actinomyces viscosus w higher at sites with early caries. Mutans streptococci could no detected at 37 percent of the sites with early caries and Veillonella were markedly reduced. Lactobacilli were rarely iso and were never recovered from caries-free surfaces. It was concluded that particular stages of lesion formation may be associated with different combinations of bacteria.
Study no. 17 Mikkelsen and Poulsen (1976)	L (1.5 yrs)	54	5-7.5 yrs	SP From proximal surfaces of primary molars.	1. Neither S. mutans nor micro-organisms producing intracellula polysaccharide were found to have a relationship with caries development. 2. No changes in the composition of the microflora were observe in Gram-stain smears during caries development. 3. The ratio of aerobic/anaerobic micro-organisms changed in connection with caries development. The ratio ranging from 7.5 to 139.5 percent prior to the period of caries developme decreased to between 0.5 and 43.5 percent.
Study no. 18 Mikkelsen et al. (1981)	C	39	14-47 yrs	S From one carious and one caries-free interproximal area.	No difference was observed in the microbial composition of dent plaque covering carious and caries-free tooth surfaces. S. muta was detected in carious as well as in caries-free areas investi The ratio of total aerobic and anaerobic micro-organisms recove did not show any difference between carious and caries-free areas.

Table 3 Epidemiological studies on dental plaque and caries (contd)

Study no. and Authors	Type of study	Sample size	Age of subjects	Type of plaque sample	Findings
Study no. 19 Schamschula and Charlton (1971)	L	388	9-10 yrs	SP From buccal surface of 6\, labial of 1\ and lingual of /6. S From a small area on the centre of labial of /2.	Statistically significant positive correlations were found bet caries prevalence (DMFT) and (a) the total number of streptococci per unit volume of plaque (b) the number of cariogenic streptococci per unit volume of p (c) cariogenic proportions of total cultivable streptococcal counts in pooled plaque samples. However, correspondingly significant associations were not fou in plaque collected from the labial surface of /2.
Study no. 20 Shklair et al. (1974)	C	232	17-22 yrs	SP Samples from all 4 quadrants for each anatomic site (occlusal, approximal and buccolingual) were pooled.	1. The approximal and occlusal surfaces of both the control gr (with some caries experience) and caries-free group had a significantly greater isolation frequency of S. mutans than buccolingual surfaces. 2. There was approximately a twofold increase in the isolation frequency of S. mutans from the control group compared to t total caries-free group.
Study no. 21 Swenson et al. (1976)	L (2 yrs)	781	6-9 yrs	P From buccal, mesial and approximal surfaces of clinically non-carious maxillary permanent molars.	Lactobacilli were frequently not recovered and when detected w found in small numbers. The relationship between the detection S. mutans (on Mitis Salivarius Agar) and the occurrence of gro dental caries was consistently associated statistically. No association was discovered between S. sanguis, S. mitior or S. salivariuis and the occurrence of dental caries.

S = Specific type of plaque sample
P = Pooled type of plaque sample
SP = Plaque collected from specific sites and pooled
C = Cross-sectional study
L = Longitudinal study

In all three groups of studies, *S. mutans* has been particularly investigated often to the exclusion of other organisms. Studies using pooled plaque samples have most often shown a relationship between plaque micro-organisms (especially *S. mutans*) and caries in cross-sectional (e.g. nos. 4, 14, 16 and 20) as well as in longitudinal studies (nos. 11, 12 and 21 in Table 3) but the relationship between other plaque organisms and the occurrence of caries has been less well established. Lactobacilli were not usually recovered and when they were cultivated, they formed only a small proportion of the plaque microflora. In one study where other organisms were investigated, no association was shown between *S. sanguis*, *S. mitior* or *S. salivarius* and caries (study no. 21). In contrast, Mikkelsen and Poulsen (1976) using pooled plaque failed to show any relationship between *S. mutans* and caries. However, the ratio of aerobic/anaerobic micro-organisms was seen to decrease with caries development in this investigation (study no. 17).

Studies employing plaque from specific sites have similarly shown a relationship between *S. mutans* and caries (nos. 2, 5, 7, 11 and 12) and all these were longitudinal studies. An inverse relationship of *S. sanguis* and caries has also been reported (study nos. 6 and 7) but neither *S. mutans* nor *S. sanguis* was found to have any relationship to caries in one of the investigations (study no. 17).

Just as with *S. mutans*, no association of lactobacilli to caries was observed when samples were drawn from specific sites (study no. 11 in Table 3). Two longitudinal studies utilised plaque from distal surfaces of upper premolars. It was reported that the microflora of plaque from carious and caries-free sites appeared to be generally

similar (study nos. 1 and 8). Although it was suggested in one of these two studies that domination of *S. mutans* and lactobacilli in plaque could produce a lesion (Bowden *et al.*, 1976; study no. 1 in Table 3), no obvious increase in either *S. mutans* or lactobacilli before development of caries was detected in the second (Hardie *et al.*, 1977; study no. 8). Similarly, in a cross-sectional study (study no. 18) where samples were drawn from specific sites, no difference was noted between the microbial composition of plaque overlying carious and caries-free sites. In addition, in this study in contrast to study no. 17, there was no difference in the ratio of aerobic and anaerobic micro-organisms between carious and caries-free areas in this study.

Using specific sampling sites was thought to be able to directly reflect the changes occurring at these sites with the development of caries but as Boyar and Bowden (1985) have pointed out, there may be data available for only a few developing lesions since lesions may fail to develop at other susceptible sites during the study period.

For this reason, these authors utilised incipient approximal lesions as sampling sites. Significantly positive associations were detected between *S. mutans*, lactobacilli, *Veillonella*, *Actinomyces odontolyticus* and the progressive lesions in this study (Boyar and Bowden, 1985; study no. 2 in Table 3).

In some studies (e.g. nos. 14, 16, 17, 19 and 20) plaque samples were collected from specific tooth sites and samples from similar sites were pooled for analysis. Plaque was collected from similar sites (occlusal, approximal and bucco-lingual surfaces in all four quadrants and pooled for analysis in one study (study no. 20).

In another study, plaque was collected with a sharpened curette from the interproximal surfaces of primary molars which had no clinically detectable caries (study no. 17).

As in studies where plaque samples were drawn in other ways, the relationship of *S. mutans* to caries has been noted in the investigations using this type of plaque sample (study nos. 19 and 20 in Table 3).

In relation to other organisms, *Actinomyces viscosus* was reported to be generally higher in early carious sites together with *S. mutans*. It has also been reported that at those sites showing earlier caries and where *S. mutans* could not be detected, the proportion of *Veillonella* was found to be markedly reduced (study no. 16 in Table 3).

When the ratios of streptococci, lactobacilli, *S. mutans*, *S. mitior*, *S. sanguis*, *A. viscosus* and *Veillonella* were studied (study no. 3), no significant differences were observed between these ratios at the beginning (day 1) or at the end (14 days) of the study.

It may thus be concluded that a relationship between plaque micro-organisms (in most cases *S. mutans*) and caries has been observed in studies utilising any of the three types of plaque samples (nos. 2, 5, 11, 12, 13, 20 and 21 in Table 3) whereas this could not be established in other studies (nos. 8, 9, 14, 17 and 18). In one study (no. 19 in Table 3), two types of plaque samples (pooled and specific) were utilised for analysis and an association was detected between caries prevalence (DMFT) and streptococcal species in pooled plaque samples. No similar relationship could be found in plaque collected from the specific site chosen in the same patients. However, choice of site may be critical when using specific samples. The chosen site in this study,

the labial surface of an upper incisor was not especially susceptible to caries in the majority of people. In one study (Chong, 1986; discussed earlier in Section 2.4.1) it was noted that there was less intra-subject variation in the number of *S. mutans* cultivated in saliva samples than in (pooled) plaque samples. In contrast, Aluuluusua and Renkonen (1983) have recommended that the evaluation of *S. mutans* in plaque would seem more practical and more reliable than the evaluation of salivary *S. mutans* levels in young children.

Most of the investigations have utilised either saliva or plaque samples and the salivary flora or plaque microflora have been related to dental caries accordingly. Fewer studies have utilised both types of samples for analysis. Since caries occurs in the oral environment closely related to both plaque and saliva, a comparative study to explore the relationship between flora derived from the two types of sample and the development of caries might yield more valuable results.

2.4.2 Isolation and culture procedures for study of oral microflora

The choice of culture media and growth conditions to be used depends upon the objective of the investigation. In order to cultivate all the viable micro-organisms present in a sample it is necessary to provide a range of different media and cultural conditions. Usually, a combination of non-selective and selective media are used for aerobic and anaerobic incubation (Hardie and Bowden, 1976).

Difficulties are usually encountered in an attempt to account for the total numbers by summating viable (cultivable) counts. It has been reported (Socransky *et al.*, 1963) that even with the best available media and methods the total viable count rarely exceeds a fifth of the

total microscopic count. Grossman (1966) pointed out that the minimum number of organisms required to initiate growth varies widely even under seemingly equally favourable conditions. Since there is such a wide range of micro-organisms in the oral cavity isolation is technically very difficult. For example, some of the species such as fusobacteria, *Bacteroides* and lactobacilli are in such a numerical minority that they are often lost in the overgrowth of the common streptococci, *Veillonella* and diphtheroids which are usually found in association (Burnett *et al.*, 1968).

In early studies (e.g. Socransky *et al.*, 1963) the quantitative plating counts would detect only about 20 per cent of the microscopic count. The employment of culture systems involving the use of transport media and maintenance of anaerobiosis during sample, inoculation and incubation of culture media have led to a threefold improvement in the recovery of bacteria from gingival plaque samples (Burnett *et al.*, 1968). Principally two approaches have been made to isolate and enumerate cultivable oral micro-organisms.

Use of non-selective media

Non-selective media are used to cultivate the maximal number and the widest variety of organisms at the same time. However, no one medium could be possibly used to meet all requirements. Blood agar is most widely used and it is incubated both aerobically and anaerobically. In general anaerobic incubation gives about twice as many colonies compared to aerobic incubation. However, an important limitation of this approach is that in order to obtain practical counts of not more than 100 to 200 colonies per plate, high dilutions of oral specimens

must be used and by doing so many organisms numbering 1 per cent or less of the total count are lost (Burnett and Scherp, 1968). Some investigators used 5% horse blood agar as the standard non-selective medium for plaque studies (Hardie and Bowden, 1976). Wilkins chalgren anaerobe agar (Sutter *et al.*, 1979) is a non-selective medium used for the general growth of anaerobes.

To determine the most suitable medium for non-selective isolation of the viable micro-organisms in dental plaque Slots (1975) compared the following five growth media:

1. "Plaque medium" (Jensen *et al.*, 1968).

2. N_2C (Nutrient-cysteine medium) (Gilmour and Poole, 1970).

3. HIA - 10% B (Heart Infusion Agar - 10% Blood) (Gordon *et al.*, 1971).

4. BHIA - Suppl. (Brain Heart Infusion Agar - Supplemented). (Holdeman and Moore, 1973).

5. MM 10 (Modified Medium 10) (Loesche and Syed, 1973).

From the results of this study it was found that MM 10 is a valuable non-selective medium with either the roll tube technique or the anaerobic jar technique (Slots, 1975).

<u>Use of selective media</u>

The second approach is to cultivate the maximal numbers of only a limited variety of micro-organisms by using restrictive media which selectively inhibit undesired organisms by such agents as dyes, enzyme

inhibitors, antibiotics, unfavourable pH and high salt content, e.g. Mitis salivarius agar and crystal violet azide agar for streptococci, Rogosa's media for lactobacilli and *Veillonella*. Selective media are used to determine the presence and number of various types of specific oral organisms (Nolte, 1977). The selective media may be helpful for the isolation of strains which are present in low concentrations and which may be consequently missed because of overgrowth by more numerous species on non-selective plates (Hardie and Bowden, 1976). Nevertheless, it must be remembered that colonies on a selective medium are not necessarily pure. At the base of the colony other organisms may be present and although they are unable to develop on the selective medium they will grow rapidly when transferred to a non-selective medium. Direct use of colonies from a selective medium for testing the properties could give misleading results. Therefore colonies from a selective medium should be replated onto a plain medium to ensure their purity before testing the biochemical, antigenic or other properties of the organism under study (Wilson and Miles, 1955). The acidogenic oral flora is principally made up of various species of lactobacilli and streptococci (Jordan, 1986). Amongst the plaque micro-organisms lactobacilli and streptococci have been most studied due to their strong relation to caries. Various selective media have been developed to cultivate these organisms. It was Hadley (1933) who described a technique for culturing aciduric organisms from saliva on tomato juice agar. Most of the organisms cultured were lactobacilli and were easily recognised on this medium. Rogosa and co-workers (1951) developed a more specific selective medium for culturing oral lactobacilli. The selectivity of this medium is based on its low pH (5.4), high acetate content and the presence of a surfactant, Tween 80. This is still used

as the medium of choice for primary culture of lactobacilli in oral samples (Jordan, 1986).

A selective medium for the primary detection and isolation of *Veillonella* species has also been described by Rogosa (1956).

Mitis Salivarius agar (Chapman, 1944) was used as a differential medium for cultivating the viridans streptococci. Krasse *et al.* (1968) demonstrated a correlation between dental caries and *S. mutans* using Mitis Salivarius agar. Although *S. mutans* was easily identified on these differential media, it could be hard to detect when it occurred in relatively low numbers compared to other streptococci. Carlsson (1967b) described a medium for selective growth of *S. mutans* based on addition of 1 gm/l sulfadimatine to a medium with a composition similar to Mitis Salivarius agar. A significant association between *S. mutans* in plaque material and dental caries experience in a group of 44 patients was demonstrated using this medium (Woods, 1971). Ikeda and Sandham (1972) reported 40 per cent sucrose in mitis salivarius agar as a selective medium for *S. mutans* although there was some inhibition of this species compared to its growth on plain mitis salivarius agar.

Mitis salivarius bacitracin agar (MSB) was developed by Gold *et al.* (1973) as a selective medium for *S. mutans* based on the combined selective activity of sucrose and bacitracin in mitis salivarius agar. However it has pointed out that MSB agar does not support full growth of all *S. mutans* strains (Emilson and Bratthall, 1976).

A selective medium (TYCSB) with improved recovery of *S. mutans* was described by van Palenstein-Helderman *et al.* (1983). TYC agar, a medium used for isolation of dextran-forming streptococci (de Stoppelaar *et*

al., 1967), was modified by addition of sucrose to a final concentration of 20 per cent and 0.1 unit/ml bacitracin.

Tanzer *et al.* (1984) described a selective medium (GSTB agar) for improved recovery of *S. mutans*. This medium contains 0.3 unit/ml bacitracin, 0.001% potassium tellurite and 5% each of glucose and sucrose added to a trypticase (BBL), yeast extract (Difco), salts, basal medium (Jordan *et al.*, 1960). Major subgroups of *S. mutans* can be differentiated on this medium.

A simplified cultural method using wooden spatulas was proposed (Kohler and Bratthall, 1979) for estimation of *S. mutans* levels in saliva. A wooden spatula was rotated in the mouth of the infant to wet it with saliva. Each side of the spatula was then pressed directly against an agar plate containing MSB agar medium.

Traynor *et al.* (1981) reported the use of Columbia agar containing 2.5 mg/litre metronidazole supplemented with 10% horse blood as a selective medium for isolation of *Actinomyces* species.

Various selective media have been used for isolation of particular organisms in the study of oral flora. However, all selective media inhibit to some degree, even the organisms they are supposed to support, and as a result an under-estimation of the numbers of particular species could occur in quantitative studies using this kind of media (Hardie, 1983).

Transport media

Reasons for using a transport medium

The human oral flora contains a large proportion of anaerobic bacteria (Gibbons *et al.*, 1963) which differ markedly in their sensitivity to oxygen (Loesche, 1969). Serious loss of cultivable bacteria could occur unless the specimens are cultured promptly or are preserved in a holding medium (Moller, 1966).

For optimum isolation of viable organisms it is desirable that plaque specimens should be received and processed in the laboratory as soon as possible after removal from the mouth (Hardie and Bowden, 1976). As this is not always possible, specimens collected from the oral cavity may be placed in a special transport medium to ensure the viability of the greatest number of organisms.

Types of transport media

The following are some of the transport media which have been used:

1. VMG II (Moller, 1966).
2. VMG IV.
3. Stuart medium.
4. Modified Stuart medium.
5. Reduced transport fluid (RTF) (Syed and Loesche, 1972).

Moller (1966) reported that VMG II transport medium was superior for demonstrating streptococci and anaerobic non-sporulating bacteria in the endodontic sample. It was Jordan *et al.* (1968) who showed that VMG II medium retained the reproductive capacity of the organisms

present in samples of dental plaque and it was also shown that the viability of most plaque streptococci was preserved at room temperature for several days.

Gastrin *et al.* (1968) investigated the effect of storage of various clinically important pathogenic bacteria in VMG II medium, Stuart medium as modified by Ringertz (1960) and modified Stuart medium (SBL). On the basis of the comparison of the comparison of the three transport media, the authors recommended SBL medium as a suitable transport medium in different types of bacteriological examinations.

Syed and Loesche (1972) compared the efficiency of the reduced transport fluid with that of VMG II and SBL media in maintaining the viability of bacterial flora present in the samples. The efficiency of the transport media was determined by comparing the quantitative recovery (expressed as a percentage of the initial viable count) from the specimens stored for various lengths of time. Results from this study suggested that VMG II and SBL served better than RTF as storage media for non-disease-associated dental plaque cultured under strict anaerobic conditions. With periodontal plaque samples higher bacterial counts were obtained for samples stored in RTF than SBL and VMG II under identical conditions. On the other hand, for carious plaque samples the organisms appeared to survive much better in RTF and VMG II than in SBL as determined by conventional culturing techniques. Higher recovery of bacteria was found with an increase in storage period in VMG II which is an indication of multiplication of the organisms. In contrast to this finding, RTF did not allow the growth of oral bacterial flora under all experimental conditions. Although the ideal transport medium is not yet available RTF appeared to be more satisfactory for dental samples (Syed

and Loesche, 1972). RTF (Table 4) is a non-selective transport and storage medium. Apart from 0.02% dithiothreitol (DTT) (reducing agent) it does not contain any compound which could support the growth of bacteria. DTT makes it more resistant to oxidation under aerobic conditions and as a result high recovery of plaque flora from carious and periodontal plaque samples are detected with the use of RTF (Syed and Loesche, 1972).

2.4.3 The importance of anaerobic incubation

It has been reported (Hardie and Bowden, 1976) that the tooth surface microflora is complex and the established dental plaque from almost any site in the mouth invariably contains a wide variety of bacterial species. These organisms include aerobic as well as facultative and obligately anaerobic species, amongst which some of the latter are very sensitive to oxygen. As a result, good anaerobiosis is required for maximum isolation of plaque micro-organisms and the highest viable counts from dental plaque could be obtained by using sophisticated anaerobic techniques (e.g. anaerobic chambers). However, these sophisticated techniques may not always be available or appropriate, particularly in epidemiological surveys. Therefore it has been recommended that the use of freshly poured pre-reduced culture plates and well maintained anaerobic jars with fresh cold catalyst may give satisfactory results in most studies (Hardie and Bowden, 1976).

Table 4 Composition of reduced transport fluid (RTF) (Syed and Loesche, 1972)

Constituent	Additions per 100 ml
0.04% Na_2CO_3*	0.5 ml of stock solution 8% Na_2CO_3
0.001 M EDTA	1 ml of 0.1 M EDTA stock solution
0.02% Dithiothreitol*	2 ml of 1% DTT stock solution
0.045% K_2HPO_4	7.5 ml of 0.6% K_2HPO_4 stock solution
0.045% KH_2PO_4	7.5 ml of stock mineral solution containing
0.09% NaCl	0.6% KH_2PO_4
0.09% $(NH_4)_2SO_4$	1.2% NaCl
0.018% $MgSO_4$	1.2% $(NH_4)_2SO_4$
	0.25% $MgSO_4$

* To be filter sterilised 0.22 um filter

2.5 PIT AND FISSURE PLAQUE STUDY

2.5.1 Sampling methods used in the study of fissure plaque

The choice of an appropriate sampling method has been reported to be the basis to any study of the oral flora, particularly where relationships between micro-organisms and disease are being sought (Hardie and Bowden, 1976).

It has already been suggested that obtaining samples from specific sites may be difficult and deriving samples of fissure plaque has presented particular differences. A number of different sampling techniques have been used by various investigators to study the occlusal fissure microbiota. These have included scraping across the fissure opening with needles (Loesche and Straffon, 1979; Svanberg and Westergren, 1983; Burt *et al.*, 1983; 1985), with dental explorers (Ikeda *et al.*, 1973; Gibbons *et al.*, 1974; Jordan and De Paola, 1974; Caufield and Gibbons, 1979; Rogers, 1973; Shklair *et al.*, 1974). Artificial fissures which can be implanted at a desired site and removed later have been used to collect plaque for accurate analysis. This involves mylar fissures (Loe *et al.*, 1973; Karring *et al.*, 1974) or human enamel fissure blocks inserted into amalgam restorations (Fejerskov *et al.*, 1976). Fissures from impacted third molars have also been used implanted in dentures (Folke *et al.*, 1973; Thott *et al.*, 1974). In addition, a method employing fissure removal has recently been introduced (Meiers *et al.*, 1982; Meiers and Schachtele, 1984).

Needles, pointed wires or dental explorers have been commonly used in the study of fissure plaque as they are readily available, easy to use and economical. In order to collect plaque from the narrow pits and fissures, explorer, needle or pointed wire is usually scraped several

times through these areas. Recently, a number of studies have shown the deleterious effects of the use of explorer for tactile examination in caries detection. These studies have shown that the use of an explorer for direct probing of demineralised enamel could lead to mechanical damage of tissue structures and subsequent demineralisation. Although there appears to be no report that scraping a probe along a fissure for plaque collection could damage sound enamel, such explorers do need to be used with great care as their use with undue pressure could produce irreversible traumatic defects (Ekstrand *et al.*, 1987; Corien *et al.*, 1988). A further disadvantage of this method is that the plaque collected may be plaque from the fissure entry point and not the base since even with a sharp probe it may be difficult to reach the fissure base. It was to overcome some of the difficulties encountered in collection of plaque samples from the surfaces of natural teeth *in situ* that a number of workers developed alternatives. Artificial devices may be inserted in the mouth to allow plaque formation to occur on the surface. These have the advantage that after an appropriate period of time they can be removed and used for laboratory investigation (Slack and Bowden, 1965). This approach may be especially applicable to the study of plaque formation in fissures, since collection of material from the base of a natural fissure is so difficult (Theilade *et al.*, 1973).

Loe *et al.* (1973) developed a method for studying plaque formation in occlusal fissures of human teeth. This method includes the implantation of "bags" prepared from Mylar foil into the occlusal surfaces of fully erupted natural molars. The bags were constructed to have dimensions corresponding to natural molar morphology.

It has been suggested that the artificial fissures constitute an experimental model system which permits study of plaque formation under conditions similar to those of natural occlusal fissures. However, with this model it was not possible to investigate the relation and the interaction between plaque and tooth tissues (Fejerskov *et al.*, 1973). Nevertheless, the age of the plaque could be recorded with this method. At varying periods of time after implantation, these artificial fissures may be removed and detailed structural and histochemical examinations or biochemical analysis could be carried out on collected material (Karring *et al.*, 1974). Using this technique, Karring *et al.* studied fissure plaque growth in a group of 10 dental students with study periods of up to 60 days. At the end of each study period, the artificial fissures were removed and processed for histologic and histochemical examination. It was concluded that the development and composition of plaque in the artificial fissures seemed to resemble that of natural fissures. The authors concluded that the location and anatomic characteristics of the occlusal fissures are important factors in the establishment of this particular ecosystem (Karring *et al.*, 1974). In contrast to these studies using artificial surface materials, Theilade *et al.* (1974) used occlusal fissures from unerupted third molars to study the nature of developing plaque. Small blocks measuring 2 x 2 x 3 mm, each containing part of the occlusal fissure were prepared from these and were implanted in occlusal fillings in molars of six dental students involved in the study. In a similar technique, crowns of fully impacted third molars were held in place in the mouths of five human subjects by the use of an acrylic appliance (Thott *et al.*, 1974). Each specimen was then exposed to the oral environment for up to 21 days and prepared under anaerobic conditions for microbiological analysis of the fissure contents. The

authors concluded that this model seemed well suited for the study of fissure plaque.

In one study, two sampling methods were directly compared. Meiers and Schachtele (1984b) reported that the needle scrape method failed to detect the specific cariogenic bacteria found to be present on fissure removal in four out of the six fissures studied. The authors reported that the needle method also failed to detect viable bacteria present in the fissures although the presence of bacteria was subsequently demonstrated on removal of the experimental fissures. It was mentioned that this could well relate to the physical limitations for needle access and penetration due to the anatomical configuration of the various fissures.

It is apparent that artificial systems may be more effective than the sampling methods using probes, needles or curettes but they do have disadvantages. In the first instance, they are artificial and the conditions they produce may not be an exact replica of the natural fissures when caries is occurring. A second disadvantage is that they are complex to use. This makes these techniques unsuitable for any but studies in small numbers of subjects.

2.5.2 Microbiology of fissure plaque

From the following 10 studies of fissure plaque using artificial fissures in humans (Loe *et al.*, 1973; Theilade *et al.*, 1973; Fejerskov *et al.*, 1976), natural fissures (Folke *et al.*, 1973; Thott *et al.*, 1974; Theilade *et al.*, 1974; 1976; 1978) and in rat molars (Huxley, 1971; Kalberer *et al.*, 1971) it has been found that fissure plaque is essentially predominated by Gram-positive cocci and short rods, a few

Gram-negative organisms, a few filaments and no fusiforms, spirils or spirochaetes. These findings are in contrast to the greater numbers of fusiforms, filaments and spirochaetes that were found in the early stages of gingival smooth surface plaque formation (Theilade *et al.*, 1966).

Plaque outside the fissure proper may be more similar to smooth surface plaque. This was noted in a study where small blocks of natural human fissures prepared from unerupted teeth were implanted into the occlusal surfaces of molars in 12 dental students. Palisaded plaque, which is typical of smooth surfaces, was found only at the entrance to the fissure. A typical smooth surface type of plaque was also identified along the enamel in the bottom of the occlusal groove at the entrance to the fissure proper by Theilade *et al.* (1976).

Changes which occurred in the proportions of micro-organisms with the development of fissure plaque were reported by Thott *et al.* (1974). In this study five human subjects were investigated. Crowns of extracted fully impacted third molars were retained in the oral cavity by means of intra-oral appliances. Each specimen was exposed to the oral environment for up to 21 days and prepared under anaerobic conditions for microbiological analysis of the fissure contents. The fissure plaque was found to consist of large proportions of Gram-positive and Gram-negative cocci (40-70 per cent of the total viable count), slightly smaller proportions of Gram-positive and pleomorphic rods (20-50 per cent) and relatively few Gram-negative rods (5-9 per cent). Filaments were seldom seen during early fissure plaque formation but were recovered (0-2.2 per cent of the total viable count) from samples of older plaque. However, certain changes were found to occur

with the aging of plaque. Gram-positive cocci decreased from 47 per cent to 13 per cent while Gram-negative cocci remained relatively constant (20 per cent) over nine plaque growth periods during 21 days.

In contrast to these, there was an increase in the proportion of Gram-positive rods (30-54 per cent). Gram-negative rods stayed much the same (4 per cent) during the nine plaque growth periods of between 1-21 days. In this study the correlation seen between decreasing Gram-positive cocci and increasing Gram-positive rods with increasing plaque age was found to be statistically significant ($p<0.005$) (Thott *et al.*, 1974). Similar findings were reported by Theilade *et al.* (1974). using small blocks of occlusal fissures (prepared from unerupted third molars) implanted in mandibular molars of six dental students with large amalgam fillings, the authors studied developing plaque in human fissures.

The microflora consisted of 81-93 per cent cocci and 7-19 per cent rods (of the total microscopic counts), and no fusiforms, filaments, spirilla and spirochaetes were seen. The anaerobic total viable counts (AnVc) were found to be 1 to 33 x 10^6 per fissure. Streptococcal counts on Mitis Salivarius Agar (MSA) constituted 29 to 60 per cent of the AnVc and *S. Mutans* constituted 0 to 40 per cent of the colonies on MSA.

Streptococci in fissure plaque

S. mutans

The occurrence of *S. mutans* has been particularly investigated in studies of fissure plaque. *S. mutans* has been reported to constitute a large proportion of streptococcal flora in several fissure plaque studies (Theilade *et al.*, 1978; Ikeda *et al.*, 1973; Loesche and Syed, 1973).

Theilade *et al.* (1978) found that the proportion of *S. mutans* increased with time to a median value of 50 per cent of the streptococcal count at the expense of other colony types present on MSA. This increase was seen in the absence of caries or at least prior to the development of caries. Similar high proportions of *S. mutans* were observed in the streptococcal flora of plaque from caries-active sites and in carious dentine in other studies (Ikeda *et al.*, 1973; Loesche and Syed, 1973). In contrast Theilade *et al.* (1974; 1976) had reported that *S. mutans* formed only a minor proportion of the streptococci in fissure plaque (using natural human fissure blocks) and suggested that this species may colonise the fissures rather slowly. This is in agreement with the slow colonisation by *S. mutans* in artificial and natural fissures observed in some other studies (Thott *et al.*, 1974; Svanberg and Loesche, 1977).

The disagreement seen in findings of *S. mutans* between these studies could be partly a result of such factors as age, number of subjects involved in the studies and study periods but also of sampling techniques used. As for example, samples taken with an explorer in the study of Ikeda *et al.* (1973) seemed to comprise only the flora of the orifice of the fissures. On the other hand, Theilade *et al.* (1974) used occlusal fissures from unerupted third molars implanted in occlusal fillings and by this means samples could have included the flora from deeper parts of the fissures.

The proportion of *S. mutans* has been found to be different in carious and non-carious fissures (Meiers *et al.*, 1982). The fissure sampling technique in this study consisted of removing the fissure using a high speed bur with water spray and then suctioning the fissure

contents into a sterile sampling container. Sixty-eight teeth (from 68 subjects) were sampled and results indicated that total counts as well as the count of colony forming units (cfu's) of the *S. mutans* were higher in carious than in non-carious fissures. All of the 48 carious fissures had detectable levels of *S. mutans* but it was also recovered from 17 of the 20 non-carious fissures. There was a difference in the proportion of *S. mutans* between carious and non-carious fissures. *S. mutans* comprised, on average, 7.3 per cent of the total flora of carious fissures compared to an average of 2.3 per cent of the flora of non-carious fissures. It was reported that there was a tendency for individuals with occlusal caries only (Group I) to have more *S. mutans* in their fissures than in those who also had occlusal and proximal caries (Group II). A difference in the percentage of *S. mutans* isolated from all these subjects was noted when values from the first group were compared with those from a further group who had non-carious fissures.

Other streptococci

Other streptococcal species have been less studied in fissure plaque. However, it has been reported that *S. sanguis* constituted a higher proportion of streptococci in fissures than they do in saliva although they form a smaller proportion than which occurred in smooth surface plaque (Theilade *et al.*, 1974). In the same study *S. sanguis* was present in high numbers (forming up to 78 per cent of the colonies recovered on MSA) in five out of six subjects involved in the study.

Thott *et al.* (1974) reported that *S. sanguis* was found in equal amounts to *S. mutans* (forming up to 89 per cent of the streptococci) in all the subjects involved in the study. It has been reported that

S. sanguis formed a smaller but statistically non-significant percentage of the total count in carious fissures than in non-carious fissures (Meiers *et al.*, 1982). On the other hand, *S. salivarius* has been reported to be recovered in only five out of 45 samples and the authors considered it to be a contaminant rather an indigenous member of fissure plaque flora (Thott *et al.*, 1974). Theilade *et al.* (1974) reported that this species was either absent or constituted less than 5 per cent of the colonies in over half of the samples (n=11) although it was recovered (forming 15-63 per cent of the streptococcal colonies) in the remaining four samples.

Lactobacilli

High percentages of lactobacilli have been reported in artificial fissures with increasing plaque age (Theilade *et al.*, 1973). This was observed when artificial mylar fissures were implanted in the molar teeth of six subjects included in the study. Nine plaque growth periods were observed ranging from 1-21 days. However, other authors have failed to detect these organisms in significant numbers. In one study (Thott *et al.*, 1974) where crowns of extracted impacted third molars were retained in the mouths of five subjects with appliances, lactobacilli were not highly recovered in all nine plaque growth periods (1-21 days). Other studies (Ikeda *et al.*, 1973; Theilade *et al.*, 1974; Meiers and Schachtele, 1982; 1984) have also reported that lactobacilli formed a small proportion of fissure plaque micro-organisms. Similarly, these organisms constituted only a minor fraction of smooth surface plaque and saliva (Van Houte *et al.*, 1972).

Other species of micro-organisms

With regard to *Actinomyces* species, Meiers *et al.* (1982) found that *A. viscosus* comprised a minor proportion of the percentage of total count in both carious and non-carious fissures. On the other hand, in a later study Meiers and Schachtele (1984) recovered *A. viscosus* from eight of the nine fissures included in the study using the same fissure removal method. In the second study it was also noted that the needle method of plaque sampling failed to detect these organisms from the same fissures.

Veillonella has been reported to form a major portion (20-70 per cent of the total viable count) of the fissure plaque microflora in all five subjects included in the study and could be recovered most predictably among the evaluated organisms (Thott *et al.*, 1974). In contrast Theilade *et al*, (1978) reported that *Veillonella* constituted only a minor and variable proportion of the cultivable flora when they studied the microbiology of fissure plaque from 10 natural occlusal fissures implanted for 200-270 days in lower molars of dental students.

It may be concluded therefore that the nature and make-up of fissure plaque is not yet fully understood. Even the presence and relative numbers of particular species has been disputed. Most but not all of the studies were carried out in relatively small numbers of subjects but they were also carried out in adults where caries is less likely to occur.

2.6 SUMMARY OF REVIEW OF THE LITERATURE

From the review of the literature it could be concluded that:

(a) Despite the essential role of bacteria in caries aetiology being well established, studies have failed to show any particular group or species to be solely responsible for the disease.

(b) A relationship between certain organisms (especially *S. mutans*, lactobacilli) and caries has been observed in a number of studies but findings are equivocal.

(c) Recently there has been increasing recognition of the complex nature of the oral ecosystem and of the numerous bacterial interactions which may occur within it. It has been anticipated from results of both animal and human studies of oral micro-organisms that inter-relationships between these organisms may play a crucial role in caries aetiology.

(d) Studies of oral microflora and caries have often been cross-sectional in design. Longitudinal studies are needed since caries occurs over a period of time.

(e) The oral microflora has been studied in relation to salivary flora and also in relation to plaque. Caries is site-specific and occurs more often in fissures. However, fissure plaque has been studied less than has smooth surface plaque. Difficulty in obtaining samples of plaque from these constricted areas may have been one reason for this. Because caries is site-specific, plaque sampled from specific sites may yield particularly valuable information about disease at that particular site. In contrast, salivary microflora may be more relevant to overall levels of caries activity.

3. MATERIALS AND METHODS

The aim of this study was to investigate plaque and salivary flora in a group of children. Following initial planning, a protocol was drawn up. This was submitted to and discussed with the Community Dental Services of Inner London Health District through the District Dental Officer and Community Dental Officers. With their agreement and that of the education authorities, the headteachers from each of two primary schools were approached personally and details of the study were again discussed as well as submitting the formal protocol. The protocol was also submitted to the Joint Research and Ethical Committee of the Institute of Dental Surgery and approval obtained (Fig. 4). Once agreement had been reached, a letter describing the study (Fig. 5) together with a form of consent (Fig. 6) was sent out to the parents of all children in the six and seven year old classes of the two selected schools (including children of 5-8 years). Letters were sent out by the schools to all children in relevant classes. A total of 100 letters were supplied for distribution in the two schools, of which 78 letters were returned. Parents of 64 children (41 from the first school and 23 from the second) gave consent to their children taking part in the study. Parents of 11 children (three from the first school and eight from the second school) did not give agreement to participate. Three more children were excluded as their consent forms were returned without indicating either consent or refusal.

3.1 SUBJECTS

A total of 63 children aged 5-8 years whose parents had given written consent, took part in the study. One child was excluded as he was absent throughout the time when visits were made to collect the first series of samples. Forty-one children (24 boys and 17 girls) were

Eastman Dental Hospital
256 Gray's Inn Road London WC1X 8LD
Telephone 01-837 3646

British Postgraduate
Medical Federation

Dean and Director of Studies: Professor G B Winter MB BDS FDS DCH

22nd February, 1989.

Dear Dr. Wilson,

re: Study of Plaque-Related Diseases in Children

I have now heard from all the members of the Joint Research and Ethical Committee who have given their approval to the above-mentioned study. It may, therefore, proceed.

Yours sincerely,

G.B. Winter

Dr. M. Wilson
Department of Clinical Pathology and Immunology

c.c. Dr. R.D. Holt
Children's Department

Mr. B.T. House

Eastman Dental Hospital
256 Gray's Inn Road London WC1X 8LD
Telephone: 01-837 3646

British Postgraduate
Medical Federation

Department of Children's Dentistry : Professor G B Winter MB BDS FDS DCH

Dear Parent,

Dentists know that one part of the cause of dental decay is the plaque present on teeth. Plaque is made up of large numbers of bacteria in a sticky film that collects on tooth surfaces. It is partly removed by brushing and by chewing. Although we know that plaque is important we do not yet fully understand how it causes decay.

To try to discover more about this process, we at the Eastman are anxious to look in more detail at plaque and dental decay over a period of time. To do this, we need to look at the plaque in children's mouths, when decay is most likely to occur. It is for this reason that I am asking you and your child for help.

To do this, we would like to take a series of simple samples of plaque and saliva from your child's mouth over a period of time.

In more detail, what it would involve would be your child being seen at school for no more than a few minutes on a total of 6 occasions over a one year period. On each occasion one of us would take a gentle scraping of plaque from the surface of one tooth. We would then ask your child to spit into a small individual plastic container after chewing a piece of special wax for not more than 3 minutes.

The school visits would be arranged with the head teachers and staff so as to cause as little disruption to school routine as possible.

I do very much hope that you will feel able to help in what promises to be a very important study.

If you would like further information, please contact one of us at the hospital. We do have the agreement and help of the Community Dental Services in this study.

If you do agree to your child taking part, please would you sign and return the enclosed slip in the envelope provided.

With thanks,

Yours sincerely,

DR. R.D. HOLT
Senior Lecturer in Children's Dentistry

DR. M. WILSON
Senior Lecturer in Microbiology

MR. M. WIN
Research Fellow in Children's Dentistry

Name of child .....

School attended .....

I agree/do not agree to my child taking part in this study. I understand that this will involve him/her having small plaque and saliva samples taken on no more than 6 occasions.

Signed ..... (parent or guardian)

Date .....

from the first school and the remaining 22 children (13 boys and nine girls) were from the second school. Details of the age and sex of the children seen at the start of the study are shown in Tables 5 and 6.

The study commenced in March 1989 and was completed by the end of May 1990. A total of 58 children were seen on three occasions, three were seen on two occasions, and two on a single occasion. Five children left school during the period of the study (Table 7).

3.2 METHOD

The procedures followed are summarised as flow diagrams in Figures 7 and 8.

3.2.1 Clinical procedures

Dental examination

Dental examination was carried out at the schools using a fibre optic light (Safo 20) with interchangeable mirror heads (Mirodent). Diagnosis of caries was based on a visual assessment. Caries was diagnosed when there was a visible cavity considered to involve the dentine. A probe was used to remove plaque and to confirm (or refute) doubtful diagnosis.

Examination of all the teeth was carried out for the children on the first and third sampling occasions before collection of samples. The sampling site was checked for caries before collection of second samples.

Table 5 Age and sex of children - first school

Subject no.	Age at beginning of study (yrs + mths)	Sex	Subject no.	Age at beginning of study (yrs + mths)	Sex
1	5 yrs 8 mths	F	22	6 yrs	M
2	Left	F	23	6 yrs 9 mths	F
3	6 yrs 11 mths	M	24	6 yrs	M
4	7 yrs 5 mths	M	25	6 yrs	M
5	6 yrs 11 mths	M	26	6 yrs	M
6	6 yrs 11 mths	F	27	6 yrs 10 mths	M
7	6 yrs 10 mths	M	28	7 yrs 1 mth	M
8	7 yrs 6 mths	M	29	6 yrs 3 mths	F
9	6 yrs 7 mths	F	30	6 yrs	M
10	6 yrs 8 mths	F	31	6 yrs 1 mth	F
11	7 yrs	M	32	6 yrs 3 mths	M
12	6 yrs 5 mths	M	33	6 yrs	M
13	6 yrs 1 mth	F	34	5 yrs 7 mths	M
14	5 yrs 11 mths	M	35	6 yrs 1 mth	M
15	6 yrs 1 mth	M	36	6 yrs	F
16	5 yrs 11 mths	M	37	6 yrs 6 mths	M
17	5 yrs 8 mths	F	38	6 yrs 8 mths	F
18	6 yrs 3 mths	M	39	6 yrs 9 mths	F
19	6 yrs 8 mths	F	40	5 yrs 7 mths	F
20	6 yrs 1 mth	M	41	7 yrs 6 mths	F
21	5 yrs 10 mths	F			

Mean = 6.42 yrs
Median = 6.45 yrs
SD = 6.15 mths

Table 6 Age and sex of children - second school

Subject no.	Age at beginning of study (yrs + mths)	Sex	Subject no.	Age at beginning of study (yrs + mths)	Sex
1	7 yrs 6 mths	F	12	8 yrs 2 mths	M
2	7 yrs 2 mths	F	13	8 yrs 5 mths	F
3	6 yrs 10 mths	F	14	8 yrs 1 mth	F
4	7 yrs	M	15	7 yrs	M
5	7 yrs 2 mths	F	16	8 yrs 4 mths	M
6	6 yrs 10 mths	M	17	8 yrs 4 mths	M
7	7 yrs 4 mths	M	18	7 yrs 9 mths	M
8	8 yrs 7 mths	M	19	7 yrs 11 mths	F
9	7 yrs 6 mths	M	20	8 yrs 7 mths	M
10	6 yrs 11 mths	F	21	8 yrs 3 mths	M
11	8 yrs 8 mths	F	22	7 yrs 11 mths	M

Mean = 7.7 yrs
Median = 7.83 yrs
SD = 7.6 mths

Table 7 Children involved in the study

	Beginning of study	Left during the study	No.of children who completed the study
First school	41	4	37
Second school	22	1	21
Total	63	5	58

Figure 7 Flow diagram for plaque sample

Plaque sample collected by running the tip of a sterile probe (10) times along the mesio-occlusal fissure of upper left first permanent molar.

Probe tip cut and aseptically transferred into container with glass beads and 1.5 ml of RTF

Vortexed (30 secs)

1 ml in RTF (neat) | 1/10 (0.5 ml RTF in prereduced BHI 4.5 ml) | 1/100 (in prereduced BHI) | 1/1000 (inprereduced BHI)

50 ul spread on to each medium in duplicate

WC	WC + Mz	GNA	Veillonella	Rogosa	Columbia agar,N/A + Mz	MSA	WC (aerobic)
		Incubation at 37°C x 5 days (anaerobic)					Incubation at 37°C x 48 hrs (aerobic)
		Gram stain subculture, aerobic incubation	Gram stain	Gram stain	Gram stain catalase test	Gram stain	
Total anaerobic count	Facultative anaerobes count	Gram-negative anaerobes count	*Veillonella* count	Lactobacilli count	*Actinomyces* count	Total strepto-cocci and differential streptococcal count Representative colonies sub-cultured on WC 37°C x 48 hrs (anaerobic incubation) Rapid identifi-cation tests	Total aerobe count

Figure 8 Flow diagram for saliva sample

Paraffin wax-stimulated saliva
collected in a sterile plastic container

1 ml of saliva

Serial dilutions using 9 ml of normal saline

1/10	1/100	1/1000		1/10,000		1/100,000	
WC	WC + Mz	GNA	Veillonella	Rogosa	Columbia agar,N/A + Mz	MSA	WC (aerobic)
		Incubation at 37°C x 5 days (anaerobic)					Incubation at 37°C x 48 hrs (aerobic)
		Gram stain subculture, aerobic incubation	Gram stain	Gram stain	Gram stain catalase test	Gram stain	
Total anaerobic count	Facultative anaerobes count	Gram-negative anaerobes count	*Veillonella* count	Lactobacilli count	*Actinomyces* count	Total strepto-cocci and differential streptococcal count Representative colonies sub-cultured on WC 37°C x 48 hrs (anaerobic incubation) Rapid identifi-cation tests	Total aerobe count

Figure 7 Flow diagram for plaque sample

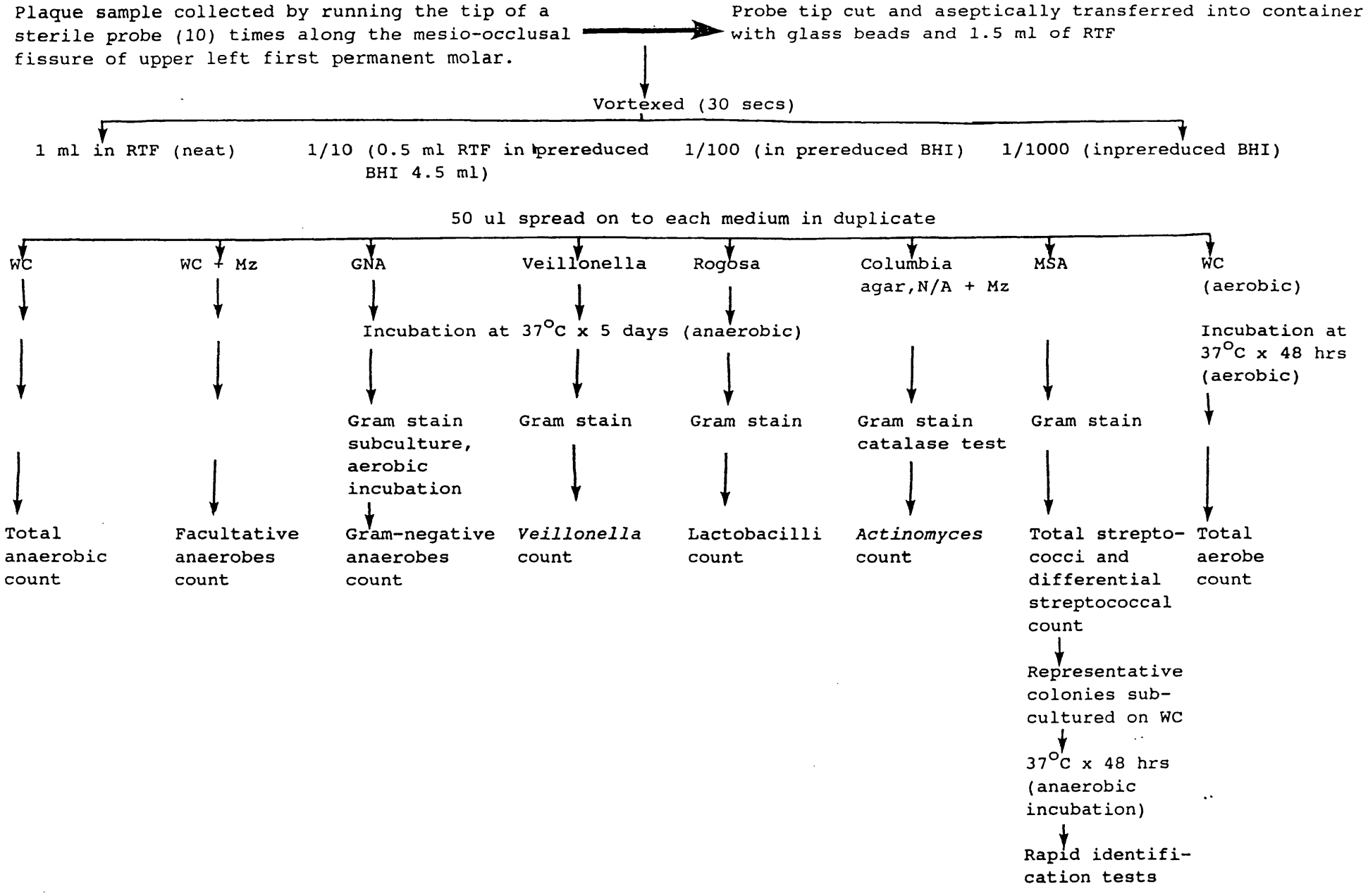
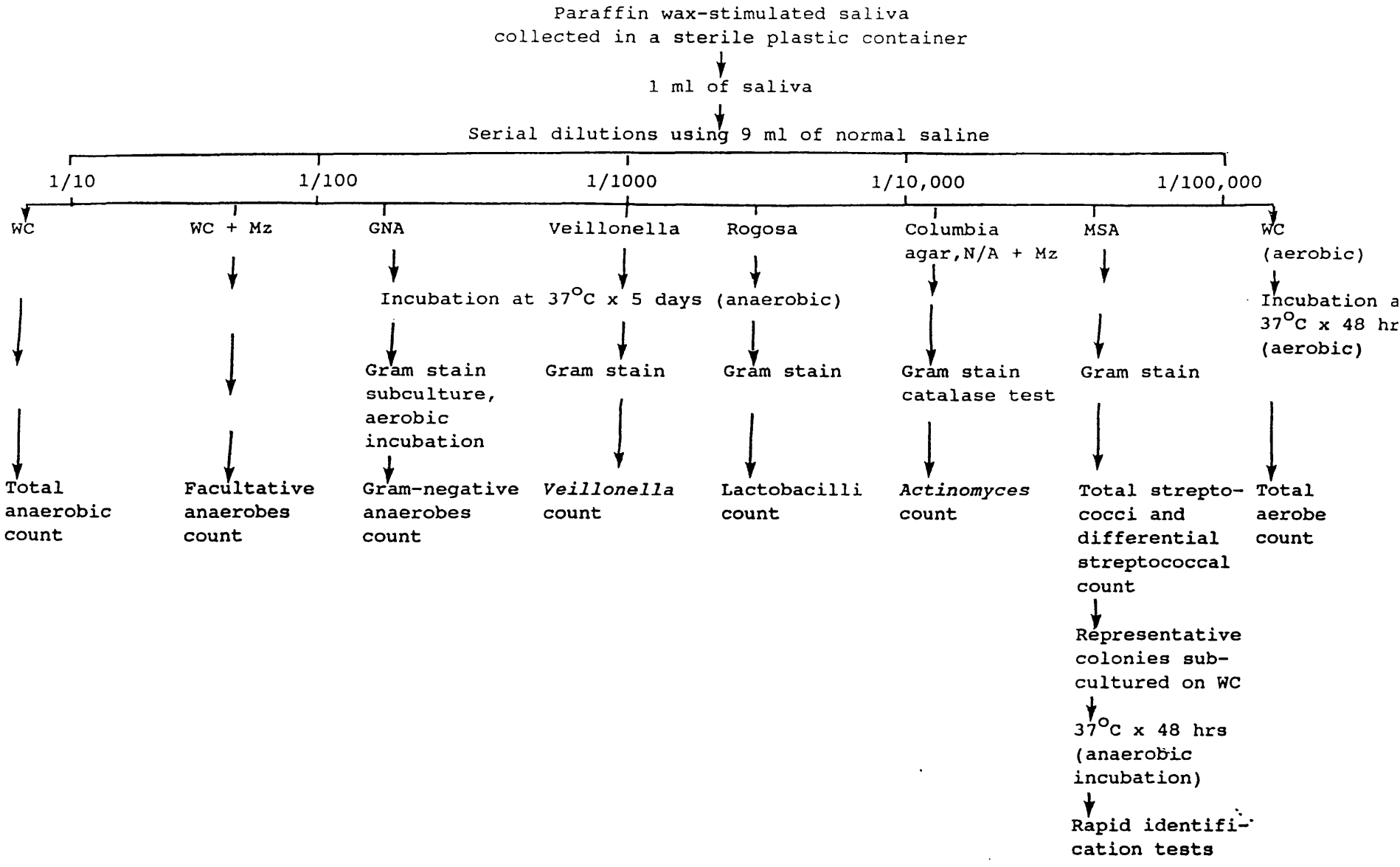


Figure 8 Flow diagram for saliva sample



Reproducibility of diagnosis

During the first study, 10 children were examined by two examiners (MW and RDH) and in the final study, all the children involved in the study were seen by both examiners. Kappa scores were 0.83 and 0.93 respectively indicating good inter-examiner reproducibility. In addition, six children were seen twice by a single examiner (MW) during the first study (Kappa = 0.91) and a total of seven randomly selected children were examined by both examiners on the third occasion (Kappa = 0.8, good agreement for both) to check intra-examiner reproducibility.

Sample collection

Plaque and saliva samples were collected on three occasions (start, six months and 12 months) over a period of one year.

Plaque collection

A plaque sample was collected from the mesial occlusal fissure of the caries-free upper left first permanent molar (/6) using a sterile probe. The probe was passed 10 times along the fissure with gentle pressure and then the tip was cut into a bottle containing glass beads and 1.5 ml reduced transport fluid (Syed and Loesche, 1972). In cases where the /6 had not yet erupted, the same method was used to collect plaque from the adjacent sound second deciduous molar (/E).

Plaque samples were collected from the /6 on three occasions for 43 children and twice for seven children. For the 11 children where this tooth had not erupted until the second sampling occasion, plaque was collected from /E for the final study.

Collection of saliva sample

A sample of stimulated mixed saliva was collected by asking the child to chew a piece of paraffin wax initially for 2-3 minutes before expectorating saliva into a sterile plastic container (Sterilin, England). Saliva collection was continued to obtain a minimum of 2 ml.

3.2.2 Laboratory procedures

Bacteriological procedures were started within 1-2 hours after collection of samples. Both the plaque as well as saliva samples were dispersed by means of a Vortex mixer for 30 seconds.

Culture media used

Total anaerobic count and total aerobic count

One set of Wilkin's Chalgren Anaerobic agar (Oxoid) with 10% horse blood (WC plates) was used for total anaerobic count (i.e. facultative anaerobes and obligate anaerobes) and another set was inoculated aerobically for total aerobic count (i.e. facultative aerobes and aerobes).

Facultative anaerobes

Wilkin's Chalgren and metronidazole 20 mg/litre with 10% horse blood (WC + Mz) was used to cultivate facultative anaerobes.

Gram-negative anaerobes (GNA)

WC agar and 10% horse blood with Gram-negative anaerobes supplement (Oxoid, SR108) was used to recover Gram-negative anaerobes.

Veillonella

Veillonella agar (Difco) with 7.5 ug/ml vancomycin (Rogosa *et al.*, 1956) was used as a selective medium for isolation of *Veillonella*.

Lactobacilli

Rogosa SL agar (Rogosa *et al.*, 1951) was used for isolation of lactobacilli.

***Actinomyces* species**

Columbia agar (Oxoid) and 10% horse blood with 50 mg/litre nalidixic acid and 2.5 mg/litre metronidazole was used as a selective medium for isolation of *Actinomyces* species (Traynor *et al.*, 1981).

Streptococci

Mitis salivarius agar (Difco) with 3.5% potassium tellurite (Oxoid) 2 ml/litre was used as a selective medium for streptococcal species.

Processing plaque samples

0.5 ml of the original plaque sample was added to 4.5 ml of pre-reduced BHI (Brain Heart Infusion, Oxoid Ltd., England) and serial tenfold dilutions were made with pre-reduced BHI to give 1/10, 1/100 and 1/1,000 dilutions of the original sample.

Duplicate 50 ul aliquots of the original sample and appropriate dilutions were spread over the surfaces of a range of selective and non-selective media as described above.

Processing saliva samples

Serial dilutions were made in saline from 1 ml of collected saliva to give 10^{-1} to 10^{-5} dilutions of the original samples. Duplicate 20 ul aliquots from each dilution were cultured on the same range of media as used for plaque samples.

Incubation

Anaerobic

All the inoculated plates apart from WC (aerobic) plates were incubated anaerobically at 37°C for five days.

Aerobic

WC (aerobic) plates were incubated aerobically at 37°C for two days.

Counting and identification of isolates

Total anaerobic, total aerobic and facultative anaerobe counts

Colonies recovered on WC (anaerobic) and WC (aerobic) plates were counted for total anaerobic and aerobic counts respectively. Facultative anaerobes count was obtained by counting colonies recovered on WC and M_3 plates.

Gram-negative anaerobe count

Gram stains were done for representative colonies recovered on GNA plates. Colonies having Gram-negative characteristics (cocci or rods) were subcultured on WC plates and incubated aerobically in the presence of 5% carbon dioxide for 48 hours. Colonies unable to be cultivated in this manner were counted as Gram-positive anaerobes.

***Veillonella* count**

Colonies on *Veillonella* selective medium were checked by Gram staining and very small Gram-negative cocci, 0.3 to 0.4 um in diameter, were counted as *Veillonella* colonies.

Lactobacilli count

Gram stains were performed on colonies recovered on Rogosa SL agar and colonies having Gram-positive characteristics (Gram-positive rods) were counted. On this selective medium, lactobacilli colonies appear as round (0.5-2 mm in diameter), opaque, white and soft colonies.

***Actinomyces* count**

On selective medium *Actinomyces* species appear as round smooth white (0.5-1 mm in diameter), translucent and soft or as irregularly-shaped, white coloured (2-4 mm in diameter) which are adherent and difficult to emulsify. The representative colonies were Gram stained and Gram-positive rods (with or without typical branching) were counted. A catalase test was performed on all colonies.

Streptococci count

Identification of isolates on MSA was based upon their respective colonial morphology on this agar (Table 8). Representative colonies were then subcultured on WC plates and incubated anaerobically for 24-48 hours. Colonies on subculture plates were checked by Gram staining and a series of short tests for streptococci as illustrated in Table 9 was performed.

Table 8 Colonial characteristics of oral streptococci on Mitis Salivarius Agar (from Michalek and McGhee, 1982)

Species	Size (diameter in mm)	Descriptive characteristics
S. mutans	0.5 - 1.5	Raised, convex, undulate opaque colonies of light blue colour; rough margins with granular frosted glass appearance, a glistening bubble often accumulates on top of the colony when excessive glucan is synthesised from sucrose.
S. sanguis	0.5 - 1.0	Raised, smooth colonies which embed into agar and create a colony which adheres to agar medium with great tenacity.
S. salivarius	5.0 - 10.00	Classical gum-drop mucoid colonies which, due to large formation of levan from sucrose, are large, raised colonies on the plate, readily visible to naked eye.
s. mitior (*S. mitis*)	0.5 - 1.5	Flat with small dome in centre, smooth, soft colonies of light blue colour which are on the surface of the agar and easily removed.
S. milleri	0.5 - 1.5	Either convex, smooth, soft colonies of light blue colour which are on the surface of the agar and are easily removed.

Table 9 Tests for identification of oral streptococci

	Mannitol	Sorbitol	Arginine	Aesculin	VP
S. mutans	+	+*	-**	+*	+
S. sanguis	-	-	+	+	-
S. mitior	-	-	-	-	-
S. milleri	-	-	+	+	+
S. salivarius	-	-	-	+*	-

* Some strains negative
** Serotype b strains positive

From Hardie and Bowden (1976b) and Beighton (1985)

Test methods for rapid identification of streptococci

A dense milky suspension from a fresh subculture of the organism to be tested was prepared in 0.25 ml of phosphate buffered saline in a sterile tube for each test. After adding one tablet of reagent (as illustrated in Table 9) to each tube, the tubes were closed and agitated vigorously for a few seconds and incubated at 37°C. Three drops of paraffin oil were added to arginine dehydrolase test tube before incubation.

Arginine dehydrolase and aesculin hydrolysis tests were incubated for four hours before reading the results but the results for rapid carbohydrates (mannitol and sorbitol) and Voges-Proskauer tests were read after 24 hours incubation. Results were interpreted by reading the colour changes as illustrated in Table 10. In cases of doubtful reaction, API 20 STREP (API-Bio Merieux, UK, Ltd.) was used to confirm the results.

Method used for API 20 STREP tests

A well isolated colony was subcultured on Columbia agar (10% horse blood) plate and incubated anaerobically for 24 hours at 35-37°C.

Preparation of the strip

An incubation box, tray and cover were prepared and 5 ml of distilled water was added to the tray in order to create a humid atmosphere.

The strain reference was recorded and the strip was removed from its packaging and placed in the tray.

Table 10 Rapid identification tests for streptococcal species

Test method

A dense milky suspension from a fresh subculture (24-48 hours) of the organism to be tested was prepared in 0.25 ml of phosphate buffered saline in a sterile tube.
After adding one tablet of reagent (A/S Rosco Diagnostic Tablets, Denmark), the tube was closed and agitated vigorously for a few seconds and incubated at 37°C.

Test performed	Incubation period	Positive reaction	Negative reaction
1.Rapid carbohydrates (mannitol and sorbitol)	24 hours	Yellow	Red
2.Arginine dehydrolase 3 drops of paraffin oil added before incubation	4 hours	Purple, red or orange	Yellow or rose
3.Esculin hydrolysis	4 hours	Brown or black	Colourless
4.Voges-Proskauer (VP)	24 hours	Red or pink on addition of 2 drops of 5% alpha napthol (VP2) and 1 drop of 40% KOH (VPI)	Colourless for 10 minutes after adding VPI and VP2

(from Lab M, 1989)

A sterile swab was used to harvest all the colonies on the subculture plate and a dense suspension of this was made in 2 ml of sterile distilled water.

Inoculation of the strip

In the first half of the strip (tests VP to ADH), the suspension was distributed with a sterile pipette, avoiding the formation of bubbles. Three drops with Pasteur pipette were used for test VP to LAP. For test ADH only the tube portion was filled.

The remaining suspension was then transferred to API 20 Strep medium and vortexed for 30 seconds. This suspension was then distributed into the second half of the strip (tests RIB to GLYG) filling the tubes only. Mineral oil was overlaid on cupules of tests ADH to GLYG.

The incubation box was closed and incubated at 37^{o}C for four hours to obtain the first reading and if necessary for a further 24 hours to obtain the second reading. After four hours of incubation the following were added:

1 drop of VP1 and VP2 to VP test

2 drops of NIN for HIP test

1 drop of ZYMA and ZYMB for PYRA, GAL, ßGUR, ßGAL, PAL, LAP tests

Reactions were recorded after 10 minutes by referring to the colour changes (Table 11).

Identification was made by using the identification table (API 20 STREP).

Table 11 API 20 STREP interpretation table

Tests	Substrates	Reactions/Enzymes	Results			
			Negative		Positive	
			VPI + VP2/ wait to 10 min			
VP	Pyruvate	Acetoin production	Colourless		Pink-red	
			NIN/ wait to 10 min			
HIP	Hippurate	Hydrolysis	Colourless/ pale blue		Dark blue/ violet	
			4 hrs	24 hrs	4 hrs	24 hrs
ESC	Esculin	ß-glucosidase	Colour-less Pale yellow	Colour-less Pale yellow Light grey	Grey Black	Black
			ZYM A + ZYM B/10 min (1) If necessary, decolourise with intense light			
PYRA	Pyrrolidonyl 2 naphthylamide	Pyrrolidonylaryl amidase	Colourless or very pale orange			Orange
GAL	6-Bromo-2-naphthyl -D-Galactopyranoside	-galactosidase	Colourless			Violet
BGUR	Naphthol AS-BI	ß-glucuronidase	Colourless			Blue
BGAL	2-naphthyl-ß-D galactopyranoside	ß-galactosidase	Colourless or very pale violet			Violet
PAL	2-naphthyl phosphate	Alkaline phosphatase	Colourless or very pale violet			Violet
LAP	L-leucine-2-naphthyl amide	Leucine arylamidase	Colourless			Orange
ADH	Arginine	Arginine dihydrolase	Yellow			Red

Table 11 API 20 STREP interpretation table (cont'd)

Tests	Substrates	Reactions/Enzymes	Results			
			Negative		Positive	
			4 hrs	24 hrs	4 hrs	24 hrs
RIB	Ribose	Acidification	Red	Orange/ red	Orange/ yellow	Red
ARA	L-Arabinose	Acidification	Red	Orange/ red	Orange/ yellow	Red
MAN	Mannitol	Acidification	Red	Orange/ red	Orange/ yellow	Red
LAC	Lactose	Acidification	Red	Orange/ red	Orange/ yellow	Red
TRE	Trehalose	Acidification	Red	Orange/ red	Orange/ yellow	Red
INU	Inulin	Acidification	Red	Orange/ red	Orange/ yellow	Red
RAF	Raffinose	Acidification	Red	Orange/ red	Orange/ yellow	Red
AMD	Starch (2)	Acidification	Red	Orange/ red	Orange/ yellow	Red
GLYG	Glycogen	Acidification	Red or orange		Bright yellow	

Gram stain method

A smear was made on a slide, dried thoroughly and fixed by flaming.

Ammonium oxide crystal violet was applied to cover the whole slide for 30 seconds.

Washed with water.

Lugols iodine solution applied over the whole slide for 30 seconds.

Iodine solution tipped off the slide.

Decolourised with a few drops of acetone (2-3 seconds).

Washed thoroughly with water.

Carbol fuchsin applied to cover the whole slide (30 minutes).

Washed with water and blot dried.

Catalase test

A small amount of the organism to be tested was picked from a subculture plate (WC) using a clean Pasteur pipette tip. This was then introduced into a drop of hydrogen peroxide on a clean glass slide. A positive reaction was noted by production of gas bubbles.

Calculation of the counts

The total number of organisms recovered per ml of sample was calculated from the average counts and the number of each type was expressed as a percentage of the total anaerobic count.

Counts were entered onto an IBM PCAT[1] computer using a DBase III software[2]. Analyses were carried out using Nanostat statistical package[3]. Data were skew and statistical comparisons were made using non-parametric tests. These involved computation of normal scores which were then used in standard 't' tests and one-way analysis of variance. These procedures are equivalent to Mann-Whitney 'U' tests, for comparison of two groups and Kruskal Wallis one-way analysis of variance for comparision of more than two groups of data.

Study of fissure plaque from extracted teeth samples

Upper first permanent molars which were extracted under general anaesthetic were collected from children attending the Children's Department of the Eastman Dental Hospital. A verbal consent was obtained from the parent(s) before any collection.

Teeth were collected from five children who needed extractions of both upper first permanent molars (one sound and one carious) (Table 12).

A sterile container was used to collect the teeth immediately after extraction and a plaque sample taken from the same site as in the in vivo study as described above. This plaque sample was subjected to bacteriological analysis as described previously. After the plaque sample had been taken, the extracted teeth were stored in 10 per cent formalin for histological analysis.

(1) IBM UK Ltd., Portsmouth, UK.
(2) Ashton Tate, Culver City, California.
(3) Alpha Bridge, London, UK.

Table 12 Extracted teeth sample (sound vs. carious clinically)

Subject no.	Age	Sex	Teeth sampled	
			Sound	Carious
1	8	F	6\	/6
2	9	F	6\	/6
3	8	M	/6	6\
4	8	M	/6	6\
5	9	M	6\	/6

Diagnosis of caries

This was carried out with the aid of fibre optic light using the same visual criteria as mentioned before.

Histological examination

Teeth were examined along the occlusal fissure in a mesio-distal vertical plane using a sectioning machine (Macrotome 2, Metals Research Ltd., Cambridge, England).

The sectioned teeth were viewed under a dissecting microscope (Zeiss, West Germany). A cavity was considered to be present when there was destruction of the enamel and either cavitation in dentine or a diffused, well demarcated discoloured area ranging in hue from light brown to black beneath the amelodentinal junction (Downer and O'Mullane, 1975).

4. RESULTS

4.1 SUBJECTS

At the start of the one year experimental period, plaque and saliva samples were taken from all of the 63 children whose parents had consented to their taking part in the study. However, two children (subject nos. 2 and 18) had left the school between the first and the second sampling occasion six months later and a further three (subject nos. 19, 35 and 43) moved schools before the third samples were collected at the end of the experimental period. The results given below relate to the 58 children who had samples taken on three occasions during the one year period.

All children had stimulated saliva samples taken on each of the three occasions during the experimental period.

With reference to the sampling site for plaque samples, the selected tooth (upper left first permanent molar) had not yet erupted in 15 of the 58 children at the start of the study. For four of these, the tooth erupted before the second sample was taken and for one child it erupted between second and third sampling occasions. This tooth was still unerupted at the time of the final sample collection in the remaining 10 children. (For those children for whom fissure samples could not be taken from the first permanent molar because the tooth was unerupted, equivalent samples were taken from the adjacent second primary molar. Data drawn from these 'substitute' samples were NOT included in results given below but are shown in the Appendix.

Of the 43 children whose upper left first permanent molars were present at the start of the study, one had his tooth restored during the experimental period and in nine cases the tooth was fissure-sealed

before the end of the study. Thus, a total of 33 children had plaque samples taken from the mesial occlusal fissure of their upper left first permanent molar on three occasions during a one year period.

4.1.1 Disease experience

A summary of caries developing during the study period in the 58 children taking part is shown in Figure 9.

At the start 32 children were diagnosed as having some caries experience and the remaining 26 as caries-free. Twelve of these 32 with some caries at the start developed no new lesions during the period of the study whereas the remaining 20 were diagnosed as having one or more new lesions at the final examination. Of the 26 who were caries-free at the start, 20 remained so and the remaining six children developed one or more new lesions. Thus a total of 26 children developed caries at one or more sites during the study.

In relation to the site used for plaque samples, this was caries-free at the start for all of the 33 children for whom the tooth was sampled on three occasions. At the examination at the end of the experimental period, caries was diagnosed at the sampling site in five children but this site was considered to have remained caries-free in the remaining 28 children.

With the exception of one group (Group IV), the groupings used to relate caries incidence to oral microflora were based on caries occurring during the one year period of the study. In Group IV, children were caries-free at the start of and during the study.

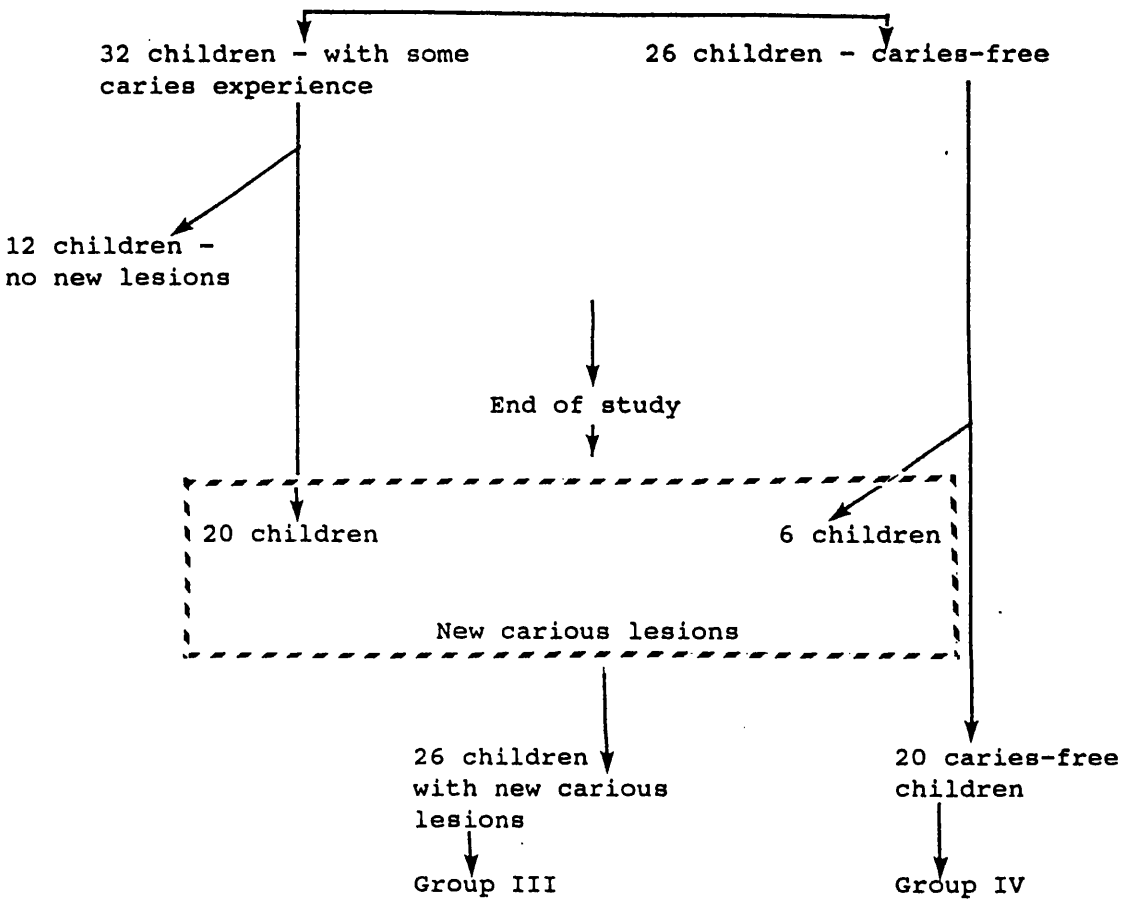


Figure 9 Flow diagram showing disease experience of the children included in the study

Groups I and II were based on caries occurring in the site used for plaque samples and were used for relating plaque microflora to caries. Groups III and IV related to caries occurring on any tooth surface and were used to relate caries to salivary microflora.

4.1.2 Group I - Plaque, children who developed caries in the site sampled during the course of the study

Five children were included in this group. Details have been presented in Table 13. The group was made up of three boys and two girls. The mean age was 7.3 years (SD = 10.9 months) for the five children. Of the five, four had some caries experience at the start of the investigation. One child (subject no. 51) had a dmf value of 2 and the dmf for subject no. 30 was 3. For subject nos. 52 and 63, the dmf values were 7 and 6 respectively. One of the children (subject no. 42) had no evidence of caries although all primary upper incisors were missing, assumed to have exfoliated.

4.1.3 Group II - Plaque, children whose sampling sites remained caries-free during the study (irrespective of caries elsewhere)

Twenty-eight children included in this group had no clinical evidence of caries at the sampling site occurring during the study group. Eighteen boys and 10 girls made up the group (Table 13). Their mean age was 7.16 years (SD = 10.7 months). There was no significant difference in age between the two groups. Sixteen were caries-free at the start and 12 had caries affecting one or more tooth surfaces. The mean dmf was 1.96 (SD = 2.95).

Table 13 Details of children included in Groups I and II

Group I (caries /6) (n=5)			Group II (caries-free /6) (n=28)					
Subject nos.	Age (yrs and mths)	Sex	Subject nos.	Age (yrs and mths)	Sex	Subject nos.	Age (yrs and mths)	Sex
30	6 yrs	M	3	6 yrs 11 mths	M	44	6 yrs 10 mths	F
42	7 yrs 6 mths	F	5	6 yrs 11 mths	M	45	7 yrs	M
51	6 yrs 11 mths	F	6	6 yrs 11 mths	F	47	6 yrs 10 mths	M
57	8 yrs 4 mths	M	7	6 yrs 10 mths	M	48	7 yrs 4 mths	M
63	7 yrs 11 mths	M	9	6 yrs 7 mths	F	49	8 yrs 7 mths	M
			17	5 yrs 8 mths	F	52	8 yrs 8 mths	F
			25	6 yrs	M	54	8 yrs 5 mths	F
			26	6 yrs	M	55	8 yrs 1 mth	F
			27	6 yrs 10 mths	M	56	7 yrs	M
			28	7 yrs 1 mth	M	58	8 yrs 4 mths	M
			29	6 yrs 3 mths	F	59	7 yrs 9 mths	M
			32	6 yrs 3 mths	M	60	7 yrs 11 mths	F
			33	6 yrs	M	61	8 yrs 7 mths	M
			38	6 yrs 8 mths	F	62	8 yrs 3 mths	M
Mean	7.3 yrs		Mean	7.16 yrs				
Median	7 yrs 6 mths		Median	6 yrs 11 mths				
S.D.	10.9 mths		S.D.	10.7 mths				

4.1.4 Group III - Children who developed caries at any site during the study (irrespective of their caries experience prior to the study)

Twenty-six children, 14 boys and 12 girls, were included in Group III as developing at least one new lesion during the study.

Eight children developed one new lesion, 10 developed two new lesions, three developed three new lesions and two children developed four new lesions. One child developed five new lesions during the study and another one had six new lesions.

The mean dmf at the start of the study was 2.88 (SD = 2.59) and the mean age of the 26 children was 6.88 years (SD = 9.53 months) at the start of the study. Details have been presented in Table 14.

4.1.5 Group IV - Children who were caries-free at the start and who remained caries-free throughout the study

Twenty children (13 boys and seven girls) were included. Their mean age was 6.98 years (SD = 12.09 months) at the start of the study (Table 14).

4.2 RECOVERY OF ORGANISMS

Recovery of organisms from each plaque sample is shown in detail in Appendix A in relation to first permanent molars (Appendix A(i) 1-13), second primary molars sampled when the first permanent molar was unerupted (Appendix A(ii) 1-13) and first permanent molars that were fissure sealed during the course of the study (Appendix A(iii) 1-13). Mean and median values for recovery of organisms from plaque of children in Group I (who developed caries at the sampling site), and Group II (where the site remained caries-free) are given in Tables 15 and 16 respectively.

Table 14 Details of children included in Groups III and IV

Subject no.	Age (yrs + mths)	Sex	No.of of new lesions	Subject no.	Age (yrs + mths)	Sex
1	5 yrs 8 mths	F	1	3	6 yrs 11 mths	M
4	7 yrs 5 mths	M	0	7	6 yrs 10 mths	M
5	6 yrs 11 mths	M	1	10	6 yrs 8 mths	F
9	6 yrs 7 mths	F	1	16	5 yrs 11 mths	M
13	6 yrs 1 mth	F	2	24	6 yrs	M
14	5 yrs 11 mths	M	4	25	6 yrs	M
17	5 yrs 8 mths	F	2	26	6 yrs	M
22	6 yrs	M	3	31	6 yrs 1 mth	F
23	6 yrs 9 mths	F	3	36	6 yrs	F
27	6 yrs 10 mths	M	1	38	6 yrs 8 mths	F
28	7 yrs 1 mth	M	2	40	5 yrs 7 mths	F
29	6 yrs 3 mths	F	2	45	7 yrs	M
30	6 yrs	M	1	47	6 yrs 10 mths	M
33	6 yrs	M	2	49	8 yrs 7 mths	M
37	6 yrs 6 mths	M	2	53	8 yrs 2 mths	M
39	6 yrs 9 mths	F	1	54	8 yrs 5 mths	F
41	7 yrs 6 mths	F	1	56	7 yrs	M
42	7 yrs 6 mths	F	2	58	8 yrs 4 mths	M
46	7 yrs 2 mths	F	4	60	7 yrs 11 mths	F
48	7 yrs 4 mths	M	2	61	8 yrs 7 mths	M
50	7 yrs 6 mths	M	2			
51	6 yrs 11 mths	F	3			
55	8 yrs 1 mth	F	2			
57	8 yrs 4 mths	M	6			
62	8 yrs 3 mths	M	1			
63	7 yrs 11 mths	M	5			
Mean	6.88 yrs		2.16	Mean	6.98 yrs	
Median	6 yrs 10 mths		2	Median	6 yrs 10 mths	
S.D.	9.53 mths		1.40	S.D.	12.09 mths	

Table 15 Mean values for the recovery of organisms - plaque - Group I (percentage of total anaerobic count)$^{\phi}$

	Start		6 months		One year	
	Mean (SD)	Median	Mean (SD)	Median	Mean (SD)	Median
Total anaerobic count*	1.02×10^7 (1.8×10^7)	750×10^3	2.6×10^5 (3.4×10^5)	348×10^2	2.8×10^6 (4×10^6)	197×10^4
Total aerobic count*	9.5×10^5 (1.5×10^6)	7.5×10^4	9.9×10^5 (1.8×10^6)	3.1×10^5	1.6×10^6 (1.9×10^6)	1.03×10^6
Facultative anaerobes	498.6 (934.7)	78.3	70.6 (51.7)	62.5	85.9 (21.6)	84.8
Gram-negative anaerobes	0.5 (0.9)	0	1.3 (0.9)	1.5	0.7 (0.5)	0.8
Veillonella	6.4 (5.1)	8.1	22.1 (38.2)	7.2	0.7 (0.5)	0.8
Lactobacilli	0.2 (0.4)	0	1.2 (2.1)	0	0.4 (0.5)	0
Actinomyces	25.0 (27.7)	8.1	5.6 (6.6)	3.9	13.4 (25.7)	0
Total streptococci	13.97 (13.0)	13.5	149.3 (191.6)	104.5	26.7 (37.0)	11.6
S. mutans	8.7 (6.8)	9.9	98.9 (122.9)	73.3	9.9 (21.4)	0.001
S. sanguis	0.5 (1.1)	0	28.3 (38.8)	4.9	3.4 (7.1)	0.01
S. milleri	1.8 (2.0)	1.6	1.7 (2.7)	0	7.1 (9.5)	0.9
S. mitior	2.0 (4.1)	0	19.3 (32.4)	1.7	6.2 (9.5)	0.2
S. salivarius	0.98 (1.9)	0	1.2 (2.4)	0	0 (0)	0

ϕ Because some counts exceeded the total anaerobic count for that sample, some percentages exceed 100.

* Results expressed as the number of colony forming units per ml of sample suspension.

Table 16 Mean values for the recovery of organisms - plaque - Group II (percentage of total anaerobic count)$^{\phi}$

	Start		6 months		One year	
	Mean (SD)	Median	Mean (SD)	Median	Mean (SD)	Median
Total anaerobic count*	5.98×10^5 (1.8×10^6)	475×10^2	2.1×10^5 (3.6×10^5	8000	6.9×10^5 (1.4×10^6)	625×10^2
Total aerobic count*	2×10^6 (4.1×10^6)	2.9×10^5	3.7×10^5 (9.6×10^5)	2.2×10^4	4.8×10^5 (1.1×10^6)	2.5×10^4
Facultative anaerobes	123.6 (277.1)	71.1	136.6 (292.6)	73.9	84.9 (47.4)	80.4
Gram-negative anaerobes	18.3 (83.7)	0.1	0.6 (1.6)	0	14.0 (30.2)	1.8
Veillonella	8.8 (11.8)	3.9	6.3 (12.2)	2.3	12.8 (29.4)	1.3
Lactobacilli	0.1 (0.3)	0	0.2 (0.6)	0	0.6 (2.2)	0
Actinomyces	120.6 (253.7)	57.1	44.1 (66.5)	25.3	75.1 (96.2)	40.8
Total streptococci	87.3 (291.7)	23.5	34.4 (47.8)	23.4	76.2 (175.6)	28.4
S. mutans	72.4 (293.9)	8.8	22.6 (40.2)	12.9	21.6 (58.2)	6.0
S. sanguis	0.1 (0.3)	0	2.8 (6.5)	0	1.4 (3.9)	0
S. milleri	7.3 (12.8)	1.75	0.6 (1.9)	0	25.0 (85.5)	0
S. mitior	6.1 (8.7)	1.05	7.8 (13.4)	1.85	22.1 (39.9)	4.4
S. salivarius	1.5 (4.1)	0	0.6 (2.0)	0	0.6 (3.2)	0

ϕ Because some counts exceeded the total anaerobic count for that sample, some percentages exceed 100.

* Results expressed as the number of colony forming units per ml of sample suspension.

Values derived from culture of saliva samples are similarly shown in detail in Appendix B with mean and median values for Group III (who developed one or more new lesions during the study) and Group IV (who were and who remained caries-free) shown in Tables 17 and 18 respectively.

With the exception of total anaerobic and aerobic counts, which are expressed as the number of colony forming units (cfu), values are given as percentages of the total anaerobic count for the same sample. In the case of saliva, counts represent numbers per ml of the sample. Some percentages exceed one hundred, this is a consequence of counts exceeding the total anaerobic count for that sample.

4.2.1 WC (anaerobic) for total anaerobic count

This count represented the total number of organisms capable of growing under anaerobic conditions, i.e. both strict anaerobes and facultative anaerobes.

In most cases some organisms were recovered on WC medium incubated anaerobically. However few or no organisms were recovered on this medium from nine plaque samples from eight patients. With regard to saliva samples, few or no organisms were not recovered on WC (anaerobic) plates for two subjects on one occasion each.

Details of anaerobic counts for each plaque sample are shown in the Appendix (Tables A(i)1, A(ii)1 and A(iii)1 for plaque and B1 for saliva).

There were some trends for anaerobic counts to be higher in relation to caries; mean values for the total anaerobic count in

Table 17 Mean values for the recovery of organisms - saliva samaples - Group III (percentage of total anaerobic count)$^{\phi}$

	Start		6 months		One year	
	Mean (SD)	Median	Mean (SD)	Median	Mean (SD)	Median
Total anaerobic count*	$3.0x10^8$ ($3.5x10^8$)	$1.7x10^8$	$2.3x10^8$ ($2.5x10^8$)	$1.8x10^8$	$2.7x10^8$ ($2.3x10^8$)	$1.9x10^8$
Total aerobic count*	$1.8x10^8$ ($2.0x10^8$)	$1.1x10^8$	$1.2x10^8$ ($1.1x10^8$)	$1.2x10^8$	$2.7x10^8$ ($2.3x10^8$)	$1.9x10^8$
Facultative anaerobes	134.4 (292.0)	72.8	74.7 (32.3)	67.4	82.6 (48.1)	67.3
Gram-negative anaerobes	46.8 (149.8)	6.3	10.4 (13.7)	4.7	15.1 (20.8)	6.9
Veillonella	53.5 (140.4)	16.3	34.0 (33.4)	22.3	256.5 (1191.6)	14.2
Lactobacilli	0.04 (0.1)	0	2.2 (9.0)	0.002	11.5 (56.6)	0.02
Actinomyces	133.1 365.9)	54.8	12.9 (19.9)	4.6	259.9 (993.1)	69.8
Total streptococci	50.9 (130.8)	15.3	22.1 (33.8)	12.9	28.8 (36.4)	14.1
S. mutans	11.5 (28.7)	2.5	6.8 (12.5)	3.0	5.3 (9.6)	0.9
S. sanguis	8.9 (25.3)	1.3	4.8 (18.3)	0	0.2 (1.3)	0
S. milleri	14.8 (27.6)	4.9	5.5 (12.2)	0.1	10.6 (16.6)	3.5
S. mitior	6.4 (22.1)	0	6.6 (10.7)	2.5	11.5 (19.0)	5.2
S. salivarius	9.9 (38.2)	0.85	1.5 (4.1)	0	1.1 (1.7)	0.01

ϕ Because some counts exceeded the total anaerobic count for that sample, some percentages exceed 100.

* Results expressed as the number of colony forming units per ml of sample suspension.

Table 18 Mean values for the recovery of organisms - saliva samples - Group IV (percentage of total anaerobic count)$^{\phi}$

	Start		6 months		One year	
	Mean (SD)	Median	Mean (SD)	Median	Mean (SD)	Median
Total anaerobic count*	$2.4x10^8$ ($2.3x10^8$)	$1.7x10^8$	$1.8x10^8$ ($1.3x10^8$)	$1.5x10^8$	$2.7x10^8$ ($1.8x10^8$)	$2.6x10^8$
Total aerobic count*	$1.5x10^8$ ($1.8x10^8$)	$1.1x10^8$	$9.2x10^7$ ($8.2x10^7$)	$9.7x10^7$	$2.7x10^8$ ($1.8x10^8$)	$2.6x10^8$
Facultative anaerobes	72.2 (18.3)	73.9	79.3 (23.1)	80.7	77.0 (32.4)	73.5
Gram-negative anaerobes	12.3 (28.7)	4.6	10.3 (13.1)	7.1	16.9 (25.5)	5.2
Veillonella	24.2 (19.8)	15.5	22.3 (17.9)	17.3	26.8 (23.1)	26.1
Lactobacilli	0.002 (0.01)	0	0.003 (0.01)	0	3.3 (12.7)	0
Actinomyces	75.9 (51.2)	63.1	9.2 (13.5)	5.4	51.6 (42.2)	57.2
Total streptococci	38.5 (54.1)	19.4	14.4 (20.9)	4.2	20.9 (18.7)	14.7
S. mutans	6.9 (8.5)	2.8	4.3 (11.9)	0.4	4.2 (5.4)	1.3
S. sanguis	4.2 (18.2)	0.4	4.0 (15.6)	0.15	3.2 (13.7)	0
S. milleri	11.0 (13.7)	5.0	2.4 (5.2)	0.05	8.8 (14.9)	2.0
S. mitior	3.6 (8.3)	0.4	3.2 (4.6)	1.4	4.2 (4.0)	2.8
S. salivarius	7.4 (9.8)	3.8	0.9 (1.8)	0.1	1.9 (5.1)	0

ϕ Because some counts exceeded the total anaerobic count for that sample, some percentages exceed 100.

* Results expressed as the number of colony forming units per ml of sample suspension.

plaque, shown in Tables 15 and 16, were found to be higher in samples from Group I than in those from Group II. The same was true of the median values. However, at no point was the difference between Groups I and II shown to be statistically significant.

For saliva samples (Tables 17 and 18), the mean values for anaerobic counts were also found to be higher for children in Group III (who developed new caries) than for those in Group IV (who were caries-free) for two of the three sampling occasions. Values were similar on the third occasion. However, where differences did occur, these were less marked than those seen between plaque samples and median values for saliva were similar in the two groups.

4.2.2 WC (aerobic) for total aerobic count

This count represented all those organisms capable of growing under aerobic conditions, i.e. strict aerobes and facultative anaerobes. Organisms failed to reach detectable levels on WC plates incubated aerobically for plaque samples of 11 children. For saliva samples, there were two cases where organisms failed to be detected in this culture. As with anaerobic counts, detailed findings are listed in the Appendix (Tables A(i)2, A(ii)2, A(iii)2 for plaque and Table B2 for saliva).

There was little evidence of any consistent difference between groups in aerobic counts in either plaque or saliva samples; the mean and median values for the total aerobic count for plaque samples (Tables 15 and 16) were higher for samples from Group I on two of the three sampling occasions. The exception was on the first occasion, where median and mean were both greater in Group II.

The mean total aerobic count for saliva samples (Tables 17 and 18) was higher for Group III than that for Group IV on the first two samples, although median values were similar. On the third occasion, the mean value was the same with the median being slightly higher in Group IV.

4.2.3 WC + metronidazole for facultative anaerobic count

Detailed results of the facultative anaerobic count in plaque expressed as the percentage of total anaerobic count are shown in the Appendix (Tables A(i)3, A(ii)3, A(iii)3 and B3). No organisms were isolated on WC + metronidazole plates for 11 plaque samples from nine children but cultures were positive for all saliva samples.

The mean counts (which are shown in Tables 15 and 16) showed facultative anaerobes in plaque as forming a more variable percentage of anaerobic counts in plaque from children in Group I than they did in Group II (Tables 15 and 16). Mean values in Group I were higher than those in Group II on the first occasion, lower on the second and broadly similar on the third, with medians following the same trend.

A similar pattern was seen in values for saliva (Tables 17 and 18), with mean values being higher in Group III on the first and third occasions and lower on the second. However, median values were found to be higher for Group III on all three occasions.

4.2.4 Gram-negative anaerobes

The following three types of colonies were cultivated on the selective medium for Gram-negative anaerobes:

(a) Circular colonies (0.5-1 mm in diameter), slightly convex, smooth, translucent and soft.

(b) Slightly bigger (1-2 mm in diameter), flatter, smooth, dry and slightly adherent to the medium. Pale white or greyish in colour.

(c) Irregularly-shaped, flat, smooth, opaque, soft and greenish in colour (1-2 mm in diameter).

No such colonies grew aerobically on WC in the presence of 5% carbon dioxide.

Occasionally, a small number of large (2-3 mm in diameter), smooth, opaque, soft, white colonies were recovered. These were found to be Gram-positive cocci with a catalase-positive reaction and were excluded from counts.

Amongst the total of 174 plaque samples collected there were 89 positive isolates and 85 negative for Gram-negative anaerobes. These organisms were isolated more frequently from saliva with positive counts for 169 of 174 saliva samples.

Details of Gram-negative anaerobic counts, expressed as a percentage of anaerobic counts, are given in the Appendix (Tables A(i)4, A(ii)4, A(iii)4 for plaque samples and Table B4 for saliva).

Gram-negative anaerobes formed a variable percentage of the total anaerobic count in plaque samples in the two groups with variations being greater in Group II than in Group I. Although mean values were higher in Group II on the first and third sampling occasions, this was not true on the second (Tables 15 and 16). In saliva samples also there

was no obvious trend for mean and median values to differ between the two groups (Tables 17 and 18). In the case of saliva, variation was greater in Group III than in Group IV.

4.2.5 *Veillonella*

Colonies recovered on the *Veillonella* selective medium were found to be opaque, greyish-white or sometimes pink in colour, circular, slightly convex and soft in consistency. Some of the colonies were small (0.5-1 mm in diameter) and others larger (1-2 mm in diameter). When checked by Gram staining, these colonies had Gram-negative characteristics (small cocci, 0.3-0.4 um in diameter).

Veillonella were cultured from the majority of plaque as well as the saliva samples studied. They were isolated from 124 of the 174 plaque samples and from 167 of the 174 saliva samples collected during the study. Recovery of *Veillonella* is shown in detail in the Appendix (Tables A(i)5, A(ii)5, A(iii)5 for plaque and B5 for saliva).

The mean number of *Veillonella* was at its highest in Group I on the second sampling occasion. However, examination of median values suggests that this high mean may have been especially distorted in the small group of subjects.

Mean values for *Veillonella* were lower in Group I than in Group II on the first sampling occasion, although the median was higher. Both mean and median were higher than in Group II on the second and lower on the third occasion.

In contrast to this finding, mean values were all higher for saliva samples of children in Group III than for those in Group IV.

Medians showed the same pattern for the first two samples but on the third, the median was higher in Group IV.

4.2.6 Lactobacilli on Rogosa agar

Lactobacilli appeared on Rogosa agar as round, opaque, white, smooth and soft colonies varying from 0.5-2 mm in diameter.

These organisms were cultured from 45 of the 174 plaque samples and 67 of the 174 samples of saliva. Details are given in the Appendix (Tables A(i)6, A(ii)6, A(iii)6 for plaque and B6 for saliva).

In plaque, median values were zero for both groups throughout the study (Tables 15 and 16). Mean numbers of lactobacilli as percentages of anaerobic count exceeded one, only in Group I on the second sampling occasion. In saliva samples also, values were small but showed some evidence of an increase with time in both Group III and Group IV. On all three occasions median values were close to or at zero, but mean values were slightly higher in Group III than in Group IV.

4.2.7 *Actinomyces* on Columbia agar (nalidixic acid and metronidazole

Two types of colonies were basically recovered from this selective medium. They appeared as round, smooth, white, translucent and soft (0.5-1 mm in diameter) or as irregularly-shaped white coloured colonies (size 2-4 um in diameter), which were slightly adherent to the medium and difficult to emulsify.

Gram stains carried out on representative colonies revealed that the majority of these colonies were of small Gram-positive rods (with no branching). In some cases, colonies having the same morphological

characteristics were found to be Gram-positive rods with typical branching. The catalase test was negative for most of the colonies recovered.

Small, white, slightly raised colonies with an irregular outline were occasionally recovered on this medium and these were difficult to emulsify. These colonies were found to be Gram-positive cocci. Details of counts, expressed as percentage of anaerobic counts are shown in detail in the Appendix (Tables A(i)7, A(ii)7, A(iii)7 for plaque and B7 for saliva).

Considering mean and median values shown in Tables 15-18, it can be seen that recovery of these organisms appeared to be consistently higher in plaque from fissures remaining free of caries (Group II) compared to samples from fissures in which lesions developed (Group I). In saliva samples mean values suggested recovery to be higher in samples from children in Group III (who had one or more new lesions develop during the study) than from those in Group IV but medians did not show the same trend, being lower than those in Group IV for two of the three samples.

4.2.8 Streptococci on Mitis Salivarius Agar (MSA)

Morphological characteristics of the colonies recovered on MSA agree broadly with the descriptive characteristics outlined in Table 8 under Section 3. To confirm identity, representative colonies were subcultured and tests performed as described earlier for identification of streptococcal species cultivated on this selective medium.

In the majority of cases, it was possible to interpret the outcome of the rapid identification tests using Table 9 but in some cases the isolate did not appear to belong to any of the defined categories. In these instances API tests were also used to confirm identity.

S. mutans, *S. mitior* and *S. milleri* were the most commonly isolated from saliva and plaque samples with *S. sanguis* and *S. salivarius* being less frequently seen in cultures from this group of children during the study period.

Details of the recovery of streptococcal species on MSA are given in the Appendix (Tables a(i)8-13, A(ii)8-13, A(iii)8-13 for plaque samples and B8-13 for saliva).

Recovery of organisms expressed as the mean and median value for plaque and saliva samples are summarised in Tables 15-18. The values suggest little consistent trend for either total streptococci or the percentage formed by any one species to relate to caries in the group of children studied. The mean and median values for total streptococci were higher in plaque samples taken from children in Group II than in Group I on the first and third sampling occasions but both mean and median were higher in Group I on the second occasion. In saliva samples mean values were less variable and were slightly higher in Group III on all three sampling occasions. This was, however, not mirrored in the median values which were lower than those of Group IV on the second sampling occasion.

Amongst individual species, the mean number of *S. mutans* expressed as a percentage of anaerobic count showed no obvious trend to relate to caries. Mean values were higher in plaque from Group II (caries-free

site) on the first and third sampling occasions but the median number of these organisms in plaque was higher in Group I on the first and second occasion. Both median and mean number of *S. mutans* in plaque had increased between first and second sampling occasions in Group I compared to a fall in mean seen in Group II (Tables 15 and 16). In saliva the mean value for *S. mutans* was consistently higher in cultures from Group III than in those from Group IV but this relatively small difference in means was not borne out by median values, which were lower in Group III on two of three occasions.

S. sanguis, *S. milleri*, *S. mitior* and *S. salivarius* showed little evidence of a relationship to caries in this study. Recovery on culture is given in detail in the relevant Appendix tables and is summarised in terms of mean and median values in Groups I-IV is included in Tables 15-18 for the sake of completeness.

4.3 RATIOS STUDIED

Two inter-relationships between bacterial species have been particularly considered in this study:

1. The relationship between anaerobic and aerobic counts, expressed as the ratio of total aerobic to total anaerobic count.

2. The relationship between *Actinomyces* and *S. mutans*, expressed as the ratio of *S. mutans* to *Actinomyces*.

As in calculation of counts, where no organisms had been cultivated on cultures for total anaerobic counts, values were excluded from statistical analysis. Where an anaerobic count had been made but a

specific micro-organism had failed to reach detectable levels, a percentage value of 10^{-7} was substituted for zero to allow computation.

For purposes of graphic representation, ratios were expressed as logarithms to the base 10 (log ratios).

4.3.1 Ratio of total aerobic to total anaerobic count

Plaque samples

The ratios of total aerobic to total anaerobic counts seen amongst children in Group I and those in Group II are shown in Table 19. Log ratios are shown graphically in Figure 10.

Median ratios in Group I, where caries developed at the sampling site showed variation during the study period. Thus the median number of aerobes to anaerobes rose from 0.04 at the first sample to 1.81 at six months before falling back to 0.45 on the third sampling occasion. In Group II the mean rose slightly from 0.60 to 0.67 and 0.82 at the end of the study period. These variations can be seen in Figure 10. It can also be seen from this Figure that, with a few exceptions, the range of values showed some evidence of a decline with time. The number of samples in Group I was small, but the effect could be seen more clearly in Group II.

Despite these trends, statistical analysis showed no evidence of a significant difference between groups.

Saliva samples

The ratio of total aerobic count to total anaerobic count for children in Groups III and IV are presented in Table 20 and log ratios are shown graphically in Figure 11.

Table 19 Ratio of total aerobic count to total anaerobic count (plaque samples)

Group I (caries /6) (n=5)				Group II (caries-free /6) (n=28)							
Subject no.	Ratio observed Start	6 months	One year	Subject no.	Ratio observed Start	6 months	One year	Subject no.	Ratio observed Start	6 months	One year
30	4.78	11.37	0.44	3	0.65	2.42	0.767	44	27.67	0.48	0.16
42	0.02	1.81	0.18	5	0.28	1.63	1.191	45	379.46	185.45	0.58
51	0.00	6.95	0.41	6	0.05	1.22	0.827	47	0.24	0.49	0.10
57	0.04	0.46	6.47	7	1.85	1003.80	0.372	48	0.33	395.45	0.37
63	19.53	0.05	1.32	9	1.27	0	0.726	49	1.21	0	0.60
				17	3.32	0.79	1.095	52	0.01	2.26	5.22
				25	0.13	0.30	0.822	54	166.30	0.004	0.87
				26	15.23	0.20	4.254	55	0.13	6.20	0.66
				27	0.48	0.46	6.700	56	0.08	0.22	1.53
				28	0.39	0.04	1.696	58	9.58	0	1.45
				29	59.52	0.30	0.207	59	0.15	6.07	0
				32	5894.70	0.38	0.990	60	0.55	0.45	1.02
				33	0.003	<0.001	<0.001	61	0.15	0.37	0
				38	2.36	471.58	1.515	62	201.15	0.67	<0.001

	Start			6 months			One year	
	Group I	Group II		Group I	Group II		Group I	Group II
Mean	4.88	241.69	Mean	4.10	74.25	Mean	1.77	1.24
Median	0.04	0.60	Median	1.81	0.67	Median	0.45	0.82
Range	0.002 to 19.53	0.003 to 5894.7	Range	0.05 to 11.38	0 to 1003.8	Range	0.18 to 6.48	<0.001 to 6.7

Non-parametric statistics showed no significant difference between Groups I and II on any of the three sampling occasions.

0 = Not recovered

Table 20 Ratio of total aerobic count to total anaerobic count (saliva samples)

Group II (caries) (n=26)				Group III (caries-free) (n=20)			
Subject no.	Ratio observed Start	6 months	One year	Subject no.	Ratio observed Start	6 months	One year
1	0.09	0.29	0.59	3	0.65	1.01	0.67
4	1.47	0.35	0.62	7	3.01	0.75	0.82
5	0.58	0.57	22.56	10	0.07	0.26	1.52
9	0.30	0.47	0.71	16	0.95	0.14	3.61
13	0.41	0.34	0.57	24	0.95	0.47	0.21
14	0.56	0.59	0.97	25	1.04	<0.001	0.40
17	0.60	0.54	1.42	26	3.1	0.57	0.52
22	0.002	0.71	<0.001	31	0.12	0.53	0.63
23	0.34	0.23	1.52	36	0.03	0.68	0.71
27	1.83	0.11	119.51	38	0.26	0.66	0.19
28	0.50	0.95	0.28	40	0.31	0.59	0.52
29	0.54	0.99	<0.001	45	1.34	0.76	0.81
30	1.48	0.68	0.41	47	0.83	0.65	0.48
33	0.38	0.73	0.44	49	0.69	0.39	0.57
37	0.28	1.36	0.91	53	0.29	0.77	1.63
39	8.64	0.64	0.35	54	0.45	0.48	0.53
41	2.25	0.71	0.53	56	0.39	0.55	0.68
42	0.80	10.22	0.68	58	0.63	0.03	<0.001
46	0.60	0.71	0.52	60	1.56	0.51	0.35
48	6.88	0.46	0.71	61	0.66	0.14	<0.001
50	0.51	0	0.73				
51	0.59	0.81	0.50				
55	0.30	0.83	0.44				
57	0.73	0.68	<0.001				
62	0.84	0.37	0.64				
Mean	1.23	0.97	6.0	Mean	0.87	0.50	0.74
Median	0.59	0.68	0.58	Median	0.64	0.54	0.053
Range	0.002 to 8.64	0 to 10.22	<0.001 to 119.51	Range	0.03 to 3.1	<0.001 to 1.01	<0.001 to 3.61

Non-parametric statistics showed no significant difference between Groups II and III on any of the three sampling occasions.

0 = Not recovered

Figure 10 Ratio of total aerobic count to total anaerobic count (plaque samples)

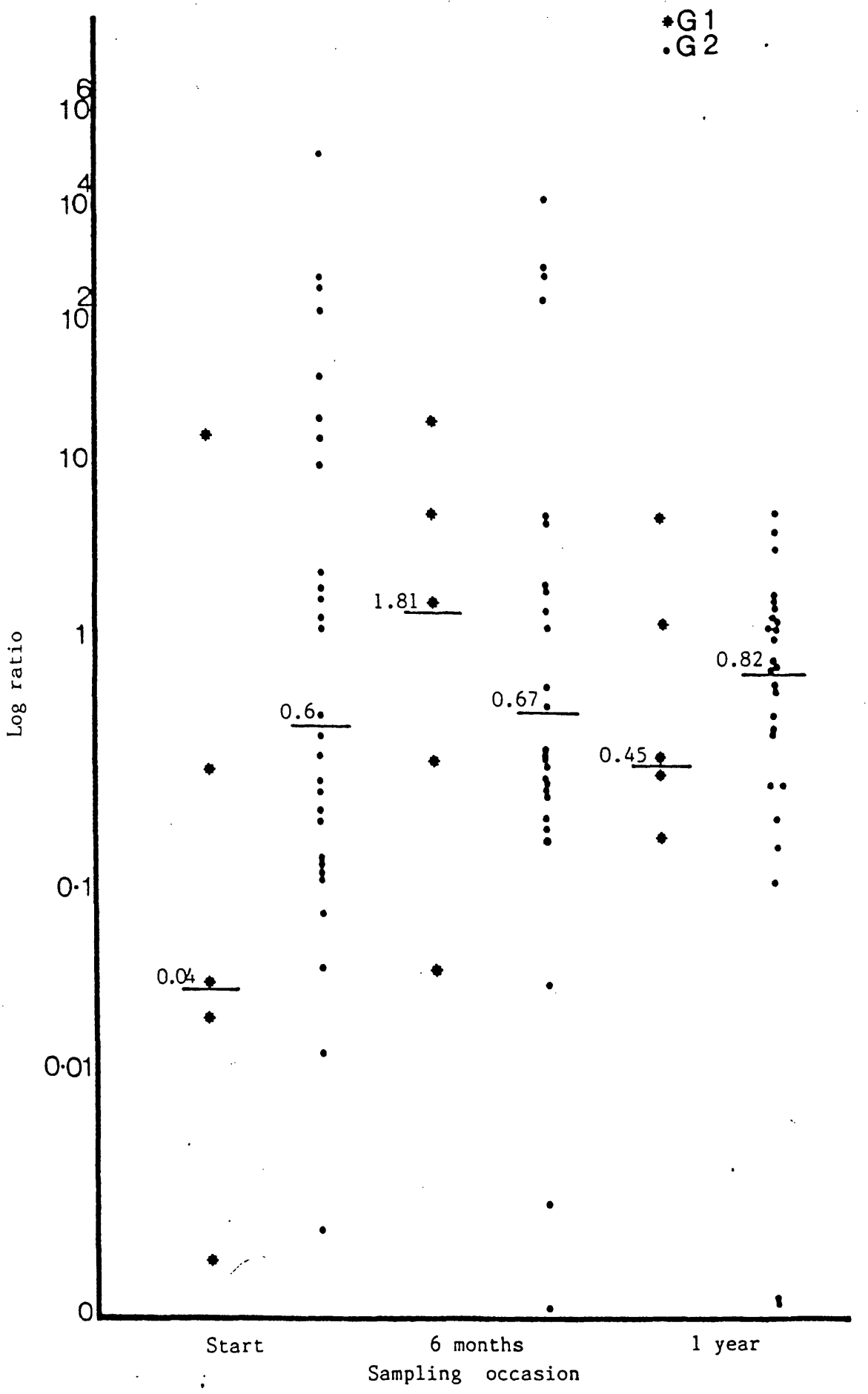
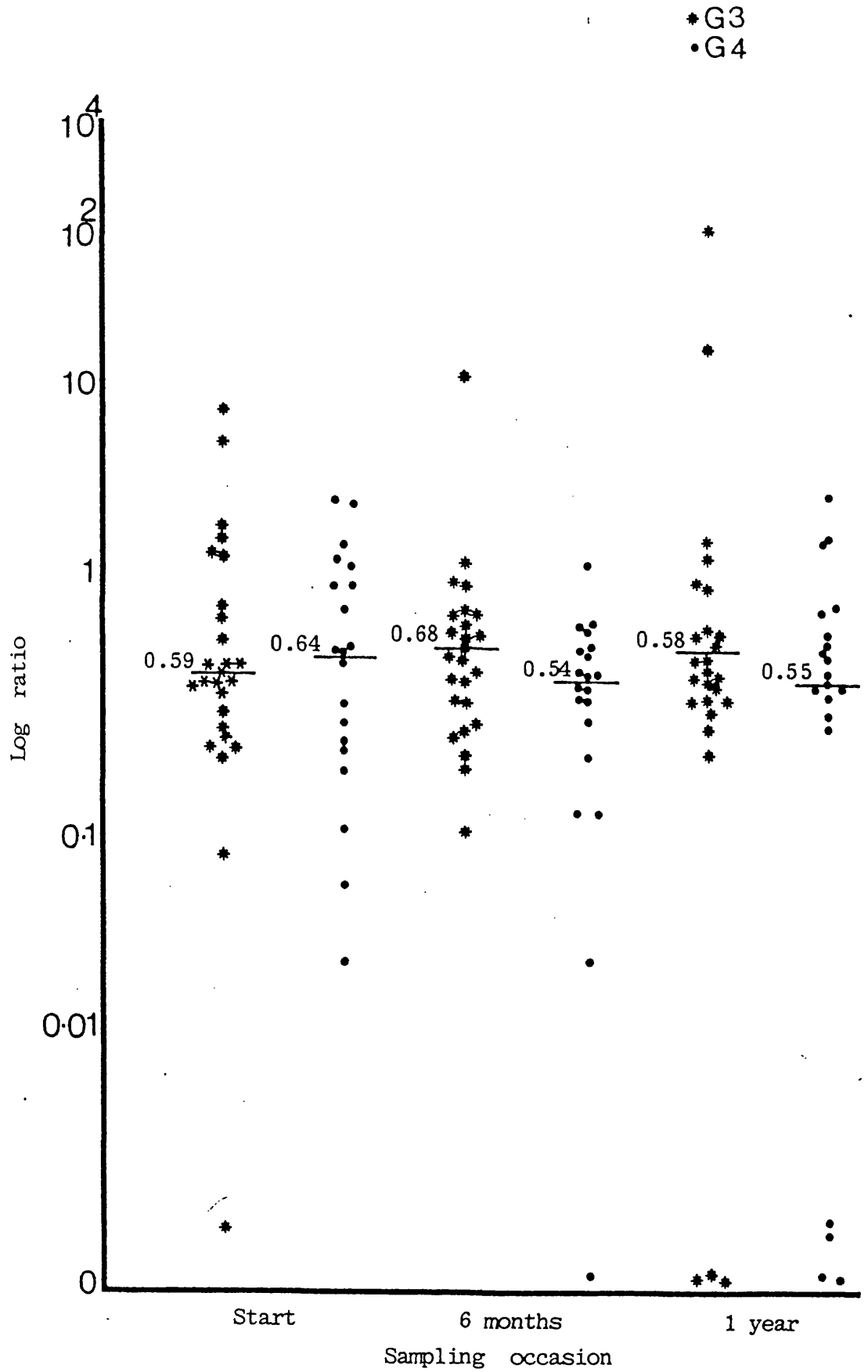


Figure 11 Ratio of total aerobic to total anaerobic count (saliva samples)



Examination of Figure 11 illustrates that median ratios showed little variation either between the groups or between sampling occasions. Median ratios for Group III were slightly higher in saliva on the second and third sampling occasions but the reverse was true on the first.

Agreement between findings in plaque and saliva

After observation of data values, agreement between the ratios observed in plaque and saliva samples from each of the children in the study was rationalised by categorising ratios into four broad groups:

a) Ratios between 0 and 0.59.

b) Ratios between 0.60 and 0.99.

c) Ratios between 1.00 and 10.00.

d) Ratios greater than 10.

Ratios calculated from counts made on the first sampling occasions have been used for this purpose and the level of agreement seen using this system is summarised in Table 21.

It can be seen that the ratio of aerobic to anaerobic organisms fell into the same category in saliva and plaque samples on 14 of 54 occasions on which ratios could be calculated for both saliva and plaque. Twenty-seven of the 40 disagreements suggested the presence of more aerobes to anaerobes in plaque than in saliva but in the remaining 13 the reverse was true.

Table 21 Ratio of total aerobic to total anerobic count (agreement and disagreement between plaque and saliva samples)

Saliva sample	Plaque sample			
	0-0.59	0.6-0.99	1-10	>10
0-0.59	10	1	5	7
0.6-0.99	8	2	5	6
1-10	5		2	3
>10				

Agreement = 14/54 = 25.9%

4.3.2 Ratio of *S. mutans* to *Actinomyces*

Plaque samples

The ratio of *S. mutans* to *Actinomyces* observed amongst children in Groups I and II is shown in Table 22 with log ratios being shown graphically in Figure 12.

Median values for this ratio were 0.71 and 0.15 for Groups I and II respectively at the start of the study, 9.62 and 0.53 for the second sampling occasion and 1 and 0.23 for the final sample. Both groups showed a similar pattern of change with time, having a peak value on the second sample with little difference between first and third occasions. However, on all occasions median values were higher in Group I than in Group II. There were large variations in the ratio within the groups (Fig. 12) and although consistent over the three samples, differences seen between Groups I and II failed to reach statistically significant levels in the 33 study subjects.

Saliva samples

The ratio of *S. mutans* to *Actinomyces* in saliva samples is given in Table 23 with log ratios shown graphically in Figure 13.

Median values for the ratio in Group III were 0.08, 0.42 and 0.03 for the three sampling occasions. Equivalent values for Group IV were 0.04, 0.36 and 0.11. In both groups the ratios for the first and third sampling occasions were similar with there being a higher median on the second sampling occasion. This pattern of change with a peak at six months is broadly similar to that seen for the ratio in plaque samples. There was, however, no evidence of a consistent or a significant difference between Groups III and IV.

Table 22 Ratio of *S. mutans* to *Actinomyces* (plaque samples)

Group I (caries /6) (n=5)				Group II (caries-free /6) (n=28)							
Subject no.	Ratio observed Start	6 months	One year	Subject no.	Ratio observed Start	6 months	One year	Subject no.	Ratio observed Start	6 months	One year
30	0.71	1.87	0.02	3	0.22	<0.001	7.1	44	<0.001	0.62	5.9×10^6
42	1000.0	7.3×10^4	4.8×10^7	5	0.10	0.11	0.82	45	5×10^6	0.56	6.9×10^5
51	1.23	1074.1	1.00	6	1.71	<0.001	<0.001	47	2.40	0.14	1.03×10^7
57	0.21	9.62	1.00	7	0.60	0.27	0.08	48	0.01	5.5×10^7	1.00
63	0.31	0.47	0.001	9	8.83	1.00	0.24	49	0.60	0.67	1.7×10^7
				17	0.10	0.20	<0.001	52	0.17	1.95×10^6	0.53
				25	0.44	0.50	0.21	54	<0.001	1.00	0.15
				26	365.56	0.10	0.76	55	<0.001	2.36	0.03
				27	0.009	12.58	2×10^7	56	0.10	0.24	1.8×10^6
				28	<0.001	2.77	0.18	58	1.06	0.06	0.02
				29	1.43	1.46	0.17	59	0.06	0.44	0
				32	0.13	0.26	<0.001	60	0.007	0.43	0.08
				33	0.05	2.43	0.22	61	0.08	13.44	0
				38	0.14	0.15	0.08	62	0.33	9.46	1.00

	Start			6 months			One year	
	Group I	Group II		Group I	Group II		Group I	Group II
Mean	200.49	178585	Mean	14873.2	0.20×10^7	Mean	0.96×10^7	0.21×10^7
Median	0.71	0.15	Median	9.62	0.53	Median	1.00	0.23
Range	0.21 to 1000.0	3.1E-09 to 5×10^6	Range	0.47 to 7.3×10^4	2.9E-09 to 5.5×10^7	Range	0.001 to 4.8×10^7	3.02E-09 to 2×10^7

Non-parametric statistics showed no significant difference between Groups I and II on any of the three sampling occasions.

0 = Not recovered

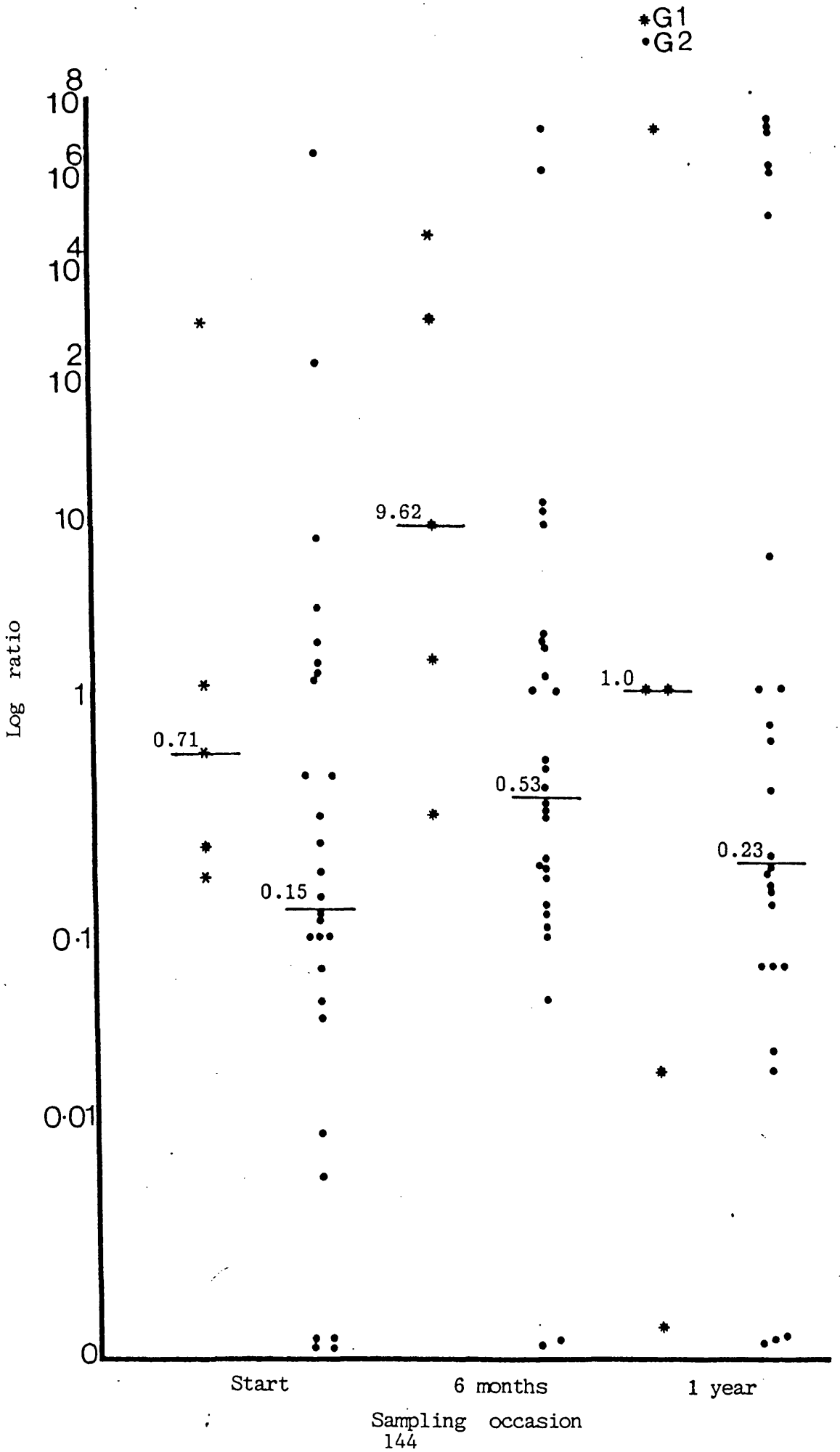
Table 23 Ratio of *S. mutans* to *Actinomyces* (saliva samples)

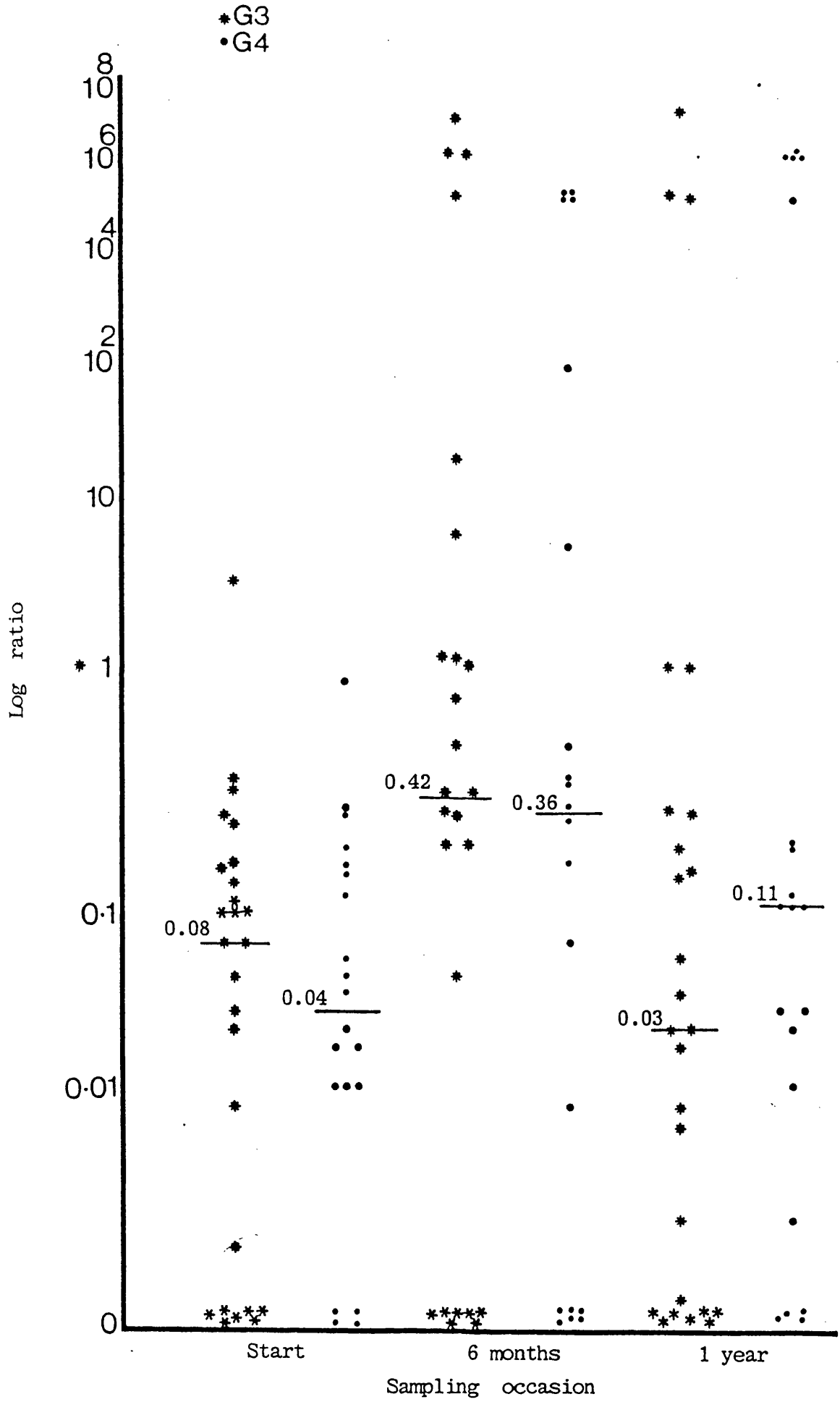
Group III (caries) (n=26)				Group IV (caries-free) (n=20)			
Subject no.	Ratio observed Start	6 months	One year	Subject no.	Ratio observed Start	6 months	One year
1	4.95	1.51×10^{7}	0.05	3	0.03	<0.001	0.01
4	0.19	<0.001	0.009	7	0.18	<0.001	0.11
5	0.18	<0.001	0.02	10	0.05	3.8×10^{5}	0.11
9	0.003	8.86×10^{6}	<0.001	16	0.22	0.48	0.22
13	<0.001	<0.001	0.03	24	0.01	3.1×10^{5}	<0.001
14	<0.001	<0.001	0.17	25	0.91	0.08	<0.001
17	0.46	1.41	<0.001	26	0.13	0.009	0.04
22	0.08	<0.001	0.03	31	<0.001	2.5×10^{5}	<0.001
23	0.37	<0.001	0.38	36	0.01	0.38	0.13
27	<0.001	0.43	<0.001	38	0.07	<0.001	<0.001
28	<0.001	0.36	0.22	40	0.06	8.4×10^{5}	0.03
29	<0.001	8.6×10^{5}	2.78×10^{7}	45	0.02	<0.001	3.09×10^{6}
30	0.31	0.23	0.36	47	0.17	<0.001	2.7×10^{5}
33	<0.001	3.04×10^{6}	0.16	49	0.02	0.50	1.2×10^{6}
37	0.009	<0.001	0.001	53	<0.001	0.18	1.25×10^{6}
39	0.08	0.06	<0.001	54	<0.001	0.33	0.11
41	0.10	0.43	<0.001	56	<0.001	6.66	1.05×10^{6}
42	0.04	1.08	4.4×10^{5}	58	0.01	<0.001	0.004
46	0.10	7.07	<0.001	60	0.37	0.64	0.04
48	0.03	0.23	1.00	61	0.39	92.85	0.24
50	0.06	0.65	4.2×10^{5}				
51	0.50	0.86	1.00				
55	<0.001	0	0.07				
57	0.15	0.38	<0.001				
62	0.11	1.25	0.008				
63	0.10	28.96	0.004				
Mean	0.30	1.07×10^{6}	1.1×10^{6}	Mean	0.13	8.9×10^{4}	2.1×10^{5}
Median	0.08	0.42	0.03	Median	0.04	0.36	0.11
Range	3.0E-09 to 4.95	1.2E-08 to 1.51×10^{7}	8.3E-09 to 2.7×10^{7}	Range	1.3E-08 to 0.91	2.0E-08 to 8.4×10^{5}	1.3E-08 to 3.09×10^{6}

Non-parametric statistics showed no significant difference between Groups III and IV on any of the three sampling occasions.

0 = Not recovered

Figure 12 Ratio of S. mutans to Actinomyces (plaque samples)




*G3
•G4
8
10
6
10
4
10
2
10
10
* 1
0·1
0·01
0
Log ratio
0.08
0.04
0.42
0.36
0.03
0.11
Start
6 months
1 year
Sampling occasion

Agreement between plaque and saliva samples

Using the same system as was defined in Section 4.3.1 in relation to the ratio of aerobic to anaerobic organisms, the level of agreement for the ratio of *S. mutans* to *Actinomyces* on the first sampling occasion is shown in Table 24. Ratios for 34 of the 58 children fell into the same broad category for saliva and plaque. Thirty-three of the "agreements" were in the range 0.0-0.59 and one was between 1 and 10. For 22 of the 24 children with a disagreement the number of *S. mutans* to *Actinomyces* was higher in their plaque than in their saliva sample.

4.5 STUDY OF PLAQUE SAMPLED FROM FISSURES OF EXTRACTED TEETH

As a further method to investigate the two bacterial inter-relationships considered, the same ratios as described under Section 4.3 were calculated for plaque sampled from five pairs of upper first permanent molars, one of each pair being sound and one carious (as diagnosed clinically using the visual criteria) and each pair being collected from the same child. Results are presented in Tables 25 and 26.

All these 10 teeth from which plaque had been sampled were afterwards sectioned and subjected to examination as described under Section 3.2. It was found that all the five teeth which had been diagnosed clinically as carious at the sampling site were also confirmed by examination on section as carious.

Of the five teeth which had been diagnosed clinically as having no visual evidence of caries involving dentine, three had no evidence of caries at all and two (those from the first and fifth pair of teeth) showed evidence of early lesion formation confined to enamel.

Table 24 Ratio of *S. mutans* to *Actinomyces* (Agreement and disagreement between plaque and saliva samples)

Saliva sample	Plaque sample			
	0-0.59	0.6-0.99	1-10	>10
0-0.59	33	4	11	7
0.6-0.99	1			
1-10	1		1	
>10				

Agreement = 34/58 = 58.62%

Table 25 Ratio of total aerobic count to total anaerobic count (plaque sampled from extracted 6/6)

	Teeth carious on visual examination	Teeth caries-free on visual examination
Subject no.	Ratio observed	Ratio observed
1	0.66	1.09
2	1.03	1.78
3	1.11	0.40
4	0.009	0.65
5	0.69	0.74
Mean	0.70	0.93
Median	0.69	0.74
Range	0.009 to 1.11	0.40 to 1.78

Table 26 Ratio of *S. mutans* to *Actinomyces* (plaque sampled from extracted 6/6)

	Teeth carious on visual examination	Teeth caries-free on visual examination
Subject no.	Ratio observed	Ratio observed
1	<0.001	0.02
2	<0.001	0.11
3	57×10^6	25×10^6
4	0.34	<0.001
5	28×10^6	26×10^5
Mean	17×10^6	5.5×10^6
Median	0.34	0.11
Range	<0.001 to 57×10^6	<0.001 to 25×10^6

Histological examination was in agreement with clinical diagnosis since the level of clinical diagnosis chosen was of caries involving dentine.

For the ratio of total aerobic to total anaerobic count (Table 25), it was found that this ratio was higher for plaque from fissures with no visual evidence of caries in four of the five pairs including in the two in which enamel caries was visible on section. Median values for the ratios seen in plaque samples from carious fissures were 0.69 compared to 0.74 in plaque from apparently caries-free sampling sites (Table 25).

The ratio of *S. mutans* to *Actinomyces* was higher for plaque from fissures of extracted teeth with clinical evidence of caries in three of the five pairs. In two the ratio exceeded $25x10^6$ in both members of the pair. The median ratio was 0.34 for plaque from carious fissures and it was 0.11 for teeth caries-free on visual examination (Table 26).

Statistical analysis was not conducted for these ratios as the number of pairs of teeth available had been very small.

5. DISCUSSION

5.1 INTRODUCTION

This study has investigated the flora of fissure plaque and of saliva in a group of children. The major aim was to investigate changes in the oral microflora with the development of caries and the investigation was designed to this end. Fissures are the most common site for caries to occur but fissure plaque has been studied less than smooth surface plaque and studies of fissure plaque have been carried out more often in older subjects than in children where caries is most likely to occur. Previous studies have often been cross-sectional but this study was carried out over a 12 month period with sequential samples taken from a specific site. The children who took part were aged between five and eight years at the start of the one year study, an age when caries is especially likely to occur. Selection of the sample site was made on the same basis; samples were taken from the upper left first permanent molar. It has been reported that, within two years of eruption (with a median age of eruption of just under six years) 37% of children in the United Kingdom had caries affecting at least one first permanent molar (Todd and Dodd, 1985). In the present study, five of the 33 children in whom the tooth was present and caries-free at the start of the study and who did not have it either filled or fissure sealed during the experimental period, developed caries at the sampling site; a finding that confirms as reasonable the choice of age group and site.

The diagnosis of occlusal caries is notoriously difficult. For the purposes of the study, diagnosis was made using visual criteria. This method has been used in other studies and has been reported to be efficient. Downer (1975) has reported that there was no statistically

significant difference between a visual method and a method in which a probe was also used in terms of validity. Sensitivity of the two methods was 0.91 and 0.92 and specificity was 0.81 and 0.85 respectively.

In considering caries diagnosis, especially at the sampling site, it must be borne in mind that dental caries formation is no longer regarded as a one-way process of demineralisation but as a two-way process which involves intermittent periods of demineralisation and remineralisation. Therefore, caries progression from ultrastructural changes to visible decay is a cumulative effect of a long series of alternate dissolution at low pH and a partial re-precipitation when the pH rises (Mellberg, 1988). In the initial stages, the lesion is sub-microscopic and not visible. Thus, by the time it can be diagnosed clinically, the lesion has progressed to a considerable size. The plaque microflora studied may thus relate to caries initiation and progression even when the site is apparently free of disease.

The study included investigation of samples of plaque and of saliva; salivary microflora may be thought to have a less close relationship to caries than that of plaque. It represents a pool of micro-organisms dislodged from soft tissues in the oral cavity such as the tongue and cheek, as well as from hard tissues. Streptococci are the predominant species amongst salivary organisms and *S. salivarius* is usually present in high numbers. Other organisms such as *Veillonella* and Gram-positive rods and filaments are also present in significant numbers. *Neisseria* and fusobacteria are also present in variable proportions in saliva. Saliva is the main fluid environment of bacteria colonising hard and soft tissue surfaces and it has a major influence on

the oral ecology. For example, alteration in salivary flow following surgery can have a dramatic effect on the composition of the oral flora. In these circumstances, the microflora changes towards one that is highly acidogenic with an increase in numbers and proportions of *S. mutans*, lactobacilli and *Actinomyces* and a decrease in *S. sanguis*, *Neisseria*, corynebacteria and *Veillonella* (Kleinberg, 1977).

Salivary microflora does not directly indicate the ecology of any one of the other compartments present in the mouth but it seems likely to provide an overview of the changes occurring in the microbial composition of the oral ecosystem as a whole.

Despite the fact that wide differences are well understood to exist between salivary microflora and those of other compartments, relatively few studies have directly compared these by taking both types of sample from the same individual as was carried out in the present study. This method makes it possible to consider variations at the level of individuals as well as in more general terms.

It is apparent from assessment of the results that few, if any statistically significant differences were present between figures for different groups of subjects. Caries varies widely in the extent and severity to which individuals are affected. Although the number of children developing caries during the study period was no lower than might be expected, the small numbers of subjects included in the groups (e.g. Group I) makes statistical interpretation of results difficult. This problem is aggravated by the very wide variations that are known to occur both within and between subjects in the numbers and proportions of micro-organisms making up the oral microflora. Despite these

difficulties some trends were apparent. Whilst the lack of statistical significance may reflect the fact that these were a consequence of sampling error, it must be remembered that real differences may have been masked by the variations seen.

5.2 RECOVERY OF ORGANISMS

As has been emphasised in reviewing the literature, the majority of studies of the role of oral micro-organisms in caries aetiology have been concerned with specific groups or species, particularly streptococci (especially *S. mutans*) and lactobacilli. A relationship between *S. mutans* and caries has been demonstrated in many, but not all investigations. However, it is now recognised that the relationship may not be straightforward. In the current study too, there was a lack of clear cut evidence of an association between *S. mutans* and caries. In saliva samples, mean values for the percentage of *S. mutans* were higher for children who developed caries than in those who had remained caries-free up to and during the period of the study, although differences between the groups failed to reach significance. However, in plaque samples, where the relationship would be thought to be closer, mean and median levels of *S. mutans* were higher in the teeth that remained apparently caries-free on the first sampling occasion, higher in teeth in which caries may well have been progressing at six months and little different in the two groups at the end of the study. Only on the second sampling occasion did *S. mutans* appear to be prominent amongst the plaque flora in teeth which developed caries. However, even at this point the difference between fissures that did and did not demonstrate clinical caries was not statistically significant, suggesting that this was by no means a consistent finding in the group.

Lactobacilli have also been especially related to caries but in this study the organism was cultured from relatively few of either the saliva or the plaque samples. Median values were all zero and mean values showed no obvious trend to differ between children who did and did not develop caries.

Two groups of organisms did however show a trend for a more consistent relationship to caries, at least in plaque samples; anaerobes and *Actinomyces*.

In the case of anaerobes, mean and median counts were consistently higher in plaque samples derived from sites in which caries developed during the study period. Anaerobes were expressed as absolute counts and it may be thought that the higher numbers represented a heavier inoculation of micro-organisms as a whole. However, aerobes, which were expressed in the same way, did not show the same pattern. Anaerobes are favoured by thicker plaque formation and have been shown to increase with increasing plaque age suggesting that thicker plaque of greater age was found in children who developed caries in the underlying tooth surface. This would agree with the conclusion of other authors who suggest not only that anaerobes are more caries conducive than aerobic organisms but also that the dental plaque must have a certain age to be able to produce carious lesions (Mikkelsen and Poulsen, 1976). It has also been suggested that as the carious lesion deepens into dentine and becomes covered by a thicker layer of plaque, facultative and obligate anaerobes are predominant in such lesions (Loesche and Syed, 1973).

No similar trend in respect of anaerobes was seen in saliva samples, but the environment of saliva may be less favourable to

anaerobes, since it contains dissolved oxygen, perhaps concealing a genuine difference in the mouth as a whole.

In plaque, mean and median counts of *Actinomyces* were consistently and markedly (although not significantly) higher in samples from the teeth which remained caries-free, suggesting a negative relationship to caries. In the case of saliva, the reverse trend was seen with mean and median values being higher in children who developed caries.

Previous work has demonstrated *Actinomyces* species to be capable of producing caries in animals (Jordan and Hammond, 1972; Jordan, 1972) and to be associated with root surface caries (Sumney and Jordan, 1974; Syed *et al.*, 1975)) and nursing caries (Milnes and Bowden, 1985). *Actinomyces* have also been isolated from deep dentinal lesions (Hoshino, 1985) and it may be that in root surface caries and nursing caries this group of organisms is associated with dentine, rather than enamel, caries. In the present study, many children who developed new lesions already had caries present elsewhere in their mouths, often involving dentine and not always satisfactorily restored. This could have contributed to the higher levels of *Actinomyces* seen in saliva samples of children who developed caries. The negative relationship seen in plaque may relate partly to caries at this site being at an earlier stage, with involved dentine (and *Actinomyces*) being confined to the deepest parts of the fissure.

No attempt was made to identify different species of *Actinomyces* and it may be also that species differed in the two sample types. In plaque, the presence and numbers of a species do not in themselves imply pathogenicity, or lack of it. They may rather provide an indirect

indicator of a given environment. *Actinomyces*, for example have been found to be resistant to sodium fluoride (Willett, White and Rasen, 1991), itself well known to be associated with an increased resistance to carious attack. A high fluoride level in plaque, or in the adjacent part of the tooth surface may thus have produced a population shift within the plaque, critically affecting the numbers of some species to the benefit of others. Some, but not all, authors have demonstrated that high concentrations of fluoride in gels or pumice pastes may cause a selective reduction in *S. mutans* (Thylstrup and Fejerskov, 1986).

The concept of the ecology of plaque being as important as the presence and numbers of any one species is assuming increasing value. With increasing recognition of the complex nature of the oral ecosystem, studies carried out in recent years have given more consideration to the interactions between plaque micro-organisms and their role in caries aetiology rather than to a particular species.

Drawing on conclusions of other authors and from trends seen in this study in respect of single organisms and groups of organisms, two particular relationships have been selected for consideration in relation to caries activity:

1. That between aerobic and anaerobic groups of micro-organisms.

2. That between *Actinomyces* and *S. mutans* (expressed as a percentage of anaerobic groups).

5.3 THE RELATIONSHIP BETWEEN AEROBIC AND ANAEROBIC COUNTS

When the relationship between aerobic and anaerobic organisms in plaque microflora was studied it was found that there was a variation in

the ratio of total aerobic count (comprising aerobes and facultative anaerobes) to the total anaerobic count (comprising anaerobes and facultative anaerobes) amongst children who developed caries at the sampling site. The median ratio for this group (Group I) was 0.04 at the first sampling occasion, rose to 1.81 on the second occasion and fell to 0.45 in samples taken at the end of the 12 month study period. The ratio in plaque samples derived from children in whom the site remained caries-free was more constant, the median being 0.60 on the first, 0.67 on the second and 0.82 on the third, with means following a similar pattern. What was also apparent was that the range of values in both groups showed some evidence of a reduction with time. It may be thought therefore that a fluctuation in the relationship between the two groups of organisms occurred with the development of caries. In contrast, the relationship between the two showed a lesser change with time in fissures that were caries-free. In both groups the ratio seemed to become less variable, perhaps suggesting some degree of consistent equilibrium with increasing time since tooth eruption. The differences seen in this ratio between the two types of site could have related to differences (and changes) in either group of organism. The difference seen in anaerobe count between children who did and children who did not develop caries has already been discussed but not the changes in the count with time. In the course of the study, the mean number of anaerobes in plaque samples from those who developed caries declined between the first and second samples and recovered by the third, whereas aerobic counts were more static. Thus, changes in the relationship between the two must have related more to change in anaerobes.

The organisms making up the total anaerobic count represented a very large group. Facultative anaerobes, which were also cultured separately during the study showed no trend to be present in greater numbers in plaque overlying surfaces in which caries developed in the same way as anaerobes as a whole, suggesting that the difference in anaerobes was a consequence of the numbers of obligate anaerobes such as *Veillonella*, *Bacteroides*, etc. Facultative anaerobes also did not show the same pattern as anaerobes in the change in mean and median with time.

At least three factors may have influenced the difference seen in plaque in the ratio between carious and sound sites. First, plaque used in the present study was from fissures and it has been reported that fissure plaque is usually composed of few aerobes, such as *Neisseria* spp. and is mainly composed of facultative anaerobes such as streptococci, lactobacilli and *Actinomyces* and obligate anaerobes (mainly *Veillonella* spp.) (Theilade *et al.*, 1974; Marsh and Martin, 1984). The relative proportions of aerobes and anaerobes may reflect differences in fissure morphology with wider and more accessible fissures harbouring higher numbers of aerobes. Such fissures may be less likely to develop caries.

Secondly, differences in the ratios may also show a difference in the site from which plaque was drawn; even in a single fissure, anaerobic organisms may form a greater proportion of the microflora present in the deeper parts of the fissure than they do in plaque nearer to its portal of entry.

Thirdly, a shift in composition of plaque from mainly aerobic to facultative anaerobic species in the early stages of colonisation to a situation where facultative anaerobes and obligate anaerobes predominate has been recognised as a normal feature of developing coronal plaque (Ritz, 1967). The relationship seen between aerobic and anaerobic groups of micro-organisms may thus be an indication of the stage of bacterial succession reached at that site at that time. Plaque that is younger and has been more recently disturbed by toothbrushing for example, may have greater numbers of aerobes present in relation to anaerobes. Children who brush their teeth more regularly and who have a more stable dietary pattern may demonstrate a more static relationship between the two types of micro-organism. Such children may also be less likely to develop caries. Children who brush the sampling site more erratically and whose dietary habits are less regular may show a more erratic pattern in the relationship as well as demonstrating a higher caries level. Such effects would seem especially likely to be apparent in samples taken at a particular time of the day and week, as were samples in the present study.

Between the second and third sampling occasions there was some evidence of a fall in the relative numbers of aerobes to anaerobes in samples overlying lesions detected at the final sampling occasion. With increasing depth of a lesion, especially once this extends into dentine, micro-organisms in the deeper parts would be deprived of saliva which is the main source of oxygen for the oral microflora. These conditions would favour anaerobic growth and discourage aerobic metabolism. However, against this explanation for changes seen in the present study are, first, that the change was not a consistent one over the study

period and secondly, the fact that caries at the sites sampled was at a relatively early stage of development even at the end of the study. At the start, all sites were apparently caries-free and the study interval of one year was short in terms of caries progression for most individuals (Parfitt, 1956; Burt *et al.*, 1983). Dentinal involvement, if it had occurred was likely to have been at a relatively early stage.

This study was not alone in showing some indication of a decrease with time in aerobic to anaerobic organisms in children who developed caries. A similar finding has been reported in another study carried out in children of similar age to those included here (Mikkelsen and Poulsen, 1976). In this latter study, the ratio was the only parameter which showed a significant decrease with the development of caries. However, Mikkelsen *et al.* (1981) reported that neither the ratio of aerobically and anaerobically grown micro-organisms nor the other ratios studied (e.g. *S. mutans* to total anaerobic count) showed any significant difference between plaque from carious and caries-free sites in a second study of adult subjects.

If it is significant, the exact manner in which differences in aerobic and anaerobic metabolism in plaque may be directly involved in caries is difficult to establish. The enzyme pyruvate formate-lyase is thought to be one factor that is especially sensitive to oxygen, being completely inactivated by exposure to oxygen for a few minutes. In streptococci the action of this enzyme is important in breakdown of pyruvate during the metabolism of glucose but its effect may still be indirect since the breakdown products of pyruvate are formate and ethanol whereas lactate is the product that is thought to be more important in caries.

When the relationship between aerobes and anaerobes was considered in samples of saliva it was apparent that it was much more consistent, showed less variation between individuals and was broadly similar in samples of children who did and did not develop caries. Median log values for the ratio varied between 0.04 to 1.81 in plaque samples but only between 0.55 and 0.68 in saliva. It has already been noted that the numbers of anaerobes in saliva samples did not show as much trend to be higher in relation to caries as did numbers in plaque samples. It was suggested earlier that this may partly relate to the fact that saliva does not provide an environment conducive to anaerobic metabolism. This effect seems likely to have influenced the inter-relationship between aerobes and anaerobes in the same way, effectively "neutralising" differences apparent at the tooth surface. Thus the relationship between the two may be partly determined by the oxygen carrying capacity of the saliva containing them, which may not necessarily relate to caries.

The salivary flora may be thought to represent a "transit station" for many bacteria moving to and from other compartments of the oral ecosystem. It is the major source for micro-organisms that colonise the tooth surface. The lack of difference between the two groups in terms of saliva samples therefore suggests that the inter-relationships seen between them at the tooth surface are not merely a result of the levels of each type of organism in saliva, ready to colonise a clean tooth surface, but are more likely to be a consequence of growth and environment in that specific site.

In the present study it was also possible to consider how this ratio differed between plaque and saliva with individuals are well as

considering trends between groups of children. In the 54 children for whom both types of sample was taken and for whom counts were available it was found that the ratio between aerobic and anaerobic counts was in some measure of agreement for only 14. For 27 individuals the proportion of aerobes to anaerobes was higher in plaque than in saliva and in 13 it was lower. This finding lacks a clear cut direction but may again be an indication of the complexity of relationships occurring between host factors and oral microflora at different sites in the oral ecosystem.

5.4 THE RELATIONSHIP BETWEEN *S. MUTANS* AND *ACTINOMYCES*

The lack of evidence to support the concept of a simple relationship between numbers of *S. mutans* and caries has already been noted. It has been suggested by other authors that other bacteria may influence the activity and numbers of this micro-organism. In the present study, the relationship between *S. mutans* and *Actinomyces* was seen to be more consistently related to caries than the numbers of *S. mutans* alone. It was found that the proportion of *S. mutans* in relation to that of *Actinomyces* was consistently higher in plaque taken from sites that developed caries in the study period. The difference between the two groups was seen within a pattern of change that was similar (Fig. 12). In both groups the median showed a peak in the six months samples. As in the case of aerobe to anaerobe relationship, results were not statistically significant and must therefore be viewed with caution. However, if real, they may suggest a balance between the two types of micro-organisms at the fissure surface which may be relate directly or indirectly to caries activity. The relationship was not

seen in streptococci as a whole but only in *S. mutans*, which has been very frequently shown to relate to caries.

It has been reported that high levels of *Actinomyces*-like organisms rarely occur with high levels of *S. mutans*, although some species have been shown to be capable of surviving in the presence of *S. mutans* and lactobacilli at the tooth surface. It has further been suggested that *S. mutans* may occupy a similar ecological niche as *Actinomyces* and it may be that relationships seen in the present study represent the outcome of competition between the two species. In the case of fissures that remained caries-free, *Actinomyces* were the "winner" whereas *S. mutans* were relatively more successful in fissures that became carious. As suggested earlier in relation to *Actinomyces* alone, such an interaction could be influenced by use of fluoride-containing agents.

Results in relation to plaque do not suggest that *Actinomyces* as a group favour or potentiate the effects of *S. mutans* or that they themselves are pathogenic at this site. However, whilst this may be true of the groups of micro-organisms as a whole, it may not be true of individual species.

In studying the relationship of *S. mutans* to *Actinomyces* in saliva in children who developed caries and in the group who remained caries-free, it was apparent that the pattern of change was broadly similar in the two groups. As a whole it was also similar to that seen in plaque, suggesting perhaps some element of correspondence between plaque and saliva in this instance and some consistency in the pattern of change within the sample of children and independent of caries activity.

However, in saliva alone the relationship to caries was a little less clear cut. On the first and second sampling occasions the median ratio of *S. mutans* to *Actinomyces* was lower in children who were caries-free, as was seen in plaque but on the third occasion it was higher in this group. In all cases the median ratios and the variation around the means were less in saliva than in plaque. In addition, on all three sampling occasions, mean values for the ratio were higher in the group of children who developed caries. This finding, of inconsistency between means and medians, again illustrates the difficulty in interpreting results as a whole in this small group of children.

Within individuals, agreement between results from plaque and those from saliva was apparently greater for the ratio of *S. mutans* and *Actinomyces* than it had been for aerobic and anaerobic counts. In 34 of the 58 children the ratio fell into the same category in both types of sample. However, in all but one case the ratio was in the same lowest, subjectively chosen category, so that true close agreement may have been simply a consequence of lower variation. Although in two cases the ratio seen in saliva was higher than that in plaque, in 22 the proportion of *S. mutans* to *Actinomyces* was greater in plaque samples. Thus, whilst these two groups of micro-organisms may occupy and perhaps compete for ecological niches on the tooth surface, *S. mutans* may be the less capable of surviving in saliva and in other compartments that form a major source of the salivary flora, such as the tongue.

5.5 STUDY OF PLAQUE FROM FISSURES OF EXTRACTED TEETH

Because of the difficulties known to be present in relation to diagnosis of caries, a small subsidiary study was carried out into the

ecology of plaque present in the fissures of pairs of extracted teeth. Teeth were upper first permanent molars, extracted from children of similar age to those taking part in the main study. They were selected such that one of each pair was sound and one carious on visual examination. The advantage of using extracted teeth was that diagnosis could be confirmed by sectioning and by histological examination.

Problems encountered with the diagnosis of occlusal caries by other authors were confirmed here in this small study of five pairs of teeth (Kidd, 1984; Rock and Kidd, 1987). Whilst the tooth of each pair that was diagnosed visually as carious was confirmed to be so on histological section, two of the five antimeres diagnosed as sound using the same criteria were found to have early enamel caries on sectioning.

Taking these results into consideration, it must be questioned whether some of the sampling sites diagnosed as caries-free on visual evidence, in reality had early enamel lesions.

Despite these problems and despite the inherent bias in using extracted teeth, results for both of the two relationships considered showed similar trends to those seen in the study of plaque in the main study. Thus, the proportion of aerobes to anaerobes present in carious fissures was lower than that seen in plaque from truly caries-free fissures in most pairs with values within the range seen in the main study.

In the case of the relationship between *S. mutans* and *Actinomyces*, the levels of *S. mutans* were higher relative to those of *Actinomyces* in plaque from teeth which were carious in three of the five pairs with median values also falling within the range of those seen in the main

study. These results from the study of extracted teeth may be thought to add some confirmation to the trends detected in the main part of the investigation, although it must be borne in mind that numbers were small, making firm conclusions difficult. It was seen in the five pairs of teeth that the ratios showed variations but the variation between teeth appeared subjectively to be less than that seen between individuals. These findings provide some small evidence that whilst the flora of fissure plaque varies within the same subject at different sites as well as between different individuals at the same site, common environmental influences within a mouth do have a marked effect.

5.6 SUGGESTED DIRECTIONS FOR FURTHER RESEARCH

This study has provided information about the relationships between aerobes and anaerobes and between *S. mutans* and *Actinomyces* in fissure plaque and saliva taken from a sample of children over a one year period. It is apparent that both relationships showed a wide variation, although some trends were detected in relation to caries. Study of plaque seems the most meaningful for events occurring at that site but results for salivary microflora are more difficult to interpret. The two bacterial inter-relationships selected for study are only two of a very great number of possibilities within the complexity of the oral ecosystem. They did nevertheless show trends to relate to caries at least as much as did single species and groups. Aspects of these relationships would seem to merit further investigation:

1. Within the field of fissure caries, research into the inter-relationships of single species within the groups studied merits further investigation. Current results suggest that aerobes and obligate anaerobes would merit study but particular species of

Actinomyces would also justify attention in relation to differing strains of *S. mutans*.

2. The present study has considered a one year period at an early stage of fissure caries development. The nature of the two selected inter-relationships at a later stage in the carious process also merits further work.

3. Investigation of fissure plaque in relation to development of caries in the underlying tooth surface is limited by the limitations of diagnostic methods for this site. Investigations using newer diagnostic methods such as electronic caries detectors could be of value in this respect but the validity of the technique used would need to be established.

6. PRINCIPAL FINDINGS

This longitudinal study has investigated fissure plaque and salivary microflora in relation to caries. Fifty-eight children aged 5-8 years took part. A single sampling site was selected for plaque; the mesial occlusal fissure of the upper left first permanent molar. Where the site was not available, plaque was taken from the adjacent molar tooth, but results in these cases were excluded from analysis. Stimulated saliva samples were taken from each child on the same occasion immediately following collection of plaque.

Samples were collected at the beginning of the study, after six months and at the end of the one year study period.

The following represents a summary of major findings:

1. Caries developed at the sample site in five of the 33 children from whom plaque samples had been taken on each of the three sampling occasions.

   New carious lesions, either at the plaque sampling site or elsewhere, developed in 26 of the 58 children from whom saliva samples were drawn on each occasion. Of the 32 in whom no new lesions were apparent, 20 had been caries-free at the start of the study.

2. No statistically significant differences were seen in isolation of organisms in relation to caries.

   There was however a consistent trend for anaerobic counts to be higher in plaque samples drawn from sites which became carious than in those from sites which remained caries-free.

There was also a trend for *Actinomyces* to form a greater proportion of the anaerobic microflora in plaque from caries-free sites.

In neither case was the trend so clearly demonstrated in saliva.

3. Bacterial interrelationships were considered in terms of ratios between groups of organisms. Based on findings summarised above and on reports of other authors, two ratios, that of aerobic to anaerobic count, and that of *S. mutans* to *Actinomyces*, were selected for consideration. As with isolation of micro-organisms, differences seen in relation to caries failed to reach statistical significance but trends were detected.

a) <u>Ratio of total aerobic to total anaerobic count</u>

i) <u>Findings in plaque</u>

Alteration in the ratio of aerobic to anaerobic count occurred amongst children who developed caries at the sampling site. The median ratio in this group of five children was 0.04 on the first sampling occasion, rose sharply to 1.81 on the second and fell to 0.45 at the end of the year. In contrast, the median value for this ratio showed a smaller increase from 0.60 to 0.62 and finally to 0.82 on the three sampling occasions in plaque samples from sites which remained apparently free of caries.

ii) **Findings in saliva**

In saliva the ratio of total aerobic to total anaerobic count was found to be more consistent and showed less variation between individuals than it did in plaque samples. The ratio was also found to be broadly similar in saliva samples from children who developed new carious lesions and in those who remained caries-free throughout the study period, with medians ranging from 0.53 to 0.68.

iii) **Agreement between findings in plaque and saliva**

When ratios were classified in a series of four broad groups, agreement between findings in plaque and those in saliva occurred in 14 of the 54 children for whom the ratio could be calculated on the first sampling occasion. No clear pattern was apparent in the disagreement, with 27 suggesting the presence of more aerobes to anaerobes in plaque than in saliva, but the remaining 13 suggesting the reverse.

b) **Ratio of *S. mutans* to *Actinomyces***

i) **Findings in plaque**

The ratio of *S. mutans* to *Actinomyces* was found to be consistently higher in plaque sampled from the sites which developed caries than in plaque from caries-free sites, although the pattern of change was similar in the two groups. Median ratios for carious sites were 0.71, 0.62 and 1.0 on the three sampling occasions and for sites which remained caries-free 0.15, 0.53 and 0.23.

ii) Findings in saliva

On the first and second sampling occasions the median ratio of *S. mutans* to *Actinomyces* was lower (at 0.04 and 0.36) in the saliva of children who were caries-free than in those who developed new lesions (0.08 and 0.42) but the opposite was true on the second occasion (0.11 for the caries-free compared to 0.03 in the group with new lesions). Mean values, in contrast to medians, were higher in the caries-free children on all three sampling occasions.

iii) Agreement between findings in plaque and saliva

Agreement between findings in plaque and saliva was seen in 34 of 58 pairs of samples where ratios could be calculated. However, all but one agreement fell within the same grouping of ratios.

5. From study of plaque samples drawn from fissures of a series of pairs of extracted upper first permanent molars from five children of similar age to those in the main study it was found that:

a) All five teeth diagnosed as carious on visual inspection were confirmed as such on section, but two of the five diagnosed as sound had histological evidence of enamel caries.

b) The findings related to the two ratios selected for consideration were similar to those seen in the main part of the investigation. The ratio of aerobic to anaerobic organisms was lower in teeth with caries than in those that

were caries-free, with values in both types of teeth falling within the range seen in the main study.

The range of *S. mutans* to *Actinomyces* was higher in plaque from carious extracted teeth with median values again falling into the range of values seen in plaque samples taken in the main study.

c) Ratios observed in the five pairs of extracted teeth showed variation but the variation between teeth from the same individual appeared to be less than the variation occurring between the individuals.

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APPENDIX A

RECOVERY OF MICRO-ORGANISMS FROM PLAQUE SAMPLES

Appendix A(i) - Table 1

Recovery from upper left first permanent molars
Total anaerobic count (cfu)

Subject no.	Start	6 months	One year
3	980	3630	730×10^2
5	1120	6500	157×10^2
6	440×10^2	619×10^3	52×10^3
7	194×10^3	2600	266×10^2
9	453×10^3	290	106×10^4
17	316×10^3	520×10^3	1370
25	576×10^3	118×10^3	617×10^4
26	630×10^2	207×10^2	7100
27	940×10^4	5920	2210
28	289×10^4	146×10^4	560×10^3
29	8400	1086×10^3	150×10^3
30	750×10^3	348×10^2	992×10^4
32	1900	426×10^3	384×10^3
33	9600	424×10^3	446×10^4
38	440×10^3	9500	660
42	424×10^5	4080	2220
44	448×10^3	390×10^2	101×10^3
45	112×10^2	550	175×10^4
47	850×10^3	3030	3890
48	7600	220	118×10^4
49	7900	300	1980
51	770×10^4	610×10^3	248×10^4
52	710×10^3	410×10^3	335×10^3
54	920×10^2	299×10^3	570
55	131×10^2	3910	234×10^3
56	8100	4040	120×10^4
57	185×10^3	667×10^3	4400
58	120×10^3	3590	3090
59	390×10^2	690	0
60	690	1780	390
61	510×10^2	81×10^3	0
62	8700	209×10^3	44×10^3
63	3840	4350	197×10^4
Mean	2.05×10^6	2.1×10^5	1.03×10^6
Median	6.3×10^4	9500	7.3×10^4
SD	7.5×10^6	3.5×10^5	2.2×10^6

Appendix A(i) - Table 2

Recovery from upper left first permanent molars
Total aerobic count (cfu)

Subject no.	Start	6 months	One year
3	640	8800	56×10^3
5	320	106×10^2	187×10^2
6	2570	758×10^3	43×10^3
7	360×10^3	261×10^4	9900
9	576×10^3	0	770×10^3
17	105×10^4	413×10^3	150
25	79×10^3	36×10^3	507×10^4
26	960×10^3	4270	302×10^2
27	451×10^4	2730	148×10^2
28	113×10^4	692×10^2	950×10^3
29	500×10^3	327×10^3	310×10^2
30	359×10^4	396×10^3	442×10^4
32	112×10^5	162×10^3	380×10^3
33	28	60	25
38	104×10^4	448×10^4	1000
42	108×10^4	7400	400
44	124×10^5	190×10^2	167×10^2
45	425×10^4	102×10^3	102×10^4
47	209×10^3	1510	400
48	2550	870×10^2	440×10^3
49	9600	0	1200
51	137×10^2	424×10^4	103×10^4
52	9800	930×10^3	175×10^4
54	153×10^5	1210	500
55	1710	242×10^2	155×10^3
56	700	890	184×10^4
57	7800	310×10^3	285×10^2
58	115×10^4	0	4500
59	6100	4190	0
60	380	810	400
61	7700	307×10^2	0
62	175×10^4	142×10^3	2390
63	75×10^3	230	260×10^4
Mean	1.9×10^6	4.6×10^5	6.3×10^5
Median	2.09×10^5	2.4×10^4	3.02×10^4
SD	3.8×10^6	1.1×10^6	1.2×10^6

Appendix A(i) - Table 3

Recovery from upper left first permanent molars
Facultative anaerobes (%)

Subject no.	Start	6 months	One year
3	73.5	99.2	135.6
5	80.4	255.4	124.8
6	72.7	61.4	100.0
7	93.8	75.0	169.9
9	39.5	17.2	54.7
17	39.6	11.2	13.1
25	16.7	1584.8	57.5
26	69.8	140.1	71.8
27	41.5	322.6	16.3
28	115.6	68.5	48.2
29	65.5	42.9	86.7
30	86.7	48.6	88.7
32	1521.1	74.7	75.0
33	28.1	59.9	25.3
38	75.0	14.8	196.9
42	78.3	86.0	79.7
44	51.1	114.1	55.5
45	72.3	74.6	99.4
47	203.5	48.1	128.5
48	78.9	77.3	40.7
49	129.1	73.3	85.9
51	70.1	147.5	58.1
52	22.5	123.9	107.2
54	20.7	0.2	91.2
55	84.7	1.8	60.7
56	114.8	75.5	50.8
57	51.4	62.5	118.2
58	65.8	33.2	106.8
59	61.5	124.6	0
60	143.5	55.1	43.6
61	12.2	153.1	0
62	67.8	42.1	161.4
63	2161.5	7.4	84.8
Mean	179.1	126.6	85.1
Median	72.3	73.3	84.8
SD	437.9	270.5	43.9

Appendix A(i) - Table 4

Recovery from upper left first permanent molars
Gram-negative anaerobes (%)

Subject no.	Start	6 months	One year
3	0	0	0.88
5	0	0	0
6	0	0.1	10.8
7	19.9	0	2.9
9	3.6	0	38.5
17	2.4	0.2	2.2
25	0.1	7.8	11.7
26	0	0	100.0
27	0	1.2	0
28	0	0.4	1.7
29	0	1.5	0
30	0	2.6	1.1
32	0	2.5	0
33	0	2.1	3.9
38	10.9	0	0
42	0	1.5	0
44	2.6	0	1.9
45	0	0	6.4
47	0.4	0	0
48	444.7	0	9.7
49	17.3	0	116.2
51	0.4	1.5	0.8
52	1.04	0	0.6
54	1.8	0	0
55	0	0	5.13
56	0.4	0	1.7
57	2.1	0.9	1.1
58	3.5.	0	50.5
59	4.7	0	0
60	0	0	0
61	0	0.4	0
62	0.001	0.98	0
63	0	0	0.3
Mean	15.6	0.7	11.9
Median	0.0001	0	1.1
SD	77.2	1.5	28.1

Appendix A(i) - Table 5

Recovery from upper left first permanent molars
Veillonella (%)

Subject no.	Start	6 months	One year
3	10.2	4.4	3.8
5	0	0	0
6	0	5.4	3.9
7	32.5	20.4	1.9
9	5.3	0	10.9
17	4.4	2.2	0
25	2.8	60.3	11.4
26	3.5	0.9	19.9
27	0.6	0	28.1
28	3.6	1.3	4.6
29	7.0	6.0	0.99
30	8.1	7.2	7.9
32	15.8	1.9	26.3
33	5.7	4.2	18.8
38	19.3	24.7	15.2
42	0	3.7	0
44	0.2	2.4	3.9
45	1.5	0	6.4
47	2.9	7.3	0
48	4.3	0	40.7
49	50.6	0	101.0
51	2.3	90.2	0.4
52	1.5	2.5	24.8
54	27.7	0	82.5
55	3.6	0	8.2
56	4.1	0	8.7
57	12.3	9.6	1.1
58	15.5	0	145.6
59	2.4	10.1	0
60	0	2.8	51.3
61	3.5	7.8	1.2
62	17.1	11.9	0
63	9.4	0	69.04
Mean	8.4	8.7	22.5
Median	4.1	2.5	8.2
SD	10.9	18.5	34.3

Appendix A(i) - Table 6

Recovery from upper left first permanent molars
Lactobacilli (%)

Subject no.	Start	6 months	One year
3	0	0	0
5	0	0	0
6	0	0	0
7	0	0	0
9	0	0	0
17	0	0	0
25	0.1	0.03	0.01
26	1.03	0.4	10.9
27	0	0	0
28	0	0	1.2
29	0.6	1.04	0
30	0	0	0.8
32	0	0	0
33	0	0	0
38	1.3	3.2	4.6
42	0.01	0	0
44	0	0	0
45	0	0	0
47	0	0	0
48	0	0	0.001
49	0	0	0
51	0	1.1	0
52	0	0	0
54	0	0	0
55	0	0	0
56	0	0	0
57	0.9	4.8	1.1
58	0	0	0
59	0	0	0
60	0	0	0
61	0	0	0.6
62	0	0	0
63	0	0	0
Mean	0.1	0.3	0.6
Median	0	0	0
SD	0.3	1.0	2.1

Appendix A(i) - Table 7

Recovery from upper left first permanent molars
Actinomyces (%)

Subject no.	Start	6 months	One year
3	154.1	336.1	42.5
5	85.7	76.9	4.2
6	1.5	39.4	75.0
7	36.1	37.3	39.1
9	101.2	0	113.2
17	31.7	5.9	74.5
25	6.8	29.1	51.9
26	4.3	125.1	28.2
27	57.4	17.1	0
28	57.4	10.9	34.3
29	25.0	16.3	110.0
30	6.8	15.8	58.9
32	217.9	31.5	330.7
33	51.0	9.4	22.4
38	56.8	115.7	333.3
42	0	0.001	0
44	176.3	75.9	0
45	0	29.1	0
47	15.9	21.5	0
48	119.7	0	0
49	101.3	20.0	0
51	8.1	0.3	0
52	7.3	0	90.8
54	54.4	0	140.4
55	320.6	9.2	166.7
56	135.8	51.9	0
57	51.9	8.1	0
58	44.2	48.5	229.8
59	18.7	59.4	0
60	1333.3	64.0	66.7
61	40.9	0.7	0
62	65.5	3.4	0
63	58.3	3.9	7.9
Mean	101.7	38.3	65.2
Median	51.0	17.1	34.3
SD	232.2	62.7	91.3

Appendix A(i) - Table 8

Recovery from upper left first permanent molars
Total streptococci (%)

Subject no.	Start	6 months	One year
3	60.2	0	847.3
5	19.6	17.1	39.2
6	6.0	0.2	98.7
7	81.4	10.8	25.2
9	91.8	20.7	55.7
17	23.0	8.8	0
25	8.5	23.6	27.6
26	1568.3	14.6	61.8
27	1.8	253.4	31.2
28	2.7	37.9	10.2
29	35.7	43.2	18.7
30	4.8	43.1	11.6
32	52.1	18.6	3.2
33	15.1	27.1	8.9
38	45.5	42.6	40.9
42	0.001	114.7	90.1
44	0.07	104.1	13.1
45	23.8	25.5	1.4
47	77.8	6.9	60.7
48	17.8	54.6	31.9
49	75.9	13.3	29.3
51	17.9	481.9	31.5
52	10.5	30.7	402.9
54	0	1.1	42.1
55	0	23.3	104.3
56	44.2	16.6	1.8
57	13.5	104.5	0
58	85.8	40.1	18.8
59	39.8	30.4	0
60	10.2	29.8	5.1
61	5.3	17.7	13.5
62	42.1	50.4	0
63	33.6	2.3	0.2
Mean	76.2	51.8	68.2
Median	19.6	25.5	27.6
SD	269.3	90.9	161.9

Appendix A(i) - Table 9

Recovery from upper left first permanent molars
S. mutans (%)

Subject no.	Start	6 months	One year
3	33.7	0	301.4
5	8.9	8.8	3.4
6	2.5	0.03	0
7	21.7	10.0	3.01
9	89.6	0	27.4
17	3.2	1.2	0
25	2.9	14.6	11.4
26	1568.3	12.6	21.3
27	0.5	214.5	20.4
28	0	30.4	6.1
29	35.7	23.8	18.7
30	4.8	29.6	1.3
32	27.4	8.2	0
33	2.4	22.9	4.9
38	7.7	17.6	25.8
42	0.001	73.3	48.2
44	0.06	47.2	5.9
45	5.0	16.4	0.7
47	38.1	2.9	10.3
48	11.5	54.6	0
49	60.8	13.3	16.7
51	9.9	311.5	0
52	1.3	1.9	47.8
54	0	0	21.1
55	0	21.7	4.3
56	14.1	12.4	1.8
57	10.7	77.9	0
58	46.7	2.8	4.5
59	10.8	26.1	0
60	8.7	27.5	5.1
61	3.3	9.1	0
62	21.7	32.2	0
63	18.2	1.9	0.01
Mean	62.7	34.1	19.7
Median	8.9	14.6	4.9
SD	271.0	63.4	53.9

Appendix A(i) - Table 10

Recovery from upper left first permanent molars
S. sanguis (%)

Subject no.	Start	6 months	One year
3	0	0	0
5	0.9	1.9	0
6	1.2	0.002	18.7
7	0	0	0
9	0	0	0
17	0.9	0	0
25	0	6.2	0
26	0	0.7	4.8
27	0	32.1	0
28	0	0	0
29	0	9.3	0
30	0	0	0
32	0	8.5	3.2
33	0	2.1	0
38	0.5	0	0
42	0	41.4	0.9
44	0	0	1.9
45	0	9.1	0
47	0	0	0
48	0	0	0
49	0	0	0
51	2.3	95.1	16.1
52	0	1.7	0
54	0	0.8	0
55	0	0	9.4
56	0	0	0
57	0	4.9	0
58	0	0	0
59	0	0	0
60	0	0	0
61	0	6.9	0
62	0	0	0
63	0	0	0.01
Mean	0.4	2.5	1.8
Median	0	0	0
SD	1.5	10.9	4.6

Appendix A(i) - Table 11

Recovery from upper left first permanent molars
S. milleri (%)

Subject no.	Start	6 months	One year
3	10.2	0	358.9
5	8.9	0.6	0
6	2.1	0	0
7	59.8	0.8	1.1
9	2.2	10.3	0
17	3.2	0	0
25	1.7	0	8.4
26	0	0.4	0
27	0	0	0
28	0	0	0
29	0	0	0
30	0	6.3	0.9
32	24.7	1.9	0
33	1.8	0	0
38	7.7	1.8	0
42	0	0	19.4
44	0	0	0.5
45	8.6	0	0
47	26.4	0	0
48	0	0	14.2
49	10.1	0	12.6
51	4.9	0	15.3
52	0	0	292.5
54	0	0	3.5
55	0	0	11.1
56	0	0	0
57	2.3	2.1	0
58	16.7	0	0
59	17.9	0	0
60	1.5	1.7	0
61	0.1	0	0
62	0	0	0
63	1.6	0	0
Mean	6.4	0.8	23.8
Median	1.7	0	0
SD	11.9	2.1	81.2

Appendix A(i) - Table 12

Recovery from upper left first permanent molars
S. mitior (%)

Subject no.	Start	6 months	One year
3	0	0	186.3
5	0	5.9	18.6
6	0	0.2	80.0
7	0	0	21.1
9	0	0	28.3
17	15.8	7.6	0
25	1.0	0	0
26	0	0	35.8
27	1.1	6.8	10.9
28	2.4	7.5	0
29	0	10.1	0
30	0	1.7	9.4
32	0	0	0
33	8.0	2.1	4.0
38	29.3	23.3	0
42	0	0	21.6
44	0	56.9	4.7
45	9.3	0	0
47	13.3	3.9	36.5
48	6.3	0	17.6
49	5.1	0	0
51	0.8	75.4	0
52	9.3	25.4	62.7
54	0	0.3	17.5
55	0	1.3	79.5
56	30.1	4.2	0
57	0	19.5	0
58	7.5	37.3	14.2
59	11.0	4.4	0
60	0	0	0
61	0.3	1.6	0
62	20.3	18.2	0
63	9.4	0	0.2
Mean	5.5	9.5	20.9
Median	0.8	1.7	4.7
SD	8.3	17.3	38.1

Appendix A(i) - Table 13

Recovery from upper left first permanent molars
S. salivarius (%)

Subject no.	Start	6 months	One year
3	16.3	0	0.7
5	0.9	0	17.2
6	0.2	0	0
7	0	0	0
9	0	10.3	0
17	0	0	0
25	2.8	2.8	0
26	0	0.9	0
27	0.2	0	0
28	0	0	0
29	0	0	0
30	0	5.5	0
32	0	0	0
33	2.9	0	0
38	0.2	0	0
42	0	0	0
44	0.01	0	0
45	0.9	0	0
47	0	0	0
48	0	0	0
49	0	0	0
51	0	0	0
52	0	1.7	0
54	0	0	0
55	0	0.3	0
56	0	0	0
57	0.5	0	0
58	15.0	0	0
59	0	0	0
60	0	0.6	0
61	1.6	0	0
62	0	0	0
63	4.4	0.5	0
Mean	1.4	0.7	0.6
Median	0	0	0
SD	3.8	2.04	3.1

Appendix A(ii) - Table 1

Recovery from upper left second primary molars
Total anaerobic count (cfu)

Subject no.	Start	6 months	One year
1	550×10^2	230	1064×10^4
10	219×10^3	790	2600
13	340×10^3	413×10^4	990×10^4
14	600×10^2	529×10^3	276×10^4
15	3310	720	333×10^4
16	2990	9000	200
20	530	740	120
21	710	0	180
24	245×10^3	737×10^3	900×10^2
31	1580	320	40
40	0	160	232×10^3
Mean	756×10^2	266×10^3	964×10^3
Median	3310	740	900×10^2
SD	115×10^3	845×10^3	252×10^4

Appendix A(ii) - Table 2

Recovery from upper left second primary molars
Total aerobic count (cfu)

Subject no.	Start	6 months	One year
1	2580	294×10^3	978×10^4
10	9500	0	9800
13	110×10^5	699×10^3	175×10^4
14	138×10^5	338×10^4	792×10^3
15	518×10^4	118×10^4	348×10^4
16	166×10^4	2510	200
20	113×10^3	740	0
21	1300	280	300
24	119×10^3	204×10^3	129×10^3
31	694×10^4	330	127×10^4
40	0	260	137×10^3
Mean	353×10^4	524×10^3	157×10^4
Median	119×10^3	251	137×10^3
SD	502×10^4	102×10^4	293×10^4

Appendix A(ii) - Table 3

Recovery from upper left second primary molars
Facultative anaerobes (%)

Subject no.	Start	6 months	One year
1	89.1	39.1	6.2
10	1.3	55.7	200
13	105.9	212.6	75.5
14	125.0	113.6	15.9
15	62.8	97.2	73.3
16	111.0	47.8	70.0
20	273.6	10.8	75.0
21	109.9	0	83.3
24	21.6	86.0	71.4
31	68.9	121.9	0
40	0	162.5	28.9
Mean	88.1	86.1	61.9
Median	89.1	86.0	71.4
SD	76.0	64.6	64.1

Appendix A(ii) - Table 4

Recovery from upper left second primary molars
Gram-negative anaerobes (%)

Subject no.	Start	6 months	One year
1	16.9	0	11.5
10	0	0	59.6
13	6.3	0	0.1
14	0	2.5	0
15	0	0	6.2
16	0	0	0
20	0	0	0
21	0	0	0
24	0.3	3.9	1.0
31	0	0	0
40	0	112.5	4.0
Mean	2.1	10.8	7.5
Median	0	0	0.1
SD	5.2	33.8	17.7

Appendix A(ii) - Table 5

Recovery from upper left second primary molars *Veillonella* (%)

Subject no.	Start	6 months	One year
1	19.3	0	11.5
10	0.2	0	59.6
13	0	34.9	0.1
14	0	35.5	0
15	0	70.8	6.2
16	0	0	0
20	0	0	0
21	26.2	0	0
24	25.3	7.9	1.0
31	0	0	0
40	0	0	4
Mean	6.5	13.6	7.5
Median	0	0	0.1
SD	11.1	23.5	17.7

Appendix A(ii) - Table 6

Recovery from upper left second primary molars Lactobacilli (%)

Subject no.	Start	6 months	One year
1	0	0	0.9
10	0	0	0
13	0	0.04	0.3
14	0	1.8	2.2
15	0	0	0
16	0	0	0
20	0	0	0
21	0	0	0
24	0.3	0	0
31	0	0	0
40	0	0	0
Mean	0.03	0.2	0.3
Median	0	0	0
SD	0.09	0.5	0.7

Appendix A(ii) - Table 7

Recovery from upper left second primary molars
Actinomyces (%)

Subject no.	Start	6 months	One year
1	14.7	0	79.9
10	22.8	31.7	807.7
13	37.4	3.7	6.8
14	156.7	51.8	17.8
15	12.4	170.8	45.1
16	287.6	622.2	0
20	32.1	332.4	0
21	64.8	10.0	0
24	4.5	0	3.7
31	0	900.0	1750.0
40	0	56.3	517.2
Mean	57.5	198.1	293.5
Median	22.8	51.8	17.8
SD	88.5	302.0	551.7

Appendix A(ii) - Table 8

Recovery from upper left second primary molars
Total streptococci (%)

Subject no.	Start	6 months	One year
1	4.0	0	93.6
10	0	10.1	306.5
13	10.0	10.6	6.9
14	62.0	90.7	24.1
15	4.0	37.5	29.4
16	435.0	34.4	20.0
20	34.0	2554.1	16.7
21	37.0	0	44.4
24	31.0	89.3	11.2
31	28.0	15.6	0
40	0	168.8	15.9
Mean	58.6	273.7	51.7
Median	28.0	34.4	20.0
SD	126.4	758.1	88.2

Appendix A(ii) - Table 9

Recovery from upper left second primary molars
S. mutans (%)

Subject no.	Start	6 months	One year
1	1.9	0	6.8
10	0.02	6.3	133.1
13	7.7	7.9	1.5
14	52.5	67.5	21.8
15	2.7	25.0	9.0
16	270.9	5.3	20.0
20	16.9	1878.4	16.7
21	15.5	0	44.4
24	1.6	0	0
31	18.9	9.4	0
40	0	100.0	15.9
Mean	35.3	190.9	24.5
Median	7.7	7.9	15.9
SD	79.6	560.6	38.3

Appendix A(ii) - Table 10

Recovery from upper left second primary molars
S. sanguis (%)

Subject no.	Start	6 months	One year
1	0.4	0	0
10	0	0	0
13	0	2.6	2.1
14	0	0	0
15	0	0	0
16	0	1.2	0
20	0	283.8	0
21	0	0	0
24	0	0	0
31	0	0	0
40	0	0	0
Mean	0.04	26.2	0.2
Median	0	0	0
SD	0.1	85.4	0.6

Appendix A(ii) - Table 11

Recovery from upper left second primary molars
S. milleri (%)

Subject no.	Start	6 months	One year
1	0.8	0	0
10	0.1	0	0
13	1.8	0	0
14	9.5	9.3	0
15	0.9	8.3	0
16	163.9	0	0
20	7.6	229.7	0
21	18.3	0	0
24	8.2	14.9	0
31	9.5	6.3	0
40	0	0	0
Mean	20.1	24.4	0
Median	7.6	0	0
SD	48.0	68.3	0

Appendix A(ii) - Table 12

Recovery from upper left second primary molars
S. mitior (%)

Subject no.	Start	6 months	One year
1	0	0	86.8
10	0	3.8	170.4
13	0	0	3.3
14	0	13.0	2.3
15	0	0	18.6
16	0	27.9	0
20	9.4	162.2	0
21	0	0	0
24	6.9	74.4	0
31	0	0	0
40	0	68.8	0
Mean	1.5	31.8	25.6
Median	0	3.8	0
SD	3.3	51.4	54.5

Appendix A(ii) - Table 13

Recovery from upper left second primary molars
S. salivarius (%)

Subject no.	Start	6 months	One year
1	0.8	0	0
10	0	0	3.1
13	0.3	0	0
14	0	0	0
15	0	4.2	0
16	0	0	0
20	0	0	0
21	2.8	0	0
24	14.7	0	0
31	0	0	0
40	0	0	0
Mean	1.7	0.4	0.3
Median	0	0	0
SD	4.4	1.3	0.9

Appendix A(iii) - Table 1

Recovery from upper left first permanent molars that were fissure sealed during the study
Total anaerobic count (cfu)

Subject no.	Start	6 months	One year
4	0	3710	3810
8	433×10^3	151×10^2	900
11	104×10^4	760×10^2	550×10^4
12	2430	363×10^3	228×10^4
22	350×10^4	572×10^2	100×10^4
23	203×10^3	3330	3900
34	185×10^3	460	5900
36	398×10^2	5400	160×10^2
37	0	2130	184×10^3
41	288×10^4	2140	0
Mean	828×10^3	528×10^2	899×10^3
Median	194×10^3	4555	109×10^2
SD	129×10^4	112×10^3	178×10^4

Appendix A(iii) - Table 2

Recovery from upper left first permanent molars that were fissure sealed during the study
Total aerobic count (cfu)

Subject no.	Start	6 months	One year
4	810	145×10^2	288×10^2
8	680×10^2	152×10^2	100×10^2
11	640×10^3	360×10^2	586×10^4
12	175×10^4	261×10^4	0
22	660×10^3	330×10^2	9280
23	840×10^2	9300	420
34	179×10^4	410	6030
36	479×10^4	7200	115×10^4
37	0	5500	150×10^4
41	108×10^4	0	30
Mean	108×10^4	273×10^3	856×10^3
Median	650×10^3	119×10^2	96×10^2
SD	147×10^4	821×10^3	184×10^4

Appendix A(iii) - Table 3

Recovery from upper left first permanent molars that were fissure sealed during the study
Facultative anaerobes (%)

Subject no.	Start	6 months	One year
4	0	108.4	73.5
8	89.8	223.2	155.6
11	93.3	69.7	86.2
12	41.2	127.0	40.8
22	35.7	60.7	1.7
23	46.3	1.9	52.8
34	38.9	252.2	254.2
36	148.2	83.3	315.0
37	0	199.1	55.4
41	0	1.4	0
Mean	49.3	112.7	103.5
Median	40.1	95.9	64.5
SD	48.3	87.8	106.1

Appendix A(iii) - Table 4

Recovery from upper left first permanent molars that were fissure sealed during the study
Gram-negative anaerobes (%)

Subject no.	Start	6 months	One year
4	0	0	0
8	7.9	0	1411.1
11	4.4	8.2	8.3
12	0	3.3	15.1
22	0.1	47.7	0
23	0	0.9	0
34	1.5	0	1.7
36	0.4	1.3	7.2
37	0	0	65.2
41	2.6	0	0
Mean	1.7	6.1	150.9
Median	0.25	0.45	4.5
SD	2.6	14.8	443.2

Appendix A(iii) - Table 5

Recovery from upper left first permanent molars that were fissure sealed during the study
Veillonella (%)

Subject no.	Start	6 months	One year
4	0	9.9	0
8	10.5	10.8	1411.1
11	6.7	18.0	8.3
12	0	13.8	15.1
22	0.9	2.9	0
23	0.1	0	0
34	18.1	0	1.7
36	3.6	11.9	7.2
37	0	0	65.2
41	2.3	0	0
Mean	4.2	6.7	150.9
Median	1.6	6.4	4.5
SD	5.9	6.9	443.2

Appendix A(iii) - Table 6

Recovery from upper left first permanent molars that were fissure sealed during the study
Lactobacilli (%)

Subject no.	Start	6 months	One year
4	0	0	0
8	0	8.7	0
11	0	0	2.1
12	0	0.5	0
22	0.1	0	0
23	0	0	3.6
34	0.03	0	0
36	0	0	0
37	0	0	0
41	0	0	0
Mean	0.01	0.9	0.6
Median	0	0	0
SD	0.03	2.7	1.3

Appendix A(iii) - Table 7

Recovery from upper left first permanent molars that were fissure sealed during the study
Actinomyces (%)

Subject no.	Start	6 months	One year
4	0	39.6	173.2
8	63.3	43.7	0
11	0	39.5	76.7
12	78.2	48.2	36.4
22	0.03	20.1	2.4
23	0.9	0	96.2
34	816.2	39.1	169.5
36	70.4	101.9	0
37	0	100.5	105.9
41	69.8	4.2	0
Mean	109.9	43.7	66.0
Median	32.1	39.5	56.6
SD	250.6	34.5	68.9

Appendix A(iii) - Table 8

Recovery from upper left first permanent molars that were fissure sealed during the study
Total streptococci (%)

Subject no.	Start	6 months	One year
4	0	5.1	4671.9
8	128.0	6.2	62.2
11	0	2.4	63.5
12	367.0	1.6	14.0
22	9.0	79.4	0.9
23	51.0	0	34.4
34	22.0	32.6	49.8
36	68.0	174.1	134.4
37	0	1.4	27.2
41	25.0	0	0
Mean	67.0	30.3	505.8
Median	23.5	3.8	42.1
SD	112.8	56.4	1464.3

Appendix A(iii) - Table 9

Recovery from upper left first permanent molars that were fissure sealed during the study
S. mutans (%)

Subject no.	Start	6 months	One year
4	0	4.3	656.2
8	18.9	4.0	62.2
11	0.01	0	17.1
12	265.4	0.6	14.0
22	0.3	75.2	0.6
23	17.2	0	29.7
34	14.4	23.9	46.1
36	33.9	20.4	129.4
37	0	1.4	13.0
41	1.8	0	0
Mean	35.2	12.9	96.8
Median	8.1	2.7	23.4
SD	81.7	23.6	200.3

Appendix A(iii) - Table 10

Recovery from upper left first permanent molars that were fissure sealed during the study
S. sanguis (%)

Subject no.	Start	6 months	One year
4	0	0	0
8	0	0	0
11	0	0	0
12	101.2	0.5	0
22	2.5	0	0
23	0	0	0
34	0	0	0
36	0	98.2	0
37	0	0	0
41	0	0	0
Mean	10.4	9.9	0
Median	0	0	0
SD	31.9	31.0	0

Appendix A(iii) - Table 11

Recovery from upper left first permanent molars that were fissure sealed during the study
***S. milleri* (%)**

Subject no.	Start	6 months	One year
4	0	0	866.1
8	109.5	0.1	0
11	0.03	0.5	0
12	0	0.3	0
22	5.6	0	0
23	27.1	0	0
34	0.9	0	0
36	6.5	7.4	0
37	0	0	0
41	0	0	0
Mean	14.9	0.8	86.6
Median	0.47	0	0
SD	34.3	2.3	273.9

Appendix A(iii) - Table 12

Recovery from upper left first permanent molars that were fissure sealed during the study
***S. mitior* (%)**

Subject no.	Start	6 months	One year
4	0	0.5	3149.6
8	0	2.1	0
11	0	1.2	46.4
12	0	0.6	0
22	0.9	4.2	0.3
23	6.4	0	4.6
34	6.3	8.7	0
36	29.9	12.9	5.0
37	0	0	14.1
41	21.3	0	0
Mean	6.5	3.02	322.0
Median	0.45	0.9	2.5
SD	10.6	4.4	993.6

Appendix A(iii) - Table 13

Recovery from upper left first permanent molars that were fissure sealed during the study

S. salivarius (%)

Subject no.	Start	6 months	One year
4	0	0.3	0
8	0	0	0
11	0	0.7	0
12	0	0.2	0
22	0.1	0	0
23	0	0	0
34	0	0	0
36	0	35.2	0
37	0	0	0
41	0	0	0
Mean	0.01	3.6	0
Median	0	0	0
SD	0.03	11.1	0

APPENDIX B

RECOVERY OF MICRO-ORGANISMS FROM SALIVA SAMPLES

Appendix B - Table 1

Recovery of micro-organisms from saliva samples
Total anaerobic count (cfu)

Subject no.	Start	6 months	One year
1	$6.3x10^8$	$8.8x10^7$	$1.7x10^8$
3	$1.6x10^8$	$1.8x10^8$	$2.7x10^8$
4	$8.0x10^7$	$4.7x10^8$	$1.9x10^8$
5	$1.8x10^8$	$2.6x10^8$	$1.1x10^7$
7	$2.3x10^7$	$1.6x10^8$	$9.8x10^7$
9	$1.4x10^8$	$8.8x10^6$	$1.5x10^8$
10	$1.9x10^8$	$1.4x10^8$	$1.2x10^8$
13	$1.6x10^9$	$8.0x10^8$	$1.8x10^8$
14	$4.9x10^6$	$6.8x10^8$	$1.6x10^8$
16	$2.1x10^8$	$1.0x10^7$	$4.5x10^7$
17	$2.5x10^8$	$2.3x10^8$	$1.5x10^8$
22	$3.3x10^8$	$2.8x10^7$	$1.6x10^8$
23	$1.0x10^8$	$1.0x10^8$	$1.5x10^8$
24	$2.0x10^8$	$3.9x10^8$	$8.3x10^8$
25	$1.3x10^8$	$2.3x10^8$	$3.4x10^8$
26	$7.5x10^7$	$2.3x10^8$	$2.8x10^8$
27	$4.4x10^7$	$2.2x10^8$	$2.1x10^6$
28	$3.9x10^8$	$9.8x10^7$	$2.8x10^8$
29	$8.6x10^8$	$2.3x10^8$	$1.8x10^8$
30	$2.7x10^8$	$2.6x10^7$	$5.3x10^8$
31	$1.3x10^8$	$2.8x10^8$	$1.5x10^8$
33	$5.1x10^8$	$3.4x10^7$	$2.2x10^8$
36	$1.7x10^8$	$1.6x10^7$	$1.9x10^8$
37	$9.1x10^8$	$9.9x10^7$	$1.1x10^8$
38	$1.1x10^8$	$1.5x10^8$	$1.1x10^7$
39	$3.3x10^7$	$2.3x10^8$	$3.9x10^8$
40	$8.7x10^8$	$2.6x10^8$	$2.9x10^8$
41	$4.6x10^7$	$1.1x10^7$	$1.5x10^8$
42	$1.6x10^8$	$1.1x10^7$	$2.9x10^8$
45	$5.9x10^8$	$1.3x10^8$	$4.9x10^8$
46	$2.2x10^8$	$1.9x10^7$	$2.4x10^8$
47	$1.9x10^6$	$1.5x10^8$	$3.7x10^8$
48	$1.2x10^8$	$2.0x10^8$	$6.7x10^8$
49	$1.6x10^8$	$6.5x10^7$	$2.4x10^8$
50	$1.9x10^8$	$1.4x10^8$	$3.4x10^8$
51	$1.5x10^8$	$1.8x10^8$	$1.2x10^9$
53	$1.3x10^8$	$1.2x10^8$	$2.6x10^8$
54	$4.8x10^8$	$3.5x10^7$	$3.1x10^8$
55	$1.2x10^8$	0	$3.7x10^8$
56	$6.9x10^8$	$7.0x10^7$	$9.5x10^7$
57	$1.0x10^8$	$3.1x10^8$	$1.9x10^8$
58	$1.8x10^8$	$2.1x10^8$	$1.8x10^8$
60	$1.3x10^8$	$1.3x10^8$	$3.6x10^8$
61	$2.1x10^8$	$5.8x10^8$	$4.3x10^8$
62	$2.6x10^7$	$2.2x10^8$	$2.7x10^8$
63	$4.5x10^8$	$9.9x10^8$	$2.9x10^8$
Mean	$2.8x10^8$	$2.0x10^8$	$2.7x10^8$
Median	$1.7x10^8$	$1.5x10^8$	$2.3x10^8$
SD	$3.0x10^8$	$2.1x10^8$	$2.1x10^8$

Appendix B - Table 2

Recovery of micro-organisms from saliva samples
Total aerobic count (cfu)

Subject no.	Start	6 months	One year
1	$5.4x10^7$	$2.6x10^7$	$1.0x10^8$
3	$1.1x10^8$	$1.8x10^8$	$1.8x10^8$
4	$1.2x10^8$	$1.6x10^8$	$1.2x10^8$
5	$1.0x10^8$	$1.5x10^8$	$2.4x10^8$
7	$7.0x10^7$	$1.2x10^8$	$8.0x10^7$
9	$4.2x10^7$	$4.1x10^6$	$1.0x10^8$
10	$1.3x10^7$	$3.6x10^7$	$1.8x10^8$
13	$6.4x10^8$	$2.7x10^8$	$1.0x10^8$
14	$2.7x10^6$	$4.0x10^8$	$1.6x10^8$
16	$1.9x10^8$	$1.5x10^6$	$1.6x10^7$
17	$1.5x10^8$	$1.2x10^8$	$2.1x10^8$
22	$6.6x10^5$	$1.9x10^7$	$9.3x10^3$
23	$3.4x10^7$	$2.4x10^7$	$2.3x10^8$
24	$1.9x10^8$	$1.8x10^6$	$1.8x10^6$
25	$1.4x10^8$	$1.1x10^4$	$1.4x10^8$
26	$2.3x10^8$	$1.3x10^8$	$1.5x10^8$
27	$8.0x10^7$	$2.5x10^7$	$2.5x10^8$
28	$1.9x10^8$	$9.3x10^7$	$8.0x10^7$
29	$4.7x10^8$	$2.2x10^8$	$8.3x10^4$
30	$3.9x10^8$	$1.8x10^7$	$2.2x10^8$
31	$1.6x10^7$	$1.5x10^8$	$9.5x10^7$
33	$1.9x10^8$	$2.5x10^7$	$9.5x10^7$
36	$4.8x10^6$	$1.1x10^7$	$1.4x10^8$
37	$2.6x10^8$	$1.4x10^8$	$1.0x10^8$
38	$2.9x10^7$	$1.0x10^8$	$2.0x10^6$
39	$2.9x10^8$	$1.5x10^8$	$1.4x10^8$
40	$2.7x10^8$	$1.5x10^8$	$1.5x10^8$
41	$1.0x10^8$	$8.0x10^6$	$8.0x10^7$
42	$1.3x10^8$	$1.2x10^8$	$1.9x10^8$
45	$7.9x10^8$	$9.5x10^7$	$3.9x10^8$
46	$1.3x10^8$	$1.4x10^7$	$1.2x10^8$
47	$1.6x10^6$	$9.8x10^7$	$1.8x10^8$
48	$8.3x10^8$	$9.3x10^7$	$4.8x10^8$
49	$1.1x10^8$	$2.6x10^7$	$1.3x10^8$
50	$1.0x10^8$	$1.2x10^8$	$2.5x10^8$
51	$8.8x10^7$	$1.5x10^8$	$5.8x10^8$
53	$3.7x10^8$	$9.3x10^8$	$4.2x10^8$
54	$2.2x10^8$	$1.7x10^7$	$1.7x10^8$
55	$3.4x10^7$	0	$1.6x10^8$
56	$2.7x10^8$	$2.2x10^6$	$6.5x10^7$
57	$7.5x10^7$	$2.1x10^8$	0
58	$1.1x10^8$	$1.1x10^8$	$3.4x10^3$
60	$1.9x10^7$	$1.9x10^7$	$1.3x10^8$
61	$1.4x10^8$	$3.2x10^8$	0
62	$2.2x10^7$	$1.8x10^8$	$1.7x10^8$
63	$2.7x10^8$	$3.7x10^8$	$1.3x10^8$
Mean	$1.7x10^8$	$1.1x10^8$	$1.5x10^8$
Median	$1.1x10^8$	$1.0x10^8$	$1.4x10^8$
SD	$1.9x10^8$	$9.8x10^7$	$1.2x10^8$

Appendix B - Table 3

Recovery of micro-organisms from saliva samples
Facultative anaerobes (%)

Subject no.	Start	6 months	One year
1	4.5	38.6	57.4
3	78.5	139.4	45.3
4	32.2	76.6	58.1
5	92.9	65.1	62.8
7	66.7	90.6	76.9
9	56.1	44.6	65.5
10	57.9	78.6	158.3
13	83.9	75.9	62.9
14	2.1	75.2	84.4
16	110.8	100.0	92.8
17	50.5	62.2	295.0
22	61.7	61.8	52.3
23	65.0	20.2	73.8
24	80.3	88.3	48.3
25	84.6	69.9	87.4
26	20.0	81.7	47.8
27	268.6	103.4	114.6
28	81.3	135.9	106.2
29	68.4	88.9	72.2
30	84.1	150.5	108.6
31	65.4	89.4	73.3
33	81.4	85.9	67.8
36	61.8	95.2	73.7
37	43.1	141.4	104.6
38	95.5	81.9	61.9
39	1545.5	57.1	66.7
40	73.9	54.9	89.6
41	159.3	73.3	50.0
42	64.1	20.0	76.3
45	67.1	84.0	79.9
46	71.6	62.0	66.3
47	76.0	83.0	48.9
48	81.3	56.3	56.1
49	69.2	76.9	76.6
50	70.5	84.2	69.0
51	115.3	65.7	63.5
53	80.0	77.1	51.9
54	47.9	76.6	94.4
55	73.9	0	125.2
56	88.0	14.3	65.8
57	78.1	65.9	80.0
58	76.1	63.9	160.0
60	74.0	61.5	47.2
61	70.6	79.76	60.6
62	89.4	67.4	57.4
63	70.4	88.9	51.3
Mean	107.4	76.8	80.2
Median	73.9	76.6	68.4
SD	220.2	28.4	41.7

Appendix B - Table 4

Recovery of micro-organisms from saliva samples
Gram-negative anaerobes (%)

Subject no.	Start	6 months	One year
1	1.4	4.3	5.9
3	10.5	57.8	3.2
4	2.4	2.8	1.4
5	0	4.3	3.0
7	0.1	7.3	3.9
9	0.3	26.3	13.5
10	5.8	8.2	100.0
13	14.8	0.3	2.4
14	0.3	1.3	4.5
16	0	9.5	1.7
17	70.7	9.0	11.5
22	2.8	3.2	16.3
23	1.8	1.8	7.9
24	7.4	2.1	1.9
25	1.2	0.9	6.2
26	1.1	0.6	41.4
27	31.4	1.1	29.0
28	6.8	12.6	0.9
29	1.5	6.6	7.2
30	6.9	38.1	14.8
31	1.6	4.1	1.0
33	42.2	39.3	17.8
36	4.9	6.3	7.4
37	19.3	13.1	90.9
38	8.4	5.7	0.4
39	757.6	50.6	3.3
40	11.9	0.3	3.3
41	33.5	0.3	0.8
42	5.9	0.1	4.5
45	24.5	0.9	48.9
46	9.1	4.7	3.8
47	1.7	11.7	4.3
48	0.8	6.4	20.8
49	4.5	7.7	11.8
50	7.8	0.6	26.1
51	7.1	7.6	26.2
53	132.0	5.2	0.01
54	2.1	19.9	33.9
55	184.8	0	66.7
56	12.4	8.6	10.0
57	2.2	1.9	0.6
58	4.2	28.9	8.6
60	8.2	6.9	3.1
61	4.4	13.9	48.2
62	4.4	15.4	6.7
63	1.9	7.6	5.3
Mean	31.8	10.3	15.9
Median	5.3	6.4	6.4
SD	114.5	13.3	22.7

Appendix B - Table 5

Recovery of micro-organisms from saliva samples
Veillonella (%)

Subject no.	Start	6 months	One year
1	3.9	12.0	13.9
3	15.1	64.8	4.9
4	1.9	22.3	10.5
5	50.7	69.9	55.8
7	51.6	60.9	34.4
9	2.1	71.1	9.8
10	44.7	20.5	0
13	26.3	1.4	4.6
14	16.8	2.9	0
16	0.9	12.2	36.7
17	74.8	36.7	7.5
22	18.8	5.0	9.2
23	9.3	143.9	14.4
24	6.9	37.7	25.1
25	15.8	2.2	59.3
26	1.5	5.7	82.9
27	20.0	10.8	6097.6
28	7.8	10.8	51.3
29	37.7	8.1	47.2
30	3.6	67.6	2.5
31	7.1	52.2	7.0
33	111.3	29.6	36.8
36	6.9	26.9	14.2
37	34.3	41.7	0
38	15.2	20.7	0
39	727.3	61.5	74.4
40	36.1	8.2	45.2
41	44.5	71.1	43.3
42	1.0	0.5	35.1
45	52.3	16.0	61.9
46	6.1	10.3	4.2
47	30.7	20.0	27.2
48	5.4	10.3	19.7
49	5.9	13.9	6.9
50	10.6	14.7	38.7
51	64.4	55.7	0.1
53	46.0	24.6	7.4
54	34.9	9.6	35.5
55	15.9	0	53.1
56	18.6	13.2	11.6
57	14.2	28.5	1.1
58	13.1	7.1	5.4
60	68.0	11.9	38.9
61	12.2	18.6	32.35
62	81.7	44.2	30.6
63	0.9	20.5	6.2
Mean	40.8	28.8	156.6
Median	15.8	20.0	17.1
SD	106.5	27.9	895.7

Appendix B - Table 6

Recovery of micro-organisms from saliva samples
Lactobacilli (%)

Subject no.	Start	6 months	One year
1	0.001	8.9	0.4
3	0	0	0.01
4	0	0	0.4
5	0	0	0
7	0	0	0
9	0	0	0.001
10	0	0	10.4
13	0.09	0.001	0.9
14	0	0.003	0.03
16	0	0	0
17	0	0	6.0
22	0.004	45.5	0.002
23	0	0.9	0.02
24	0	0	0
25	0.004	0	0.002
26	0.01	0.001	0.002
27	0.02	0.002	289.0
28	0	0	0.4
29	0.04	0.002	0.2
30	0	0	0.3
31	0	0	0
33	0	0.002	0.002
36	0	0	0
37	0	0	0
38	0.04	0.03	0.2
39	0.04	1.03	0.2
40	0	0	0
41	0	0	0
42	0	0.1	0.2
45	0	0	0.001
46	0.5	0.1	0.001
47	0	0.002	0.001
48	0	0	0.001
49	0	0	0
50	0.01	0.02	0.01
51	0.4	0.02	0.001
53	0	0.03	0
54	0	0	0.001
55	0	0	0
56	0	0	0.003
57	0.01	0.001	0
58	0	0	0
60	0	0	56.3
61	0	0	0
62	0.03	0.002	0.3
63	0.002	0.8	0.01
Mean	0.03	1.2	7.9
Median	0	0	0.001
SD	0.09	6.8	43.2

Appendix B - Table 7

Recovery of micro-organisms from saliva samples
Actinomyces (%)

Subject no.	Start	6 months	One year
1	4.0	0	79.9
3	180.0	43.7	78.1
4	30.0	34.6	86.5
5	24.9	27.2	251.2
7	103.2	48.4	61.5
9	68.4	0	62.1
10	59.2	0	112.5
13	31.9	0.8	82.9
14	103.1	0.001	93.8
16	112.1	7.6	70.6
17	54.6	4.9	120.0
22	63.2	4.6	69.2
23	2.7	9.0	95.1
24	80.3	0	26.6
25	19.0	3.9	48.2
26	200.0	5.6	73.9
27	331.4	5.6	5121.9
28	75.5	16.9	106.7
29	51.5	0	0
30	51.1	15.8	26.7
31	55.8	0	40.0
33	41.2	0	20.7
36	42.7	10.5	61.8
37	84.5	80.8	70.5
38	35.9	0.6	76.2
39	1901.5	4.1	66.7
40	123.9	0	60.9
41	99.5	0.1	66.7
42	56.3	53.3	0
45	127.4	10.6	0
46	76.1	4.3	75.8
47	72.0	6.2	0
48	6.5	40.0	0
49	43.1	10.8	0
50	37.2	8.6	0
51	81.4	4.3	0
53	76.0	5.2	0
54	45.8	17.0	120.9
55	78.3	0	55.8
56	66.9	8.9	0
57	41.5	2.1	1.2
58	28.2	3.4	137.1
60	32.0	0.6	9.8
61	16.5	1.2	53.5
62	34.6	0.3	112.0
63	26.3	6.1	94.0
Mean	108.3	11.3	169.4
Median	56.0	4.9	64.4
SD	276.2	17.3	748.1

Appendix B - Table 8

Recovery of micro-organisms from saliva samples
Total streptococci (%)

Subject no.	Start	6 months	One year
1	68.8	34.9	13.7
3	20.9	0.1	6.1
4	18.1	3.9	8.1
5	7.6	0.4	14.7
7	59.1	0.7	26.7
9	11.2	19.7	4.8
10	16.2	2.6	18.3
13	0.003	41.3	16.0
14	28.6	0.9	24.1
16	46.9	35.6	38.3
17	25.3	13.3	10.0
22	142.9	12.1	19.9
23	7.5	3.6	100.0
24	17.9	0.8	16.3
25	188.5	2.5	59.3
26	186.7	2.8	12.7
27	14.7	9.0	4.8
28	9.4	14.1	56.6
29	3.7	4.3	156.9
30	49.5	16.9	41.4
31	49.6	2.2	7.3
33	46.1	4.2	11.6
36	2.1	21.1	17.6
37	20.4	27.5	0.1
38	13.4	1.9	15.2
39	674.2	2.9	42.9
40	30.1	1.2	4.7
41	40.1	0.03	26.0
42	10.8	128.9	5.1
45	39.2	29.4	51.5
46	15.9	129.1	0.7
47	45.3	14.3	6.1
48	2.1	15.8	27.1
49	2.1	8.5	4.9
50	9.6	17.7	5.5
51	76.3	19.6	66.7
53	26.2	14.5	14.2
54	0.9	39.0	59.7
55	3.9	0	72.1
56	0.4	0.8	7.6
57	19.0	6.7	7.5
58	2.5	5.5	0.9
60	12.4	86.5	9.8
61	9.1	17.3	40.0
62	11.4	12.2	6.3
63	8.8	12.9	5.6
Mean	45.6	18.7	25.3
Median	17.0	12.1	14.4
SD	103.8	28.8	29.9

Appendix B - Table 9

Recovery of micro-organisms from saliva samples
S. mutans (%)

Subject no.	Start	6 months	One year
1	20.0	15.1	4.1
3	5.2	0	0.9
4	5.6	0	0.8
5	4.5	0	6.1
7	18.3	0	6.7
9	0.2	8.9	0
10	3.0	0.4	12.9
13	0	0	2.9
14	0	0	15.8
16	24.1	3.7	15.6
17	25.3	6.9	0
22	5.3	0	2.2
23	1.0	0	36.1
24	0.9	0.3	0
25	17.3	0.3	0
26	26.7	0.1	2.8
27	0	2.4	1.1
28	0	6.2	23.0
29	0	0.9	27.8
30	16.8	3.7	9.5
31	0	0.3	0
33	0	3.0	3.3
36	0.5	4.0	7.9
37	0.8	0	0.1
38	2.5	0	0
39	143.9	0.2	0
40	7.5	0.8	1.7
41	10.4	0.03	0
42	2.2	57.8	0.4
45	2.1	0	3.1
46	7.9	30.4	0
47	12.0	0	0.3
48	0.2	9.3	0
49	0.8	5.4	1.3
50	2.3	5.6	0.4
51	40.7	3.7	0
53	0	0.9	1.3
54	0	5.7	13.7
55	0	0	4.1
56	0	0	1.1
57	6.3	0.8	0
58	0.4	2.2	0.5
60	11.8	53.9	0.4
61	6.5	7.8	12.9
62	3.7	7.5	0.9
63	2.7	7.6	0.3
Mean	9.6	5.7	4.8
Median	2.6	0.9	1.1
SD	22.2	12.2	7.9

Appendix B - Table 10

Recovery of micro-organisms from saliva samples
S. sanguis (%)

Subject no.	Start	6 months	One year
1	9.6	0	0
3	4.5	0	0
4	1.3	0	0
5	1.6	0	0
7	0	0	0
9	2.9	0	0
10	6.6	1.1	0
13	0	0	0
14	0	0	0
16	0	16.8	0
17	0	5.7	0
22	104.5	0	0
23	4.3	0	0
24	5.6	0.1	0
25	119.2	0.2	0
26	36.7	1.2	0
27	4.7	0	0
28	2.7	0	0
29	0.3	0	0
30	0	0	0
31	8.1	0	0
33	0	0	0
36	0.2	7.3	0
37	5.5	11.6	0
38	1.6	0.8	0
39	83.3	0	6.4
40	1.2	0	0
41	4.4	0	0
42	0.9	0	0
45	4.6	0	0
46	0	0	0
47	0	0	0.9
48	0.5	0	0
49	0.1	0	0
50	0	0	0
51	0	0	0
53	0	0	0
54	0	19.2	0
55	0	0	0
56	0	0	0
57	2.2	0	0
58	0.6	0.9	0
60	0	17.3	0
61	0	6.1	11.8
62	0	1.9	0
63	2.9	0	0
Mean	9.1	1.9	0.4
Median	0.8	0	0
SD	25.8	4.8	1.9

Appendix B - Table II

Recovery of micro-organisms from saliva samples
S. milleri (%)

Subject no.	Start	6 months	One year
1	39.2	0	1.3
3	10.3	0	0
4	11.3	0.2	0.4
5	1.6	0.02	0
7	10.8	0	0
9	8.1	0	3.8
10	0	0.8	0
13	0.002	37.2	2.6
14	7.1	0	0
16	19.3	10.9	11.7
17	0	0	3.5
22	20.3	0	7.7
23	0	1.8	27.9
24	5.7	0	0
25	26.9	0.7	59.3
26	53.3	0.6	4.9
27	7.3	3.8	0
28	5.3	3.3	5.3
29	2.7	2.6	38.5
30	32.7	5.5	5.2
31	28.7	0	5.0
33	32.8	0	5.3
36	0.5	4.5	0
37	6.6	8.8	0
38	3.6	0.6	11.2
39	136.4	2.1	19.2
40	4.3	0.3	1.7
41	11.5	0	26.0
42	3.8	22.2	1.2
45	16.5	20.2	26.8
46	4.4	49.4	0.2
47	20.0	8.7	2.2
48	0.5	3.5	2.2
49	0.7	0	1.8
50	4.1	0	3.5
51	22.03	0	60.7
53	15.8	0	11.4
54	0.6	0	31.5
55	2.9	0	51.7
56	0.4	0.1	0
57	2.7	2.8	4.8
58	0.6	0	0
60	0	0	8.7
61	2.1	0	0
62	3.2	0	1.3
63	3.1	0	3.6
Mean	12.8	4.1	9.8
Median	4.9	0.1	3.5
SD	22.2	9.8	15.7

Appendix B - Table 12

Recovery of micro-organisms from saliva samples
S. mitior (%)

Subject no.	Start	6 months	One year
1	0	16.0	5.0
3	0	0.1	3.0
4	0	1.7	5.5
5	0	0.03	6.1
7	0	0.5	11.3
9	0	8.3	1.03
10	0	0.4	5.6
13	0	4.1	5.4
14	11.2	0.7	6.4
16	0	4.2	11.1
17	0	0.8	6.5
22	9.0	6.8	10.0
23	0	1.8	36.1
24	4.9	0	4.8
25	9.6	0	0
26	36.7	0	5.1
27	0	2.6	3.7
28	0	4.6	28.3
29	0	0.9	91.7
30	0	6.7	26.7
31	0	1.9	2.3
33	0	1.2	2.9
36	0.8	0.8	8.4
37	3.0	0	0
38	1.8	0.5	1.9
39	113.6	0.3	17.3
40	3.2	0	1.2
41	7.7	0	0
42	2.2	28.9	1.9
45	5.1	3.0	0
46	2.7	49.4	0.4
47	9.3	5.7	2.6
48	0.3	3.0	20.1
49	0.3	2.3	1.9
50	2.4	12.1	1.6
51	8.5	14.3	0
53	0	9.4	1.6
54	0.1	14.2	13.7
55	0	0	13.6
56	0	0	4.2
57	0	1.3	2.1
58	0	2.4	0.1
60	0.6	15.4	0.7
61	0.5	3.5	4.7
62	4.6	2.7	4.5
63	0	2.5	1.7
Mean	5.2	5.1	8.3
Median	0.05	2.1	4.4
SD	17.4	8.9	14.9

Appendix B - Table 13

Recovery of micro-organisms from saliva samples
S. salivarius (%)

Subject no.	Start	6 months	One year
1	0	0.6	3.2
3	0.9	0.03	2.2
4	0	1.2	1.4
5	0	0.3	2.6
7	30.1	0.2	8.7
9	0	2.6	0
10	6.6	0	0
13	0.001	0	5.1
14	10.2	0.2	1.9
16	3.6	0	0
17	0	0	0
22	3.8	0	0
23	2.3	0	0
24	0.9	0	0
25	15.4	0.3	0
26	33.3	0.9	0
27	2.7	0.2	0
28	1.4	0	0
29	0.7	0	0
30	0	1.1	0
31	12.9	0	0
33	13.2	0	0
36	0.2	4.5	1.3
37	4.4	7.1	0.01
38	3.9	0	2.1
39	196.9	0.3	0
40	13.9	0.1	0
41	6.0	0	0
42	1.7	20.0	1.5
45	10.9	6.2	21.7
46	0.8	0	0.1
47	4.0	0	0
48	0.6	0	2.2
49	0.1	0.8	0
50	0.8	0	0
51	5.1	1.6	6.0
53	10.4	4.2	0
54	0.3	0	0.8
55	0.9	0	2.7
56	0.01	0.8	2.4
57	7.8	1.9	0.5
58	0.9	0	0
60	0	0	0
61	0	0	0
62	0	0	0.8
63	0	2.8	0
Mean	8.9	1.3	1.5
Median	1.2	0.02	0
SD	29.3	3.3	3.5