VARIATIONS IN THE MICROFLORA OF DENTAL PLAQUE AT DEFINED SUB-SITES ON APPROXIMAL TOOTH SURFACES

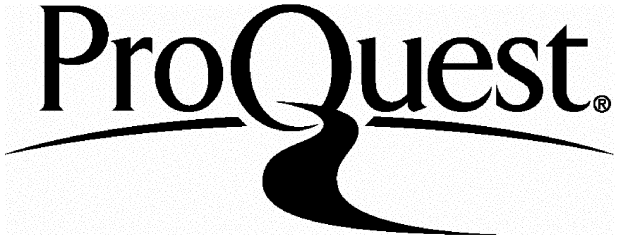
A THESIS SUBMITTED IN FULFILMENT OF THE DEGREE
OF DOCTOR OF PHILOSOPHY, UNIVERSITY OF LONDON

KABOUTAR GHOLAMI BABAAHMADY
B. Sc (JODISHAPOUR, IRAN), M. Phil. (WALES, UK)

1995

DEPARTMENT OF PERIODONTOLOGY
EASTMAN DENTAL INSTITUTE
LONDON WC1X 8LD

DEPARTMENT OF ORAL MEDICINE AND PATHOLOGY
UNITED MEDICAL AND DENTAL SCHOOLS
GUY'S AND ST THOMAS' HOSPITALS
LONDON SE1 9RT



Abstract

Dental caries is most prevalent in sites which retain plaque. Environmental conditions vary markedly over such caries-prone sites (e.g. fissures and approximal surfaces), and this may influence the distribution and levels of the predominant bacteria. The primary aim of this study was to compare the microflora of plaque from discrete, small sub-sites around the contact area on approximal surfaces of clinically sound teeth extracted for orthodontic reasons. The study was divided into three main sections:

1) Investigation of the distribution and composition of the microflora in approximal surface gingival margin plaque. Small plaque samples were removed from three sub-sites, away from (A), to the side of (S), and below (B) the contact area of clinically sound approximal surfaces. An average of 7-9 species were cultured from each plaque sample, but the species recovered varied between the sub-sites. The isolation frequency and proportions of *S. mutans* and *Veillonella* spp. were significantly higher at sub-site B.

2) A comparison of culture and immunofluorescence methods of bacterial detection. The use of immunofluorescence for identification proved to be a more rapid alternative to culture techniques. Both *S. mutans* 'c' and *S. sobrinus* 'd' showed a site preference in the order of B>S>A. An overall positive association was found between the presence of *S. mutans* 'c' and *S. sobrinus* 'd', and between mutans streptococci and lactobacilli. The results of the culture study were compared with those of immunofluorescence from the same samples, and the latter proved to be a more feasible method than conventional culture techniques.

3) Comparative analysis by restriction fragment length polymorphism (RFLP) of *S. mutans* 'c' and *S. sobrinus* 'd', and between *S. oralis*, *S. mitis* I and *S. mitis* II in order to improve discriminations among these species. Type strains and representative clinical isolates were compared for heterogeneity. 16s rRNA genes from these species were amplified by the polymerase chain reaction and digested with restriction enzymes and the fragments separated by agarose gel electrophoresis. The restriction patterns obtained for *S. mutans* could be used to differentiate this species from other streptococci in human dental plaque. For other streptococci tested, the restriction profiles were similar with the exception of *S. mitis* I and II.

Acknowledgements

I am grateful to my supervisors Professor S. J. Challacombe, Professor P. D. Marsh and Professor H. N. Newman for their guidance and constant encouragement in the work and preparation of this thesis, in addition I would like to thank Professor Newman for obtaining the grants which made this work possible.

I am also grateful to:
Dr. D. Beighton and Ms S. Brailsford for their advice on the molecular techniques
Dr. J. Bulman for his advice on statistical analysis.
Dr. D. Rahman for raising polyclonal antisera, and Professor J. J. de Soet for providing anti-*S. sobrinus* 'd' monoclonal antibody.

Professor M. Kilian for providing human IgA, and streptococcal strains.
My colleagues in the Departments of Microbiology and Maxillofacial Surgery at the Eastman Dental Institute and in the Department of Oral Medicine and Pathology at Guy's Hospital for their cooperation, and the staff of the Department of Paediatric Dentistry at the Eastman Dental Hospital for their cooperation concerning the collection of teeth.

This research was supported by the Biscuit, Cake, Chocolate and Confectionery Alliance. London, UK., and by GABA International, Basel, Switzerland.

To my late father, my dear mother,
and my native motherland

CONTENTS

LIST OF FIGURES

clinical isolates of *S. oralis* with *Hae*III and *Hin*dIII, followed by electrophoresis on 1.8% Metaphor agarose gels. 123

LIST OF TABLES

CHAPTER ONE

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Dental caries remains a public health problem in western societies and is an increasing problem in developing countries (Johnson 1991). Over many centuries numerous theories concerning the aetiology of dental caries have been proposed, of which perhaps the most widely accepted was tooth worm (according to a cuneiform clay tablet in the British Museum, dated from 4000 B.C., Levine 1977). More than one hundred years ago, the chemico-parasitic theory (Miller 1890) proposed that dental caries was caused by demineralisation of enamel as a result of acid production from bacterial activity. This basic concept has remained essentially unchanged from Miller's time, but the search has since continued for specific micro-organisms responsible for dental caries.

The present pattern of caries incidence shows that coronal decay is still primarily a disease of young children. While extensive data are available on caries in children, little is known about trends in adults (Johnson 1991). Some studies have reported an increase in root caries, which is characteristically a disease of the elderly (Mandell 1985). A great concern at present is the possibility of a vast expansion in the incidence of dental caries in many parts of the third world due to increased access to processed food and sucrose. According to the World Health Organization Data Bank, countries that have shown dramatic rises in caries incidence in 12 year old children include Chile, Mexico, Iran, Jordan, Morocco, India, Indonesia, and the African continent (World Health Organization 1989). Some 3700 million people (74% of the world population, rising to 79% by the year 2000) live in such countries (Sreebny 1982, Mandell 1985).

1.2 Microbial ecology of the mouth

More than three hundred bacterial species are found in the oral cavity (Moore 1987). The oral cavity presents a series of distinct environments, each of which may be suitable for

colonisation by different species (Hardie and Bowden 1974). The composition of the microflora varies from site to site because of the range of micro-environments in the mouth. Variation may occur in the microbial composition of plaque on different surfaces of the same tooth, and on the same surface both between and within mouths. The microbial composition of plaque may also change with time at the same site (Marsh and Martin 1992).

1.3 Factors affecting the distribution of the oral flora

Colonization and growth of micro-organisms in the mouth are dependent on several factors and some of them are discussed here.

1.3.1 Temperature

Bacterial metabolism and enzyme activity are dominated by temperature. Other significant environmental factors, such as pH, ion activity, aggregation of macromolecules and the solubility of gases may also be affected by temperature. The temperature of the mouth is approximately 36°C, and this is optimal for the growth of a wide range of micro-organisms (Marsh and Martin 1992).

1.3.2 Redox potential

The majority of the oral microflora are either facultative or obligate anaerobes. Oxygen concentration is considered the main factor limiting the growth of obligately anaerobic bacteria. Oxygen can raise the redox potential and become an inhibitor. However, some species can survive high concentrations of oxygen if Eh is sustained at low levels. In general, the distribution of anaerobes in the mouth will be related to the redox potential

at a particular site.

The redox potential has been shown to fall during plaque development on clean enamel surfaces (Ritz 1963). This is associated with a specific succession of micro-organisms. Early colonizers will utilize O_2 and produce CO_2; later colonizers may produce H_2 and other reducing agents, such as sulphur-containing compounds and volatile fermentation products. Thus, the Eh may be gradually lowered during plaque formation making it suitable for the survival and growth of a changing pattern of organisms.

Variations in Eh are found in the gingival crevice in health and disease, and also at the same site in different subjects (Kenney and Ash 1969). Approximal areas (between teeth) would be expected to have a relatively low Eh because of the prevalence of anaerobes although values for the redox potential at these sites have not been reported. It is anticipated that gradients of O_2 and Eh will exist in the mouth, particularly in plaque. Thus, plaque will be suitable for the growth of bacteria with a range of oxygen tolerances. Modifications to the habitat that disturb such gradients may influence the composition of the microbial community. Similarly, the metabolism or properties of particular bacteria might be influenced by the Eh of the micro-environment.

1.3.3 pH

Many micro-organisms are sensitive to an excess of acid or alkali, and a pH around neutrality is required for their growth. The pH of most surfaces of the mouth is affected by saliva (Mandel 1987). The mean pH of unstimulated whole saliva is in the range 6.7-7.5 (Nikiforuk 1985) so that, in general, optimum pH values for microbial growth will be

provided at sites bathed by this fluid. Bacterial population shifts within the plaque microflora can occur following marked fluctuations in environmental pH. After sugar consumption, the pH in plaque can fall rapidly to below pH 5.0 by the production of acids (predominantly lactic acid) by bacterial metabolism; the pH then recovers slowly to the baseline value (Schachtele and Jensen 1982).

Many of the predominant bacteria of dental plaque associated with healthy sites can tolerate brief conditions of low pH (for review see van Houte 1980), but are inhibited or killed by more frequent or prolonged exposure to acidic conditions. Few oral bacteria are able to tolerate acidic conditions for a long time, but mutans streptococci and lactobacilli are not only able to remain viable at low pH, but are able to continue to metabolize and multiply, albeit slowly (Donoghue and Newman 1976). Therefore, the microbial composition of a given micro-environment could be dependent on pH and vice-versa.

The end products of sugar fermentations by such streptococci and lactobacilli at neutral pH are acetic and formic acids, but at acid pH is almost entirely lactic acid (de Ley 1962). Such changes in bacterial metabolism could lead to increased acid formation and dental caries. In contrast, the pH can become more alkaline during the host inflammatory response in chronic inflammatory periodontal disease (Eggert et al. 1991).

1.3.4 Nutrients

A microbial community is reliant on its habitat for the nutrients essential for the growth of its constituent species. Therefore, the existence of an organism in a given habitat is direct evidence that all of the necessary nutrients required for growth are present. The

reason that the mouth can support many nutritionally-demanding bacteria is due to the provision of endogenous or exogenous nutrients.

a) Endogenous nutrients

These are nutrients provided directly by the host. The main oral sources of endogenous nutrients are saliva and gingival crevicular fluid (GCF). Saliva contains amino acids, peptides, proteins, vitamins, gases and glycoproteins. De Jong et al. (1984) have shown that saliva is a complete, carbohydrate-limited growth medium for oral *Streptococcus* spp. and for *Actinomyces viscosus.*

GCF contains albumin and other host proteins and glycoproteins, including haemin-containing molecules (Cimasoni 1983). GCF is one of the factors that may be uniquely available to the microflora of the gingival crevice compared with other oral sites.

b) Exogenous nutrients (diet)

From the complex array of foodstuffs in the diet, only fermentable carbohydrates have been found to have a significant influence on the ecology of the mouth.

Carbohydrates can be readily broken down to acids; in addition, sucrose can be converted into two main classes of polymers, glucans and fructans, which can serve to consolidate bacterial attachment to the teeth or may act as extracellular nutrient storage compounds, respectively. The frequent consumption of fermentable dietary carbohydrate is associated with a decrease in plaque pH (Firestone 1982), and may cause shifts in the proportions of the microflora of dental plaque. Bradshaw et al. (1989) showed that the mechanism was the low pH rather than the availability of sugar *per se.*

The influence of carbohydrates on flora was demonstrated clearly by Huxley (1973). During a dietary carbohydrate-free period of 17 days, the percentage of *S. mutans* in the total cultivable flora decreased to a very low or undetectable level, while simultaneously the percentage of *S. sanguis* increased. Similarly, several comparable studies have reported a rise in the proportions of *S. mutans* and *Lactobacillus* spp. with a reduction in *S. sanguis* following a rise in the intake of sucrose. This is accompanied by a simplification of the metabolism of plaque from a heterofermentative pattern to one where most carbohydrate is converted to lactate (de Stoppelaar et al. 1970, Minah et al. 1985, Bradshaw et al. 1989).

1.3.5 Adhesion

For successful colonization, populations must first adhere to, and be retained at a surface and then be able to multiply (Gibbons and van Houte 1973, 1980, Gibbons 1980). Cell surface molecules on bacteria, known as adhesins, are thought to interact with specific receptors on the substrate surface.

Lipoteichoic acid is one well recognised non-specific factor promoting bacterial adhesion (Alkan et al. 1977, Ofek and Beachey 1980). Adhesive properties have been associated with various streptococci that colonise tooth surfaces (Gibbons and van Houte 1980).

Adhesins which are important for the interaction of *S. mutans* with the acquired enamel pellicle have not been found in *S. sobrinus* (Gibbons et al. 1986). *S. sobrinus* can adhere to surfaces when sucrose is present. Many species are known to attach avidly to saliva-treated hydroxyapatite, which showed different numbers of binding sites for each species.

It seems likely that bacteria interact with different receptors within the pellicle (Clark et al. 1978, Liljemark and Schauer 1977, Liu et al. 1991). Such interactions may involve sialic acid-reactive streptococcal adhesins (Levine et al. 1978, Liljemark et al. 1989, Demuth et al. 1990a, b) other adhesins for proline-rich proteins (Gibbons et al. 1991, Ligtenberg et al. 1992) and lectins, i.e. non-antibody proteins that bind specifically to sugar moieties (Cisar et al. 1985). Oral bacteria often attach to surfaces by specific adhesion appendages, known as fimbriae and fibrils (Handley 1990).

1.3.6 Host defences

The host has a number of defence mechanisms which play an important role in maintaining the integrity of the oral surfaces. The defence system is divided into specific (acquired) and non-specific components which are maintained by immune and innate factors, respectively. Innate factors are always present and their functions are independent of the type of foreign bodies that invade the system. The immune factors are raised when they are needed, and their actions are specific and depend on the type of foreign body (antigen) which has invaded the system. Defence factors are listed in Table 1.1.

1.3.7 Antimicrobial agents and inhibitors

The mouth is challenged regularly with modest concentrations of antimicrobial agents and inhibitors (Addy et al. 1990). Antimicrobials are delivered mainly from toothpastes (dentifrices) and mouthwashes (Gjermo 1989), while many natural inhibitors such as lactoferrin (Cole et al. 1981), lysozyme (Tortosa et al. 1981, Pollock et al.1981, 1987), salivary peroxidase (Tenovuo et al. 1985, 1986, Carlsson 1987) and sialic acid (McBride and Gisslow 1977) are present in saliva. Jalil et al. (1993) studied the association between

Table 1.1 Specific and non-specific host defence factors of the mouth.

Defence factor	Main function
Non-specific	
Normal flora	Preventing the growth of pathogenic micro-organisms
Saliva flow	Physical removal of micro-organisms
Mucins/agglutinins	Physical removal of micro-organisms
Lysozyme-protease-anion system	Cell lysis
Lactoferrin	Iron sequestration
Apo-lactoferrin	Cell killing
Sialoperoxidase system	Hypothiocyanite production (neutral pH) Hypocyanous acid production (low pH)
Histidine-rich peptides	Antibacterial and antifungal activity
Specific	
Intra-epithelial lymphocytes	Cellular barriers to penetrating bacteria and/or antigens
Langerhans cells	Antigen-presenting cell*
sIgA	Prevents microbial adhesion and metabolism
IgG, IgA, IgM	Prevent microbial adhesion; opsonins; complement activators
Complement	Activates neutrophils
Neutrophils/macrophages	Phagocytosis

* Roitt et al. 1985
Modified after Marsh and Martin (1992).

are present in saliva. Jalil et al. (1993) studied the association between the concentration of some of these salivary factors, plaque accumulation and gingival inflammation in children. They found that thiocyanate concentration indirectly and lysozyme concentration directly were related to the amount of plaque and gingival inflammation. Fluoride is

present in most toothpastes and, although its primary beneficial anti-caries action is due to its incorporation into enamel and its influence on remineralization, it can inhibit bacterial metabolism, particularly glycolysis, even at low concentrations, especially under acidic conditions (Hamilton and Bowden 1988, Bradshaw et al. 1990, Li and Bowden 1994).

1.4 The normal oral flora

The normal oral microflora of humans includes bacterial species capable of colonising the oral cavity as their primary habitat and subsequently of establishing themselves as common residents of the mouth. Over 325 species of micro-organism have been isolated from the mouth (Moore 1987), including Gram-positive and Gram-negative bacteria (Table 1.2), mycoplasmas, yeasts and protozoa (Theilade 1989).Over recent years, numerous taxonomic studies have resulted in major changes in the nomenclature of most oral bacteria. This can cause difficulties when attempting to compare studies in which different taxonomic schemes have been used. Consequently, throughout this thesis, the original names of micro-organisms used prior to the recent reclassification will be cited to avoid confusion.

1.4.1 *Actinomyces*

The oral cavity of man and animals is the principal natural habitat of the genus *Actinomyces*; species of actinomyces can be isolated from dental plaque, calculus and saliva (Schaal 1986).

Actinomyces are Gram-positive, pleomorphic rods, that are among the predominant bacteria found in plaque of healthy adult humans (Bowden et al. 1975, Ellen 1976, Socransky et al. 1977, Sanyal and Russell 1978, Liljemark et al. 1993), although they have also been associated with disease (Jordan 1982). *Actinomyces viscosus* and

Table 1.2 Bacterial genera commonly found in the oral cavity.

Gram-positive	Gram-negative
Cocci	
Peptostreptococcus	*Branhamella*
Streptococcus	*Neisseria*
Stomatococcus	*Veillonella*
Rods	
Actinomyces	*Actinobacillus*
Bifidobacterium	*(Bacteroides)**
Corynebacterium	*Campylobacter*
Lactobacillus	*Centipeda*
Propionibacterium	*Eikenella*
Rothia	*Fusobacterium*
	Haemophilus
	Leptotrichia
	Mitsuokella
	Porphyromonas
	Prevotella
	Selenomonas
	Simonsiella
	Treponema (helical rod)

* The genus *Bacteroides* has been reclassified to include "*B. fragilis*-group" (Shah and Collins 1990). After Marsh and Martin (1992).

Actinomyces naeslundii have attracted particular attention because they have been implicated in periodontitis in rodents (Jordan and Hammond 1972, van der Hoeven et al. 1975), and in gingivitis in man (Loesche and Syed 1978, Moore et al. 1982, Moore et al. 1984). Species of *Actinomyces* have also been found in root surface caries (Syed et al. 1975, Ellen et al. 1985, Bowden 1990), and in nursing caries (Milnes and Bowden 1985).

Actinomyces species have been studied in developing plaque (Nyvad and Kilian 1987). In their study, plaque was allowed to form on enamel in 4 subjects and samples were taken every 4 hours up to 12 hours. *Actinomyces* spp. were isolated as part of the predominant cultivable flora on a non-selective agar medium. A decrease in the proportions of *A. viscosus* and *A. naeslundii* was found from 4 to 8 and 8 to 12 hours of plaque formation. The majority of *A. viscosus* strains were found in the 2 hour plaque samples, but very few were found later, showing their role in early plaque formation. The description of *Actinomyces* taxa found in humans (Schaal 1992) is given below:

A. israelii: this was the first strain of *Actinomyces* which was isolated, from lesions of actinomycosis in man. Wolff and Israel (1891) published a bacteriological description of this species, and hence its current designation.

A. naeslundii: Thompson and Lovestedt (1951) proposed the name *A. naeslundii* for this filamentous bacterium. Often found in the mouth, *A. naeslundii* is facultatively anaerobic and grows best anaerobically in 5% CO_2.

A. odontolyticus: this is a Gram-positive, predominantly rod-shaped bacterium, and isolated from advanced human caries lesions.

A. viscosus: Pine and George (1969) used modern approaches to the taxonomy of

Actinomyces. George and colleagues (1969) modified the genus description to include both catalase-positive and catalase-negative bacteria, to accommodate the species *A. viscosus* (catalase positive).

According to the most recent classification of human strains of *Actinomyces,* the genospecies *A. naeslundii* includes strains of *A. naeslundii* serotypes II and III and *A. viscosus* serotype II, while *A. viscosus* is retained for the animal strains of this species (Johnson et al. 1990). However, to avoid confusion with earlier literature *A. viscosus* will not be referred to by its new classification (*A. naeslundii*) in this thesis.

1.4.2 *Lactobacillus*

Lactobacilli are aciduric microorganisms which constitute a small proportion of the total oral microbiota (Socransky and Manganiello 1971). Lactobacilli have been isolated from the oral cavity of infants during the first few days of life (Socransky and Manganiello 1971, Carlsson et al. 1975).

Oral lactobacilli seem to have difficulty in attaching themselves to smooth surfaces (van Houte et al. 1972), while extracellular polymers from sucrose, produced by other bacteria like *S. mutans*, may be a prerequisite for their colonisation (Socransky and Manganiello 1971, van Houte et al. 1972, Burt et al. 1985, Crossner et al. 1989). They prefer retention sites (Theilade and Budtz-Jorgensen 1988), hence, they are frequently isolated interdentally (van Houte et al. 1981, Crossner et al. 1989).

The presence of lactobacilli may reflect a caries-inducing environment, and this may explain their importance as a predictor of future caries (Hardie et al. 1977, Boyar and Bowden 1985, Crossner et al. 1989), compared with their rather limited aetiologic importance in caries initiation (Klock and Krasse 1978, Crossner 1989).

1.4.3 *Neisseria*

Neisseria spp. are Gram-negative cocci which together with "*S. oralis* group" (*S. oralis* and closely related species) are found among the early colonizers of a clean tooth surface (Ritz 1967). Some strains of *Neisseria* could be considered as caries-reducing factors in plaque since they are able to utilize lactic acid (Mikx et al. 1972).

1.4.4 *Veillonella*

Veillonellae are Gram-negative, obligately anaerobic cocci, which are non-sporing and non-motile. Bowden and Hardie (1992) have considered veillonellae to be amongst the most numerous anaerobic organisms in the human mouth, since they are isolated from most sites of the oral cavity and from saliva. *Veillonella* spp. colonize teeth by aggregating with other bacteria already there, as they appear unable to adhere to the teeth directly on their own (Theilade 1989).

Veillonellae lack glucokinase and fructokinase and do not ferment carbohydrates, but degrade lactate produced by *Streptococcus* and *Actinomyces species* to weaker acids (acetic and propionic). Since they use lactate as an energy source, they may play an important role in the ecology of dental plaque and in the aetiology of dental caries. Lactic acid is the strongest acid produced by oral bacteria and is implicated in the dissolution of enamel. It has been proposed that *Veillonella* in plaque might reduce the harmful effects of potentially cariogenic bacteria by metabolizing lactic acid (Mikx et al. 1972, 1976), although this hypothesis is not always supported by clinical evidence (Marsh and Martin 1992).

1.4.5 *Streptococcus*

Streptococci are Gram-positive, facultative anaerobic cocci, which are non-motile and non-sporing. The oral viridans streptococci form an important component of the normal microbial flora of the mouth (Jones 1978). They have been associated with infections at

different sites in humans and animals, e.g., dental caries (Drucker et al. 1984a, b), infective endocarditis, and septicaemia (Parker and Ball 1976, Horaud and Delbos 1984). These streptococci are also abundant in saliva from predentate and dentate children and adults (Tappuni and Challacombe 1993).

Many studies have been carried out to detect and classify the species of oral streptococci (Carlsson 1968, Bridge and Sneath 1983, Schmidhuber et al. 1987, Kilian et al. 1989, Beighton et al. 1991a, Hardie and Whiley 1992). However, there have been problems in assiging strains to species.

1.4.6 Taxonomy of oral streptococci

In 1972 a biochemical scheme for the identification of oral streptococci was proposed by Colman and Williams, by which *S. mitior*, *S. milleri*, *S. sanguis*, *S. salivarius* and *S. mutans* were recognised. However, the classification of this group has still remained ambiguous, as the same strain have been assigned to different species, depending on the author.

Kilian et al. (1989) studied taxa published in The Approved List of Bacterial Names (Skerman et al. 1980), They considered *S. constellatus, S. intermedius* and "*S. milleri* group" to be synonyms of *S. anginosus,* in line with a report by Coykendall et al. (1987). Welborn et al. (1983) had shown that *S. constellatus, S. intermedius* and *S. anginosus* are three distinct species, which were confirmed by Whiley and Hardie (1989), Whiley et al. 1990a, these species have been differentiated biochemically (Whiley et al. 1990a).

In addition to the species described above, Whiley and Hardie 1988 identified *S. vestibularis*, which resembles *S. salivarius* phenotypically but differs from it as identified by deoxyribonucleic acid (DNA)-DNA homology and cell wall analyses. Additional species include *S. parasanguis* (Whiley et al. 1990b, c) and *S. crista* (Handley et al.

1985).

The scheme used for speciating oral streptococci in the present study was based on the identification schemes of both Kilian et al. (1989) and Beighton et al. (1991a). The former scheme has the advantage of including IgA_1 protease activity (Kilian and Holmgren 1981), which is a unique test to differentiate *S. sanguis* and *S. oralis* , while the latter could be used to differentiate *S. sobrinus* from *S. mutans.*

The results of the tests they proposed are reproducible and offer reasonable discrimination between oral streptococcal species. A brief outline of each of the recognised species of oral streptococci is given below.

1.4.6.1. *S. sanguis*

The taxonomic history of *S. sanguis* provides perhaps the best example of the of a change from a predominantly serological approach to the use of biochemical and physiological data, cell wall studies, and finally to the application of genotypic criteria (Hardie and Whiley 1992).

S. sanguis was the name given to α-haemolytic streptococci that produce an extracellular dextran from sucrose (Hehre and Neill 1946). Kilian et al. (1989) proposed two groups within "*S. sanguis*" on the basis of their biochemical and serological differences. The first group is the type strain of *S. sanguis*, and retained the name *S. sanguis*. The second group, included strains that were designated as *S. sanguis* subsp. *sanguis*. This also included the type strain for *S. mitis* (NCTC 3165) (Skerman et al. 1980), which was then assigned to *S. gordonii*. Finally, Beighton et al. (1991a) designated *S. gordonii* as a separate species, and also they suggested three biotypes for *S. sanguis* rather than the four proposed by Kilian et al. (1989).

1.4.6.2 *S. gordonii*

S. gordonii was named by Kilian at al. (1989) to group strains that closely resemble *S. sanguis*. subsp. *S. sanguis* as described by Coykendall and Specht (1975). It also included strain NCTC 3165, which is the designated type strain of *S. mitis* (Skerman et al. 1980). This group (*S. gordonii*) is characterised by arginine and aesculin hydrolysis, inulin fermentation and production of extracellular polysaccharides from sucrose. They differ, however, from *S. sanguis* in lacking IgA_1 protease activity, in their ability to ferment amygdalin, and in having ß-glucosaminidase, ß-mannosidase, α-fucosidase and strong alkaline phosphatase activity. The results of DNA base composition and DNA-DNA homology studies support the specific separation of this group into a distinct species (Kilian et al. 1989).

1.4.6.3 *S. oralis*

S. oralis was the name given by Bridge and Sneath (1983). In a study incorporating cell wall analysis, physiological data and DNA hybridisation, those strains which resembled *S. sanguis* were excluded to give an amended description of *S. oralis* (Kilpper-Baltz et al. 1985). Bridge and Sneath (1982) proposed the type strain which was later found to be a typical strain of "*S. mitior*" (Kilpper-Balz et al. 1985, Kilian et al. 1989).

1.4.6.4 *S. mitis*

In the past similarities in the cell wall composition of *S. oralis* and *S. mitis* have probably contributed to the inability to distinguish between them (Colman and Williams 1972). Due to the small number of tests later used to identify strains as *S. mitis* this species was often poorly differentiated from *S. salivarius*.

There have been two recent proposals for the rejection of strain NCTC 3165 as the type species of *S. mitis* (Kilian et al. 1989). Pending a decision on this by the Judicial

Commission of the International Committee on Systematic Bacteriology, Kilian et al. (1989) have proposed a new type strain for *S. mitis* NS51(= NCTC 12261). These strains sometimes hydrolyse arginine, do not produce extracellular polysaccharide from sucrose, and produce IgA1 protease less frequently than do strains of *S. oralis.* The strain NCTC 12261 was subsequently proposed as the valid type strain of *S. mitis*, based on genetic (Gilmour et al. 1987) and taxonomic studies (Kilian et al. 1989). The latter study also subdivided this species into two groups, biovar I (*S. mitis* I) and biovar II (*S. mitis* II).

1.4.6.5 *S. salivarius*

S. salivarius was named by Andrewes and Horder (1906) for relatively easily recognised streptococci commonly isolated from human saliva. Strains of *S. salivarius* are typically non-haemolytic, produce acid from inulin, lactose, raffinose, salicin and trehalose, but not from mannitol, sorbitol or melibiose, and can hydrolyse aesculin but not arginine. Most strains produce levan as an extracellular polysaccharide from sucrose.

1.4.6.6 "*S. milleri* group"

The name *S. milleri* was given to a group of non-haemolytic streptococci from active oral infections which were able to hydrolyse aesculin and arginine, were able to grow on 40% bile agar at 45 ^{0}C, but could not ferment mannitol or sorbitol.

S. anginosus is the term used to cover the "*S. milleri* group" by Andrewes and Horder (1906). These were a heterogeneous group of strains including *S. milleri*, *S. intermedius*, *S. constellatus* and *S. MG-intermedius* (Facklam 1977). Facklam also noted a close similarity among several species previously described by different authors as *S. anginosus* and *S. constellatus* (Holdeman and Moore 1974). However, no natural subdivisions were possible on the basis of the data. Consequently, they considered "*S. milleri*", *S. constellatus,* and *S. intermedius* to be later synonyms of *S. anginosus* as proposed

previously by Coykendall et al. (1987). However, studies have shown that *S. intermedius*, *S. constellatus* and *S. anginosus* represent three genetically distinct groups (Whiley and Hardie 1989, Whiley et al. 1990a) that can be differentiated biochemically (Whiley et al. 1990a).

1.4.6.7 *S. vestibularis*

S. vestibularis (Whiley and Hardie 1988), constitutes a group of alpha haemolytic streptococci that had been isolated mainly from the vestibular mucosa of the human mouth (Hardie and Whiley 1992). Chemotaxonomic data suggested that *S. vestibularis* shares homology with *S. salivarius* (Whiley and Hardie 1988). However, DNA-DNA hybridization studies confirmed that *S. vestibularis* strains represented a distinct species (Whiley and Hardie 1988, Hardie and Whiley 1992).

1.4.6.8 Other streptococci

There are obligately anaerobic species of streptococci which have been difficult to isolate and classify from oral samples, the most important of which are *Peptostreptococcus*. Several peptostreptococcal species have been recovered from dental plaque e.g. *Ps. micros*, *Ps. magnus* and *Ps. anaerobius* (Marsh and Martin 1992).

1.4.7 Mutans streptococci (MS)

The originally described "*S. mutans* group" in humans was genetically heterogeneous, and has been divided into five species: *S. mutans, S. sobrinus*, *S. rattus, S. cricetus* and *S. ferus* (Coykendall 1974, 1977, 1983). *S. cricetus* and *S. rattus* were first isolated from hamsters and rats, respectively, but they are also found in the human mouth, although rarely (Loesche 1986). Subsequent studies increased the number of mutans-like species to seven, with the addition of *S. macacae* (Beighton et al. 1984) and *S. downei* (Whiley et al. 1988). The taxonomic position of *S. ferus* is less certain. It has been included in the

mutans streptococcus group only on the basis of DNA homology (Schleifer et al. 1984) and appears to be more closely related to the *S. oralis* group (Gilmour et al. 1987).

On the basis of serological heterogeneity within the mutans streptococci, 8 different serological groups can be recognized (Bratthall 1970, Perch et al. 1974, Beighton et al. 1981). These are: *S. cricetus* (serotype 'a'), *S. rattus* (serotype 'b'), *S. sobrinus* (serotypes 'd' and 'g'), *S. downei* (serotype 'h') and *S. mutans* (serotypes 'c', 'e' and 'f') (Coykendall 1977, 1983).

1.4.7.1 *S. mutans*

S. mutans was originally isolated from carious teeth (Clark 1924). The main habitat of *S. mutans* in the oral cavity is on the tooth surface (Krasse and Edwardsson 1966, Carlsson 1967). It was later found in interdental areas and colonises mainly the pits and fissures of the teeth (Loesche et al. 1975, Keene et al. 1981, Theilade et al. 1982). The cariogenicity of *S. mutans* is partly due to its ability to synthesise extracellular polysaccharide from sucrose (Krasse 1965), due to its ability to convert dietary sugars to acid, and to withstand the acidic environment that this sugar metabolism induces (Donoghue and Newman 1976, Newman et al. 1976).

S. mutans produces extracellular glucosyltransferase (GTF) and fructosyltransferase (FTF) (Wenham et al. 1979), which allows the synthesis of water-soluble, adherent glucans in addition to a certain amount of fructans from sucrose (Hamada and Slade 1980). There are two distinct types of glucan, one which contains mainly α-(1-3) linkages and is water-insoluble. The other is α-(1-6) linkage-rich, is relatively water-soluble (Walker and Hare 1977), and is the main glucan produced by *S. mutans*.

1.4.7.2 *S. sobrinus*

This species was distinguished from other members of MS on the basis of genetic, antigenic and biochemical characteristics by Coykendall (1974). He showed that *S. sobrinus* is the species least related to *S. mutans*. Electron micrographs of *S. sobrinus* K1R, 6715, and OMZ174 show cocci with an outer dendritic layer, or "fuzz", which has not been observed in *S. mutans, S. rattus,* or *S. cricetus*. Most *S. sobrinus* strains react with the Bratthall 'd' antiserum. Some strains did not give a strong 'd' reaction and were put into a separate serotype, designated 'g' by Perch et al. (1974). Strain SL1 did not react with either 'd' or 'g' antibody. *S. sobrinus* cells appear incapable of synthesizing or catabolizing significant amounts of intracellular polysaccharide (Freedman and Coykendall 1975). The habitat of *S. sobrinus* is the human tooth surface (Bratthall 1972, Perch et al. 1974). It has been frequently isolated from human dental plaque and is reported to be associated with approximal caries (Huis in't Veld et al. 1979).

S. sobrinus can synthesise extracellular polysaccharide from sucrose.These species can produce two types of glucan, one containing mainly α-(1-3) linkage water-insoluble and an α-(1-6) linkage-rich type which is water-soluble (Freedman et al. 1983, Murchison et al. 1985). The glucans are produced by two types of glucosyltransferase, GTF-S and GTF-I, which catalyze the production of α-(1-6) branched polymers and α-(1-3) polymers, respectively (Hamada and Slade 1980, Inoue 1982, Koga et al. 1986). Four different types of GTF are reported to be present in *S. sobrinus* cell walls. Most need a polymer primer to initiate the polymerization reaction. However, a primer independent enzyme, GTF-S4 of *S. sobrinus*, is able to initiate the polymerization reaction and plaque formation in the absence of polysaccharides. *S. sobrinus* can produce acids faster than other MS, and is capable of active glycolysis at low pH values (Stosser and Kneist 1988, de Soet et al. 1989).

1.4.8 Other species of oral micro-organisms

Bifidobacterium dentium, *Corynebacterium* (formerly *Bacterionema*) *matruchotii*, *Propionibacterium* spp. and *Rothia dentocariosa* (Gerencser and Bowden 1986) are Gram-positive species which have been regularly isolated from dental plaque (Bowden et al. 1975). A major group of Gram-negative bacteria is the genus *Fusobacterium.* Bacteria belonging to the genus have the characteristic morphology of long filaments or pleomorphic rods. Species of *Fusobacterium* have been isolated from the normal gingival crevice and from periodontal pockets and from approximal plaque (Bowden et al. 1975).

In addition, species of *Actinobacillus*, "*Bacteroides*", *Campylobacter*, *Centipeda*, *Eikenella*, *Haemophilus* (Sims 1970, Kilian and Schiott 1975), *Leptotrichia*, *Mitsuokella*, *Porphyromonas* (Shah and Collins 1988), *Prevotella* (Shah and Collins 1990), *Selenomonas*, *Simonsiella* and *Treponema* are found in dental plaque, depending upon the sites sampled and the clinical state of the site (Table 1.2).

1.5 Plaque formation

The diversity of the flora on the tooth surface is evident from the above. The tooth microflora is not merely a mixture of microorganisms, but it is rather a biofilm. Dental plaque is the term given to the complex microbial biofilm which forms on any part of the tooth surface, embedded in a matrix of polymers of bacterial and salivary origin (Newman and Poole 1974). Impetus for much research on plaque has been due to an association between the microflora of plaque and diseases such as caries and periodontitis. The basic mechanisms of plaque formation are similar to those underlying the general adhesion of bacteria to surfaces. A micro-organism is attracted to a surface by ionic attractions (Gibbons and van Houte 1980), which are of low specificity and allow only loose attachment of the micro-organisms to the substrate surface. Adhesins are cell surface molecules on the bacteria that interact with specific receptors on the surface (Alkan et al.

1977) to increase the strength and specificity of attachment.

Bacteria can adsorb either non-specifically to the salivary pellicle or interact specifically with receptors or polymers present in the pellicle. The results of these interactions are reversible and irreversible adhesions respectively (Marsh 1986).

1) Reversible adhesion: Macroscopic cell surface properties relevant for reversible microbial adhesion include surface free energy (SFE), zeta potential and hydrophobicity:
a) High SFE surfaces promote more bacterial adhesion than surfaces of initially low SFE, indicating an underlying influence of the surface through the pellicle. Support for this assumption has been found in *in vivo* comparative studies of plaque formation (Quirynen et al. 1989). The influence of surface roughness was found to be more important for plaque formation *in vivo* than SFE (Quirynen et al. 1990).

b) The zeta potential of an organism has also been described as an important surface characteristic in adhesion (Olsson et al. 1976). The zeta potential is determined by the nature and the number of ionic groups on the cell surface and depends also on the pH and ionic strength of the suspending medium.

c) The acquired salivary pellicle is a hydrophobic film (Doyle et al. 1982), interacting with hydrophobic oral bacteria. There is a correlation between the hydrophobicity of bacterial strains and their ability to adhere to experimental pellicle. However, hydrophobicity alone was insufficient to account for the specific requirements of adhesion (Gibbons and Etherden 1983).

2) Irreversible adhesion

Several salivary components have been shown to attract micro-organisms, supporting a role for such components in irreversible microbial adhesion to the pellicle-covered tooth

surface. These could confer specificity on the adhesive process of the early colonizers (Ericson and Magnusson 1976). For instance, salivary oligosaccharide-containing glycoproteins may serve as receptors for oral streptococci in the salivary pellicle (Gibbons and Qureshi 1978). Gibbons et al. (1990) reported that conformational changes may occur in proteins upon adsorption to surfaces. This provides the bacteria with a mechanism for efficiently attaching to teeth when they are suspended in saliva. The generation of cryptitopes due to conformational changes or enzymatic modifications appears to be involved in the colonization of several bacteria on mucosal and tooth surfaces (Gibbons and Hay 1989). In addition, there is evidence which suggests that elevated levels of neuraminidases and proteases associated with poor oral hygiene and gingivitis may also generate cryptitopes which promote colonization of certain Gram-negative bacteria associated with destructive periodontal disease. These enzymes concurrently destroy receptors required for attachment of relatively benign species such as *S. mitis* and *S. sanguis* (Childs and Gibbons 1990). Thus, the elevated levels of enzymes appear to have the potential for modulating bacterial colonization.

Intergeneric coaggregation is defined as cell-cell recognition and adhesion between bacterial pairs from different genera and is exhibited by nearly all human oral bacteria tested to date (Kolenbrander et al. 1989, Kolenbrander 1991). Cisar et al. (1985) suggested that interbacterial adhesion mediated by lectin-carbohydrate interactions is a central mechanism in the colonization of tooth surfaces.

Intrageneric coaggregation has been reported for coaggregation among actinomyces and in particular streptococci. Extensive coaggregation was observed for *S. sanguis, S. oralis* and *S. gordonii.* The interactions among streptococci were highly specific in that only certain paired strains were coaggregation partners, and all of the coaggregations were inhibited by galactosides. Only one strain of each pair was inactivated by heat (85 °C for

30 min) or protease treatment. Intragenereic coaggregation among the streptococci and possibly actinomyces could be important during early plaque formation, since the most numerous colonizers 4 h after cleaning of a human enamel surface are streptococci, which constitute 78% of the total viable count (Nyvad and Kilian 1990a). The predominant streptococci are *S. oralis*, *S. mitis* I and *S. sanguis*.

However, Skopek et al. (1993) found that co-aggregation had only a limited effect on *in vivo* plaque formation. Their results pointed to the importance of other environmental factors, such as microbial growth.

3) Environmental conditions

First, environmental conditions and microbial interactions affect microbial growth rate, which in turn may affect the surface structures involved in microbial adhesion (van der Hoeven et al. 1984, 1985). Second, such factors may affect subsequent plaque accumulation, as shown by the well-established microbial shifts taking place within dental plaque with time (Ritz 1967).

Sucrose is an important environmental factor, for example, in combination with glucosyltransferases in saliva and pellicle (Scheie et al. 1987). Glucan may provide receptors for a number of micro-organisms possessing glucan-binding proteins. Non-adhering micro-organisms may possibly be entrapped in the glucan meshwork formed in the presence of sucrose (Scheie 1994).

Macpherson et al. (1991) found that sucrose increases the initial number of micro-organisms per unit surface area of enamel slabs in humans. These micro-organisms comprised Gram-positive and Gram-negative cocci and rods, including *S. sanguis* and "*Bacteroides*" spp. They also showed that, in the absence of sucrose, the number of

colonizing micro-organisms increases steadily, whereas colonization seems to reach a plateau after 30h during sucrose exposure. Scheie (1994) stated that a "climax community" is attained within a shorter period in the presence of sucrose. However, Li and Bowden (1994) studied the effect of environmental factors such pH and fluoride on the development of biofilm. They showed *in vitro* that fluoride liberated from the substratum can inhibit the growth of biofilm cells, but only under excess of glucose and low pH conditions.

1.6 Microflora of plaque

The development of plaque with the establishment of pioneer species and subsequent colonisation by secondary species has been studied from tooth eruption onwards for different periods of time. For example, an early study on development of coronal plaque showed a broad progressive shift from mainly aerobic and facultative anaerobic flora in the early stages, to a situation where facultative and strictly anaerobic organisms become dominant (Ritz 1967). Streptococci are important pioneer species, together with aerobic species such as *Neisseria* and *Rothia* in the early stages. After nine days, streptococci, *Actinomyces, Veillonella* and "*Corynebacterium*" were predominant (Ritz 1967). The results of this study indicated that the growth of anaerobes such as *Veillonella* and *Fusobacterium* depended upon prior growth of aerobic and facultatively anaerobic organisms and, as plaque thickness increased with age, conditions became more suitable for anaerobes.

A number of studies (Socransky et al. 1977, Syed and Loesche 1978, Kilian et al. 1979, Theilade et al. 1982, Liljemark et al. 1986, Nyvad and Kilian 1987, 1990a) have concluded that streptococci, in particular *S. sanguis, S. oralis, "S. mitior"* and *S. mitis*, predominate during the initial stages of plaque formation on enamel.

Once the initial, predominantly streptococcal layer has formed, secondary plaque formers such as *A. viscosus* are able to colonize the developing plaque. Maturing plaque increases in complexity and Gram-negative anaerobes such as *Veillonella* spp., *Fusobacterium* and *Prevotella* spp. appear in increasing numbers while, in the climax community, spirochaetes can be detected (Theilade and Theilade 1985).

The maturation of plaque involves an increase in both the amount and diversity of micro-organisms on the tooth. *F. nucleatum* may play a key role in the plaque maturation process, since it is able to adhere to bacteria from many different genera (Kolenbrander et al. 1989).

1.7 Microbial interactions in plaque

Microbial interactions play an important role in the ecology of dental plaque. For example, bacteriocin-producing *S. mutans* strains appear to colonize plaque better than non-producing strains (van der Hoeven and Rogers 1979). In addition to competition for substrates, interbacterial adhesion, and competition for adhesion sites, the development of food chains among different species is known to play a role in maintaining the microbial diversity of plaque. The food chain best investigated in dental plaque is the association between the lactate-producing streptococci and the lactate-utilizing veillonellae (Mikx et al. 1972, van der Hoeven et al. 1985).

The association between species may be complex since, when Mikx et al. (1976) inoculated the oral cavity of specific pathogen-free (SPF) rats with *A. viscosus* and *S. sanguis*, it was difficult for *S. mutans* to establish itself. It was also noted that pre-inoculation of *A. viscosus* and *S. mutans* 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 may be affected by their sequence

of introduction into the oral cavity. The effect of early colonizers on the formation of subsequent communities was complex, since pre-inoculation of either of these two species alone failed to inhibit the establishment of *S. mutans* in SPF rats (Mikx et al. 1975).

Svanberg and Loesche (1977) discovered that the experimental reduction of salivary levels of *S. mutans* around the time of insertion of artificial fissures prevented the colonisation of *S. mutans* in these fissures, even though the salivary concentrations of *S. mutans* subsequently were allowed to increase. Once *S. mutans* was established, however, experimental reduction in salivary *S. mutans* did not influence the proportional distribution of *S. mutans* in the fissures. The same results were observed for *S. sanguis*. Pre-inoculation with *Actinomyces viscosus* and *S. mutans* reduced the subsequent proportions of *S. sanguis* (Mikx et al. 1976), indicating that this inter-relationship system can be influenced by more complex interactions. Consequently, in the study of the plaque microflora in relation to caries, the entire plaque community and bacterial inter-relationships should be considered. It would be anticipated that numerous interactions could take place within plaque which could modify caries activity.

The characteristic tendency of the streptococci to grow in close association with other bacteria within dental plaque may depend on the expression of specific bacterial lectins mediating adhesion and their receptors. The lectin-mediated cell-cell recognition favours other interbacterial interactions, such as the mutual utilisation of different substances, or antagonistic activities, resulting in the formation of characteristic niches within the oral environment (Morris and McBride 1984). In fact, the specific initial recognition of one bacterium by another would be expected to favour additional types of interactions which would contribute to the establishment of microbial communities.

1.8 Bacterial metabolism in dental plaque

1.8.1 Carbohydrate metabolism

One of the most important biochemical processes in plaque is the formation of acid or base from fermentable carbohydrates or urea (Kleinberg 1970).

The catabolism of carbohydrates is an important process in plaque, because of the relationship between the metabolism of sugars, low pH, and dental caries. Sucrose is the most widely used sweetening agent. It has been intensively studied for its influence on the composition of the oral flora, notably *S. mutans* and *Lactobacillus* spp. Sucrose can be:

a) broken down by extracellular bacterial invertases (α-glucosidases) to glucose and fructose molecules, which are then taken up by plaque organisms (Gibbons 1972, Fukui et al. 1974),

b) transported intact as a disaccharide phosphate and cleaved inside the cell by an intracellular invertase or a sucrose phosphate hydrolase (Tanzer et al. 1977),

c) utilized extracellularly by glycosyltransferases. Glycosyltransferases (GTF) produce both soluble and insoluble glucans (with the release of fructose), which are important in plaque formation and in the consolidation of bacterial adhesion, while fructosyltransferases (FTF) produce fructans (and liberate glucose) which are frequently labile and can be utilized by other plaque organisms (Marsh and Keevil 1986).

Glucose enters bacterial cells either via the phospho-enol pyruvate-sugar phosphotransferase (PEP-PTS) system or alternatively through the energised membrane, a proton-linked active transport mechanism (Carlsson et al. 1985).

The phosphotransferase system is activated when sugar is in low concentration, whereas the active transport mechanism predominates when sugar is in excess and the environmental pH is low. The uptake of some sugars into the cell can be modified by these membrane carriers to prevent flooding with nutrient, or through the PEP-PTS to

scavenge for the small quantities of sugars available in times of "famine" (Marsh and Martin 1992).

Once glucose is inside the cell it is phosphorylated and degraded by the glycolytic enzymes of the Embden-Meyerhof pathway, each molecule of glucose forming two molecules of pyruvate. The fate of pyruvate is regulated by two enzymes, lactate dehydrogenase (LDH) and pyruvate formate lyase (PFL). These are the triggers to homofermentation resulting in production of lactic acid or heterofermentation by which formate, acetate and ethanol are formed (Fig. 1.2). Yamada and Carlsson (1975) studied mutans streptococci grown in continuous culture with excess glucose, and found that lactate was the main anion formed. However, when glucose was restricted the major products were those of heterofermentation. The major acids formed from fermentable carbohydrates are lactic, acetic and propionic; small amounts of other acids such as butyric, isobutyric and valeric have been identified (Guggenheim et al. 1965).

1.8.2 Metabolism of nitrogenous compounds

Nitrogenous compounds are also used by oral bacteria. The major sources of nitrogenous material for oral bacteria are saliva and gingival crevicular fluid. Saliva contains low amounts of carbohydrate but a wide range of amino acids, peptides, protein and urea (20 mg/100 ml) (Jenkins 1984). Many oral bacteria can degrade urea to carbon dioxide, and to ammonia which can raise the plaque pH. A number of bacteria are dependent on the protein fraction of saliva for growth. Therefore, hydrolysis of glycoproteins and other proteins and peptides by extracellular bacterial proteases will generate free amino acids and small peptides essential for growth. Carlsson and Griffith (1974) examined *S. sanguis*, *S. mutans*, *S. salivarius* and *S. bovis* grown anaerobically in continuous culture at pH 7.0 under glucose-limitation and nitrogen-limitation. They found a significantly higher yield of bacterial mass under glucose limited conditions than under conditions of nitrogen limitation. *S. sanguis* is more proteolytic than *S. mutans* and analysis of proteins before

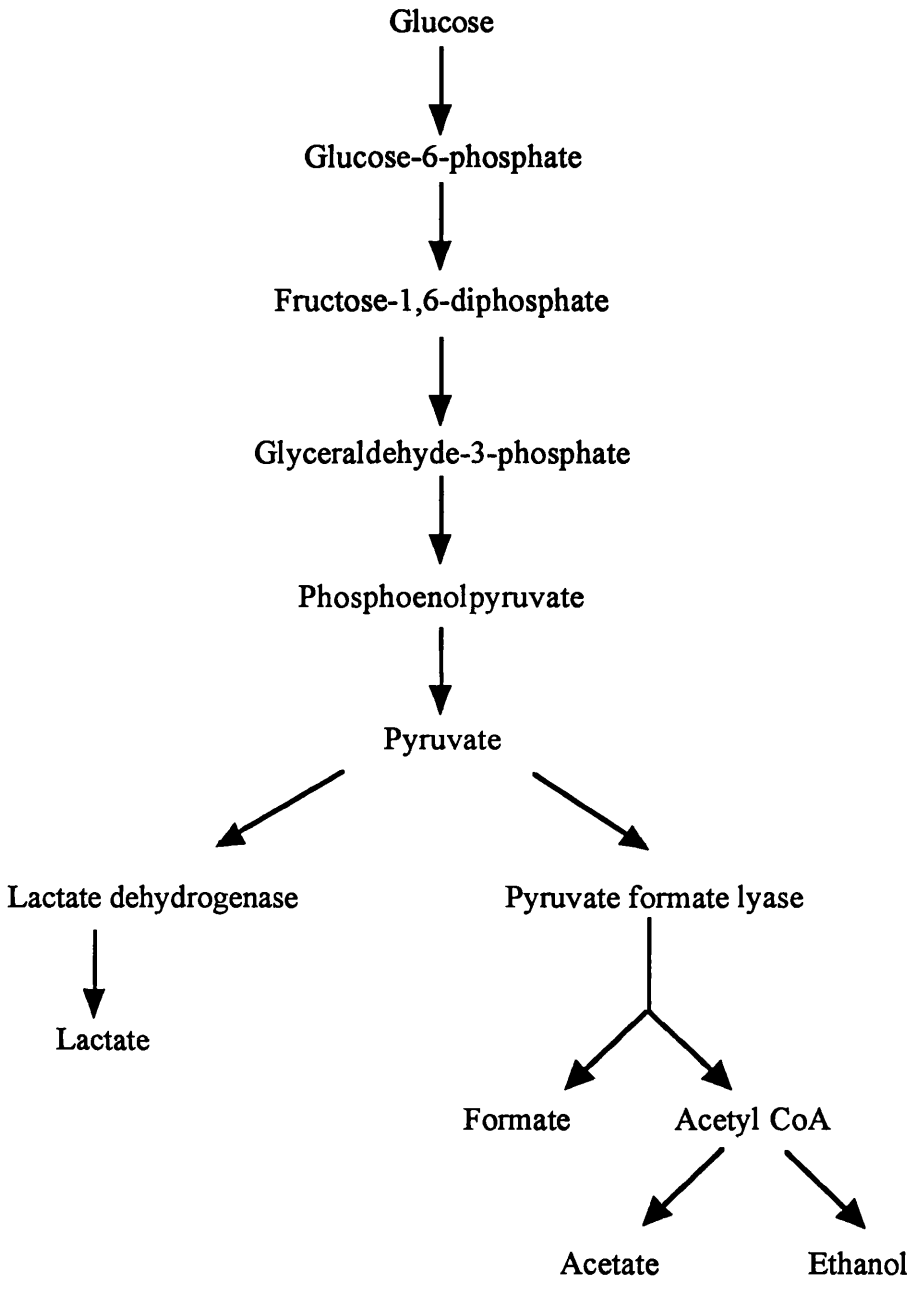


Fig.1.1 Metabolism of glucose by *S. mutans* and the formation of acid end products (After Tanzer et al. 1969)

and after incubation with cells suggests that the pattern of protein metabolism of the two species is different (Cowman et al. 1974, Cowman and Fitzgerald 1975).

Glycolytic activity of plaque can be enhanced by peptides and low molecular weight proteins. Kleinberg et al. (1973) reported one peptide that can increase lactic acid production by plaque bacteria, but can also increase base formation, thereby counteracting the enhanced glycolysis, and therefore could be important in reducing the risk of caries development. This will result in (a) a more rapid clearance of dietary carbohydrate from the mouth, and (b) the generation of base which will both neutralize the acid formed from the increase in glycolytic activity and raise pH to values around neutrality (Kleinberg et al. 1976, 1979). Species including *S. mutans* 'b', *A. naeslundii* and heterofermentative lactobacilli have pH rise activity when incubated with a combination of glucose and either arginine or lysylarginine (Kleinberg et al. 1982).

1.9 Aetiology of dental caries

Dental caries is considered to be an infectious disease of multifactorial aetiology determined by interactions between the host microflora, substrate and time and the tooth surface (Fitzgerald and Keyes 1960, Keyes and Fitzgerald 1962, Newbrun 1978).

Clarke (1924) isolated *S. mutans* from human caries lesions. He concluded that *S. mutans* was associated with dental caries, but other investigators failed to recover this species from lesions. Studies with experimental animals confirmed the essential role of bacteria in dental caries (Orland et al. 1954, 1955). Germ-free rats of a susceptible strain did not develop caries even when they were fed a diet rich in fermentable carbohydrate (Orland et al. 1954). However, if the same animals were infected with enterococci (*S. faecalis*) together with a proteolytic anaerobic rod or an anaerobic pleomorphic rod, then caries

developed (Orland et al. 1955). Subsequently studies showed the transmissible nature of dental caries (Keyes 1960, Fitzgerald and Keyes 1960, Fitzgerald 1968).

Animal and clinical studies have shown that although several species have been isolated from caries lesions, the most cariogenic species are mutans streptococci (Klock and Krasse 1977, 1978, Togelius and Bratthall 1982, Carlsson et al. 1985) see (Table 1.3).

The properties of mutans streptococci that contribute to its cariogenicity include its marked aciduricity (Harper and Loesche 1984), acidogenicity (Minah and Loesche 1977), and ability to synthesize intracellular and extracellular polysaccharides (ECP) from sucrose. However, despite considerable investigation, the relationship between *S. mutans* and caries is not wholly understood. Some studies of dental plaque in relation to caries have shown that various organisms are able to produce caries (Table 1.3), in particular *S. mutans* and lactobacilli were present in higher proportions at carious surfaces than non-carious surfaces (Hardie et al. 1977, Boyar and Bowden 1985, Burt et al. 1985, Milnes and Bowden, 1985). Duchin and van Houte (1978) found that the prevalence of mutans streptococci (MS) could vary markedly between plaque from a "white-spot" lesion and that from the neighbouring sound enamel on the same tooth surface.

However, this organism has been isolated from individuals prior to caries initiation, and caries has occurred in some subjects in the absence of detectable *S. mutans* (Ikeda et al. 1973, Mikkelsen and Poulsen 1976, Swenson et al. 1976). In addition, Thylstrup and Fejerskov (1986) stated that acidogenic bacteria as a whole are responsible for caries rather than a single group or species. These bacteria are present in plaque of caries-active, caries-inactive and caries-free individuals. In support of this view, Marsh et al. (1989a) compared the bacterial composition of plaque from sites with early stages of enamel demineralization with that of sound surfaces, and suggested that formation of lesion may

Table 1.3 Selected studies which associated bacterial species with caries incidence.

References	Micro-organisms
Bratthall (1972)	*S. mutans*
Jordan and Hammond (1972)	*A. viscosus*
Bowden et al. (1976)	*S. mutans*, *Lactobacillus* spp.
Jordan (1976)	*A. viscosus*
Drucker and Green (1981)	*S. faecalis*, *S. milleri*, *S. mutans*, *S. salivarius*, *S. sanguis*
Kohler et al. (1981)	*S.mutans*
Loesche et al. (1982)	*S. mutans*
Boyar and Bowden (1985)	*S. mutans*, *Lactobacillus* spp., *Actinomyces* spp. and *Veillonella*
Milnes and Bowden (1985)	*L. acidophilus*, *L. brevis*, *L. casei*, *L. fermentum*, *L. plantarum*, *L. salivarius*
Thylstrup and Fejerskov (1986)	*A. viscosus*, *L. fermentum*, *L. salivarius*
Boyar et al. (1989)	*A. viscosus*, *S. mutans*, *Lactobacillus* spp.
de Soet et al. (1989)	*S. sobrinus*

be associated with different combinations of bacteria rather than a specific pathogen. Acid produced from dietary carbohydrates is the most important factor in the pathogenesis of dental caries. The production of acid will alter the bacterial environment and reduce the local pH. Although many plaque organisms may produce acids at the critical pH value at which significant amounts of enamel are considered to dissolve (pH 5.4-4.4), only a few species are able to survive and continue to produce acid (Scherp 1971). In addition, van Houte et al. (1991) studied *in vitro* the pH-lowering potential of suspensions of fresh human dental plaque from tooth surface areas with incipient "white spot" caries and from sound tooth surfaces. It was shown that a very rapid pH drop and a very low plaque pH

minimum could occur in the apparent absence of MS or lactobacilli. This suggests that organisms other than MS or lactobacilli can make a critical contribution to a fast pH drop and low pH minimum in dental plaque, and therefore could play a significant role in the aetiology of caries. These non-mutans streptococci generally can constitute a high proportion of the human dental plaque flora (Loesche 1986), and many strains are acidogenic at low pH, as indicated by their low final pH in sugar broth (Carlsson 1967, Kilian et al. 1989). Milnes and Bowden (1985) have investigated the progressing lesions of nursing caries, and they have isolated different species of *Lactobacillus* spp. (Table 1.3), and stated that veillonellae could not modify caries attack. Matee et al. (1992) reported no significant difference in the number of MS between plaque overlying cavities and over adjacent sound enamel, which is in support of the above studies. In contrast, the *Lactobacillus* count was approximately 100-fold higher in plaque overlying cavities than on sound enamel. These findings suggest that the high number of lactobacilli in cavities is due to the provision of favourable local conditions such as a retentive area with a low pH. The increase in number of lactobacilli in cavities and their ability to produce high concentrations of lactic acid at an acidic pH indicates a role for lactobacilli in producing cavitation once initial lesions have formed.

1.10 Site specificity of caries

Caries does not occur randomly on the tooth surface but is localised to certain sites (Kidd and Joyston-Bechal 1987). Pits and fissures in the teeth are the most common sites of dental caries and provide areas where prevention of caries is most difficult (Marthaler 1975). Another commonly affected area is the approximal surface, where carious attack occurs cervical to the contact area. Other smooth tooth surfaces, e.g. buccal, labial and lingual, are less commonly affected. When these sites become carious, it is usually the surface adjacent to the gingival margin that is affected (Schafer et al. 1983, Kidd and

Joyston-Bechal 1987).

1.10.1 Root caries

Nyvad and Kilian (1987) reported that, irrespective of potential differences in the physico-chemical properties and pellicle composition of enamel and root surfaces (a factor which has been suggested to determine the amino acid composition of the acquired pellicle) the bacteria colonizing the two surfaces were identical. However, as caries develops or when the lesion is 'active', the proportions of species can change, and mutans streptococci and *Lactobacillus* spp. may increase significantly in such lesions (Nyvad and Kilian 1990b, Beighton et al. 1993). *Lactobacillus* spp. together with *S. mutans* on a surface may represent a specific situation indicating caries risk (Ellen et al. 1985, Bowden et al. 1990), or a specific stage in lesion development (Bowden 1990).

Adults currently retain more of their natural teeth into the later years of life. For ageing people who continue to be dentate, caries remains a problem, despite the fact that caries is mainly a disease of the young. Several investigations have shown that caries continues into later life, confirming that the disease may not necessarily decline with increasing age in adults (Mandell 1985, Beck et al. 1988, Beighton et al. 1993).

1.10.2 Fissure plaque and caries

The high caries susceptibility of this area relates to the morphology of the pit or fissure (Newbrun 1989). The effects of dental plaque, age and bacterial composition on the pH of artificial fissures in human volunteers were studied by Igarashi et al. (1990). Streptococci and actinomyces dominated the fissure plaque, and their levels were related to the minimum pH of plaque. Fissure plaque of all ages contains high concentrations of acidogenic bacteria (Minah and Chu 1984). A decreasing acidogenic response at the base

of the fissure was observed with increasing plaque age, suggesting that maturing fissure plaque created a diffusion barrier to fermentable carbohydrate (Igarashi et al. 1990). Alternatively it could be due to all the fermentable carbohydrate being used up before it reached the base of the fissure.

Microbiological studies of fissure plaque have shown a wide range in numbers and types of bacteria, although the dominant species are streptococci, especially those producing extracellular polysaccharide (Minah and Chu 1984). Anaerobic bacteria including *Veillonella* and *Propionibacterium* are found in low numbers, as are *Neisseria* spp., and facultative anaerobic Gram-negative rods (Theilade et al. 1982).

1.10.3 Approximal plaque and caries

Caries and chronic gingivitis or periodontitis are usually initiated interdentally. The lack of movement between contiguous approximal surfaces produces a stagnant site for plaque accumulation. Such sites are at increased risk of caries, chronic gingivitis and chronic periodontitis. The increase in plaque thickness resulting from its accumulation at such stagnant sites is associated with increasing anaerobic conditions and reduced rates of diffusion (Newman 1980). The association between plaque accumulation and caries initiation suggests that caries is more likely to occur beneath thick rather than beneath thin plaques (Mellberg et al. 1991). Approximal caries is initiated in relation to the sub-contact portion of plaque (Newman and Morgan 1980). This may be due to the fact that the predominant species which colonize this portion of gingival margin plaque on children's teeth include saccharolytic, acidogenic organisms, which are mainly Gram-positive and Gram-negative polysaccharide-containing cocci, in a largely polysaccharide matrix (Newman 1979).

1.11 Plaque sampling

The hard, non-shedding nature of teeth provides unique conditions for adhesion and proliferation of bacteria to form an immobilised biofilm. Biochemical investigations have suggested that sites near each other may have plaques with different metabolic capabilities (Charlton et al. 1971). This variation could be due to differences in the bacterial composition of plaque on neighbouring surfaces. In order to minimise any effects of such variations, the sampling area should be small and well-defined, approximately 4.0mm^2 (Bowden et al. 1975, Duchin and van Houte 1978). Samples derived from a whole tooth surface might obscure variations in the bacterial composition at different sites on that surface (Loesche and Syed 1973).

Histological studies have shown that bacteria often exist in plaque as discrete micro-colonies (Newman and McKay 1973). Only by selecting the smallest practicable area for sampling can any progress be made by cultural or other methods of examination towards the study of the distribution of bacteria on a given tooth surface. Various means of obtaining suitably small samples have been evaluated (Hardie and Bowden 1974). Samples removed from teeth *in vivo* with dental floss (Edman et al. 1975, Loesche and Laughon 1982), excavator (Ikeda and Sandham 1971) or probes have inherent inaccuracies, because it is difficult to exclude contamination by saliva, and to ensure that the entire thickness of plaque is removed. It is also impossible to define the condition of the underlying enamel or the exact location of the sample when it was *in situ*. A possible solution to this problem is to obtain specimens from carefully extracted teeth. The plaque flora of freshly extracted premolars has been studied by cutting slices of enamel from different surfaces and removing the plaque by scraping (Donoghue 1974). The punch technique has also been used to remove plaque samples from defined areas of freshly extracted teeth (Sidaway 1979, Lindquist and Emilson 1991a).

Marsh et al. (1989a) demonstrated the potential of sampling small sites of approximal plaque on extracted teeth for studies relevant to the earliest stages of enamel demineralisation. In this study it was possible to culture the predominant flora from different sites around the contact area. This method has also been used for the study of approximal plaque on extracted teeth using immunofluorescence techniques (Bush et al. 1989, 1990, Gill et al. 1991).

1.12 Plan of investigation

In order to clarify the microbial ecology of the gingival margin plaque in relation to health and disease, the **entire** cultivable microflora from three sub-sites around the contact area has been examined. Microbiological studies on approximal plaque have indicated previously that the closer the plaque is to the caries-prone sub-contact area, the higher the count of streptococci in general and of MS in particular (Bush et al. 1989, Gill et al. 1991). It is possible that the proportions of a number of the predominant species of plaque organisms are affected by discrete local ecological conditions. Such a shift in the proportions of species could be a more important factor than the mere presence or absence of mutans streptococci. Furthermore, studies by van Houte et al. (1991a, 1991b) have suggested the importance of streptococci other than MS in the initiation of dental caries.

The classification schemes of Kilian et al. (1989), Beighton et al. (1991b) and Johnson et al. (1990) were used to analyze the microflora of dental plaque at three sub-sites in relation to the contact area. Isolates of *Streptococcus* spp. and *Actinomyces* spp. received particular attention. The environmental conditions of these sub-sites may vary from one to another; therefore, the effect this may have on the balance of the plaque microflora was studied.

Not all of the bacteria in a sample are able to grow equally well on a particular growth medium. Such factors as variation in growth rates, inter-species antagonism, inhibitory effects of selective media, and the vast expense of identifying all bacteria present by laborious biochemical tests have limited the practical use of the culture method. A major improvement in this approach would be if colonies could be speciated in a single step. The most promising approach to this is to identify species directly from samples by probing with species-specific antibody or DNA probes. For example, the recovery of *S. sobrinus* has been greatly underestimated by conventional cultural techniques (de Soet et al. 1987). Immunolabelling has overcome some of the problems of identification of selected plaque species (Bush et al. 1989, 1990, Gill et al. 1991). Thus, in the second part of this study immunolabelling was used to study local variations in distribution of selected cariogenic species at the three approximal sub-sites.

Nevertheless, traditional methods such as serological typing and biotyping tests may also be considered to have limited utility in identifying strains, and may not enable variations in the physiology and function of these strains to be identified. For instance, different strains of MS vary in their rate of acid production during glycolysis (Harper and Loesche 1983, de Soet et al. 1989). This may be one of the reasons why correlations between counts of MS and caries lesions are often not made at individual sites. It is apparent that *S. mutans* and other oral bacteria, have a clonal population structure (Lenski 1993). Because each clonal line within a species is slightly different genetically, each will have a slightly different set of physiological and structural properties. Hence, they are likely to differ in their preference for a particular ecological niche or in their virulence (Russell 1994).

Molecular techniques can provide more precise methods of strain identification and

differentiation. Molecular methods such as restriction fragment length polymorphism (RFLP) have been used successfully for studying genetic diversity, transmission, and stability within populations of *S. mutans* (Caufield and Walker 1989, Alaluusua et al. 1994). Kulkarni et al. (1989) have used RFLP to show transmission, proportions and diversity of MS species within members of one family. Rudney et al. (1992) studied strain identification, ecology and transmission of oral streptococci by RFLP pattern.

Nevertheless, despite the broad applicability of this method, its use in clinical microbiology is limited by the prolonged time needed for southern blot transfer and specific hybridization to gene or oligonucleotide probes and the large number of chromosomal bands to be analyzed. Analysis of a specifically defined genetic region provides a more precise method for distinguishing similar strains (DiRienzo et al. 1990).

Nucleotide sequences found in rRNAs vary in an orderly fashion through the phylogenic interrelationship of microorganisms (Woese 1987). Because of their high information content and differing degrees of sequence conservation, ranging from very conserved to highly variable regions, rRNA can be used to measure distant as well as close genealogical relationships (Woese 1987). Therefore, in the third part of the study, the identification system was based on amplification of 16S rRNA genes from clinical isolates of *S. mutans*, *S. sobrinus*, *S. mitis* I, *S. mitis* II and *S. oralis* using the polymerase chain reaction (PCR) (Saiki et al. 1985) and restriction enzyme analysis. This was done in order to find any variation in the pattern of 16S rRNA gene of these species which could be related to their discrete ecological sites of isolation.

1.13 Aims of the thesis

It is possible that the composition of the plaque flora can vary at neighbouring sites. Therefore the aims of the present study were to:

a) Sample discrete sub-sites on approximal surfaces that are known to vary in their disease susceptibility, in order to determine whether any differences in composition of the flora might correlate with the clinically observed pattern of caries and gingivitis.

b) Determine the level of individual species at discrete small sites in gingival margin plaque in relation to their location relative to the contact area.

c) Compare immunofluorescence and culture techniques for their ability for identification and quantification of selected caries-related species in plaque.

d) To establish whether relationships exist between the predominant ecological factors prevailing at different sites on the approximal surface and the microbial composition of plaque.

e) To evaluate and develop existing molecular biological approaches to improve discrimination between some species and biovars of study streptococci that were difficult to discriminate using conventional methods.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Clinical Material and Plaque Sampling

2.1.1 Subjects and sampling sites

Plaque samples (270) were obtained from approximal surfaces of 90 teeth from 60 schoolchildren (46 girls and 14 boys; mean age 12.5 ± 1.4 year) from different areas of London. All the 90 teeth were clinically sound premolars (extracted for orthodontic reasons). The number of teeth sampled per patient varied from one to four. Out of the above subjects, 21 teeth from 21 children (16 girls, 5 boys; mean age = 12 ± 1.8 years) were chosen for the culture study (Table 2.1). Both the child and parent or guardian had given their voluntary consent for the teeth to be used in the study.

Table 2.1 Patient and site details, culture study.

Number of patients	Number of teeth
21	21
Sex	**Number of sub-sites**
F = 16 M = 5	N = 63
Mean age	**away side below**
12 ± 1.8 years	12 12 12

F = Female, M = Male

2.1.2 Sampling technique

The freshly extracted teeth were placed in sterile universal bottles (Bibby-Sterilin, Stone, UK) containing 5 ml pre-reduced transport fluid (RTF, Syed and Loesche, 1972) and immediately transferred to the laboratory for bacteriological analysis. The teeth were

rinsed with sterile phosphate-buffered saline (PBS) (Oxoid, Basingstoke, UK) to remove blood and loosely attached bacteria. The teeth were held by sterile forceps, and the plaque was stained with a few drops of 0.5% (w/v) indigo carmine solution (ICI, Cheshire, UK) which had previously been shown not to affect bacterial viability (Marsh et al. 1989b). About 1-2 mm^2 of gingival margin plaque was removed with a sterile dental curette from each of three sub-sites: away from (A), to the side of (S), and below (B) the contact area (Fig. 2.1), and suspended in 800 µl RTF in a sterile microcentrifuge tube (Philip Harris Scientific, London, UK) containing small amount of glass beads (approximately 0.2 mm diameter, BDH, Poole, UK). The teeth were then washed with a few drops of PBS to visualise the contact area. Only extracted teeth with an intact full ring of plaque around the contact area were selected. Plaque samples were processed within 5 minutes of extraction for culture analysis. The teeth were dried at room temperature and the sample sites marked with red nail varnish (Fig. 2.1). Profiles of the teeth were drawn and photographed to record the locations of the sampled sites (Fig. 2.1). The teeth were then stored in 70% aqueous ethanol.

2.1.3 Preparation of teeth for caries diagnosis

The teeth were immersed in water for 2 hours, then the plaque was removed with a soft toothbrush. This procedure also tended to remove the varnish markings from the teeth. Therefore, before brushing, the original markings were replaced with a water-resistant red marker (Staedtler, Germany). The teeth were photographed and the resultant images compared with previous photographs of the stained surface to ensure that the locations of the markings had not changed (Fig. 2.2). The integrity of enamel was studied by visual examination using low-power magnification.

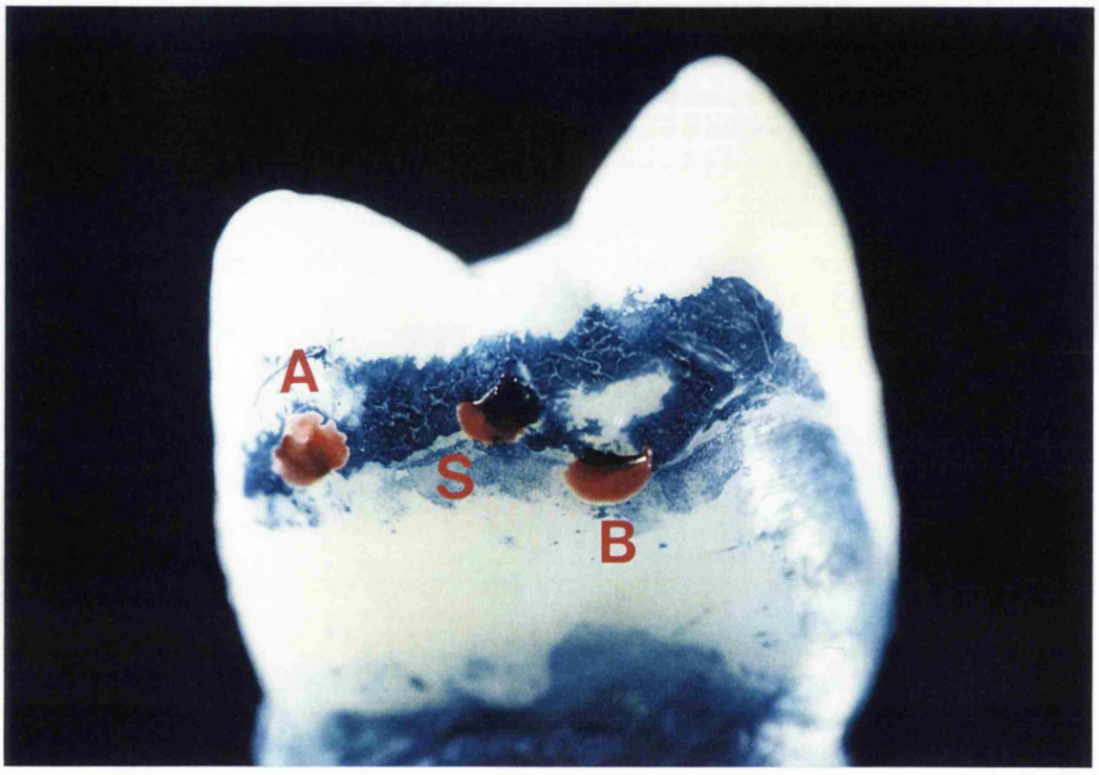


Fig. 2.1 Location of sub-sites around the contact area.

A = Away from contact area.

S = To the side of contact area.

B = Below contact area.

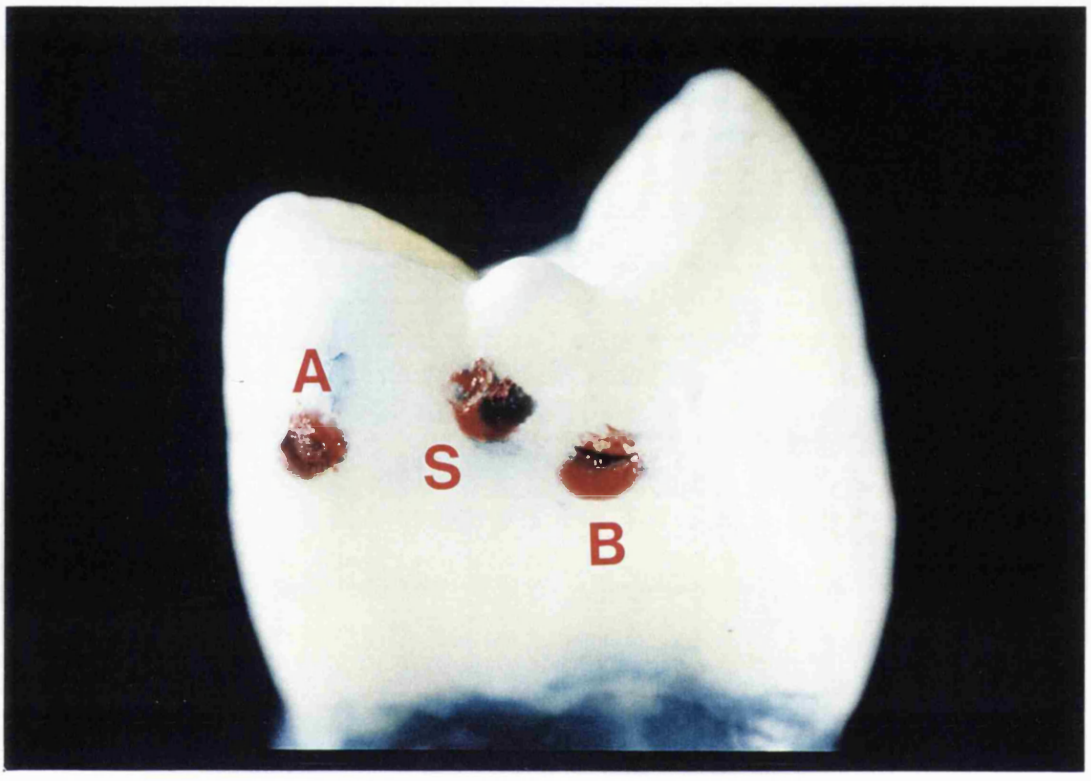


Fig. 2.2 Location of sub-sites around the contact area after plaque was removed.

A = Away from contact area.

S = To the side of contact area.

B = Below contact area.

2.2 Culture procedures

2.2.1 Quality control for media

Several bacterial strains were used as positive controls to evaluate the selective media (Appendix A) and each strain was simultaneously cultured on non-selective media (Columbia blood agar). These strains included, *S. mutans* NCTC 10449, *S. sobrinus* NCTC 27351, *S. oralis* NCTC 11427, *S. oralis* NCTC 7864, *S. salivarius* HHT 76, *L. casei* NCTC 10302, *N. lactamica* NCTC 10618, *V. parvula* NCTC 11463, *A. israelii* NCTC 4860 and *A. viscosus* NY 1B.

In order to test for the sterility of the reduced transport fluid, a Columbia blood agar plate was inoculated with 100 µl of this fluid. In addition, to check for the sterility of both selective and non-selective media, a non-inoculated plate was regularly incubated with each batch of plaque samples.

2.2.2 Reproducibility of culture techniques

Twelve plaque samples were prepared as explained in section 2.1.2 (Bush et al. 1990) and divided into two parts. Each part was treated as an individual sample. These twenty four samples were then serially diluted to 10^{-3} in pre-reduced transport fluid (RTF). Aliquots (100µl) were spread over the surface of duplicate selective and non-selective media (Table 3.1).

2.2.3 Bacterial Analysis of Plaque Samples

Plaque samples were dispersed by vortexing with sterile glass beads, approximately 0.2 mm diameter (BDH, Poole, UK) for 1 min followed by aspiration (12 times) with a sterile syringe (Becton Dickinson, Dublin, Ireland) and a 25G sterile needle (Becton Dickinson, Dublin, Ireland) (Bush et al. 1990). Samples were then serially diluted to 10^{-3} in RTF, and 100 µl aliquots were spread over the surface of pre-reduced selective and non-selective

media. Columbia Agar Base (Oxoid, Basingstoke, UK) supplemented with 7% (v/v) horse blood (Oxoid) was used to enumerate the total cultivable flora, also *Actinomyces*. Tryptone Yeast Cystine (TYC) agar and TYC supplemented with 20% (w/v) sucrose (BDH, Poole, UK) and 0.1 unit/ml bacitracin (TYCSB) (van Palenstein Helderman et al. 1983) were used to recover streptococci and mutans streptococci, respectively. *Veillonella* spp. were isolated from Veillonella vancomycin agar. Rogosa agar was used for isolation of *Lactobacillus* spp. and nutrient agar used to grow isolates for the catalase test and for aerobic growth.

All media were incubated in an atmosphere of 80% N_2, 10% H_2, and 10% CO_2 in an anaerobic chamber for 5 days at 37°C. A summary of the full bacterial analysis of the plaque samples is given in Table 2.2.

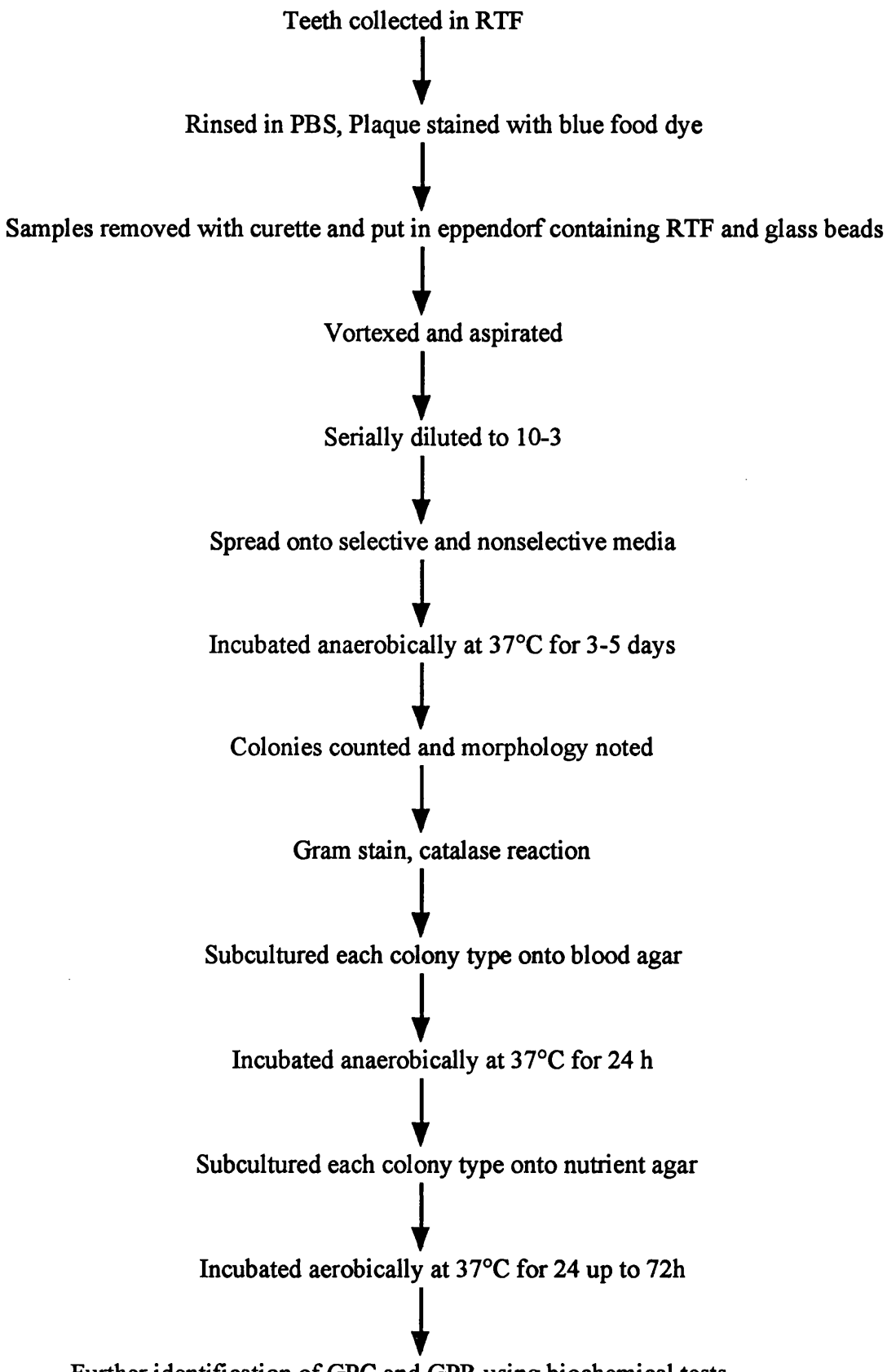
Viable counts of bacteria were expressed as a percentage of the total cultivable microflora, and also as a percentage of the total number of sites colonised (isolation frequency).

The media used for the primary isolation of bacteria, together with details of their preparation, composition and manufacturer are listed in Appendix A.

2.2.4 Bacterial identification

All materials used for biotyping were obtained from Sigma Chemicals Ltd (Poole, UK) or Lab M (Bury, Lancs, UK) unless otherwise stated. Tests were carried out either according to the manufacturers' instructions (Diagnostic Tablets, Rosco Diagnostica 1990), or by the method of Beighton et al. (1991a).

Table 2.2 Plaque sampling scheme.



2.2.5 Streptococci

Several representative colonies of all catalase-negative Gram-positive cocci from TYC and TYCSB agar were isolated and grown overnight on blood agar and Todd-Hewitt broth. Mutans streptococci were distinguished from other streptococci biochemically and sub-divided by their fermentation of N-acetylglucosamine, amygdalin, inulin, mannitol, melibiose, sorbitol and raffinose, the hydrolysis of arginine and aesculin, and on the production of H_2O_2, α-glucosidase and acetoin (Voges-Proskauer; V. P. test) (Hardie and Bowden 1976, Coykendall and Gustafsen 1986, Kilian et al. 1989, Beighton et al. 1991a, b). Substrates were obtained either from Lab M (Bury UK) or Sigma (Poole UK); fermentation tests and enzyme assays were performed according to the manufacturers' instructions. IgA_1 protease activity was demonstrated using human myeloma IgA_1 (kindly donated by Professor M. Kilian, Aarhus, Denmark) as the substrate (Kilian and Holmgren 1981). Neuraminidase was detected using a 4-methylumbelliferyl-linked substrate (Beighton et al. 1991a, b) (Table 2.3).

2.2.6 *Actinomyces*

Actinomyces were identified by a combination of catalase, α-fucosidase and urease activity, nitrate reduction and the ability to grow in the presence or absence of air (Scharfen 1973) (Table 2.4).

According to the recent classification of human strains of *Actinomyces,* the genospecies *A. naeslundii* included strains of *A. naeslundii* and *A. viscosus* (Johnson et al. 1990).

2.2.7 *Neisseria* and *Veillonella* spp.

Neisseria and *Veillonella* sp. were identified by catalase and peroxidase tests, fermentation of sugars, nitrate reduction and the ability to grow in the presence or absence of air (Cowan 1974).

Table 2.3 Identification scheme for oral streptococci

	S. mutans	*S. sobrinus**	*S. sanguis*	*S. gordonii*	*S. mitis* I	*S. mitis* II	*S. oralis*	*S. salivarius*	"*S. milleri*"-group
α-Fucosidase	-	n.d.	_	+	-	-	-	-	-
α-Galactosidase	+	+	-	v	88	77	60	-	-
IgA_1 protease	-	n.d.	+	-	-	-	+	-	-
Neuraminidase	-	-	-	-	20	62	+	-	-
Acid from:									
Arbutin	+	-	+	+	-	-	_	+	+
Amygdalin	81	-	-	+	-	-	-	+	59
Inulin	+	84	+	+	-	-	-	60	-
Mannitol	+	61	-	-	-	-	-	-	-
Melibiose	+	v	v	v	v	+	+	-	-
N-acetyl-glucosamine	+	-	+	+	+	+	+	+	+
Raffinose	+	-	v	v	70	+	56	-	-
Sorbitol	+	-	v	-	-	39	-	-	-
Trehalose	+	n.d.	+	+	30	-	+	70	+
Hydrolysis of:									
Aesculin	+	-	v	+	-	-	-	+	+
Arginine	-	-	+	+	-	+	-	-	+
Voges-Proskauer reaction	+	n.d.	-	-	-	-	-	+	+
EPS production	+	+	+	+	-	-	78	-	-
H_2O_2	-	+	+	+	+	+	+	-	-

+ = > 80% of strains are positive, - = > 80% of strains are negative; figures relate to percentage of strains positive or negative.
EPS production = Extracellular polysaccharide producing, n.d. = Not determined. V = The fermentation reaction results differed with different biovars.
From Kilian et al. (1989), *Beighton et al. (1991b).

Table 2.4 Identification scheme for *Actinomyces* spp.

Species	Colony pig[a] and morph[c]	O_2 Growth	Catalase	αFuc[b]	Urease	NO_3 Reduction
A. israelii	small white	-	-	-	-	+
A. naeslundii	fawn	±	±	-	+	+
A. odontolyticus	red/brown	±	-	(80%)	-	+

[a] = pigmentation.
[b] = α-Fucosidase.
[c] = morphology
( ) = Percentage of strains positive.
± = The reaction to this test was variable.

2.2.8 *Lactobacillus* spp.

Lactobacillus spp. were catalase negative, unbranched, regular or curved Gram-positive rods. They formed large white colonies and were recovered from Rogosa SL agar (Rogosa et al. 1951). Criteria for identification of oral bacteria are given in Table 2.5.

In addition there were some isolates that could not be identified further other than Gram-staining reaction. These were grouped under: Other (Unidentified) GPC; Other GPR; Other GNC and Other GNR.

Table 2.5 Criteria for identification of oral bacteria.

Organism	Identification test	
Streptococcus	Growth morphology on TYC and TYCSB from plaque samples.	
	Gram stain	GPC
	O_2 growth	+
	Catalase test	-
	Biochemical tests (Table 2.3).	
Actinomyces	Growth morphology on blood agar (BA)	
	Gram stain	GPR
	Biochemical tests (Table 2.4)	
Lactobacillus	Growth morphology on Rogosa SL	
	Gram stain	GPR
	O_2 growth	+
	Catalase	-
Neisseria sp.	Growth morphology on BA	
	Gram stain	GNC
	O_2 growth	+
	Catalase test	+
Veillonella sp.	Growth morphology on Veillonella agar	
	Gram stain	GNC
	O_2 growth	-
	Catalase test	±
Fusobacterium sp.	Growth morphology on BA	
	Gram stain	GNR
	O_2 growth	-
	Catalase test	-
Other	Gram stain	±
	O_2 growth	±
	Catalase test	±

GPC= Gram-positive coccus, GPR= Gram-positive rod, GNC= Gram-negative coccus, GNR= Gram-negative rod. Other = GPC, GPR, GNC and GNR.

2.3 Immune labelling technique and direct microscopic count

2.3.1 Sample preparation for IF study

Plaque samples were prepared as illustrated in sections 2.1.1 and 2.1.2. The plaque suspensions were vortexed for a few seconds before aliquots (15 µl) of each were dispensed onto multi-spotted (12 wells) slides (Hendley, Essex, UK), and dried in a 37°C oven for 2h. The films were then flame-fixed and the slides placed in special boxes containing copper sulphate crystals to exclude water, and stored at +4°C until required. The four antisera (anti-*S. mutans* serotype 'c' NCTC 10449, anti-*S. sobrinus* serotype 'd' NCTC 27351, anti-*L. casei* NCTC 10302 and anti-*L. acidophilus* NCTC LA 435) used for this investigation were raised in BALB/c mice at UMDS Guy's Hospital, London, by the method described by Bush et al. (1990).

2.3.2 Working dilution

The optimum working dilution was determined by titration. Each new batch of antiserum was diluted decimally from 1:10 to 1:800 and the highest dilution which gave a 4+ intensity of staining was chosen. The intensity of staining of the cells was graded on a 0 to 4+ basis, with 4+ representing brilliant staining (Moody et al. 1958).
The working dilutions of each antiserum were: *S. mutans* 'c' 1:200, *S. sobrinus* 'd' 1:600, *L. casei* 1:250 and *L. acidophilus* 1:600.

Each high-titre polyclonal mouse antiserum was used in conjunction with a rabbit anti-mouse IgG (H + L chains) fluorescein isothiocyanate (FITC) conjugate (ICN Biomedicals, High Wycombe, UK), diluted 1:30 in PBS.

2.3.3 Indirect IF labelling

The method of staining and optimal conditions for use were as described by Bush et al. (1989). Briefly, aliquots (15µl) of the antiserum diluted 1:200 in PBS were applied to the films, which were then incubated in a moist chamber for 30 minutes at room temperature. Films were then rinsed with PBS, slotted into a slide rack and washed in a buffer (PBS) tank, continuously stirred for 30 minutes, then air-dried. Rabbit anti-mouse IgG (H+L chains) FITC conjugate (ICN Biomedicals, High Wycombe, UK) was diluted 1:30 in PBS, and aliquots (15µl) applied to the films which again were incubated in a humid chamber

at room temperature for 30 minutes. After a further wash, films were carefully dried and mounted in Citifluor AF1 (Citifluor Ltd. The City University, London, UK). The slides were covered with a glass cover slip, excess mounting solution removed with tissue paper, and the edges of the coverslips sealed with glyceel (Gurr, BDH, Poole, UK).

2.3.4 Specificity of antisera

The specificity of each high-titre antiserum (primary antisera) was tested against an existing panel of 75 strains of oral micro-organisms (Table 2.6). The antiserum raised against *L. casei* showed cross-reactivity only with *S. salivarius* HHT 76, whilst the anti-*L. acidophilus* antiserum cross-reacted with *S. salivarius* HHT 76, *S. oralis* NCTC 7864, *S. oralis* NCTC 11427 and *S. mitis* BM 1296. Therefore, the anti-*L. casei* and anti-*L. acidophilus* antisera were adsorbed with *S. salivarius* by the method of Moody et al. (1958). The cross-reactivity of anti-*L. acidophilus* antisera with *S. salivarius* HHT 76, *S. sanguis* 7864, *S. oralis* 11427 and *S. mitis* BM 1296 was found to be caused by a common antigen. After adsorbing with *S. salivarius* HHT 76 or *S. sanguis*, the serum was no longer cross-reactive; however, the titre was reduced from 1:600 to 1:300 dilution. The anti-S. *mutans* and anti-*S. sobrinus* antisera showed no cross reactions, reacting with only the homologous strains and none of the other 75 strains that were tested (Table 3.7). The rabbit anti-mouse antiserum cross-reacted with *Staphylococcus aureus* and some of the filamentous rods (Table 3.7). Strains which cross-reacted with any of the antisera were photographed using both phase contrast and fluorescence and their morphology noted.

The anti-*L. casei* and anti-*L. acidophilus* antisera were pooled, giving a final titre of 1:200 for anti-*L. casei* and 1:300 for anti-*L. acidophilus*. This mixture was used as a primary antiserum for IF staining of lactobacilli.

Table 2.6 List of 75 oral bacteria used to test for cross-reactivity of antisera.

No	Species	Strain No.	Gram stain and cell morphology
1	*Actinobacillus actinomycetemcomitans*	9709 (NCTC)	G^- rod
2	*Actinobacillus actinomycetemcomitans*	10980 (NCTC)	G^- rod
3	*Actinobacillus actinomycetemcomitans*	Y4 (NCTC)	G^- rod
4	*Actinomyces odontolyticus*	9935* (NCTC)	G^+ rod
5	*Actinomyces viscosus*	10951* (NCTC)	G^+ rod
6	*Actinomyces viscosus*	WV U1371 (NCTC)	G^+ rod
7	*Actinomyces viscosus*	NY 1B (NCTC)	G^+ rod
8	*Actinomyces naeslundii*	10301 (NCTC)	G^+ rod
9	*Actinomyces israelii*	4860 (NCTC)	G^+ rod
10	*Arachnia propionica*	11666 (NCTC)	G^+ rod
11	*Actinomadura madurae*	5654* (NCTC)	G^+ rod
12	*Branhamella catarrhalis*	11020* (NCTC)	G^- coccus
13	*Bifidobacterium dentium*	11816 (NCTC)	G^+ rod
14	*Bacteroides fragilis*	9343* (NCTC)	G^- rod
15	*Campylobacter concisus*	11485* (NCTC)	G^- rod
16	*Capnocytophaga gingivalis*	11654 (ATCC)	G^- rod
17	*Campylobacter rectus*	11489* (NCTC)	G^- rod
18	*Capnocytophaga* sp. (*sputigena*)	11654 (NCTC)	G^- rod
19	*Capnocytophaga sputigena*	33612 (ATCC)	G^- rod
20	*Corynebacterium hofmanii*	plaque isolate	G^+ rod
21	*Corynebacterium matruchotii*	10254 (NCTC)	G^+ rod
22	*Eikenella corrodens*	10596 (NCTC)	G^- rod
23	*Eikenella corrodens*	10647 (NCTC)	G^- rod
24	*Eubacterium alactolyticum*	23263 (ATCC)	$G^\pm$ rod**
25	*Eubacterium saburreum*	33271 (ATCC)	$G^\pm$ rod**
26	*Fusobacterium nucleatum*	10562 (NCTC)	G^- rod
27	*Fusobacterium naviforme*	11464 (NCTC)	G^- rod
28	*Haemophilus aphrophilus*	11098 (NCTC)	G^- rod
29	*Haemophilus parainfluenzae*	7857* (NCTC)	G^- rod
30	*Lactobacillus acidophilus*	LA 435 (NCTC)	G^+ rod
31	*Lactobacillus casei*	10302 (NCTC)	G^+ rod
32	*Lactobacillus odontolyticus*	1406 (NCTC)	G^+ rod
33	*Leptotrichia buccalis*	10249* (NCTC)	G^- rod
34	*Micrococcus mucilaginosus*	10663 (NCTC)	G^+ coccus
35	*Neisseria* sp.	BM1305/A1078	G^- coccus
36	*Neisseria lactamica*	10618 (NCTC)	G^- coccus
37	*Neisseria pharyngis*	4590 (NCTC)	G^- coccus
38	*Nocardia asteroides*	11293* (NCTC)	G^+ rod
39	*Peptostreptococcus anaerobius*	11460* (NCTC)	G^+ coccus

Table 2.6 Continued

No	Species	NCTC No.	Gram stain and cell morphology
40	*Prevotella corporis*	33547 (ATCC)	G^- rod
41	*Prevotella denticola*	33184 (ATCC)	G^- rod
42	*Prevotella loescheii*	15930 (ATCC)	G^- rod
43	*Prevotella nigrescens*	9336 (NCTC)	G^- rod
44	*Prevotella melaninogenica*	11321 (NCTC)	G^- rod
45	*Prevotella oralis*	11459* (NCTC)	G^- rod
46	*Porphyromonas gingivalis*	W50 (NCTC)	G^- rod
47	*Propionibacterium acnes*	737* (NCTC)	G^+ rod
48	*Rothia dentocariosa*	10917* (NCTC)	G^+ rod
49	*Selenomonas sputigena*	33150 (ATCC)	G^- rod
50	*Simonsiella crassa*	10283* (NCTC)	G^- rod
51	*Staphylococcus aureus*	11561 (NCTC)	G^+ coccus
52	*Streptococcus intermedius*	11324 (NCTC)	G^+ coccus
53	*Streptococcus mitior (mitis)*	BM 1296 (NCTC)	G^+ coccus
54	*Streptococcus mitis* I	NS51 (NCTC)	G^+ coccus
55	*Streptococcus mitis* II	SK132 (NCTC)	G^+ coccus
56	*Streptococcus milleri*	10709 (NCTC)	G^+ coccus
57	*Strep. mutans* 'a' (*S. cricetus*)	AHT (NCTC)	G^+ coccus
58	*Strep. mutans* 'b' (*S. rattus*)	RAT 78 (NCTC)	G^+ coccus
59	*Strep. mutans* 'c' (*S. mutans*)	10449* (NCTC)	G^+ coccus
60	*Streptococcus mutans* 'c'	Guys 78 (NCTC)	G^+ coccus
61	*Streptococcus mutans* 'c'	R9 (NCTC)	G^+ coccus
62	*Strep. mutans* 'd' (*S. sobrinus*)	OMZ176 (NCTC)	G^+ coccus
63	*Strep. mutans* 'e' (*S. mutans*)	T 93 (NCTC)	G^+ coccus
64	*Strep. mutans* 'f' (*S. mutans*)	OMZ 175 (NCTC)	G^+ coccus
65	*Strep. mutans* 'g' (*S. sobrinus*)	6715 WT (NCTC)	G^+ coccus
66	*Strep. mutans* 'h' (*S. downei*)	11391 (NCTC)	G^+ coccus
67	*Streptococcus oralis*	11427 (NCTC)	G^+ coccus
68	*Streptococcus salivarius*	HHT 76 (NCTC)	G^+ coccus
69	*Streptococcus sanguis*	10904 (NCTC)	G^+ coccus
70	*Streptococcus sanguis*	7864 (NCTC)	G^+ coccus
71	*Streptococcus vestibularis*	12166 (NCTC)	G^+ coccus
72	*Treponema denticola*	35405 (ATCC)	G^- rod
73	*Treponema vincentii*	35580 (ATCC)	G^- rod
74	*Veillonella dispar*	17745 (ATCC)	G^- coccus
75	*Veillonella parvula*	11463 (NCTC)	G^- coccus

* Type strain.
** *Eubacterium* spp. are often Gram-variable.
ATCC American Type Culture Collection.
NCTC National Collection of Type Cultures.
Strep. = *Streptococcus*

2.3.5 Adsorption of antisera

The anti-*L. acidophilus* antiserum was adsorbed with *S. salivarius* HHT 76. *S. salivarius* HHT 76 was grown in Tryptone Soya Broth anaerobically for 24h and washed once with sterile saline. After centrifugation, the cells were mixed with four volumes of the serum and incubated at 37°C for 3h. The suspension was stirred periodically during the incubation period. The adsorbed serum was separated from the cells by centrifugation. After adsorbing anti-*L. acidophilus* antiserum with *S. salivarius* HHT 76 it was found that the cross-reaction with *S. sanguis* 7864, *S. oralis* 11427 and *S. mitis* BM 1296 also deteriorated, indicating the possibility of a common antigen as a cause of this cross-reaction.

2.3.6 Sensitivity of antisera

This experiment was performed to establish whether *S. mutans, S. sobrinus* or lactobacilli, when pooled together, could each be accurately identified and enumerated. A number of pure cultures of bacteria (species known not to cross-react with the given antiserum) were prepared by suspending cells in 10 ml Wilkens-Chalgren Anaerobe broth (Oxoid, Basingstoke, UK), and incubating anaerobically for 2 days at 37° C. Loops of the culture were Gram-stained to ensure purity and the broth was separated from the cells by centrifuging at 4000 g for 15 min. Pellets were washed twice by resuspending in PBS (pH 7.2), and centrifuging and decanting off the supernatant. Final suspensions were prepared with a turbidity similar to No. 5 on the MacFarland scale (Elmer et al. 1988). Mixtures of cells of *S. mutans* 'c' NCTC 10449 with *S. oralis* NCTC 7864 and of *S. mutans* with *L. casei* NCTC 10302 in the ratios 1:10 to 1:100,000 were prepared. Aliquots of 15 µl of each mixture were dispensed onto multi-spotted slides, dried and IF-stained, and then examined by immunofluorescence and phase contrast microscopy. Total numbers of fluorescing cocci were compared to the total number observed by phase contrast in randomly selected microscopic fields (0.01 mm^2); fluorescent cells were enumerated

similarly using appropriate illumination. This procedure was repeated for anti-*S. sobrinus*, anti-*L. casei* and anti- *L. acidophilus* antisera.

Paired t tests were performed on the data. The sensitivity of the method is limited by the number of cells easily counted (500 per specimen in this study). This procedure was repeated for *S. sobrinus* NCTC 27351, *L. casei* LA 435, and *L. acidophilus* NCTC 10302.

2.3.7 Positive and negative controls for IF staining

Cultures of *S. mutans* serotype 'c' (NCTC 10449) and *S. sobrinus* serotype 'd' (NCTC 27351) were grown in Tryptone Soya Broth with and without 5% (w/v) sucrose for up to 5 days in order to investigate the effect of any extracellular polysaccharide on IF staining. The possible inhibition of IF staining by *in vivo* factors relevant to natural plaque (cell-bound Ig, Emilson et al. 1974) was also tested by treating films of pure cultures of the homologous strain, grown in Tryptone Soya Broth with and without 5% (w/v) sucrose (BDH, Poole, UK), and with and without human serum (HS) for 1 h and followed by IF staining (Bush et al. 1990).

Duplicate films were treated with PBS and anti-mouse FITC conjugate as negative controls. *S. mutans* serotype 'c' (NCTC 10449), *S. sobrinus* serotype 'd' (NCTC 27351), *L. casei* (NCTC 10302) and *L. acidophilus* (LA 435) were IF labelled with their specific antiserum (section 2.3.3) as positive controls for antisera.

2.3.8 Monoclonal antibody (Mab) studies

The FITC-conjugated rabbit anti-mouse antiserum cross-reacted with *S. aureus* and some filamentous rods (Table 3.7). This was countered by using negative controls to note the morphology of the cells. However, it is possible that during the dispersion of plaque some of these filamentous rods had been fragmented, so that the remnants resembled cocci.

Two slides were prepared from each of sixty plaque samples, one was treated with anti-*S. sobrinus* polyclonal antiserum and the other with a monoclonal antibody that specifically stains *S. sobrinus* (OMVU10, de Soet et al. 1987) which was kindly donated by Dr. J. de Soet, Amsterdam, Netherlands.

Monoclonal antibody was diluted 1:500 in PBS, and 40 µl applied to each well, and then incubated for 1h at room temperature. After washing, 40 µl of FITC-conjugated rabbit anti-mouse antiserum (ICN Biomedicals, High Wycombe, UK), diluted 1:30 in PBS, was added into each well and the slides incubated for 1h at room temperature (de Soet et al. 1987). The slides were washed, covered with a cover slip and then examined (see section 2.3.10). Rabbit anti-mouse FITC was used as a negative control. To avoid false positive results, the samples were carefully screened. Where the negative control showed some reaction, very few single cocci were positive and there were no chains of cocci, this was taken as a false positive and not counted.

2.3.9 Direct microscopic counting

All stained films were examined by incident light fluorescence using a Zeiss standard 16 microscope (Zeiss, Oberkochen, Germany) equipped with phase contrast and fluorescence optics. A HBO 50 W mercury lamp and filter set 487716 giving narrow band excitation illumination (485-505 nm) and fluorescence <520 nm was used. Determination of individual species in plaque samples was carried out using a Plan x100 objective and a x10 eye piece incorporating a grid graticule, 0.02 mm^2 (Zeiss, Oberkochen, Germany). Fluorescence microscopy was used to determine the number of IF positive cells. Randomly selected fields were first examined by fluorescence microscopy, then the field was examined by phase contrast microscopy to determine the total cell numbers per field, at least 20 fields for each well (each well contained one sample) were scanned. This procedure was repeated until 400 to 500 cells per sample were counted and the percentage

of fluorescing cells was then calculated. Fluorescence intensity was recorded on a scale of 0 to 4+ (Moody et al. 1958).

2.3.10 Reproducibility test on plaque counting data

Duplicate films of a sample of approximal plaque which was positive for *S. mutans* 'c' were prepared and 10 fields of each sample were counted. Paired t tests on the differences between the percentage values for the two films were then performed. Similar reproducibility assays were performed for *S. sobrinus.*

2.3.11 Photographic processing of slides

Different types of film were tried as there were disadvantages with both fast and slow films. Fast films do not have fine grain and so, although the exposure time is shorter, the detail is poor when projected. Slow films have fine grain and convey the detail, but the exposure times are longer and this can alter colour balance and lead to vibration problems during exposure.

a) Black and white photography

Kodak TMAX 100 and 400 ASA films were compared as follows:

15 seconds exposure, reciprocity 3, 4, 5, or 6, total exposure 30 to 52 seconds, and development in TMAX developer, diluted 1 in 4. Kodak TMAX 400 yielded greater detail, with a 2 - 2.5 minutes exposure. Negatives were processed in neat Acutol developer (Paterson Products, Herts, UK) for 10 min. at 20 °C. Black and white negatives were printed on Kodak Professional paper (F4 M 192 2293).

b) Colour photography

Ektachrome 400x Kodachrome 200 and Kodachrome 25 ASA films were evaluated. The grain size of 400 and 200 ASA films resulted in poor detail. However, Ektachrome 400x

professional film exposed for 15 - 30 seconds (reciprocity = 3) yielded good contrast.

2.4 Statistical analysis

a) Culture: Viable counts of bacteria were expressed as colony forming units (cfu)/ml and as percentage of the total cultivable microflora. Due to the nature of the samples and on the basis of the different sites sampled it was appropriate to consider the samples independent. As the data (cfu/ml) distribution was highly skewed, it was normalised, where possible by $\log_{10}$ transformation of colony forming units/ml and percentage viable counts, and then analyzed for differences between species at each sub-site, using one-way analysis of variance. When a species was not detected, half of the minimum level of detection was used. The half minimum level of detection was 5 cfu/ml and 0.05% of the viable count. However, when species occurred with low frequency resulting in distributions which were not only highly skewed but also not amenable to $\log_{10}$ transformation, the Kruskal-Wallis test was used instead of one-way analysis of variance. If this test proved positive the Mann-Whitney test was then used to identify significantly differing groups.

McNemar's test for comparing two proportions of the paired data (Armitage and Berry 1987), was used to compare the proportion of each species at the three sub-sites. Associations between species were investigated using the comparison of two proportions for unpaired data (Armitage and Berry 1987). The standard error (SE) is given, as this provides a way of predicting the true population mean from the sample mean, i.e., there is a 95% chance that the true population mean will lie within the range: sample mean ± 1.96 x SE sample mean.

The Wilcoxon matched pairs signed ranks test (Siegel 1956) was used for comparison of percentage viable counts or cfu/ml of species at the same sites. However, where a large

number of comparisons are made with a set of data it is possible that differences significant at $p < 0.05$ may occur by chance in a proportion of cases. This would normally be corrected by the method of Newman-Keuls, Duncan and Scheffe or Bonferroni. However, as the number of comparisons to be made here is very large, these above methods might give too conservative a response (Altman 1991). In these circumstances the best possible solution was to adopt a more stringent level of significance. Therefore, as a precaution, a more stringent level of significance, $p < 0.025$, was selected for comparison of species within the sub-sites (section 3.1.2.7).

b) IF: The IF data were presented as a percentage of total microscopic cell counts (see 2.3.10). One fluorescing cell determined in 500 cells gave a percentage count of 0.2%. This was taken as the minimum limit of detection (Bush et al. 1989). Therefore, comparison of proportions of each species at the three sub-sites was carried out using McNemar's test. Associations between species were determined using the comparison of two proportions for unpaired data (Armitage and Berry 1987).

c) Caries: Chi-square analysis (Siegel 1956) was used to test for associations between the presence of individual organisms and caries (white spot lesions).

2.5 Restriction enzyme analysis of 16S rRNA

2.5.1 Preparation of DNA

Genomic DNA from *S. mutans* 'c', *S. sobrinus* 'd', *S. mitis* I (NS51), *S. mitis* II (SK132) *S. oralis* (LVG1) and clinical isolates of these species were obtained using a modification of the Marmur method (Marmur, 1961). Pre-reduced brain heart infusion (BHI) broth (Oxoid, Basingstoke, UK) was made up with DL-threonine (Sigma, Poole, UK) to a final concentration of 10 mM and pre-reduced by overnight incubation in an anaerobic chamber. Cell cultures were prepared by inoculating 100 ml of the pre-reduced BHI broth with 10 ml of an overnight inoculum. Cultures were incubated in an anaerobic chamber at 37° to exponential phase (2h-3h), until the broth turned cloudy, then solid glycine (BDH, Poole, UK) was added slowly to the culture to a final concentration of 5% w/v. The cultures were allowed to continue to grow for a further 1h - 2h. Cells were harvested at 4000 g and 4°C for 15 min.

2.5.2 DNA extraction

Cell pellets were resuspended in 1 ml TE buffer (Tris-HCl, EDTA, pH 7.2) in two sterile microcentrifuge tubes (500µl each) centrifuged at 13000 g for 1 min, and the pellet resuspended in 300µl of 10mg/ml RNAase (Sigma, Poole, UK), see Appendix B. 50µl of 20mg/ml lysozyme (Sigma L-2879) (see Appendix B) was added and the mixture incubated at 37°C for 20 min, followed by the addition of sodium dodecyl sulphate (SDS, Sigma, Poole, UK) to a final concentration of 1% (15µl of 20% w/v) and a further 10 min incubation. The final volume was made up to 600µl with TE and mixed very gently 50 times, then 300µl of phenol saturated with TE (See Appendix B) (BDH, Poole, UK) and 300 µl of chloroform (BDH, Poole, UK) were added and mixed gently 20 - 50 times.

The tubes were then centrifuged for 10 - 15 min in an MSE bench microcentrifuge (13,000 g). The upper layer was removed with a wide bore (2.5 - 3.5 mm diameter) sterile pipette tip. This stage was repeated 3 times.

The DNA was precipitated with one tenth of the volume of 5 M ammonium acetate and two volumes of cold (-20°C) ethanol, and mixed gently 5 - 10 times. The mixture was stored at -20°C overnight to precipitate the DNA.

Chromosomal DNA was spooled with a sealed bent glass pasteur pipette and washed in 70% (w/v) ethanol, then air dried for a few minutes at room temperature (while still adhering to the glass pasteur pipette), and resuspended in 500µl of TE, allowing the DNA to dissolve slowly.

2.5.3 Quantification of DNA

The concentration of extracted DNA was determined from the absorbance values obtained at 260nm. An OD_{260} reading of 1 is equivalent to 50 µg/ml double stranded DNA (Sambrook et al. 1989). Purity was estimated by taking the ratio of the OD_{260}: OD_{280} readings. Ratios between 1.8 and 2.0 indicate the sample is pure. The OD_{260} and OD_{280} of the DNA samples were measured in quartz cuvettes using a spectrophotometer (Ultraspect III, Phamacia, UK).

2.5.4 Agarose gel preparation

Agarose gel (No. A-6013 Agarose type I Low EEO M_r 0.12; Sigma, Poole, UK) was made up to 0.8 % w/v with 1x TBE (Appendix B) and melted by heating, followed by

cooling in a water bath at 65°C. Subsequently, 30 ml of the mixture was poured into a mini gel tank (100 x 70 mm^2, Flowgen, Kent, UK) and the gel allowed to set at room temperature for 30 minutes. The two end brackets were removed and the gel tank was filled with 50 ml TBE (1 x concentration).

2.5.5 Agarose gel electrophoresis for examination of DNA

The DNA sample (10 µl) was mixed with (2µl) loading buffer (Appendix B) in a sterile microcentrifuge tube, and 8 µl of this was loaded in one well of the gel. A measured amount of 2 µl of molecular weight size marker (section 2.5.6) was used as a control. The gel was run for 5 min on 100 V then 70 V for 15 min, followed by examination of the gel by UV light using ethidium bromide to enhance the visualization of DNA. The gels were photographed under ultraviolet illumination (Chromato-vue, model TM 36, Inc, USA) using Polaroid 665 film (Sigma UK) and a Polaroid CU-5 Land camera (5 inch lens, Oxford UK). The exposure time was 30 seconds.

2.5.6 Polymerase chain reaction (PCR)

PCR enables the 10^8 fold amplification of a specific region of DNA *in vitro* in a matter of hours (Saiki et al. 1985). The process involves repeated cycles of heat denaturation, annealing of 2 oligonucleotide primers designed to define the fragment to be amplified, and extension of the annealed primers with the four dNTPs and DNA polymerase (Fig. 2.3).

Using PCR, 16S rRNA genes of *S. mutans* 'c', *S. sobrinus* 'd', *S. mitis* I (NS51), *S. mitis* II (SK132) and *S. oralis* (LVG1) were amplified using the following reagents.

buffer (10x)	10µl
sterile distilled water	84µl
dNTPs	1µl (100 mM)
primer A (the 5'primer)	1.5µl (0.1mg/ml)
primer B (the 3'primer)	1.5µl (0.1mg/ml)
DNA	1µl (0.3mg/ml)
Taq polymerase	1µl (2U/µl)

All the reagents used were obtained from Promega, Southampton, UK, except where otherwise noted.

The reagents were added to a 0.5 ml sterile microcentrifuge tube (Sigma, Poole, UK) in the order shown above. The 10 x buffer contained 15 mM Mg Cl_2, 1µl of 100 mM dNTPs (25 mM of each of dATP, dCTP, dGTP and dTTP). The oligonucleotide primers used in this study were synthesized by Molecular Medicine, King's College London, UK. Sequences corresponded to those within the 16S rRNA genes of streptococci, the forward primer and the reverse primers were 5'-AGAGTTTGATCCTGG CTCAG-3' and 5'-GGTT ACCTTGTTACGACTT-3', respectively (Eden et al. 1991). These were used at a concentration of 150 ng/µl. 300ng DNA (phenol and chloroform extracted DNA) was added last. The total volume of reaction mixture was 100µl. In addition to the samples two controls were prepared. The negative control comprised 1 µl sterile distilled water instead of DNA. The positive control consisted of 300ng of previously PCR tested DNA. Sixty µl of light weight mineral oil (Sigma, Poole, UK) was added to each reaction mixture and the microcentrifuge tubes pulsed for several seconds in the microcentrifuge. The microcentrifuge tubes were placed in a PCR machine (Techne PHC3, Cambridge, UK) which was programmed as follows:

5 minutes at 92°C for denaturing (resulting in single stranded DNA), then 1 minute at

80°C. The Taq polymerase (1µl=5U) was added at this point (Faloona et al. 1990). The thermal profile used included 30 cycles, denaturing at 92°C for 1.5 min, primer annealing at 55°C for 1.5 min, and nucleotide extension at 72°C for 1.5 min (Fig. 2.3). Approximately 8µl of the reaction volume was then analysed by gel electrophoresis (section 2.4.9).

2.5.7 Examination of PCR products (Agarose gel)

Metaphor agarose (Flowgen, Kent, UK) was made at a concentration of 1.8% with TBE (1x concentration) in a conical flask covered with perforated cling film and then melted in a microwave oven (850 W, Sharp, Watford, UK) for 2 min, then cooled to 65°C in a water bath. 30ml of the melted gel was poured in a mini gel tank (100 x70 mm^2, Flowgen, Kent, UK). This gel was kept for 1h at room temperature, and then placed for a further 1h in the fridge at 4°C to develop solidly.

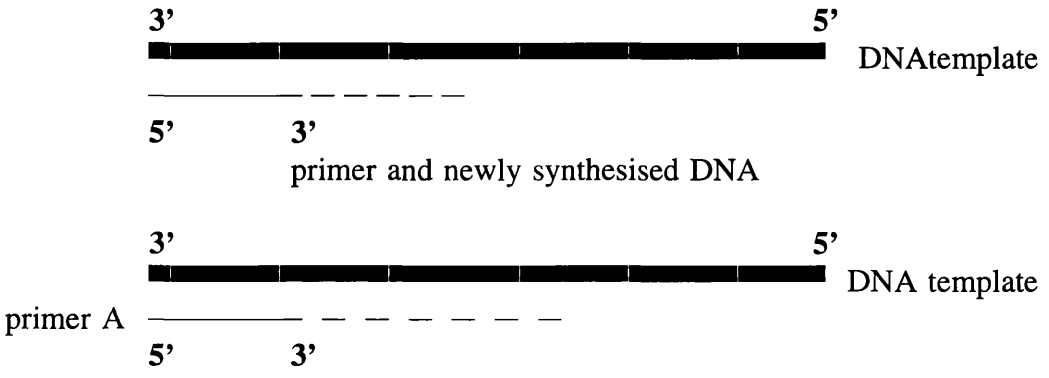
2.5.8 Agarose gel (Metaphor) electrophoresis

After the gel had set, the gel tank was loaded with 50 ml of TBE buffer (x1, Appendix B), the comb removed and the first, middle and last wells loaded, each with 2µl of the molecular weight markers (see section 2.5.6). Eight µl of each sample were mixed with loading buffer as in section 2.5.4 and loaded on the gel and the gel was then run for 1h at 50V. The gel was then stained in a solution of ethidium bromide (2.5 µg/ml water) with gentle shaking for 10 minutes.

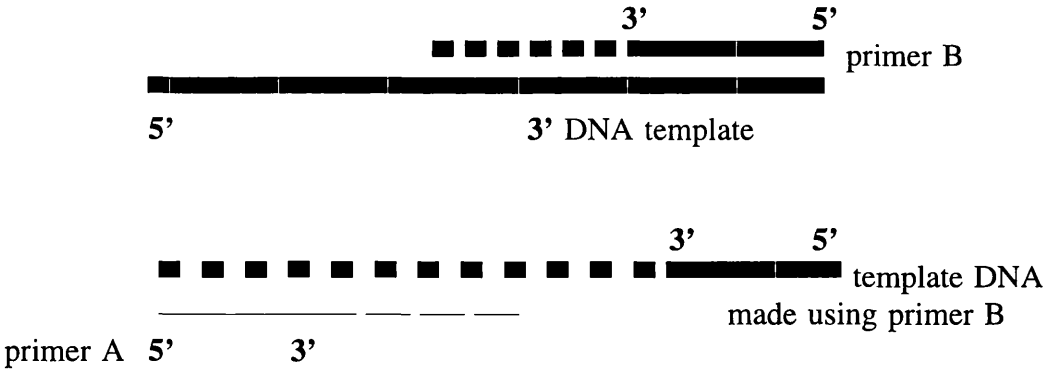
The gels were photographed under ultraviolet illumination using Polaroid film and a Polaroid camera (see section 2.5.2). The optimum exposure time was 30 seconds.

Fig. 2.3 Illustration of the polymerase chain reaction

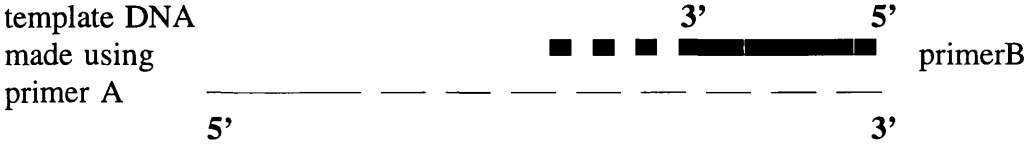
A primer which is complementary to a DNA sequence will base pair with that region if the double stranded DNA is heated to separate the strands, and allowed to cool in the presence of the primer. By adding the components necessary for DNA synthesis (dNTPs and DNA polymerase) a new strand of DNA, complementary to the original strand, will be made using the 3' end of the primer as starting point.



After primers anneal to opposite strands the following will occur.



Subsequently the strand newly synthesised from one primer acts as a template for the second primer.



After many such cycles the product is a single fragment (total yield 1 -10 μg).

2.5.9 Restriction endonuclease analysis

All the materials used in this part of the study were obtained from Promega (Promega, Southampton, UK) unless otherwise stated. The amplified 16S rRNA genes of *S. mutans* 'c', *S. sobrinus* 'd', *S. mitis* biovar I (NS51), *S. mitis* biovar II (SK132) and *S. oralis* (LVG1) were digested with eight restriction enzymes (Table 2.7). A group of six-bases recognition enzymes which included *Bam*HI, *Hin*dIII, *Eco*RI, *Sma*I, *Kpn*I, *Pst*I, *Pvu*II and the four base cutter *Hae*III were used (Table 2.7). All these enzymes were used with multi- core buffer but *Hae*III, *Pvu*II and *Sma*I were used with the specific buffers which were provided with them. The size of restriction fragments was then estimated by running, on the same gel, polynucleotide markers of known molecular weight, such as lambda DNA (ϕ x 174, digested with the enzyme *Hae*III, section 2.5.6).

Table 2.7 Restriction enzymes and their recognition sequences (target sites).

Source	(Name)	Recognition sequence	U/ml	λ cut
Escherichia coli RY13	(*Eco*RI)	G↓AATTC	25000	5
Bacillus amyloliquefaciens H	(*Bam*HI)	C↓GATCC	2000	5
Klebsiella pneumoniae	(*Kpn*I)	GGTAC↓C	15000	2
Haemophilus influenzae	(*Hin*dIII)	A↓AGCTT	18000	6
Haemophilus aegyptius	(*Hae*III)	GG↓CC	10000	>50
Proteus vulgaris	(*Pvu*II)	CAG↓CTG	12000	4
Providencia stuartii	(*Pst*I)	CTGCA↓G	15000	18
Serratia marcescens	(*Sma*I)	CCC↓GGG	90000	3

λ = Lambda
↓ The restriction site.

The reaction was performed in a 1.5 ml sterile microcentrifuge tube. A measured amount (8.5 µl) of PCR product of each species was mixed with 1µl of the buffer. Nine of such tubes were prepared. *Bam*HI, *Hin*dIII, *Eco*RI, *Sma*I, *Kpn*I, *Pst*I and *Pvu*II were used at a concentration of 10U/µl. 0.5 µl of each enzyme was added to each microcentrifuge tube, except *Bam*HI which was provided in a relatively low concentration (2U/µl), 1µl of it was used to digest the same amount of DNA. A negative control containing only PCR product and buffer was also prepared. All the microcentrifuge tubes were incubated in a water bath at 37°C for 3h, but the tube with *Sma*I was incubated at room temperature. For comparative purposes, DNA (PCR product) of *S. mitis* I (NS51), *S. mitis* II (SK132) and *S. oralis* (LVG1) was digested with *Hae*III and *Hin*dIII.

The reaction mixture of each species was run on Metaphor agarose (see 2.6.2 and 2.6.4). The restriction patterns of each species were compared in order to attempt to obtain a unique restriction pattern for each species. These patterns were compared to determine the degree of heterogeneity or relatedness of strains.

CHAPTER THREE

RESULTS

3.1 Culture study

3.1.1 Reproducibilty of plaque sampling and bacterial counts

The results of reproducibility tests for plaque dispersion and primary cultures (section 2.2.2) showed a mean percentage variation of 5.2, with a range of 1.3% to 13%; therefore, the reproducibilty of the techniques was 94.8% (Tables 3.1).

The samples removed from the three sub-sites on each premolar were of small size, as indicated by the low total numbers of bacterial viable count (cfu/ml) (Table 3.2). In general, 20 species were identified, but not all of them were present in each sample. An average of 7-9 species was found at each sub-site. The largest range of isolates was present at sub-site B. Quantitative differences (cfu/ml and percentage of the total viable count) were found between the sub-sites from different subjects and also within subjects (Appendix D, Tables 1-20). Overall, *Actinomyces* spp. and *Streptococcus* spp. accounted for the majority of all the isolates, as is clear from the examination of mean percentage isolation frequencies (Tables 3.3).

3.1.2 Cultivable microflora of plaque from different sub-sites

3.1.2.1 *Actinomyces* spp.

Species of *Actinomyces* were isolated from sub-sites (A), (S) and (B) with mean proportions of 27.2%, 39.9% and 29.2%, respectively. The majority of the 63 surfaces examined had at least one species of actinomyces; only five sub-sites did not have any, four from the site away (A) and one of the below (B) sites. There was no substantial increase in the numbers of other species (e.g. streptococci) at these sites. The most prevalent *Actinomyces* spp. at all sites was *A. naeslundii* with mean percentage isolation frequency of A = 76.2%, S = 85.7% and B = 90.5%. The second most prevalent *Actinomyces* spp. was *A. israelii*, with a mean percentage isolation frequency at sub-sites

Table 3.1 Reproducibility of culture techniques. Duplicate samples of approximal plaque were cultured on blood agar.

Sample Number	Count (CFU) Plate 1	Count (CFU) Plate 2	Dilution factor	% Variation
1	320	290	10^{-2}	9.4
2	230	225	10^{-2}	2.2
3	315	311	10^{-2}	1.3
4	314	301	10^{-2}	4.4
5	75	81	neat	7.7
6	260	265	neat	1.9
7	220	214	10^{-2}	2.3
8	285	300	10^{-3}	5.0
9	80	83	10^{-3}	9.5
10	345	300	neat	13.0
11	264	258	neat	2.3
12	132	129	10^{-2}	3.0

(CFU) = Numbers of colony forming units.
Mean percentage variation ($\bar{x}$ = 5.2%).

A = 47.6%, S = 66.7% and B = 61.9% (Table 3.3), although their proportions were low (Table 3.2). *A. odontolyticus* was recovered with a mean percentage isolation frequency, A = 38.1, S = 33.3 and B = 57.1 (Table 3.3) again as a low proportion of the total viable count (Table 3.2). *A. naeslundii* and *A. odontolyticus* both were isolated more often at sub-site B (Table 3.4), although the mean percentage viable count showed an increase for sub-sites A and S for *A. naeslundii* and *A. odontolyticus* respectively, when compared to the other two sub-sites (Table 3.2).

Table 3.2 Percentage viable counts (mean and ranges; N = 21) of bacteria from small samples of approximal plaque taken at sites away from (A), to the side of (S) and below (B) the contact area.

Bacteria	Away (A) Mean±SE*	Range	Side (S) Mean±SE	Range	Below (B) Mean±SE	Range
	[a]					
S. mutans	9.9±4.8	(0.0-78)	7.7±2.3	(0.0-28)	13.3±4.0	(0.0-54)
S. sobrinus	1.9±8.4	(0.0-38)	3.9±3.0	(0.0-63)	4.9±3.9	(0.0-83)
S. sanguis	4.4±1.3	(0.0-32)	8.3±2.7	(0.0-48)	7.8±2.9	(0.0-42)
S. gordonii	2.1±1.0	(0.0-10)	1.6±0.7	(0.0-13)	2.6±1.3	(0.0-14)
S. mitis I	13.2±3.6	(0.0-51)	7.6±2.3	(0.0-39)	8.4±2.4	(0.0-44)
S. mitis II	2.7±1.5	(0.0-30)	0.7±0.4	(0.0-90)	1.5±0.8	(0.0-14)
S. oralis	8.7±2.5	(0.0-31)	3.2±1.0	(0.0-16)	7.6±1.6	(0.0-28)
"S. milleri"-group	5.0±2.4	(0.0-43)	3.7±1.5	(0.0-22)	2.9±1.3	(0.0-22)
S. salivarius	3.6±1.3	(0.0-41)	1.2±0.7	(0.0-11)	2.0±1.3	(0.0-23)
Unidentified GPC	3.8±2.1	(0.0-40)	6.2±2.3	(0.0-38)	8.1±4.3	(0.0-84)
A. naeslundii	20.6±4.2	(0.0-78)	20.1±4.5	(0.0-71)	18.3±4.6	(0.0-50)
A. odontolyticus	4.1±2.4	(0.0-50)	7.9±4.0	(0.0-78)	5.8±2.8	(0.0-60)
A. israelii	4.3±2.9	(0.0-50)	11.6±4.2	(0.0-70)	4.7±2.9	(0.0-60)
Lactobacillus	0.4±0.3	(0.0-7.0)	0.2±0.1	(0.0-20)	0.04±0.0	(0.0-1.0)
Unidentified GPR	0.7±0.4	(0.0-6.0)	1.6±0.4	(0.0-27)	1.1±0.8	(0.0-14)
	♦ [b]					
Neisseria	5.9±2.2	(0.0-28)	2.3±0.9	(0.0-15)	0.2±0.1	(0.0-8.0)
	♦ [c]			♦ [d]		
Veillonella	3.3±1.7	(0.0-30)	2.3±1.2	(0.0-24)	10.7±4.7	(0.0-83)
Fusobacterium	0.1±0.05	(0.0-1)	0.7±0.4	(0.0-7)	0.4±0.4	(0.0-8.0)
Facultative anaerobes (GNR)	0.7±0.6	(0.0-13)	2.5±2.4	(0.0-50)	1.2±0.8	(0.0-14)
Obligate anaerobes (GNR)	2.2±1.0	(0.0-20)	4.1±1.6	(0.0-29)	3.4±0.6	(0.0-25)
Mean total count cfu/ml	31300±14300		102700±38100		155800±102700	

*SE = Standard error of the mean. **[a]** One-way analysis of variance of log-transformed raw data, and subsequent t test on the differences between the log-transformed data of each two sites, $p<0.05$ for B>A. ♦ Mann-Whitney test (section 2.4), **[b]** $p<0.05$ for A>B, **[c]** $p<0.05$ A<B and **[d]** $p<0.05$; for B>S. Viable count (cfu/ml) was used for these analyses; when organisms were not found, half of the minimum level of detection (5 cfu/ml) was used (data in Appendix D). GNR = Gram negative rods.

Table 3.3 Mean percentage isolation frequency of species at different sites in relation to the contact area.

Organism	Away (N=21)	Side (N=21)	Below (N=21)
Gram-positive cocci (GPC)			
S. mutans	42.9	62.0	85.7*
S. sobrinus	4.8	19.4	33.3**
S. sanguis	57.1	66.7	61.9
S. gordonii	33.3	33.3	38.8
S. mitis I	66.7	76.2	66.7
S. mitis II	33.3	28.6	33.0
"S. milleri"-group	38.1	42.9	42.9
S. oralis	57.1	47.6	62.0
S. salivarius	33.3	38.1	33.3
Unidentified (GPC)	47.6	52.3	42.9
Gram-positive rods (GPR)			
A. naeslundii	76.2	85.7	90.5
A. odontolyticus	38.1	33.3	57.1
A. israelii	47.6	66.7	61.9
Lactobacillus spp.	14.3	14.3	9.5
Unidentified (GPR)	19.0	23.8	23.8
Gram-negative cocci (GNC)			
Neisseria sp.	47.6	42.3	28.6
Veillonella sp.	28.5***	38.1	76.2****
Gram-negative rods (GNR)			
Fusobacterium	9.5	19.0	14.3
Facultative anaerobes	9.5	9.5	9.5
Obligate anaerobes	28.6	42.9	52.3

Comparison of two proportions for unpaired case was significant for:
S. mutans *$p<0.01$; A<B, SND = 2.9, 95% C.I. (14.0% to 85.7%).
S. sobrinus **$p<0.05$; A<B, SND, 95% C.I. (4.8% to 52.33%).
Veillonella sp. ***$p<0.01$; A<B and ****$p<0.05$; B>S, SND = 0.15, 95% (C.I. 7.8 to 87.4%) and SND = 0.5, 95% (C.I. 8.6% to 67.8 %) respectively.

3.1.2.2 Gram-negative rods

Facultative and obligately anaerobic Gram-negative rods were occasionally isolated from all sites. The mean percentage viable counts of these species were A = 0.7, S = 2.5, B = 1.2, and A = 2.2, S = 4.1, B = 3.4, respectively. The mean percentage isolation frequency of obligate anaerobes also increased at sub-site B (Table 3.3).

Gram-negative morphotypes were rare. For example, the mean percentage viable counts of *Fusobacterium* spp. at sub-sites (A), (S) and (B) were 0.1%, 0.7% and 0.4%, respectively. The mean percentage isolation frequency, nevertheless, was also similarly low at all sub-sites (Table 3.3).

3.1.2.3 *Lactobacillus* spp.

Lactobacillus spp. were isolated at only very low levels; their mean percentage viable counts were relatively low at all the sites (A = 0.4%, S = 0.2%, and B = 0.04%). Therefore, due to the low number of isolates, comparisons between the sites would not give statistically valid results. Their mean percentage isolation frequencies were A = 14.3%, B = 9.5% and S = 14.3% (Table 3.3).

3.1.2.4 Gram-negative cocci

Neisseria spp. comprised 5.9, 2.3, and 0.2% of the total viable counts of sub-sites A, S, and B, respectively. The differences between sub-sites A and B were statistically significant, A>B, $p<0.03$ (Table 3.2). The lowest mean percentage isolation frequency was also found at sub-site B (Table 3.2).

The mean percentage counts of *Veillonella* spp. were: A = 3.3%, S = 2.3% and B = 10.7%. The cfu/ml counts of sub-sites were compared using Mann-Whitney U test (B>A, $p<0.05$, B>S, $p<0.05$, Table 3.2). *Veillonella* spp. were isolated significantly more often

at sub-site B (B>A, $p<0.01$, Table 3.3). The remaining species and groups (see Table 3.4) showed no such trend with sampling site, with the possible exception of *Neisseria* spp. which tended to decrease at the sub-site below the contact area.

3.1.2.5 *Streptococcus* spp.

Streptococcus was the dominant genus at all sites (Table 3.2). An average of 4-5 species was found at each sub-site. All 63 sub-sites harboured at least one species of streptococcus (Appendix D, Tables 1-20). The most frequently isolated species at sub-sites (A) and (S) were *S. mitis* I, *S. sanguis*, and *S. mutans* (Table 3.2). The mean percentage isolation frequencies of these species at sub-sites (A) and (S) showed a similar trend, in the order *S. mutans* < *S. sanguis* < *S. mitis* I (*S. mutans* A = 43%, S = 62%, *S. sanguis* A = 57%, S = 67% and *S. mitis* I A = 66.7%, S = 76.2%) (Table 3.3). However, at sub-site (B) the trend changed in the order *S. mutans*, 85.7%> *S. mitis* I 66.7%> *S. sanguis*, 61.9%. The least frequently isolated species at sub-sites (A) and (S) was *S. sobrinus*, with a mean isolation frequency of 5% and 19%, respectively, and *S. salivarius* at sub-site B (B = 33.3%, Table 3.3).

S. mitis I was the numerically predominant streptococcal species at sub-sites A, *S. sanguis* at sub-site S, and *S. mutans* at sub-site B (Table 3.2). The mean percentage viable counts for *S. mitis* I were A = 13.2%, S = 7.6%, and the median values were 7.5% and 3.0%, respectively, and for *S. mutans* B = 13.4%, median 4.9% (Table 3.4).

The distribution of *S. sanguis* and *S. gordonii* was very little affected by the site of isolation. The mean percentage viable counts and median values for *S. sanguis* and *S. gordonii* were at sub-sites (A) 4.4 and 3.6% respectively at sub-sites (S) 8.3% and 2.1%, respectively, and at sub-sites (B) 7.8% and 0.7%, respectively (Table 3.4).

S. oralis also showed a decrease in mean percentage viable counts in the order A>B>S (Table 3.2). Despite the apparently large mean percentage viable counts at the three sub-sites (A = 8.7%, B = 7.6% and S = 3.2%) the differences between the sites were not statistically significant, probably because of the large numbers of zero values (when species were not detectable) at sub-site S (Table 3.2).

Table 3.4 Mean and median percentage viable counts of streptococcal species from samples of approximal plaque taken away from (A), to the side of (S) and below (B) the contact area.

Species	Away (N=21)	Side (N=21)	Below (N=21)
S. mutans	┌ 9.9±4.8[a]	┌ 7.7±2.3	┌13.3±4.0
	* 0.0[b]	** 0.0	*** 4.9
S. sobrinus	└ 1.9+1.8	└ 3.9±3.0	└ 4.9±3.9
	0.0	0.0	0.0
S. sanguis	4.4±1.3	8.3±2.7	7.7±2.9
	3.6	2.1	0.7
S. gordonii	2.1±1.0	1.6±0.7	2.7±1.0
	0.0	0.0	0.0
S. mitis I	┌13.2±3.6	┌ 7.6±2.3	┌ 8.4±2.4
	♦ 7.5	♦♦3.0	♦♦♦ 3.9
S. mitis II	└ 2.7±2.7	└ 0.7±0.4	└ 0.5±0.8
	0.0	0.0	0.0
S. oralis	8.6±2.1	3.2±1.0	7.6±1.6
	5.1	0.0	5.7
"S. milleri"-group	5.0±2.4	3.7±1.5	2.5±1.2
	0.0	0.0	0.0
S. salivarius	3.6±1.3	1.2±0.8	2.0±1.1
	0.0	0.0	0.0
Unidentified	3.8±2.3	5.9±2.3	8.1±4.0
	0.0	0.4	0.0
IgA_1 protease producers	13.0±2.0	14.2±4	15.3±3.5

a = Mean ± standard error, b = median.
Vertical brace : Wilcoxon matched pair signed rank test, *p = 0.019, **p = 0.023, ***p<0.01, ♦p<0.05, ♦♦p<0.01, ♦♦♦p<0.05.
The mean values have been transferred from Table 3.2 as it was not possible to write the medians in that table.

The proportions of IgA_1 protease-producing species were: 13.0%, 14.2% and 15.3% at sub-sites A, S and B, respectively. At each sub-site, IgA_1 protease-producing species were recovered at a lower isolation frequency and proportion than those lacking this activity $p<0.05$ (Table 3.4).

The "*S. milleri*"-group were recovered with the same frequency at sub-sites B and S (Table 3.3).

S. salivarius was present in one third of the children, the highest mean isolation frequency being at sub-site (S) (Table 3.3). Mean percentage viable counts of *S. salivarius* were at (A) 3.6%, at (S) 1.2% and at (B) 2.0%, confirming that *S. salivarius* frequently colonised sub-site A (Table 3.2).

Mutans streptococci (MS) were isolated preferentially from the sub-site below the contact area (B) (91%). Comparison of the distribution of *S. mutans* and *S. sobrinus* at the three sub-sites revealed a significant difference between (A) and (B) (B>A, $p<0.05$). When *S. mutans* or *S. sobrinus* were isolated alone, there was no significant difference in the percentage of sub-sites colonized (Table 3.5) although, for MS in general and *S. mutans* in particular, there was a clear trend for the isolation frequency to increase in the order A<S<B. *S. mutans* was the predominant and frequently the only species of mutans streptococcus recovered from a site (Table 3.5). *S. sobrinus* was rarely isolated in the absence of *S. mutans* and, when it was recovered with *S. mutans*, it was located preferentially at sub-site B (Table 3.5).

One-way analysis of variance on the log-transformed percentage viable counts of *S. mutans* was performed at each sub-site; the F value was 3.15, which showed there was a difference between the concentration of *S. mutans* from three sub-sites, which was

significant at the 5% level. Further analysis and a paired t test on the differences between the log-transformed data for sub-sites (B) and (A) yielded p = 0.025 (Table 3.2). There were no statistically significant differences between *S. sobrinus* levels at any sub-site, even though the trend was for proportions to increase in the order A<S<B. The percentage viable count of *S. mutans* was significantly higher than that of *S. sobrinus* at all three locations (p=0.019, p=0.034 and p=0.004 for sub-sites A, S and B, respectively; Table 3.5).

Table 3.5 Number and percentage of premolars and sub-sites colonised by mutans streptococci (MS) and lactobacilli.

Species	Premolars colonised (n = 21)	Sub-sites colonised			
		Away (n=21)	Side (n=21)	Below (n=21)	Total (n=63)
			♦		
MS	20 (95)	10 (48)	14 (67)	19 (91)	43 (68)
S. mutans alone	11 (52)	9 (43)	10 (48)	12 (57)	31 (49)
S. sobrinus alone	1 (5)	1 (5)	1 (5)	1 (5)	3 (5)
S. mutans + *S. sobrinus*	8 (38)	0 (0)	3 (14)	6 (29)	9 (14)
			*		
Lactobacillus spp. alone	1 (5)	0 (0)	1 (5)	0 (0)	1 (2)
Lactobacillus spp. + MS	4 (19)	3 (14)	4 (19)	2 (10)	9 (14)

( ) = percentage
Comparison of two proportions (unpaired cases) was highly significant for A<B; ♦p<0.01, SND = 3; 99% C.I. (6.1% to 79.7%). Similarly, *P<0.01; SND = 2.64, and 99 % C.I. (2.7 to 54.4).

There was no pattern in the relative concentrations of *S. mutans* and *S. sobrinus* when

they were recovered together at the same sub-site. There were no statistically significant differences between other streptococcal species levels at any sub-site, even though the trend was for proportions to increase in the order B>S>A for some species and for others in the order A>B>S (Table 3.2).

S. mutans and *A. naeslundii* yielded the highest counts at most of the sites, and *S. mutans* and *Veillonella* spp. were significantly abundant below the contact area. Overall isolation frequency data indicated that each species could be isolated from all three sites, and that no species was specific to any of the sub-sites.

Using the current methodology, it was possible to isolate frequently a number of Gram-positive cocci (GPC) and rods (GPR) that did not fit conventional identification schemes. The mean percentage counts and proportions of unidentified GPR were generally less than the GPC. GPR were more prevalent at sub-site S than sub-sites A and B (Tables 3.2). The proportion of unidentified GPC increased in the order: B>S>A, although their mean isolation frequencies were similar at all sub-sites (Tables 3.2 and 3.4).
This study did not sub-classify *S. salivarius* and closely related species (Whiley and Hardie 1988), nor the "*S. milleri*-group" (Whiley and Hardie 1989). Also, lactobacilli and veillonellae have been identified only to genus level, since the physiological tests used for the study were limited.

3.1.2.6 Bacterial associations

The McNemar's test was applied to see if there was an association between the species isolated at each of the sub-sites. The comparison of proportions for unpaired data was applied to see if there was an association between the isolation of species from sub-sites (A), (S) and (B). For example, the proportion of *S. sobrinus* in samples with *S. mutans* was 0.23 and 0.33 at sub-sites S and B, respectively, and they were not found together

at sub-site A. The proportion of *S. sobrinus* in samples without *S. mutans* was 0.08, 0.13 and 0.33 at sub-sites A, S, and B, respectively, indicating no statistical association between the two species. Similarly, there were no significant positive or negative intergeneric or intrageneric associations between other species (for the complete list of other species), (Table 3.2) at any of the sites.

3.1.2.7 Variations in proportions of species within each sub-site

An average of 8 species was found at each sub-site. The minimum number of species was found at sub-site A, and the maximum number was at sub-site B (A = 3 and B = 14).

Wilcoxon matched pair signed ranks tests were used to compare the percentage viable counts of different species within each sub-site. At each sub-site, the percentage viable count of *S. mutans* was significantly higher than that of *S. sobrinus* at all the three locations (P = 0.019, 0.023 and p = 0.008 for sub-sites A, S and B, respectively). *S. mitis* I was also found in a significantly higher proportion than *S. mitis* II at each sub-site, p = 0.02, p = 0.001 and p = 0.01, respectively (Table 3.5). The percentage viable counts of each species were compared with nineteen other species. Species for which their percentage viable counts were significantly higher than the percentage viable counts of others presented three patterns, a, b and c as indicated in Table 3.6a-3.6c.

Pattern a: Species for which percentage viable counts were significantly higher than others (level of significance $p<0.025$, see section 2.4) at only one of the three sub-sites. At sub-site A, the percentage viable counts of *A. israelii* and *Neisseria* were statistically significantly higher than *Fusobacterium*, p = 0.006 and 0.008, respectively. Similarly, *S. salivarius* was significantly higher than *Fusobacterium* and lactobacilli; p = 0.021, p = 0.024, respectively. Also, sub-site A was the only sub-site at which *A. naeslundii* was significantly higher than *A. israelii*, p = 0.003 (Table 3.6a).

At sub-site S, *S. gordonii* was significantly higher than *S. sobrinus*; *S. mitis* II was significantly higher than unidentified GPC and *S. sanguis* was significantly higher than *A. odontolyticus* ($p < 0.001$, $p = 0.008$, and $p = 0.002$, respectively) (Table 3.6a).

At sub-site B, aciduric and acidogenic bacteria such as *S. mutans* and *Veillonella* were significantly higher than five and nine other species, respectively (Table 3.6a). These included facultative and obligate anaerobes (Table 3.6a).

Pattern b: Species for which the percentage viable counts were significantly different from others at two of the three sub-sites. At sub-sites A and S, the proportions (%) of *A. naeslundii* were significantly greater than those of *S. oralis* and *S. sobrinus* (Table 3.6b). Also, *S. oralis* and *S. mitis* I were significantly higher than unidentified GPR and *S. oralis* (Table 3.6b).

At sub-sites A and B, which were the greatest distance apart, *A. odontolyticus* was significantly higher than *Fusobacterium* spp., *S. mitis* II was higher than *S. oralis*, and *S. oralis* was higher than *Fusobacterium* spp. (Table 3.6b).

Table 3.6a Comparison of percentage viable counts of bacterial species isolated from small samples of approximal plaque (N = 63). Species levels (%) which were significantly different ($p < 0.025$) from each other at only one of the three sub-sites: away from (A) to the side of (S) and below (B) the contact area.

Species	**p (A)	p (S)	p (B)
A. naeslundii > *A. israelii*	0.003		
A. naeslundii > Unidentified GPC	0.005		
A. naeslundii > *S. sanguis*	0.000		
A. israelii > lactobacilli	0.006		
A. israelii > *Fusobacterium*	0.006		
S. oralis > Obligate anaer.***	0.017		
S. oralis > *S. gordonii*	0.023		
S. oralis > Facultative anaer.***	0.004		
S. mitis I > *S. gordonii*	0.010		
S. mitis I > Obligate anaer.***	0.014		
S. sanguis > *S. sobrinus*	0.017		
S. salivarius > *Fusobacterium*	0.021		
S. salivarius > lactobacilli	0.024		
"*S. milleri*" > *Fusobacterium*	0.009		
Neisseria > *Fusobacterium*	0.008		
S. gordonii > *S. sobrinus*		0.000	
S. mitis II > Unidentified GPC		0.008	
S. sanguis > *A. odontolyticus*		0.002	
S. sanguis > *S. mitis* II		0.002	
A. naeslundii > *S. gordonii*			0.004
A. israelii > Unidentified GPC			0.020
A. israelii > *S. salivarius*			0.001
S. gordonii > lactobacilli			0.014
S. oralis > "*S. milleri*"			0.010
S. mitis I > *Neisseria*			0.001
S. mutans > *S. salivarius*			0.008
S. mutans > *S. gordonii*			0.017
Obligate anae > lactobacilli			0.014
S. mutans > *A. odontolyticus*			0.021
S. mutans > "*S. milleri*"			0.010
S. sobrinus > lactobacilli			0.020
Veillonella > Facultative anaer.***			0.007
Veillonella > *S. mitis* II			0.012
Veillonella > Unidentified GPC			0.008
Veillonella > *Neisseria*			0.005
Veillonella > Obligate anaer.***			0.017
Veillonella > Unidentified GPR			0.005

** Wilcoxon matched pair signed rank test was applied, GPC = Gram-positive coccus, GPR = Gram-positive rod, *** anaer. = anaerobes.

Table 3.6b Comparison of percentage viable counts of bacterial species isolated from small samples of approximal plaque (N = 63). Species levels (%) which were significantly different ($p < 0.025$) from each other at two of the three sub-sites: away from (A) to the side of (S) and below (B) the contact area.

Species	**p (A)	p (S)	p (B)
A. naeslundii > *S. oralis*	0.020	0.016	
A. naeslundii > *S. sobrinus*	0.000	0.002	
S. oralis > Unidentified GPR	0.004	0.003	
S. mitis I > *S. oralis*	0.008	0.019	
S. sanguis > Facultative anaer.***	0.008	0.012	
Unidentified GPC > *Fusobacterium*	0.012	0.011	
S. mitis I > Unidentified GPR		0.010	0.003
S. mutans > *S. mitis* II		0.002	0.002
S. sanguis > *S. gordonii*		0.002	0.007
S. mutans > Facultative anaer.***		0.010	0.020
A. odontolyticus > *Fusobacterium*	0.018		0.014
"*S. milleri*"> *Lactobacillus* spp.	0.013		0.024
S. mitis II > *S. oralis*	0.024		0.013
S. oralis > *Fusobacterium*	0.003		0.002
S. oralis > *Lactobacillus* spp.	0.003		0.001

** Wilcoxon matched pair signed rank test was applied.
GPR = Gram-positive rod, ***anaer. = anaerobes.

Pattern c: Species for which the percentage viable counts were significantly different from others at all three of the sub-sites. *A. naeslundii* was the dominant species, the percentage viable counts were found significantly higher than seven of the eighteen species (Table 3.6c). The second most dominant species was *S. mitis* I which was significantly higher than five of the other species (Table 3.6c). The third species of this group was *S. mutans* which was significantly higher than four other species (Table 3.6c). Finally, obligate anaerobes were significantly higher than *Fusobacterium* at all of the three sub-sites (Table 3.6c).

Table 3.6c Comparison of percentage viable counts of bacterial species isolated from small samples of approximal plaque (N = 63). Species counts which were significantly different ($p<0.025$) from each other at three sub-sites: away from (A) to the side of (S) and below (B) the contact area.

Species*	**p (A)	p (S)	p (B)
A. naeslundii > *Neisseria*	0.004	0.001	0.000
A. naeslundii > *"S. milleri"*	0.009	0.004	0.002
A. naeslundii > lactobacilli	0.000	0.000	0.000
A. naeslundii > Obligate anaer.***	0.000	0.005	0.002
A. naeslundii > Facultative anaer.***	0.000	0.003	0.003
A. naeslundii > *A. odontolyticus*	0.005	0.023	0.003
A. naeslundii > *S. salivarius*	0.000	0.000	0.000
S. sanguis > *Lactobacillus* spp.	0.002	0.001	0.003
S. sanguis > *Fusobacterium*	0.007	0.007	0.008
S. mitis I > *S. mitis* II	0.017	0.001	0.012
S. mitis I > *Lactobacillus* spp.	0.001	0.001	0.008
S. mitis I > *"S. milleri*-group"	0.021	0.024	0.010
S. mitis I > *S. salivarius*	0.010	0.003	0.021
S. mitis I > Facultative anaer.***	0.002	0.009	0.007
S. mutans > *Lactobacillus* spp.	0.000	0.000	0.000
S. mutans > *S. sobrinus*	0.019	0.023	0.008
S. mutans > *Fusobacterium*	0.007	0.010	0.000
S. mutans > Unidentified GPR	0.019	0.011	0.004
Obligate anae. > *Fusobacterium*	0.014	0.009	0.012

* For the complete list of bacterial species cultured from plaque samples see Table 3.2.
** Wilcoxon matched pair signed rank test was applied.
*** anaer. = anaerobes, GPC = Gram-positive coccus, GPR = Gram-positive rod.

3.2 Direct microscopic count

3.2.1 Mutans streptococci (MS) and lactobacilli

The control quantification experiments, using films of pure cells of *S. mutans*, *S. sobrinus* and *L. casei*, showed that all of the cells present could be detected by IF staining (Fig. 3.1a). Mutans streptococci and lactobacilli were distinguished from other species of dental plaque by fluorescence microscopy (Fig. 3.1b). This was confirmed as the count of fluorescing cells (fifty fields) showed 98% agreement with the total cell counts of the same fields as examined by phase contrast. Although some cells appeared to be single by phase contrast illumination, they could be distinguished as two cells by fluorescence, and vice versa. Variation between the counts was only 2%, which was similar to the value reported by Bush et al. (1990).

The specificity of the primary antisera (anti-*S. mutans*, anti-*S. sobrinus*, anti-*L. acidophilus* and anti-*L. casei*) and of the rabbit anti-mouse FITC-conjugated antiserum used was evaluated against 75 different oral species and the results are shown in Table 3.7. The full list of test species was shown in Table 2.6).

The reproducibility tests for counting techniques showed a mean percentage variation of 4.7% for *S. mutans*, with a range of 0.65 to 10.5 (Table 3.8). For *S. sobrinus* the mean percentage variation was 4.9 with a range of 1.3% to 9.7%; therefore, the overall mean percentage variation was less than 5% and the reproducibility was 95% (Tables 3.8 and 3.9).

Mutans streptococci were preferentially identified by IF from the sub-site below the contact area (B = 90%), compared with isolation frequencies of MS at sub-site A of 45% and at sub-site S of 62% ($p<0.01$).

S. mutans was identified by IF at 41% of sub-sites A, 51% of sub-sites S and 70% of sub-

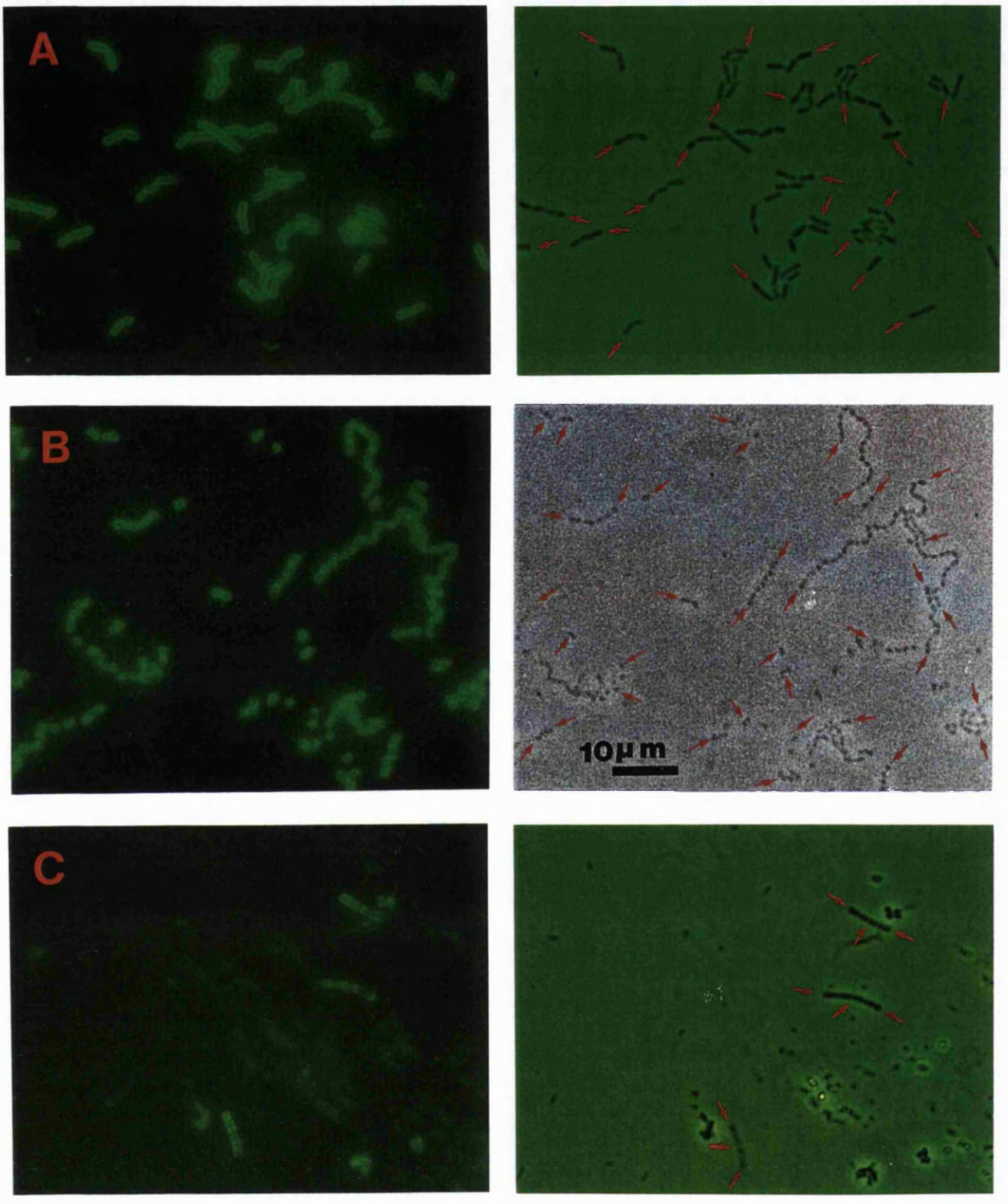


Fig. 3.1a Transmission light micrographs: fluorescence (left) and phase contrast (right) pictures of the same microscope field. Arrows indicate labelled cells. The scale bar applies to all the figures; A = pure culture of *L. casei* NCTC 10302, B = pure culture of *S. sobrinus* NCTC 27351, C = cross-reacting bacillus in a sample of approximal plaque.

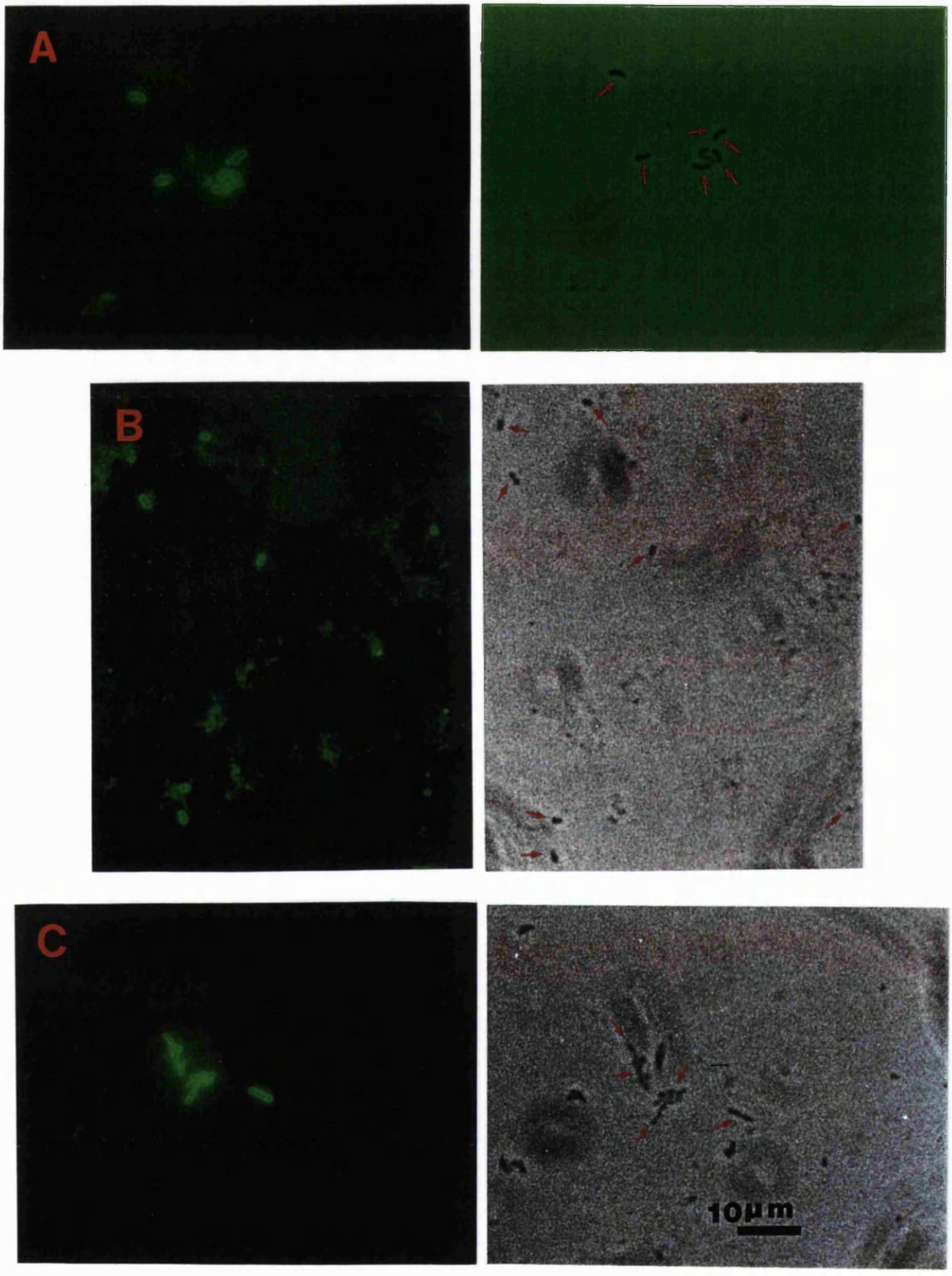


Fig. 3.1b Transmission light micrographs: fluorescence (left) and phase contrast (right) pictures of the same microscope field. Arrows indicate labelled cells. The scale bar applies to all the figures; A = *Lactobacillus* spp., B = *S. mutans* 'c' and C = *S. sobrinus* 'd' all in samples of approximal plaque.

Table 3.7 Analysis of specificity of primary (p) and secondary (s) antisera used in IF studies with 75 species of oral bacteria.

α^a-sera Dilution	[α-*S. mutans* 1:200	α-*S. sobrinus* 1:600	α-*L. acidophilus* 1:600	α-*L. casei*]^p 1:200	[α-mouse FITC]^s 1:30
Strain	fluorescence intensity				
S. mutans 'c', 'e', 'f', 'h'	4+	-	-	-	-
S. mutans 'b'	-	1+	-	-	-
S. sobrinus 'd'	-	4+	-	-	-
S. mitis	-	-	3+		
S. oralis	-	-	3+	-	-
S. sanguis	-	-	3+	-	-
S. salivarius	-	-	3+	3+	-
Staph. aureus	4+	4+	4+	4+	
L. acidophilus	-	-	4+	-	-
L. casei	-	-	-	4+	-
L. odontolyticus	-	-	3+	-	-
C. ochracea	-	3+	-	-	-
M. mucilaginosum	-	2+	-	-	2+
R. dentocariosa	-	3+	-	-	-
Simonsiella crassa	-	2+	-	-	2+
Leptotrichia dentium	-	2+	-	-	2+
Bifidobacterium dentium	-	-	2+	+3	
Other species*	-	-	-	-	-

[a] α = anti; [p]Primary antisera = antiserum to each of following species: *S. mutans* 'c', *S. sobrinus* 'd', *L. acidophilus* and *L. casei*.
[s]Secondary antiserum = rabbit anti-mouse FITC-conjugated antiserum; *Staph. aureus* = *Staphylococcus aureus*.
4+ = bright yellow-green, very sharp; 3+ = bright yellow-green, sharp and clear; 2+ = definite fluorescence, colour definitely yellow-green but low intensity; 1+ = minimal fluorescence, colour nondescript, not definitely green and - = borderline reaction between 1+ and negative (Moody et al. 1958). *For the complete list of other bacteria and their sources used in this study, see Table 2.6.

Table 3.8 Reproducibility of counting technique using duplicate films of approximal plaque samples labelled by anti-*S. mutans 'c'* antiserum.

Well No.	*S. mutans*	Film 1 total	%	*S. mutans*	Film 2 total	%	Variation
1	42[a]	410[b]	10.24	47	483	9.73	5.10
2	48	449	10.69	45	418	10.76	0.65
3	38	356	10.67	49	488	10.04	6.08
4	40	409	9.78	44	445	9.88	1.02
5	46	463	9.38	47	451	10.42	10.50

Mean percentage of variations ($\bar{x}$ = 4.67).
[a] Counts of fluorescing cells, [b] Total cell counts, phase contrast microscopy, 10 fields per film.

Table 3.9 Reproducibility of counting technique using duplicate films of approximal plaque samples labelled by anti-*S. sobrinus 'd'* antiserum.

Well No.	*S. sobrinus*	Film 1 total	%	*S. sobrinus*	Film 2 total	%	Variation
1	12[a]	410[b]	2.93	14	483	2.9	1.30
2	15	449	3.34	15	418	3.58	4.1
3	16	356	4.49	13	488	4.71	4.78
4	9	409	2.2	11	455	2.41	9.65
5	22	500	4.4	19	451	4.2	4.65
6	11	480	2.3	12	495	2.62	5.08

Mean percentage of variations ($\bar{x}$ = 4.92).
[a] Counts of fluorescing cells in 10 fields, [b] Total cell counts, phase contrast microscopy, 10 fields per film.

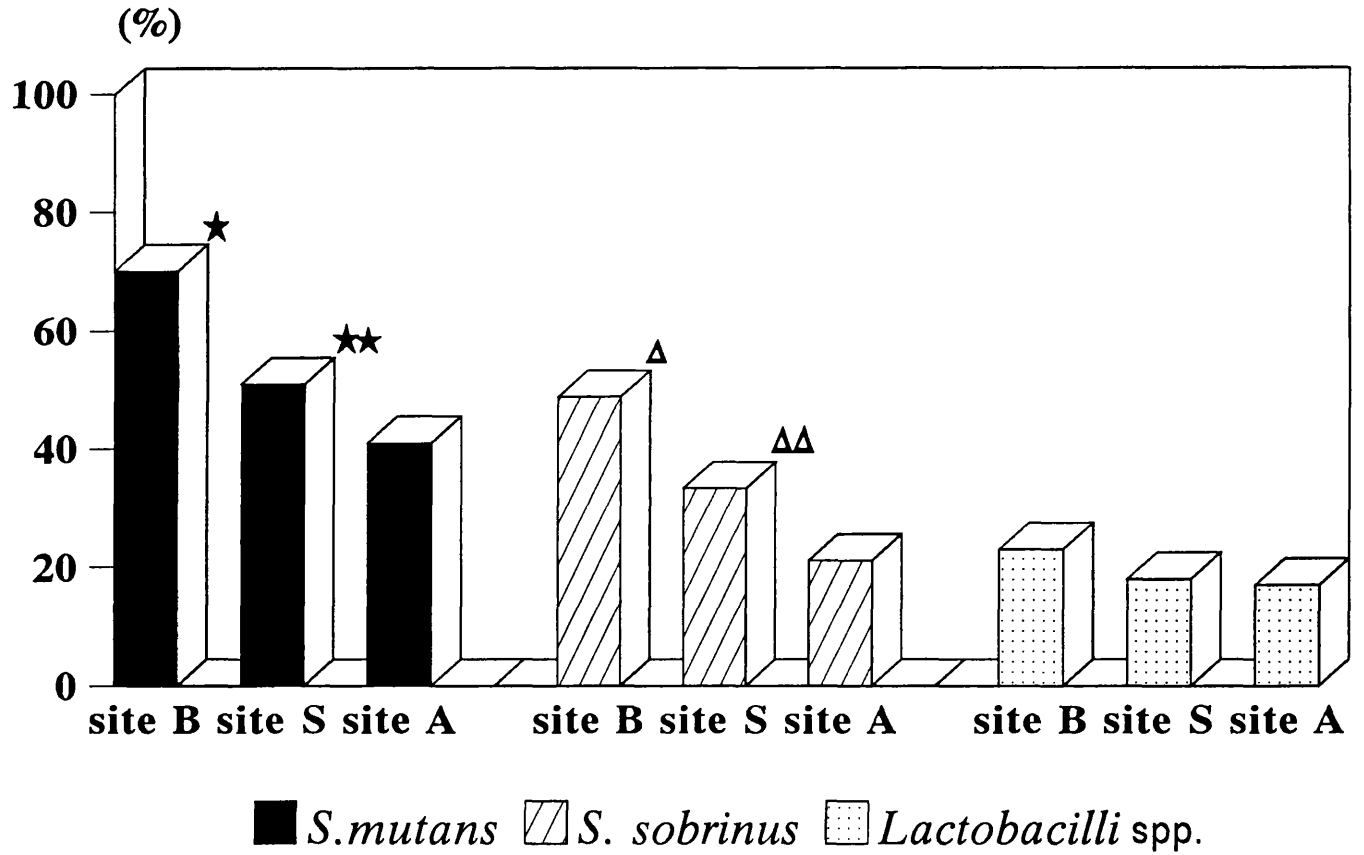
sites B. The detection frequency at sub-site B was significantly greater than at sub-sites A (SND = 4.1) and S (SND = 2.69); $p<0.01$ (for both B>A and B>S). The difference between the mean detection frequency at sub-sites A and S was not statistically significant. Similarly, *S. sobrinus* was detected in total from 21% of sub-sites A, 33% of sub-sites S and 49% of sub-sites B. The differences in mean detection frequencies of *S. sobrinus* at these three sub-sites were statistically significant, B>A (SND = 3.9) $p<0.01$, and B>S (SND = 2.1) $p<0.05$ (Fig. 3.2).

S. mutans and *S. sobrinus* were identified together in 12% of A sub-sites, 38% of B sub-sites and 22% of S sub-sites. The percentage isolation frequencies of MS from B sub-sites were significantly greater than from A sub-sites and also from S sub-sites ($p<0.01$ and $p<0.05$, respectively). *S. mutans* was identified on its own more often than *S. sobrinus*, with detection frequencies for *S. mutans* of 27%, 29% and 32% from sub-sites A, S and B, respectively (Table 3.10). When *S. mutans* or *S. sobrinus* were isolated alone, there was no statistically significant difference in the percentage of sub-sites colonized (Table 3.10). *S. sobrinus* was identified infrequently in the absence of *S. mutans*. There were instances where neither species was found; only nine of the 90 teeth were MS-free (Table 3.10).

S. mutans and *S. sobrinus* were both identified in greater numbers at sub-site B, compared with sub-sites A and S ($p<0.05$) (Fig. 3.3). The mean percentage of proportional counts for *S. mutans* at sub-sites A, S and B was: 0.60, 0.82 and 1.63, respectively. The equivalent values for *S. sobrinus* were 0.42, 0.66 and 0.97 at sub-sites A, S and B, respectively. The count at B was significantly greater than at A or S ($p<0.01$). Similarly, for *S. mutans* the mean microscopic counts at B were significantly greater than at S and A ($p<0.01$ for both, Fig. 3.3).

At each sub-site there were no statistically significant differences between the microscopic

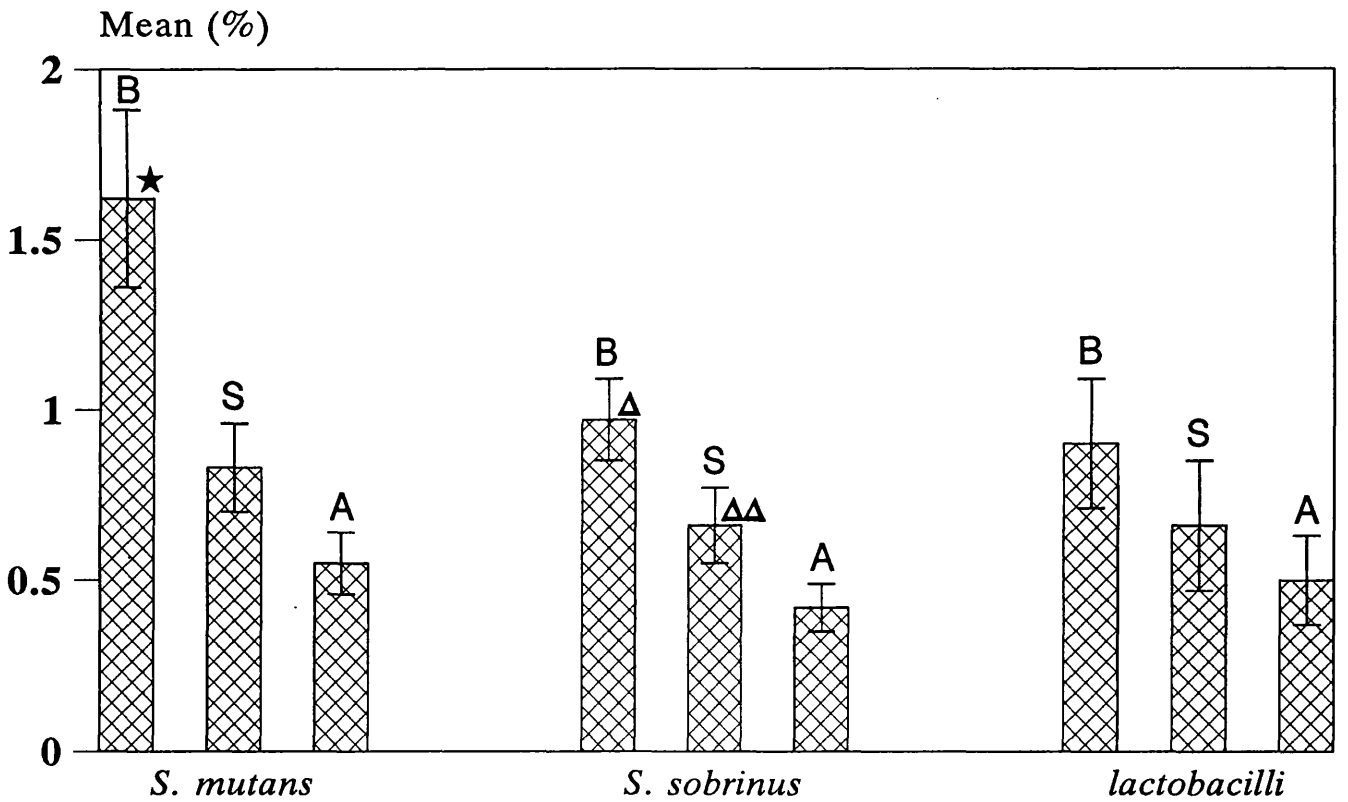
Fig. 3.2 Relationship between sites of isolation and detection frequencies of mutans streptococci (N = 90) and lactobacilli (N = 70) in plaque from three sub-sites, determined by immunofluorescence.



A = away; B = below and S = side of contact area.

Comparison of two proportions; for *S. mutans* B>A ★p<0.01, B>S ★★p<0.01 for *S. sobrinus* B>A ΔpΔ<0.01 and B>S ΔΔpΔΔ<0.01.

Fig. 3.3 Mean percentage counts (± SEM) of mutans streptococci (N = 90) and lactobacilli (N = 70) at three sub-sites of approximal plaque, determined by direct microscopic count using immunofluorescence.



SEM = Standard error of the mean.
A = away; B = below and S = side of contact area.
Wilcoxon signed-ranks test; for S. mutans B>A and B>S ★$p<0.01$, S. sobrinus B>A and B>S $^{\Delta}p<0.01$ and S>A $^{\Delta\Delta}p<0.05$ respectively.

counts of *S. sobrinus* and *S. mutans*, even though the trend was for proportions to increase in the order *S. mutans* > *S. sobrinus* at all the three sub-sites. The pattern for the relative concentrations of each species when they were recovered together at each sub-site was in the order of B>S>A. There was a positive association between the frequency of detection of *S. mutans* and *S. sobrinus* at sub-site S (Table 3.11).

In some instances more than one tooth per patient was sampled (Table 3.12); this made it possible to compare the proportional microscopic counts of MS from upper teeth with those of opposite teeth from the lower jaw. The MS counts of upper teeth were higher than those of the opposite lower teeth. *S. mutans* counts were higher at the below (B) contact area sub-site of the upper teeth compared to the opposing teeth in the lower jaw ($p<0.05$). When the average counts of *S. mutans* from sub-sites A, S and B of the upper teeth were compared with those from lower teeth, the upper counts remained significantly higher than the lower jaw counts, $p<0.05$ (Table 3.12).

3.2.2 Lactobacilli

Lactobacillus spp. were identified on average from 21% of the 210 sub-sites examined (at sub-sites A 17%, sub-sites S 19% and at sub-sites B 23%) (Fig. 3.2) . However, when *Lactobacillus* spp. were present, they were often found in relatively high proportions compared to MS, with a range from not detectable (ND) to 8%. Their mean proportional counts were A = 0.5, S = 0.7 and B = 0.9. There were no statistically significant differences between the isolation frequency or the mean percentage proportional counts of lactobacilli recovered from the three sub-sites (Fig. 3.2 and Fig.3.3).

The proportions of *Lactobacillus* spp. in samples with MS were 0.38, 0.24 and 0.33 at sub-sites A, S and B, respectively. By using the comparison of two proportions (McNemar's test; fifty seven samples from each sub-site) a positive association between the detection frequencies of these species and those of MS was found at sub-sites A and

Table 3.10 Numbers and percentage of approximal premolar surfaces and sub-sites colonised by mutans streptococci and lactobacilli, using indirect immunofluorescence and direct microscopic count.

Bacterium	APS[a] colonised (N=90)	Sub-sites colonised			
		Away (A) (N=90)	Side (S) (N=90)	Below (B) (N=90)	Total (N=270)
MS	81 (90)	43 (48)	56 (62)	73 (81)	172 (64)
S. mutans alone	28 (31)	24 (27)	26 (29)	29 (32)	81 (30)
S. sobrinus alone	6 (7)	8 (9)	10 (11)	10 (11)	28 (10)
		* ┌ b ┐			
S. mutans + *S. sobrinus*	47 (52)	11 (12)	20 (22)	34 (38)	65 (24)
			└ c ┘		
		┌ d ┐			
MS-free	9 (36)	47 (52)	34 (38)	17 (19)	96 (36)
[n]*Lactobacillus* spp.	22 (31)	12 (17)	13 (19)	16 (23)	44 (21)
Lactobacillus spp. + MS	19 (27)	9 (13)	8 (11)	15 (21)	33 (15)

( ) Percentage in parenthesis. [n] Number of samples; N= 70, and total number of samples N = 210.

[a]APS = approximal premolar surfaces. * Comparison of two proportions for B, S and A, were:

b for B>A; SND = 3.9; P <0.01 and 99 % C.I. (9% to 42%),

c for B>S; SND = 2.2; P <0.05 and 95 % C.I. (2.1 to 28.9).

d for MS free surfaces A>B; SND = 2.2; p<0.05 and 95% C.I. (12.3% to 15.4%).

Table 3.11 McNemar's test for comparison of the detection of *S. sobrinus* in the absence (-) or presence (+) of *S. mutans* at sub-site S.

		S. sobrinus		
		+	-	Total
S. mutans	+	20	10	30
	-	26	34	60
		46	44	90

SND = 2.0 ; 95% C.I. (1.29% to 39%) and p<0.05.

B ($p<0.01$). However, there was no correlation between the direct microscopic counts of lactobacilli and MS. *Lactobacillus* spp. were also found in the absence of MS (0.1, 0.17 and 0.1% at sub-sites A, S and B, respectively).

3.2.3 Comparison of IF and culture

The data from sixty samples of 20 teeth processed by culture and IF techniques were in general agreement with one another. However, there were lower values of mean percentage counts for IF (Table 3.13). Both methods showed that prevalence of MS varied with location in relation to the contact area, with a tendency to higher MS counts at the B and S sites (Tables 3.14). *S. mutans* was detected by culture from 40%, 60% and 85% of the sub-sites A, S and B, respectively, and by IF from 75%, 70% and 80%, of the sites respectively.

S. sobrinus was cultured from 5%, 40% and 35% of sub-sites A, S and B, respectively. Significantly higher counts were obtained using IF: 30, 40 and 60% at sub-sites A, S and B, respectively ($p<0.01$, Table 3.14). There were some instances in which species were identified by only one of the methods. The results for *S. mutans* and *S. sobrinus* (N = 60, by IF only) were 75.0 and 40.0%, respectively, and by culture only 61% and 20.0% respectively (Table 3.14). The detection levels for *Lactobacillus* spp. were identical for both methods (Table 3.14).

To find out whether the significantly lower detection of *S. sobrinus* by culture possibly is related to an inhibitory effect of the selective media (TYC and TYCSB) used for their growth and isolation, growth of MS on TYC and TYCSB was compared with their growth on blood agar. Compared with the colony count on blood agar, *S. mutans* was inhibited by 13.9% and 16.4% on TYC and TYCSB, respectively, while *S. sobrinus* was inhibited by 19.8% and 32.0% on TYC and TYCSB, respectively (Table 3.16). Therefore, the higher detection levels using IF could be accounted for by the inhibitory effect of TYC

and TYCSB.

The possibility of false positive detection of *S. sobrinus* (see section 2.3.9) by polyclonal antibody was studied by treating duplicate samples of 21 teeth (63 sub-sites) with a specific monoclonal antibody. The first set of samples was treated with anti-*S. sobrinus* 'd' polyclonal and the second set with monoclonal (OMVU10, de Soet et al. 1987) antibodies. The results showed no statistically significant differences between the counts obtained by the use of two anti-*S. sobrinus* 'd' antibodies, and there was a strong correlation between the counts (Table 3.17).

3.2.4 Prevalence of *Lactobacillus* spp. and MS on approximal surfaces

a: Culture study

Mutans streptococci (MS) were isolated from 95% of the 21 tooth surfaces, and from 68% of the 63 sub-sites (Table 3.5). On an individual approximal surface, *S. mutans* could be recovered on its own from one gingival margin sub-site (A = 9; B = 12; S = 10), and *S. sobrinus* alone from another. Similarly, *S. mutans* and *S. sobrinus* were recovered together from sub-sites: B = 6 and S = 3. There were instances where neither species or only one species might be found at one sub-site and not at the other two sub-sites (Table 3.6). Of the 63 sub-sites on the 21 approximal tooth surfaces examined, only one had no MS at any sub-site. Three, ten and seven teeth had one, two or three sub-sites, respectively, colonised by MS.

b: IF study

Although a much larger number of teeth were studied by IF the overall results were similar to those obtained by culture. MS were identified from 90% of the 90 tooth surfaces. However, MS were detected on 64% of the 270 sub-sites studied by IF, and only 36% of these sub-sites had no detectable MS (Table 3.10). In many cases MS were found at one of the three sub-sites but not at the other two sub-sites on the same tooth surface.

The MS-free sub-sites were 50% of A, 38% of S and 21% of B (data in Appendix E, Table 1).

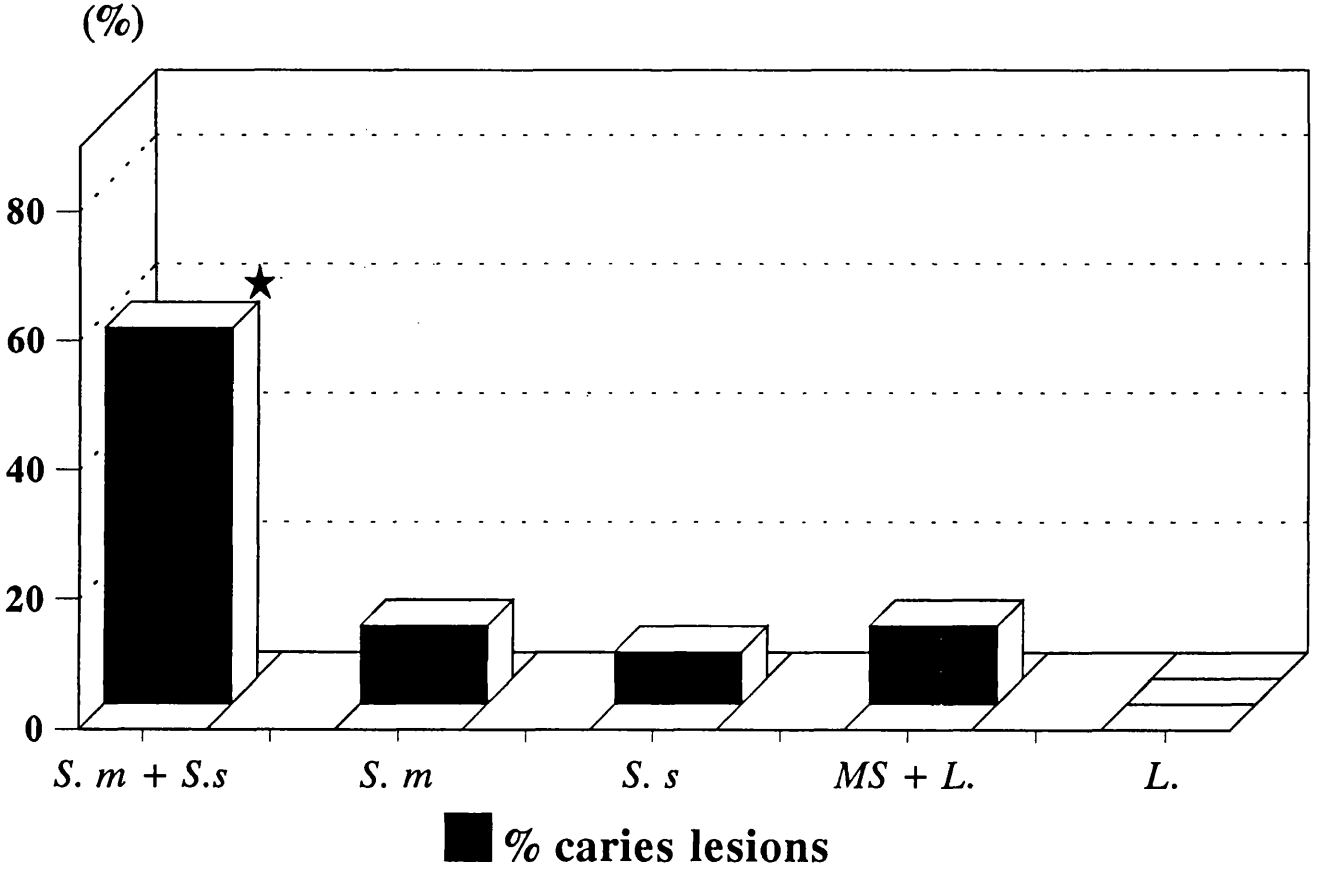
3.2.5 Relationship between bacterial species and early caries lesions

Visible white spot lesions were found in 21 of the 90 teeth and in 25 of 270 sub-sites processed by IF for MS and lactobacilli; 90% of these early lesions were at the sub-site below the contact area (B). All the plaque samples from these sites contained MS. Lactobacilli were found on 7 of the 25 white spot lesions (Fig. 3.4).

An overall statistically positive association (see section 2.4 **c**) was found between the presence of *S. mutans* 'c' and *S. sobrinus* 'd' and caries lesions ($p<0.001$). The trend of B>S>A for the mean proportional counts of *Lactobacillus* spp. correlated with the finding that all of the 7 sub-sites that had lactobacilli had white spot lesions. A similar positive association was found between the isolation frequency of *Lactobacillus* spp. and the presence of white spot lesions (Fig. 3.4).

Four of the 21 teeth studied by culture had minor white spot lesions: two at sub-sites B, two at sub-site S and one at sub-site A. *Lactobacillus* spp. were isolated together with MS and *A. naeslundii,* and exclusively from teeth that had white spot lesions. However, two of these teeth had no detectable lactobacilli (two lesions at sub-sites B and one at sub-site S), but 3 of these teeth were also colonised by *A. odontolyticus.*

Fig. 3.4 Association between presence of mutans streptococci, lactobacilli determined by immunofluorescence, and percentage of caries lesions.



S. m = *S. mutans*, *S. s* = *S. sobrinus*, *L.* = lactobacilli, MS = mutans streptococci.
Percentage of lesions which had *S. m* + *S. s* > *S. m*, *S. s*, *L.* or *L.* + MS; ★$P < 0.01$.

Table 3.12 The distribution of *S. mutans* 'c' as percentage proportional microscopic counts (IF) at various sites from ipsilateral upper and lower teeth.

Patient No.	UPPER TOOTH				LOWER TOOTH			
	Below♦ (B)	Side (S)	Away (A)	*Total	Below (B)	Side (S)	Away (A)	Total
127	0.0	4.0	0.5	4.5	0.0	0.0	0.0	0.0
150	1.4	0.0	0.0	1.4	0.0	0.0	0.0	0.0
152	3.1	0.7	0.0	3.8	0.5	0.0	0.0	0.5
183	1.5	1.3	0.0	2.8	0.9	1.2	0.3	2.4
187	0.0	0.9	0.0	0.9	0.0	0.0	0.2	0.2
191	0.5	0.0	0.3	0.8	0.0	0.0	0.2	0.2
193	0.9	0.0	0.0	0.9	0.0	0.0	0.0	0.0
200	0.6	0.6	0.0	1.2	1.6	0.0	0.0	1.6
164	2.1	0.5	0.0	2.6	0.7	0.0	1.2	1.9
244	4.9	2.6	1.3	8.8	3.4	2.45	0.2	4.0
246	0.7	0.0	0.0	0.7	0.8	0.0	0.8	1.6
255	3.8	4.7	1.5	10.0	0.0	0.0	0.0	0.0
156	0.2	0.9	0.0	0.9	0.0	0.9	0.0	1.9
158	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.2

♦ Wilcoxon matched pair signed rank test, upper teeth B>lower teeth B; $p<0.05$.

* Total upper B>total lower B, $p<0.05$.

Table 3. 13 Comparison of mean percentage counts of *S. mutans, S. sobrinus* and *Lactobacillus* spp. determined by culture and IF at different sub-sites around the contact area.

	Sub-sites colonised					
	Away (A) (N = 20)		Side (S) (N = 20)		Below (B) (N = 20)	
	CU	IF	CU	IF	CU	IF
Bacterium	Mean % ± SE		Mean % ± SE		Mean % ± SE	
S. mutans	5.5±3.0	1.0±0.2	6.7±2.0	1.3±0.4	11.4±3.7	2.9±0.7
S. sobrinus	2.0±0.9	0.3±0.1	4.1±3.2	0.5±0.2	5.2±4.1	1.1±0.3
Lactobacillus spp.	0.4±0.3	0.3±0.2	0.2±0.1	0.2±0.2	0.04±0.0	0.1±0.1

CU = Culture IF = immunofluorescence

Mann-Whitney test applied on percentage viable counts for:
S. mutans $^{\bullet}$B>A; $p<0.01$; by culture and by IF for B>A and B>S; $^{\bullet\bullet}p<0.02$ and $^{\bullet\bullet\bullet}p<0.04$, respectively.
S. sobrinus B>A; $^{\bullet}p<0.02$ by IF.

Table 3.14 Comparison of percentage isolation frequency and number of sub-sites colonised by *S. mutans, S. sobrinus* and *Lactobacillus* spp. as identified by culture and IF.

	Sub-sites colonised											
	Away (N=20)			Side (N=20)			Below (N=20)			Total (N=60)		
Bacterium	CU	IF	*CUIF	CU	IF	CUIF	CU	IF	CUIF	CU	IF	CUIF
MS	9	16	7	13	15	10	19	18	15	41	49	37
S. mutans +	0	5	0	3	7	2	5	8	5	8	20	12
S. sobrinus	(0)	(25)	(0)	(15)	(35)	(10)	(25)	(40)	(25)	(13)	(33)	(20)
S. mutans alone	8	10	7	9	7	7	12	8	8	29	25	22
	(40)	(50)	(35)	(45)	(35)	(35)	(60)	(40)	(40)	(48)	(42)	(37)
S. sobrinus alone	1	1	0	1	1	1	2	2	2	4	4	3
	(5)	(5)	(0)	(5)	(5)	(15)	(10)	(10)	(10)	(7)	(7)	(5)
Lactobacillus spp.	3	3	3	3	3	3	2	2	2	8	8	8
	(15)	(15)	(15)	(15)	(15)	(15)	(10)	(10)	(10)	(13)	(13)	(13)

CU = culture IF = immunofluorescence * CUIF = Culture + IF ( ) = Percentage isolation frequency
Comparison of two proportions for B, S and A, were:
SND of (B) and (A) = 2.8; 95% CI = (4% to 85%); *S. mutans* culture.
SND of (B) and (A) = 2.0; 95% CI = (1.6% to 58%); *S. mutans* + *S. sobrinus* culture.

Table 3.15 Comparison of numbers of colonies of *S. mutans* 'c' and *S. sobrinus* 'd' on blood agar, TYC and TYCSB on twelve consecutive subcultures.

	S. mutans			*S. sobrinus*		
	BA*	TYC	TYCSB	BA	TYC	TYCSB
1	45	40	44	45	36	28
2	190	160	158	190	145	126
3	86	81	62	215	170	101
4	250	220	152	41	39	35
5	39	34	40	120	105	99
6	140	134	128	120	110	84
7	85	79	70	116	86	79
8	39	38	38	49	40	39
9	80	69	57	135	90	85
10	58	50	52	52	44	29
11	32	26	30	50	32	42
12	80	78	48	220	184	113
DM^d	-	13.9%	16.4%	-	19.8%	32.0%

* Columbia agar base supplemented with 7% v/v horse blood.
DM^d = Differences between mean percentage counts (cfu) on TYC and TYCSB from counts on blood agar.

Table 3.16 Comparison of percentage proportional counts of *S. sobrinus* 'd' using anti-*S. sobrinus* 'd' polyclonal and monoclonal antibodies.

	Polyclonal antibody			Monoclonal antibody*		
	Away	Side	Below	Away	Side	Below
1	0.10	0.10	0.82	0.10	0.10	0.00
2	0.10	0.10	0.10	0.10	0.10	0.10
3	0.10	0.41	0.80	0.10	0.81	0.10
4	0.10	0.10	0.40	0.10	0.10	0.10
5	0.10	0.10	0.10	0.10	0.10	0.10
6	0.10	0.80	0.10	0.10	0.10	0.10
7	0.10	0.10	0.10	0.10	0.10	0.10
8	0.10	0.60	1.96	0.10	0.10	1.40
9	0.80	2.20	1.43	0.20	2.80	2.04
10	0.10	0.25	2.00	0.81	0.10	1.22
11	0.10	1.00	0.65	0.10	1.00	0.88
12	0.10	0.10	1.25	0.10	1.05	1.99
13	1.80	0.10	0.10	1.20	0.10	1.80
14	0.10	0.40	0.10	0.10	0.10	0.10
15	0.20	0.10	0.40	0.10	0.10	0.10
16	0.10	1.10	0.83	0.10	0.80	0.10
17	0.10	0.42	8.00	0.10	0.82	2.50
18	0.10	0.40	0.10	0.10	0.10	0.10
19	0.10	0.40	0.80	0.10	0.10	0.87
20	0.43	0.10	0.50	1.40	0.10	0.60
21	0.10	0.10	0.60	0.10	0.10	0.25

Correlation between counts at sub-sites; A ($r = 0.64$), S ($r = 0.82$) and B ($r = 0.66$), and for total ($N = 63$) sub-sites, $r = 0.66$.
Counts were expressed as a proportion of total microscopic count.
* = Monoclonal antibody, OMVU10 (de Soet et al. 1987).

3.3 Restriction analysis of 16S rRNA genes

Restriction analysis of 16S rDNA of selected streptococci including *S. mutans*, *S. sobrinus*, *S. mitis* I, *S. mitis* II, *S. oralis* and *S. intermedius* were performed together with a limited RFLP analysis of *S. crista*, *S. sanguis S. salivarius* and *S. vestibularis*. The DNA extracted was intact (>2200 bp), and the size of amplified 16 S rDNA by PCR for most of the test species was approximately 1500 bp for all strains (Fig. 3.5). The amplified 16S rDNA of the laboratory strains was each digested with eight restriction enzymes (Table 2.7).

3.3.1 Mutans streptococci

The amplified 16S rDNA fragment of *S. mutans* 'c' (NCTC 10449) was digested with eight restriction endonuclease enzymes. Restriction endonuclease sites for *Bam*HI (C↓GATCC), *Hin*dIII (A↓AGCTT), *Pvu*II (CAG↓CTG), *Pst*I (CTGCA↓G) and *Sma*I (CCC↓GGG) were not detected for 16S rDNA of *S. mutans* 'c', whereas for *Eco*RI there was one restriction site and for *Hae*III four restriction sites (Table 3.17).

One restriction endonuclease site was detected for *S. sobrinus* 'd' with *Eco*RI, *Hin*dIII and *Kpn*I. Only *Hae*III had two restriction sites giving three fragments for each. The *Hae*III third fragment had a low molecular weight, which made its restriction pattern different from that of *Eco*RI, and also from the pattern of *Hae*III-digested *S. mutans* 'c' (Table 3.17). Therefore, it was possible to easily distinguish *S. mutans* 'c' from *S. sobrinus* 'd' by using *Hae*III only. The amplified 16S rDNA of other serotypes of "*S. mutans*-group" e.g. *S. sobrinus* 'g', *S. cricetus* 'a', *S. mutans* 'e' and 'f' were digested with *Hae*III. Serotype 'g' had the same pattern as serotype 'd', and serotypes 'a', 'e' and 'f' showed a pattern the same as for *S. mutans* 'c'.

3.3.2 *S. mitis* I and *S. mitis* II

The results of restriction fragment length polymorphism (RFLP) of 16S rDNA of *S. mitis* I with the selected restriction enzymes are shown in table 3.17. *S. mitis* I had one site for *Eco*R1 and three sites for *Hae*III (Table 3.17). The results were the same for *S. mitis* II, and neither of them had any restriction sites for the other six enzymes (Table 3.17).

3.3.3 *S. oralis*

The results of RFLP showed that *S. oralis* (LVG1) 16S rDNA had restriction sites for *Eco*RI and *Hae*III, *Hin*dIII and *Kpn*I. However, no restriction endonuclease sites for *Pst*I, *Bam*HI, *Sma*I and *Pvu*II were detected for *S. oralis* (LVG1) (Table 3.17).

3.3.4 Other species of viridans streptococci

Under the set conditions, the PCR of 16S rDNA of *S. intermedius* produced three copies with the given primers. The results of digestion with eight enzymes were positive for *Eco*RI with one restriction site, and *Hae*III with 5 restriction sites (Table 3.17). Since the most effective restriction enzymes were *Eco*RI, *Hin*dIII and *Hae*III, it was decided to use a combination of enzymes, and then re-test the other species.

3.4.5 Double digestion

An attempt was made to achieve greater specificity by using combinations of restriction endonuclease enzymes.

*Eco*RI and *Hae*III together produced three fragments for *S. mitis* I, *S. mitis* II and *S. oralis* (Table 3.18). Similarly, when *Hae*III and *Hin*dIII were used together, the fragment of <194 bp appeared to be specific for *S. oralis* (LVG1) (Table 3.18). Therefore, on the basis of the results shown in Table 3.18, *Hae*III and *Hin*dIII are the most appropriate enzymes to be used together for examination of 16S rDNA of *S. mitis* I, *S. mitis* II and *S. oralis*.

These enzymes were used to restrict other species of viridans streptococci but all of them showed the same pattern as *S. mitis* I or *S. mitis* II (Table 3.18). Therefore, the sequences of AAGCTT (*Hin*dIII) and GGCC (*Hae*III) appear to be part of conserved regions of the oral streptococci tested, and no combination of the two enzymes was found that was capable of distinguishing between the species.

Table 3.17 Number of restriction endonuclease sites and approximate fragment sizes following digestion of 16 rDNA of *S. mutans* 'c', *S. sobrinus* 'd', *S. mitis* I, *S. mitis* II and *S. oralis*.

Species	Enzymes	*No. of sites	Fragment size [bp]
S. mutans (NCTC 10449)	*Eco*RI	1	800, 750
	*Hae*III	4	550, 500, 300, <100, <100
S. sobrinus (NCTC 27351)	*Eco*RI	1	840, 750
	*Kpn*I	1	840, 680
	*Hae*III	2	600, 500, 280
	*Hin*dIII	1	800, 750
S. mitis I (NS51)	*Eco*RI	1	872, 700
	*Hae*III	3	620, 400, 320,200
S. mitis II (SK 132)	*Eco*RI	1	872, 700
	*Hae*III	3	620, 400, 320,200
S. oralis (NCTC 1142)	*Eco*RI	1	872, 700
	*Kpn*I	1	1070, 500
	*Hin*dIII	1	1070, 480
	*Hae*III	2	600, 500, 280
S. intermedius (ATCC 27335)	*Eco*RI	1	870, 750
	*Hae*III	5	650, 450, 300, 230, 150, <100

*No. of sites = Number of restriction sites detected.
() = strain number

Table 3.18 Number of restriction endonuclease sites and approximate fragment sizes following double digestion of amplified 16S rDNA of *S. mitis* I, *S. mitis* II and *S. oralis*.

Species	Enzymes	*No. of sites	Fragment size [bp]
S. mitis I (NS 51)	*Eco*RI and *Hae*III	2	603, 400, 310
	*Hae*III and *Hin*dIII	3	600, 500, 330, 200
S. mitis II (SK 132)	*Eco*RI and *Hae*III	2	603, 400, 310
	*Hae*III and *Hin*dIII	3	600, 500, 330, 200
S. oralis (NCTC 12166)	*Eco*RI and *Hae*III	2	605, 400, 281
	*Hae*III and *Hin*dIII	4	500, 260, 210, <194, <100
S. anginosus (NCTC 10713)	*Hae*III and *Hin*dIII	3	600, 500, 330, 200
S. crista(CR311)	*Hae*III and *Hin*dIII	3	600, 500, 330, 200
S. gordonii (NCTC 12166)	*Hae*III and *Hin*dIII	3	600, 500, 330, 200
S. sanguis (SK 7)	*Hae*III and *Hin*dIII	3	600, 500, 330, 200
S. salivarius (NCTC 8618)	*Hae*III and *Hin*dIII	3	600, 500, 330, 200
S. vestibularis (NCTC 12166)	*Hae*III and *Hin*dIII	3	600, 500, 330, 200

*No. of sites = Number of restriction sites detected.
() = strain number

3.4.6 Clinical samples

Species of MS, *S. mitis* (I and II) and *S. oralis* were recovered from 57 sub-sites (19 teeth from 11 patients). Forty nine isolates of *S. mutans* and 13 isolates of *S. sobrinus* (from 10 sub-sites) were ribotyped using *Hae*III to digest their amplified 16S rDNA. All species of *S. mutans* showed the same pattern as *S. mutans* 'c' with three distinctive bands (550 bp, 500 bp and 300 bp). There was no deviation from the pattern for different subjects or different sub-sites.

All species of *S. sobrinus* presented the same pattern obtained for *S. sobrinus* 'd' or 'g' with three bands (600 bp, 500 bp and 280 bp). There were no differences between the isolates from different sub-sites or from different subjects (Fig. 3.6, Table 3.19).

Thirty nine strains of *S. mitis* (I, II) from 18 sub-sites and 14 strains of *S. oralis* from 9 sub-sites were processed for ribotyping. Discrimination between clinical isolates of *S. mitis* (I, II) and *S. oralis* was not easy, since additional ribotypes were seen for some of these *S. mitis* (I, II) strains. A greater consistency in pattern was observed for *S. oralis* strains (Fig. 3.7, Table 3.19), whereas several isolates of *S. mitis* deviated from the pattern presented previously by type strains of this species. These profiles were not specific to any of the sub-sites.

Table 3.19 Restriction endonuclease pattern of amplified 16S rDNA of plaque samples of: *S. mitis* I, *S. mitis* II and *S. oralis* with *Hae*III and *Hin*dIII, and mutans streptococci with *Hae*III.

Species	Number of strains	Ribotype	Prevalence
S. mitis I/II	28	I	72%
	7	II	18%
	4	II	10%
S. oralis	14	I	100%
S. mutans	49	I	100%
S. sobrinus	13	I	100%

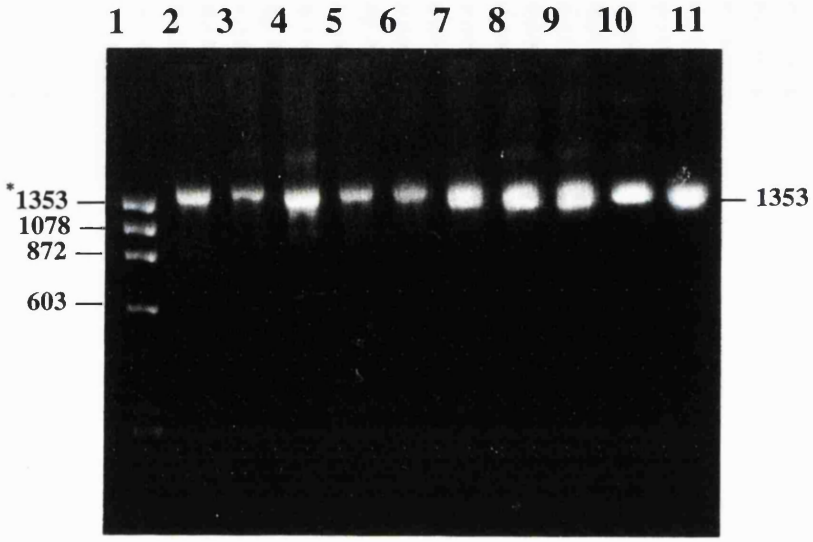


Fig. 3.5 The PCR products of 16S rDNA of *S. mutans* 'c'(10449 NCTC), *S. sobrinus* 'd'(NCTC 27351), *S. mitis* I (NS51), *S. mitis* II (SK132), *S. oralis* (NCTC 11427), *S. intermedius* (ATCC 27335), *S. anginosus* (NCTC 10713), *S. crista* (CR 311), *S. gordonii* (NCTC 7865), *S. sanguis* (SK7), *S. salivarius* (NCTC 8618) and *S. vestibularis* (NCTC 12166) followed by electrophoresis on 8% agarose gels, lanes 2 to 11 respectively. Lane 1 is *Hae*III-digested ϕ X 174 DNA.

* Standard molecular weight markers [Promega Corporation, UK].

( ) = Strain number.

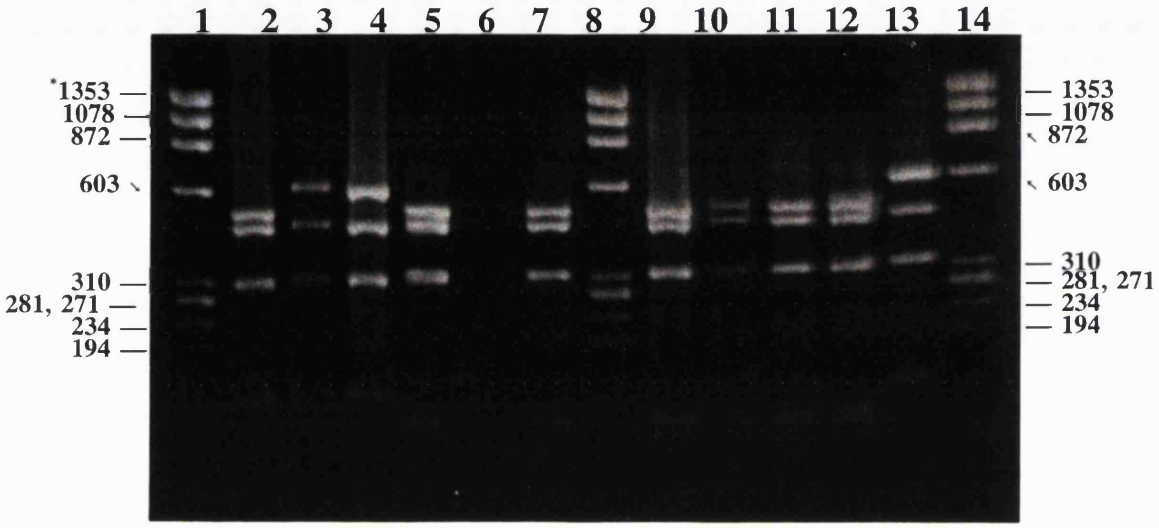


Fig. 3.6 Restriction endonuclease digestion patterns of 16S rDNA of 11 clinical isolates (*S. mutans* 'c' and *S. sobrinus* 'd') with *Hae*III, followed by electrophoresis on 1.8% Metaphor agarose gels. Lanes are: 2 and 3 = *S. mutans* 'c' (Tsb IV B5) and *S. sobrinus* 'd' (Tsb VB) from sub-site B of subject 223, respectively. Lanes 4 and 5 = *S. sobrinus* 'd' (Ba VI B) and *S. mutans* 'c' (Tsb I B) from sub-site B of subject 225; 7 and 9 = *S. mutans* 'c' (Tsb II S2, TC II A5) from sub-sites S and A of subject 261. Lanes 10 and 11 = *S. mutans* 'c' (Tsb II A4 and Tsb IV S3) from sub-sites A and S of subject 272; 12 and 13 = *S. mutans* 'c' (Tc I B3) and *S. sobrinus* 'd' (Tc II B3) from sub-site B of subject 266. Lanes 1, 8 and 14 = *Hae*III-digested ϕ X 174 DNA; fragment sizes are given in base pairs.

* Standard molecular weight markers [Promega Corporation, UK].

( ) = Strain number.

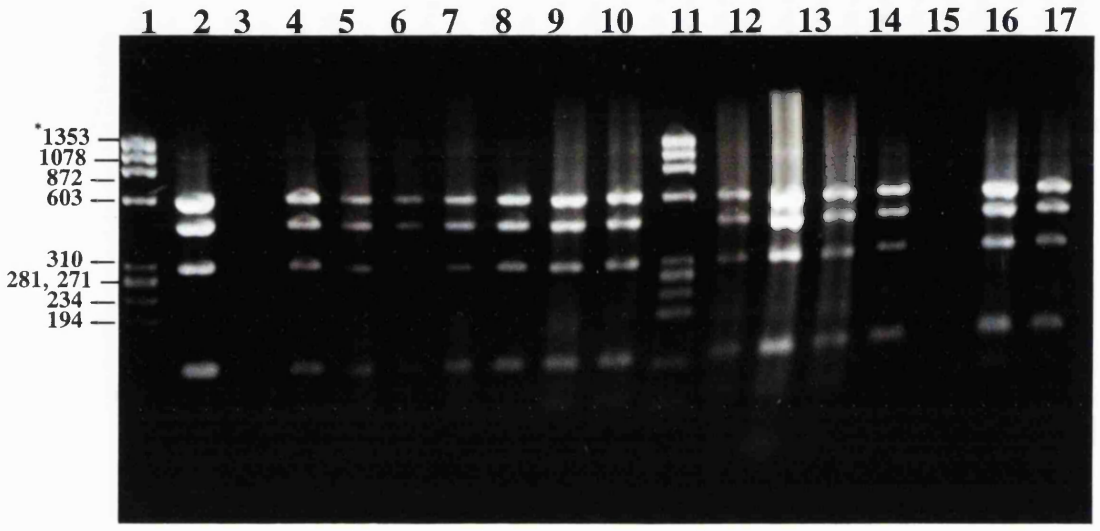


Fig. 3.7 Restriction endonuclease digestion pattern of 16S rDNA of 16 clinical isolates of *S. oralis* with *Hae*III and *Hin*dIII, followed by electrophoresis on 1.8% Metaphor agarose gels. Lanes 2 to 10 and 12 to 18 are profiles of digested DNA [2 = (N6 11/o), 3 = (N6 18/o), 4 = (N5 29/o), 5 = (N2 26/o), 6 = (N2 26/o), 6 = (N2 25/o), 7 = (N6 1/o), 7 = (N6 1/o), 8 = (N6 22/o), 9 = (N4 3/o), 10 = (N5 30/o), 12 = (N5 4/o), 13 = (Have /B1), 14 = (Have /B2), 15 = (N5 3/o), 16 = (N3 4/o), 17 = (Tc II S) and 18 = (Tc I A)]. Due to the low concentration of the DNA in wells 3 and 15 bands in these lanes did not appear in the photograph. Lanes 1 and 11 are HaeIII-digested ϕ X 174 DNA; fragment sizes are given in base pairs. All the strains appear to have the same pattern.

* Standard molecular weight markers [Promega Corporation, UK].

( ) = Strain number.

CHAPTER FOUR

DISCUSSION

4.1 Introduction

The present investigation comprises a systematic study of the microbiology of small samples of plaque from discrete sites on human teeth, in an attempt to explain the microbial ecology of approximal human dental plaque. Although small samples were analyzed in an earlier study (Marsh et al. 1989a), no attempt was made at that time to standardise the sampling sites for comparative purposes. To give enough space between the sample sites, the sample size was reduced. Hence, the present study provides data concerning the distribution of plaque bacterial species from comparative locations increasing in stagnation away from to below the contact area, which is the most stagnant site, and which is thought to be caries and gingivitis-susceptible.

Although it is now generally accepted that mutans streptococci are closely associated with the aetiology of caries at a variety of enamel surfaces (Loesche 1986), little is known of the microbiology of the very early stages of enamel caries development. However, evidence is beginning to emerge that suggests that the microflora of plaque as a whole and perhaps shifts in their proportions may be involved in different stages of disease development.

4.2 Culture aspects

The first part of the present study concerned the distribution of bacterial populations, as obtained by culture at the different sub-sites. The three sub-sites which were chosen for the present work would differ from each other due to their physical and biological properties. For example, sub-site A might be more accessible to saliva and be more aerobic than sites B and S. Increasing stagnation at sub-site B could result in the formation of thicker plaque which might be more anaerobic, with a lower Eh and different

rate of diffusion than the plaque at sub-sites A and S.

The approximal surface is a site with considerable microbial, biochemical, immunological and mechanical interactions, therefore, plaque formed in this site is expected to accommodate different micro-environments. Marsh and Martin (1992) suggested a general model for plaque, where either sharp or gentle gradients extending over small distances will exist in plaque for many key parameters (physical and chemical). These parameters include: concentration of essential nutrients, pH, Eh, concentration of toxic products of metabolism and rate of diffusion. All these would influence microbial growth and survival. Therefore, in the present work the results are discussed on the basis of expected differences in microflora composition due to different factors at each micro-environmental site.

The isolation frequency of *Actinomyces israelii* showed a varying trend, although this did not reach statistical significance. The mean percentage viable count and mean percentage isolation frequency of *A. israelii* (Tables 3.2 and 3.3, respectively) were markedly higher at sub-site S compared with sub-sites A and B. This is in general agreement with the results of a longitudinal study of approximal plaque from premolars of 13 to 14-year old children (Bowden et al. 1975, 1976), in which they identified *Actinomyces* spp. using more complicated physiological and serological tests compared with the present study (Table 2.4). They also reported large differences between the mean percentage viable counts of *A. israelii* recovered from different sites in the same subject. However, in a study of plaque from children in South America, Thompson et al. (1980) reported a mean isolation frequency of 96.8%, and mean percentage viable count of 10.4% for *A. israelii*. The high mean isolation frequency in the latter study could be due to cross-reactivity of fluorescent antibody with other species in the plaque sample.

A. naeslundii was found in at least at one sub-site on all of the premolars (Appendix D Tables 1-20). Species of *A. naeslundii* had the highest mean percentage viable counts and mean percentage isolation frequency at all the sub-sites (Tables 3.2 and 3.3, respectively). Comparison of the data for this species with previous studies proved problematical due to changes in classification (Johnson et al. 1990). Therefore, the data for *A. naeslundii* has been compared with the sum of strains of *A. naeslundii* and *A. viscosus* from previous reports. On this basis, *A. naeslundii* data (mean percentage viable counts and isolation frequency) were in agreement with previous reports (Bowden et al. 1975, Boyar and Bowden 1985, Milnes and Bowden 1985, Milnes et al. 1993). Although the mean percentage isolation frequency and mean percentage viable counts for total *Actinomyces* spp. are in general agreement with the above studies, the mean percentage viable counts of *A. naeslundii* (Tables 3.2 and 3.3) were lower than those reported previously by Marsh et al. (1989a). This could be due to the changes in the taxonomy of *A. naeslundii* mentioned above (Johnson et al. 1990).

Similarly, variations between mean percentage counts at different sub-sites in the present study were smaller than those reported by Bowden et al. (1975). This is expected since they compared samples of approximal plaque from the contralateral (two teeth on opposite sides of the mouth, same jaw) upper first molars. However, in the present work, samples were taken from sub-sites on the same surface of the same tooth. The high viable counts of *A. naeslundii* at sub-sites A and S could be due to its ability to utilize lactate under aerobic conditions. Van der Hoeven and van den Kieboom (1990) have proposed that lactate consumption may be an important factor for *Actinomyces* spp. (*A. naeslundii* and *A. viscosus*) to survive in an environment limited in energy sources for most of the time. This is more likely to happen at sub-sites A or S rather than sub-site B, since oxygen is more likely to be in excess at the former sites.

At each sub-site the mean percentage of *A. naeslundii* was statistically higher than the mean percentage of many of the other species (Table 3.6 c). At sub-sites at which *A. naeslundii* was high, the total number of some streptococci (*S. mitis* I/II and *S. sanguis*) was also high. One of the elements which could have contributed to such an increase is lectin-carbohydrate interactions among *A. naeslundii*, *S. mitis* I/II and *S. sanguis* (Cisar et al. 1979). The results of this *in vitro* study are also supported by other *in vivo* investigations of the microbiological development of supragingival plaque (Socransky et al. 1977, Liljemark et al. 1993), and studies of the early microbial colonization of human enamel (Nyvad and Kilian 1987). Therefore, the high counts of *A. naeslundii* at all sub-sites could be due to different physicochemical and biological factors controlling the growth of this species at each sub-site.

A. odontolyticus had its highest mean isolation frequency at sub-site B whilst its greatest mean percentage viable count was at sub site S. Small variations were observed for the mean percentage count and mean isolation frequency of *A. odontolyticus* (Tables 3.2 and 3.3). However, these values for both variables were substantially higher than those previously reported (Bowden et al. 1975, Boyar and Bowden 1985, Boyar et al. 1989), but not in other studies (Kilian et al. 1979, Milnes and Bowden 1985); in all these studies Rogosa SL agar was used, whereas Columbia agar supplemented with 7% horse blood was used in the present study, which could be one of the reasons for these differences in viable counts of *A. odontolyticus*.

The present study showed that *Veillonella* spp. could be recovered with significant differences in isolation frequency and mean percentage counts at each sub-site (B>S $p<0.03$, and S>A, $p<0.007$, Table 3.2). The mean percentage count and isolation frequency of *Veillonella* spp. at sub-sites B was in line with the findings of Bowden et al. (1975), however, the overall mean (mean of three sub-sites) isolation frequency and viable counts

were less than the values reported by Bowden et al. (1975). This could be due to the larger sample size and lower number of subjects processed in that longitudinal study, suggesting that to obtain representative results concerning ecology of plaque it is absolutely essential to use mini-size plaque samples from different subjects.

There is some controversy over the precise role of *Veillonella* spp. in the aetiology of dental caries (Noorda et al. 1988). *Veillonella* spp. are unable to ferment dietary carbohydrates, but they can utilize lactic acid generated by other plaque bacteria during glycolysis, converting it to the weaker propionic and acetic acids. van der Hoeven et al. (1978) have shown a symbiotic relationship between *V. alcalescens* and *S. mutans* in dental plaque of gnotobiotic rats, resulting in significantly lower caries.

Other studies have shown higher proportions of *Veillonella* in progressing incipient lesions (Boyar and Bowden 1985), and at sites with nursing (bottle) caries (Milnes and Bowden 1985). Also, *Veillonella* have proliferated along with *S. mutans* and *L. casei*, at the expense of "non-cariogenic" species, in mixed culture studies during growth at low pH (McDermid et al. 1986). Furthermore, no inhibition of demineralization was observed *in vitro* when *V. alcalescens* was cultured with *S. mutans* in an "artificial mouth" (Noorda et al. 1988). The probable explanation for these findings is the likely higher lactic acid concentration at the sites studied. High levels of *Veillonella* may act as markers for sites with a high lactate concentration. In the present study, the highest proportions of *Veillonella* were found at sites with the highest counts of mutans streptococci (sub-site B, Table 3.2). Similarly, Marsh et al. (1989a) found the lowest proportions of *Veillonella* at those sites that had developed early caries in the absence of cultivable mutans streptococci.

Neisseria spp. were isolated in significantly higher proportions at sub-site A (A>B, p =

0.03). The higher counts of *Neisseria* spp. at this sub-site may be attributed to their aerobic requirements. Surprisingly, the isolation frequency of *Neisseria* spp. did not appear to be influenced by the site of isolation. The high mean percentage count of *Neisseria* spp. at sub-site A, which had the highest count of *S. mitis* I/II, is in contrast with the findings of Dajani et al. (1976a, b). They found that bacteriocins produced by *S. mitis* and *S. sanguis* inhibited many Gram-negative species including *Neisseria* spp.

Lactobacillus spp. were recovered only infrequently, and their proportions were not affected by the site of isolation. This may be because they do not form part of the main resident plaque microflora of the approximal surface. While established caries lesions were not a feature of the teeth sampled (only four white spot lesions were observed on three of the teeth in the culture study), nevertheless an attempt was made to clarify whether microbial composition at a specific site correlated with the clinically-observed pattern of approximal caries. Lactobacilli were found without MS only once at one of the sub-sites, but MS were found commonly without lactobacilli (Table 3.5). Since, lactobacilli become more predominant only following establishment of mutans streptococci and the development of early lesions (Marsh et al. 1989a), it is not surprising that the former were isolated infrequently in the present study. Their isolation frequencies and proportions appeared to be much higher from sites undergoing demineralisation. Indeed, in one study, lactobacilli were only recovered from such approximal surfaces (Boyar and Bowden 1985).

In the present study, identification of streptococci was carried out according to the latest taxonomic schemes (Kilian et al. 1986, Beighton et al. 1991) which provided a more precise definition of the various species. This study has apparently for the first time elucidated some qualitative and quantitative changes in the composition of approximal plaque from discrete sites. Some of these differences were to a certain extent dependent

upon changes in the taxonomy. In this connection one of the main differences between these and previous studies is the subdivision of *S. sanguis* into two separate species, *S. sanguis* and *S. gordonii*, and in addition, division of *S. oralis* from *S. mitis,* and subdividing *S. mitis* into two distinct biovars (*S. mitis* I and *S. mitis* II). Ecological significance is attached to these findings since different taxonomic groups presumably have evolved to fill characteristic niches within the oral environment.

Of the streptococcal species considered above, all are found in mature dental plaque but only some participate in the initial colonisation of the tooth surface. Nyvad and Kilian (1987) used enamel squares for various time periods and found that after 12 h, *S. mitis* II (arginine-negative), *S. oralis* and *S. sanguis* were in the greatest numbers. Because of the changes in the taxonomy of the oral streptococci, particularly with regard to *S. sanguis*, *S. mitis* I and *S. mitis* II, it is difficult to compare directly the results from different workers. Nevertheless, in the present study, the results concerning streptococcal composition of approximal plaque are in overall agreement with other studies (Ikeda et al. 1973, Bowden 1975, Hardie et al. 1977, Mikkelesen et al. 1981, Boyer and Bowden 1985, Marsh et al. 1989a). Small differences can be explained by changes in nomenclature. Furthermore, the present study has described a broad range of species in approximal plaque some of which (e.g. *S. mitis* and *S. oralis*) have not been reported (in detail) in previous studies.

The mean viable counts (cfu/ml) of total streptococci were: $13.8x10^3$ at sub-site (A), $32.6x10^3$ at sub-site (S) and $40.5x10^3$ at sub-site (B), and their mean percentage viable counts were 53.6 %, 37.2% and 49.2%, respectively. There was a decrease in mean percentage viable count of streptococci at sub-site (S), although the recovery (mean percentage isolation frequency) of streptococci at sub-site (S) was not significantly different from those at sub-sites (A) and (B) but was consistent with an increase in non-

streptococcal species at this sub-site (S). It is not possible to determine whether this change in the relative proportions of organisms was due to an increase in the numbers of some, a decrease in others, or a combination of both. Alternatively, it may be because this site is more suitable for the co-existence of a wide spectrum of physiologically-related genera.

S. mitis has recently attracted much attention. This is due to the fact that it is an early coloniser of the tooth surface and usually can be found in many different sites in the mouth. In this study, *S. mitis* had the highest median and percentage isolation frequency at sub-sites (A) and (S) and the third highest at sub-site (B) (Tables 3.4 and 3.5). These results are in general agreement with other studies of approximal plaque, for example, Boyar et al. (1989). In the study of microflora associated with the development of initial enamel decalcification below orthodontic bands *in vivo* which was proposed to be similar to protected areas on approximal surfaces, they found that "*S. mitior*" (*S. mitis* I/II and *S. oralis*) had the highest mean percentage viable counts, median and isolation frequencies. In an earlier study, Boyar and Bowden (1985) reported an isolation frequency of 80% for "*S. mitior*". Also, Hardie et al. (1977) reported "*S. mitior*" as the most prevalent streptococcal species isolated from approximal surfaces in 11 to 12 year old children.

Similarly, *S. mitis* was reported as the most prevalent species at 4 sites (maxillary approximal, labial, mandibular approximal and labial) by McNamara et al. (1979). In the more detailed studies of Nyvad and Kilian (1987), *S. mitis* I was the most prevalent streptococcal species from root surfaces and enamel after 12 and 24 h.

The differences between the mean percentage viable counts of *S. mitis* I at these sub-sites were not statistically significant, although a trend of A> B> S was observed (Table 3.2). The higher recovery of *S. mitis* I at sub-site A could suggest that sub-site (A) is a better

habitat for *S. mitis* I. On a microscopic scale this sub-site is more accessible to saliva, and this sub-site is less stagnant compared with the other sub-sites examined, and therefore plaque at sub-site A might be less anaerobic and perhaps have a higher pH. Liljemark and Gibbons (1972) found that *S. mitis* ("*S. mitior*") could adhere better than the other streptococci to buccal mucosa and to teeth this attachment being mediated by a "fuzzy surface" coat. Nevertheless, Hsu et al. (1994), could not directly correlate the adhesion and colonisation properties of *S. mitis* I.

S. mitis II was recovered at a significantly lower frequency and in lower numbers than *S. mitis* I (Table 3.4). Since the levels of *S. mitis* II were mainly lower than *S. mitis* I in pre-dentate and dentate children (Smith et al. 1993), during the early colonisation of human enamel and root surfaces after 8 hours (Nyvad and Kilian 1987) and on dental enamel in caries-active and caries-inactive individuals (Nyvad and Kilian 1990a), this could imply that the *S. mitis* II population decreases a short time after the formation of plaque (8 hours) and stays low irrespective of local environmental conditions or the state of the tooth surface. However, Frandsen et al. (1991) reported that *S. mitis* II constituted a surprisingly large proportion of the streptococci on the dorsum of the tongue, even outnumbering *S. salivarius.*

Another streptococcus closely related to *S. mitis* I/II is *S. oralis.* The mean percentage viable counts of *S. oralis* at the three sub-sites were in the order A>B>S, and there was a significant difference between sub-sites B and S (Table 3.2). An increase in proportion of *S. oralis* was found within the initial 24 h of dental plaque formation on enamel and root surfaces (median 1% to 27%) (Nyvad and Kilian 1989). Milnes et al. (1993) reported mean percentage counts of 8.6% in maxillary supragingival plaque and 13.2% in mandibular supragingival plaque in preschool children.

Nyvad and Kilian (1990a) found that *S. oralis* was primarily associated with initial dental plaque, where it constituted 28% of the total cultivable streptococci. When present on other surfaces, it amounted to only a small part of the flora. There was no difference in the recovery of *S. oralis* from caries-active and caries inactive sites (Nyvad and Kilian 1990a), leading to the conclusion that the level of *S. oralis* is not affected by caries-related environmental factors. The overall percentage count of *S. oralis* (mean of the three sub-sites) in the present study is higher than that reported by Nyvad and Kilian (1987, 1990a) but lower than by Milnes et al. (1993). This may be due to differences in sampling techniques, e.g. sample size.

The range of physiological tests used in the present study did not allow distinction between species of the "*S. milleri*"-group. The findings are in general agreement, however, with previous studies which also reported on the "*S. milleri*"-group (Kilian et al. 1979, Marsh et al. 1989a, Milnes et al. 1993). The inconsistency between the recovery of the "*S. milleri*-group" in the present study and in those of Bowden et al. (1975), Hardie et al. (1977) and Boyar and Bowden (1985), which reported much lower isolation frequencies and viable counts again could be due to the use of different media and sampling techniques.

S. sanguis and *S. gordonii* (formerly included within *S. sanguis*) were recovered from all the sub-sites, and their distributions at sub-sites B and S were similar (Table 3.2, 3.3). *S. sanguis* was the second most dominant streptococcus recovered from sub-sites A and S, but the third at sub-site B in frequency of isolation. Furthermore, *S. sanguis* had the highest mean percentage viable count at sub-site S, and the third highest mean percentage viable count at sub-sites A (Table 3.2). The mean percentage viable count and mean isolation frequency of *S. sanguis* are in general agreement with Marsh et al. (1989a) and Milnes et al. (1993), but differ from earlier work which reported that *S. sanguis* is the

predominant streptococcus in supragingival dental plaque (Carlsson et al. 1970, 1975, Loesche et al. 1972). This discrepancy probably can be explained by differences in nomenclature.

The high counts and persistence of *S. sanguis* could be due to the fact that this species can produce an IgA_1 protease (Kilian and Holmgren 1981), and also can utilize carbohydrates and arginine as a carbon and energy source (van der Hoeven et al. 1984). Production of the IgA_1 protease facilitates colonisation, and the latter nutritional properties enable this species to co-exist with other species and utilize arginine in the absence of carbohydrate.

S. salivarius was present in one third of the children, and there was no attempt to identify the newly recognized species of *S. vestibularis* (Whiley and Hardie 1988). Variations were found between the proportions of *S. salivarius* at all the sub-sites, though none of them were statistically significant (Tables 3.2 and 3.3).

The mean isolation frequency of *S. salivarius* at the various sub-sites of about 30% was similar to those reported by Macpherson et al. (1990). However, a comparison of present *S. salivarius* data with a similar study in terms of site analysis (Marsh et al. 1989a), showed that the mean percentage viable counts found in the present study were higher. These inconsistencies could be due to the use of different tests and identification schemes in the two studies.

The proportions of *S. salivarius* and *S. sanguis* have been investigated during the initial phase of plaque formation by van Houte et al. (1970). They found that the percentage of *S. salivarius* on tooth surfaces was much lower than in saliva and on the tongue tip. In contrast, the percentages of other extracellular polysaccharide-producing (EPS)

streptococci, particularly, *S. sanguis* were very high on the tooth surfaces, and simultaneously much lower in the saliva and tongue tip samples. The observed low proportions of *S. salivarius* and high proportions of *S. sanguis* in dental plaque are mainly the result of differences in the ability of cells to adhere to, rather than in their ability to grow on the tooth surface. The data for *S. salivarius* and *S. sanguis* from the present work are consistent with the above findings.

The frequency of isolation of MS from premolar approximal surfaces in the present study was higher than that reported in a larger survey of older individuals (Lindquist and Emilson 1991b). In agreement with other studies (for reviews, see Loesche 1986, Bratthall 1991), *S. mutans* was found significantly more often, and in higher proportions, than *S. sobrinus*. The recovery of *S. sobrinus* was similar to that reported by Lindquist and Emilson (1991b), but the isolation of *S. mutans* was more frequent. This might reflect the use of different media in the two studies to isolate mutans streptococci. Higher viable counts of *S. mutans* have been obtained on TYC and TYCSB compared with MSB agar (Schaeken et al. 1986), although others have not found such marked differences (Beighton 1991). The difference might also relate to the narrow range of age group in the present study, to differences in sampling, or to the use of a wide range of physiological tests for distinguishing between *S. mutans* and *S. sobrinus*, rather than a reliance on colonial morphology (as was used by Emilson 1983 and Lindquist and Emilson 1991a, b).

Ideally, any association between plaque composition and caries should be determined at defined sub-sites (Marsh et al. 1989a, Bush et al. 1989, 1990, Gill et al. 1991). Approximal surfaces are particularly prone to caries, especially just apical to the contact area (Leigh 1927, Newman and Morgan 1980). It is not feasible to study the distribution of individual bacterial species around the contact area on teeth *in situ*, and correlations between approximal plaque and caries have had to be based on radiographs and samples

of the microflora of the entire macro-area. Therefore, possible important sub-site differences in microflora (especially in MS) could have been obscured.

In the present study the most frequent recoveries and highest proportions of MS (*S. mutans* and *S. sobrinus*) were from the sub-site below (B) the contact area. This was also the sub-site from which *S. mutans* was detected most commonly by immunofluorescence (Gill et al. 1991). Although *S. mutans* and *S. sobrinus* were found together on 38% of teeth, there was no evidence of a statistically significant positive association in their presence at any sub-site. This was in contrast to the findings of Lindquist and Emilson (1991a), and also the results obtained by applying immunofluorescence in the second part of the present investigation. This in turn could be due to the limited number of samples used in the culture study, whereas in both the Lindquist and Emilson (1991a) and the present immunofluorescence studies a much larger number of samples were processed. Also, it may be due to the probable inhibitory effect of TYC and TYCSB on the growth of these species (Table 3.15). Furthermore, one or both of these species could be absent from the other sites sampled on the same tooth surface.

Factors responsible for regulating the distribution of these two species at a given site are only poorly understood. Indeed, there have been few reports in which small plaque samples have been studied in relation to clearly-defined sub-sites (Bush et al. 1989, Boyar et al. 1989, Marsh et al. 1989a, Gill et al. 1991). The preferential location of MS below the contact area might be related to stagnation in this location as in occlusal fissures, and to the impaired substrate clearance effect of saliva. In such an environment, the pH may be lower for longer periods, and such conditions would favour the growth of MS at the expense of less acidogenic and aciduric species (Donoghue and Newman 1976, Newman et al. 1976, Bradshaw et al. 1989). Although *S. mutans* is more bacteriocinogenic than *S. sobrinus*, and growth of the latter species can be suppressed by bacteriocin production by

S. mutans (Ikeda et al. 1988), such inhibitors are not considered a major factor in determining whether an individual species would colonise or predominate a particular sub-site in buccal plaque (Lindquist and Emilson, 1991b). Differences in the pattern of colonisation by *S. mutans* and *S. sobrinus* might rather be related to the fact that they possess different abilities to metabolise locally available endogenous nutrients (Homer and Beighton 1991).

From the results of studies carried out in experimental animals and in man it has been anticipated that numerous interactions could take place in plaque which could modify caries activity. However, concerning caries, relationships among bacterial species are likely to be at least as complex and variable as the presence and number of any one of the species. From the results given in Tables 3.6a and b, relationships between the different species could be affected by ecological factors that dominate their micro-environment. From the pattern a (Table 3.6a) the least variation between species was found at sub-sites S. This may be explained by the fact that this site is located somewhere between sub-sites A and B, and may provide conditions intermediate, or less extreme than those present at sub-sites A and B, which are more different from each other as reflected in species detected (Table 3.a). However, some species were isolated in significantly greater numbers than others at all three sub-sites (Table 3.6c). This may be due to the ability of these species to adapt to the different environments better than the others.

The present cultural study is limited since it has failed to sub-classify *S. crista* (Handley et al. 1985, Whiley and Hardie 1988). Also, the number of samples was limited due to the time demanding and other requirements of cultural microbiology. Since more data are needed to validate the above results, it was decided to focus on some of the caries-related species which varied in their proportions at different sub-sites. Therefore, in the second part of the study *S. mutans*, *S. sobrinus* and lactobacilli were studied in a large number

of samples by immunofluorescence, as it is fast and potentially cheap.

4.3 Immunofluorescence studies

An important factor to be considered in relation to the ecology of mutans streptococci and lactobacilli is the location of cells in relation to caries-prone sites. Few studies have considered the relevance of the precise location of plaque samples, and all have been limited either by the number of samples (culture) or species (IF) studied.

Using high-titre polyclonal antisera in IF, *S. mutans* 'c', *S. sobrinus* 'd' and *Lactobacillus spp.* (all human caries-related species) were shown to be detectable in discrete approximal plaque samples at levels as low as 0.2% of a proportional direct microscopic count (Bush et al. 1990) when this procedure was used to analyze 270 discrete small samples.

S. mutans has been identified previously in plaque by IF, but the antiserum usually gave troublesome cross-reactions which could either be adsorbed out (Bratthall 1972) or masked by counter-staining (Grenier et al. 1973). In the present study, very high-titre polyclonal mouse antisera (anti-*S. mutans* 'c' and anti-*S. sobrinus* 'd') helped to overcome the minimum cross-reactivity found simply by diluting the antiserum. However anti-*L. casei* and anti-*L. acidophilus* antisera required adsorption with *S. salivarius*. The adsorbed sera retained a very strong specific reaction, sufficient to permit their use at a dilution of 1:300 (anti- *L. acidophilus* and anti-*L. casei*) and gave a 4+ intensity for homologous strains.

The (3+) reactions with *S. salivarius* and members of strains closely related to *S. oralis* (*S. mitis* and *S. sanguis*) could be due to a single antigen common to these four species and *L. acidophilus*. The strong 3+ reactions given by *L. odontolyticus* to the anti-*L. acidophilus* antiserum was regarded as genus-specific.

The FITC-conjugated rabbit anti-mouse antiserum (used as secondary antibody) cross-reacted with *Staph. aureus* and some filamentous rods (Table 3.7). Application of monoclonal antibody (OMVU10, de Soet et al. 1987) did not help to avoid this problem, and showed similar cross-reactions. Therefore, there was no reason for not using anti-*S. sobrinus* polyclonal antiserum, or any obvious advantage in using monoclonal antibodies compared with the polyclonal antiserum. In addition, monoclonal antibodies may be too exquisitely specific and so fail to recognize isolates of the target species that have undergone only minor variation in one antigen (Russell 1991). The loss of antigens can occur as a result of subculturing, as reported by Russell and Smith (1986). In that study they reported the release of antigens A, B, C, and lipoteichoic acid (LTA) of *S. mutans* in cell suspensions and supernatants. They suggested that the increased shedding of surface antigens as a result of subculturing may be a secondary consequence of changes in the underlying wall structure to which antigens A, B, C and LTA are linked, or to a 'domino' effect in which a change in any one of the surface components could destabilise the others (Russell and Smith 1986).

However, using SDS-PAGE and immunoblot analysis only small differences in the surface antigens were found between the fresh and laboratory isolates of *S. mutans* 'c', and *S. sobrinus*, and also the only *S. sobrinus* strain showing a protein of 190 kDa in SDS-PAGE was a subcultivated serotype 'g' reference strain (Widerstrom et al. 1994). Similarly Hamilton et al. (1989) reported no antigenic changes between fresh isolates and their homologous laboratory strains.

In the present study the antisera (anti-*S. sobrinus* 'd' and anti- *S. mutans* 'c') appeared to be specific in spite of shared antigens (Russell et al. 1986). For instance, LTA is common to all streptococci and many other Gram-positive species. If the antiserum was reacting with any of the known antigens such as LTA the cross-reactions would be expected to

have been more extensive. However, weak cross-reactions observed with filamentous rods (Table 3.7) could be due to a common chemical grouping in a cell wall molecule shared by different species, or otherwise to non-specific binding. Cross-reactions with filamentous rods (Table 3.7) were easily distinguished from the 4+ results with *S. mutans* or *S. sobrinus* on the basis of simple morphology and did not pose a problem.

It is possible that serum antibodies could interfere with the sensitivity and specificity of detection of bacteria using IF. Antibodies to *S. mutans* have been detected in human serum (Challacombe 1974) and these can reach the gingival crevice via the crevicular fluid (Challacombe et al. 1978). Serum Ig has been demonstrated by IF staining in the same apical plaque border on the approximal tooth surfaces of premolars in children, as used in the present study (Newman et al. 1979). Emilson et al. (1974) suggested that such host Ig could affect the reliability of the IF technique if it masked bacterial antigens. It has been shown by Pekovic et al. (1987) and Bush et al. (1990) that Ig and complement were associated with plaque species. In the present work incubation of *S. mutans* 'c' and other species with human serum did not inhibit or affect the intensity of IF-staining (section 2.3.8). This suggests that host antibodies are unlikely to have affected the efficacy of the IF technique used in this study. One explanation for the finding of no inhibition of staining is that the host Ig may be displaced by the greater avidity (due to immunization) of the antiserum IgG (Bush et al. 1990). Alternatively, the antiserum raised by immunization may be detecting additional or alternative antigens to those induced by natural immunization in man.

Sucrose is a common dietary sugar and its presence stimulates the synthesis of soluble and insoluble glucans by *S. mutans* GTF-S and GTF-I enzymes, respectively (Marsh 1986). These enzymes are normally extracellular but they, and a glucan binding protein (GBP), become cell-bound when sucrose is present (Russell et al. 1986). The effect of this is that

the cell becomes surrounded by a layer of extracellular polysaccharides (EPS) which could mask antigens (Newman et al. 1976). The results of the present study and that of Bush et al. (1990) confirmed that this does not occur under the conditions used. In fact, cells exposed to sucrose may be expected to have a greater density of cell-bound GTF (an antigen to the antiserum) and glucan binding protein, so possibly potentiating the staining reaction.

The highest isolation frequency and mean percentage counts of *S. mutans*, *S. sobrinus* and lactobacilli were obtained at the sub-site below the contact area (Figs 3.2 and 3.3). The IF study confirmed the results of the culture study (Tables 3.14 and 3.15) and showed a significant difference between counts and isolation frequency of mutans streptococci at the three sub-sites studied (Tables 3.14 and 3.15). An overall comparison of the results with the results of previous studies indicates general agreement (Table 4.1).

In the present study, mini-sampling allowed detection of high numbers of *S. sobrinus* at sub-site B. Previous studies have not used samples from discrete sites below the contact area. The average mean percentage value for the isolation frequencies at sub-sites A, B and S; 53.3% for *S. mutans* and 34.3% for *S. sobrinus* are in good agreement with those of de Soet et al. (1990, 1993) and Beighton et al. (1989) although they did not use the direct microscopy method. The discrepancy between the results of the present study and those of Bratthall (1972), Thomson et al. (1976, 1980), and Keene et al. (1977) could be due in part to sampling and identification techniques. All these studies showed that *S. mutans* is isolated more frequently than *S. sobrinus* serotype 'd'. This could be due partly to fundamental differences between the mechanisms by which *S. mutans* and *S. sobrinus* attach to pellicles of tooth surface. It may also be that *S. sobrinus* attachment is enhanced to pellicle which has been exposed to sucrose (Gibbons et al. 1986). Recently Wennerholm and Emilson (1995) studied the relationship between sucrose retention and colonization by mutans streptococci at different sites (buccal surface) of the dentition. The

Table 4.1 Comparison of frequency distribution of *S. mutans* and *S.sobrinus* in plaque or saliva from different countries.

Investigator	No. of Samples	Prevalence of Serotype(%)		Method of Detecting Serotypes	Source of Samples	Country
		S. mutans 'c'	*S. sobrinus* 'd'			
Present study	90(B)	70.0	49.0	IF	HDP	UK, London
(1995)	90(S)	51.0	33.0	IF	HDP	"
	90(A)	39.0, 53.3[m]	21.0, 34.6[m]	IF	HDP	"
Previous reports:						
Sigurjons et al. (1995)	56	97	30.0	Cu	HDP	Iceland
de Soet et al.	125	51.0	43.0	IBT	HDP	Iceland
(1990)	72	81.0	35.0	IBT	HDP	Netherlands
Beighton et al. (1989)	183	94.0	34.0	Cu, IF	saliva	Kenya
Thomson et al. (1980)	55	70.0	1.9[g]	IF	HDP	USA, Maine
Keene et al.	64	32.7	11.2 (22)	Bio	HDP	Saudi Arabia
(1977)	169	87.1	1.5 (3)	Bio	HDP	USA, Great Lakes
	25	83.3	6.7 (2)	Bio	HDP	USA, Orlando
	17	70.8	4.2 (1)	Bio	HDP	Chile, San Diego
	41	82.0	6.0 (3)	Bio	HDP	USA, Hawaii
Thomson et al.	10	80.0	10.0[g]	IF	HDP	USA
(1976)	186	87.0	4.0[g]	IF	HDP	USA
	55	76.1	ND[g]	IF	HDP	USA
Shklair and Keene (1974)	216	88.0	7.0[g]	Bio.	HDP	USA
Loesche et al. (1973)	139	80.0	31.0[g]	IF	HDP	USA
Ella et al.(1973)	24	8.0	100.0[g] (43)	IF	HDP	USA
Bratthall	69	73.0	10.0[g]	IF	HDP	USA
(1972)	307	41.0	12.0[g]	IF	HDP	"

HDP = Human dental plaque, IBT = Immune blotting, CU, IF = Culture and then identified by IF, Bio = Biochemical, ND = Not detected, [m] = mean, ( ) = Sample site or Number of samples, [g] = Serotypes 'd' and 'g' identified together.

frequency of mutans streptococci decreased towards the anterior teeth with *S. sobrinus* predominating over *S. mutans*. This was not related to sucrose concentration, but it could be that molars receive less effective cleaning than anterior teeth and removal of an acidic environment is less effective, thereby favouring the growth of mutans streptococci (Bradshaw et al. 1989). In this context the increased percentage of *S. sobrinus* at sub-site B is in line with the results reported by Wennerholm and Emilson (1995). Similarly, the positive association between *S. mutans* and *S. sobrinus* (Table 3.11), was in agreement with the findings of Lindquist and Emilson (1991a). This was in contrast to the observation of Wennerholm and Emilson (1995) that *S. sobrinus* predominated over *S. mutans*.

The frequency of MS in maxillary approximal sites was greater than in mandibular sites ($p<0.05$). The frequency of MS at maxillary sub-site B was significantly higher than at mandibular sub-site B ($p<0.01$). This is consistent with the results of McNamara et al. (1979), and could be explained by the relatively limited access of saliva to maxillary sites. Where the access of saliva to plaque sites is reduced, a decrease in the local pH will occur. In addition, the gingival crevice region, especially in protected approximal areas, is bathed by the nutritionally-rich gingival crevicular fluid (GCF) particularly in the case of upper sites, since GCF tends to flow downward. A more nutritionally-rich GCF environment and a low pH at the site below the contact area could lead to an increase in the proportions of lactobacilli and streptococci in plaque at this site. This may be attributed not only to saliva exclusion but also to carbohydrate retention in stagnant sites, especially below the contact area (Kleinberg 1977, Weatherell et al. 1989). Also, it could be related to differences in sugar intake, as suggested by Wilson and Ashley (1990) for free smooth surface and approximal plaque. At the same time, it may be that the early colonisers are also more resistant to the antimicrobial components of GCF.

The present IF study suggest a definite positive association of MS with early visible lesions (Fig. 3.4). *S. mutans* and *S. sobrinus* were detected at 64% of the 270 sites, often over visibly apparently healthy sites. Although Marsh et al. (1989a) reported that very early caries could not be related to the presence or numbers of any one organism, these species were detected in the few plaque samples which were taken from tooth surfaces with early caries, where the lesion could be seen as a white spot. The association may be even stronger at the histological level. Some investigators have stated that at this level there are no caries-free approximal surfaces (J. M. ten Cate, personal communication). A large increase in the number of *S. mutans* over white spot lesions was noted by Duchin and van Houte (1978). They found one hundred fold differences in the concentration of *S. mutans* in samples taken from a single white spot lesion compared to adjacent healthy enamel. Many studies have shown a correlation between plaque counts of MS and both caries prevalence and incidence (Zickert et al. 1982, Loesche 1986, Lang et al. 1987). There have also been investigations that found no correlation between the MS count and the presence of caries (Hardie et al. 1977, Carlsson et al. 1985, Marsh et al. 1989a, Russell et al. 1990, Macpherson et al. 1992).

Local factors such as differences in exposure to saliva, or variations in fluoride levels of surface enamel could be implicated in the association of MS with healthy sites. The water fluoride content was <0.3 ppm for all patients (Thames Water). Hamilton and Buckley (1991) showed that prolonged growth of *S. mutans* in an acidic environment, such as might be found at retention sites or in relation to caries lesions, resulted in significant changes in cell physiology that conferred increased aciduricity. Also, other species present in the plaque may modify the effects of *S. mutans*. Lang et al. (1987) found that the proportion of *S. mutans* at sites that later developed lesions would increase significantly 6-9 months prior to the clinical diagnosis of a lesion, suggesting that local colonisation with *S. mutans* precedes the demineralisation process.

The very low frequency and lack of statistically significant differences between the sites colonised by *Lactobacillus* spp. is generally consistent with the findings of Ikeda et al. (1973) (Figs. 3.2 and 3.3). In the present study, lactobacilli were found together with MS and mainly where white spot lesions were present on the tooth surface (Fig. 3.4). This could indicate that these organisms appear at a later stage of caries development and then spread on the site. In agreement with this Matee et al. (1992) have shown the relative increase in the numbers of lactobacilli in cavities and suggested that their ability to produce large amounts of acid indicates a role for lactobacilli in producing cavitation once initial lesions have been formed.

Previous studies suggested that *S. mutans* has an unequal distribution on smooth surfaces (Ikeda et al. 1973). An overall decrease in its prevalence from molar to anterior teeth has also been demonstrated (Keene et al. 1981, Kristofferson et al. 1984, Lindquist and Emilson, 1990). The results of the present study show that proportions can vary even at neighbouring sites in the gingival margin plaque on the same surface. Ultrastructural studies have demonstrated the presence of microcolonies of similar cocci juxtaposed in the same subcontact area plaque, also on children's premolars (Newman 1975). This indicates the necessity of obtaining small plaque samples from precise areas, to demonstrate possible ecological differences in plaque location, which could be related to its pathogenicity at that specific site.

Comparing the results obtained by IF with those of culture, IF proportional counts of *S. mutans* were lower than cultural, in contrast to the results of Emilson et al. (1974) who determined 7.3% by IF and 6.1% by culture for *S. mutans*. This could be due to the greater specificity of antisera and efficiency of techniques used in the present work. Also, there was no masking of antigen, and the existence of dead cells in the plaque samples could have increased the total IF count. The higher percentages with culture could be due

to the fact that the number of colony forming units represents only a fraction of the cell counts obtained by direct microscopic techniques. Apart from non-viable cells, differences may also be due to problems in isolation and growth (Colwell et al. 1985).

Organisms affected by environmental factors or distortion of their community may not grow. Some organisms are more sensitive to these factors than others. It is possible that the lower isolation frequency of *S. sobrinus* by culture compared with IF (Table 3.15) may at least be attributed partly to the higher inhibitory effect of TYC and TYCSB on *S. sobrinus* than on *S. mutans* (Table 3.15). This is in line with the results previously reported by Schaeken et al. (1986) and Wade et al. (1986) who compared the growth of *S. mutans* and *S. sobrinus* on different media. Also, de Soet and de Graaff (1990) compared the recovery of *S. sobrinus* from blood plates and from TYCSB by immunoblotting, and reported a higher proportion of *S. sobrinus* when blood plates were used.

It is clear that microbiological studies (by culture) of *S. sobrinus* on approximal surfaces have underestimated the frequency and levels of this organism. The present study confirms this having shown by two different approaches that there is a significant increase in MS isolation in gingival margin approximal surface plaque as one proceeds from a cleansable aspect towards the most stagnant and, therefore, most caries-prone site on the approximal surface, below the contact area.

The pattern observed in the present investigation for the distribution of MS is consistent with the clinically observed pattern of initiation of caries and gingivitis. In view of previous evidence and the present findings it seems reasonable to propose that the presence of MS together with underlying ecological factors that created the microbial community are responsible for the pathogenicity of plaque, particularly at the site below

the contact area, at which both caries and chronic gingivitis are initiated approximally.

Different strains of MS vary in their rate of acid production during glycolysis (Harper and Loesche 1983, de Soet et al. 1989). This variation among strains may occur also intraorally in humans and may be one of the reasons why correlations between counts of MS and caries lesions are often not seen in individual sites. Culture, serotyping and biotyping can only show limited variations concerning the physiology of these species. More powerful techniques may reveal further differences at the molecular level. Therefore, the next part of the study was directed, as an initial stage, at assessing the utility of ribotyping MS and the most prevalent streptococci, *S. mitis* I/II and *S. oralis*, in an attempt to develop a more discriminating system for microbial identification in plaque.

4.4 Analysis of 16S rRNA of streptococci

Methods that yield a precise strain identification are needed to track strains among closely related species, to study potential differences in pathogenicity and strains of species from geographically different locations. Phenotypic characteristics such as serotype and biotype have been used in the present study as useful markers. However, they provide limited information for species identification. This is a critical issue in studies of plaque ecology, especially when results from different laboratories are to be compared. Molecular genetic approaches that rely on DNA sequence differences provide more powerful methods for fingerprinting closely related species. Restriction endonuclease fragment patterns have been used for strain identification in studies related to the epidemiology and transmission of a number of species including *S. mutans* (Alaluusua et al. 1994). However, performing southern blot analysis of a whole genomic digestion is a long procedure. Alternatively, one may use restriction endonuclease enzymes on a specific region within a gene, that contains variable sequences among strains. This has proved to be a successful tool for

strain speciation and species identification (Woese 1987). PCR and restriction fragment length polymorphism of 16S rRNA has been successfully used to differentiate *S. uberis* from *S. prauberis* (Jayarao et al. 1991). Although variable regions of the 16S ribosomal genes have frequently been used as the target for DNA probes to identify microorganisms, in some situations there is very little sequence variation observed between the 16S rRNA of closely related species (Rogall et al. 1990, Barry et al. 1991).

In an attempt to determine the 16S rRNA restriction pattern of selected streptococci, the amplified 16S rDNA fragment of *S. mutans* 'c' (NCTC 10449) and *S. sobrinus* 'd'(NCTC 27351) were digested with eight restriction endonuclease enzymes (Table 2.7). The digestion with *Hae*III gave three fragments of different molecular weight for each species of *S. mutans* 'c' and *S. sobrinus* 'd', which made it possible to easily distinguish *S. mutans* 'c' from *S. sobrinus* 'd' (Table 3.18 and Fig. 3.8). To date, this was the easiest method to distinguish these two species by using only one restriction enzyme (*Hae*III). The present results on *S. mutans* 'c' and *S. sobrinus* 'd' confirm the previous reports of Coykendall (1974), and Coykendall and Lizotte (1978), which showed considerable lack of DNA-homology between certain serotypes.

The present examination of other serotypes ('a', 'e', 'f' and 'g') of MS showed no more RFLP profiles of 16S rDNA with the *Hae*III. Serotypes 'd' and 'g' had an identical pattern. Similarly serotypes 'a', 'e' and 'f' showed a pattern similar to that of serotype 'c'. This was in contrast with the findings of Schleifer et al. (1984), who showed that by ribosomal RNA homologies, *S. sobrinus* 'd' was more related to *S. cricetus* 'a' and *S. mutans* 'c' was more related to *S. rattus* 'b'. The reason for this discrepancy might be in the different methods used in the two studies. In the present study a minimum number of restriction sites were probed, and resulted in fragments of similar sizes for some of the serotypes (Fig. 3.6). However, restriction fragments of the same size can have sequence

variations that cannot be detected unless new restriction sites are generated. Therefore, it should be possible to increase the number of ribotypes and thereby the specificity of the results, by using several restriction enzymes to confirm the identity of the ribotypes (Griffen et al. 1992).

S. mitis I and II showed the same restriction profiles (Figs. 3.10 and 3.11). However, application of enzymes on amplified 16S rDNA of *S. oralis* (LVG1) showed variations with *Hae*III and *Hind*III (Table 3.18). Therefore, a combination of the two enzymes was used which resulted in fragments of different lengths. The differences between these patterns was enough to discriminate the species of *S. oralis* (LVG1) from *S. mitis* I/II (Table 3.19). The same enzymes were applied to other species of streptococci (*S. intermedius*, *S. crista*, *S. sanguis*, *S. salivarius* and *S. vestibularis*) and mainly they showed a pattern which was the same as that observed for *S. oralis* (LVG1). Hence, sequences of GGCC (*Hae*III) and AAGCTT (*Hind*III) are likely to be parts of the conserved region of 16S rDNA of streptococci. The variations in clinical samples of *S. mitis* I/II (Table 3.19) could be due to minor DNA rearrangements (Hall 1994), or to the fact that they may be atypical *S. mitis* I/II strains. Also, it should be noted that the 16S rDNA is highly conserved for phylum Gram positive (Tanner et al. 1994), and a number of species other than streptococci may have some homology similar to those described in the present study, therefore such studies require the use of several restriction enzymes.

The data obtained by analysis of 16S rDNA genes of streptococci have been limited due to: a) technical problems involved in obtaining reproducible amplification of genes by PCR.

b) lack of information about the sequences of 16S rDNA,

c) the rather large size of restriction enzymes (six base cutter) and the limited number of restriction enzymes used,

d) and perhaps by the limited ability of ethidium bromide to stain small fragments.

CHAPTER FIVE

CONCLUSIONS AND FUTURE WORK

5.1 Conclusions

In this study dental plaque has been sampled from small sites from different locations increasing in stagnation away from to below the contact area on human approximal tooth surfaces. Microbiological analysis of these samples showed that not every species that had been isolated was found at all of the sub-sites, and none of the species cultivated was unique to a given site. Local variations in the prevalence of particular species were evident at different sub-sites. Some species were found more often and in higher levels at a particular sub-site. The predominant *Actinomyces* spp. and streptococcal species at mostof the sub-sites were *A. naeslundii* and *S. mitis* I, respectively. Variations in the plaque microflora were demonstrated at the different sub-sites, both with respect to species prevalence at each site, and by variations in the proportion of species within each sub-site.

A trend was found for *A. odontolyticus* to be isolated more often at sub-site B, below the contact area, and *A. israelii* from sub-site S to the side of the contact area. *S. mutans* was also isolated by culture significantly more often at sub-site B, while both *S. mutans* and *S. sobrinus* were identified by IF significantly more often and in higher proportions from sub-site B. Similarly, *Veillonella* spp. were isolated significantly more often and in higher proportions at sub-site B when compared to sub-sites S and A. However, in contrast, *Neisseria* spp. were isolated significantly more often at sub-site A than sub-site B. This difference in site specificity for *Neisseria* and *Veillonella* spp. may reflect the more aerobic status of sub-site A compared to sub-site B.

There was also a trend for some other species and groups of bacteria (*A. naeslundii*, *A. odontolyticus*, obligate anaerobes, *S. gordonii* and *S. oralis*) to be isolated more frequently from the most stagnant site B, compared with the least stagnant site A. However, *S.mitis* I was isolated more frequently from site S, compared with sites A and B. IgA_1 protease-

producing species were found at each sub-site, but they formed a small proportion of the total *Streptococcus* spp.

Lactobacillus spp. were isolated rarely, and were usually found together with mutans streptococci. There was a positive relationship between the presence of lactobacilli and caries (white spot lesions only), but this was determined mainly only on the basis of immunofluorescence (IF), probably because a larger number of teeth were processed in this part of the study. A similar positive association between *S. mutans* 'c' and *S. sobrinus* 'd' was confirmed by IF but not by culture. While this study confirmed the association between mutans streptococci (MS) and caries, it showed that these species could also frequently be isolated from non-carious sites.

Comparison of data obtained by IF and by culture showed general agreement. Mean percentage counts obtained by IF were lower than those obtained by culture. The possibility of false positive detection by IF of *Lactobacillus* spp. and low or not detectable growth of *S. sobrinus* 'd' by culture was observed. Anti-*S. sobrinus* 'd' polyclonal antibody proved to be as efficient as a specific monoclonal antibody (OMVU10, de Soet et al. 1987) for detection of *S. sobrinus* 'd'. Both culture and IF methods showed that the prevalence of MS varied with location in relation to the contact area, and the detection levels for *Lactobacillus* spp. were identical for both methods.

The frequency of isolation of MS species at all sites indicated that not even *S. mutans* or *S. sobrinus* are specific pathogens in the classical microbiological sense. They may represent examples of oral opportunistic pathogens responding to change in the local oral environment. The data obtained from culture and IF indicate that in any antimicrobial approach to caries the aim should be, not the elimination of a given species but rather its control. On the basis of the results from this and previous work this disease is unlikely to be caused by a specific pathogen.

In any treatment of plaque-based disease it should be remembered that the plaque flora is also important as a host defence factor. It is essential to maintain the factors which control plaque ecology close to its status found during health. Also, in any administration of special diet or oral treatment the importance of micro-environments and stagnant sites should be considered. Low pH generated in plaque from fermentable carbohydrate can lead to the selection of acidogenic and aciduric bacteria. Such changes in pH may be modulated by other dietary components. Therefore, restoration of plaque composition to one compatible with an oral microbial ecology associated with health could help in the prevention of caries. The overall results indicate that approaches such as vaccination are inadvisable for prevention of plaque-based disease as we are dealing, not with a specific pathogen-based infection, but with a shift in the balance of the resident oral flora.

Problems were encountered in the identification of certain streptococci (e.g. distinguishing *S. mitis* I from *S. mitis* II). As an alternative approach, therefore, PCR and RFLP were used for the analysis of 16S ribosomal DNA genes of streptococci digested with the two enzymes (*Hea* III and *Hind* III). However little variation in sequence was found. The reproducibility of amplification of 16S rDNA genes was improved by using a standard amount of genomic DNA from these species for PCR. *S. mutans* could be distinguished from *S. sobrinus* using restriction enzyme *Hae*III on amplified 16S rDNA genes of these species. Combination of two restriction enzymes (*Hae*III and *Hind*III) yielded a better resolution between the bands.

The restriction fragments obtained (550 bp) from the analysis of 16S rDNA genes of *S. mutans* can be sequenced and used to develop a DNA probe or to make species specific primers for identification of these species. The results from PCR and RFLP of 16S rDNA genes of *S. mutans* and *S. mitis* (I, II) made it clear that if appropriate restriction enzymes are chosen, the similarities and differences among strains can be easily determined by visual examination.

5.2 Future work

Proceeding from this study, further research is suggested as follows. Microbiological data obtained in this study could become more meaningful in relation to the status of enamel underlying the site of plaque sampling by cutting these teeth through the sample sites and examining the resultant sections by polarised light microscopy for evidence of early caries lesions. As mutans streptococci were found in plaque over carious and non-carious enamel the aim would be to determine whether other species are involved in early colonisation or shift in proportion prior to an increase in the levels of mutans streptococci. A longitudinal study could follow up sequential changes in proportions of plaque flora at risk sites and sites which are less at risk, in order to establish whether, and at what stage shifts in proportions of species occur,with a view to earlier intervention to prevent caries.

Further research is indicated to identify those species that could not be speciated in the present study. This possibly could be achieved by using PCR, RFLP and other molecular techniques.

In relation to further possible developments involving the use of IF the possibility of simultaneously counting two species at a time should be considered. This could be achieved by raising primary antibodies in different species such as mouse and goat or rabbit; also, the secondary antibodies could be labelled by different fluorescent dyes.

More work on the analysis of 16S rDNA genes of different *Streptococcus* spp. is required as digestion of this region with the selected enzymes showed relatively little variation. For example, the PCR and RFLP of 16S rDNA genes of *S. mitis* (I, II) requires more work using *Hea* III and *Hind* III restriction enzymes. This would help to distinguish these strains from each other, and might also reveal relationships between the genetic structure and sites that these species are isolated from the mouth. Polymerase chain reaction and restriction length polymorphism analysis of ribosomal DNA genes could be used to

distinguish between streptococci. This would be facilitated by applying at least two of the following methods:

a) direct sequencing the 16S rDNA encoding gene,

b) digestion with several restriction enzymes to identify variable sequences,

c) using PCR for random amplification of polymorphic DNA (RAPD), and alternatively

d) using the spacer (intergenic) region between the 16S and 23S rDNA genes, which have been reported to possess variable sequences and can be used to obtain distinctive profiles for strains tested (Barry et al. 1991, Griffen et al. 1992).

REFERENCES

Addy M, Dummer PMH, Hunter ML (1990). The effect of tooth brushing frequency, tooth brushing hand, sex and social class on longitudinal cohort study. Community Dent Health. 7: 237-247.

Alaluusua S, Alaluusua SJ, Karjalainen J, Saarela M, Holttinen T, Kallio M (1994). The demonstration by ribotyping of the stability of oral *Streptococcus mutans* infection over 5 to 7 years in children. Arch Oral Biol. 39: 467-471.

Alkan M, Ofek I, Beachey EH (1977). Adherence of pharyngeal and skin strains of group A streptococci to human skin and oral epithelial cells. Infect Immun. 18: 555-557.

Altman DG (1991). Practical Statistics for Medical Research. Chapman and Hill, London, p 211.

Andrewes FW, Horder TJ (1906). A study of the streptococci pathogenic for man. Lancet, part ii: 708-713, 775-782, 852-855.

Armitage P, Berry G (1987). Statistical Methods in Medical Research, 2nd. ed. Oxford, Blackwell, pp 123-125.

Barry T, Colleran G, Glennon M, Dunican LK, Gannon F (1991). The 16S /23S ribosomal spacer region as a target for DNA probes to identify eubacteria. PCR Methods and Applications. 1: 51-56.

Beck JD, Kohout F, Hunt RJ (1988). Identification of high caries risk adults: attitudes, social factors and diseases. Int Dent J. 38: 231-238.

Beighton D, Russell RR, Hayday H (1981). The isolation and characterization of *Streptococcus mutans* serotype h from dental plaque of monkeys (*Macaca fascicularis*). J Gen Microbiol. 124: 271-279.

Beighton D, Hayday H, Russell RRB, Whiley RA (1984). *Streptococcus macacae* sp. nov. from dental plaque of monkeys (*Macaca fascicularis*). Int J Syst Bacteriol. 34: 332-335.

Beighton D, Manji F, Baelum V, Fejerskov O, Johnson NW, Wilton JM (1989). Associations between salivary levels of *Streptococcus mutans*, *Streptococcus sobrinus*, lactobacilli, and caries experience in Kenyan adolescents. J Dent Res. 68: 1242-1246.

Beighton D (1991). The value of salivary bacterial counts in the prediction of caries activity; In Johnson NW. (ed): Risk Markers for Oral Diseases, Dental Caries, Vol 1, Dental Caries: Markers of High and Low Risk Groups. Cambridge University Press. Cambridge, pp 313 -326.

Beighton D, Hardie JM, Whiley RA (1991a). A scheme for the identification of viridans streptococci. J Med Microbiol. 35: 367-372.

Beighton D, Russell RR, Whiley RA. (1991b) A simple biochemical scheme for the differentiation of *Streptococcus mutans* and *Streptococcus sobrinus*. Caries Res. 25: 174-178.

Beighton D, Lynch E, Heath MR (1993). A microbiological study of primary root-caries lesions with different treatment needs. J Dent Res. 72: 623-629.

Bowden GH, Hardie JM, Slack GL (1975). Microbial variations in approximal dental plaque. Caries Res. 9: 253-277.

Bowden GH, Hardie JM, McKee AS, Marsh PD, Fillery ED, Slack GL (1976). The microflora associated with developing carious lesions of the distal surfaces on the upper first premolars in 13-14 year old children. In: Stiles HM, Loesche WJ, O'Brien TJ, (eds.), Microbial Aspects of Dental Caries. Information Retrieval Inc, Washington DC and London, pp 223-241.

Bowden GH (1990). Microbiology of root surface caries in humans. J Dent Res. 69: 1205-1210.

Bowden GH, Ekstrand J, Challacombe SJ (1990). The association of selectedbacteria with the lesions of root surface caries. Oral Microbiol Immunol 5: 346-351.

Bowden GH, Hardie JM (1992). Anaerobic organisms from the human mouth. In: Shapton DA, Board GR (eds.). Isolation of Anaerobes, Academic Press, London, pp 177-199.

Boyar RM, Bowden GH (1985). The microflora associated with the progression of incipient carious lesions of children living in a water-fluoridated area. Caries Res. 19: 298-306.

Boyar RM, Thylstrup A, Holmen L, Bowden GH (1989). The microflora associated with the development of initial enamel decalcification below orthodontic bands *in vivo* in children living in a fluoridated-water area. J Dent Res. 68: 1734-1738.

Bradshaw DJ, McKee AS, Marsh PD (1989). Effects of carbohydrate pulses and pH on population shifts within oral microbial communities *in vitro*. J Dent Res. 68: 1298-1302.

Bradshaw DJ, McKee AS, Marsh PD (1990). Prevention of population shifts in oral microbial communities *in vitro* by low fluoride concentrations. J Dent Res. 69: 436-441.

Bratthall D (1970). Demonstration of five serological groups of streptococcal strains resembling *Streptococcus mutans*. Odont Revy. 21: 143-152.

Bratthall D (1972). Immunofluorescent identification of *Streptococcus mutans*. Odont Revy. 23: 181-196.

Bratthall D (1991). The global epidemiology of mutans streptococci. In Johnson NW. (ed): Risk Markers for Oral Diseases, Vol 1, Dental Caries: Markers of High and Low Risk

Groups. Cambridge University Press, Cambridge, pp 287-312.

Bridge PD, Sneath PH (1982). *Streptococcus gallinarum* sp. nov. and *Streptococcus oralis* sp. nov. Int J Syst Bacteriol. 32: 410-415.

Bridge PD, Sneath PH (1983). Numerical taxonomy of *Streptococcus*. J Gen Microbiol. 129: 565-597.

Burt BA, Loesche WJ, Eklund SA (1985). Stability of selected plaque species and their relationship to caries in a child population over 2 years. Caries Res. 19: 193-200.

Bush MS, Challacombe SJ, Newman HN (1990). A method for the identification of *Streptococcus mutans* in gingival margin plaque by immunofluorescence. Caries Res. 24: 23-29.

Bush MS, Challacombe SJ, Newman HN (1989). A quantitative immunofluorescence study of the association between *Streptococcus mutans* and approximal caries. Microb Ecol Health Dis. 2: 261-266.

Caldwell J, Challacombe SJ, Lehner T (1977). A sequential bacteriological and serological investigation of *Rhesus* monkeys immunised against dental caries with *Streptococcus mutans*. J Med Microbiol. 10: 213-224.

Carlsson P, Olsson B, Bratthall D (1985). The relationship between the bacterium *Streptococcus mutans* in the saliva and dental caries in children in Mozambique. Arch Oral Biol. 30: 265-268.

Carlsson J (1967). Presence of various types of non-haemolytic streptococci in dental plaque and in other sites of the oral cavity in man. Odont Revy. 18: 55-74.

Carlsson J (1968). A numerical taxonomic study of human oral streptococci. Odont Revy. 19: 137-160.

Carlsson J, Grahnen H, Jonsson G, Wikner S (1970). Establishment of *Streptococcus sanguis* in the mouths of infants. Arch Oral Biol. 15: 1143-1148.

Carlsson J, Griffith CJ (1974). Fermentation products and bacterial yields in glucose-limited and nitrogen-limited cultures of streptococci. Arch Oral Biol. 19: 1105-1109.

Carlsson J, Grahnen H, Jonsson G (1975). Lactobacilli and streptococci in the mouth of children. Caries Res. 9: 333-339.

Carlsson J, Kujala U, Edlund MB (1985). Pyruvate dehydrogenase activity in *Streptococcus mutans*. Infect Immun. 49: 674-678.

Carlsson P, Gandour IA, Olsson B, Rickardsson B, Abbas K (1987). High prevalence of mutans streptococci in a population with extremely low prevalence of dental caries. Oral

Microbiol Immunol 2: 121-124.

Carlsson J (1987). Salivary peroxidase: an important part of our defense against oxygen toxicity. J Oral Pathol. 16: 412-416.

Caufield WP, Walker TM (1989). Genetic diversity within *Streptococcus mutans* evident from chromosomal DNA restriction fragment polymorphisms. J Clin Microbiol. 27: 274-278.

Cole MF, Hsu SD, Baum BJ, Bowen WH, Sierra LI, Aquirre M, Gillespie G (1981). Specific and nonspecific immune factors in dental plaque fluid and saliva from young and old populations. Infect Immun. 31: 998-1002.

Challacombe SJ (1974). Serum complement-fixing antibodies in human dental caries. Caries Res. 8: 84-95.

Challacombe SJ, Russell MW, Hawkes J (1978). Passage of intact IgG from plasma to the oral cavity via crevicular fluid. Clin Exp Immunol 34: 417-422.

Charlton G, Fitzgerald RJ, Keyes PH (1971). Determination of saliva and dental plaque pH in hamsters with glass micro-electrodes. Arch Oral Biol. 16: 649-654.

Childs WC, Gibbons RJ (1990). Selective modulation of bacterial attachment to oral epithelial cells enzyme activities associated with poor oral hygiene. J Periodont Res. 25: 172-178.

Cimasoni G (1983). Crevicular Fluid Updated. Monographs in Oral Science. 12: 1-152.

Cisar HO, Kolenbrander PE, McIntire FC (1979). Specificity of coaggregation reaction between human oral streptococci and strains of *Actinonomyces viscosus* or *Actinomyces naeslundii*. Infect Immun 24: 742-752.

Cisar JO, Brennan MJ, Sandberg AL (1985). Lectin-specific interaction of *Actinomyces* fimbriae with oral streptococci. In: Mergenhagen SE, Rosan B (eds.). Molecular Basis of Oral Microbial Adhesion. American Society for Microbiology. Washington DC, pp 159-163.

Claesson R, Crossner CG (1985). Presence of *Lactobacillus casei* in saliva from children and adults using a new medium. Scand J Dent Res. 93: 17-22.

Clark JK (1924). On the bacterial factor in the aetiology of dental caries. Brit. J. Exp. Path. 5: 141-147.

Clark WB, Bammann LL, Gibbons J (1978). Comparative estimates of bacterial affinities and adsorption sites on hydroxyapatite surfaces. Infect Immun. 19: 846-853.

Colman G, Williams RE (1972). Taxonomy of some human viridans streptococci. In: Wannamaker LW, Satsen JM (eds.). Streptococci and Streptococcal Disease, Academic

Press, Inc, New York, pp 281-299.

Colwell RR, Brayton PR, Grimes DJ, Roszak DR, Huq SA, Palmer LM (1985). Viable but non-cultivable *Vibrio cholerae* and related pathogens in the environment : implication for release of genetically engineered microorganisms. Biotechnol. 3: 817-820.

Cowman RA, Perella MM, Fitzgerald RJ (1974). Influence of incubation atmosphere on growth and amino acid requirements of *Streptococcus mutans*. Appl Microbiol. 27: 86-92.

Cowman RA, Fitzgerald RJ (1975). The effect of oral streptococci on electrophoretic properties of human salivary anionic proteins. J Dent Res. 55: 298-303.

Cowan ST (1974). In: Cowan's and Steel's Manual for the Identification of Medical Bacteria. 2nd ed. Cambridge University Press, Cambridge, pp 94-101.

Coykendall AL (1974). Four types of *Streptococcus mutans* based on their genetic, antigenic and biochemical characteristics. J Gen Microbiol. 83: 327-338.

Coykendall AL, Specht PA (1975). DNA base sequence homologies among strains of *Streptococcus sanguis*. J Gen Microbiol. 91: 92-98.

Coykendall AL, Bratthall D, O'Connor K, Dvarskas RA (1976). Serological and genetic examination of some nontypical *Streptococcus mutans* strains. Infect Immun. 14: 667-670.

Coykendall AL (1977). Proposal to elevate the subspecies of *Streptococcus mutans* to species status based on their molecular composition. Int J Syst Bacteriol. 27: 26-30.

Coykendall AL, Lizotte PA (1978). *Streptococcus mutans* isolates identified by biochemical tests and DNA base contents. Arch Oral Biol. 23: 427-428.

Coykendall AL (1983). *Streptococcus sobrinus* nom. rev. and *Streptococcus ferus* nom. rev: habitat of these and other mutans streptococci. Int J Syst Bacteriol. 33: 883-885.

Coykendall AL, Gustafson KB (1986). Taxonomy of *Streptococcus mutans*. In: Hamada S, Michalek SM, Kinyino H, Menaker L, McGhee JL, (eds.). Molecular Microbiology and Immunology of *Streptococcus mutans*. Amsterdam, Elsevier, pp 21-28.

Coykendall AL, Wesbecher PM, Gustafson KB (1987). " *Streptococcus milleri*" *Streptococcus constellatus*, and *Streptococcus itermedius* are later synonyms of *Streptococcus anginosus*. Int J Syst Bacteriol. 37: 222-228.

Crossner CG, Claesson R, Johansson T (1989). Presence of mutans streptococci and various types of lactobacilli in interdental spaces related to development of proximal carious lesions. Scand J Dent Res. 97: 307-315.

Dajani AS, Law DJ, Bollinger RO, Ecklund PS (1976a). Ultrastructural and biochemical alterations effected by viridin B, a bacteriocin of alpha-hemolytic streptococci. Infect

Immun. 14: 776-782.

Dajani AS, Tom MC, Law DJ (1976b). Viridans bacteriocins of alpha haemolytic streptococci: isolation, characterization, and partial purification. Antimicrob Agents Chemother. 9: 81-88.

Demuth DR, Lammey MS, Huck M, Lally ET, Malamud D (1990). Comparison of *Streptococcus mutans* and *Streptococcus sanguis* receptors for human salivary agglutinin. Microb Pathogen. 9: 199-211.

de Jong MH, van der Hoeven JS, van Os JH, Olijve JH (1984). Growth of oral *Streptococcus* species and *Actinomyces viscosus* in humans. Appl Environ Microbiol. 47: 901-904.

de Ley J (1962). Comparative biochemistry and enzymology in bacterial classification. In: de Ley J, Microbial Classification 12th. Symp Soc Gen Microbiol. Cambridge Press, Cambridge, pp 164-195.

de Soet JJ, van Dalen PJ, Appelmelk BJ, de Graaff J (1987). Identification of *Streptococcus sobrinus* with monoclonal antibodies. J Clin Microbiol. 25: 2285-2288.

de Soet JJ, van Dalen PJ, de Graaff J (1989). Reliable identification of mutans streptococci with monoclonal antibodies. Caries Res. 23: 110-111.

de Soet JJ, de Graaff J (1990). Monoclonal antibodies for enumeration and identification of mutans streptococci in epidemiological studies. Arch Oral Biol. 35: 165S-169S.

de Soet JJ, Holbrook WP, van Amerongen WE, Schipper E, Homburg CHE, de Graaff J (1990). Prevalence of *Streptococcus sobrinus* in relation to dental caries in children from Iceland and the Netherlands. J Dent Child. 57: 337-342.

de Soet JJ, Holbrook WP, Magnusdottir MO, de Graaff J (1993). *Streptococcus sobrinus* and *Streptococcus mutans* in a longitudinal study of dental caries. Microbial Ecol Health Dis. 6: 237-243.

de Stoppelaar JD, van Houte J, Moore CE (1967). The presence of dextran-forming bacteria, resembling *Streptococcus bovis* and *Streptococcus sanguis*, in human dental plaque. Arch Oral Biol. 12: 1199-1201.

de Stoppelaar JD, van Houte J, Dirks OB (1970). The effect of carbohydrate restriction on the presence of *Streptococcus mutans, Streptococcus sanguis* and iodophilic polysaccharide-producing bacteria in human dental plaque. Caries Res. 4: 114-123.

DiRienzo JM, Cornell S, Kazoroski L, Slots J (1990). Probe-specific DNA fingerprinting applied to the epidemiology of localized juvenile periodontitis. Oral Microbiol Immunol. 5: 49-56.

Donoghue HD (1974). Composition of dental plaque obtained from eight sites in the mouth of a ten-year-old girl. J Dent Res. 53: 1289-1293.

Donoghue HD, Newman HN (1976). Effect of glucose and sucrose on survival in batch culture of *Streptococcus mutans* C67-1 and a noncariogenic mutant, C67-25. Infect Immun. 13: 16-21.

Doyle RJ, Nesbitt WE, Taylor KG (1982). On the mechanism of adherence of *Streptococcus sanguis* to hydroxylapatite. FEMS Microbiol Lett. 15: 1-5.

Drucker DB, Green RM (1981). The relative cariogenicity of different streptococci in the gnotobiotic WAG/RIJ rat. Arch Oral Biol. 26: 599-606.

Drucker DB, Shakespeare AP, Green RM (1984a). The production of dental plaque and caries by the bacterium *Streptoccocus salivarius* in gnotobiotic WAG/RIJ rats. Arch Oral Biol. 29: 437-443.

Drucker DB, Shakespeare AP, Green RM (1984b). *In vivo* dental caries plaque-forming ability and relative cariogenicity of the bacteria *Streptococcus mitis* and *Streptococcus sanguis* I and II in mono-infected gnotobiotic rats. Arch Oral Biol. 29: 1023-1031.

Duchin S, van Houte J (1978). Relationship of *Streptococcus mutans* and lactobacilli to incipient smooth surface dental caries in man. Arch Oral Biol. 23: 779-786.

Eden PA, Schmidt TM, Blakemore RP, Pace NR (1991). Phylogenetic analysis of *Aquaspirillum magnetotacticum* using polymerase chain reaction-amplified 16 S rRNA-specific DNA. Int J Syst Bacteriol. 41: 324-325.

Edman DC, Keene HJ, Shklair IL, Hoerman KC (1975). Dental floss implantation and sampling of *Streptococcus mutans* from approximal surfaces of human teeth. Arch Oral Biol. 20: 145-148.

Eggert FM, Drewell L, Bigelow JA, Speck JE, Goldner M (1991). The pH of gingival crevices and periodontal pockets in children, teenagers and adults. Arch Oral Biol. 36: 233-238.

Ella MG, Eveland WC, Loesche WJ (1973). Identification of *Streptococcus mutans* serotypes in dental plaque by fluorescent antibody techniques. Arch Oral Biol. 18: 707-715.

Ellen RP (1976). Microbiological assays for dental caries and periodontal disease susceptibility. Oral Sci Rev. 8: 3-23.

Ellen RP, Banting DW, Fillery ED (1985). Longitudinal microbiological investigation of a hospitalized population of older adults with a high root surface caries risk. J Dent Res. 64: 1377-1381.

Emilson CG, Kohler B, Bratthall D (1974). Immunofluorescence determination of the relative proportions of *Streptococcus mutans* in human dental plaque: a comparison with cultural techniques. Arch Oral Biol. 20: 81-86.

Emilson CG (1983). Prevalence of *Streptococcus mutans* with different colonial morphologies in human plaque and saliva. Scand J Dent Res. 91: 26-32.

Ericson T, Magnusson J (1976). Affinity for hydroxyapatite of salivary substances inducing aggregation of oral streptococci. Caries Res. 10: 8-18.

Facklam RR (1977). Physiological differentiation of viridans streptococci. J Clin Microbiol. 5: 184-201.

Faloona F, Weiss S, Ferre F, Mullis K (1990). Paper presented at the Sixth International Conference on AIDS, 20 to 24 June 1990, San Francisco, CA, 1990. Cited in: Erlich HA, Gelfand D, Sninsky JJ (1991). Recent advances in the polymerase chain reaction. Science. 252: 1643-1651.

Firestone AR (1982). Effect of increasing contact time of sucrose solution or powdered sucrose on plaque pH *in vivo*. J Dent Res. 61: 1243-1244.

Fitzgerald RJ, Keyes PH (1960). Demonstration of the etiologic role of streptococci in experimental caries in the hamster. J Am Dent Assoc 61: 9-19.

Fitzgerald RJ (1968). Dental caries research in gnotobiotic animals. Caries Res. 2: 139-146.

Frandsen EVG, Pedrazzoli V, Kilian M (1991). Ecology of viridans streptococci in the oral cavity and pharynx. Oral Microbiol Immunol. 6: 129-133.

Freedman ML, Coykendall AL (1975). Variation in internal polysaccharide synthesis among *Streptococcus mutans* strains. Infect Immun. 12: 475-479.

Freedman M, Tanzer J, Swayne E, Allenspach-Petrzilka G (1983). Colonization and virulence of *Streptococcus sobrinus:* the roles of glucan-associated phenomena reveald by the use of mutans In: Doyle RJ, Ciardi JE (eds.). Glucosyltransferases Glucan Sucrose and Dental Caries. Information retrieval Ltd. Washington DC and London, pp 39-50.

Fukui K, Fukui Y, Moriyama T (1974). Purification and properties of dextransucrase and invertase from *Streptococcus mutans*. J Bacteriol. 118: 796-804.

Gerencser MA, Bowden GH (1986). Genus *Rothia* Georg and Brown 1967. In: Sneath PHA, Sharpe ME, Holt JG (eds.). Bergey's Manual of Systematic Bacteriology Vol. 2. Williams and Wilkins, Baltimore, pp 1342-1346.

Gibbons RJ (1972). Presence of an invertase-like enzyme and a sucrose permeation system in strains of *Streptococcus mutans*. Caries Res. 6: 122-131.

Gibbons RJ, van Houte J (1973). On the formation of dental plaques. J Periodontol. 44: 347-360.

Gibbons RJ, Qureshi JV (1978). Selective binding of blood group-reactive salivary mucins by *Streptococcus mutans* and other oral organisms. Infect Immun. 22: 665-671.

Gibbons RJ (1980). Adhesion of bacteria to surfaces of the mouth. In: Berkeley RCW, Lynch JM, Melling J, Rutter PR, Vincent B (eds.). Microbial Adhesion to Surfaces. Ellis Horwood Ltd, Chichester, England, pp 351-388.

Gibbons RJ, van Houte J (1980). Bacterial adherence and the formation of dental plaque. In: Beachey EH (eds.). Bacterial Adherence. London Chapman and Hall. pp 61-104.

Gibbons RJ, Etherden I (1983). Comparative hydrophobicities of oral bacteria and their adherence to salivary pellicles. Infect Immun. 41: 1190-1196.

Gibbons RJ, Cohen L, Hay DI (1986). Strains of *Streptococcus mutans* and *Streptococcus sobrinus* attach to different pellicle receptors. Infect Immun. 52 : 555-561.

Gibbons RJ (1989). Bacterial adhesion to oral tissues: a model for infectious diseases. J Dent Res. 68: 750-760.

Gibbons RJ, Hay DI (1989). Adsorbed salivary acidic proline-rich proteins contribute to the adhesion of *Streptococcus mutans* JBP to apatitic surfaces. J Dent Res. 68: 1303-1307.

Gibbons RJ, Hay DI, Child WC, Davis G (1990). Role of cryptic receptors (crytitopes) in bacterial adhesion to oral surfaces. Arch Oral Biol. 135: 107S-114S.

Gibbons RJ, Hay DI, Schlesinger DH (1991). Delineation of a segment of adsorbed salivary acidic proline-rich proteins which promotes adhesion of *Streptococcus gordonii* to apatitic surfaces. Infect Immun. 59: 2948-2954.

Gill S, Newman HN, Challacombe SJ, Bulman J (1991). An immunofluorescence study of the distribution of *Streptococcus mutans* on children's teeth. Microb Ecol Health Dis 4: 253-257.

Gilmour MN, Whittam TS, Kilian M, Selander RK (1987). Genetic relationships among the oral streptococci. J Bacteriol. 169: 5247-5257.

Gjermo P (1989). Chlorhexidine and related compounds. J Dent Res. 68: 1602-1608.

George LK, Pine L, Gerencser MA (1969). *Actinomyces viscosus*, comb. nov., a catalase-positive facultative member of the genus *Actinomyces*. Int J Syst Bacteriol. 19: 291-293.

Grenier EM, Eveland WC, Loesche WJ (1973). Identification of *Streptococcus mutans* serotypes in dental plaque by fluorescent antibody techniques. Arch Oral Biol. 18: 707-715.

Griffen AL, Leys EJ, Fuerst PA (1992). Strain identification of *Actinobacillus actinomycetemcomitans* using the polymerase chain reaction. Oral Microbiol Immunol. 7: 240-243.

Guggenheim B, Konig KG, Muhlemann HR (1965). Modifications of the oral bacterial flora and their influence on dental caries in the rat. 1. The effect of inoculating 4 labelled strains of streptococci. Helv Odont Acta. 9: 121-129.

Hall LMC (1994). Are point mutations or DNA rearrangements responsible for the restriction fragment length polymorphisms that are used to type bacteria. Microbiol 140: 197-204.

Hamada S, Slade HD (1980). Biology, immunology and cariogenicity of *Streptococcus mutans*. Microbiol Rev. 44: 331-384.

Hamilton IR, Bowden GH (1982). Response of freshly isolated strains of *Streptococcus mutans* and *Streptococcus mitior* to change in pH in the presence and absence of fluoride during growth in continuous culture. Infect Immun. 36: 255-262.

Hamilton IR, Bowden GH (1988). Effect of fluoride on oral microorganisms. In: Ekstrand J, Fejerskov O, Silverstone ML (eds.). Fluoride in Dentistry. Munksgaard, Copenhagen, pp 77-103.

Hamilton IR, Gauthier L, Desjardins B, Vadeboncoeur C (1989). Concentration- dependent repression of the soluble and membrane components of the *Streptococcus mutans* phosphoenolpyruvate: sugar phosphotransferase system by glucose. J Bacteriol. 171: 2942-2948.

Hamilton IR, Buckley ND (1991). Adaptation by *Streptococcus mutans* to acid tolerance. Oral Microbiol Immunol. 6: 65-71.

Handley PS, Carter PL, Wyatt JE, Hesketh LM (1985). Surface structures (peritrichous fibrils and tufts of fibrils) found on *Streptococcus sanguis* strains may be related to their ability to coaggregate with other oral genera. Infect Immun. 47: 217-227.

Handley PS (1990). Structure, composition and functions of surface structures on oral bacteria. Biofouling. 2: 239-264.

Hardie JM, Bowden GH (1974). Cell wall and serological studies on *Streptococcus mutans*. Caries Res. 8: 301-316.

Hardie JM, Bowden GH (1976). Physiological classification of oral viridans streptococci. J Dent Res. 55: A166-176.

Hardie JM, Thomson PL, South RJ, Marsh PD, Bowden GH, McKee AS, Fillery ED, Slack GL (1977). A longitudinal epidemiology study on dental plaque and development of dental caries-interim results after two years. J Dent Res. 55: C90-C98.

Hardie JM, Whiley RA (1992). The genus *Streptococcus*-Oral in: Balows A. Truper HG. Dworkin M, Harder W, Schleifer KH (eds.). The Prokaryotes 2nd ed. Springer-Verlag, pp 1421-1449.

Harper DS, Loesche WJ (1983). Effect of pH upon sucrose and glucose catabolism by the various genogroups of *Streptococcus mutans*. J Dent Res. 62: 526-531.

Harper DS, Loesche WJ (1984). Growth and acid tolerance of human dental plaque bacteria. Arch Oral Biol 29: 843-848.

Hayes ML, Carter EC, Griffiths SJ (1983). The acidogenic microbial composition of dental plaque from caries-free and caries-prone people. Arch Oral Biol. 28: 381-386.

Hehre EJ. Neill JM (1946). Formation of serologically reactive dextran by streptococci from subacute bacterial endocarditis. J Exp Med. 83: 147-161.

Holdeman LV, Moore WEC (1974). New genus, *Coprococcus*, twelve new species and emended descriptions of four previously described species of bacteria from human faeces. Int J Syst Bacteriol. 24: 260-277.

Homer KA, Beighton D (1991). N-acetylglucosamine metabolism by mutans streptococci. J Dent Res. 70: 672-672.

Horaud T, Delbos F (1984). Viridans streptococci in infective endocarditis: species distribution and susceptibility to antibiotics. Eur Heart J. (Suppl C): 39-44.

Hsu SD, Cisar Jo, Sandberg AL, Kilian M (1994). Adhesive properties of viridans streptococcal species. Microb Ecol Health Dis. 7: 125-137.

Huis in't Veld JH, van Palenstein Helderman WH, Dirks OB (1979). *Streptococcus mutans* and dental caries in humans: a bacteriological and immunological study. Antonie van Leeuwenhoek. 45: 25-33.

Huxley HG (1973). The effect of inoculating strains of *Sreptococcus mutans* and *Streptococcu sanguis* upon caries incidence and bacterial content of plaque in rats. Arch Oral Biol. 18: 1215-1226.

Igarashi K, Lee IK, Schachtele CF (1990). Effect of dental plaque age and bacterial composition on the pH of artificial fissures in human volunteers. Caries Res. 24: 52-58.

Ikeda T. Sandham HJ (1971). Prevalence of *Streptococcus mutans* on various tooth surfaces in Negro children. Arch Oral Biol. 16: 1237-1240.

Ikeda T, Sandham HJ, Bradley EL Jr (1973). Changes in *Streptococcus mutans* and lactobacilli in plaque in relation to the initiation of dental caries in Negro children. Arch Oral Biol. 18: 555-566.

Ikeda T, Kurita T, and Hirasawa M (1988). Suppression of *Streptococcus sobrinus* 6715 (g) in plaques by *Streptococcus mutans* 32k (c). J Oral Pathol. 17: 471-474.

Inoue M, Shibata H, Morioka T (1982). Serotype specific for some biochemical characteristics of *S. mutans*. Microbios 33: 7-14.

Jalil RA, Ashley FP, Wilson RF, Wagaiyu EG (1993). Concentrations of thiocyanate, hypothiocyanite, 'free' and 'total' lysozyme, lactoferrin and secretory IgA in resting and stimulated whole saliva of children aged 12-14 years and the relationship with plaque accumulation and gingivitis. J Periodont Res. 28: 130-136.

Jayarao BM, Dore JJE, JR, Baumbach GA, Matthews KR, Oliver SP (1991). Differentiation of *Streptococcus uberis* from *Streptococcus parauberis* by polymerase chain reaction and restriction fragment length polymorphism analysis of 16S ribosomal DNA. J Clin Microbiol. 29: 2774-2778.

Jenkins GN (1984). The recent fall in dental caries incidence. Ontrio Dentist. 61: 29-32.

Jones D (1978). Composition and differentiation of the genus *Streptococcus*. In: Skinner FA, and Quesnel LB (eds.). Streptococci. Academic Press Inc, London, pp 1-49.

Johnson JL, Moore LVH, Kaneko B, Moore WEC (1990). *Actinomyces georgiae* sp. nov., *Actinomyces gerencseriae* sp. nov., designation of two genospecies of *Actinomyces naeslundii*, and inclusion of *A. naeslundii* serotypes II in *naeslundii* genospecies 2. Int J Syst Bacteriol. 40: 273-286.

Johnson NW (1991). The nature of the caries process and the need for markers of risk. In: Johnson NW (ed) Risk Markers for Oral Diseases, Dental caries, Vol 1, Dental Caries: Markers of High and Low Risk Groups. Cambridge University Press, Cambridge, pp 1-12

Jordan HV, Hammond BF (1972). Filamentous bacteria isolated from human root surface caries. Arch Oral Biol. 17: 1333-1342.

Jordan HV (1976). Cariogenic flora: establishment, localization, and transmission. J Dent Res. 55 Spec: 10-14.

Jordan HV (1982). Pathogenicity of *Actinomyces* species. In: Genco RJ, Mergenhagen SE (ed.). Host Parasite Interactions in Periodontal Disease. Amer Soc Microbiol. Washington DC, pp 169-178.

Keene HJ, Shklair IL (1974). Relationship of *Streptococcus mutans* carrier status to the development of carious lesions in initially caries-free recruits. J Dent Res. 53: 1295.

Keene HJ, Irving L, Shklair IL, Mickel GJ, and Wirthlin MR. (1977) Distribution of *Streptococcus mutans* biotypes in five human populations. J Dent Res. 56: 5-10.

Keene HJ, Horton IM, Handler SF (1981). *Streptococcus mutans* approximal plaque index

as a new epidemiologic tool for defining the parameters of *Streptococcus mutans* infection in human populations. Arch Oral Biol. 26: 345-355.

Kenney EB, Ash MM (1969). Oxidation reduction potential of developing plaque, periodontal pockets and gingival sulci. J Periodontol. 40: 630-633.

Keyes PH, Fitzgerald RJ (1962). Dental caries in the Syrian hamster-IX. Arch Oral Biol. 7: 267-278.

Keyes PH (1960). The infectious and transmissible nature of experimental dental caries. Findings and implications. Arch Oral Biol. 1: 304-320.

Kidd EAM, Joyston-Bechal S (1987). Essentials of Dental Caries: The Disease and its Management. Dental Practitioners Handbook, Wright Bristol, pp 8-15.

Kilian M, Schiott CR (1975). Haemophili and related bacteria in the human oral cavity. Arch Oral Biol. 20: 791-796.

Kilian M, Thylstrup A, Fejerskov O (1979). Predominant plaque flora of Tanzanian children exposed to high and low water fluoride concentrations. Caries Res. 13: 330-43.

Kilian M, Holmgren K (1981). Ecology and nature of immunoglobulin A1 protease producing streptococci in the human oral cavity and pharynx. Infect Immun. 31: 868-873.

Kilian M, Nyvad B, Mikkelsen L (1986). Taxomonic and ecological aspects of some oral streptococci. In: Hamada S, Michalek SM, Kiyono H, Menaker L, McGhee RJ (eds.). Molecular Microbiology and Immunology of *Streptococcus mutans*. Elsevier Science, Amsterdam. 391-399.

Kilian M, Mikkelsen L, Henrichsen J (1989). Taxomonic study of viridans streptococci: Description of *Streptococcus gordonii* sp. nov. and emended description of *Streptococcus sanguis* (White and Niven 1946), *Streptococcus oralis* Bridge and Sneath 1982), and *Streptococcus mitis* (Andrewes and Horder 1906). Int J Syst Bacteriol. 39: 471-484.

Kilpper-Balz R, Wenzig P, Schleifer KH (1985). Molecular relationship and classification of some viridans streptococci as *Streptococcus oralis* and amended description of *Streptococcus oralis* (Bridge and Sneath 1982). Int J Syst Bacteriol. 35: 482-488.

Kleinberg I (1970). Regulation of the acid-base metabolism of the dento-gingival plaque and its relation to dental caries and periodontal disease. Int Dent J. 20: 451-471.

Kleinberg I, Craw D, Komiyama K (1973). Effect of saliva supernatant on the glycolytic activity of the bacteria in salivary sediment. Arch Oral Biol. 18: 787-812.

Kleinberg I, Kanapka JA, Craw D (1976). Effects of saliva and salivary factors on the metabolism of mixed oral flora. In: Stiles HM, Loesche WJ, O'Brien TC (eds.). Microbial Aspects of Dental Caries. Information Retrieval Inc, Washington DC and London, pp 433-464.

Kleinberg I (1977). Effects of fluoride on the metabolism of the mixed oral flora. Caries Res. 11 (Suppl.). 293-320.

Kleinberg I, Kanapka JA, Chatterjee R, Craw D, D'Angelo N, Sandham HJ (1979). Metabolism of nitrogen by the oral mixed bacteria. In: Kleinberg I, Ellison SA, Mandel ID (eds.). Saliva and Dental Caries. Information Retrieval, New York, pp 357-377.

Kleinberg I, Jenkins GN, Chatterjee R, Wijegeweera L (1982). The antimony pH electrode and its role in the assessment and interpretation of dental plaque pH. J Dent Res. 61: 1139-1982.

Klock B, Krasse B (1977). Microbial and salivary conditions in 9-12 year old children. Scand J Dent Res. 85: 56-63.

Klock B, Krasse B (1978). Effect of caries-preventive measures in children with high numbers of *S. mutans* and lactobacilli. Scand J Dent Res. 86: 221-230.

Koga T, Asakawa H, Okahashi N, Hamada S (1986). Sucrose-dependent cell adherence and cariogenicity of serotype 'c' *Streptococcus mutans*. J Gen Microbiol. 132: 2873-2883.

Kohler B, Pettersson BM, Bratthall D (1981). *Streptococcus mutans* in plaque and saliva and the development of caries. Scand J Dent Res. 89: 19-25.

Kolenbrander PE, Andersen RN, Moore LV (1989). Coaggregation of *Fusobacterium nucleatum*, *Selenomonas flueggei*, *Selenomonas infelix*, *Selenomonas noxia*, and *Selenomonas sputigena* with strains from 11 genera of oral bacteria. Infect Immun. 57: 3194-3203.

Kolenbrander PE (1991). In: Coaggregation: adherence in the human oral microbial ecosystem. In: Dworkin M (ed.). Microbial Cell-Cell Interaction. Ame Soc Microbiol. Washington DC, pp 303-329.

Koneman EW, Stephen DA, Dowell VR, William MJ, Herbert MS, Washington CW (1988). Test for determining inhibitory activity of antibiotics. In: Elmer WK, Stephen DA, Dowell VR, Wiliam MJ, Herbert MS, Washington CW (eds.). Diagnostic Microbiology 3rd ed. Lippincott Company, Philadelphia, Pennsylvania, pp 479-481.
Krasse B (1965). The effect of nutrition on saliva and oral flora. Sym Swed Nutr Found. 3: 21-29.

Krasse B, Edwardsson S (1966). The proportional distribution of caries-inducing streptococci in various parts of the oral cavity of hamsters. Arch Oral Biol. 11: 1137-1142.

Kristoffersson K, Axelsson P, Bratthall D (1984). Effect of a professional tooth cleaning programme on interdentally localized *Streptococcus mutans*. Caries Res. 18: 385-390.

Kulkarni GV, Chan KH, Sandham HJ (1989). An investigation into the use of restriction endonuclease analysis for the study of transmission of mutans streptococci. J Dent Res.

68: 1155-1161.

Lang NP, Hotz PR, Gusberti FA, Joss A (1987). Longitudinal clinical and microbiological study on the relationship between infection with *Streptococcus mutans* and the development of caries in humans. Oral Microbiol Immunol. 2: 39-47.

Leigh RW (1927). Studies of the enamel. J Am Dent Assoc. 14: 592-600.

Lenski RE (1993). Assessing the genetic structure of microbial populations. Proc Nat Acad Sci. USA. 90: 4334-4336.

Levine MJ, Herzberg MG, Levine MS, Ellison SA, Stinson MW, Li HC. van Dyke T (1978). Specificity of salivary-bacterial interactions, Role of terminal sialic acid residues in the intraction of salivary-bacterial interactions of salivary glycoprotein with *Streptococcus sanguis* and *Streptococcus mutans*. Infect Immun. 19: 107-115.

Levine RS (1977). The aetiology of dental caries-an outline of current thought. Int Dent J. 27: 341-348.

Li YH, Bowden GH (1994). The effect of environmental pH and fluoride from the substratum on the development of biofilms of selected oral bacteria. J Dent Res. 73: 1615-1626.

Ligtenberg AJ, Walgreen-Weterings E, Veerman EC, de Soet JJ, de Graaff J, Amerongen AV (1992). Influence of saliva on aggregation and adherence of *Streptococcus gordonii* HG 222. Infect Immun. 60: 3878-3884.

Liljemark WF, Gibbons RJ (1972). Proportional distribution and relative adherence of *Streptococcus miteor (mitis)* on various surfaces in the human oral cavity. Infect Immun. 8: 846-849.

Liljemark WF, Schauer SV (1977). Competitive binding among oral streptococci to hydroxyapatite. J Dent Res. 56: 157-165.

Liljemark WF, Fenner LJ, Bloomquist GC (1986). *In vivo* colonization of salivary pellicle by *Haemophilus, Actinomyces* and *Streptococcus* species. Caries Res. 20: 481-497.

Liljemark WF, Bloomquist CG, Fenner LJ, Antonelli PJ, and Coulter MC. (1989) Effect of neuraminidase on the adherence to salivary pellicle of *Streptococcus sanguis* and *Streptococcus mitis*. Caries Res. 23: 141-145.

Liljemark WF, Bloomquist CG, Bandt CL, Pihlstrom BL, Hinrichs JE, Wolff LF (1993). Comparison of the distribution of *Actinomyces* in dental plaque on inserted enamel and natural tooth surfaces in periodontal health and disease. Oral Microbiol Immunol. 8: 5-15.

Lindquist B, Emilson CG (1990). Distribution and prevalence of mutans streptococci in human dentition. J Dent Res. 69: 1160-1166.

Lindquist B, Emilson CG (1991a). Dental location of *Streptococcus mutans* and *Streptococcus sobrinus* in humans harbouring both species. Caries Res. 25: 146-152.

Lindquist B, Emilson CG. (1991b). Interactions between and within *Streptococcus mutans* and *Streptococcus sobrinus* isolated from humans harbouring both species. Scand J Dent Res. 99: 498-504.

Liu T, Gibbons RJ, Hay DI, Skobe Z (1991). Binding of *Actinomyces viscosus* to collagen: association with the type 1 fimbrial adhesin. Oral Microbiol Immunol. 6: 1-5.

Loesche WJ, Hockett RN, Syed SA (1972). The predominant cultivable flora of tooth surface plaque removed from institutionalized subjects. Arch Oral Biol. 17: 1311-1326.

Loesche WJ, Syed SA (1973). The predominant cultivable flora of carious plaque and carious dentine. Caries Res. 7: 201-216.

Loesche WJ, Rowan J, Straffon LH, Loos PJ (1975). Association of *Streptococcus mutants* with human dental decay. Infect Immun. 11: 1252-1260.

Loesche WJ, Syed SA (1978). Bacteriology of human experimental gingivitis: effect of plaque and gingivitis score. Infect Immun. 21: 830-839.

Loesche WJ, Syed SA, Laughon BE, Stoll J (1982). The bacteriology of acute necrotizing ulcerative gingivitis. J Periodontol. 53: 223-230.

Loesche WJ, Laughon B (1982). Role of spirochetes in periodontal disease. In: Genco RJ, Mergenhagen SE (eds.). Host Parasite Interactions in Periodontal Disease. Amer Soc Microbiol. Washington DC. pp 62-75.

Loesche WJ (1986a). Role of *Streptococcus mutans* in human dental decay. Microbiol Rev. 50: 353-380.

Loesche WJ (1986b). The identification of bacteria associated with periodontal disease and dental caries by enzymatic methods. Oral Microbiol Immunol. 1: 65-72.

Macpherson LMD, MacPherson TW, Stephen KW (1990). An intra-oral appliance study of plaque microflora associated with early enamel demineralization. J Dent Res. 69: 1712-1716.

Macpherson LMD, MacFarlane TW, Stephen KW (1991). An *in situ* microbiological study of the early colonisation of human enamel surfaces. Microb Ecol Health Dis. 4: 39-46.

Macpherson LMD, MacFarlane TW, Geddes DAM, Stephen KW (1992). Assessment of the cariogenic potential of *Streptococcus mutans* strain and its relationship to *in vivo* caries experience. Oral Microbiol Immunol. 7: 142-147.

Mandell ID (1985). Changing patterns of dental caries. Quintess Int J. 61: 27-33.

Mandell ID (1987). The function of Saliva. J Dent Res. 66: 623-627.

Marmur J (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J Mol Biol. 3: 208-218.

Marsh PD, Hunter JR, Bowden GH, Hamilton IR, McKee AS, Hardie JM, Ellwood DC (1983). The influence of growth rate and nutrient limitation on the microbial composition and biochemical properties of a mixed culture of oral bacteria grown in a chemostat. J Gen Microbiol. 129: 755-770.

Marsh PD (1986). Microbiology of dental plaque in health and disease. In: Ivanyi L (ed.). Immunological Aspects of Oral Diseases. MTP Press Ltd, London, pp 13-46.

Marsh PD, Keevil CW (1986). The metabolism of oral bacteria in health and disease. In: Hill MJ (ed.). Microbial Metabolism in the Digestive Tract. CRC Press, Inc. Florida, pp 155-181.

Marsh PD, Featherstone A, McKee AS, Hallsworth AS, Robinson C, Weatherell JA, Newman HN, Pitter AFV (1989a). A microbiological study of early caries of approximal surfaces in schoolchildren. J Dent Res. 68: 1151-1154.

Marsh PD, Bevis RA, Newman HN, Hallsworth AS, Robinson C, Weatherell JA, Pitter AF (1989b). Antibacterial activity of some plaque-disclosing agents and dyes. Caries Res. 23: 348-350.

Marsh PD, Martin M (1992). Oral Microbiology, 2nd ed. Chapman and Hall, London, pp 12, 14, 45, 53, 82-94, 104-107.

Marthaler TM (1975). Improved oral health of schoolchildren of 16 communities after 8 years of prevention. II. Findings in different types of caries predilection sites. Helv Odontol Acta. 19: 2-12.

Matee MIN, Mikx FHM, Maselle SYM, van Palenstein Helderman WH (1992). Mutans streptococci and lactobacilli in breast-fed children with rampant caries. Caries Res. 26: 183-187.

McBride BC, Gisslow MT (1977). Role of sialic acid in saliva-induced aggregation of *Streptococcus sanguis*. Infect Immun. 18: 35-40.

McDermid AS, Mckee AS, Ellwood DC, Marsh PD (1986). The effect of lowering the pH on the composition and metabolism of a community of nine oral bacteria grown in a chemostat. J Gen Microbiol. 132: 1205-1214.

McNamara TF, Friedman BK, Kleinberg I (1979). The microbial composition of human incisor tooth plaque. Arch Oral Biol. 24: 91-95.

Mellberg RJ, Petrou ID, Grote NE (1991). The effect of plaque thickness on progression of artificial caries lesion formation. J Dent Res. 69: 865-867.

Mikkelsen L, Poulsen S (1976). Microbiological studies on plaque in relation to development of dental caries in man. Caries Res. 10: 178-188.

Mikkelsen L, Jensen SB, Jakobsen I. (1981). Microbial studies on plaque from carious and caries free proximal tooth surfaces in a population with high caries experience. Caries Res. 15: 428-435.

Mikx FH, van der Hoeven JS, Konig KG, Plasschaert AJ, Guggenheim B (1972). Establishment of defined microbial ecosystems in germ-free rats. I. The effect of the interactions of *Streptococcus mutans* or *Streptococcus sanguis* with *Veillonella alcalescens* on plaque formation and caries activity. Caries Res. 6: 211-223.

Mikx FH, van der Hoeven JS (1975). Symbiosis of *Streptococcus mutans* and *Veillonella alcalescens* in mixed continuous cultures. Arch Oral Biol. 20: 407-410.

Mikx FH, van der Hoevn SJ, Plasschaert AJM, Konig KG (1975). Effect of *Actinomyces viscosus* on the establishment and symbiosis of *Streptococcus mutans* and *Streptococcus sanguis* in SPF rats on different sucrose diets. Caries Res. 9: 1-20.

Mikx FHM, van der Hoeven JS, Walker GJ (1976). Microbial symbiosis in dental plaque studied in gnotobiotic rats and in the chemostat. In: Stiles HM, Loesche WJ and O'Brien TC. Microb Aspects of Dental Caries. Information Retrieval, Washington DC, 3: pp 763-771.

Miller WD (1890). Microorganisms of the Human Mouth. The Local and General Diseases Which are Caused by Them. SS. White Dental Manufacturing Co. Philadelphia, p 364.

Milnes AR, Bowden GH (1985). The microflora associated with developing lesions of nursing caries. Caries Res. 19: 289-297.

Milnes AR, Bowden GH, Gates D, Tate R (1993). Normal microbiota on the teeth of preschool children. Microb Ecol Health Dis. 6: 213-227.

Minah GE, Loesche WJ (1977). Sucrose metabolism in resting-cell suspensions of caries associated and non-caries-associated dental plaque. Infect Immun. 17: 43-54.

Minah GE, Chu N (1984). Sucrose metabolism *in situ* by dental plaque in appliance-borne bovine enamel tooth fissure inserts in man. Arch Oral Biol. 29: 467-471.

Minah GE, Solomon ES, Chu .K (1985). The association between dietary sucrose consumption and microbial population shifts at six oral sites in man. Arch Oral Biol. 30: 387-401.

Moody MD, Ellis EC, Updyke EL (1958). Staining bacterial smears with fluorescent antibody. IV: grouping streptococci with fluorescent-antibody. J Bacteriol. 75: 553-560.

Murchison H, Larrimore S, Curtiss R (1985). *In vitro* inhibition of adherence of *Streptococcus mutans* strains by nonadherent mutants of *S. mutans* 6715. Infect Immun. 50: 826-832.

Moore WE, Holdeman LV, Smibert RM, Good IJ, Burmeister JA, Palcanis KG, Ranney RR (1982). Bacteriology of experimental gingivitis in young adult humans. Infect Immun. 38: 651-667.

Moore WE, Holdeman LV, Smibert RM, Cato EP, Burmeister JA, Palcanis KG, Ranney RR (1984). Bacteriology of experimental gingivitis in children. Infect Immun. 46: 1-6.

Moore WEC (1987). Microbiology of periodontal disease. J Periodont Res. 22: 335-341

Morris EJ, McBride BC (1984). Adherence of *Streptococcus sanguis* to saliva-coated hydroxyapatite: evidence for two binding sites. Infect Immun. 43: 656-663.

Newbrun E (1978). Dietary fluoride supplementation for the prevention of caries. Pediatrics. 62: 733-737.

Newbrun E (1989). Effectiveness of water fluoridation. J Public Health Dent. 49: 279-289.

Newman HN (1972). Structure of approximal human dental plaque as observed by scanning electron microscopy. Arch Oral Biol. 17: 1445-53.

Newman HN, McKay GS (1973). An unusual microbial configuration in human dental plaque. Microbios. 8: 117-128.

Newman HN (1974). Diet, attrition, plaque and dental disease. Brit Dendt J. 136: 491-497.

Newman HN, Poole DFG (1974). Structural and ecological aspects of dental plaque. In: Skinner FA, Carr JG (eds.). The Normal Microbial Flora of Man. Academic Press, New York and London, pp 111-134.

Newman HN (1975). The gingival border of plaque. Morphological studies in 8 to 15-year-old children. Brit Dent J. 138: 335-345.

Newman HN, Donoghue HD, Britton AB (1976). Effect of glucose and sucrose on the survival in batch culture of *Streptococcus mutans* C67-1 and a non-cariogenic mutant C67-25. Morphological studies. Microbios. 15: 113-125.

Newman HN (1979). The approximal apical border of plaque on children's teeth. 2. Adhesion, interbacterial connections and carbohydrate metabolism. J Periodontol. 50: 568-576.

Newman HN, Seymour GJ, Challacombe SJ (1979). Immunoglobulins in human dental plaque. J Periodont Res. 14: 1-9.

Newman HN, Morgan WJ (1980). Topographical relationship between plaque and

approximal caries. Caries Res. 14: 428-433.

Newman HN (1980). Update on plaque and periodontal disease. J Clin Periodontol. 7: 251-258.

Nikiforuk G (1985). Understanding Dental Caries. Etiology and Mechanisms Basic and Clinical Aspects. Karger S, London, pp 150-154.

Noorda WD, Purdell-Lewis DJ, van Montfort AMAP, Weerkamp AH (1988). Monobacterial and mixed bacterial plaques of *Streptococcus mutans* and *Veillonella alcalescens* im an artificial mouth: Development, metabolism, and effect on human dental enamel. Caries Res. 22: 342-347.

Nyvad B, Kilian M (1987). Microbiology of the early colonization of human enamel and root surfaces *in vivo*. Scand J Dent Res. 95: 369-380.

Nyvad B, Kilian M (1990a). Comparison of the initial streptococcal microflora on dental enamel in caries-active and in caries-inactive individuals. Caries Res. 24: 267-272.

Nyvad B, Kilian M (1990b). Microflora associated with experimental root surface caries in humans. Infect Immun. 58: 1628-1633.

Ofek I, Beachey EH (1980). Bacterial adherence. Adv Intern Med. 25: 503-532.

Olsson J, Glantz PO, Krasse B (1976). Surface potential and adherence of oral streptococci to solid surfaces. Scand J Dent Res. 84: 240-242.

Orland FJ, Blayney JR, Harrison RW (1954). Use of the germ-free animal technique in the study of experimental dental caries. J Dent Res. 33: 147-174.

Orland FJ, Blayney JR, Harrison RW, Reyniers JA, Trexler PC, Ervin RF, Gordon HA, Wagner M (1955). Experimental caries in germ-free rats inoculated with enterococci. J Am Dent Assoc. 5: 259-272.

Parker MT, Ball LC. (1976). Proceedings: Streptococci and aerococci associated with systemic disease in man. J Med Microbiol. 9: 275-302.

Pekovic DD, Adamkiewicz VW, Shapiro A (1987). Identification of bacteria in association with immune components in human carious dentine. J Oral Pathol. 16: 223-233.

Perch B, Kjems E, Ravn T (1974). Biochemical and serological properties of *Streptococcus mutans* from various human and animal sources. Acta path. et Microbiol. Scand. (B). 82: 357-370.

Pine L, Georg LK (1969). Reclassification of *Actinomyces propionicus*. Int J Syst Bacteriol. 19: 267-272.

Pollock JJ, Katona LI, Goodman H, Cho MI, Iacono VJ (1981). Bacteriolysis of

Streptococcus mutans BHT by lysozyme and inorganic anions normally present in human saliva. Arch Oral Biol. 26: 711-716.

Pollock JJ, Lotardo S, Gavai R, Grossbard BL (1987). Lysozyme-protease-inorganic monovalent anion lysis of oral bacterial strains in buffers and stimulated whole saliva. J Dent Res. 66: 467-474.

Poole PM, Wilson G (1976). Infection with minute-colony-forming beta-haemolytic streptococci. J Clin Pathol. 29: 740-745.

Quirynen M, Marechal M, Busscher HJ, Weerkamp AH, Arends J, Darius PL (1989). The influence of surface free-energy on planimetric plaque growth in man. J Dent Res. 68: 796-799.

Quirynen M, Marechal M, Busscher HJ, Weerkamp AH, Darius PL, van Steenberghe D. (1990). The influence of surface free energy and surface roughness on early plaque formation. J Clin Periodontol. 17: 138-144.

Reinholdt J, Tomana M, Mortensen SB, Kilian M (1990). Molecular aspects of immunoglobulin A1 degradation by oral streptococci. Infect Immun. 58: 1186-1194.

Ritz HL (1963). Localization of *Nocardia* in dental plaque by immunofluorescence. Proc Soc exp Biol Med. 113: 925-929.

Ritz HL (1967). Microbial population shifts in developing human dental plaque. Arch Oral Biol. 12: 1561-1568.

Rogall T, Wolters J, Flohr T, Bottger EC (1990). Towards a phylogeny and definition of species at the molecular level within the genus Mycobacterium. Int J Syst Bacteriol. 40: 323-330.

Rogosa M, Mitchell JA, Wiseman RF (1951). A selective medium for the isolation and enumeration of oral and faecal lactobacilli. J Dent Res. 30: 682-689.

Rogosa M (1956). A selective medium for the isolation and enumeration of the *Veillonella* from the oral cavity. J Bacteriol. 72: 533-536.

Roitt I, Brostoff J, Male D (1985). Adaptive and innate immunity. In: Roitt I, Brostoff J, Male D. (ed) Immunology. Gower Medical, London, pp 1-11.

Rudney JD, Neuvar EK, Sberay AH (1992). Restriction endonuclease fragment polymorphisms of oral viridans streptococci, compared by conventional and field inversion gel electrophoresis. J Dent Res. 71: 1182-1188.

Russell RR, Abdulla E, Gilpin ML, Smith L (1986). Characterisation of *Streptococcus mutans* surface antigens. In: Hamada S, Michalek S, Kiyono H (eds): Molecular Microbiology and Immunology of *Streptococcus mutans*. Oxford, Elsevier Science.

Russell RR, Smith K (1986). Effect of subculturing on location of *Streptococcus mutans* antigens. FEMS Microbiol Lett. 35: 319-323.

Russell RR (1990). Molecular genetics of glucan metabolism in oral streptococci. Arch Oral Biol. 35: 53S-58S

Russell RR (1991). Genetic analysis and genetic probes for oral bacteria: In Ferguson DB. (ed): Frontiers of Oral Physiology-Aspects of Oral Molecular Biology. Karger, Basel, pp 57-76.

Russell RR (1994). The application of molecular genetics to the microbiology of dental caries. Caries Res. 28: 69-82.

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N (1985). Enzymatic amplification of ß—Globin genomic sequences and restriction site analysis for diagnosis of sickle cell anaemia. Science, 230: 1350-1354.

Sanyal B, Russell C (1978). Nonsporing, anaerobic, gram-positive rods in saliva and the gingival crevice of humans. Appl Environ Microbiol. 35: 670-8.

Sambrook J, Frich EF, Maniatis T. (1989). Molecular Cloning: a Laboratory Manual 2nd ed. Cold Spring Harbor Laboratory Press, New York, pp E5.

Schaal KP (1986). *Actinomyces*. In: Sneath MS. Marr, Sharpe ME. and Holt JG. Bergeys Manual of Systematic Bacteriology. Williams and Wilkins, Baltimore, pp 1383-1418.

Schaal KP (1992). The Genera *Actinomyces*, *Arcanobacterium*, and *Rothia*. In: Balows A. Truper HG, Dworkin M, Harder W, Schleifer KH (eds.). The Prokaryotes 2nd ed. Springer-Verlag, pp 850-905.

Schachtele CF, Jensen ME (1982). Comparison of methods for monitoring changes in the pH of human dental plaque. J Dent Res. 61: 1117-1125.

Schaeken MJM, van der Hoeven JS, Franken HCM (1986). Comparative recovery of *Streptococcus mutans* on five isolation media, including a new selective medium. J Dent Res. 65: 906-908.

Shafer WG, Hine MK, Levy BM (1983). Textbook of Oral Pathology, 4th edition. WB Saunders Co. Philadelphia, London, pp 436-437.

Scharfen J (1973). Urease as a useful criterion in the classification of microaeropilic *Actinomyces*. Zbl. Bakt. Hyg. 1. Abr. Orig. A 225, 89-94.

Scheie AA (1994). Mechanisms of dental plaque formation. Adv Dent Res. 8: 246-253.

Scheie AA, Eggen KH, Rolla G (1987). Glucosyltransferase activity in human *in vivo* formed enamel pellicle and in whole saliva. Scand J Dent Res. 95: 212-215

Scherp HW (1971). Dental caries: prospects for prevention. Science. 173: 1199-1205

Schleifer KH, Killpper-Balz R, Kraus J, Gehring F (1984). Relatedness and classification of *Streptoccus mutans* and mutans-like streptococci. J Dent Res. 63: 1047-1050.

Schmidhuber S, Kilpper-Balz R, Schleifer KH (1987). A taxonomic study of *Streptococcus mitis, S. oralis* and *S. sanguis*. Syst Appl Microbiol. 10: 74-77.

Shah HN, Collins DM (1988). Proposal for reclassification of *Bacteroides asaccharolyticus, Bacteroides gingivalis*, and *Bacteroides endodontalis* in a new genus, *Porphyromonas*. Int J Syst Bacteriol. 38: 128-131. Int J syst Bacteriol.

Shah HN, Collins DM (1990). *Prevotella*, a new genus to include *Bacteroides melaninogenicus* and related species formerly classified in the genus *Bacteroides*. Int J Syst Bacteriol. 40: 205-208.

Shklair IL, Keene HJ (1974). A biochemical scheme for the separation of the five varieties of Streptococcus mutans.
Arch Oral Biol. 19: 1079-81.

Sidaway DA (1979). A comparison of the *in vitro* calcification of viable and non-viable miroorganisms. J Periodont Res. 14: 167-172.

Siegel S (1956). Non-Parametric Statistics for the Behavioral Sciences. McGraw-Hill, New York, pp 166-172.

Sigurjons H, Magnusdottir MO, Holbrook WP (1995). Cariogenic bacteria in a longitudinal study of approximal caries. Caries Res. 29: 42-45.

Sims W (1970). The concept of immunity in dental caries I. General considerations. Oral Surg. 30: 670-677.

Skerman VBD, McGowan V, Sneath PHA (1980). Approved list of bacterial names. Int J Syst Bacteriol. 30: 225-420.

Skopek RJ, Liljemark WF, Bloomquist CG, Rudny JD (1993). Dental plaque development on defined streptococcal surfaces. Oral Microbiol Immunol. 8: 16-23.

Smith DJ, Anderson JM, King WF, van Houte J, Taubman MA (1993). Oral streptococcal colonization of infants. Oral Microbiol Immunol. 8: 1-4.

Socransky SS, Manganiello AD (1971). The oral microbiota of man from birth to senility. J Periodontol. 42: 485-496.

Socransky SS, Manganiello AD, Propas D, Oram V, van Houte J (1977). Bacteriological studies of developing supragingival dental plaque. J Periodont Res. 12: 90-106.

Sreebny LM (1982). Sugar availability, sugar consumption and dental caries Commun Dent Oral Epidemiol. 10: 1-7.

Stosser L, Kneist S(1988). Acidogenic and aciduric properties of *Streptococcus mutans* and their cariogenic significance. Caries Res. 22: 118-118.

Svanberg M, Loesche WJ (1977). The salivary concentration of *S. mutans* and *S. sanguis* and their colonization of artificial tooth fissures in man. Arch Oral Biol. 22: 441-447.

Swenson JI, Liljemark WF, Schuman LM (1976). A longitudinal epidemiologic evaluation of the association between the detection of plaque streptococci and development of dental caries in children. In: Stiles HM, Loesche WJ, O'Brien TC (eds.). Microbial Aspects of Dental Caries. Washington DC. Information Retrieval Inc, pp 211-222.

Syed SA, Loesche WJ, Pape HL Jr, Grenier E (1975). Predominant cultivable flora isolated from human root surface caries plaque. Infect Immun. 11: 727-731.

Syed SA, Loesche WJ (1978). Bacteriology of human experimental gingivitis: effect of plaque age. Infect Immun. 21: 821-829.

Syed SA, Loesche WJ (1972). Survival of human dental plaque flora in various transport media. Appl Microbiol. 24: 638-644.

Tanzer JM, Wood WI, Krichevsky MI (1969). Linear growth kinetics of plaque-forming streptococci in the presence of sucrose. J Gen Microbiol. 58: 125-133.

Tanzer JM, Brown AT, McInerney MF, Woodiel FN (1977). Comparative study of invertases of *Streptococcus mutans*. Infect Immun. 16: 318-327.

Tanner A, Maiden MFJ, Paster BJ, Dewhirst FE (1994). The impact of 16S ribosomal RNA-based phylogeny on the taxonomy of oral bacteria. Periodontol 2000. 5: 25-51

Tappuni AR, Challacombe SJ (1993). Distribution and isolation frequency of eight streptococcal species in saliva from predentate and dentate children and adults. J Dent Res 72: 31-36.

Tenovuo J, Soderling, Sievers G (1985). The peroxidase system in human tears. Protides Biol. Fluids Proc. Colloq. 32: 107-110.

Tenovuo J, Lehtonen OP, Aaltonen AS, Vilja P, Tuohimaa P (1986). Antimicrobial factors in whole saliva of human infants. Infect Immun. 51: 49-53.

Theilade E, Fejerskov O, Karring T, Theilade J (1982). Preodominant cultivable microflora of human dental fissure plaque. Infect Immun. 36: 977-982.

Theilade E, Theilade J, Mikkelsen L (1982). Microbial studies of early dentogingival plaque on teeth and mylar strips in humans. J Periodont Res. 17: 12-25.

Theilade E, Theilade J (1985). Formation and ecology of plaque at different locations in the mouth. Scand J Dent Res. 93: 90-95.

Theilade E, Budtz-Jorgensen E (1988). Predominant cultivable flora of plaque on removable dentures in patients with denture-induced stomatitis. Oral Microbiol Immunol. 3: 8-13.

Theilade E (1989). Advances in oral microbiology. Ann R Australas Coll Dent Surg. 10: 62-71.

Thompson L, Lovestedt SA (1951). An *Actinomyces* like organism obtained from the human mouth. Proceedings of the Staff Meeting of the Mayo Clinic 26: 169-175.

Thompson LA, Little WA, Hageage GJ (1976). Application of fluorescent antibody method in the analysis of plaque samples. J Dent Res. 55: 80-86.

Thompson LA, Little WA, Bowen WH Sierra LI, Aguirrer M, Gillespie G (1980). Prevalence of *Streptococcus mutans* serotypes, *Actinomyces* and other bacteria in the plaque of children. J Dent Res. 59: 1581-1589.

Thylstrup LA, Fejerskov O (1986). Textbook of Cariology, 1st ed. Munksgaard Copenhagen, p 309.

Togelius J, Bratthall D (1982). Frequency of the bacterium *S. mutans* in the saliva of selected human populations. Arch Oral Biol. 27: 113-116.

Tortosa M, Cho MI, Wilkens TJ, Iacono VJ, Pollock JJ (1981). Bacteriolysis of *Veillonella alcalescens* by lysozyme and inorganic anions present in saliva. Infect Immun. 32: 1261-1273.

van Houte J, Gibbons RJ, Banghart SB (1970). Adherence as a determinant of the presence of *Streptococcus salivarius* and *Streptococcus sanguis* on the human tooth surface. Arch Oral Biol. 15: 1025-1034.

van Houte J, Gibbons RJ, Pulkkinen AJ (1972). Ecology of human oral lactobacilli. Infect Immun. 6: 723-729.

van Houte J (1980). Bacterial specificity in the etiology of dental caries. Int Dent J. 30: 305-326.

van Houte J, Aasenden R, Peebles TC (1981). Lactobacilli in human dental plaque and saliva. J Dent Res. 60: 2-5.

van Houte J, Sansone C, Joshipura K, Kent R (1991a). *In vitro* acidogenic potential and mutans streptococci of human smooth surface plaque associated with initial caries lesions and sound enamel. J Dent Res. 70: 1497-1502.

van Houte J, Sansone C, Joshipura K, Kent R (1991). Mutans streptococci and non-mutans

streptococci acidogenic at low pH, and *in vitro* acidogenic potential of dental plaque in two different areas of the human dentition. J Dent Res. 70 :1503-1507.

van der Hoeven JS, Mikx FH, Plasschaert AJ, Konig KG (1972). Methodological aspects of gnotobiotic caries experimentation. Preliminary investigations into the microbial ecology of dental plaque. Caries Res. 6: 203-210.

van der Hoeven JS, Mikx FH, Plasschaert AJ, Maltha JC (1975). Experimental periodontal disease in rats induced by plaque-forming microorganisms. J Periodont Res. 10: 143-177.

van der Hoeven JS, Toorop AI, Mikx FHM. (1978). Symbiotic relationship of *Veillonella alcalescens* and *Streptococcus mutans* in dental plaque in gnotobiotic rats. Caries Res. 12: 142-147.

van der Hoeven JS, Rogers AH (1979). Stability of the resident microflora and the bacteriocinogeny of *Streptococcus mutans* as factors affecting its establishment in specific pathogen-free rats. Infect Immun. 23: 206-213.

van der Hoeven JS, de Jong MH, Rogers AH, Camp PJM (1984). A conceptual model for the co-existence of *Streptococcus* spp. and *Actinomyces* spp. in dental plaque. J Dent Res. 63: 389-392.

van der Hoeven JS, de Jong MH, Camp PJM, van den Kieboom CWA (1985). Competition between oral *Streptococcus* species in the chemostat under alternating conditions of glucose limitation and excess. FEMS Micobiol Ecol 31: 373-379.

van der Hoeven JS, van den Kieboom CWA (1990). Oxygen-dependent lactate utilization by *Actinomyces viscosus* and *Actinomyces naeslundii.* Oral Microbiol Immunol. 5: 223-225.

van Palenstein Helderman WH, Ijsseldijk M, Huis in't Veld JH (1983). A selective medium for the two major subgroups of the bacterium *Streptococcus mutans* isolated from human dental plaque and saliva. Arch Oral Biol. 28: 599-603.

Wade WG, Aldred MJ, Walker DM (1986). An improved medium for isolation of *Streptococcus mutans.* J Med Microb. 22: 319-323.

Walker GJ, Hare MD (1977). Metabolism of the polysaccharides of human dental plaque. Part II. Purification and properties of *Cladosporium resinae* (1 leads to 3)- alpha-D-glucanase, and the enzymic hydrolysis of glucans synthesised by extracellular D-glucosyltransferases of oral streptococci. Carbohydrate Res. 58: 415-432.

Walker GW (1978). Dextrans. Int Rev Biochem. 16: 75-126.

Weatherell JA, Strong M, Robinson C, Nakagaki H, Ralph JP (1989). Retention of glucose in oral fluid at different sites in the mouth. Caries Res. 23: 399-405.

Welborn PP, Hadley KW, Newbrun E, Yajko DM (1983). Characterization of strains of viridans streptococci by deoxyribonucleic acid hybridization and physiological tests. Int J Syst Bacteriol. 33: 293-299.

Wenham DG, Hennessey TD, Cole JA (1979). Regulation of glucosyl- and fructosyltransferase synthesis by continuous cultures of *Streptococcus mutans*. J Gen Microbiol. 114: 117-124.

Wennerholm K, Emilson CG (1995). Sucrose retention and colonization by mutans streptococci at different sites of the dentition. Caries Res. 29: 396-401.

Whiley RA, Hardie JM (1988). *Streptococcus vestibularis* sp. nov. from the human oral cavity. Int J Syst Bacteriol. 38: 335-339.

Whiley RA, Russell RRB, Hardie JM, Beighton D (1988). *Streptococcus downei* sp. nov. for strains previously described as *Streptococcus mutans* serotype *'h'* Int J Syst Bacteriol. 38: 25-29.

Whiley RA, Hardie JM (1989). DNA-DNA hybridization studies and phenotypic characteristic of strains within "*Streptococcus milleri* group". J Gen Microbiol. 135: 2623-2633.

Whiley RA, Fraser H, Hardie JM, Beighton D (1990a). Phenotypic differentiation of *Streptococcus intermedius*, *Streptococcus constellatus*, and *Streptococcus anginosus* strains within the "*Streptococcus milleri* group". J Clin Microbiol. 28: 1497-1501.

Whiley RA, Fraser HY, Douglas CW, Williams AM, Collins MD (1990b). *Streptococcus parasanguis* sp. nov., an atypical viridans streptococcus from human clinical specimens, FEMS Microbiol Lett. 68: 115-122.

Whiley RA, Fraser HY, Douglas CW, Hardie JM, Williams AM, Collins MD (1990c). *Streptococcus parasanguis* sp. nov., an atypical viridans *Streptococcus* from human clinical specimens. FEMS Microbiol Lett. 56: 115-121.

Whiley RA, Beighton D (1991). Emended descriptions and recognition of *Streptococcus constellatus*, *Streptococcus intermedius*, and *Streptococcus anginosus* as distinct species. Int J Syst Bacteriol. 41: 1-5.

Widerstrom L, Bratthall D, Hamberg K (1994). Immunoglobulin A antibodies to mutans streptococci in human saliva and serum comparing fresh and subcultivated strains and activity in repeated saliva samples. Oral Microbiol Immunol. 9: 278-283.

Wilson RF, Ashley FP (1990). Relationships between the biochemical composition of both free smooth surface and approximal plaque and salivary composition and a 24-hour retrospective dietary history of sugar intake in adolescents. Caries Res. 24: 203-210.

Woese CR (1987). Bacterial evolution. Microbiol Rev. 51:221-271.

Wolff M, Israel J (1891). Uber Reincultur des *Actinomyces* und seine Ubertragbarkeit auf Thiere. Archiv der Pathologischen Anatomie, Physiologie and Klinischen Medizin. 126: 11-59. Cited in: Schaal KP (1992). The Genera *Actinomyces*, *Arcanobacterium*, and *Rothia*. In: Balows A. Truper HG, Dworkin M, Harder W, Schleifer KH (eds.). The Prokaryotes 2nd ed. Springer-Verlag, pp 850-905.

World Health Organization (1989). Dental caries levels at 12 years (mimeograph of data from Global Oral Data Bank). Geneva: World Health Organization.

Yamada T, Carlsson J (1975). Glucose-6-phosphate-dependent pyruvate kinase in *Streptococcus mutans*. J Bacteriol. 124: 562-563.

Zickert I, Emilson CG, Krasse B (1982). *Streptococcus mutans*, lactobacilli and dental health in 1-14 year old Swedish children. Commun Dent Oral Epidemiol. 10: 77-81.

Zylber LJ, Jordan HV (1982). Development of a selective medium for detection and enumeration of *Actinomyces viscosus* and *Actinomyces naeslundii* in dental plaque. J Clin Microbiol. 15: 253-259.

Appendix A

List of media

1- Enriched horse blood agar

Columbia agar Base (Oxoid, Basingstoke, UK) supplemented with 7% (v/v) horse blood (Oxoid, Basingstoke, UK) was used to enumerate the total cultivable flora.

2- TYC agar

TYC (Lab M, Bury, England) was used for the primary isolation of streptococcal strains (de Stoppelaar 1967).

3- TYC sucrose bacitracin agar (TYCSB)

This selective medium was used for the isolation of mutans streptococci (Van Palenstein Helderman et al. 1983). TYC agar was supplemented with 15% (w/v) sucrose (BDH, Poole, UK) to give a final concentration 20% of sucrose and 0.1 unit/ml bacitracin (Sigma, Poole, UK).

4- Veillonella vancomycin agar

Veillonella agar (Rogosa 1956) was used for the isolation of *Veillonella* SPP. Veillonella agar (Oxoid, Basingstoke, UK)) was prepared and autoclaved then allowed to cool to 50° C before adding 7.5 μg/ml filter sterilised vancomycin (Eli Lilly, Basingstoke, England).

5- Rogosa agar

Rogosa SL media (Difco, Mosely, England) was used as a selective medium to recover lactobacilli. Glacial acetic acid was added to the agar after boiling to decrease the pH (Rogosa et al. 1951).

6- Nutrient agar

Nutrient agar (Oxoid, Basingstoke, UK) was used for aerobic growth.

All media were made up according to manufacturers' instructions and autoclaved at 121°C for 15 minutes, then cooled to 50-55°C before adding any supplements, and before pouring into petri dishes. The plates were then dried at 37°C for 45-60 minutes and stored at 4°C for a maximum of one week. All plates were incubated anaerobically overnight before they were inoculated, and then incubated for 5 days at 37°C. Different colony types were counted and sub-cultured for identification.

Appendix B

List of reagents used.

	gram/100 ml
Reduced transport fluid (RTF)	
Sodium carbonate Na_2CO_3	0.04
di-potassium orthophosphate K_2HPO_4	0.045
Magnesium sulphate $MgSO_4$	0.018
Potassium di-hydrogen phosphate KH_2PO_4	0.045
Sodium chloride NaCl	0.09
Ammonium sulphate $(NH_4)_2SO_4$	0.09
Dithiothreitol	0.02
EDTA	0.001 M
Distilled water to make volume to 100 ml	
Phosphate buffered saline (PBS) pH 7.2	
Sodium chloride	8.0 g
Potassium chloride	0.2 g
Disodium hydrogen phosphate	1.5 g
Sodium dihydrogen phosphate	0.2 g
Distilled water to make volume to one litre.	
PBS + azide	
Sodium azide powder	0.2 g
PBS one litre	
PBS + bovine serum (BSA) + Tween 20 (T20)	
List of solutions used in DNA extraction	
Ammonium acetate	5.0 M
Ammonium acetate (BDH, Poole, UK)	38.54 g
Dissolve in 100 ml distilled water.	
GBx Loading buffer (6x)	
Glycerol (BDH, Poole, UK))	30.0 ml
Bromophenol blue (BDH, 44305)	0.25 g
Xylene cyanol (BDH 20123)	0.25 ml

Make up volume to 100 ml with distilled water.
Store at 4°C.

Lysozyme 20.0 mg/ml

Made up in 1 ml sterile distilled water.

20 % SDS

Sodium dodecyl sulphate (SDS) (BDH, Poole, UK) 20.0 g

Dissolve in 100 ml water.

Sterilize by filtration through a 0.2 μm filter.

5 M Sodium chloride

Sodium chloride (NaCl) (Sigma, Poole, UK) 29.0 g

Dissolve in 100ml autoclaved distilled water.

TBE buffer x10

Tris base 108.0 g

Boric acid 55.0 g

EDTA 7.44 g

Make up volume to 1000 ml with distilled water.

EDTA

EDTA 7.449g

1000 ml distilled water pH 8

TE buffer

10 mM Tris-HCl + 1 mM EDTA, pH 8.0

TES Buffer

50 mM Tris (Tris, BDH, Poole, UK)

15 mM Ethylenediaminetetra-acetic acid disodium salt (EDTA, BDH, Poole, UK) pH 8

Preparation of RNAase

Pancreatic RNAase (RNAase A) (Sigma, Poole, UK) was prepared at a concentration of 10 mg/ml in 10 mM Tris-HCl (pH 7.5) and 15 mM NaCl. In order to make the solution

free from any DNAase it was boiled for 15 minute in a water bath then cooled slowly to room temperature. The mixture was then centrifuged (13000 g) for 3 minutes in order to remove denatured DNAase, the supernatant transferred to a sterile tube and stored at -20°C. The prepared RNAase solution was diluted 1:10 before used.

Preparation of molecular weight size markers

Molecular weight markers were prepared by adding 0.5 µl of ϕ X 174, *Hae*III digested (Promega, Southampton, UK) to 9.5 µl TE and 2µl loading buffer in a sterile micro-centrifuge tube.

Appendix C

List of abbreviations

A	adenine
ADP	adenosine diphosphate
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
EDTA	ethylenediamine tetra-acetic acid
E. coli	*Escherichia coli*
FITC	fluorescein isothiocyanate
GCF	gingival crevicular fluid
GTF	glucosyltransferase
h	hour
IF	immunofluorescence
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
kb	kilobase (number of bases in thousands)
λ	lambda bacteriophage DNA (used for testing restriction enzymes)
M	molar
Mab	monoclonal antibody
MS	mutans streptococci
$MgCl_2$	magnesium chloride
µg	microgram
µl	microlitre
mM	millimolar
µM	micromolar
min	minute
NaCl	sodium chloride
NaOH	sodium hydroxide

OD	optical density
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNAase	ribonuclease
rRNA	ribosomal ribonucleic acid
RTF	reduced transport fluid
sIgA	secretory immunoglobulin A
SE	standard error
SEM	standard error of the mean
Tris	tris (hydroxymethyl) aminomethane
Tris-HCl	tris (hydroxymethyl) aminomethane hydrochloride
TYC	Tryptone Yeast Cystine
TYCSB	Tryptone Yeast Cystine; sucrose and bacitracin
U	unit
uv	ultraviolet (light)
v/v	volume per volume (%v.v, the volume in ml in 100ml total volume)
w/v	weight for volume (%w/v, the weight in gram in 100 ml total volume)

APPENDIX D
TABLES 1-20 CULTURE DATA

Table 1 Distribution of *Streptococcus mutàns* as cfu/ml and as a percentage of the viable count at various sites in relation to the contact area.

Patient N°	Tooth N°	Away		Side		Below	
		cfu/ml	(% vc)	cfu/ml	(% vc)	cfu/ml	(% vc)
211	UL4	0.0	(0.0)	0.0	(0.0)	980.0	(37.24)
212	LR4	0.0	(0.0)	0.0	(0.0)	200.0	(4.9)
215	LR5	200.0	(0.4)	$1.0x10^3$	(10.1)	10.0	(0.12)
216	LR4	$8.9x10^3$	(78.0)	$4.9x10^4$	(27.4)	$9.6x10^4$	(52.5)
217	LR5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
218	UR5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
219	LR4	1000.0	(2.9)	420.0	(2.7)	300.0	(1.8)
220	UR4	0.0	(0.0)	8000.0	(1.9)	$3.0x10^3$	(1.24)
221	LR4	0.0	(0.0)	0.0	(0.0)	900.0	(8.41)
222	LLR	0.0	(0.0)	100.0	(0.1)	$1.2x10^4$	(22.9)
223	UR5	6000.0	(1.9)	$1.43x10^4$	(24.0)	$2.25x10^3$	(0.6)
224	UR4	3000.0	(3.2)	0.0	(0.0)	$1.0x10^3$	(3.8)
225	UR4	200.0	(0.4)	0.0	(0.0)	200.0	(0.1)
226	UL4	0.0	(0.0)	200.0	(1.6)	$1.0x10^3$	(9.4)
227	UR4	0.0	(0.0)	300.0	(9.0)	700.0	(9.7)
228	UR4	0.0	(0.0)	$4.2x10^3$	(14.9)	800.0	(9.4)
229	UL5	0.0	(0.0)	$5.2x10^3$	(28.9)	$8.0x10^3$	(7.3)
230	LL5	0.0	(0.0)	$2.0x10^3$	(28.2)	$3.x10^3$	(51.6)
231	UL4	11500.0	(53.2)	$1.3X10^4$	(10.9)	$2.2X10^4$	(1.0)
232	LR4	240.0	(17.4)	0.0	(0.0)	$1.9x10^3$	(53.5)
233	UL4	30.0	(30.0)	100.0	(0.7)	0.0	(0.0)

N° = number
% vc = percentage of viable count.

Table 2 Distribution of *Streptococcus sobrinus* as cfu/ml and as a percentage of the viable count at various sites in relation to the contact area.

Patient N°	Tooth N°	Away		Side		Below	
		cfu/ml	(% vc)	cfu/ml	(% vc)	cfu/ml	(% vc)
211	UL4	40.0	(0.7)	0.0	(0.0)	0.0	(0.0)
212	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
215	LR5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
216	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
217	LR5	0.0	(0.0)	0.0	(0.0)	$1.95x10^3$	(83.0)
218	UR5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
219	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
220	UR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
221	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
222	LL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
223	UR5	0.0	(0.0)	$10.0x10^3$	(0.2)	$1.6x10^4$	(4.7)
224	UR4	0.0	(0.0)	0.0	(0.0)	1000.0	(0.4)
225	UL4	0.0	(0.0)	0.0	(0.0)	$2.2x10^4$	(11.7)
226	UL4	0.0	(0.0)	400.0	(3.1)	600.0	(5.6)
227	UR4	0.0	(0.0)	500.0	(15.1)	0.0	(0.0)
228	UR4	200.0	(38.5)	0.0	(0.0)	20.0	(0.2)
229	UL5	0.0	(0.0)	0.0	(0.0)	100.0	(0.1)
230	LL5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
231	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
232	LR4	0.0	(0.0)	$5.0x10^3$	(63.4)	0.0	(0.0)
233	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)

N° = number
% vc = percentage of viable count.

Table 3 Distribution of *Streptococcus sanguis* as cfu/ml and as a percentage of the viable count at various sites in relation to the contact area.

Patient N°	Tooth N°	Away		Side		Below	
		cfu/ml	(% vc)	cfu/ml	(% vc)	cfu/ml	(% vc)
211	UL4	0.0	(0.0)	20.0	(0.1)	0.0	(0.0)
212	LR4	450.0	(12.7)	0.0	(0.0)	300.0	(7.3)
215	LR5	$2.7x10^3$	(5.9)	$2.4x10^3$	(24.3)	$3.0x10^3$	(35.3)
216	LR4	0.0	(0.0)	$2.1x10^4$	(11.8)	$1.1x10^4$	(6.0)
217	LR5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
218	UR5	20.0	(3.6)	800.0	(7.3)	$4.0x10^3$	(13.1)
219	LR4	0.0	(0.0)	20.0	(0.1)	0.0	(0.0)
220	UR4	20.0	(5.6)	0.0	(0.0)	0.0	(0.0)
221	LR4	200.0	(9.9)	60.0	(1.5)	600.0	(5.6)
222	LL4	50.0	(3.7)	$4.0x10^4$	(29.5)	$1.0x10^3$	(1.9)
223	UR5	$2.0x10^4$	(6.8)	$22.4x10^4$	(47.8)	300.0	(0.1)
224	UR4	4000.0	(4.2)	$1.12x10^4$	(15.5)	100.0	(0.4)
225	UL4	900.0	(2.0)	$2.5x10^4$	(4.2)	$2.6x10^4$	(13.9)
226	UL4	$2.0x10^3$	(16.7)	0.0	(0.0)	200.0	(1.9)
227	UR4	0.0	(0.0)	0.0	(0.0)	$5.0x10^3$	(52.4)
228	UR4	0.0	(0.0)	0.0	(0.0)	60.0	(0.7)
229	UL5	$4.0x10^3$	(6.2)	400.0	(2.2)	$2.4x10^4$	(21.9)
230	LL5	0.0	(0.0)	900.0	(12.7)	0.0	(0.0)
231	UL4	0.0	(0.0)	$1.7x10^4$	(14.2)	0.0	(0.0)
232	LR4	330.0	(14.3)	0.0	(0.0)	0.0	(0.0)
233	UL4	0.0	(0.0)	300.0	(2.1)	0.0	(0.0)

N° = number
% vc = percentage of viable count.

Table 4 Distribution of *Streptococcus gordonii* as cfu/ml and as a percentage of the viable count at various sites in relation to the contact area.

Patient N°	Tooth N°	Away		Side		Below	
		cfu/ml	(% vc)	cfu/ml	(% vc)	cfu/ml	(% vc)
211	UL4	0.0	(0.0)	20.0	(0.0)	80.0	(3.1)
212	LR4	350.0	(10.2)	0.0	(0.0)	0.0	(0.0)
215	LR5	0.0	(0.0)	0.0	(0.0)	$2.0x10^3$	(23.6)
216	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
217	LR5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
218	UR5	0.0	(0.0)	200.0	(1.8)	$1.0x10^3$	(3.3)
219	LR4	0.0	(0.0)	0.0	(0.0)	200.0	(1.2)
220	UR4	10.0	(2.8)	0.0	(0.0)	0.0	(0.0)
221	LR4	100.0	(5.0)	0.0	(0.0)	0.0	(0.0)
222	LL4	0.0	(0.0)	$3.6x10^3$	(2.7)	$1.4x10^3$	(0.8)
223	UR5	$2.0x10^3$	(0.7)	$5.8x10^4$	(12.4)	0.0	(0.0)
224	UR4	0.0	(0.0)	$7.2x10^3$	(10.0)	0.0	(0.0)
225	UL4	800.0	(1.7)	$1.5x10^4$	(2.5)	$2.0x10^3$	(1.1)
226	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
227	UR4	0.0	(0.0)	0.0	(0.0)	$1.0x10^3$	(10.5)
228	UR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
229	UL5	0.0	(0.0)	0.0	(0.0)	$1.2x10^4$	(10.9)
230	LL5	0.0	(0.0)	200.0	(0.4)	0.0	(0.0)
231	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
232	LR4	260.0	(19.4)	0.0	(0.0)	0.0	(0.0)
233	UL4	0.0	(0.0)	400.0	(2.8)	0.0	(0.0)

N° = number
% vc = percentage of viable count.

Table 5 Distribution of *Streptococcus mitis* I as cfu/ml and as a percentage of the viable count at various sites in relation to the contact area.

Patient N°	Tooth N°	Away		Side		Below	
		cfu/ml	(% vc)	cfu/ml	(% vc)	cfu/ml	(% vc)
211	UL4	$2.7x10^3$	(47.4)	$4.0x10^3$	(14.8)	600.0	(23.5)
212	LR4	80.0	(2.3)	30.0	(33.4)	0.0	(0.0)
215	LR5	$2.4x10^3$	(5.3)	300.0	(3.0)	$1.7x10^3$	(20.0)
216	LR4	0.0	(0.0)	$3.0x10^4$	(10.1)	$8.9x10^4$	(19.1)
217	LR5	500.0	(26.1)	10.0	(7.7)	0.0	(0.0)
218	UR5	100.0	(17.8)	0.0	(0.0)	$9.0x10^3$	(29.6)
219	LR4	0.0	(0.0)	350.0	(2.3)	$2.6x10^3$	(15.1)
220	UR4	0.0	(0.0)	$5.6x10^3$	(1.3)	$6.0x10^3$	(2.7)
221	LR4	400.0	(19.9)	290.0	(7.1)	600.0	(5.6)
222	LL4	10.0	(0.7)	$6.3x10^3$	(4.7)	900.0	(1.9)
223	UR5	$2.68x10^4$	(8.8)	0.0	(0.0)	$1.55x10^5$	(43.6)
224	UR4	$7.5x10^3$	(7.8)	$5.0x10^3$	(6.9)	$4.8x10^3$	(18.4)
225	UL4	$5.0x10^3$	(10.9)	$1.4x10^4$	(2.6)	$9.0x10^3$	(4.8)
226	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
227	UR4	20.0	(40.0)	500.0	(15.1)	0.0	(0.0)
228	UR4	0.0	(0.0)	$11.0x10^3$	(39.0)	400.0	(4.7)
229	UL5	$33.0x10^4$	(50.0)	600.0	(3.3)	$9.0x10^3$	(8.2)
230	LL5	$1.8x10^3$	(30.0)	900.0	(12.7)	40.0	(0.8)
231	UL4	$1.51x10^3$	(7.5)	500.0	(0.4)	0.0	(0.0)
232	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
233	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)

N° = number
% vc = percentage of viable count.

Table 6 Distribution of *Streptococcus mitis* II as cfu/ml and as a percentage of the viable count at various sites in relation to the contact area.

Patient N°	Tooth N°	Away		Side		Below	
		cfu/ml	(% vc)	cfu/ml	(% vc)	cfu/ml	(% vc)
211	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
212	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
215	LR5	$1.6x10^3$	(3.5)	100.0	(1.0)	300.0	(3.54)
216	LR4	0.0	(0.0)	$1.6x10^4$	(9.0)	$1.1x10^4$	(2.35)
217	LR5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
218	UR5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
219	LR4	400.0	(1.2)	150.0	(1.1)	0.0	(0.0)
220	UR4	10.0	(3.0)	0.0	(0.0)	500.0	(0.2)
221	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
222	LL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
223	UR5	$2.12x10^4$	(4.7)	0.0	(0.0)	$1.5x10^4$	(0.23)
224	UR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
225	UL4	0.0	(0.0)	$6.6x10^3$	(1.2)	0.0	(0.0)
226	UL4	$1.0x10^3$	(8.3)	0.0	(0.0)	0.0	(0.0)
227	UR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
228	UR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
229	UL5	0.0	(0.0)	0.0	(0.0)	$1.18x10^3$	(1.1)
230	LL5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
231	UL4	$9.9x10^3$	(4.9)	400.0	(0.3)	0.0	(0.0)
232	LR4	0.0	(0.0)	0.0	(0.0)	500.0	(14.08)
233	UL4	30.0	(30.0)	250.0	(1.7)	10.0	(10.0)

N° = number
% vc = percentage of viable count.

Table 7 Distribution of *Streptococcus oralis* as cfu/ml and as a percentage of the viable count at various sites in relation to the contact area.

Patient N°	Tooth N°	Away		Side		Below	
		cfu/ml	(% vc)	cfu/ml	(% vc)	cfu/ml	(% vc)
211	UL4	0.0	(0.0)	$3.0x10^3$	(11.0)	400.0	(15.3)
212	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
215	LR5	$2.3x10^3$	(5.0)	0.0	(0.0)	1000.0	(11.8)
216	LR4	0.0	(0.0)	$10.0x10^3$	(5.6)	$63.0x10^3$	(13.3)
217	LR5	300.0	(15.7)	0.0	(0.0)	0.0	(0.0)
218	UR5	90.0	(16.1)	0.0	(0.0)	0.0	(0.0)
219	LR4	0.0	(0.0)	$1.48x10^3$	(9.7)	2000.0	(12.0)
220	UR4	0.0	(0.0)	$4.4x10^3$	(1.0)	$3.5x10^3$	(1.5)
221	LR4	300.0	(14.9)	400.0	(9.8)	500.0	(5.7)
222	LL4	0.0	(0.0)	0.0	(0.0)	1200.0	(2.6)
223	UR5	$3.5x10^4$	(11.8)	0.0	(0.0)	$10.0x10^4$	(28.2)
224	UR4	$6.5x10^3$	(6.9)	$3.0x10^3$	(4.2)	4000.0	(15.3)
225	UL4	$4.0x10^3$	(8.8)	$1.0x10^5$	(16.6)	7000.0	(3.0)
226	UL4	$3.0x10^3$	(25.0)	0.0	(0.0)	0.0	(0.0)
227	UR4	10.0	(20.0)	0.0	(0.0)	0.0	(0.0)
228	UR4	0.0	(0.0)	$1.8x10^3$	(6.3)	400.0	(4.7)
229	UL5	$2.0x10^4$	(30.8)	0.0	(0.0)	2000.0	(5.5)
230	LL5	$1.2x10^3$	(20.0)	0.0	(0.0)	0.0	(0.0)
231	UL4	$1.5x10^3$	(5.1)	200.0	(0.2)	0.0	(0.0)
232	LR4	0.0	(0.0)	0.0	(0.0)	300.0	(8.5)
233	UL4	0.0	(0.0)	250.0	(1.7)	0.0	(0.0)

N° = number
% vc = percentage of viable count.

Table 8 Distribution of *Streptococcus milleri* group as cfu/ml and as a percentage of the viable count at various sites in relation to the contact area.

Patient N°	Tooth N°	Away		Side		Below	
		cfu/ml	(% vc)	cfu/ml	(% vc)	cfu/ml	(% vc)
211	UL4	$2.0x10^3$	(35.1)	$3.0x10^3$	(11.0)	320.0	(12.1)
212	LR4	$1.5x10^3$	(42.5)	20.0	(22.2)	900.0	(22.0)
215	LR5	$1.2x10^3$	(2.6)	40.0	(0.4)	20.0	(0.2)
216	LR4	0.0	(0.0)	$3.7x10^4$	(20.8)	0.0	(0.0)
217	LR5	0.0	(0.0)	10.0	(7.7)	0.0	(0.0)
218	UR5	0.0	(0.0)	0.0	(0.0)	$3.9x10^3$	(12.8)
219	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
220	UR4	10.0	(2.8)	0.0	(0.0)	$1.0x10^4$	(4.15)
221	LR4	100.0	(5.0)	400.0	(9.8)	100.0	(2.2)
222	LL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
223	UR5	$4.0x10^4$	(13.5)	$4.68x10^4$	(10.0)	0.0	(0.0)
224	UR4	0.0	(0.0)	$1.0x10^3$	(1.4)	$1.0x10^3$	(3.8)
225	UL4	500.0	(1.1)	$2.0x10^4$	(3.3)	$5.0x10^3$	(2.7)
226	UL4	0.0	(0.0)	0.00	(0.0)	0.0	(0.0)
227	UR4	0.0	(0.0)	0.00	(0.0)	0.0	(0.0)
228	UR4	0.0	(0.0)	400.0	(1.4)	0.0	(0.0)
229	UL5	$5.0x10^3$	(7.7)	0.0	(0.0)	0.0	(0.0)
230	LL5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
231	UL4	0.0	(0.0)	0.0	(0.0)	$6.0x10^3$	(0.3)
232	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
233	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)

N° = number
% vc = percentage of viable count.

Table 9 Distribution of *Streptococcus salivarius* as cfu/ml and as a percentage of the viable count at various sites in relation to the contact area.

Patient N°	Tooth N°	Away		Side		Below	
		cfu/ml	(% vc)	cfu/ml	(% vc)	cfu/ml	(% vc)
211	UL4	$1.0x10^3$	(17.5)	10.0	(0.1)	0.0	(0.0)
212	LR4	0.0	(0.0)	10.0	(11.1)	400.0	(9.7)
215	LR5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
216	LR4	0.0	(0.0)	0.0	(0.0)	$1.0x10^3$	(0.6)
217	LR5	0.0	(0.0)	10.0	(7.1)	0.0	(0.0)
218	UR5	70.0	(12.5)	0.0	(0.0)	120.0	(0.4)
219	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
220	UR4	60.0	(16.6)	0.0	(0.0)	0.0	(0.0)
221	LR4	0.0	(0.0)	40.0	(1.0)	0.0	(0.0)
222	LL4	100.0	(6.8)	100.0	(0.1)	0.0	(0.0)
223	UR5	$2.4x10^4$	(8.1)	0.0	(0.0)	$2.0x10^3$	(0.6)
224	UR4	$1.2x10^4$	(12.9)	$1.0x10^3$	(1.4)	0.0	(0.0)
225	UL4	0.0	(0.0)	0.0	(0.0)	$5.1x10^4$	(27.2)
226	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
227	UR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
228	UR4	0.0	(0.0)	400.0	(1.4)	0.0	(0.0)
229	UL5	$3.0x10^3$	(1.5)	0.0	(0.0)	0.0	(0.0)
230	LL5	0.0	(0.0)	0.0	(0.0)	90.0	(1.7)
231	UL4	0.0	(0.0)	$1.3x10^4$	(10.9)	$4.0x10^4$	(1.9)
232	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
233	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)

N° = number
% vc = percentage of viable count.

Table 10 Distibution of unidentified gram positive cocci as cfu/ml and as a percentage of the viable count at various sites in relation to the contact area.

Patient N°	Tooth N°	Away		Side		Below	
		cfu/ml	(% vc)	cfu/ml	(% vc)	cfu/ml	(% vc)
211	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
212	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
215	LR5	$4.2x10^3$	(9.24)	200.0	(2.0)	0.0	(0.0)
216	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
217	LR5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
218	UR5	100.0	(17.9)	0.0	(0.0)	300.0	(1.8)
219	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
220	UR4	0.0	(0.0)	$1.0x10^3$	(0.2)	0.0	(0.0)
221	LR4	10.0	(0.5)	0.0	(0.0)	0.0	(0.0
222	LL4	50.0	(3.4)	100.0	(0.1)	0.0	(0.0)
223	UR5	0.0	(0.0)	$4.8x10^4$	(10.2)	0.0	(0.0)
224	UR4	400.0	(0.4)	$1.5x10^4$	(20.6)	$1.1x10^3$	(4.21)
225	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
226	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
227	UR4	20.0	(40.0)	400.0	(12.1)	10.0	(0.1)
228	UR4	20.0	(3.8)	$7.4x10^3$	(25.9)	$7.2x10^3$	(84.3)
229	UL5	20.0	(0.1)	$1.8x10^3$	(10.0)	$5.0x10^3$	(4.6)
230	LL5	0.0	(0.0)	$2.7x10^3$	(38.1)	$2.14x10^3$	(40.3)
231	UL4	0.0	(0.0)	$6.0x10^3$	(0.5)	$6.0x10^4$	(2.8)
232	LR4	50.0	(2.2)	0.0	(0.0)	300.0	(8.5)
233	UL4	20.0	(20.0)	500.0	(3.5)	30.0	(30.0)

N° = number
% vc = percentage of viable count.

Table 11 Distribution of *Actinomyces naeslundii* as cfu/ml and as a percentage of the viable count at various sites in relation to the contact area.

Patient N°	Tooth N°	Away		Side		Below	
		cfu/ml	(% vc)	cfu/ml	(% vc)	cfu/ml	(% vc)
211	UL4	0.0	(0.0)	3030.0	(11.2)	80	(3.3)
212	LR4	140.0	(4.0)	10.0	(11.1)	$1.8x10^3$	(43.9)
215	LR5	$2.0x10^4$	(44.0)	100.0	(1.0)	330.0	(3.9)
216	LR4	$1.0x10^3$	(8.7)	$6.0x10^3$	(3.4)	$5.0x10^3$	(2.7)
217	LR5	460.0	(24.1)	20.0	(15.4)	180.0	(48.1)
218	UR5	70.0	(12.5)	$4.9x10^3$	(44.6)	$1.18x10^4$	(8.8)
219	LR4	$8.0x10^3$	(22.9)	0.0	(0.0)	$6.0x10^3$	(36.14)
220	UR4	80.0	(23.6)	$1.2x10^4$	(2.8)	0.0	(0.0)
221	LR4	500.0	(24.9)	$1.5x10^3$	(36.6)	$4.8x10^3$	(44.8)
222	LL4	$1.13x10^3$	(76.9)	$3.9x10^4$	(35.5)	$4.0x10^3$	(19.7)
223	UR5	$5.5x10^4$	(17.5)	$1.04x10^5$	(17.4)	$2.0x10^4$	(5.6)
224	UR4	$1.5x10^4$	(15.8)	$5.0x10^3$	(6.9)	200.0	(0.8)
225	UL4	$7.2x10^3$	(15.7)	$1.3x10^5$	(21.6)	$4.5x10^4$	(8.9)
226	UL4	$6.0x10^3$	(50.0)	$9.2x10^3$	(65.6)	$2.42x10^3$	(22.8)
227	UR4	0.0	(0.0)	$1.3x10^3$	(40.0)	0.0	(0.0)
228	UR4	200.0	(38.5)	0.0	(0.0)	0.0	(0.0)
229	UL5	$2.2x10^4$	(11.3)	$2.2x10^3$	(12.2)	$1.8x10^4$	(16.4)
230	LL5	0.0	(0.0)	60.0	(0.8)	500.0	(8.6)
231	UL4	50.0	(0.23)	$7.0x10^4$	(58.6)	$1.7x10^6$	(78.5)
232	LR4	330.0	(24.7)	0.0	(0.0)	150.0	(4.2)
233	UL4	20.0	(20.0)	$5.5x10^3$	(38.2)	60.0	(54.5)

N° = number
% vc = percentage of viable count.

Table 12 Distribution of *Actinomyces odontolyticus* as cfu/ml and as a percentage of the viable count at various sites in relation to the contact area.

Patient N°	Tooth N°	Away		Side		Below	
		cfu/ml	(% vc)	cfu/ml	(% vc)	cfu/ml	(% vc)
211	UL4	0.0	(0.0)	$7.0x10^3$	(25.9)	0.0	(0.0
212	LR4	0.0	(0.0)	0.0	(0.0)	200.0	(4.8)
215	LR5	500.0	(1.1)	0.0	(0.0)	0.0	(0.0)
216	LR4	100.0	(0.9)	0.0	(0.0)	0.0	(0.0)
217	LR5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
218	UR5	40.0	(7.1)	$2.0x10^3$	(18.2)	200.0	(1.2)
219	LR4	$3.0x10^3$	(8.6)	$1.2x10^4$	(78.43)	$1.0x10^3$	(6.0)
220	UR4	0.0	(0.0)	0.0	(0.0)	$1.0x10^3$	(0.4)
221	LR4	0.0	(0.0)	0.0	(0.0)	300.0	(2.8)
222	LL4	0.0	(0.0)	$8.0x10^3$	(5.9)	$4.0x10^3$	(8.3)
223	UR5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
224	UR4	$7.0x10^3$	(7.6)	$6.0x10^3$	(8.3)	100.0	(0.4)
225	UL4	$2.28x10^4$	(49.9)	0.0	(0.0)	$1.3x10^4$	(18.7)
226	UL4	0.0	(0.0)	0.0	(0.0)	$6.4x10^3$	(60.3)
227	UR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
228	UR4	0.0	(0.0)	0.0	(0.0)	0.0	(0. 0)
229	UL5	0.0	(0.0)	$5.2x10^3$	(28.89)	$1.0x10^4$	(9.1)
230	LL5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
231	UL4	$2.2x10^4$	(10.2)	800.0	(0.67)	$32.13x10^4$	(14.9)
232	LR4	20.0	(1.9)	0.0	(0.0)	200.0	(5.63)
233	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)

N° = number
% vc = percentage of viable count.

Table 13 Distribution of *Actinomyces israelii* as cfu/ml and as a percentage of the viable count at various sites in relation to the contact area.

Patient N°	Tooth N°	Away		Side		Below	
		cfu/ml	(% vc)	cfu/ml	(% vc)	cfu/ml	(% vc)
211	UL4	0.0	(0.0)	$7.0x10^3$	(25.9)	20	(0.8)
212	LR4	0.0	(0.0)	20.0	(22.2)	0.0	(0.0)
215	LR5	$1.2x10^3$	(2.63)	80.0	(57.15)	90.0	(1.0)
216	LR4	0.0	(0.0)	$7.0x10^3$	(3.9)	0.0	(0.0)
217	LR5	70.0	(3.6)	80.0	(57.15)	40.0	(1.7)
218	UR5	0.0	(0.0)	0.0	(0.0)	$1.0x10^3$	(3.3)
219	LR4	$5.0x10^3$	(14.5)	0.0	(0.0)	0.0	(0.0)
220	UR4	0.0	(0.0)	$3.0x10^4$	(69.6)	$9.0x10^3$	(3.73)
221	LR4	400.0	(19.9)	0.0	(0.0)	0.0	(0.0)
222	LL4	0.0	(0.0)	$2.0x10^3$	(1.5)	100.0	(2.0)
223	UR5	$3.3x10^4$	(11.14)	0.0	(0.0)	$2.66x10^4$	(7.5)
224	UR4	$5.0x10^3$	(5.4)	$5.0x10^3$	(6.9)	0.0	(0.0)
225	UL4	$1.7x10^3$	(3.7)	$2.9x10^5$	(48.2)	$1.0x10^3$	(0.5)
226	UL4	0.0	(0.0)	$3.0x10^3$	(23.4)	$6.4x10^3$	(60.3)
227	UR4	0.0	(0.0)	0.0	(0.0)	150.0	(1.97)
228	UR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
229	UL5	0.0	(0.0)	0.0	(0.0)	$1.6x10^4$	(14.57)
230	LL5	$1.0x10^3$	(16.7)	300.0	(4.24)	0.0	(0.0)
231	UL4	$1.0x10^3$	(4.97)	100.0	(0.08)	$1.0x10^4$	(0.46)
232	LR4	108.0	(46.75)	$2.74x10^3$	(34.73)	0.0	(0.0)
233	UL4	0.0	(0.0)	100.0	(0.67)	0.0	(0.0)

N° = number
% vc = percentage of viable count.

Table 14 Distribution of *Lactobacillus* spp. as cfu/ml and as a percentage of the viable count at various sites in relation to the contact area.

Patient N°	Tooth N°	Away		Side		Below	
		cfu/ml	(% vc)	cfu/ml	(% vc)	cfu/ml	(% vc)
211	UL4	0.0	(0.0)	10.0	(0.1)	0.0	(0.0)
212	LR4	0.0	(0.0)	0.0	(0.0)	0.0	0.0)
215	LR5	140.0	(0.32)	100.0	(1.02)	0.0	(0.0)
216	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
217	LR5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
218	UR5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
219	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
220	UR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
221	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
222	LL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
223	UR5	$2.12x10^3$	(6.7)	$5.0x10^3$	(1.7)	$2.22x10^3$	(0.72)
224	UR4	0.0	(0.0)	0.0	(0.0)	0.0	0.0)
225	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
226	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
227	UR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
228	UR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
229	UL5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
230	LL5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
231	UL4	20.0	(0.1)	$2.71x10^3$	(2.28)	245.0	(0.11)
232	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
233	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)

N° = number
% vc = percentage of viable count.

Table 15 Distibution of unidentified gram positive roads as cfu/ml and as a percentage of the viable count at various sites in relation to the contact area.

Patient N°	Tooth N°	Away		Side		Below	
		cfu/ml	(% vc)	cfu/ml	(% vc)	cfu/ml	(% vc)
211	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
212	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
215	LR5	190.0	(0.42)	150.0	(1.5)	0.0	(0.0)
216	LR4	0.0	(0.0)	40.0	(0.1)	10.0	(0.1)
217	LR5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
218	UR5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
219	LR4	0.0	(0.0)	620.0	(4.1)	0.0	(0.0)
220	UR4	60.0	(5.9)	0.0	(0.0)	0.0	(0.0)
221	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0
222	LL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
223	UR5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
224	UR4	0.0	(0.0)	0.0	(0.0)	$3.4x10^3$	(13.0)
225	UL4	0.0	(0.0)	40.0	(0.1)	0.0	(0.0)
226	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
227	UR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
228	UR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
229	UL5	10.0	(0.1)	0.0	(0.0)	340.0	(0.3)
230	LL5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
231	UL4	$1.0x10^3$	(3.7)	250.0	(0.2)	$1.0x10^3$	(0.1)
232	LR4	0.0	(0.0)	120.0	(1.5)	0.0	(0.0)
233	UL4	0.0	(0.0)	$3.9x10^3$	(27.1)	10.0	(10.0)

N° = number
% vc = percentage of viable count.

Table 16 Distribution of *Neisseria* as cfu/ml and as a percentage of the viable count at various sites in relation to the contact area.

Patient N°	Tooth N°	Away		Side		Below	
		cfu/ml	(% vc)	cfu/ml	(% vc)	cfu/ml	(% vc)
211	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
212	LR4	$1.0x10^3$	(28.3)	0.0	(0.0)	100.0	(2.4)
215	LR5	0.0	(0.0)	0.0	(0.0)	30.0	(0.4)
216	LR4	$1.5x10^3$	(21.7)	0.0	(0.0)	0.0	(0.0)
217	LR5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
218	UR5	0.0	(0.0)	300.0	(2.7)	0.0	(0.0)
219	LR4	600.0	(1.7)	20.0	(0.0)	0.0	(0.0)
220	UR4	110.0	(30.5)	$3.0x10^4$	(7.0)	0.0	(0.0)
221	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
222	LL4	210.0	(14.3)	$8.0x10^3$	(8.0)	200.0	(0.4)
223	UR5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
224	UR4	$8.0x10^3$	(8.6)	0.0	(0.0)	0.0	(0.0)
225	UL4	300.0	(0.7)	100.0	(0.1)	0.0	(0.0)
226	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
227	UR4	0.0	(0.0)	300.0	(9.0)	0.0	(0.0)
228	UR4	90.0	(17.3)	0.0	(0.0)	20.0	(0. 2)
229	UL5	$5.0x10^3$	(0.8)	$2.6x10^3$	(14.5)	80.0	(0.1)
230	LL5	0.0	(0.0)	30.0	(0.4)	0.0	(0.0)
231	UL4	$1.3x10^3$	(6.0)	700.0	(0.6)	50.0	(0.1)
232	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
233	UL4	0.0	(0.0)	800.0	(5.5)	0.0	(0.0)

N° = number
% vc = percentage of viable count.

Table 17 Distribution of *Veillonella* as cfu/ml and as a percentage of the viable count at various sites in relation to the contact area.

Patient N°	Tooth N°	Away		Side		Below	
		cfu/ml	(% vc)	cfu/ml	(% vc)	cfu/ml	(% vc)
211	UL4	0.0	(0.0)	0.0	(0.0)	120.0	(4.6)
212	LR4	0.0	(0.0)	0.0	(0.0)	200.0	(4.9)
215	LR5	0.0	(0.0)	10.0	(0.1)	20.0	(0.2)
216	LR4	0.0	(0.0)	0.0	(0.0)	$5.0x10^3$	(2.8)
217	LR5	580.0	(30.4)	0.0	(0.0)	120.0	(5.1)
218	UR5	40.0	(7.1)	$2.6x10^3$	(23.6)	$6.3x10^3$	(20.7)
219	LR4	7000.0	(20.0)	0.0	(0.0)	0.0	(0.0)
220	UR4	0.0	(0.0)	$1.0x10^4$	(2.3)	$2.0x10^5$	(83.0)
221	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
222	LL4	10.0	(0.8)	$1.4x10^4$	(10.4)	$9.0x10^3$	(17.6)
223	UR5	$1.9x10^4$	(6.4)	$1.4x10^4$	(2.99)	$1.6x10^4$	(4.5)
224	UR4	(0.0)	0.0	0.0	(0.0)	$4.5x10^3$	(17.2)
225	UL4	0.0	(0.0)	530.0	(0.08)	0.0	(0.0)
226	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
227	UR4	0.0	(0.0)	20.0	(0.6)	270.0	(2.82)
228	UR4	20.0	(3.9)	0.0	(0.0)	20.0	(0.23)
229	UL5	0.0	(0.0)	0.0	(0.0)	80.0	(0.07)
230	LL5	0.0	(0.0)	0.0	(0.0)	40.0	(0.75)
231	UL4	0.0	(0.0)	0.0	(0.0)	$2.4x10^3$	(0.15)
232	LR4	0.0	(0.0)	0.0	(0.0)	200.0	(5.6)
233	UL4	0.0	(0.0)	$1.4x10^3$	(9.72)	0.0	(0.0)

N° = number
% vc = percentage of viable count.

Table 18 Distribution of *Fusobacterium* as cfu/ml and as a percentage of the viable count at various sites in relation to the contact area.

Patient N°	Tooth N°	Away		Side		Below	
		cfu/ml	(% vc)	cfu/ml	(% vc)	cfu/ml	(% vc)
211	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
212	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
215	LR5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
216	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
217	LR5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
218	UR5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
219	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
220	UR4	0.0	(0.0)	$3.0x10^4$	(7.0)	0.0	(0.0)
221	LR4	0.0	(0.0)	200.0	(4.9)	900.0	(8.4)
222	LL4	0.0	(0.0)	0.0	0.0	0.0	(0.0)
223	UR5	2000.0	(1.0)	0.0	(0.0	0.0	(0.0)
224	UR4	0.0	(0.0)	2000.0	(2.7)	100.0	(3.8)
225	UL4	300.0	(0.7)	0.0	(0.0)	0.0	(0.0)
226	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
227	UR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
228	UR4	0.0	(0.0)	0.0	(0.0)	20.0	(0.2)
229	UL5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
230	LL5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
231	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
232	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
233	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)

N° = number
% vc = percentage of viable count.

Table 19 Distribution of Obligate Anaerobes as cfu/ml and as a percentage of the viable count at various sites in relation to the contact area.

Patient N°	Tooth N°	Away		Side		Below	
		cfu/ml	(% vc)	cfu/ml	(% vc)	cfu/ml	(% vc)
211	UL4	0.0	(0.0)	0.0	(0.0)	20.0	(0.80)
212	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
215	LR5	$9.0x10^3$	(19.8)	400.0	(4.1)	0.0	(0.0)
216	LR4	0.0	(0.0)	0.0	0.0	0.0	(0.0)
217	LR5	0.0	(0.0)	10.0	(7.14)	50.0	(2.1)
218	UR5	30.0	(5.4)	0.0	(0.0)	0.0	(0.0)
219	LR4	$1.0x10^3$	(2.9)	180.0	(1.18)	4200.0	(25.3)
220	UR4	0.0	(0.0)	$3.0x10^4$	(7.0)	8000.0	(3.3)
221	LR4	0.0	(0.0)	$1.2x10^3$	(29.3)	2000.0	(18.7)
222	LL4	0.0	(0.0)	$1.4x10^4$	(10.3)	8000.0	(15.7)
223	UR5	$9.0x10^3$	(2.9)	0.0	(0.0)	0.0	(0.0)
224	UR4	$1.0x10^4$	(10.8)	$1.1x10^4$	(15.2)	$1.2x10^3$	(4.9)
225	UL4	800.0	(1.8)	250.0	(0.04)	$6.0x10^3$	(3.2)
226	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
227	UR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
228	UR4	0.0	(0.0)	$3.4x10^3$	(11.89)	0.0	(0.0)
229	UL5	0.0	(0.0)	0.0	(0.0)	10.0	(0.01)
230	LL5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
231	UL4	0.0	(0.0)	0.0	(0.0)	400.0	(0.1)
232	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
233	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)

N° = number
% vc = percentage of viable count.

Table 20 Distribution of Facultative Anaerobes as cfu/ml and as a percentage of the viable count at various sites in relation to the contact area.

Patient N°	Tooth N°	Away		Side		Below	
		cfu/ml	(% vc)	cfu/ml	(% vc)	cfu/ml	(% vc)
211	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
212	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
215	LR5	0.0	(0.0)	$5.0x10^3$	(50.6)	0.0	(0.0)
216	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
217	LR5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
218	UR5	0.0	(0.0)	200.0	(1.8)	0.0	(0.0)
219	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
220	UR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
221	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
222	LL4	0.0	(0.0)	0.0	(0.0)	6000.0	(11.8)
223	UR	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
224	UR4	$1.3x10^4$	(13.6)	0.0	(0.0)	$3.6x10^3$	(13.7)
225	UL4	300.0	(0.7)	0.0	(0.0)	0.0	(0.0)
226	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
227	UR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
228	UR4	0.0	(0.0)	0.0	(0.0)	0.0	(0. 0)
229	UL5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
230	LL5	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
231	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
232	LR4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)
233	UL4	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)

N° = number
% vc = percentage of viable count.

APPENDIX E
TABLES 1-2

Table 1 Distribution of *S. mutans 'c'* and *S. sobrinus 'd'* as percentage of proportional count at various sites in relation to the contact area, using IF-staining.

Tooth No.	Tooth location	Away (A)		Side (S)		Below(B)	
		*S. m**	*S. s**	*S. m*	*S. s*	*S. m*	*S.s*
Single tooth							
212[c1]	LR4	2.8	ND	0.2	ND	ND	ND
215	LR4	0.8	0.5	0.9	0.8	0.38	0.4
217	LR5	ND	ND	0.6	0.7	ND	3.3
218	UR5	0.2	ND	ND	ND	0.4	ND
219	LR4	2.4	ND	2.0	ND	1.2	ND
220	UR4	0.2	ND	1.3	ND	0.9	ND
221	LR4	ND	ND	ND	ND	4.8	0.8
222	LL4	0.7	ND	1.4	ND	4.6	ND
223	UR5	1.9	1.6	2.8	3.1	0.6	4.4
224	UR4	2.7	0.8	0.25	2.2	3.3	1.5
225	UL4	1.3	ND	0.2	ND	2.4	0.6
226	UL4	ND	ND	1.0	ND	1.4	2.5
227	UR4	1.6	0.4	2.2	0.4	1.9	ND
228	UR4	ND	ND	ND	ND	ND	ND
229	UL5	1.4	0.5	6.9	0.9	5.1	0.4
230	LL5	ND	0.6	2.2	1.0	2.1	0.8
231	UL4	1.5	ND	4.0	ND	6.8	ND
232	LR4	3.2	ND	ND	1.1	12.3	ND
233[c2]	UL4	0.3	ND	ND	ND	6.9	ND
238	UL4	ND	ND	ND	1.4	0.4	1.7
241	LL4	ND	ND	ND	ND	ND	ND
250	LL4	0.4	ND	ND	ND	1.6	ND
253	LR4	ND	ND	0.4	1.1	1.2	ND
202	UR4	0.2	ND	ND	1.3	0.2	2.0
204	UL5	ND	1.5	ND	2.2	2.0	2.2
195	UR5	0.6	ND	1.0	ND	1.3	ND
160	UR5	0.6	ND	1.2	ND	1.0	1.9
133	UR4	ND	ND	ND	ND	ND	1.9
20	LR5	1.0	ND	ND	ND	0.9	2.4
27	UL5	ND	ND	ND	ND	0.3	ND
33	UL5	4.7	ND	ND	1.2	0.4	ND
45	UR4	ND	ND	0.2	ND	0.2	1.3
52	LL5	ND	ND	1.0	ND	4.1	ND
53	LR4	ND	ND	0.2	ND	1.65	0.3
61	UR4	ND	1.3	0.9	1.3	0.4	1.8

Table 1 Continued

Tooth No.	Tooth location	Away (A)		Side (S)		Below(B)	
		*S. m**	*S. s***	*S. m*	*S. s*	*S. m*	*S.s*
Two (ipsilateral) teeth							
127	UL4	0.5	2.0	4.0	1.7	ND	4.2
128	LL4	ND	ND	ND	ND	ND	3.1
150	UR4	ND	ND	ND	ND	1.4	ND
151	LR4	ND	ND	ND	ND	ND	ND
152	LR5	ND	ND	0.7	2.6	3.1	3.4
153	LR5	ND	ND	ND	0.7	0.5	0.8
155	LL5	ND	ND	0.3	ND	ND	ND
183	UL5	ND	1.9	1.3	ND	1.5	ND
184	LL5	0.3	1.2	1.2	1.2	0.9	1.8
187	UR4	ND	ND	0.9	ND	ND	ND
189	LR4	ND	ND	4.7	ND	ND	1.3
191	UR4	ND	ND	ND	ND	0.3	ND
192	LR4	0.2	ND	ND	ND	ND	ND
193	UR4	ND	0.7	ND	1.7	0.9	1.4
194	LR4	ND	ND	ND	ND	ND	ND
200	UR4	ND	ND	0.6	ND	0.6	1.1
201	LR4	ND	ND	ND	ND	1.6	ND
214	LR5	0.8	0.5	0.9	0.7	ND	2.2
Two (contralateral) teeth							
7	UR5	ND	ND	1.5	ND	0.8	1.0
9	LL5	ND	ND	ND	ND	ND	ND
10	UR5	0.6	ND	0.2	ND	0.2	2.0
11	UL5	0.9	2.4	ND	2.5	1.0	2.8
12	UR4	ND	ND	ND	ND	ND	ND
13	UL4	ND	ND	ND	ND	ND	ND
41	LR4	ND	ND	ND	ND	ND	ND
42	LL4	ND	ND	ND	ND	ND	0.8
206	UL4	ND	ND	ND	ND	ND	ND
207	LR5	ND	2.5	1.7	4.1	2.5	4.3
210	UR4	ND	ND	1.8	ND	1.5	ND
211	UL4	ND	ND	ND	ND	0.2	ND
234	UR4	ND	ND	0.4	ND	ND	1.8
235	UL4	ND	ND	ND	ND	1.3	3.3
236	UR4	ND	ND	ND	ND	ND	ND
237	UL4	ND	ND	ND	ND	ND	ND
239	UR5	0.8	2.6	1.3	1.2	14.9	1.6
240	LL5	ND	ND	0.4	ND	ND	0.9
242	LL5	ND	0.2	0.2	2.2	0.4	ND
243	UR5	ND	ND	0.2	ND	1.6	ND
248	UR5	2.1	ND	2.2	2.8	2.8	0.7

Table 1 Continued

Tooth No.	Tooth location	Away (A)		Side (S)		Below(B)	
		S. m[*]	*S. s*[**]	*S. m*	*S. s*	*S. m*	*S.s*
249	UL5	1.2	2.8	0.7	2.6	7.3	ND
Three teeth							
163	LR5	ND	ND	ND	ND	1.3	ND
164	UL5	ND	1.5	0.5	1.7	2.1	3.2
165	LL5	ND	ND	ND	ND	0.6	ND
242	LL5	ND	ND	ND	2.2	0.4	0.4
243	UR5	ND	ND	ND	ND	1.5	ND
244	UL5	1.3	ND	2.6	1.9	4.9	2.4
245	UL4	2.0	2.4	2.4	ND	3.4	3.1
246	UR4	ND	ND	ND	ND	0.7	ND
247	LR4	0.8	ND	ND	ND	0.8	2.1
255	UL4	1.5	0.4	4.7	ND	3.8	0.9
256	LL4	ND	ND	ND	ND	ND	ND
257	UR4	1.9	ND	2.0	0.8	2.7	1.1
Four teeth							
156	UR4	ND	ND	0.9	ND	0.2	ND
157	LR4	ND	ND	ND	ND	ND	ND
158	UL4	ND	ND	ND	ND	ND	ND
159	LL4	0.2	ND	ND	ND	ND	ND

[*] *S. m* = *S. mutans*,[**] *S. s* = *S. sobrinus*.
ND = <0.2

[C1] TO [C2] and C, culture data available in appendix D.

Table 2 Distribution of *Lactobacillus spp.* as percentage of proportional count at various sites in relation to the contact area, using IF-staining.

Tooth No.	Tooth location	Away (A)	Side (S)	Below(B)
7	UL4	ND	ND	2.8
8	LR5	ND	ND	ND
9	LL5	ND	ND	3.2
18	LR5	3.8	ND	ND
24	LR4	ND	ND	ND
25	LL5	ND	ND	ND
27	UL5	1.9	0.7	3.2
31	UL4	ND	0.9	0.4
35	UR4	ND	ND	ND
36	LL5	ND	ND	ND
41	LR4	ND	ND	ND
45	UR4	ND	ND	ND
52	LL5	ND	ND	3.6
53	LR4	ND	ND	ND
59	UR4	ND	ND	ND
61	UR4	1.8	5.4	5.2
118	LR4	ND	ND	ND
133	UL5	ND	ND	ND
135	UL4	ND	ND	ND
143	UL4	ND	ND	ND
144	LL5	ND	ND	ND
145	LR5	ND	ND	ND
161	UL5	ND	3.6	ND
163	LR5	ND	ND	3.6
162	UR5	ND	ND	ND
164	UL5	0.6	5.9	8.0
165	LL5	2.9	3.3	4.5
174	UR4	ND	ND	ND
181	UL4	1.0	ND	ND
183	UL5	4.7	ND	3.6
187	UR4	ND	ND	ND
195	UR5	ND	ND	ND
196	UL5	ND	ND	ND
200	UR4	ND	ND	ND
202	UR4	ND	ND	ND
210	UR4	ND	ND	ND
211	UL4	ND	ND	ND
212	LR4	ND	ND	ND
215	LR5	2.2	0.6	2.6
217	LR5	ND	ND	ND
218	UR5	ND	ND	ND

Table 2 continued

Tooth No.	Tooth location	Away (A)	Side (S)	Below(B)
219	LR4	ND	0.9	ND
220	UR4	ND	ND	ND
221	LR4	ND	ND	ND
222	LL4	ND	ND	ND
223	UR5	0.4	0.6	4.0
224	UR4	ND	ND	ND
225	UL4	ND	ND	ND
226	UL4	ND	ND	ND
227	UR4	ND	ND	ND
228	UR5	ND	ND	ND
229	UL5	ND	ND	ND
230	LL5	ND	ND	ND
231	UL4	0.3	8.0	1.9
232	LR4	ND	ND	ND
233	UL4	ND	ND	ND
234	UR4	0.5	1.0	2.8
235	UL4	ND	ND	4.8
236	UR4	ND	ND	ND
237	UL4	ND	5.6	ND
238	UL4	ND	ND	ND
240	LL5	ND	1.9	ND
241	LL4	ND	ND	ND
242	LL5	ND	ND	ND
245	UL4	ND	ND	ND
248	UR5	ND	ND	ND
250	LL4	ND	ND	ND
253	LR4	ND	ND	ND
255	UL4	ND	ND	ND
257	UR4	2.0	ND	3.9
258	LU4	ND	ND	ND

ND = <0.2

PUBLICATIONS

Ahmady K, Marsh PD, Newman HN (1993). Distribution of *Streptococcus mutans* and *Streptococcus sobrinus* at sub-sites in human approximal dental plaque. Caries Res. 27: 135-139.

Abstracts

Ahmady K, Marsh PD, Newman HN (1992). Distribution of mutans streptococci at sub-sites in approximal plaque. J Dent Res. 71: 645.

Ahmady K, Marsh PD, Newman HN (1993). Streptococci in children: an ecological study of approximal plaque using microsite sampling. J Dent Res. 72: 735.

Ahmady K, Marsh PD, Newman HN (1993). Microbial ecology of plaque from caries-prone sites on approximal tooth surface in schoolchildren. Caries Res. 27: 238.

Ahmady K, Marsh PD, Challacombe SJ, Newman HN (1994). Culture and immunofluorescence studies of mutans streptococci and lactobacilli in schoolchildren. J Dent Res. 73: 851.

Ahmady K, Challacombe SJ, Marsh PD, Newman HN (1994). Ecological study of mutans streptococci and lactobacilli at sub-sites in approximal dental plaque of schoolchildren. Caries Res. 28: 219.

Ahmady K, Challacombe SJ, Beighton D, Marsh PD, Newman HN (1995). Analysis of 16S rRNA genes of human dental plaque streptococci. Submitted for BSDR 1996.

aries Research
ditor-in-Chief: J.M. ten Cate, Amsterdam

eprint
ublisher: S. Karger AG, Basel
rinted in Switzerland

Basic Sciences

Caries Res 1993;27:135–139

Distribution of *Streptococcus mutans* and *Streptococcus sobrinus* at Sub-Sites in Human Approximal Dental Plaque

K. Ahmady[a]
P. D. Marsh[c]
H. N. Newman[a]
J. S. Bulman[b]

[a] Department of Periodontology, Institute of Dental Surgery, British Postgraduate Medical Federation, University of London,
[b] Department of Public Dental Health, Institute of Dental Surgery, London,
[c] Pathology Division, PHLS Centre for Applied Microbiology and Research, Salisbury, UK

Key Words
Approximal plaque
Bacterial distribution
Streptococcus mutans
Streptococcus sobrinus

Abstract
The distribution and prevalence of *Streptococcus mutans* and *Streptococcus sobrinus* were determined at three sub-sites in human approximal plaque: away from (A), to the side of (S) and below (B) the contact area. Small plaque samples were taken from all three sub-sites on clinically sound approximal surfaces of a single premolar from each of 21 schoolchildren. *S. mutans* was detected significantly more often and in higher proportions than *S. sobrinus* from sub-sites A ($p = 0.019$), S ($p = 0.034$) and B ($p = 0.004$). *S. mutans* was detected in highest proportions from the B site compared to the A site ($p = 0.025$); there were no significant differences in the isolation frequency or prevalence of *S. sobrinus* from any of the sub-sites. *S. mutans* and *S. sobrinus* were never isolated together from the A sub-sites and were recovered together most commonly from the B sub-sites ($p<0.01$). It is concluded that *S. mutans* and *S. sobrinus* preferentially colonise the most caries-prone site apical to the contact area.

Mutans streptococci do not colonise the dentition uniformly. Their presence varies from tooth to tooth and even on different surfaces of the same tooth [Gibbons et al., 1974]; an overall decrease in their prevalence from molar to anterior teeth has also been demonstrated [Keene et al., 1981; Kristoffersson et al., 1984; Lindquist and Emilson, 1990].

The two species of mutans streptococci most commonly isolated from human tooth samples are *S. mutans* and *S. sobrinus*. Numerous studies of different populations have found that *S. mutans* is isolated more often than *S. sobrinus* from teeth [Loesche, 1986; Bratthall, 1991] and from individual tooth surfaces [Lindquist and Emilson, 1991a]. However, there have been few studies of the distribution of bacteria at different sub-sites on an individual tooth surface. Duchin and van Houte [1978] found that the prevalence of mutans streptococci could vary markedly between plaque from a 'white-spot' lesion and from neighbouring sound enamel on the same tooth surface. More recently, wide variations were reported in the colonisation of small discrete areas on the buccal surfaces of clinically sound maxillary teeth by *S. mutans* and *S. sobrinus* [Lind-

Received:
June 16, 1992
Accepted after revision:
November 16, 1992

Professor H. N. Newman
Institute of Dental Surgery
University of London
256 Gray's Inn Road
London WC1X 8LD (UK)

0008–6568/93/0272–0135
$2.75/0

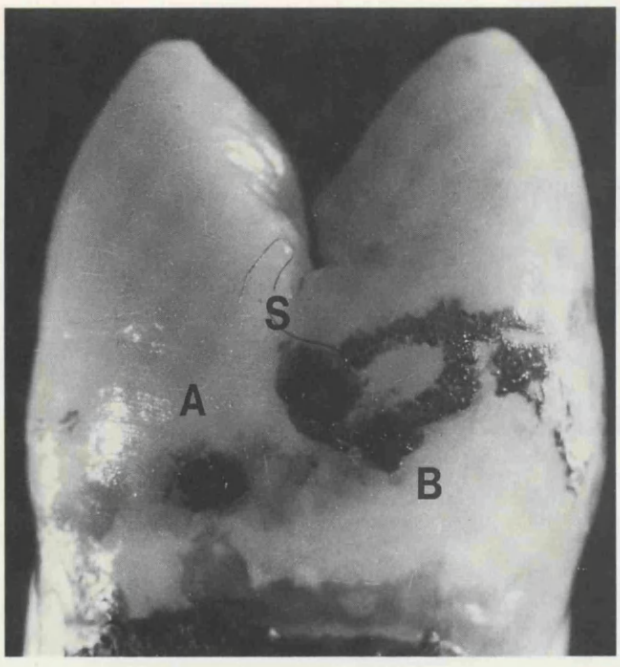


Fig. 1. Location of dental plaque around the contact area of a premolar to indicate the sampling sites away (A) from, to the side (S) of, and below (B) the contact area.

quist and Emilson, 1991b]. However, as these surfaces have a low risk of caries, the aim of the present study was to determine the distribution and prevalence of these two species of mutans streptococci at three sub-sites on the more caries-susceptible human approximal tooth surface.

Materials and Methods

Clinical Material and Plaque Sampling

From each of 21 schoolchildren (16 girls, 5 boys; mean age = 12±1.6 years), one clinically sound premolar tooth (extracted for orthodontic reasons) was used for the study. Both the child and parent or guardian had given their consent for the teeth to be used in the study.

The freshly extracted teeth were collected in sterile reduced transport fluid (RTF) [Syed and Loesche, 1972] and processed within 5 min of extraction for cultural analysis. Teeth were rinsed with phosphate-buffered saline, pH 7.2, to remove blood and loose debris, and plaque was stained with 0.5% (w/v) indigo carmine (ICI, Cheshire, UK), which has been shown not to affect bacterial viability [Marsh et al., 1989a]. Teeth were then washed again with phosphate-buffered saline to visualise the contact area on the approximal surfaces as outlined by the staining procedure. All 21 teeth studied exhibited an intact full ring of plaque around the contact area. About 1–2 mm^2 of gingival margin plaque was removed with a sterile dental curette from three sub-sites: away from (A), to the side of (S), and below (B) the contact area (fig. 1), and suspended in 800 µl RTF in a sterile microcentrifuge tube (Alpha Laboratories, Eastleigh, UK). The teeth were dried and the sample sites marked with red nail varnish and photographed to record their location.

Bacterial Analysis of Plaque Samples

Plaque samples were dispersed by vortexing with glass beads for 1 min, followed by aspirating (12 times) with a syringe and 25-gauge needle [Bush et al., 1990]. Samples were then serially diluted to 10^{-3} in RTF. One-hundred-microlitre aliquots were spread over the surface of pre-reduced selective and non-selective media. Columbia Agar Base (Oxoid, Basingstoke, UK) supplemented with 7% (v/v) horse blood (Oxoid) was used to enumerate the total cultivable flora. TYC (Lab M, Bury, UK) and TYC supplemented with 20% (w/v) sucrose and 0.1 unit/ml bacitracin (TYCSB) [van Palenstein Helderman et al., 1983] were used to recover streptococci and mutans streptococci, respectively. All media were incubated in an atmosphere of 80% N_2, 10% H_2, 10% CO_2 in an anaerobic chamber for 5 days at 37 °C. Viable counts of *S. mutans* and *S. sobrinus* were expressed as a percentage of the total cultivable microflora and also as a percentage of the total number of sites colonised (isolation frequency).

Bacterial Identification

All colonies on TYC and TYCSB were gram-stained and tested for catalase production; cells that were gram-positive cocci and catalase-negative were identified as streptococci. Two representative colonies of each morphological type were identified. Mutans streptococci were distinguished from other streptococci biochemically and subdivided by their fermentation of N-acetylglucosamine, amygdalin, inulin, mannitol, melibiose, sorbitol and raffinose, the hydrolysis of arginine and aesculin and on the production of H_2O_2 and α-glucosidase [Hardie and Bowden, 1976; Coykendall and Gustafsen, 1986; Beighton et al., 1991]. Substrates were obtained either from Lab M or Sigma (Poole, UK); fermentation tests and enzyme assays were performed according to the manufacturer's instructions.

Statistical Analysis

As the distribution of mutans streptococci data was highly skewed, it was normalised by means of a log transformation of the colony-forming units per millilitre and percentage viable counts. Where microorgansisms were not detected, half of the minimum level of detection was used. The half minimum level of detection for mutans streptococci was 5 cfu/ml and 0.05% of the viable count. Analysis was based on using these log-transformed data, using one-way analysis of variance. However, due to the lower isolation frequencies involved, it was impossible to normalise the data for *S. sobrinus* using a similar transformation and, therefore, analysis of the latter was carried out using the Friedman test [Siegel, 1956]. This meant that isolation frequencies of *S. sobrinus* and *S. mutans* at each sub-site were compared using the Wilcoxon matched pairs signed ranks test [Siegel, 1956].

Comparison of mean isolation frequency of the sites was carried out using McNemar's test for comparing two proportions for the paired data [Armitage and Berry, 1987]. Any association between the two species was tested using the comparison of two proportions for unpaired data [Armitage and Berry, 1987].

Table 1. Mean and percentage viable counts of the total cultivable microflora, *S. mutans* and *S. sobrinus* from small samples of approximal plaque taken at sites away from (A), to the side of (S) and below (B) the contact area

Bacterium	Mean viable count±SE					
	A		S		B	
	(mean cfu/ml±SE)×10^3	%	(mean cfu/ml±SE)×10^3	%	(mean cfu/ml±SE)×10^3	%
Total anaerobic count	3.1±14.3 (50–296,100)	100	102.7±38.1 (90–601,600)	100	155.8±102.7 (100–2,165,300)	100
	a					
	b					
S. mutans	6.4±5.5 (ND–11,500)	* 9.9±4.8 (ND–78)	4.7±2.4 (ND–49,000)	** 7.6±2.3 (ND–28.9)	7.3±4.6 (ND–96,000)	*** 13.3±4.1 (ND–53.5)
S. sobrinus	0.01±0.009 (ND–200)	* 1.9±8.4 (ND–78)	0.3±0.24 (ND–5,000)	** 3.9±14.0 (ND–67.4)	2.0±1.3 (ND–22,000)	*** 4.7±18.0 (ND–83)

Figures in parentheses represent ranges. ND = Not detected (see 'Materials and Methods'). Horizontal braces: one-way analysis of variance of log-transformed raw data, and subsequently t test on the differences between the log-transformed data for the B and A; a = log-transformed cfu/ml, p = 0.004; b = log-transformed percentage of viable count, p = 0.02.

Vertical braces: Wilcoxon matched pair signed ranks test, *p = 0.019, **p = 0.034, ***p = 0.004.

Results

The samples removed from the three sub-sites on each premolar were of small size as indicated by the low numbers of cultivable bacteria recovered from each sub-site (table 1). Only three surfaces had (minor) white spot lesions, with no clear association with higher levels of mutans streptococci.

Mutans streptococci were isolated from 95% of the 21 tooth surfaces, and from 68% of the 63 sub-sites (table 2) Mutans streptococci were isolated preferentially from the sub-sites below the contact area (B, 91%). Comparison of the distribution of the two species at the three sub-sites revealed a significant difference between A and B when *S. mutans* and *S. sobrinus* were recovered together (A = 0%, B = 29%; p<0.01). When *S. mutans* or *S. sobrinus* were isolated alone, there was no significant difference in the percentage of sub-sites colonized (table 2), although there was a trend for the isolation frequency of mutans streptococci and *S. mutans* to increase in the order A < S < B. When isolates were identified, *S. mutans* was the predominant and frequently the only species of mutans streptococci recovered from a site (table 2). *S. sobrinus* was rarely isolated in the absence of *S. mutans* and, when it was recovered with *S. mutans*, it was located preferentially at sub-sites B (table 2).

Table 2. Number and percentage (in parentheses) of premolars and sub-sites colonised by mutans streptococci (ms)

Bacterium	Premolars colonised (n = 21)	Sub-sites colonised			
		A (n = 21)	S (n = 21)	B (n = 21)	total (n = 63)
ms	20 (95)	10 (48)	14 (67)	19 (91)	43 (68)
S. mutans alone	11 (52)	9 (43)	10 (48)	12 (57)	31 (49)
S. sobrinus alone	1 (5)	1 (5)	1 (5)	1 (5)	3 (5)
S. mutans + *S. sobrinus*	8 (38)	0 (0)	3 (14)	6 (29)	9 (14)
		*		*	

McNemar's test highly significant, *p<0.01. The limits for the difference between the proportions of isolations: SND = 2.64; 95% CI (7.40–49.74).

The comparison of two proportions for unpaired data was applied to see if there was an association between the isolation of both species from sub-sites A, S and B. The proportion of *S. sobrinus* in samples with *S. mutans* was 0.0, 0.23 and 0.33 at sub-sites A, S and B, respectively, and

for *S. sobrinus* in samples without *S. mutans* it was 0.08, 0.13 and 0.33 at sub-sites A, S, and B, respectively, indicating no statistical association between the two species.

A one-way analysis of variance on the log-transformed percentage viable count of *S. mutans* was performed at each sub-site; the F value was 3.15, which was significant at the 5% level, and a paired t test on the differences between the log-transformed data for the sub-sites B and A gave $p = 0.025$ (table 1). There were no statistically significant differences between *S. sobrinus* levels at any sub-site, even though the trend was for proportions to increase in the order $A < S < B$ (table 1). At each sub-site, the percentage viable count of *S. mutans* was significantly higher than that of *S. sobrinus* at all three locations ($p = 0.019$, $p = 0.034$ and $p = 0.004$ for sub-sites A, S and B, respectively; table 1).

On an individual surface, *S. mutans* could be recovered on its own from one sub-site ($A = 9$, $B = 12$, $S = 10$), and *S. sobrinus* alone from another. Similarly, *S. mutans* and *S. sobrinus* could be recovered together from one sub-site ($B = 6$, $S = 3$), and there were instances where neither species or only one species might be found at the other two sub-sites on that surface (table 2). Of the 63 sub-sites on the 21 approximal tooth surfaces examined, only one had no mutans streptococci at any sub-site. Four, nine and seven teeth had one, two or three sub-sites, respectively, colonised by mutans streptococci.

There was no pattern for the relative concentrations of each species when they were recovered together at the same sub-site. At the 9 sub-sites where both species were found, *S. mutans* was present in greater numbers at 4 sub-sites, *S. sobrinus* predominated at 4 sub-sites, and one sub-site had identical levels of each species.

Discussion

The frequency of isolation of mutans streptococci from premolar approximal surfaces in our study was higher than that reported in a larger survey of older individuals [Lindquist and Emilson, 1991a]. In agreement with other studies [for reviews, see Loesche, 1986; Bratthall, 1991], we found *S. mutans* significantly more often, and in higher proportions, than *S. sobrinus*. The colonisation level of *S. sobrinus* in our study was similar to that of Lindquist and Emilson [1991a], but our isolation of *S. mutans* was greater. This might reflect the use of different media in the two studies to isolate mutans streptococci. Higher viable counts of *S. mutans* have been obtained on TYC and TYCSB compared with MSB agar [Schaeken et al., 1986], although others have not found such marked differences [Beighton, 1991]. The difference might also relate to the narrow range of age group in our study, to differences in sampling, or to our use of a wide range of physiological tests for distinguishing between *S. mutans* and *S. sobrinus*, rather than a reliance on colonial morphology [Emilson, 1983; Lindquist and Emilson, 1991a, b].

Ideally, associations between plaque composition and caries should be determined at defined sub-sites [Marsh et al., 1989b; Bush et al., 1989, 1990; Gill et al., 1991]. Approximal surfaces are particularly prone to caries, especially just apical to the contact area [Leigh, 1927; Newman and Morgan, 1980]. It is not feasible to study the distribution of individual bacterial species around the contact area on teeth in situ, and correlations between approximal plaque and caries have had to be based on radiographs and samples of the microflora of the entire macroarea. Therefore, possible important sub-site differences in microflora (especially in mutans streptococci) and caries could have been obscured.

In this study, we used teeth that had been extracted for orthodontic reasons to investigate the distribution of mutans streptococci in plaque in the gingival margin area in several locations in relation to the contact area. The most frequent recoveries and highest proportions of mutans streptococci were from the sub-site below (B) the contact area. This was also the sub-site from which *S. mutans*, alone or in combination with *S. sobrinus*, was isolated most commonly, and in the highest proportions. It was also the sub-site from which *S. mutans* was detected most commonly by immunofluorescence [Gill et al., 1991]. Although we found *S. mutans* and *S. sobrinus* together on 38% of teeth, there was no evidence of a positive association in their presence at any sub-site, in contrast to the findings of Lindquist and Emilson [1991a]. Indeed, one or both of these species could be absent from the other sites sampled on the same tooth surface.

Factors responsible for regulating the distribution of these two species at a given site are only little understood. Indeed, there have been few reports in which small plaque samples have been studied in relation to clearly defined sub-sites [Bush et al., 1989; Boyar et al., 1989; Marsh et al., 1989b; Gill et al., 1991]. The preferential location of mutans streptococci below the contact area might be related to the stagnation in this location (as in occlusal fissures), the ability of such species to avoid antibacterial factors at this site or, more likely, to this site being less accessible to the buffering and substrate clearance effect of saliva. In such an environment, the pH may be lower for longer periods, and such conditions would favour the

growth of mutans streptococci at the expense of less acidogenic and aciduric species [Donoghue and Newman 1976; Newman et al., 1976; Bradshaw et al., 1989]. *S. mutans* is also more bacteriocinogenic than *S. sobrinus* [Lindquist and Emilson, 1991b], and growth of the latter species can be suppressed by bacteriocin production by *S. mutans* [Ikeda et al., 1988]. Alternatively, differences in the pattern of colonisation by *S. mutans* and *S. sobrinus* might be related to the fact that they possess different abilities to metabolise locally available indigenous nutrients [Homer and Beighton, 1991]. Further work will be necessary to determine whether other oral species show similar patterns of distribution at sub-sites in plaque.

Acknowledgements

This research was supported by the UK Biscuit, Cake, Chocolate, and Confectionary Alliance.

References

Armitage P, Berry G: Statistical Methods in Medical Research, ed 2. Oxford, Blackwell, 1987, pp 123–125.

Beighton D: The value of salivary bacterial counts in the prediction of caries activity, in Johnson NW (ed): Risk Markers for Oral Diseases: Dental Caries, Cambridge, Cambridge University Press, 1991, vol 1: Dental Caries: Markers of High and Low Risk Groups, pp 313–326.

Beighton D, Hardie JM, Whiley RA: A scheme for the identification of viridans streptococci. J Med Microbiol 1991;35:367–372.

Boyar RM, Thylstrup A, Holmen L, Bowden GH: The microflora associated with the development of initial enamel decalcification below orthodontic bands in vivo in children living in a fluoridated-water area. J Dent Res 1989;68: 1734–1738.

Bradshaw DJ, McKee AS, Marsh PD: Effects of carbohydrate pulses and pH on population shifts within oral microbial communities in vitro. J Dent Res 1989;68:1298–1302.

Bratthall D: The global epidemiology of mutans streptococci; in Johnson NW (ed): Risk Markers for Oral Diseases. Cambridge, Cambridge University Press, 1991, vol 1: Dental Caries Markers of High and Low Risk Groups, pp 287–312.

Bush MS, Challacombe SJ, Newman HN: A quantitative immunofluorescence study of the association between *Streptococcus mutans* and approximal caries. Microb Ecol Health Dis 1989; 2:261–266.

Bush MS, Challacombe SJ, Newman HN: A method for the identification of *Streptococcus mutans* in gingival margin plaque by immunofluorescence. Caries Res 1990;24:23—29.

Coykendall AL, Gustafson KB: Taxonomy of *Streptococcus mutans;* in Hamada S, Michalek SM, Kinyino H, Menaker L, McGhee JL (eds): Molecular Microbiology and Immunology of *Streptococcus mutans*. Amsterdam, Elsevier, 1986, pp 21–28.

Donoghue HD, Newman HN: Effect of glucose and sucrose on survival in batch culture of *Streptococcus mutans* C67 – 1 and a noncariogenic mutant, C67-25. Infect Immun 1976;13: 16–21.

Duchin S, van Houte J: Relationship of *Streptococcus mutans* and lactobacilli to incipient smooth surface dental caries in man. Arch Oral Biol 1978;23:667–673.

Emilson CG: Prevalence of *Streptococcus mutans* with different colonial morphologies in human plaque and saliva. Scand J Dent Res 1983;91: 26–32.

Gibbons RJ, Cohen L, Hay DI: Strains of *Streptococcus mutans* and *Streptococcus sobrinus* attach to different pellicle receptors. Infect Immun 1986;52:555–561.

Gibbons RJ, Depaola PF, Spinell DM, Skobe Z: Interdental localization of *Streptococcus mutans* as related to dental caries experience. Infect Immun 1974;9:481–488.

Gill S, Newman HN, Challacombe SJ, Bulman J: An immunofluorescence study of the distribution of *Streptococcus mutans* on children's teeth. Microb Ecol Health Dis 1991;4:253–257.

Hardie JM, Bowden GH: Physiological classification of oral viridans streptococci. J Dent Res 1976;55:A166–A176.

Homer KA, Beighton D: N-acetylglucosamine metabolism by mutans streptococci. J Dent Res 1991;70:672.

Ikeda T, Kurita T, Hirasawa M: Suppression of *Streptococcus sobrinus* 6715 (*g*) in plaques by *Streptococcus mutans* 32k (*c*). J Oral Pathol 1988;17:471–474.

Keene HJ, Horton IM, Handler SF: *Streptococcus mutans* approximal plaque index as a new epidemiologic tool for defining the parameters of *Streptococcus mutans* infection in human populations. J Dent Res 1981;56:5–10.

Kristoffersson K, Axelsson P, Bratthall D: Effect of a professional tooth cleaning program on interdentally localized *Streptococcus mutans*. Caries Res 1984;18:385–390.

Leigh RW: Studies of the enamel. J Am Dent Assoc 1927;14:592–600.

Lindquist B, Emilson CG: Distribution and prevalence of mutans streptococci in human dentition. J Dent Res 1990;69:1160–1166.

Lindquist B, Emilson CG: Dental location of *Streptococcus mutans* and *Streptococcus sobrinus* in humans harbouring both species. Caries Res 1991a;25:146–152.

Lindquist B, Emilson CG: Interactions between and within *Streptococcus mutans* and *Streptococcus sobrinus* isolated from humans harbouring both species. Scand J Dent Res 1991b; 99:498–504.

Loesche WJ: Role of *Streptococcus mutans* in human dental decay. Microbiol Rev 1986;50:353–380.

Marsh PD, Bevis RA, Newman HN, Hallsworth AS, Robinson C, Weatherell JA, Pitter AFV: Antibacterial activity of some plaque-disclosing agents and dyes. Caries Res 1989a;23:348–350.

Marsh PD, Featherstone A, McKee AS, Hallsworth AS, Robinson C, Weatherell JA, Newman HN, Pitter AFV: A microbiological study of early caries of approximal surfaces in schoolchildren. J Dent Res 1989b;68:1151–1154.

Newman HN, Donoghue HD, Britton AB: Effect of glucose and sucrose on the survival in batch culture of *Streptococcus mutans* C67-1 and a non-cariogenic mutant C67-25: Morphological studies. Microbios 1976;15:113–125.

Newman HN, Morgan WJ: Topographical relationship between plaque and approximal caries. Caries Res 1980;14:428–433.

van Palenstein Helderman WH, IJsseldijk M, Huis in't Veld JHJ: A selective medium for the two major subgroups of the bacterium *Streptococcus mutans* isolated from human dental plaque and saliva. Arch Oral Biol 1983;28:599–603.

Schaeken MJM, van der Hoeven JS, Franken HCM: Comparative recovery of *Streptococcus mutans* on five isolation media, including a new simple selective medium. J Dent Res 1986;65:906–908.

Siegel S: Non-Parametric Statistics for the Behavioral Sciences. New York, McGraw-Hill, 1956, pp 166–172.

Syed SA, Loesche WJ: Survival of human dental plaque flora in various transport media. Appl Microbiol 1972;24:638–644.