A LONGITUDINAL INVESTIGATION OF THE MICROFLORA ASSOCIATED WITH DEVELOPING LESIONS OF NURSING CARIES

BY

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DEDICATION

Sometimes my mind was crazy
Sometimes my mind was numb
Sometimes it sang like angel wings
And beeped like kingdom come.

This poem, by Dennis Lee, aptly describes the range of emotions through which I traversed during this project. The loving support of my wife, Sylvia, helped me to deal with many of the conflicts of schedules and priorities which arose during this period and she never tired of my asking 'how does this sound?'. Although they didn't understand why their dad couldn't be involved in many of their activities, my daughters, Adrienne and Laura, accepted my absence of both body and mind without complaint.

Going, going, gone
No, daddy won't be long.
I have to go to work.
Wasn't I a jerk?
Going, going, gone.

I am indebted to my supervisor, Dr. George Bowden, for his supreme patience and unsurpassed wisdom. Everyone can think of individuals in their lives who have made a significant contribution to their personal growth. George is such an individual in my life.

Thanks and appreciation should also be extended to Nora, Anna, Ingrid and Bev, 'the gang in the lab', to my secretary Ira Sliwinski, who 'took the wheel' during the final stages of my writing and to those faculty members who provided valuable advice at various time during this research. Doug Gates deserves special recognition because of the extra effort he always made to complete lab work on schedule. In addition, the University of Manitoba deserves credit for allowing faculty members to pursue higher education within the University proper.

Lastly, I would like to thank the children and their parents who participated in this study. Without their cooperation this work would not have been possible.

It is to all those I have mentioned that I dedicate this work.

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ABSTRACT

Nursing caries is a pattern of rampant dental caries affecting the maxillary primary incisors of infants and young children who engage in non-nutritive sucking habits from the bottle or breast. Because the tooth surfaces at risk are readily accessible, facilitating reproducible sampling and accurate diagnosis of lesions which develop rapidly, nursing caries is an excellent human model for the study of the ecology of the microflora associated with dental caries. A longitudinal analysis of the bacterial communities on selected tooth surfaces was made in two groups of nine children aged 10-16 months, a control group of children from Winnipeg who were at low risk of nursing caries and a test group of children from the Cross Lake Indian Reserve who were at high risk of nursing caries. Two susceptible sites, the labial and palatal surfaces of a maxillary primary incisor and a non-susceptible control site, the labial surface of a mandibular primary incisor, were sampled at approximately 6-week intervals in all children for one year. In control subjects , the central occlusal fissure of a maxillary primary molar and the dorsal surface of the tongue were also sampled on the same schedule.

Plaque samples were cultured on a range of media including selective media for some genera and species. Each of the 390 samples was analyzed for the presence, isolation frequency and percentage contribution to the microflora of a total of

thirty-four species within *Streptococcus, Actinomyces, Rothia, Bacterionema, Lactobacillus, Neisseria, Veillonella, Fusobacterium, Leptotrichia, Micrococcus, Hemophilus, Actinobacillus, Bacteroides, Capnocytophyga* and Yeasts. The data were analyzed on a species-by-species and site-by-site comparison basis within and between the children by parametric and non-parametric statistical methods. The Lambda of Good permutation calculation was used to measure the similarity of populations between sites.

The plaque samples obtained from the control children provided unique data on the commensal microflora on dental surfaces and the tongue in preschool children. The oral microflora of preschool children was found to be much more complex than has been reported. Comparisons between the flora of control and test children showed that each site was qualitatively similar, although quantitative variations did exist. However, few of these were statistically significant. Some surfaces were consistently free of specific organisms. For example, *Lactobacillus* was never isolated from the upper or lower primary incisor teeth in control children or from the lower primary incisor teeth in test children.

Five of the test children developed lesions at susceptible sites and four remained caries-free. Comparisons of the microfloras between caries-active test children, caries-free test children and control children were made. Shifts

occurred in the microbial populations in the communities associated with susceptible surfaces as lesions developed. The microflora at these surfaces in caries-active test children became increasingly dissimilar to the microflora from susceptible surfaces in both caries-free test children and control children. Significant increases in the levels and isolation frequencies of *S. mutans, Lactobacillus, Veillonella* and *A. viscosus* occurred at the sites which developed lesions compared to the control sites in the same mouths and susceptible and control sites in the control children. An important observation was that the susceptible sites which remained caries-free in caries-active test children supported a flora which was not significantly different from that of sites which developed lesions. This suggests that the environment at these sites allowed the establishment of a potentially pathogenic flora and that other factors are involved in the production of a lesion.

There were no significant differences in the microflora between susceptible sites and control sites in the four test children who remained caries-free.

The results of this study confirm previous work which has shown that localized increases of *S. mutans* and *Lactobacillus* are closely associated with the caries process. However, the results also show a positive association of *A. viscosus* with the development of lesions. An inverse relationship with the caries process was noted

between *S. mutans* and *S. mitior* and between *A. viscosus* and *A. naeslundii*. The variations in the composition of the microflora between children with caries were such that no one organism could be implicated as the sole cause of a carious lesion. Considering that caries is the end result of a complex series of ecologic and metabolic interactions between the microflora , the tooth surface and the oral environment, it is likely that, given appropriate conditions, any combination of aciduric and acidogenic organisms could produce a carious lesion. The results of this study support this hypothesis.

CHAPTER ONE

I. INTRODUCTION

'The destruction of the enamel as it occurs in the decay process must be regarded as essentially a parasitico-chemical process. The loosening of the enamel prisms is caused by acids concerning whose origin there can be no doubt: they arise in the mouth by fermentation of the carbohydrates.... The bacteria directly participate in the process, inasmuch as they invade the broken down enamel, perhaps drive the prisms further apart, and destroy the remnant of organic matter. Microorgansims do not exert a direct influence on normal enamel,' (Miller 1890).

With statements like this, and others similar to it, W.D. Miller has generally been credited to be the originator of the chemico-parasitic theory of dental caries, despite his acknowledgement that Professor Erdl in 1843 and Leber and Rottenstein in 1867, followed later by Miles and Underwood in 1881, had proposed much the same theory. Perhaps Miller's close association with the eminent microbiologist Robert Koch and his voluminous outpouring of 164 scientific articles over 25 years contributed to the recognition he has received as the father of oral microbiology.

Although Miller recognized the importance of bacteria in the caries process he failed to note the importance in the etiology of dental caries of the 'gelatinous microbic plaques' described by both Williams (1897) and Black (1899). Miller's lack of appreciation of this evidence can be noted in his statement:

'I am not convinced that the nature of this film has been determined with sufficient clearness, or whether its significance and importance may not have been somewhat overrated',(1890).

With the development of a new method for grinding enamel

sections, Williams (1897) was able to show that bacteria were always associated with the decaying enamel and concluded that they must be the source of organic acids which were acting on the enamel. The thickness of the microbial film prevented the saliva from washing away the acids. He concluded:

'There seems to be no reason why any rational man should continue to doubt that acid-forming bacteria are the sole active cause of dental caries....if the environing conditions of the teeth are such as to favour the development and activity of acid-producing bacteria, and if those bacteria are permitted to become attached to the surface of the enamel, it is doomed, although it may be the most perfect that was ever formed. On the other hand, if those environing conditions are not present the worst enamel will not decay', (1897).

While this was one of the first references to the importance of the environment in the development of dental caries it did not prevent dental scientists from searching for a specific pathogen in the caries process. The influence of Koch's postulates led many researchers to look for a specific cariogenic pathogen and most to ignore the latent pathogenic potential of many of the microorganisms indigenous to and ubiquitously found within the oral cavity. Only recently have investigators turned their attention to the importance of microbial ecology in the etiology of dental caries. It is ironic that today's researchers, well financed and using sophisticated methods and equipment unavailable until recently, have, in some measure, turned away from Koch's postulates and returned to a research direction established over one hundred years ago by investigators who had comparatively little with which to work and whose efforts have been

largely forgotten.

II. The Mouth as an Environment

In early investigations of the oral microflora, the mouth was regarded as a single, uniform environment (Black 1898, 1899, Bloomfield, 1920, Cornelison, 1946, Harrison, 1948, Miller, 1890). Investigators assumed that the composition of dental plaque was homogeneous and that the oral microflora varied little from one area of the mouth to another (Kligler, 1915). Bibby (1938) was one of the first to show that the oral microflora did indeed vary from site to site, although he relied on a smear method because of the poor knowledge at that time of the cultural requirements of oral bacteria. He correctly perceived many of the problems which still plague investigators today, namely that a classification system be used that satisfactorily distinguishes the types of bacteria isolated, that methods of collecting material must be devised which will more effectively exclude organisms from adjacent surfaces/sites, that we recognize the possibility the oral flora may vary with time at each site/surface and that a system to diagnose accurately clinical conditions under study be devised. Harrison,(1948), stated that 'An unpublished study by Hemmens, Blayney, and Harrison revealed that except for some variation in proportionate distribution, there is little difference between the bacterial flora of the saliva and that of the dental surfaces.'

Krasse (1953, 1954) refuted these findings with a series of studies which showed that *S. salivarius* was present in much greater numbers in saliva than in dental plaque. He reasoned that since dental plaque formed on 'inactive dental surfaces', i.e. surfaces not mechanically cleaned by mastication, it was not unreasonable to assume that the method of collection (chewing paraffin wax) would remove more bacteria from exposed surfaces such as the tongue and thus determine to a greater extent the bacterial flora of saliva. Subsequent studies confirmed that the source of salivary bacteria is most often the tongue (Gibbons et al, 1964; Gordon and Jong, 1968; Richardson and Jones, 1958). Despite Bibby's findings, as late as 1969, Handelman and Hess, (1969), using sophisticated techniques unavailable to Bibby in 1938, were unable to show any significant differences in the overall bacterial profile between dental sites although they did find that the percentage distribution varied with the site sampled. They concluded that the use of pooled plaque samples was a legitimate method.

Others have confirmed Bibby's findings of dissimilarity between microfloras in different areas of the mouth. Donoghue (1972) showed that the composition of dental plaque from eight sites in a ten-year-old female differed from site to site. Although the method of sampling was questionable and may have introduced artifact into the results, (teeth were first extracted, washed, and sectioned

before the plaque was removed) differences between sites were still demonstrable. Reasons suggested for site to site variations in microflora included differences in oral hygiene between sites and anatomic variations between sites leading to differences in plaque thickness with its effect on anerobiosis and pH. Huis In't Veld et al, (1979), showed that the microbial composition of plaque obtained from the same clinically sound tooth surface in different individuals was less heterogeneous than the composition of approximal and fissural plaques obtained from the same individual. Even plaque from different areas on the same tooth surface has been found to be quite dissimilar. The levels of *S. mutans* in the centre of white spot lesions can be remarkably high while sound enamel 100 um away harbours negligible levels (Duchin et al, 1978). Considerable site-to-site variations have also been reported for the composition of mature approximal dental plaque (Bowden et al, 1975). The bacterial composition of 58 samples of approximal plaque from 10 individuals was similar at the generic level but qualitative and quantitative differences detected at the species level were so pronounced that each site could be considered unique.

Given the variety of surfaces available for colonization and the extreme variability between similar surfaces, it is reasonable to assume that environmental conditions will fluctuate from surface to surface or even on the same surface from time to time making each surface habitable by only

certain organisms at certain times. The environmental conditions in deep fisures- slow diffusion of nutrients, relatively low oxygen tension- are quite different from conditions on the buccal and lingual surfaces of a tooth where soluble carbohydrates from the diet and proteins from the gingival crevice are readily available to the microflora. This reasoning in conjunction with the realization that a significant portion of the oral flora is facultatively or obligately anerobic and coincident improvements in sampling, transport and cultivation techniques have fostered the concept that the mouth is actually comprised, in environmental terms, of a series of microecosystems, each with its own environmental determinants and characteristic microflora.

Although a great deal is known about the microflora of the oral cavity, little is known about the mouth as an environment or how the properties of that environment affect the composition and activity of the microflora. It is known that environmental determinants can and probably do change with time. This is particularly true of the growing child whose oral cavity changes dramatically from an edentulous state at birth, offering only a shedding epithelial surface to bacteria, to a dentulous state within the first year of life and provision of a non-shedding enamel surface. With increasing age and maturation of the child, the physical, chemical and biological environmental determinants of the mouth may change numerous times. Changes in externally

applied environmental factors such as diet (Alfano, 1982; Carlsson and Egelberg, 1965; Krasse, 1965; Morhart and Fitzgerald, 1976), antibiotic therapy (Bibby, 1970), dental treatment (Sakamaki and Bahn, 1968; Shklair et al, 1956), and oral hygiene procedures (Axelsson et al, 1978; Bellini et al, 1981; Kerebel et al, 1985; Palin-Palokas et al, 1984; Togelius et al, 1984) have all been demonstrated to have an effect on the composition of the oral microflora. However, it is important to note that the relationship of the environment to the microflora is not unidirectional. By its metabolism the microflora can alter the physical and chemical properties of an environment and induce changes within a community (Dirksen et al, 1963; Donoghue et al, 1975; Holmberg et al, 1973; Huis In't Veld, 1980; Iwami et al, 1972; Liljemark et al, 1973; Mikx et al, 1978; Weerkamp et al; 1977).

III. The Distribution of Oral Microorganisms

The areas within the oral cavity which are considered to support different bacterial populations include:

a) lips, palate, buccal mucosa

b) tongue

c) saliva

d) gingiva/gingival crevice

e) teeth: (i) occlusal- pits/fissures

(ii) smooth surfaces- buccal/lingual

- approximal surfaces

- gingival areas.

(see table 1-1 for summary)

In this section, the composition of the microflora of the areas outlined above will reviewed. Most of the reported studies which will serve as the source of data for this section were conducted using a variety of selective media, different sampling procedures and different age groups of subjects. This makes comparison of studies somewhat difficult. In addition, most studies have not been able to overcome many of the technical problems associated with the handling of a mixed microbial flora, the components of which have very different growth requirements and different sensitivities to the dispersion method employed for inoculating media. The taxonomy of the oral flora is still imprecise, hence the identification of all isolates in a study of a particular region or regions of the mouth is not always possible. There remain a substantial number of oral organisms which have not been adequately described and classified. To overcome this, some investigators have chosen to allocate isolates to broad categories based on morphological descriptions, Gram stain reactions and cultural characteristics. This has advantages in that the entire flora can generally be categorized but it is inaccurate because many oral bacteria are pleomorphic and may vary in their Gram reaction.

Table 1-1
APPROXIMATE DISTRIBUTION OF THE CULTIVABLE BACTERIA IN VARIOUS ORAL HABITATS

Group	Coronal *Plaque*	Gingival *Crevice*	Tongue *Dorsum*	Buccal *Mucosa*	*Saliva*
1. Gram-positive cocci	40%	35%	50%	80%	55%
S. mutans	R	V	V	–	T
S. mitior	R	R	R	R	T
S. milleri	R	R	V	V	T
S. sanguis	R	R	R	R	T
S. salivarius	V	–	R	R	T
Staphylococcus	R	R	R	–	T
Micrococcus	V	V	R	–	T
Enterococcus	–	V	–	–	T
Peptostreptococcus	R	R	R	–	T
2. Gram-negative cocci	10%	10%	20%	2%	17%
Neisseria	R	V	R	V	T
Veillonella	R	R	R	V	T
3. Gram-positive rods	40%	30%	20%	<0.1%	16%
Actinomyces spp.	R	R	R	–	T
Nocardia spp.	R	V	V	–	T
Rothia	R	V	V	–	T
Bacterionema	R	V	V	–	T
Lactobacillus spp.	V	–	V	–	T
Arachnia spp.	R	V	V	–	T
Eubacterium spp.	R	R	V	–	T
Bifidobacterium	R	V	V	–	T
Propionibacterium	R	V	V	–	T
4. Gram-negative rods	9%	20%	10%	<0.1%	7%
Bacteroides spp.	R	R	R	V	T
Fusobacterium spp.	R	R	V	–	T
Haemophilus spp.	R	R	R	V	T
Leptotrichia	R	R	V	V	T
Eikenella	R	R	V	V	T
Vibrio	V	R	–	–	T
Actinobacillus spp.	R	R	V	–	T
Capnocytophyga	R	R	T	–	T
5. Spirochetes	1%	1-3%	<0.1%	<0.1%	–
Treponema spp.	V	R	–	–	T
Borrelia	V	R	–	–	T
6. Yeasts	V	V	V	V	T

R = resident to site, V = variable to site, T = transient to site, – = present in low numbers or absent

Sources: Bowden et al, (1975); Carlsson, (1967a); Gallagher et al, (1981); Gordon and Gibbons, (1966); Gordon et al, (1968); Handelman et al, (1969); Hardie and Bowden, (1974); Howell et al, (1965); Kilian et al, (1979); Krasse, (1970); Krasse, (1953); Loesche et al, (1972); Loesche et al, (1973); Loesche et al, (1985); McCarthy et al, (1965); Milnes and Bowden, (1982); Moore et al, (1982); Richardson and Jones, (1958); Socransky and Manganiello, (1971); Socransky et al, (1977); Theilade et al, (1982).

a) Lips, palate and buccal mucosa

Few studies have examined the microflora which colonizes the soft tissues of the oral cavity. Those which have, were concerned primarily with investigating adherence as an ecological determinant and focused on *Streptococcus* species (Gibbons and Van Houte, 1971, 1975; Gibbons et al, 1972). It is known that vestibular and palatinal surfaces are sparsely populated by bacteria and that few epithelial cells have areas with confluent bacterial films. Continuous cell desquamation appears to prevent large bacterial accumulations since the time available for cell reproduction is limited. Van Houte (1982) has proposed that the proportions of organisms found on mucosal surfaces are probably a reflection of the organisms' innate ability to adhere to the surface as well as their numbers in the saliva which bathes the exposed surfaces. *S. mitior, S. sanguis and S. salivarius* have all been found frequently and in high numbers on the buccal mucosa (Gibbons, 1984). *Veillonella* and *Neisseria* have also been found on the buccal mucosa and lips but in low numbers, usually less than 1% of the total cultivable flora (Liljemark and Gibbons, 1971).

Yeasts, in particular *Candida albicans*, are frequently found on damaged mucosa of the lips, at the commissures in children and the elderly and on the palates of denture wearers who do not maintain adequate oral hygiene and develop denture stomatitis (Sabiston et al, 1976). Carlsson et al (1975) found low numbers of lactobacilli on the

palates of normal children but the organisms did not persist on this surface, which is in keeping with the finding of Van Houte et al (1972) who found that lactobacilli do not adhere well to exposed surfaces and require a depression of some sort for mechanical retention.

Haemophili are frequently isolated in low numbers, $5.7 * 10^3$ colony forming units $cm.^{-2}$, from the soft tissues of the oral cavity (Kilian and Schiott, 1975).

b) Tongue

Krasse (1953) was one of the first researchers to investigate carefully the influence of the tongue microflora on the microflora isolated from other areas of the mouth. He felt that because the tongue has such a large surface area, due in part to its papillary structure, it could retain considerable quantities of food and therefore support a large and varied microflora. Gordon and Gibbons (1966) considered it necessary to isolate and study the predominant cultivable organisms of the tongue because of the inference that the microorganisms of the tongue influence the flora of the entire oral cavity. They sampled the tongues of six adults aged 21-32 years, taking care to remove saliva from the dorsal surface of the tongue prior to taking the sample. Their results are summarized in **table 1-2** as are the results of Milnes and Bowden (1982) for preschool children. Of interest was the finding that the levels of *S. salivarius* differed between aerobic and anerobic cultivation (53.5% of the total facultative streptococci after aerobic cultivation

versus 30% of the total facultative streptococci after anaerobic cultivation; not shown in table).

Table 1-2
THE PREDOMINANT CULTIVABLE FLORA OF THE TONGUE

	Organisms	Gordon & Gibbons 1966	Milnes & Bowden 1982
Gram +	facultative cocci	44.8*	-
	Total Streptococci	38.3	-
	S. milleri	-	8.0
	S. mitior	-	14.1
	S. mutans	-	17.2
	S. salivarius	8.2	18.7
	S. sanguis	-	0.4
	M. mucilaginosus	3.6	4.2
Gram +	anaerobic cocci (*Peptostreptococcus*)	4.2	ND
Gram +	facultative rods (Diphtheroids)	13.0	-
	A. naeslundii	-	5.1
	A. viscosus	-	3.4
	Rothia	-	0.3
Gram +	anaerobic rods	7.4	ND
Gram -	anaerobic cocci (*Veillonella*)	16.0	6.1
Gram -	facultative cocci (*Neisseria*)	3.4	12.3
Gram -	anaerobic rods (*Fusobacterium*) (*Vibrio*) (*Bacteroides*)	8.2	0.5
Gram -	facultative rods	3.2	<0.01
Total number of samples		6	15
Number of Subjects		6	4
Age range of Subjects		21-32 yrs.	22-32 mos.

* = % of total cultivable flora
\- = Not reported
ND = Not detected

After - Gordon and Gibbons (1966)
Milnes and Bowden (1982)

As well, the levels of this organism in relation to the total streptococcal count were lower (21.5%) than those previously reported by Krasse (1954), 67%, and Gibbons et al (1964), 55.3% of the total facultative streptococci on the tongue. The reasons for this discrepancy are apparently due to different isolation techniques employed by Gordon and Gibbons. Krasse and Gibbons et al cultured samples directly onto mitis-salivarius agar which was then incubated aerobically. The proportion that *S. salivarius* represented of the total streptococcal count could be determined directly from plate counts since *S. salivarius* forms large mucoid colonies on this medium and is, therefore, readily identifiable. In the later study by Gordon and Gibbons, samples were first inoculated on blood agar which was then incubated anaerobically. The higher counts of facultative streptococci on anaerobically incubated blood agar plates indicates that other facultative streptococci are favoured by anerobic incubation, whereas *S. salivarius* is favoured by aerobic incubation (Gordon and Gibbons, 1966). Although it was not discussed in the paper by Gordon and Gibbons, another reason for the difference in levels between papers is the likelihood that MS agar is selectively inhibitory to the different species of streptococci, favouring isolation of *S. salivarius* (van der Hoeven and Franken, 1986). Finally, the taxonomy of *S. salivarius* has not been clearly defined. Other streptococcal species are known to produce large mucoid colonies, considered characteristic of *S. salivarius* (Edwardsson, 1968) making identification by

colonial morphology inaccurate. The other predominant streptococcal species found on the tongue is *S. mitior* although it is found in lower numbers than *S. salivarius*. *Veillonella* is also reported to be a prominent member of the community (Gibbons and van Houte, 1975; Milnes and Bowden, 1982). These organisms comprise between 5 and 10% of the total cultivable flora of the tongue. Gordon and Gibbons (1966) described an unusual Gram-positive, catalase-variable coccus which was subsequently identified as *Micrococcus mucilaginosus* by Kocur et al (1971). Bowden (1969) has shown that this organism produces an extensive extracellular slime independent of the presence of carbohydrate and proposed that the slime could play a role in the lubrication of the tongue. Bergen and Kocur (1982) have proposed that *Micrococcus mucilaginosus* be renamed *Stomatococcus mucilaginosus*. Baird-Parker (1974) had placed *M. mucilaginosus* among species *incertae sedis* because several phenotypic characters of this organism were significantly different from those of micrococci. Since there are striking differences between the fatty acid composition of the cell wall of this organism and the cell walls of other micrococci and staphylococci (Jantzen et al, 1974) and the G+C content of the DNA of the new species is 56-60.4 mol% versus 64-75 mol% for other micrococci (Rubin et al, 1978), the proposal by Bergen and Kocur appears justifiable.

Although anaerobic microorganisms are not found in large

numbers on the tongue it is likely that, given the surface topography of the tongue, a low redox potential suitable for some anaerobes could develop. This may explain why frequently organisms such as *B. melaninogenicus* and fusobacteria can be isolated from the tongue (Milnes and Bowden, 1982). Other organisms which are commonly isolated from the tooth surface are seldom if ever isolated from the tongue, indicating that the tongue harbours a characteristic flora of its own.

c) Saliva

Saliva which is collected directly from the ducts of the various glands is free of bacteria. It is only when the saliva comes into contact with the oral tissues that one is able to find bacteria in whole saliva. It is for this reason that many do not consider saliva to have an indigenous microflora of its own. The number of bacteria in unstimulated saliva is in the order of 10^8 per millilitre, the total aerobes being slightly fewer than anaerobes (Handelman and Mills, 1965). Mechanically stimulated saliva contains more bacteria, possibly because the method of stimulation dislodges more bacteria from the oral surfaces (Krasse, 1954). It is now known that the bacteria found in saliva are neither quantitatively nor qualitatively representative of the oral flora found on any other surface in the mouth. It has been assumed that the surfaces in the mouth with the greatest area would be the largest contributors to the numbers and kinds of bacteria found in

saliva. Hence, since the tongue and buccal mucosa have by far the greatest surface area and are more accessible to the effects of mastication, oral hygiene procedures and normal muscular activity, the microflora in saliva most closely mirrors the microflora indigenous to these surfaces (Krasse, 1954; Gibbons et al, 1964). In all the studies which have examined the microflora of saliva, streptococci have predominated. Others have shown that the levels of particular organisms can increase when individuals refrain from oral hygiene procedures, indicating that dental plaque may contribute to the bacteria found in saliva but this is by no means a universal finding. Carlsson (1968) found that the salivary levels of *S. sanguis* increased on cessation of oral hygiene procedures in adult volunteers. He concluded that dental plaque could contribute equally with the tongue to the microbiota of saliva. Togelius et al (1984) examined a similar group of individuals to assess short term variation in salivary levels of *S. mutans*, the effect of discontinued oral hygiene on salivary *S. mutans* and the relation between salivary levels of *S. mutans* and the number of colonized approximal and occlusal sites. They could not demonstrate short-term variation in salivary levels and found that cessation of oral hygiene had little or no effect on the salivary levels of *S. mutans*. Since *S. mutans* preferentially colonizes protected areas of the tooth surface, making it relatively inaccessible to saliva, the collection procedure employed (chewing paraffin) would be

Table 1-3
THE PREDOMINANT CULTIVABLE FLORA OF HUMAN SALIVA

Organism	Gordon & Jong	Slack & Bowden	Richardson & Jone
Streptococci	41.0	56.3	16.3
S. salivarius	11.3	19.7	10.0
S. sanguis		42.0[a]	
S. mitis		20.0[b]	
Anaerobic Gram + cocci	13.0	ND	ND
Neisseria sp.	1.2	29.2	1.8
Veillonella sp.	15.9	2.9	15.4
Gram + rods and filaments	16.6	8.3	-
Gram - anaerobic rods	4.8	3.6	-
Gram - facultative rods	2.3	-	0.01

Organisms expressed as a percentage of the total viable count
ND = not detected
- = not reported
a = data from Carlsson, (1968)
b = data from Gibbons, (1980)
Table modified from Hardie and Bowden, (1974)

unlikely to increase shedding of this organism from the tooth surface. They did find that salivary samples reflected the number of colonized approximal surfaces but not the number of colonized occlusal surfaces. It is possible, therefore, that salivary samples could be helpful in assessing approximal caries risk in an individual. This finding is reinforced by the results of an earlier study by Emilson (1983) who showed that the salivary concentration of *S. mutans* was highly correlated with the relative proportion of the organism found in dental plaque. At salivary concentrations of between 10^5 and 10^6 colony-forming units/ml., *S. mutans* could be detected in plaque samples cultured on MS agar. This finding agrees with the study by van Houte and Green (1974), which examined the relationship between the salivary concentration of bacteria and their colonization of the tooth surface and found that

the critical level for detection of *S. mutans* on pre-cleaned tooth surfaces was 50 * 10^3 organisms per ml. of saliva. Other organisms have been similarly examined and it has been determined that each organism has its own threshold salivary level below which colonization is unlikely to occur (van Houte and Green, 1974).

The influence of saliva on the colonization and metabolism of the oral flora has been extensively investigated. One reason for undertaking investigations of this sort has been to determine whether saliva is capable of supporting its own flora. Until recently it was believed that whole saliva is a poor culture medium for oral microorganisms (Hardie, 1983). However De Jong et al (1986) studied the growth of microorganisms from dental plaque on plates prepared from filter-sterilized saliva and compared the compositions of microflora growing on non-specific saliva agar and blood agar. They found that whole saliva supported the growth of predominant species from supragingival plaque, though in somewhat lower numbers than on blood agar. The one exception was *A. odontolyticus* which was recovered frequently from saliva agar but not from blood agar. The authors stated that this organism was probably favoured by the reduced conditions of the saliva plate. Dithiothreitol, a commonly used reducing agent, was added to the saliva plates because it reduces disulfide bridges in glycoprotein solutions, thereby leading to a decrease in the viscosity of saliva, making filter sterilization of pooled saliva easier.

d) Gingival Crevice

The microflora of the gingival crevice has received considerable attention recently. This seems to have occurred partly because many investigators believe that the etiologic agents in dental caries have been delineated and, with no apparent end in sight to the recent caries decline in the western world, many would have the rest of us believe that caries is a disease, presently, of little consequence.

The bulk of investigation has centered around the microflora associated with the various forms of periodontal disease in the adult (Dzink et al, 1985; Holdeman et al, 1985; Loesche et al, 1985; Moore et al, 1982; Muller and Flores-de-Jacoby, 1985; Newman et al, 1984; Savitt and Socransky, 1984; Slots, 1976; 1977; Slots et al, 1978; Slots et al, 1980; van Palenstein Helderman, 1975; Wolff et al, 1985a; Wolff et al, 1985b; Zambon et al, 1985). Few investigations have been undertaken to examine the microflora of the healthy or diseased gingival crevice in the child. This, despite the hypothesis put forward by some that periodontal disease susceptibility in adulthood is linked with the acquisition of periodontopathic microorganisms in childhood (van der Velden et al, 1985).

While the microflora which is associated with gingivitis, periodontitis or periodontosis (juvenile periodontitis) is distinctly different from the microflora found supragingivally on the tooth surface, there is evidence to show that

the microflora in a healthy gingival sulcus is quite similar to that on the tooth surface. Slots examined the gingival microflora of seven dentists and found that it was predominately Gram-positive and facultatively anaerobic (Slots, 1977). Gram-positive rods were primarily *Actinomyces*, including *A. naeslundii, A. viscosus and A. israelii. Rothia* and *Arachnia propionica* were also isolated but in low numbers. *Streptococcus* was the predominant genus in all samples with *S. sanguis* and *S. mitis* being the major representatives. *Bacteroides* and *Fusobacterium* were regularly isolated and comprised 12.7% of the cultivable flora. *Veillonella* was found only in low numbers. The findings of this study are supported by later studies which examined a variety of age groups and used different techniques (Moore et al, 1982; 1984; Loesche and Syed, 1978; Syed and Loesche, 1978). De Araujo and Macdonald (1964) examined the microflora of the gingival crevice of 5 children aged 3-7 years and found that 52% of the isolates were Gram-positive and either facultative or anaerobic. As disease develops however, the gingival microflora undergoes transition from a facultative Gram-positive flora to predominately an anaerobic Gram-negative flora including many motile bacteria (Slots, 1977; Loesche et al, 1985).

Of interest is the finding by Moore et al (1984) that the gingival microflora of the child (4-6 years old) is frequently more complex and diverse than that of the adult. In exhaustive studies of experimental gingivitis in young

adults and children, they found significant differences between the bacteria isolated from the children and those isolated from the adults, particularly in reference to the development of gingivitis. Children examined had a flora predominated by *Actinomyces naeslundii , A. israelii, Streptococcus anginosus,* and *Capnocytophyga.* Other Gram-negative and Gram-positive species were isolated infrequently and in very low numbers. A total of 160 species and subspecies were isolated. Children who were resistant to the development of gingivitis had 2-3 fold higher proportions of *Capnocytophyga* and anaerobic vibrios, (*Campylobacter, Selenomonas, Wolinella*) than did the adults. In an examination of four young adults (Moore et al, 1982), 166 bacterial species and subspecies were isolated and identified. The predominant genera isolated were *Actinomyces*, *Streptococcus* and *Veillonella*. Anaerobic Gram-negative rods and filaments were regularly isolated from the healthy sulcus but only in low numbers. With appearance of gingivitis, *Actinomyces naeslundii*, *A. odontolyticus, Fusobacterium nucleatum, Veillonella* and *Streptococcus anginosus* increased significantly in relation to other inhabitants of the sulcus.

Since the primary purpose of this dissertation is to discuss and define the microflora associated with dental caries, an extensive review of the microflora associated with gingival crevice is unnecessary. However, in terms of dental plaque development the gingival microflora does provide some inter-

esting contrasts to the supragingival dental flora. The conditions under which the gingival or, more correctly, subgingival flora develops are quite different from those to which the supragingival flora is exposed. The gingival crevice and its inhabitants are protected from the various oral hygiene procedures practiced by the host and innate oral cleansing forces, allowing organisms with weak adherent capabilities to persist in the crevice. Relative protection from saliva with its numerous antibacterial components is afforded to crevicular inhabitants. Unique growth conditions - a low oxidation-reduction potential (Eskow and Loesche, 1971; Kenney and Ash, 1969), gingival fluid and inflammatory exudate- are also provided. The provision of surfaces different from those available to supragingival dental plaque bacteria is another way in which the gingival environment encourages the development of a unique microflora. Cementum, epithelium and other microorganisms all are available for adherent interaction. In fact microbial adherent interaction may be the only mechanism available to some periodontopathic bacteria to ensure their persistence at a particular site (Cisar, 1982; Kolenbrander et al, 1983; 1984; 1985; Lancy et al, 1983). Another form of microbial interaction of some importance is the provision of special nutrients to crevicular bacteria by other crevicular bacteria. Examples include synthesis of Vitamin K and its use by *B. melaninogenicus* (Gibbons and Macdonald, 1960), and the dependency of *Treponema microdentium* on polyamines and a controlled oxidation reduction potential created by

other microorganisms (Socransky et al, 1964)

e) Tooth Surface

It has been known since the time of Antonie von Leeuwenhoek that teeth are covered with bacteria (Bardell, 1982). That the bacteria contributed in some way to the development of dental caries was suspected in the mid-nineteenth century but a concerted effort to determine exactly how they participated in the caries process was not undertaken until the middle of this century. Since then numerous studies have been made on the structure, composition, development and pathogenicity of dental plaque (Stiles et al, 1976).

Unlike oral mucosal surfaces which constantly shed superficial epithelial cells, reducing the resident microbial mass in the process, the tooth surface is nonrenewable, thereby allowing a considerable microbial mass to develop if left undisturbed. In addition, a tooth does not provide a single uniform habitat but possesses several distinct surfaces each of which is suitable for colonization by only certain microorganisms. Specifically the tooth offers smooth enamel surfaces above (supragingival) and below (subgingival) the gingival margin which can be further subdivided into approximal, buccal and lingual surfaces as well as retentive areas in the fissures of the occlusal surfaces of posterior teeth. The approximal and fissural areas offer the greatest protection for microbial communities on the teeth and therefore these areas frequently show the greatest diversity in the

resident populations. In order to provide an overview of the distribution of microorganisms in a climax community of dental plaque each of the unique areas on the tooth surface will be considered separately.

(i) Occlusal- pits/fissures

Given that pits and fissures are regarded as being the most susceptible to the development of dental caries (National Dental Caries Prevalence Survey, 1981; Hardwick, 1960; Parfitt, 1955; Lewis and Hargreaves, 1975), it is surprising that more studies have not been completed using the fissure as a model for caries development. The main problem investigators have had to overcome is the difficulty of sampling a narrow fissure. Several model systems involving the implantation of natural or artificial fissures into teeth in humans have been developed to overcome this problem (Löe et al, 1973; Theilade et al, 1973; Theilade et al, 1974; Thott et al, 1974). These models allow for sampling of the fissure to its depth and in the case of natural fissures allow for a visual, microscopic or microradiographic investigation of the enamel surface of the fissure for caries.

In all studies the predominant cultivable bacteria were Gram-positive. In general the flora was much less complex than that at other sites and considerable variation in the composition of the fissural flora was noted between individuals and between sites within the same fissural system. This suggests that different fissure morphology

could lead to areas of heavy infection of specific fissures or specific locations within a fissure. As well, since it is well known that diet influences the colonization of enamel by oral microorganisms (Carlsson and Egelberg, 1965), the intimate contact that the fissure experiences with different food types consumed by an individual could have an effect on the colonization of the fissure by certain organisms. Svanberg (1980) has shown that the salivary concentrations of *S. mutans* and *S. sanguis* are crucial to their establishment in artificial fissures. When the levels of *S.mutans* were lowered (10^3 CFU/ml)at the start of the experimental period around the time of insertion of the artificial fissure, the organism did not colonize the fissures even though salivary levels were allowed to rise during the experimental period. Once *S. mutans* was established in a fissure, experimental reduction in the salivary levels of this organism had no effect on the proportional distribution of *S. mutans* in the fissure. This agrees with the results of a study by Burt et al (1983) who have shown in a two year study of 473 occlusal fissures of first permanent molars in 279 6-7 year-old children that initial levels of *S. mutans* were fairly stable over the duration of the study. This indicates that the initial inoculum may be an important determinant for colonization of fissures and that once infected with *S. mutans* the fissure will remain infected.

At least one study showed a considerable quantitative change in the plaque flora as the plaque aged, with a decrease in

the numbers of Gram-positive cocci and an increase in Gram-positive rods (Thott et al, 1974).

In all studies streptococci was the dominant genus. Species representing the predominant flora and isolated with regularity included *S. sanguis, S. mutans, S. salivarius, A. naeslundii, A. viscosus* and *Veillonella. Lactobacillus, Haemophilus* and staphylococci were isolated less frequently and in lower numbers, (**see table 1-4 for summary**).

A comparison between studies of the actual species isolated is difficult since different models and experimental protocols were used. In artificial mylar fissures Theilade et al (1973) found that the numbers of lactobacilli increased as the plaque aged and in 7-day-old plaque were regularly isolated. Thott et al (1974) using the natural fissure system of surgically extracted third molar crowns which had been embedded in an acrylic appliance, was unable to demonstrate this. Since mylar is unlike enamel it could be argued that an artificial fissure made of mylar would favour colonization by a qualitatively different flora from that which would be expected on enamel. A comparison of the results of these studies shows that there is little difference. However, the examination of the microflora in these early studies was not very detailed and a more extensive investigation of both fissural systems would be necessary for adequate comparisons. In any event, all studies since have used natural teeth implanted in the oral

cavity as the model. Unfortunately comparison of studies is still difficult. While most studies have not interfered with oral hygiene or dietary patterns of subjects, in the most comprehensive investigation of fissural microflora to date, Theilade et al (1982) had each subject place a drop of 20% sucrose solution at the entrance of the implanted fissures twice daily during the experimental period in order to favour the development of a cariogenic flora. As expected, over the experimental period of 200-270 days the levels of *S. mutans* and *Lactobacillus (L. casei and L. plantarum)* increased to levels well in excess of those in studies where a similar practice was not used. This has lead several to propose that a retentive site such as a fissure is the natural habitat of *S.mutans* in the oral cavity (Ikeda and Sandham, 1971; Gibbons et al, 1974; Berkowitz et al, 1975; Svanberg and Loesche, 1978).

An additional problem with all studies has been the selection of adult subjects who are generally beyond the most caries-susceptible childhood years. While the data may represent the microflora of the fissural system of permanent teeth in adults, extrapolation to the child and in particular, the primary dentition with its shallower and less retentive fissures, may not be possible. As yet there are no data which delineate the **entire** microflora of the fissures of permanent or primary teeth in children.

Table 1-4
THE PREDOMINANT CULTIVABLE FLORA OF HUMAN DENTAL FISSURE PLAQUE

	Theilade et al			Thott et al
Organism[5]	1973[1]	1974[2]	1982[3]	1974[4]
Total Strep.	30.3	36.0	39.4	28.5
S. mitior	NR	NR	2.1	NR
S. sanguis	9.4	11.5	4.2	NR
S. salivarius	4.6	6.0	ND	NR
S. mutans	1.1	0.2*	29.6	19.3
Veillonella	NR	NR	12.2	40.3
Neisseria	NR	NR	NR	3.3
Lactobacillus	10.0	2.0	3.9	0.7
A. naeslundii	NR	NR	15.0	NR
A. viscosus	NR	NR	5.2	NR
Arachnia prop.	NR	NR	4.7	NR
Propionibacteria	NR	NR	2.2	NR
Eubacteria	NR	NR	2.7+	NR
Haemophilus	NR	2.2	0.3	NR
Staphylococci	NR	NR	9.0	NR

1. Artificial fissure; 6 subjects/samples; 7 day results
   Theilade et al, (1973)
2. Third molar fissure implanted in occlusal filling; 6 subjects/11 samples; 7 day results
   Theilade et al, (1974)
3. Third molar fissure implanted in occlusal filling; 10 subjects/samples; 20% sucrose applied to fissure twice each day; 200-270 day results
   Theilade et al, (1982)
4. Third molar fissure implanted on acrylic appliance; 5 subjects/44 samples; 21 day results
   Thott et al, (1974)
5. Results expressed as percentage of total viable flora on non-selective medium; in papers where results were not expressed as a percentage of the total viable flora they were recalculated to this format

* results from one subject only
\+ isolated from one fissure only
NR not reported
ND not detected

(ii) Smooth Surfaces- buccal/lingual, approximal, gingival

Numerous studies have examined the development of dental plaque on smooth dental surfaces and the structure and microbial composition of deposits found on these surfaces (Bowden et al, 1975; Carlsson, 1967a; Gallagher et al, 1981; Howell et al, 1965; Kilian et al, 1979; Krasse, 1970; Listgarten et al, 1975 Loesche et al, 1972; McCarthy et al, 1965; Saxton, 1973; Schroeder and de Boever, 1970; Socransky et al, 1977; Theilade and Theilade, 1970).

The approximal areas are more retentive in relation to the exposed buccal and lingual surfaces and provide the greatest protection to the microflora. In this sheltered enclave bacterial communites can attain a climax state of relative stability (Alexander, 1971) unlike exposed surfaces which are open to dramatic environmental changes caused by, for example, oral hygiene activities, muscular action and diet. An example of the dietary influence on the composition of dental plaque is illustrated in a paper by Hoover et al (1980) who sampled the dental plaque of subjects with hereditary fructose intolerance as well as control subjects. Occlusal fissures of permanent molars and interproximal spaces between permanent molars were sampled in both groups. While the quantity and chemical composition of plaque did not differ between groups, the isolation of *S. mutans* and *Lactobacillus* was 3-4 times more frequent in the control group than in the hereditary fructose intolerant group. *S. sanguis* was isolated regularly from both groups, indicating

that the restriction of sucrose in the hereditary fructose intolerant group had little impact on its ability to colonize and persist at each of the sites.

On a macroscopic level the approximal areas, being the most well protected, will, if left undisturbed, accumulate a more substantial microbial mass than exposed smooth surfaces. However on a microscopic level, the level of the micro-organism, studies which have examined the initial phase of plaque formation on smooth surfaces have shown that micro-organisms will initially adhere to relatively protected areas on any tooth surface such as along the gingival margin or in imperfections in the enamel (Lie and Gusberti, 1979; Lie, 1979). Consequently, differences between surfaces in the composition of the microflora are small in the early stages of plaque formation. The early microflora is comprised of primarily Gram-positive organisms, mostly streptococci and *Actinomyces*. *S. mitior* and *S. sanguis* have been found to be among the first streptococci to colonize the tooth surface (Socransky et al, 1977). With time the complexity of the community will increase as environmental conditions change making conditions favourable for colonization by other microorganisms (Ritz, 1967; 1970; Socransky et al, 1977). The accumulation of inhibitory metabolic products, a shortage of nutrients or the lowering of the E_h in the plaque (Kenney and Ash, 1969) will facilitate microbial succession within the community. This kind of succession is called autogenic succession

(Alexander, 1971). Ritz (1967), over a nine day period, showed that a progressive shift from an aerobic and facultatively anerobic microflora to a preodominately facultatively anerobic and obligate anerobic microflora occurred in developing coronal plaque.

In mature plaque, Gram positive rods and filaments occupy equal prominence with the streptococci (Bowden et al, 1975; Socransky et al, 1977). *Actinomyces viscosus* and *A. naeslundii* are the predominant Gram-positive rods in dental plaque and frequently become the dominant organisms in an established community (Bowden and Hardie, 1978; Holmberg, 1976). *S. sanguis, S. mitior* (Liljemark and Gibbons, 1972), and *S. milleri* (Mejare and Edwardsson, 1975) are generally the most dominant streptococci in dental plaque and *S. salivarius* is usually found in low numbers in contrast to its levels on the dorsum of the tongue. *S. mutans* can be a prominent member of dental plaque with frequent ingestion of fermentable carbohydrate, in particular sucrose, but its numbers tend to be lower than those of other streptococci when carbohydrate restriction occurs (Carlsson and Sundstrom, 1968; Emilson, 1983; Fitzgerald, 1976). *S. sanguis, S. milleri* and *S. mutans* all have an affinity for hard surfaces and usually do not appear in the oral cavity prior to tooth eruption (Edwardsson and Mejare, 1978; Catalanotto et al, 1975; Berkowitz et al, 1975; van Houte, 1980). It has been known for some time that *S. mutans*, while not dependent on sucrose for attachment to the tooth

surface, will not accumulate in large numbers in the absence of sucrose (Gibbons, 1980). It has also been shown that glucosyltransferase-defective mutants of *S. mutans* are able to colonize the teeth of rodents although they display reduced cariogenicity (Murchison et al, 1981). *Lactobacillus* has a relatively low affinity for the tooth surface and is usually only isolated from retentive areas such as fissures or carious lesions (van Houte et al, 1972). Under conditions of frequent sugar ingestion the levels of lactobacilli may increase and some investigators have used the levels of these organisms for an evaluation of dietary control and caries risk (Klock and Krasse, 1978; 1979). Other Gram positive rods such as *Rothia, Bacterionema, Arachnia, Propionibacterium*, and *Eubacterium* are infrequently isolated and in low numbers (Bowden and Hardie, 1978; Holmberg, 1976). *Veillonella* represent the dominant Gram negative cocci in dental plaque. It has been proposed that *Veillonella* occupies a specific niche in the community in that it may depend on lactate for its survival (van der Hoeven et al, 1978). This may account for higher levels of *Veillonella* in dental plaque frequently exposed to carbohydrate. Ritz (1970) has proposed that *Neisseria* may be an important pioneer in the development of dental plaque since in individuals with high levels of this organism the ensuing development of plaque is more rapid than in individuals with lower levels. In established communities however the levels of *Neisseria* are generally low (Bowden et al, 1975). Gram-negative facultative rods make up a small part

of supragingival dental plaque. *Haemophilus segnis, H. aphrophilus, H. paraphrophilus* and *H. parainfluenzae* are the dominant members of this genus found in dental plaque, albeit in low numbers and infrequently (Kilian, 1976; 1980). Other Gram-negative facultative coccobacilli include *Actinobacillus* (Weaver and Hollis, 1980), and *Branhamella* (Morello and Bohnhoff, 1980) both found infrequently and in low numbers in dental plaque. *Actinobacillus actinomycetemcomitans* has been implicated as a primary causative agent in juvenile periodontitis (Zambon, 1985). *Capnocytophyga* (Holt et al, 1979; Leadbetter et al, 1979) is a Gram-negative facultative/capnophilic gliding organism found in both supragingival and subgingival plaque. Its numbers in supragingival dental plaque are generally low and it too has been implicated in several types of periodontal disease (Dzink et al, 1985) although others feel that its role here has been overestimated (Holdeman et al, 1985). In established dental plaque Gram-negative anaerobic rods and filaments can comprise a large proportion of the community (Bowden et al, 1975; Moore et al, 1984; 1982). *Bacteroides, Fusobacterium* and *Leptotrichia* represent the majority of isolates but there is considerable variation between the relative numbers of these organisms reported in different studies. Since the isolation of these organisms requires exacting methods and they do not appear to be important in the development of dental caries many studies have chosen to ignore them. In addition, the taxonomy of the Gram-negative

anerobic rods and filaments is changing rapidly making accurate identification of this group of organisms difficult.

Unfortunately, few comprehensive studies of the composition of established microbial communities on the tooth surface have been completed. With the realization that dental caries is a disease of bacterial origin, many investigators began to focus on specific organisms which had been shown to be capable of causing caries in animals. They lost sight of the role which the entire community could play in the initiation and progression or modification of disease. Studies thus far which have characterized the entire microflora of coronal plaque have been completed by the MRC Dental Epidemiology Unit in England. They proposed several parameters for the characterization of the bacterial communities:

a) the qualitative bacterial composition in terms of genera and species,

b) the quantitative bacterial composition,

c) the stability of community over a period of time,

d) the persistence of specific bacterial species (Bowden et al, 1975).

The application of these parameters to samples taken from well-defined areas indicate that each site harbours its own characteristic microflora with some organisms demonstrating fairly constant levels over time and others fluctuating up or down in number in the same period (Bowden et al,1975).

Tables 1-1 and 1-5 summarize the microflora found on the

smooth surfaces of the tooth crown.

Table 1-5
THE PREDOMINANT CULTIVABLE FLORA OF HUMAN SMOOTH SURFACE DENTAL PLAQUE

Organism[4]	Bowden et al 1975[1]	Kilian et al 1979[2]	Gallagher et al 1981[3]
Streptococci	22.9	39.1	13.1
S. milleri	0.5	3.2	0.7
S. mitior	NR	21.6*	1.4
S. mutans	2.2	2.2	1.0
S. sanguis	5.9	8.0	1.0
S. salivarius	0.7	0.7	3.0
Gemella	NR	2.6	1.1
Gram-positive rods	42.1	11.4	34.4
A. visc./naeslundii	19.1	4.7	20.5
A. israelii	16.5	0.4	NR
Lactobacillus	0.5	0.7	0.7
Rothia	0.4	0.6	NR
Propionibacterium	NR	2.4	3.0
Eubacterium	NR	NR	5.4
Bifidobacterium	NR	NR	0.3
Veillonella	13.1	36.2	9.5
Neisseria	1.5	1.2	NR
Gram-negative rods	8.0+	3.7!	23.5
Fusobacterium	0.4	NR	7.7
Bacteroides	NR	NR	8.9
Haemophilus	NR	1.6	0.4

* Total of dextran + and - strains
! Anerobic species only
\+ Primarily *Bacteroides*
NR Not reported

1. 58 samples of approximal plaque from ten 13/14-year-old children, all subjects caries-free; Bowden et al (1975).
2. 15 samples of supragingival plaque from buccal surface of mandibular first permanent molar from fifteen caries-free 12-year-old Tanzanian children, five of whom were exposed to water with 3-21 ppm fluoride; Kilian et al (1979).
3. 6 samples of smooth surface plaque from premolar or molar from six New Zealand subjects aged 11-41 years, all caries-free; Gallagher et al (1981).
4. Results expressed as percentage of total viable flora on non-selective medium.

CHAPTER TWO

Development of the Oral Microflora in the Infant

The mouth of the infant is usually devoid of microoganisms at birth but rapidly acquires an indigenous microflora within the first day of life (Brailovsky-Lounkewitch, 1915). Some have postulated that the newborn derives its initial flora from the rectovaginal region of the mother. Carlsson and Gothefors (1975) in a study of 13 newborns and their mothers, found that lactobacilli, *L. jensenii* and *L. acidophilus*, could frequently be recovered from both the vagina of the mother and the oral cavity of her newborn. Lactobacilli have been shown to increase in number in the vagina during pregnancy (Werner and Seeliger, 1963) and it is known that the preferential site for colonization of *L. acidophilus* is the lower part of the ileum (Lerche and Reuter, 1962). Carlsson and Gothefors (1975), proposed that such an increase in the mother may facilitate colonization of the infant's gastrointestinal tract at the time of birth. Since it is known that *L. acidophilus* is commonly found in the oral cavity, this may indicate that similar conditions for adherence to mucous membrane exist in both the vagina and the oral cavity. However it is also known that the species *L. acidophilus* represents a heterogeneous group of organisms (Efthymiou and Hansen, 1962). Therefore, it is probable that different strains colonize each site. In the study by Carlsson and Gothefors, both species of lactobacilli did not persist beyond a few days indicating that

lactobacilli are mostly transients in the mouths of edentulous infants. Gareau et al (1959) found that vaginally delivered infants frequently acquired *E. coli* from their mother and demonstrated the same serotype in infant-mother pairs in both the oral cavity and rectum of the infant.

McCarthy et al (1965) studied the development of the oral flora in 51 newborns and 44 four-month-old infants, gathering samples at birth in the first group and at 4, 8, 12, 18 and 36 months of age in both groups. Samples from 9 of the newborns showed no growth within the first 24-hour-period indicating that either the mouth of these infants was still sterile, that the sensitivity of the media employed was not sufficient to elicit all possible organisms or that the genera which the examiners were looking for did not represent the entire oral microflora. *Streptococcus* was the dominant genus present in the mouths of all newborns and remained so throughout the study period, although its numbers fell from 98% of the total cultivable flora in the first day or two of life to 70% by the time the child was 12 months of age. Of the streptococci isolated, *S. salivarius* was the predominant species in over half of the subjects. Staphylococci were also isolated from approximately half of the subjects but in very low numbers. Other organisms isolated in the newborn group included *Neisseria, Candida, Lactobacillus, Veillonella*, and coliforms. These groups of organisms were isolated infrequently and in low numbers and

were regarded as transients. In both study groups, McCarthy et al found that the complexity of the oral microflora increased as the child aged. All children were colonized by *Streptococcus, Staphylococcus, Neisseria*, and *Veillonella* by 12 months of age. *Actinomyces, Lactobacillus, Rothia*, and *Fusobacterium* species were isolated from more than half of the infants at 12 months of age while *Bacteroides, Leptotrichia, Candida*, and *Corynebacterium* were found in less than half of the subjects. Unfortunately, they were unable to draw any conclusions regarding the relationship between the eruption of teeth and alteration in the oral microflora. Although *Fusobacterium, Leptotrichia, Actinomyces* and *Rothia* were all isolated on occasion from pre-dentate infants, no information was given regarding the persistence of these groups of bacteria in the mouths or on the tooth surfaces of individual subjects. This failure could also be related to the method of sampling employed in the study, a swab rubbed over the mucosa and ,if present, teeth. It is unlikely that this method would accurately reflect the communities residing on the teeth particularly those in the gingival crevice. Only with *Fusobacterium* and *Actinomyces* was there an increase in the frequency of isolation as teeth erupted but this was considered across the subject group and not within individual subjects. A shorter sampling interval would have been necessary to investigate properly the question of establishment of each of the species detected in this study. This, as well as other features, was evaluated

in a study of the development of the oral flora of infant monkeys (*Macaca irus*) where samples were obtained from the mouths of these animals immediately after birth and at daily intervals thereafter (Cornick and Bowen, 1971). A similar sampling method to that used by McCarthy et al was employed. A direct comparison of the colonization of the oral cavity of human and monkey infants is not possible because of the more rapid development of the monkeys, different dietary regimes and post-natal handling. However the results of both studies do reveal several interesting similarities and differences. In both studies, the infants were born with a sterile oral cavity and, in some instances in both humans and monkeys, several days passed before organisms were isolated from the mouth. Unlike the study by McCarthy et al, it was possible to demonstrate a profound change in the oral microflora coincidental with the eruption of teeth. This may have been possible because of the shorter but more frequent sampling schedule employed in the examination of the monkeys. Both studies found that as the infant aged the microflora increased in complexity and changed from a primarily aerobic to anerobic flora. Other differences have been reported by Colman and Hayday (1980) who found that dental plaque collected from children contained, proportionately, larger numbers of *S. sanguis* than dental plaque from monkeys and reported that, as yet in their studies with a successful primate colony, they have not isolated *S. salivarius* from the dental plaque of their monkeys.

Ellen (1976) showed, in a cross-sectional study of subjects ranging in age from young infants to adults, that catalase-negative Gram-positive rods resembling *A. naeslundii* colonized most infants, including 40% of the pre-dentate infants, and maintained predominance among facultatively anerobic *Actinomyces* in saliva and on the tongue. In contrast the colonization of bacteria resembling catalase-positive *A. viscosus* was delayed until after teeth had erupted and their rise to predominance over *A. naeslundii* in dental plaque was delayed for several years.

Since both human and animal studies have shown that the greater proportion or organisms found in dental plaque do not appear with any regularity until teeth have erupted, eruption of teeth must represent a significant environmental determinant in the development of the oral flora. Most of the studies which have examined the acquisition of an oral microflora in the newborn have focused on streptococci, primarily *S. mutans*, because of its association with dental caries. The importance of teeth in the colonization of *S. sanguis*, *S. milleri*, and *S. mutans* has been demonstrated (Carlsson et al, 1970; Berkowitz et al, 1975; Catalanotto et al, 1975; Edwardsson and Mejare, 1978; Stiles et al, 1976) and all three have been shown to be transients in the oral cavity prior to tooth eruption (Edwardsson and Mejare, 1978; Stiles et al, 1976) . Carlsson et al (1975), in a 5 year longitudinal investigation of 30 infants from birth,

found that *S. salivarius* was a regular inhabitant of the oral cavity from the second day of life. *S. mutans* appeared in the mouths of some infants after one year and in consistently low numbers in the majority of the children after five years although it was established and consistently isolated from only half of the infants under study. Stiles et al (1976) in an examination of 253 children, aged 3-24 months, did not find *S. mutans* in oral or fecal samples prior to the eruption of teeth. Following tooth eruption, the most prevalent serotype of *S. mutans*, identified with fluorescent antibody, was **c**, although serotype **e** and **d** were isolated from several children. *S. sanguis* was isolated from two children without erupted teeth but its frequency of isolation from the children rose to 80% following the eruption of teeth. Berkowitz et al (1975) in a study of children with cleft palates who were wearing palatal acrylic obturators, found that the highest recoveries of *S. mutans* came from the gingival third of the labial surface of the maxillary incisor teeth. The predominant serotype in the infants and their mothers was type **c**.

Since *S. salivarius* prefers to colonize epithelial surfaces (Krasse, 1954; Long and Swenson, 1976), *S. sanguis*, the smooth surfaces of teeth (Carlsson, 1967a) and *S. mutans* the pits and fissures and approximal areas (Ikeda and Sandham, 1971; Catalanotto et al, 1975), the ecological determinants for the establishment of these three organisms

must differ although the organisms appear to share many similarities such as nutritional requirements and polysaccharide production (Stiles et al, 1976).

Both animal (Keyes, 1960; Beighton et al, 1982; Jordan et al, 1985) and human studies (Berkowitz and Jordan, 1975; Berkowitz et al, 1980; Kohler and Bratthall, 1978; Rogers, 1981) have confirmed that the most likely source of microorganisms found in the mouth of the infant is the mother or primary care-giver. Serotyping (Berkowitz et al, 1975) and bacteriocin-typing (Berkowitz and Jordan, 1975) of *S. mutans* strains isolated from mother-infant pairs, and primary care-giver-infant pairs in the case of animals (Jordan et al, 1985), have confirmed that maternal reservoirs of bacteria are available for primary infant oral infection. The parameters for successful transfer of the organisms from mother to infant have not been clearly defined. Important parameters may include the number of cells transferred and available for attachment and the frequency of transfer. van Houte and Green (1974) concluded that a minimum level of *S. mutans* in saliva was necessary for colonization of the teeth. Kohler and Bratthall (1978) have shown that children of parents with high salivary levels of *S. mutans* are at greater risk of being infected earlier in life and have a definite risk for high caries experience. Berkowitz et al (1981) have extended this finding and, in attempting to quantify the risk, have shown that mothers with high levels of salivary

S. mutans, greater than 10^5 **CFU** per ml. of saliva, are nine times more likely to transmit this organism and infect their offspring than mothers whose salivary reservoirs were less than or equal to 10^3 **CFU** per ml. of saliva.

Another parameter which has been investigated is the difficulty with which *S. mutans* is implanted in or transferred to the human mouth (Jordan et al, 1972). Sucrose ingestion has been shown to facilitate implantation although it is no longer regarded as essential for attachment to the tooth surface (Clark et al, 1978; Gibbons, 1980). Since it is known that the pattern of infant sugar consumption is most frequently influenced by the mother's sugar consumption habits and that most infants are introduced to sucrose-containing food and drinks at the time of eruption of the first tooth (King, 1978; Gordon and Reddy, 1985), this parameter of sucrose-aided implantation would be satisfied. The scenario of a mother or father with high numbers of salivary *S. mutans*, who uses his/her own spoon to feed an infant food which may have been sucrose-supplemented would quite probably prove adequate to infect the infant with *S. mutans* early in life. A study by Alaluusua and Renkonen (1983) showed that the early establishment of *S. mutans* in the plaque of primary incisors was predictive of early and extensive caries attack in the young primary dentition. This finding was confirmed in two studies by Kohler et al (1983; 1984) which examined the effect of caries-preventive measures in mothers on caries development and the oral

presence of *S.mutans* and lactobacilli in their infant children. In the test group of mothers, a preventive regimen was used which successfully lowered their salivary levels of *S. mutans* and thereby delayed transmission and implantation of this organism in their children. In the control group by comparison, more children were infected with *S. mutans* and subsequently more children developed caries. The differences between test and control groups in *S. mutans* levels and the development of caries were statistically significant. Therefore, in terms of the development of the oral microflora, measures directed against the source of infection can be successful in preventing the establishment of potentially harmful bacteria.

The role of diet and immunological factors in the acquisition of an oral microflora are pertinent to this discussion as there are several differences in these areas between the young child and the older child/adult which could have profound effects on the colonization of specific organisms and the development of disease. It is known that fermentable carbohydrate in general and sucrose in particular enhance the colonization of the oral cavity by cariogenic bacteria (van Houte, 1982). The specific effect of sucrose on the colonization of *S. sanguis*, and *S. salivarius* is less dramatic than for *S. mutans* (Kuramitsu and Ingersoll, 1977). Carlsson and Sundstrom (1968) and Skinner and Woods (1984) have shown that the composition of dental plaque is affected by exposure to different dissacharides. Sucrose

encouraged a shift to a flora dominated by streptococci. As well the acidogenicity and aciduricity of the plaque increased. Replacement of dietary sucrose with xylitol in the Turku sugar studies (Scheinin and Makinen, 1975) did not affect the proportions of major bacterial groups in dental plaque but did reduce the numbers of acidogenic and aciduric organisms, in particular *S. mutans*. providing additional evidence that diet plays a major role in the composition of microbial deposits on the teeth. An extension of this effect, in terms of the development of disease, is evident since it has been clearly shown that increasing the frequency of sugar intake leads to the development of dental caries (Gustafsson et al, 1954; König, 1970). The feeding habits of most children are such that frequent eating is the rule rather than the exception (King, 1978). An example of some pertinence to the topic of this dissertation is the finding by Short (1976a; 1976b) that children who are breast fed on demand will suckle between 30-60 times in any 24-hour period. A similar scenario exists for children who are bottle fed in an ad lib fashion (Milnes, unpublished data). This type of frequent eating may represent a significant environmental pressure on the developing bacterial communities of the tooth surface and encourage rapid colonization. Once the flora is established, frequent ingestion of lactose in human or cow's milk may produce conditions favourable for colonization by aciduric and acidogenic populations since it is known that both human and bovine milk contain sufficient quantities of lactose to depress the pH

in localized areas on the tooth surface (Rugg-Gunn et al, 1985). Hence the dietary habits of the child may have a direct effect on the acquisition of the oral microflora. Interaction of the child with a parent having high salivary levels of aciduric and acidogenic organisms improves the likelihood of colonization of the child's mouth with a similar flora.

While the immune system of the young child is not developed to the same level as that of the adult, the B cell population of the term newborn appears capable of producing a normal antibody response at birth (Stiehm and Fulginiti, 1980). The T cell population is mature by 40 weeks gestation and the term newborn has normal total numbers in the peripheral blood. T cells obtained from term newborns have increased suppressor activity and/or decreased helper activity resulting in the decreased production of immunoglobulin production by B cells of the term newborn (Stiehm and Fulginiti, 1980). This low level of immunological activity may help in the establishment of an indigenous flora in the newborn.

Other forms of immunological control may be exerted by the breast milk from the nursing mother or by immunological constituents of cow's milk. Human milk and colostrum contain IgA as the predominant immunoglobulin and very little IgG which is transferred across the placenta during pregnancy (Arnold et al, 1976; Harmon, 1978). Cow's milk, on the other

hand, contains IgG as the predominant antibody since this mammal does not transmit IgG across the placenta (Harmon, 1978). Local antigenic stimulation of the gut-associated lymphoid tissue (GALT) as a consequence of swallowing oral organisms will produce a population of lymphocytes committed to IgA synthesis which will home in sufficient numbers to distant sites, such as the mammary gland and result in a significant local production of IgA (Brandtzaeg, 1983). Widerstrom and Bratthall (1984) have shown in pregnant women that a marked increase in salivary levels of IgA occurs in late pregnancy and postpartum. Since a parallel increase in the level of serum IgA does not occur (Rocklin et al, 1979), this observation by Widerstrom and Bratthall appears to represent a local effect. This observation is consistent with observations made concerning the levels of IgA in breast milk (Stiehm and Fulginiti, 1980). The breast-fed infant therefore receives IgA antibodies specific for many of the mother's oral organisms. Eggert and Gurner (1984) have demonstrated the presence of specific antibodies in human colostrum against *S. mutans, S. sanguis, S. salivarius* and *S. mitis*. Others have demonstrated a wide range of colostral antibodies against a variety of viruses, enterobacteria, and enterotoxins (cited in Chandra, 1979), indicating that maternal secretory antibodies are involved in the development of a normal human infant gastrointestinal tract flora (Nagy et al, 1979). The observation by Eggert and Gurner mentioned previously indicates that the immune system of a mother does not appear

to be involved in the selective provision of colostral antibodies for the inhibition or removal of potentially pathogenic bacteria . Rather it seems that the mother provides colostral antibodies against a broad range of organisms that the infant may encounter. Local antibody production by the mother could therefore represent a means of controlling the establishment of an indigenous flora in a newborn and later, on tooth eruption, the colonization of organisms such as *S. mutans*.

Recently, however, several investigators have shown that *S. sanguis, S. mitior, B. asaccharolyticus, B. intermedius* and *Capnocytophyga* as well as other mucosal pathogens contain an enzyme, IgAl protease, which cleaves SIgA and serum IgA to form Fab and Fc fragments (Kilian, 1981; Plaut, 1983). Hsu and Cole (1985) have demonstrated that dental plaque from two groups of Colombian children contained components of degraded SIgA and serum IgA and only small amounts of intact IgA and IgG. Thus, the reason that several oral streptococcal species are not recognized as being pioneer colonizers of the oral cavity may be related to their ability to destroy IgA directed against them. The infant fed either cow's milk or formula would not receive this protection and colonization of the oral cavity would probably proceed much differently. A study to compare how colonization of the oral cavity differs between bottle-fed and breast-fed children could provide valuable information about the development of an indigenous bacterial flora in

the human.

As the infant ages and develops, antigenic stimulation of the GALT occurs and the infant begins secreting specifically directed salivary SIgA. Selner et al (1968) found IgA in the saliva of infants by the age of seven days and measurable quantities in 97% of samples taken from infants six weeks old. They concluded that secretory immunity develops faster than humoral immunity. Ben-Aryeh et al (1984) confirmed this finding and showed that salivary concentrations of IgA were significantly lower in infants than adults, concluding that the maturation of the secretory immune system was incomplete at the end of the infants' first year. They also showed similar levels of IgA in the saliva of breast-fed and bottle-fed infants. However no attempt was made to determine the specificity of the antibodies isolated. Thus lower levels of sIgA could allow for bacterial colonization and the development of an indigenous flora.

As well, with tooth eruption and the development of eruption gingivitis, the infant could begin synthesizing IgG serum antibodies against organisms on the tooth surface including *S. mutans*, which may interfere with their colonization and establishment. It is not known what influence these antibodies would have at this stage of development since the level of IgG in saliva is very low. In addition, the level secreted through the gingival crevice would be quite low and

probably ineffective if an extrapolation can be made from studies of older children and adults which have examined this mechanism (Sims, 1985).

Antibodies are only part of the salivary antimicrobial defense and it is possible that other nonimmunoglobulin factors in saliva may affect colonization of the oral cavity by microorganisms in the infant or young child. The report by Tenovuo et al (1986) represents the first comprehensive analysis of these factors in the saliva of infants. They showed that the innate nonimmunoglobulin defense systems (lysozyme, salivary peroxidase and hypothiocyanate) present in whole saliva are at the adult level when the primary teeth erupt. Immunoglobulin, lactoferrin and myeloperoxidase were all found in smaller quantities than in adult saliva.

Since these factors possess many antimicrobial properties such as inhibition of acid production, bacteriostatic and bactericidal effects as well as the capacity to agglutinate bacteria and viruses, it is possible that they could influence bacterial colonization when antibody systems are immature.

In summary, many questions pertaining to the acquisition of an indigenous oral flora remain unanswered. Longitudinal studies should be carried out to investigate the effects of immune and non-immune factors in the development of the oral flora, mechanisms of transfer of organisms to the infant during susceptible periods for colonization and methods of

interfering with or preventing the transfer and colonization of potential dental pathogens.

CHAPTER THREE

The Microbiology of Caries

I. Historical perspective.

Despite many theories having been advanced to explain the cause of dental caries, a satisfactory, albeit simplified, explanation did not emerge until the latter part of the nineteenth century when Miller proposed his 'Chemoparasitic Theory' (Miller, 1973). This work paralleled many advances which had been made in other fields of science and medicine, particularly the work of Robert Koch and Louis Pasteur in microbiology, which enabled Miller to propose that microorganisms present in the oral cavity, by converting dietary sugars to various acids, were responsible for the demineralization of teeth and the development of tooth decay. Unlike many who were to follow him, Miller believed that caries was related to multiple microbial activity involving acid production and proteolytic activity.

G. V. Black (1898) extended Miller's theory with investigations into the importance of 'gelatinous microbic plaques' in the caries process. He recognized that plaques which formed in secluded areas on the teeth such as pits and fissures, along the gingival margin and in the approximal regions were more likely to cause caries provided the environmental conditions were correct for plaque formation. In *in vitro* experiments with oral bacteria, he was able to study plaque development by manipulating the culture medium

although the changes necessary to influence plaque development were never reported by him. An important observation during his work, that plaques which were associated with caries differed from plaques formed in caries-free mouths, led him to conclude that a specific microbial plaque was necessary for disease to occur.

Since a great deal of Miller's work had been morphological and he was unable either to grow or identify organisms which he had isolated, McIntosh et al (1922) set out

'to discover a bacterium or group of bacteria present in dental caries which can produce sufficient acid to decalcify enamel and dentine'.

They isolated two organisms, one, a long thin bacillus, which had a marked tendency to parallelism and palisade formation in dried films and a second much shorter 'bacillus' which usually occurred in chains. They proposed the name *Bacillus acidophilus odontolyticus* type I and II for these organisms. Based on their description it seems likely that type I was probably a *Lactobacillus* and type II a *Streptococcus*. This is consistent with other reports from this era wherein some investigators have identified organisms as *Lactobacillus* and others have identified similarly described organisms as *Streptococcus*. Since it is now known that both genera can be pleomorphic it is not surprising that this occurred.

Shortly after this work Clarke (1924) consistently isolated from carious lesions a streptococcus which displayed a pleomorphism dependent upon the growth conditions

to which it was exposed. For this reason he named the organism *Streptococcus mutans*. In his investigations of the microorganisms present in carious teeth he noted:

'The fact that the colonies of *S. mutans* adhere closely to the surface of the teeth appears to be of great importance, as a local concentration of acid in contact with enamel is thereby produced, and this could obviously give rise to rapid decalcification, independently of the acidity of the medium as whole.'

Although Clarke is generally credited with the first description of this bacterium, Colman (1976) reported that Heim had described a similar organism slightly earlier in 1924 which he named *Streptococcus halitus*. Maclean (1927) was also able to isolate organisms resembling *S. mutans* from carious teeth of children and adults and, in retrospect, made an important summary of all the work which had been done to that point on the microbiology of dental caries when he said:

'Thus far we can say from the results of *in vitro* experiments, both *Strep. mutans* and *Bacillus acidophilus* are possible factors in the production of natural dental caries, but in the absence of a laboratory animal in which we can easily induce dental caries, it is impossible to say exactly what is the role of bacteria in dental caries.'

Although sporadic reports of similar organisms continued to appear after Clarke's description (Tunnicliff and Hammond, 1938a; 1938b), his work was largely ignored until the 1960's when in depth investigations of the role of *S. mutans* in dental caries were undertaken.

Bunting et al (1928) in an investigation of dental caries in several groups of children with different degrees of caries susceptibility found, in attempting to apply Koch's

postulates to dental caries, that the third and fourth postulates, namely that a pure culture when inoculated into an animal causes the typical disease with which it is associated and that from such experimentally induced disease the microorganism must again be isolated, 'did not apply because of the unusual nature of dental caries.' They reasoned that as techniques for isolation and cultivation of oral bacteria had improved, the group of organisms proposed by Miller to be responsible for caries in humans could in fact be narrowed down to one category- *Bacillus acidophilus*. They also proposed that the presence of *B. acidophilus* was predictive of future caries attack since they demonstrated in a large number of subjects that those caries-free subjects who harboured the organism eventually developed caries and those from whom *B. acidophilus* could not be isolated did not develop lesions. This finding was confirmed by Enright et al (1932). An additional observation common to both of these studies confirmed that the caries attack was not constant but intermittent. These results indicated that to assess properly the microflora associated with the initiation and progression of caries longitudinal studies are preferable to cross-sectional studies.

To address this concern, Hemmens et al (1946) conducted what, up to that time, was the most comprehensive study of the microflora associated with caries development. They reasoned that if caries occurs as a result of bacterial action the search for the bacterial agents responsible for

caries should be made in and under the plaque associated with a carious lesion and not in the saliva or in pooled plaque samples. They hypothesized that microbial succession could occur in a carious lesion as it does in other ecosystems. Since the bacterial agent which initiated the caries activity may not have survived the evolution of the lesion, an examination of more advanced lesions might reveal other organisms associated with progression. Hence, an assessment of the stage of lesion development was an important part of this investigation.

In their examination of 269 approximal sites of newly erupted premolars in 85 children, samples were gathered at 6 week intervals, radiographs at 3 month intervals, and sites were followed for 6 months after the appearance of a lesion. At the study's conclusion, 939 plaque samples from 87 sites in 44 children were available for analysis. The average length of observation was one-and-one-half years but some sites were followed for as long as four years.

The microbiological analysis was very detailed and it appears that the investigators went to great lengths to identify all isolates. Unfortunately the taxonomy of oral bacteria was poorly understood at this time and hence many bacteria could only be broadly classified. As a measure of the degree of challenge to a surface, the frequency of isolation was calculated for all categories of bacteria isolated in the precarious, transitional and carious periods

of the study. This parameter had not previously been considered in other studies. Generally it was determined that the majority of microorganisms recovered showed some degree of negative correlation with the carious process. Alpha-hemolytic streptococci, *Leptotrichia, Actinomyces*, anaerobic fusiform bacilli and *Neisseria* decreased markedly in number with the onset of caries. Micrococci, *Veillonella* and Gram negative filaments all occurred in low numbers and showed little change with the onset of caries. In the precarious and transitional period, that period immediately preceeding the diagnosis of a lesion, *Veillonella*, Gram negative filaments, diphtheroids, aciduric streptococci and *Staphylococcus albus* were all found more frequently. Given the lack of knowledge concerning the metabolism of dental plaque and microbial interactions in plaque it is not surprising that Hemmens et al saw no significance in the increased level of *Veillonella* in the face of increased lactate levels preceeding lesion development. Of all the categories of organisms isolated, the aciduric streptococci were the most frequently found in the caries period. The organisms which showed the largest increase in numbers during the carious period were the lactobacilli. This increase led the investigators to propose that these bacteria were associated with the development of a lesion to greater degree than any other type which was found in the cultures. The fact that all groups of organisms were sporadically isolated in all three of the stages of the investigation clearly perplexed the authors. They suggested

that either the microorganisms were not continuously present or were present in such small numbers as to make isolation impossible.

Other research initiatives have confirmed that bacteria are essential to the development of carious lesions. The work by McClure and Hewitt (1946) and later Keyes (1960) demonstrated that the addition of penicillin to a high-sucrose diet of rats and hamsters will prevent caries in the animal consuming the experimental diet as well as in the litter mates of caries-susceptible females whose penicillin-sensitive flora had been depressed during pregnancy and lactation. Similar findings have been reported in children who are taking antibiotics on a long-term basis for medical reasons (Loesche, 1986).

II. Caries studies - experimental animals

With the recognition that dental caries in humans was a staggeringly complicated process, investigators began looking for a suitable animal model in which various factors thought to be important in the caries process could be either individually or collectively varied, providing some measure of control in the experimental process. The demonstration by Orland et al (1955) that germ-free rats did not develop caries even when fed a high-sucrose cariogenic diet but that caries rapidly developed when an 'enterococcus' was implanted in the mouths of animals fed a similar diet was the first major study of its type and

established a basic method for the investigation of caries in experimental animals.

This method and variations of it; conventional animals harbouring a normal oral flora, the relative gnotobiotic animal wherein the normal flora has been suppressed by pretreatment with an antibiotic and specific-pathogen-free animals which carry a conventional flora but are guaranteed to be free of particular pathogenic microorganisms, have been used to study many aspects of the caries process. This includes the transmissibility of specific bacteria known to be cariogenic (Keyes, 1960) the effect of various dietary regimes and fermentable carbohydrates on the implantation, colonization and cariogenicity of many organisms thought to play a role in the caries process in the human (Krasse, 1965; 1965; König, 1970; Fitzgerald et al, 1983; Gibbons and Keyes, 1969; Larson, 1976; van Houte et al, 1976; van Houte and Upeslacis, 1976; van Houte and Russo, 1985) and the effect of microbial interactions in dental plaque on plaque development, plaque metabolism and dental caries (Mikx et al, 1975; 1976; 1972).

Although a great deal of useful information has been gleaned from animal studies, fundamental differences between the animal species used and the human must be carefully considered before direct extrapolation of experimental findings to the human situation can be made. The morphology of the rat molar is not comparable with that of the human tooth. Rat molars possess deep fissures and have a thin layer of

enamel, (one twentieth the thickness of human enamel (König, 1966) which does not cover the cusp tips. On tooth eruption, rat enamel is hypomineralized particularly in fissures, unlike human enamel which is more highly mineralized at the time of tooth eruption. As well, the post-eruptive maturation of enamel may continue for up to two months in rodents (Fitzgerald and König, 1968). Therefore, the selection of animals based on age is an important consideration since younger animals with recently erupted hypomineralized teeth are more likely to be caries prone than older animals. Furthermore, the indigenous oral flora of the rat differs from that of the human (Hall et al, 1984). Since rats are coprophagic, recycling up to 35% of their feces daily, this habit could introduce organisms to the oral cavity normally associated with fecal material such as coliforms, enterococci and Gram negative rods and giving proportionately higher counts in the rat as compared to the human (Hall et al, 1984). Most studies employing experimental animals in caries studies make no attempt at oral or general hygiene procedures. Hygiene procedures could inflict major disruptions on dental plaque and should therefore be considered as major ecologic determinants. The lack of this substantial environmental pressure in the animal model represents a significant departure from the human experience. Furthermore, most experimental animals are fed ad libitum with extremely high concentrations of sucrose, a situation which predisposes the animal to

frequent eating which in itself may be caries conducive (Loesche, 1982) and which is quite different from the human experience. However, a similarity may exist in the young human infant who bottle- or breast-feeds ad libitum. This parallel has been noted by van Houte et al (1982) in a study of nursing caries.

In the gnotobiotic animal the influence of microbial interactions is absent. Mikx et al (1978) have shown that this can be a powerful environmental determinant influencing colonization and disease production in the oral cavity. Hence, another illustration that the experimental animal model does not reflect the clinical situation. Despite these shortcomings, many organisms have been shown to be cariogenic in rats and hamsters. **Table 3-1** summarizes these data.

An animal model much more similar to the human is the primate. Their dentition and pattern of caries development are quite similar to those of the human and it is possible to maintain the animal on a diet similar to that consumed by man. In addition, the oral flora of old world primates is quite similar to that of man (Cornick and Bowen, 1971). The major acids found in monkey plaque are quite similar to those found in the human and following sucrose ingestion the pattern of acid production parallels the human with a significant increase in the level of lactic acid (Bowen, 1980). The development of carious lesions is a slow process in the monkey, much like it is in the human,and the

lesions which develop are indistinguishable from the those in the human (Bowen, 1980).

Table 3-1

MICROORGANISMS CAPABLE OF PRODUCING CARIES IN TWO ANIMAL MODELS

	RAT*			HAMSTER@		
Organism	Fissure	Smooth Enamel	Root	Fissure	Smooth Enamel	Root
Streptococcus						
mutans	+	+	+	+	+	+
faecalis	+	–	–	–	–	–
salivarius	+	+	+	+	–	–
sanguis	+	+	–	+	+	–
mitior	+	–	–	–	–	–
milleri	+	+	–	–	–	–
Lactobacillus						
casei	+	–	+	–	–	–
acidophilus	+	–	–	–	–	–
fermentum	–	–	–	+	–	–
salivarius	–	–	–	+	–	–
Actinomyces						
viscosus	+	+	+	–	–	+
naeslundii	+	–	+	–	–	+
israelii	+	–	+	–	–	–
Peptostreptococcus	+	–	–	–	–	–
Rothia	–	–	–	–	–	–
Neisseria	–	–	–	–	–	–
Yeasts	–	–	–	–	–	–
Gram - rods	–	–	–	–	–	–

* gnotobiotic, relatively gnotobiotic and conventional rats
@ specific pathogen free and conventional hamsters
\+ caries produced
– no caries produced

After: van Houte (1980); Gibbons and van Houte (1978); Edwardsson (1986).

Colman and Hayday (1980) conducted a study to examine the microbiology related to the onset of dental caries in *Macaca fascicularis*. The results of this study are summarized in **Table 3-2 and 3-3**. Generally these results are very similar to those of human studies in that the levels of *S. mutans*

increased just prior to caries onset. *Lactobacillus* increased in a similar fashion and remained elevated in the caries active animals although numbers were low. *Actinomyces* showed a decline prior to caries onset but increased after the lesion was established. *Veillonella, S. sanguis, S. mitior*, and *Fusobacterium* all decreased as caries developed. Significantly, the animals which remained caries-free showed almost identical changes in the microflora inhabiting the caries-susceptible study site. Since the ecosystems in both locations must have undergone similar environmental changes such that similar microbial populations could be found, this indicates that other, as yet, unknown non-microbiological factors must have been involved at the sites which developed lesions.

In a similar but much more limited study, Beighton et al (1985) longitudinally examined the effect of dietary sucrose supplementation on the total number of bacteria and proportions of *S. mutans, S. sanguis* and total streptococci in the palatal groove of a maxillary deciduous tooth in *Macaca fascicularis*. The sample site and sampling method were virtually identical to that employed by Colman and Hayday in 1980. The introduction of the sucrose-containing diet induced a rapid increase in the total numbers of bacteria isolated from each groove and a rapid decrease in the numbers and frequency of isolation of *S. sanguis*. Concomittant with the decline of *S. sanguis* was an increase in the numbers and frequency of isolation of *S. mutans*.

Table 3-2
THE MICROFLORA ASSOCIATED WITH THE LINGUAL DEVELOPMENTAL GROOVE IN *MACACA FASCICULARIS* CARIES-FREE ANIMALS

	Sample number			
Organism*	1	2	3	4
Yeasts	0.01	0.01	0.04	0.0
Lactobacillus	0.09	0.01	0.7	0.01
Total Streptococci	66.5	49.6	69.7	53.5
Total *S. mutans*	5.1	14.2	41.7	12.0
S. mutans (c)	2.6	0.5	36.7	11.9
S. sanguis/mitior	7.0	2.1	0.01	0.02
Neisseria	0.3	2.3	0.03	1.0
Gram- aerobic rods	0.4	0.5	0.5	1.7
Actinomyces-aerobic	1.1	16.6	12.3	13.9
Actinomyces-anaerobic	3.5	11.0	14.6	19.6
Bacteroides	0.0	0.03	0.01	0.0
Fusobacterium	9.6	16.2	1.2	9.4
Veillonella	18.5	2.8	0.9	0.01

Data from Colman and Hayday (1980)

Table 3-3
THE MICROFLORA ASSOCIATED WITH THE LINGUAL DEVELOPMENTAL GROOVE IN *MACACA FASCICULARIS* CARIES-ACTIVE ANIMALS

	Sample number			
Organism*	1	2	3	4
Yeasts	0.03	0.02	0.09	0.03
Lactobacillus	0.01	0.08	0.8	1.1
Total Streptococci	66.4	43.1	62.9	37.9
Total *S. mutans*	11.6	18.1	31.9	7.1
S. mutans (c)	11.5	7.8	26.9	6.5
S. sang./mitior	9.4	2.8	0.2	0.6
Neisseria	0.3	0.6	0.03	0.2
Gram- aerobic rods	0.2	2.0	2.2	1.4
Actinomyces-aerobic	2.0	20.0	9.9	20.0
Actinomyces-anaerobic	2.7	11.5	15.4	37.1
Bacteroides	0.0	0.4	0.7	0.5
Fusobacterium	3.6	19.4	3.5	0.6
Veillonella	24.7	2.7	3.8	1.3

* results expressed as mean percentage of total viable flora on nonselective medium
Samples gathered from lingual developmental groove of maxillary deciduous molar
Carious lesions became apparent at this site between sample 3 and 4
Samples spaced 6 months apart
N = 11 animals
Data from Colman and Hayday (1980)

Grooves which subsequently became carious harboured significantly more bacteria and had higher levels of *S. mutans* at each sampling period than grooves which remained caries free. They concluded that the level of *S. mutans* at a site could be a useful predictor of future caries activity at individual sites on the dentition. Despite similar methods the results of this study appear to conflict with those of Colman and Hayday with respect to shifts in bacterial populations on carious and caries-free surfaces.

The argument supporting bacterial specificity in the caries process has been made by many (Gibbons and van Houte, 1978; Loesche, 1982; van Houte, 1980). The results of these animal studies and others conducted in humans illustrate the confusion which still exists relative to caries etiology. The following section will examine the concept of bacterial specificity more thoroughly in a discussion of human caries studies.

III. Caries studies - humans

In any caries study involving humans the evidence gathered to support a specific hypothesis is generally indirect since the investigator cannot control, for ethical and practical reasons, all the factors which may affect the data to be collected. An unfortunate result of animal studies has been an over-emphasis on controlling experimental conditions such that the role played by microorganisms in the caries process has been disproportionately emphasized at the expense of other contributing factors. Following the observation that

the Gram-positive flora of the oral cavity appeared to play a central role in the caries process, attention was directed away from studies of the oral flora as a whole. The search for a specific pathogen ignored the ecologic aspects of the disease. Animal models employing gnotobiotic animals or exaggerated levels of sucrose in the diet unrealistically distorted the disease process and failed to consider the effects of other factors in the ecosystem, such as other constituents of the diet which are anti-cariogenic, bacterial interactions involving competition or antagonism, immunological components and oral hygiene pocedures which may modify the cariogenic challenge in the human situation. Similarly, clinical studies which have limited the investigation to the role of specific organisms in caries have ignored the effect of the entire ecosystem. It is more appropriate to consider dental caries as a disease of altered ecology (Morhart and Fitzgerald, 1976) rather than focusing on the narrow question of bacterial specificity in dental caries. It is the purpose of this section to consider the microbiology of dental caries as determined from studies of human subjects and to discuss briefly some of the ecologic determinants which may regulate the microflora involved.

Most studies of the etiology of dental caries in humans have focused on *S. mutans* and *Lactobacillus* since it has been shown in animal studies that these organisms can be involved in both the initiation and progression of caries (Tanzer,

1981). Human studies, on the whole, have shown that plaque removed from carious lesions contains elevated levels of *S. mutans* and *Lactobacillus* in comparison with plaque from tooth surfaces which are disease-free (Bowden et al, 1976; Duchin and van Houte, 1978; Hardie et al, 1977; Huis In't Veld et al, 1979; Ikeda et al, 1973; Krasse et al, 1968; Loesche and Syed, 1973; Loesche and Straffon, 1979; Loesche et al, 1975, 1984; Milnes and Bowden, 1985; Swenson et al, 1976). Several of these studies were cross-sectional epidemiological surveys of randomly selected populations and they demonstrated a positive correlation between the presence of *S. mutans* and a carious lesion (Duchin and van Houte, 1978; Krasse et al, 1968; Littleton et al, 1970; Loesche and Syed, 1973; Loesche et al, 1975). However, it is now recognized that a major deficiency of cross-sectional, association type studies is the inability to demonstrate a cause-and-effect relationship between the isolation of specific bacteria and the initiation of disease at a specific site (Duchin and van Houte, 1978). The presence of an organism such as *S. mutans* at a specific site may reflect a change in the environmental conditions at that site which favour colonization and eventual prominence by the organism. Furthermore, since it is known that the development of a carious lesion in humans is a slowly progressive process involving periods of demineralization followed by disease remission and remineralization (Berman and Slack, 1973; Craig et al, 1981; Grondahl, 1979; Shwartz et al, 1984), in

cross-sectional association studies it is impossible to determine whether the samples were taken at times of progression or regression of a lesion. In other words, the effect of time in the disease process is ignored.

Although the evidence from cross-sectional studies regarding the association of *S. mutans* with caries is convincing it is by no means conclusive. Loesche et al (1975) found a significant association between percentage levels of *S. mutans* and caries in plaques removed from single occlusal fissures and in pooled plaques removed from representative occlusal and approximal molar surfaces present in children who were either caries-free or had rampant caries (DMFT=>10). 71% of the carious fissures sampled had levels of *S. mutans* accounting for 10% or more of the total cultivable flora in the fissures whereas 70% of the caries-free fissures had no detectable *S. mutans*. However they found carious fissures without detectable levels of *S. mutans* as well as high levels of *S. mutans* with a mean value of 33% of the total cultivable flora in caries-free fissures of 8 children. In comparison, the mean percentage of *S. mutans* in the fissures of children with rampant caries was 18%. These data indicate that in a chronic disease such as dental caries, it is difficult, on the basis of cross-sectional data, to assign overt pathogenicity to only one member of a very complex flora.

In order to overcome the doubt which resulted from cross-sectional studies, several longitudinal examinations of the

microbiology of caries have been completed recently (Bowden et al, 1976; Boyar and Bowden, 1985; Hardie et al, 1977; Huis In't Veld et al, 1979; Ikeda et al, 1973; Loesche and Straffon, 1979; Loesche et al, 1984; Mikkelsen and Poulsen, 1976; Mikkelsen et al, 1981; Milnes and Bowden, 1985; Swenson et al, 1976). The advantage of a longitudinal study is that variations in the composition of the populations under study can be compared with changes which may occur over time on initially clinically sound tooth surfaces. More precisely, if a specific organism is suspected of initiating disease, its numbers should increase at the susceptible site prior to the development of a lesion (Ikeda et al, 1973). However, longitudinal studies are not without their own special problems. These will discussed in a subsequent section which will review problems in the methods of data collection and analysis.

Earlier longitudinal studies of Ikeda et al and Loesche and Straffon were concerned primarily with the role of *S. mutans* and lactobacilli in the development of dental caries. Ikeda et al (1973), in an 18-month study of dental plaque associated with initially caries-free occlusal fissures, approximal and buccal surfaces of the lower first permanent molar of 12 Negro children, found that the initiation of caries was preceeded by elevated levels of both *S. mutans* and lactobacilli. Caries was found in the absence of lactobacilli but not in the absence of *S. mutans*. When lactobacilli were found the numbers were usually low, 0.1%

of the total cultivable flora or less. As well the frequency of isolation for *S. mutans* was much higher than for lactobacilli throughout the study. The authors concluded that their results supported a role for *S. mutans* but not lactobacilli in the initiation of carious lesions. They suggested that lactobacilli instead were involved in furthering the progression of the lesion once initiated.

Loesche and Straffon (1979) provided support for these findings in a report from a two-year longitudinal study of the role of *S. mutans* in fissure decay. In an examination of 52 children aged 5-12 years, 195 primary and permanent molar teeth were examined and occlusal fissures were sampled at 6 month intervals for two years. During this period 42 teeth developed fissure caries. In 37 of the 42 teeth which became carious a longitudinal analysis of the data revealed that the percentage of *S. mutans* increased significantly at the time of caries diagnosis. Cross-sectional comparisons showed that the proportions of *S. mutans* were signifcantly higher in the carious fissures than in caries-free fissures. Unlike the results of the study by Ikeda et al (1973) five new lesions developed which were associated with low or undetectable levels of *S. mutans* but high levels of lactobacilli (25.2% of the total cultivable flora six months prior to caries diagnosis and 4% at the time of caries diagnosis). By comparison, the proportions of lactobacilli were low to zero in all other subjects at all times. An additional interesting finding was that in 92 teeth which

remained caries-free for the duration of the study from children who were either caries active during the study or showed evidence of previous high caries activity, the level of *S. mutans* averaged 10% of the total cultivable flora from these sites, levels which are consistent with caries development in fissural and approximal sites (Ikeda et al , 1973; Boyar and Bowden, 1985). The authors concluded that although the results implicate *S. mutans* in fissure decay, they show that decay can occur in the absence of *S. mutans* and that consistently high levels of *S. mutans* can persist on sound tooth surfaces without initiating caries. For example, in one caries active subject, three premolars which erupted and were sampled during the study showed an increase in *S. mutans* from zero to proportions consistent with caries development on teeth of other individuals. No decay occurred over a three year period in these teeth but the individual developed four new approximal lesions during the same time period. This observation suggests that factors which promote the development of carious lesions were operative in the mouth of this individual during the study but that the caries attack in the fissures was somehow modified. It also demonstrates the uniqueness of microecosystems associated with tooth surfaces, indicating that they should be considered individually rather than collectively. Other environmental factors must be operating to allow similar microfloras to produce disease in one area and not another. Others have made similar observations

regarding consistently high levels of *S. mutans* on tooth surfaces which remain caries free (Burt et al, 1983; Mikkelsen and Poulsen, 1976; Mikkelsen et al, 1981; Hardie et al, 1977; Bowden et al, 1976; Milnes and Bowden, 1985).

Two studies from Loesche's group at Michigan furthered the earlier investigation by Loesche and Straffon into the etiology of fissure caries. Burt et al (1983) in a two year study of the occlusal fissures of 573 mandibular first permanent molars in 279 children aged 6-7 years, found that the teeth under study rapidly became infected with *S. mutans* and only 4.4% of the teeth remained uninfected at the end of the two year study period. Once infected, the levels of *S. mutans* isolated remained quite stable; those teeth infected with high levels of *S. mutans* tended to stay high and vice versa. High levels of infection were associated more often with the development of caries than were low levels of infection although the majority of occlusal surfaces (approximately 75%) with high levels of *S. mutans* remained sound. This may have been related to the fact that teeth which developed lesions were excluded from further analysis. Hence, the teeth left for subsequent analysis may have represented the more resistant teeth.

Loesche et al (1984) reporting on the same study population, studied the microbiology of fissure plaque isolated from 368 children over a three year period to determine the relationship between the proportions of *S. mutans*, *S. sanguis*, *Veillonella*, lactobacilli and an unidentified

actinomyces-like organism and the diagnosis of dental caries. Of 115 children who were categorized as exhibiting a low caries activity, 93 developed an average of 1.2 new lesions per child. In contrast, 176 of 183 children in the highly caries active group developed an average of 7.9 new lesions per child. The highly caries active group had, at all time periods, significantly higher fissural levels of *S. mutans* than did children who remained caries free. However, in those fissures which developed decay, the levels of *S. mutans* at the time of caries diagnosis for both the low and high caries groups were virtually identical at 24% and 25% of the total cultivable flora, respectively. Interestingly, decay appeared to develop more rapidly in the less caries-active children (6-12 months after the increase in *S. mutans* levels) than in the highly caries-active children (18-30 months after the increase in *S. mutans* levels). As in their previous study of fissure caries (Loesche and Straffon, 1979) there were teeth on which the proportions of *S. mutans* were high and decay did not develop. Loesche et al suggested that this may have been related to several factors: the spatial relationship of *S. mutans* to the enamel surface, that a 'cocariogen' such as *Lactobacillus* was necessary for the lesion to develop and progress, or that the caries attack was modified by other organisms in the dental plaque and salivary factors.

Lactobacilli were sporadically isolated from the teeth of children in all groups. At the time of caries diagnosis,

lactobacilli represented 7.1% of the total cultivable flora in the less caries-active children and 5.2% in the plaques taken from highly caries-active children. These values were significantly higher than the corresponding values for caries-free teeth. In both groups of children the levels of lactobacilli increased 6 months prior to the diagnosis of caries and they were invariably isolated from teeth which were destined to be either decayed or filled. They appeared therefore to play a role in the progression of the lesion. This is in agreement with the results of a study by Boyar and Bowden (1985), who showed a positive correlation between the isolation of lactobacilli from a carious lesion and progression of the lesion.

The levels of *Veillonella* in carious and caries-free fissures were relatively stable and generally not significantly different until the diagnosis of caries was made, when the levels were 5.0% in the decayed fissures and 1.4% in the caries-free fissures. This may have reflected a continuing supply of lactate in the carious fissures due to higher proportions of lactic-acid-producing bacteria such as *S. mutans* and lactobacilli. Since these fissures were carious however, it does not appear likely that *Veillonella* modified the caries attack as suggested by animal studies (Mikx and Svanberg, 1978).

The association of *S. sanguis* with caries, as expressed by the ratio of the levels of *S. mutans* to *S. sanguis*, has been

reported to be negative. This agrees with earlier results from Loesche and Straffon (1979). However, the results of Boyar and Bowden (1985), Milnes and Bowden (1985) and Swenson et al (1976), suggest that this relationship exists between *S. mutans* and *S. mitior* rather than *S. sanguis*. This discrepancy can be explained by the identification methods employed by Burt et al and Loesche et al in their respective studies since colonies resembling *S. mitior* were included in the *S. sanguis* counts. The actinomyces-like organism showed no remarkable changes in the plaque with time.

The data presented in the papers of Burt et al and Loesche et al show that *S. mutans* and, to lesser extent, *Lactobacillus* are important pathogens in the caries process. However they do not rule out the possibility that other groups of organisms could be involved in mounting a cariogenic challenge to a tooth surface.

The sampling interval of six months used by Loesche and Straffon and Loesche et al in the studies previously cited may have been too long to define accurately the changes which occur in the microflora as disease develops. This is supported by the observation that in the low caries group, lesions developed quickly (6-12 months), suggesting that with only one or a maximum of two samples during this period, successional changes in the microflora at these sites may have been missed. Similarly, the increase in the levels of lactobacilli could only be demonstrated 6 months prior to caries diagnosis. Shorter sampling intervals may

have identified children within the arbitrarily designated groups who displayed variations in the pattern of microbial succession reported in this paper. Furthermore, it is curious that the authors did not identify the actinomyces-like organism which appears to have been regularly isolated.

As well, the criterion for caries used in these studies represents a terminal event in that the surface of the tooth had to display softness or a definite break in the enamel. Presumably they chose this time to make the diagnosis as it is difficult to diagnose incipient fissure caries accurately even under ideal conditions. It is possible that in their examinations with mirror and explorer the surface of a lesion in an incipient state could have been damaged, creating conditions favouring colonization and eventual dominance of the ecosystem by more aciduric organisms such as *S. mutans* and lactobacilli, with resultant cavitation.

Lastly, besides indicating that a 26 gauge needle was used, the authors stated that the entire fissure length was sampled. Since it is well known that fissure caries develops in isolated areas of the fissure system (Miers and Jensen, 1984), this procedure would effectively eliminate the separateness of individual ecosystems. Sampling specific locations within each fissure would have preserved the differences between ecosystems although this would have been much more time consuming and labour-intensive.

While it has been conceded that *S. mutans* is a significant

pathogen in dental caries, many studies have found that dental caries can occur in the presence of either low or undetectable levels of *S. mutans* (Bowden et al, 1976; Hardie et al , 1977; Loesche and Straffon, 1979; Mikkelsen and Poulsen, 1976; Mikkelsen et al, 1981; Swenson et al, 1976).

Swenson et al (1976) longitudinally examined and sampled, over a two-year period, the buccal and mesial approximal surfaces of clinically caries-free maxillary and mandibular first permanent molars in 781 6-9-year-olds. Plaque samples were gathered at six month intervals and pooled. They found that, although the microbiological results generally reflected the caries state of an individual's dentition, the detection of *S. mutans* alone was not sufficiently sensitive to predict either individuals who would develop caries or specify those surfaces which would become carious. This could be attributed partly to the method of detection of specific organisms (identification of colony-forming units based on their colonial morphologies), and the use of pooled plaque samples, both of which could underestimate the actual levels of specific organisms at specific sites.

Based on the results of animal caries studies with *S. mutans*, many of the human caries studies investigated the relationship of this one organism only to caries initiation, with the conclusion that *S. mutans* was the principal organism responsible. More thorough examinations of the

entire microflora associated with carious lesions have been completed by others in recent years. Mikkelsen and Poulsen (1976), and Mikkelsen et al (1981) examined two populations, pre-school children in Denmark and highly caries-susceptible adults in Greenland. In the longitudinal study of Danish pre-school children, the prevalence of *S. mutans* was low both on surfaces which developed lesions and those which did not. However, in 6 of the 18 children who did get caries, *S. mutans* was isolated in higher numbers approximately 6 months prior to lesion development. In the others no clear relationship between *S. mutans* and caries development could be established. The only parameter which showed any change in relation to lesion development was an increase in the anaerobic microflora and a coincident decline in the aerobic microflora. In a later cross-sectional investigation of a highly caries susceptible adult population in Greenland, a situation not unlike that of Canada's native population, quite different results were obtained. *S. mutans* was present in nearly 100% of samples from carious and non-carious surfaces. Unlike their previous study, Mikkelsen et al found no difference in the aerobe/anaerobe ratio between carious and non-carious surfaces. They were also unable to discern any difference between the microbial composition of dental plaque covering carious and non-carious tooth surfaces as determined by Gram stain. Thus, in both studies, no association between *S. mutans* and the development of caries could be demonstrated although it could be easily argued that the microbiological

methods employed were inadequate to make such a conclusion.

By far, the most thorough examination to date of the microflora associated with the development of carious lesions has come from the MRC Dental Epidemiology Unit of the The London Hospital Medical College (Bowden et al, 1976; Hardie et al, 1977). This study appeared to take up where the study by Hemmens et al (1946) had left off, in that similar subjects, children in the late mixed or early permanent dentitions, and similar sites, approximal surfaces of erupting premolar teeth, were used. In examining 19 children, plaque samples were collected from the distal surfaces of the maxillary first premolar. Caries developed at 15 target sites during the study. These data were further subdivided into smaller groups based on the time of caries diagnosis and the number of samples taken before and after caries was detected. This made statistical analysis impossible because of the small numbers in each group. In general the composition of the microflora at the generic level was similar between carious and non-carious sites both before and after the diagnosis of caries. *Actinomyces* were predominant at all sites. High numbers of *Streptococcus, Veillonella* and *Bacteroides* were also found. Others commonly isolated included *Neisseria, Lactobacillus, Haemophilus, Rothia, Bacterionema, Leptotrichia, Eubacterium* and *Fusobacterium*, although they were present in small numbers. In only 2 carious sites did the levels of *S. mutans* increase prior to the diagnosis of caries. In the other

sites the levels of *S. mutans* and lactobacilli increased after caries diagnosis. *S. mutans* was not isolated in 2 other sites which developed caries. *S. mutans* could also persist on many surfaces without subsequent development of a carious lesion. Hardie et al concluded that domination of the microflora by *S. mutans* at a specific site was not essential for the development of a carious lesion. Furthermore, they could not associate any species with the onset of caries.

Although found in low numbers, the most commonly isolated species of lactobacilli was *L. casei*. *L. acidophilus*, *L. buchneri* and *L. fermentum* were also found but infrequently.

It was thought that progressive lesions were those associated with increasing numbers of *S. mutans* after caries diagnosis. This agrees with the results of a study by Huis In't Veld et al, (1979) in caries-active 18-20-year olds. 71% of lesions which progressed into dentin had levels of *S. mutans* in excess of 5% of the total cultivable flora. High levels of this organism were also found on surfaces which remained caries-free. *S. mutans* serotype d was the most common serotype in caries-active individuals but serotype c was found in both caries-active and caries-free subjects.

Boyar and Bowden (1985) have recently shown that the progression of incipient lesions is almost invariably associated with lactobacilli and, to lesser extent, *A. odontolyticus* as well as *S. mutans*.

Not surprisingly, the proponents of a specific bacterial etiology for dental caries have attacked the results of studies which have failed to come to the same conclusion. The choice of surfaces sampled, the sampling procedure, and the handling of plaque samples have all been cited as potential reasons for the difference in results. Given that fissures and approximal surfaces are morphologically different and that they therefore represent different environments it does not seem unreasonable to propose that the initiation of caries in each location may have a different etiology. It is known that approximal plaque supports a more diverse microflora than fissures (Bowden et al, 1975) and that many of the organisms isolated from plaque are capable of producing acid following exposure to carbohydrate. Within the mutans streptococci exist 6 'subspecies', each demonstrating varying degrees of cariogenicity in humans and animals (Gibbons, 1986). *S. mutans* (serotype c,e,f), *S. rattus* (serotype b), *S. cricetus* (serotype a), and *S. sobrinus* (serotype d,g,h) have all been shown to be cariogenic in animals (Gibbons, 1986). *S. mutans* and *S. sobrinus* have been shown to be cariogenic in humans (Gibbons, 1986). Since this species designation for the mutans streptococci is now accepted (Coykendall and Gustafson, 1986; Bergey's Manual of Determinative Bacteriology, 1986), perhaps it is time to begin looking for these species in microbiological studies of caries. This may help to explain differences in results between many

carefully conceived and conducted studies. Another major problem in any of the studies cited is the standardization of the sampling procedure. Since it is known that the proportions of a given organism may vary on different teeth in the same mouth (van Houte and Green, 1974), and in different locations on a given tooth surface (Duchin and van Houte, 1978), accurate sampling of the same site must occur at each sample collection. However, none of the studies discussed have indicated, nor can they guarantee, that all sites were reproducibly sampled. Therefore, this defect must be common to all longitudinal studies.

Thus far, the discussion has centered around the initiation of caries and a few organisms found to be associated with this event. Since it is known that a pH gradient exists in a carious lesion, with the lowest pH recorded at the depth of the cavity (Dirksen et al, 1963), and that the buffering effect of saliva is negligible at the depth of a cavity (Edgar, 1976), extension of a lesion into dentin may involve several phases of succession in the microflora resulting in dominance by aciduric organisms at the advancing front of a lesion (Loesche and Syed, 1973). Edwardsson (1974), in a detailed study of this kind of situation, removed samples from the pulpal side of lesions in 46 extracted teeth. He found a mixed flora dominated by Gram-positive rods. *Propionibacterium, Actinomyces, Bifidobacterium, Eubacterium* and *Lactobacillus* were the major genera in this group. This group collectively accounted for more than 50% of the total

cultivable flora found in the carious lesions sampled. *Lactobacillus* was the genus found most frequently. The high incidence of *L. casei* and *L. acidophilus* in carious dentin agrees with results of Loesche and Syed (1973). Edwardsson could not find any specific combination of organisms which was associated with carious lesions.

A more recent investigation by Hoshino (1985), using an anaerobic glove box system to sample 4 extracted teeth in a similar manner to Edwardsson, found that the overwhelming majority of the microorganisms isolated were obligate anaerobes, suggesting that conditions in the dentin were strictly anaerobic. The results generally agreed with those of Edwardsson. However, in addition to those genera reported by Edwardsson, Hoshino also found *Arachnia, Clostridia, Peptococcus, Peptostreptococcus, Streptococcus, Fusobacterium* and *Bacteroides* as relatively minor components of the flora. A significant negative finding was the absence of *S. mutans* in the carious lesions sampled. Although Edwardsson found this organism in carious lesions, its isolation was sporadic and its numbers small when isolated. Hence there is some agreement between these two studies about relative importance of *S. mutans* at this stage of the caries process. However, Loesche and Syed (1973) found that *S. mutans* accounted for 24% of the dentinal isolates. A reason for this difference could be related to the methods of sampling used in the studies cited. Edwardsson and Hoshino both sampled the lesions from their pulpal side to

avoid contamination from either the superficial layers of the lesion or the overlying dental plaque. On the other hand, Loesche and Syed sampled the lesions during cavity preparation. This would increase the likelihood of contamination of the carious dentin with organisms from carious enamel or the more superficial layers of carious dentin.

In any event, the small numbers to complete absence of *S. mutans* in carious dentin of teeth sampled by Edwardsson and Hoshino indicates that conditions for growth exist which are hostile to this particular organism. The low pH found in the depth of a cavity may therefore select for more aciduric bacteria such as lactobacilli.

Table 3-4 summarizes the results of these investigations. A striking feature of this table is the large number of different organisms which have been found in carious dentin. Interestingly, many of these organisms have not been tested for their capacity to cause either caries in humans or animals or modify the caries attack in a regressive or progressive fashion once it is initiated.

Table 3-4

THE MICROFLORA ASSOCIATED WITH CARIOUS DENTIN

Organism	Edwardsson(1974)	Hoshino(1985)
Propionibacterium	+	+
P. acnes	+	+
P. avidum	−	+
P. lymphophilum	−	+
P. granulosum	−	+
P. jensenii	−	+
Eubacterium	+	+
E. alactolyticum	+	+
E. aerofaciens	−	+
E. saburreum	−	+
Arachnia propionica	+	+
Lactobacillus	+	+
L. minutus	−	+
L. plantarum	−	+
L. catenforme	−	+
L. crispatus	−	+
L. brevis	+	+
L. cellobiosus	+	+
L. caseii	+	−
L. acidophilus	+	−
L. salivarius	+	−
L. fermentum	+	−
L. buchneri	+	−
Bifidobacterium	+	+
B. breve-like	−	+
B. bifidum	−	+
B. eriksonii	+	+
Actinomyces	+	+
A. israelii	+	+
A. naeslundii	+	+
A. viscosus	+	−
A. odontolyticus	+	+
Peptococcus	+	+
P. magnus	−	+
P. saccharolyticus	−	+
Peptostreptococcus	+	+
P. anerobiosis	−	+
P. parvulus	−	+
Clostridium	−	+
C. difficle	−	+
C. beijerinckii	−	+
C. ramosum	−	+
Streptococcus	+	+
S. intermedius	+	+
S. morbillorum	−	+
S. milleri	+	−
S. mutans	+	−
S. mitior	+	−
S. sanguis	+	−
Veillonella	+	−
Fusobacterium	−	+
Bacteroides	+	+

In summary, when caries is viewed from an ecological perspective, it is clear that specific ecosystems, each with its own unique combination of etiologic agents, are involved in the development of carious lesions. This disease, therefore, is unlike other infectious diseases in that, rather than being associated with a specific organism which invades the host, the indigenous microflora assumes a pathogenic role as a result of an ecological imbalance which develops because of internal or external environmental pressures applied to the ecosystem. The attractiveness of this approach is that the multifactorial nature of dental caries is acknowledged without precluding a more prominent role for some factors than others. For example, this model is consistent with observations made in both cross-sectional and longitudinal studies regarding the role played in caries development by acidogenic and aciduric organisms like *S. mutans* and *Lactobacillus*, which under normal homeostatic conditions occupy a narrow niche. However, when dietary pressure is applied through increased intake of refined carbohydrate, these organisms can assume a larger niche and colonize more sites, thereby increasing the risk for the initiation or progression of carious lesions. Only those organisms which are ecologically fit will survive in the new low-pH-environment. The harmonious relationship which existed before between the host and the microflora becomes a pathogenic relationship as a result of the ecological imbalance which has occurred. However, this hypothesis does not preclude the possibility that other components of

different ecosytems could produce disease. In comparison with the relatively simple flora of the fissure, the microflora of the approximal area has been shown previously to be more complicated. For example, the higher numbers of *Actinomyces* in approximal plaque coupled with their high level of fluoride resistance may allow them to assume an important role in the initiation or progression of approximal lesions.

These observations prompted Bowden et al (1976) to propose that the oral cavity supports a 'basic plaque' which generally lives in harmony with the host. Since environmental conditions of the oral cavity have, over time, selected for bacterial populations which are ideally suited for growth in this location, the basic plaque can be considered to be comprised of autochthonous bacterial species. This hypothesis is supported by evidence from a variety of animals (Dent and Marsh, 1981). Under normal conditions lesions could be produced by the basic plaque, but because of the weak cariogenicity of the plaque these lesions would progress slowly, allowing for periods of remineralization and hence some measure of control by the host. This suggestion is consistent with animal studies in which a variety of organisms have been found to be capable of causing caries (see previous discussion). It also does not discount the opinion regarding microbial specificity in caries etiology. Rather, it extends this concept by recognizing that, in individual ecosystems, other organisms

or groups of organisms can cause caries.

CHAPTER FOUR

Nursing Caries

I. The Clinical Picture

Nursing caries has been described as a pattern of rampant decay affecting the maxillary primary incisors of infants and young children (Ripa, 1978). Nursing bottle caries, nursing bottle mouth, nursing bottle syndrome, night bottle syndrome, baby bottle caries and bottle caries are synonyms for this condition. They have been replaced by the term nursing caries since a similar pattern of decay has been described in demand breast-fed children (Gardner et al , 1977).

Nursing caries was first described by Jacobi, an American pediatrician (1862). Fass is most often credited with the first modern description of 'nursing bottle mouth' in 1962 (Fass, 1962). Since then many papers have been published describing various aspects of the condition. In all of these the unique distribution of decay on the teeth of affected children was similar and all the authors have attributed it to non-nutritive sucking habits which persist beyond twelve months of age (Crawford et al, 1974; Kroll, 1967; Picton and Wiltshear, 1970; Robinson, 1963; Winter et al, 1971; 1971).

II. Epidemiology and Clinical Characteristics

Many articles about nursing caries describe only its clinical management. Since many of these are written by

individuals who frequently treat the condition, they often give a biased view of its prevalence. Only a handful of epidemiological studies have been published in recent years and these indicate that only a small proportion of infants can be expected to develop nursing caries (**Table 4-1**). These studies were carried out on predominantly white urban populations having, for the most part, easy access to dental care. Such studies have masked a much higher prevalence of nursing caries in specific populations. For example, a serious situation exists in Canada's North in the Native population of children under four years of age. Albert et al, in an as yet unpublished survey of 200 Inuit children in the North-west Territories of Canada, found that 90% of them had evidence of nursing caries, most of it rampant.

Table 4-1

NURSING CARIES PREVALENCE STUDIES

Author	Country	Year/reference	Prevalence %
Beltrami and Romieu	France	1939	2.5
Goose	Britain	1967	6.8
Goose and Gittus	Britain	1968	5.9
Winter et al	Britain	1971	5.2
Currier and Glinka	U.S.A.	1977	4.9
Richardson et al	S. Africa	1981	11.7 rural 12.0 urban
Derksen and Ponti	Canada	1982	3.2

In the opinion of many who work with Natives, infants and preschoolers are the poorest nourished of the Native family (Riley,1975). This is partly as a result of the fairly lenient attitude Indian parents have towards their children which includes allowing the child to feed whenever she/he so

desires. In addition, traditional Native methods of food preparation and many traditional Native foods have been discouraged by whites and displaced by nutritionally inadequate, commercially prepared convenience foods, many of which are high in carbohydrates (Smith, 1975). Thus when the infant or young child fusses, many Indian mothers will feed their child to pacify him/her. Because the promotion of breast feeding has not been very successful in many Native communities, most infants are bottle-fed. Therefore, before (and often after) the introduction of solid foods, many infants and young children spend a significant portion of their day sucking on a bottle which is filled with milk, formula, juice or any variety of liquids to which the mother frequently adds sugar. The Hudson Bay Company, the major, and frequently only, supplier of foodstuffs to the Native community, is also partly responsible for this serious dental condition. Recognizing that young Native children often eat nutritionally inadequate diets, The Bay has promoted the use of citrus juices supplemented with vitamin C. Since these liquids have a pH range of 3.22-3.65, and they are consumed by the young child over a long period of time, they could promote rapid demineralization of the teeth (Smith and Shaw, 1987).

Additional at-risk populations are recent third world immigrants to Canada, who, on arrival from countries where bottle feeding is prohibitively expensive and water supplies unsafe, find that it is affordable and can be easily adopted

in this country, freeing both parents to go to work. The additional stress of having both parents working and the guilt that some parents feel as a result of having to be away from their families for many hours each day is usually sufficient to postpone an equally stressful task of weaning the young child from the bottle. Thus the scenario of prolonged, and frequently ad libitum, bottle feeding is established. This observation is supported by results from the Camden study (Holt et al, 1982) of 555 British children 12-60 months of age and a study by Gordon and Reddy (1985), which examined, among other things, the mode of feeding and the prevalence of dental caries in infancy in 100 South African children of mixed racial descent aged 12-24 months. In both these studies bottle feeding appeared to be related to socioeconomic status, with mothers from the lower strata more likely to bottle feed.

In the vast majority of cases, the lesions of nursing caries involve the primary maxillary incisor teeth in the infant and later spread to the posterior teeth depending on how long the habit continues. Teeth are attacked according to their sequence of eruption. The age range of children affected falls between 6-48 months. Band-like carious lesions develop first on the gingival third of the labial surface or in the lingual fossa of the primary incisors (Figures 4-1 - 4-4). The next most susceptible teeth are the primary maxillary first molars which erupt at 12-18 months. These teeth frequently develop fissure caries and later

smooth surface caries involving the facial and lingual surfaces. In most cases the mandibular incisors are not affected. Coverage of these teeth by the tongue during sucking appears to offer some measure of physical protection. The close proximity of these teeth to the submandibular and sublingual salivary gland ducts may also protect them since they are bathed in saliva which may both dilute and buffer organic acids produced in the plaque. The severity of the decay will vary according to the age of the child, the feeding pattern and the type of carbohydrate contained in the liquid. It is not uncommon to learn from the parent that they frequently add sucrose to their child's bottle.

Figure 4-1

Illustration of early lesions of nursing caries- white spots and small cavities- on the labial surfaces of primary maxillary incisors adjacent to the gingival margin.

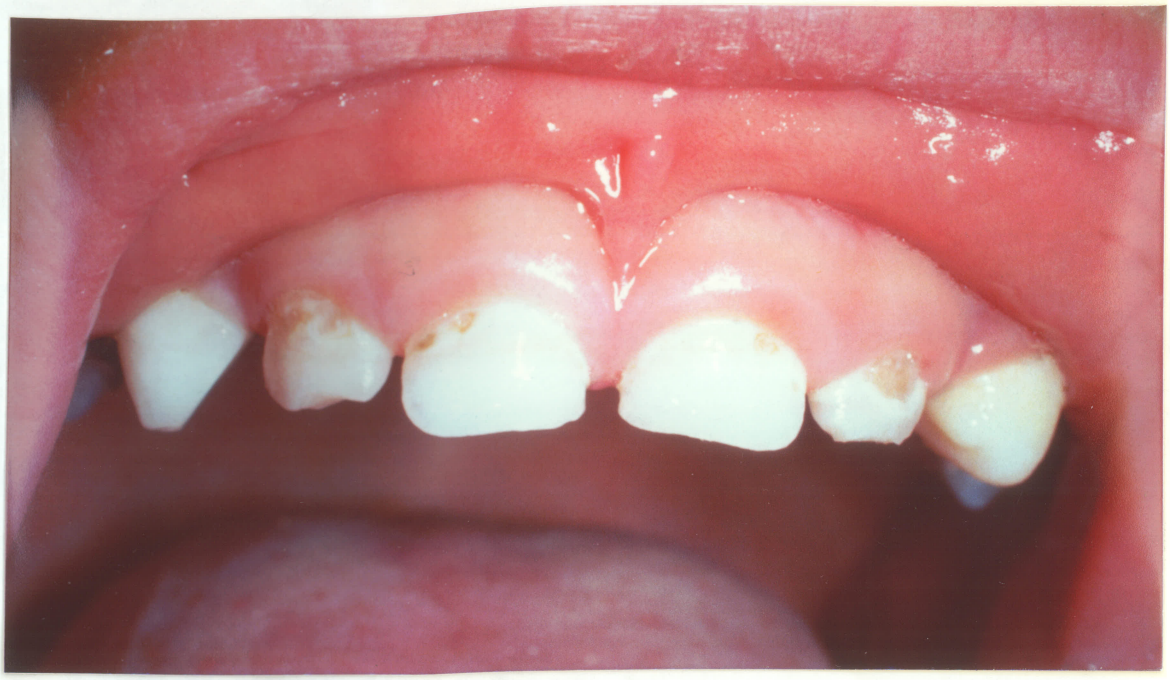


Figure 4-2

Advanced nursing caries of the primary maxillary incisors illustrating the band-like nature of the decay which encircles the teeth.

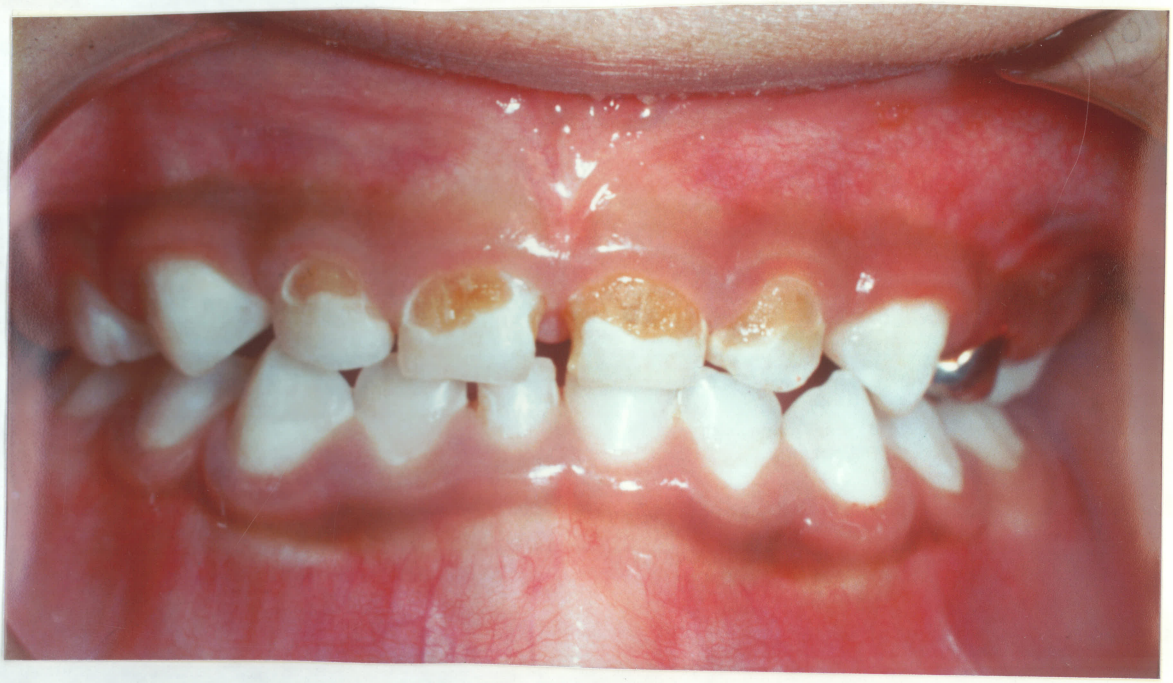


Figure 4-3

Nursing caries involving the lingual fossae of the primary maxillary incisors. The labial surfaces along the gingival margin are caries-free.

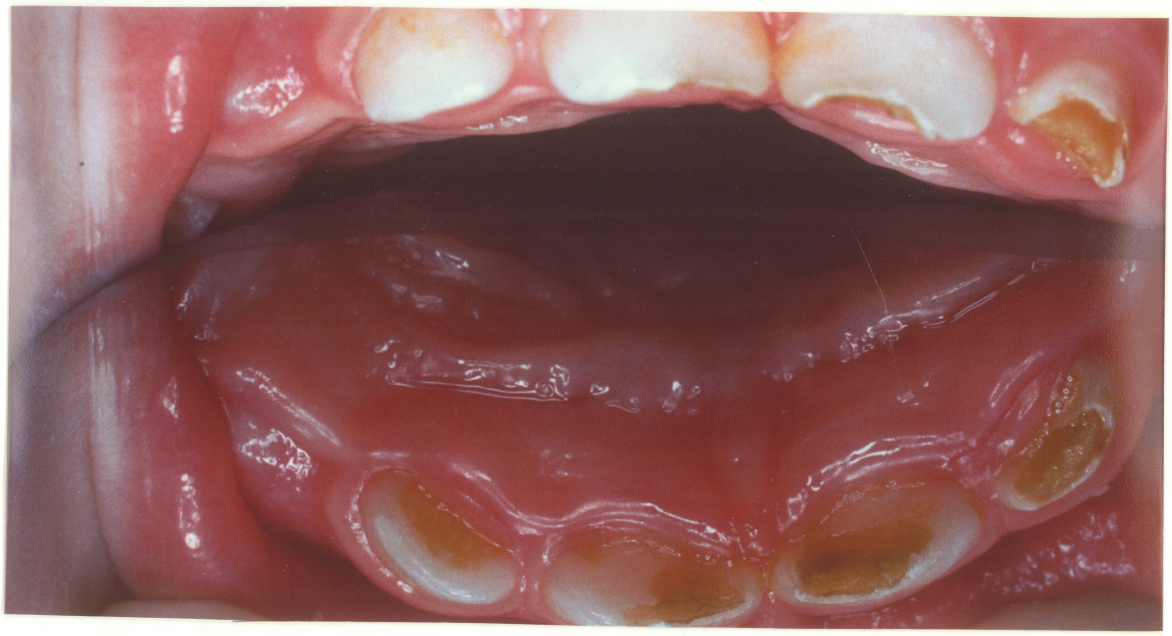
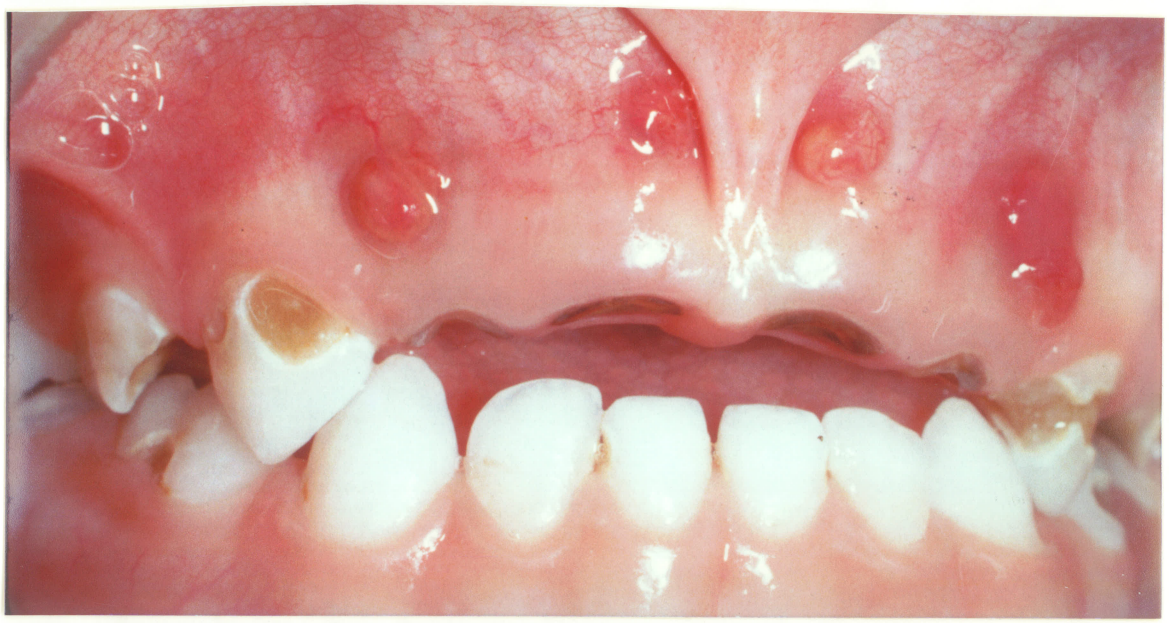


Figure 4-4

Gross destruction of the primary maxillary incisors due to prolonged nursing bottle abuse and resultant periapical abscess formation. The primary canines and first molars are also cariously involved.



Several studies have recently appeared describing the characteristics and backgrounds of children affected by nursing caries. The importance of such information is that it may help the dental health team to direct preventive measures to susceptible children. Johnsen (1982), Johnsen et al (1984), and Dilley et al (1980), found that children who develop nursing caries slept with a bottle or were put to bed with it until they fell asleep. Milk was the predominant liquid found in the bottle but other liquids, including juices, sweetened tea, Kool-Aid and other sweetened drinks were also found with decreasing frequency. Parents of affected children were less likely to have attended university, were often obese, over-indulgent, did not know when weaning should occur and when oral hygiene procedures should begin. Most parents were unaware of the cariogenic potential of the liquids contained in the bottle. The problem with extrapolating the results of these studies to those children who are most seriously affected in Canada is that the studies were completed on predominantly urban populations with relatively few barriers to dental care. In Canada, the most seriously affected children live in remote communities, receive very little health education and, most significantly, live in societies which are in a crisis of transition from a nomadic lifestyle to a sedentary existence. Many of the values of white society, including health care, are encroaching on the traditional values by which Natives have lived for thousands of years. Unfortunately, some Native communities are not adapting well

and their general ill-health is a reflection of this lack of adaptability.

III. Etiology

Nursing caries is a particularly interesting type of caries because of the rapidity with which it develops in the presence of improper feeding practices. Frequent ingestion of foods containing carbohydrate represents a significant environmental pressure on the oral flora and invariably leads to an ecological explosion in the population of cariogenic bacteria, most notably *S. mutans* and *Lactobacillus*, in localized sites on the teeth. In many ways, nursing caries represents the kind of caries produced in gnotobiotic animals which are fed, ad libitum, a high sucrose diet (Orland et al, 1955; Keyes, 1960; Tanzer, 1981).

Coincidental with tooth eruption, the infant is usually acquiring increasing voluntary motor control including the dexterity to place objects, such as a nursing bottle, into the oral cavity. These activities can and often do occur without the help of the parent. A favourite time of many parents to give their child a bottle is at bedtime. Since many children will protest at being put to bed, providing a pleasurable device, like a nursing bottle, will help to soothe not only the child's feelings, but also those of the parents who may not be able to tolerate the nightly crying associated with bedtime.

What makes this type of decay so devastatingly rampant can

be related to the time of day when fermentable carbohydrate is present in the mouth. During sleep, salivation virtually ceases (Schneyer et al, 1956), and so does deglutition (Lear et al, 1965). The buffering and diluting capabilities of saliva and oral clearance on deglutition are therefore absent. Since the child is most frequently given a bottle at bedtime, constant dripping of fluid into an oral cavity in which salivation and deglutition have ceased allows the oral microflora to metabolize fermentable carbohydrate freely producing organic acids and rapid enamel dissolution. Obviously the thinnest enamel, the cervical enamel, is the first to breached and the typical pattern of cervical caries and later fissural caries results.

A further, quite provocative extension of the logic associated with decreased salivation and nursing caries relates to the massive smooth surface decay associated with linear hypoplasia in the primary teeth of children from all over the world, most notably malnourished children. In these populations, the amount of decay in the permanent dentition is low while it is high in the primary dentition, the reverse of what is usually found in our society. The feeding practices in these developing societies usually involve demand breastfeeding for prolonged periods of up to two to three years. If the same logic holds true, that the child falls asleep with breast milk in the mouth, and this is repeated many times during the day and night, the same resultant decay may occur. In this instance the thinnest

enamel would be at the linear hypoplastic defect instead of the gingival margin; hence the different clinical pictures (Sweeney, 1979).

While there is little doubt that fruit juices and other sweetened juices or drinks are cariogenic (Birkhed, 1984), controversy exists as to the ability of milk to foster enamel decalcification proceeding to caries initiation and progression. Several investigators have concluded that milk is not a causative agent and exerts a protective effect on the enamel due to its calcium and phosphate content (Jenkins and Ferguson, 1966; Shaw et al, 1959). Other anticariogenic components of milk include casein. In human milk the concentrations of calcium, phosphate and casein are somewhat lower (Vianna, 1971), but other constituents which are present, notably antibody and lipid, may have protective effects. In rat caries experiments, bovine milk has repeatedly been demonstrated to be non-cariogenic and to reduce the cariogenicity of sucrose-containing diets (Reynolds and Johnson, 1981). Others have found that milk is indeed a causative agent and, even without the addition of carbohydrate, has cariogenic potential (Powell, 1976; Vianna, 1971). Rugg-Gunn et al (1985) have shown that human milk causes a greater fall in plaque pH and more enamel dissolution *in vitro* than does bovine milk. However, they also showed that these effects were less for both bovine and human milk when compared with solutions containing similar lactose and sucrose concentrations. Lactose has been

implicated as the major factor in the decalcification potential of milk although the plaque pH does not drop to the same extent on exposure to lactose as it does when exposed to sucrose or glucose (Neff, 1967). The lactose content of human milk (7.2%) is higher than that of bovine milk (4.5%) or milk formula (7.0%) (Vianna, 1971). It is readily metabolized by many oral microorganisms *in vitro*, in particular by *S. mutans* (Hamilton and Lebtag, 1979), and probably serves as a substrate *in vivo* although it may not be as cariogenic as other common sugars in the human diet.

Given that nursing caries occurs so rapidly on surfaces which are readily accessible to the investigator for both accurate caries diagnosis and sampling of both sound and carious areas, it is surprising that the only comprehensive investigation of the microflora associated with the lesions forms the subject of this thesis (Milnes and Bowden, 1985). Berkowitz et al (1984), and van Houte et al (1982) both examined the microflora from carious lesions in young children but only in a limited fashion and after lesions had developed. van Houte et al (1982) sampled carious lesions in six children aged 18-42 months on one occasion each. The sample sites were lesions on the upper anterior primary teeth, the white spot margins of those lesions and clinically sound enamel in the gingival areas of the same or other upper anterior teeth at least 3 mm or more removed from the lesion(s). In addition, samples were obtained from clinically sound enamel on the buccal, lingual or approximal

surfaces of posterior teeth distant from the highly carious anterior teeth.

They found that carious and non-carious anterior tooth surfaces contained unusually high concentrations of *S. mutans* (61% of the total viable flora). Sound posterior tooth surfaces also had high levels of *S. mutans* (27% of the total viable flora). This high level of infection with *S. mutans* bears a striking resemblance to that observed in conventional rodents infected experimentally with *S. mutans* and fed a high sucrose diet ad libitum, resulting in the development of rampant decay (Tanzer, 1981). van Houte et al also found lactobacilli in most plaque samples although the numbers were very low. The concentration of lactobacilli was higher in carious lesions (4.0% of the total viable flora). Unlike other studies where lactobacilli have been found in association only with progressive carious lesions (Boyar and Bowden, 1985; Ikeda et al, 1973), in this study they were also found in low numbers (0.5% of the total viable flora), in very early white spot lesions. Berkowitz et al (1984), claimed to have extended the study by van Houte et al (1982). In fact, they merely duplicated the methods and reported on a lesser range of organisms than did van Houte et al, limiting their results to the numbers of *S. mutans* and *Lactobacillus* from sound enamel, white spot lesions and cavities in seven children who had been hospitalized for dental treatment of nursing caries. The mean concentration of *S. mutans* determined on MSB agar and

expressed as a percentage of the total colony count on blood agar was 12.58% for sound enamel, 58.58% for white spot and 17.67% for carious lesions. The corresponding figures for *Lactobacillus* were 0.066%, 0.88% and 14.66% respectively.

While both van Houte et al and Berkowitz et al state that their findings provide further evidence that *S. mutans* is the primary etiologic agent in dental caries and reemphasize the concept that *Lactobacillus* has limited involvement in the initiation of dental caries (van Houte, 1980), they fail to demonstrate a cause-and-effect relationship as samples were gathered only after rampant caries had developed. It is important to remember that caries is the result of acid attack on the enamel and not the result of a specific bacterial toxin. Data such as these can only demonstrate that *S. mutans* has an affinity for decalcified enamel.

CHAPTER FIVE

Review of Experimental Methods and Methods of Data Analysis

I. Longitudinal versus cross-sectional studies

Although it is known that most biological values vary over time, only recently has this been taken into consideration when studies to investigate the microbiology of caries have been designed. It is now generally agreed that, despite the need for greater resources, the returns from longitudinal studies are proportionately greater than those from association or cross-sectional studies (Loesche et al, 1984; Loesche and Straffon, 1979; Bowden et al, 1976; Swenson et al, 1976). The advantage of longitudinal observation is that changing trends in variables under investigation can be more accurately linked with the development of disease. In other words, cause-and-effect relationships can be more clearly demonstrated.

II. Site selection, sampling procedure and caries diagnosis

It is known that the composition of the dental microflora can vary:

1. at different sites on the same tooth;
2. at the same site in different mouths;
3. at the same site, same mouth but contralateral side;
4. at the same site, same mouth, same side, but different time (Bowden et al, 1975; Duchin and van Houte, 1978; Marsh and Martin, 1984).

For these reasons it is important to select carefully a site

which is small and well defined, is accessible for reproducible sampling and allows accurate caries diagnosis. Histological studies by Hardie and Bowden (1974) have shown that bacteria often exist in plaque as discrete micro-colonies. By selecting only the smallest area for sampling an accurate assessment can be made regarding flora distribution and the development of caries.

Sampling of the accessible smooth surfaces of the tooth presents few problems in sample collection or site definition. However reproducible sampling of the more caries-susceptible fissures and approximal areas is much more difficult. A variety of methods have been used to sample fissures but most are a variation on the dental probe since the morphology of the fissure will not allow other devices to penetrate. The development of appliances into which tooth crowns can be implanted has simplified fissure sampling (Theilade et al, 1974; Thott et al, 1974). Sampling of approximal surfaces has proven much more difficult and a variety of methods have been used including dental floss (Loesche et al, 1975; Gibbons et al, 1974; Swenson et al, 1976), scalers (Hemmens at al, 1946; Littleton et al, 1970; Mikkelsen and Poulsen, 1976; Mikkelsen et al, 1981), dental probes (Duchin and van Houte, 1978), and abrasive strips (Bowden et al, 1975; 1976, Boyar and Bowden, 1985). The main problem in sampling the approximal surface is that it cannot be viewed directly. Therefore, the investigator cannot be precise about the area

sampled, no matter what sampling procedure is used.

Another problem relates to the diagnosis of caries. This has been based on either clinical or radiographic examinations, or both, of the test sites by the investigator. For approximal surfaces, the diagnosis of incipient lesions has depended almost entirely on the radiograph for reasons mentioned in the previous paragraph. It is well known that the radiographic image of a carious lesion does not accurately reflect its clinical or histological status. Bille and Thylstrup (1982) demonstrated the inaccuracy of radiographic diagnosis of caries in a study which compared the radiographic image with the appearance of the lesion at restoration. Of the lesions found on the radiograph, only 20% of those which extended into the amelodentinal junction and 50% of those which had extended into dentin exhibited cavitation of the enamel surface as determined at restoration. Gwinnett (1971), in an examination of approximal carious lesions in permanent teeth, found that when a lesion was first detected on a bite-wing radiograph, histologically, alteration of the underlying dentin had already occurred. Thus, reliance on the radiograph for diagnostic purposes would overestimate the clinical severity of the lesion regarding cavitation and underestimate the histological severity of the lesion in terms of its depth of progression into the enamel and dentin. Since the actual state of a lesion would be unknown, this diagnostic inaccuracy could affect the interpretation of the results of

a microbiological examination of approximal carious lesions. The investigator would have no idea whether the organisms isolated and identified were associated with an intact or broken enamel surface. Hence, the relation of the microflora to a lesion at a particular stage of development, a hallmark of longitudinal studies, would be lost because of diagnostic inaccuracy. This problem can be overcome if the nursing caries model is used to study the microflora associated with the development of caries. The susceptible areas in this model are on the labial surface of the maxillary incisors which can be readily viewed by the investigator, allowing accurate sampling and, more importantly, diagnosis of caries from initiation to cavitation.

Standardization of samples has been an important part of many studies. Weighing of the sample has been one of the more common forms of standardization employed in studies which have used pooled plaque (Carlsson, 1967, Krasse, 1954). However, when samples are removed from areas where the sample obtained is too small to be weighed (< 1 mg., Loesche and Syed, 1973), eg. approximals, where exposure to room air during the weighing process may jeopardize the viability of strict anerobes in the sample (Moore et al, 1982), or in field studies where the transport of heavy or bulky pieces of equipment is expensive and impractical (Kilian et al, 1979), this procedure is usually bypassed. Instead, it seems reasonable to standardize the sampling technique rather than the sample, so that the sample becomes

the material carefully removed by a standardized technique rather than a specific weight of plaque which has been randomly collected (Bowden et al, 1975).

Consideration should also be given to the population to be selected for study. In order to reduce unwanted effects, the investigator should examine the homogeneity of the population to be studied. For example, the condition of the tooth surfaces under study and tooth age must be considered in study design and subject selection. Different levels of risk are associated with major variations in morphological features such as the presence of enamel defects and the shape and depth of pits and fissures. An examination of individuals with similar tooth age and hence similar caries susceptibility for those teeth under study will reduce the heterogeneity of the population.

Although the effect of the many variables or risk factors in the caries process have been identified, the exact mechanism by which they operate either to reduce or amplify the caries process is unknown in many cases. For example, although it is known that increasing carbohydrate exposure will lead, in most cases, to increased caries attack (Gustafsson et al, 1954), it is not clear at what level of carbohydrate exposure this becomes an important variable. Similar examples can be illustrated for fluoride and antibiotic exposure. As well, many risk factors are examined in isolation or in tandem (microbiological examinations are an example of this kind of experimental behaviour) when, in

reality, they function simultaneously and jointly (Lu, H.K., cited in Bibby and Shern, 1978). Thus to contrast risk factors under study and compare susceptible tooth surfaces, homogeneity of the other risk factors which are not under study will help to provide more explicit results.

III. Methods of Data Analysis

Many methods exist to analyse the data from a microbiological investigation of dental caries. Unfortunately, the most caries susceptible areas, the pits and fissures and the approximals, are also the most difficult to sample in a reproducible fashion as has been previously discussed. This means that the investigator is often dealing with unstandardized samples. The investigator must then decide how to express data which in many scientific circles would be unacceptible because of its lack of both standardization and reproducibility.

Direct microscopic counts of the dispersed but undiluted sample have been regarded as the best way to determine how much of the flora is detected by the isolation medium. Although the methods are relatively simple, debris in the sample precludes an accurate count in many cases. Moore et al (1982) have found that cultural recoveries ranged from 15-185% of the direct microscopic count.

Most studies have relied on the total cultivable flora or total viable count (usually anaerobic), which is determined from a non-selective medium such as blood agar, as the basis

against which all other colony counts are expressed. Since plaque contains a large variety of bacterial species representing a wide range of optimum growth requirements, it is usually necessary to provide a variety of media and gaseous conditions when attempting a complete count. This ensures that organisms which may be unable to compete with faster growing organisms on the non-selective medium are recovered from the sample. The success of this method depends to a large extent on the anaerobic techniques used in the initial culturing of the sample. Manganiello et al (1977) found that failure to recover organisms could be attributed to inadequate dispersion of the sample, adhesion of organisms to glassware during dilution and spreading procedures and an inability of any single cultural environment to support the growth of all types of bacteria present in the sample. The greatest recovery of organisms occurred when samples were dispersed by anaerobic sonic oscillation in pre-reduced anaerobically-sterilized one-quarter strength Ringer's solution which was supplemented with 1% sodium metaphosphate, 0.05% L-cysteine and 0.0001% resazurin. The resulting suspension was anerobically serially diluted and spread onto sheep blood agar which was incubated in an atmosphere of 80% N_2, 10% H_2 and 10% CO_2. 60% of the total microscopic count could be recovered. An additional 15% of the flora could be recovered using additional media and cultural conditions.

The choice of selective media is important since some media

have been shown to be more inhibitory than others to the same organism. Schaeken et al (1986) compared the recovery of *S. mutans* from five media, Mitis-Salivarius agar (MS), Mitis-Salivarius sucrose bacitracin agar (MSB, Gold et al, 1973), Trypticase Yeast-Extract Cysteine (TYC, De Stoppelaar et al, 1969), TYC plus sucrose and bacitracin (TYCSB, van Palenstein Helderman et al, 1983), and a new medium TSY20B developed by the authors. The *S. mutans* counts on TYC and TYCSB were significantly higher than on MS and MSB by almost a factor of 10. Notably, samples with high levels of *S. mutans* serotype d/g gave low *S. mutans* counts on MSB. The authors concluded that MSB was inhibitory to *S. mutans* and should not be used. This finding was confirmed in an earlier study by Colman and Hayday (1980). No significant differences could be detected between TYCSB and TSY20B.

Once the initial cultural period is over the colonies must be counted and representatives of each selected for identification. Many authors have chosen to identify selected organisms, in particular streptococci, on the basis of their colonial morphology on selective media, usually MSB (Krasse et al, 1968; Littleton et al, 1970; Loesche and Straffon, 1979; Swenson et al, 1976). Other authors are reluctant to believe that an accurate identification of colonies can be made simply by inspection of colonies (Bowden et al, 1976; Colman and Hayday, 1980; Moore et al, 1982). To attempt to isolate all colonies from a sample

would be an almost impossible task. However, for accurate statistical analysis of the bacterial composition of a sample, it is necessary to obtain a representative cross-section of the cultivable flora. Some groups choose to isolate representatives of each colony type from both non-selective and selective media (Bowden et al, 1976). Others choose to isolate a predetermined number of colonies in a random and unbiased manner (Moore et al, 1982). This latter method allows for examination of more patients and more sites than the previous method and statistically, if nothing else, should provide a more representative analysis in terms of the entire population under study. However, this method obscures individual variations which may occur between both sites and subjects and hence, may not have as much clinical relevance. The selection of colonies is, therefore, at the discretion of the investigator. Moore et al (1982) have suggested that the number of colonies to be picked from each sample is dictated by the purpose of the study and the available resources. Although it is known that total viable counts and counts of individual colony types are subject to measurement error by the investigator, this may be offset to some degree by choosing to count plates with a relatively high number of colonies since the higher the number of observations made the lower the sampling error and the greater the statistical precision of the count (Meynell and Meynell, 1965).

Socransky et al (1983), have examined a variety of methods

for handling microbiological count data. The analysis of this data presents several problems. Absolute counts of plaque samples often span as many as nine orders of magnitude and thus are subject to unusally high variability. As well, there is a threshold of detection below which microorganisms cannot be accurately enumerated. For microscopic counts this threshold is approximately 10^5 and for cultural counts approximately 10^3. Therefore a zero count may represent any number from zero (it was not there) to as high as 10^5. The handling of zero counts in the analysis of the data causes concern. It is evident from many microbiologic studies of dental plaque that microbiological counts of dental plaque do not have a normal distribution. Considerable variability exists in that in many samples a particular species may be absent but in samples where it is detected it may be present in very large numbers. This problem can be diminished by data transformation (log, square root) but as Socransky et al (1983), have shown, transformation of data such that it will be normalized is not successful for most of the categories of organisms enumerated, i.e. differences between means of raw counts will not necessarily be the same as differences between means of transformed data. Both are mathematically correct but which is biologically correct is unclear.

Having obtained the total viable count and because of the difficulties involved in obtaining accurate and reproducible plaque samples, it is most common to express the

quantitative data in terms of percentages of the total viable count (Hardie and Bowden, 1976). However, viable counts of bacteria are known to be subject to wide variation (Poole and Gilmour, 1971; Jordan et al, 1968) and since the relative concentrations of organisms are often obtained from different dilutions on different media, consideration of the percentages alone can be misleading. Jordan et al (1968) have suggested that it may be more appropriate to think in terms of low, medium or high levels of an organism at a site because of this inaccuracy. Not all agree that the expression of bacterial numbers as a percentage is correct. Denepitiya and Kleinberg (1982) have stated that this method can be misleading and that a better method would be based on the amount or volume of plaque. In their study of a comparison of the composition of pooled human dental plaque and salivary sediment, they expressed the numbers of bacteria as a function of total nitrogen, which is directly related to the mass of the plaque or salivary sediment. They felt that this would be useful for most plaque enumeration studies because as little as 1 ug. of nitrogen can be estimated. This amount of nitrogen is equivalent to approximately 60 ug. wet weight of plaque or 10 ug. dry weight.

If percentage concentrations of an organism are to be used as a way of expressing results, it is important that they be expressed in a similar fashion, otherwise comparisons are difficult, if not impossible. An example where this was not

done can be found in the study by Ikeda et al (1973), in which *S. mutans* is expressed as a percentage of streptococci and *L. casei* is expressed as a percentage of the total cultivable flora. This has the effect of overestimating the contribution made by *S. mutans* to the total flora and the development of a lesion and diminishing the contribution of *L. casei* to the same process.

Another parameter which can be used in the characterization of a bacterial community is isolation frequency (Bowden et al, 1975). This can be interpreted as a measure of the persistence of a particular species at a site and may give some indication of the exposure of a site to specific bacteria as well as the stability of the community over time (Bowden et al, 1976; Svanberg and Westergren, 1986).

Statistical methods employed in the analysis of microbiological data for the most part, lack precision. This relates partly to problems discussed previously but also to the use of parametric statistics which are based on underlying assumptions such as a normally distributed population and equal variances for each subpopulation. Non-parametric statistics are less restrictive and are a powerful alternative to parametric tests when the data are non-normal or involve few subjects. In addition, use of non-parametric procedures in the analysis of microbial count data obviates the need for special handling of zeros, data transformation and the concern of excessive variability in

the data (Socransky et al, 1983).

Because of the lack of precision of the current statistical methods, several groups have developed new methods to characterize the microflora associated with healthy or diseased sites. Moore et al (1982; 1984; 1985) have described the use of Good's 'coverage analysis' (Good, 1953). This has served as a useful indicator of the complexity of the microflora in the sample and as an aid to determine the number of isolates to be analysed. It represents a prediction factor that the investigator has isolated all possible species from the site under study. Although this method has been applied primarily to periodontal microfloras, it could also be applicable to microfloras associated with dental plaque in general or dental caries specifically. The formula is:

[1-(number species seen once/number isolates analysed)] X 100 = % coverage.

Example:

In a sample from which 30 isolates were taken, 13 species were found. 7 species occurred once. The coverage was [1-(7/30)] X 100 = 77%. The estimate indicates that the 13 observed species accounted for 77% of the total colonies in the sample. The remaining 23% of the colonies that were not represented by these species might include many other species which occurred in very low numbers (Moore et al, 1982). Similarly, if from the same sample 30 different

isolates were taken, then the probability that all species had been accounted for in the sample would be 0%

The coverage analysis can also be used with summations of data from many samples in order to compare the coverage between large groups. A refined version of the coverage analysis also includes consideration of the frequencies of those species that are observed more than once (Good, 1953). The validity of the coverage analysis is dependent upon the care which is taken to identify correctly each isolate. As the flora becomes more complex, the coverage estimate will decrease. In addition, the relative homogeneity of the different microflora can be determined by examining the number of species or taxa observed and the coverage estimate (coverage percent) (Moore et al, 1982).

Socransky et al (1981) have described a simple method of comparing complex microflora. Termed the coefficient of similarity, it measures the similarity of the composition of flora as the sum of the percentages of each species in common in the two sites. The variation between samples is emphasized by using only the lower percentage in either sample should the shared species occur more than once in the two sites.

Example:

Two sites contain the same five species in the following proportions:

Site 1 - 22.2%, 3.7%, 0%, 55.6%, 18.5%

Site 2 - 22.2%, 35.9%, 40.1%, 0%, 1.8%

The sum of the lower percentage for each species between the two sites, 22.2% + 3.7% + 0% + 0% + 1.8% = 27.7% represents the minimum similarity between site 1 and site 2. Socransky et al felt that this method of pattern recognition would be useful in defining the microflora associated with different sites, different states of disease activity or different clinical syndromes. Once more, like the methods of Moore et al and Good, the usefulness of the coefficient of similarity is directly dependent on the care taken in the identification of the isolates.

Because of the complexity of the various microflora under examination by Moore and his group at the Virginia Polytechnic Institute as well as the non-normal distribution of bacterial species in periodontal microflora precluding the use of standard statistical tests, Good (1982) developed a simulation analysis to determine the statistical significance of differences between the similarities of the samples within and between subjects (similarities determined by the method of Socransky et al as above). The simulation calculation is based on the mean similarity between two subsets (e.g. subject 1 samples versus subject 2 samples) divided by the mean similarity within the subsets, to obtain a ratio value which is called the Lambda value. This analysis has, in turn, been called the 'lambda test of Good'. It provides an index of separateness between groups by comparing the variation in microflora between subjects within groups with differences in the microflora

between groups and tests the statistical significance of any differences.

Example:

24 samples from subject 1 (subset 1) are compared with 24 samples from subject 2 (subset 2). A total of 576 between-subset minimum similarity comparisons can be made. This is divided by the mean similarity of all 552 possible comparisons within each of the two subsets. The value obtained is the lambda. The 48 samples are then randomly redistributed into two new subsets 10^3 times and reanalysed each time to determine the probability of detecting a ratio as low as the observed ratio. The greater the difference between the subsets, the lower the mean between-subset similarity and the resulting between/within ratio, (i.e. the lambda).

This method appears to be applicable to studies which have isolated extremely complicated microflora and may be of some assistance in defining subtle differences between these.

In summary, a variety of considerations in study design, experimental methodology and data analysis have been presented. The era of the experiment in which only one sample was collected from subjects of differing age or disease susceptibility and/or in which risk factors were examined in isolation in relation to the disease process appears to be over. At present, the methods of data analysis most expressive of the bacterial community on the tooth

surface include:

1. the presence or absence of an organism at the test or study site;

2. the frequency of isolation of organisms from a site as a measure of community stability;

3. the numerical value, expressed as a percentage of the total viable count, of each species isolated and identified;

4. numerical fluctuations of species with time both in disease-free and diseased states;

5. the coefficient of similarity between microflora;

6. the lambda test of Good.

7. non-parametric statistical tests which have a two-way design.

Continued development of new methods of data analysis may help to unravel the complicated puzzle that is dental disease.

CHAPTER SIX

Rationale for the present study

a) Experimental rationale

Reasons for selecting pre-school children as subjects and nursing caries for a study of dental caries are related to problems which were addressed in chapter 5. Since nursing caries develops rapidly on accessible tooth surfaces, it was possible to design a longitudinal study in which initially caries-free but susceptible tooth surfaces could regularly be visually examined and sampled until lesion formation occurred. The tactile methods associated with the diagnosis of fissure caries were unnecessary. Therefore, because it was possible to diagnose accurately the state of each surface and carefully and reproducibly sample each site, an accurate correlation could be made between the status of the tooth surfaces under study at each sampling period and the microflora isolated from each. This is the first study of caries etiology which is able to demonstrate this kind of correlation.

b) Clinical rationale

Although the incidence of dental caries has been recently declining in the western world, some individuals still experience extensive carious activity. This is especially true of the young child who develops nursing caries. The young child is particularly susceptible to this disease because he/she is required to rely on parents who often do not have all the necessary information to prevent dental

disease. Furthermore, the ages of the children at risk falls below the age at which most parents take their child for his/her first dental visit. Thus, the transfer of important preventive health information from dental personnel to parents cannot occur. This situation is further complicated by the geographical and/or cultural isolation of many groups of children most at risk to this disease; native and immigrant children. Therefore, in selecting a group of native children as study subjects from a remote community of Northern Manitoba , it was anticipated that a start could be made in raising the awareness of at least one native community about the seriousness of nursing caries and educating both resident health care workers and parents about methods to prevent this disease.

As well, if significant differences existed in the microbiology of carious and non-carious surfaces, specific microbiologic tests could be designed which would aid the dentist or other health care personnel working in remote areas in predicting which surfaces might progress to cavitation. With this information, more intensive preventive therapy could be provided to those children at higher risk of disease.

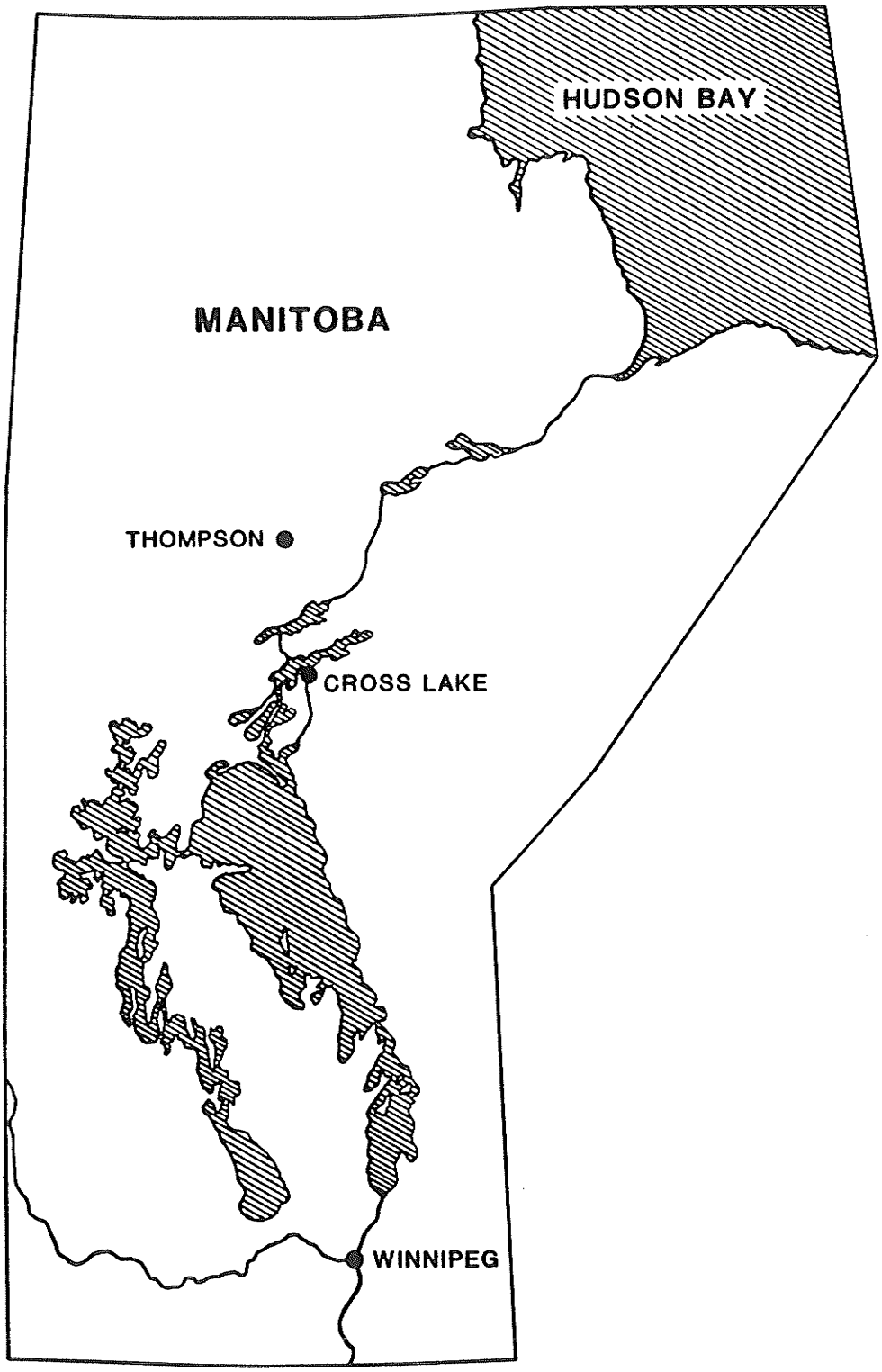
CHAPTER SEVEN

Materials and Methods

Two groups of children, a control group and a test group, were selected for study. The control group consisted of ten children, 2 boys and 8 girls, aged 8-13 months, all resident in Winnipeg. The control group was selected to provide a baseline on the nature of the flora which was present in children within this age group who had a negative or extremely low risk of developing nursing caries. The test group also consisted of ten children, 6 boys and 4 girls, aged 10-16 months, chosen from the infant population of the Cross Lake Indian Reserve in Manitoba (Figure 7-1). These children were selected because of the likelihood that some or all of them would develop nursing caries during the study period despite cautions given to the mothers by myself about the hazards of prolonged bottle feeding. Both groups were examined for a period of 16 months.

The nature and purpose of the study were explained to a parent of each child. Following this, parents were asked to sign a consent form to allow their child to participate. Medical, dental and feeding histories were obtained for all children at the first sampling period and updated at subsequent sampling periods. All children underwent an initial clinical examination under standardized conditions using an examining light, mirror and explorer. This was updated at each sampling period. On initial examination all children were caries free. Both 5-day and 24-hour recall

Figure 7-1



dietary records were used in an attempt to correlate carbohydrate exposure with the development of dental caries. 5-day dietary record sheets were distributed to parents at each sampling period for collection at the subsequent sampling period. Unfortunately, this was perceived by some parents to be an arduous task and in many cases the 5-day record sheets were either filled out incorrectly or not filled out at all. Therefore, although the quality of the data received was inferior, greater use was made of the 24-hour recall method.

I. Sampled Sites and Plaque Collection

The surfaces most susceptible to disease in the nursing caries syndrome are the labial and palatal surfaces of the upper anterior teeth. Therefore, in both groups, the labial surface of a maxillary primary incisor (**Site 1**) and the palatal surface of the same or another maxillary primary incisor (**Site 2**) were selected for sampling. Selection was dictated somewhat by the eruption pattern of these teeth. A third surface, the labial surface of a mandibular primary incisor (**Site 3**), was selected as a non-susceptible control site internal to each subject. In control children only, samples were also collected from the central occlusal fissure of a maxillary first primary molar (**Site 4**). An important feature of the sampling was that each site was examined longitudinally. This provided an insight into both the composition and also the changes in the population ratios that occurred at a given site as the child grew

older. Samples were taken at approximately 6-week intervals. A minimum of eight samples were collected from control children over a 16-month period and a maximum of six samples were gathered from test children over a 12-month period. A slightly different sampling schedule for the test group was necessitated by travel arrangements to and from Cross Lake and the availability of technical assistance in Winnipeg during the trips to Cross Lake. In view of the rapidity with which carious lesions developed in this group, this did not appear to affect the results adversely.

Using sterile orthodontic bands bent into the shape of a small spoon, plaque samples were removed from the gingival third of the labial surface of both the maxillary and mandibular primary incisors and from the lingual fossa of the maxillary primary incisor. The central fissure of the primary molar was sampled by means of a sterile disposable dental probe. In contrast to most studies of the microbiology of a site on a tooth, the ready access to the tooth surfaces in this study made possible reproducible sampling and accurate diagnosis of a white spot or carious lesion. In both groups samples were removed from the anterior two-thirds of the dorsal surface of the tongue (**Site 5**). This area was sampled in control children to determine the nature of the indigenous flora and in test children to determine whether this surface could serve as a reservoir for *Streptococcus mutans* and *Lactobacillus*. The tongue may have a particular significance in nursing

children as it rests against the upper incisor teeth during feeding. After carefully drying the tongue with sterile gauze, samples were collected with a sterile tongue depressor which was firmly scraped over the dorsal surface four times. All samples were transferred to screw-capped glass vials filled to capacity with reduced transport fluid (RTF) (Loesche et al, 1972), to minimize oxygen diffusion into the transport medium. Samples from Cross Lake were flown to Winnipeg for processing within 24 hours. Winnipeg samples were processed within two hours of collection.

II. Microbiological Methods

Upon arrival in the laboratory, plaque samples were dispersed in the transport fluid by sonication using a Kontes sonifier at a power setting of 4 for 30 seconds. Serial 10-fold dilutions from 1:10 to 1:5000 were made in RTF and a volume of 0.02 ml. of each dilution was cultured on blood agar (Oxoid CM 271 Blood Agar Base No. 2) supplemented with haemin, Vitamin K_1 and 5% sheep's blood (Atlas Laboratories, Winnipeg, Manitoba), blood agar plus vancomycin, (7.5 µg/ml, Eli Lilly), TYC agar and TYC plus bacitracin (0.2 units/ml.) (de Stoppelaar et al, 1969) and Rogosa SL agar (Difco Laboratories). Two sets of plates of supplemented blood agar and blood agar plus vancomycin were used with one set incubated aerobically at 37°C for 48 hours and the other set incubated in an anaerobic chamber (Coy Man Co., Ann Arbor, Michigan) at 37 °C in an atmosphere of 85% N_2, 5% CO_2, and 10% H_2 for 5-7 days. The TYC, TYC

plus bacitracin and Rogosa SL plates were incubated for 4 days in anaerobic jars (Oxoid Canada) each fitted with a palladium catalyst and an anaerobic indicator and filled with the same gas mixture as the anaerobic chamber. Total colony counts were determined from plates containing between 20-300 colonies and representatives of each colony type were subcultured onto supplemented blood agar for purification and identification. Colonies that touched each other and had the same morphology were counted as one. Counts of individual genera and species were calculated as a percentage of the total cultivable flora on supplemented blood agar which had been anaerobically incubated for 5-7 days.

III. Identification of Isolates

Purified isolates were initially characterized by their Gram reaction (Hucker and Conn, 1923), cell morphology, atmospheric requirements and catalase production (Paik, 1980). They were then grouped on the basis of this preliminary data into 5 groups:

Group I Gram positive facultative cocci

Group II Gram negative cocci: aerobic, anaerobic

Group III Gram positive rods: aerobic, anaerobic

Group IV Gram negative anaerobic rods, filaments

Group V Gram negative facultative rods, filaments

Table 7-1 lists the references which were consulted during the identification of the isolates.

TABLE 7-1

SELECTED REFERENCES FOR IDENTIFICATION OF ISOLATES

GENUS	REFERENCE
Streptococcus	Hardie and Bowden, 1976 Shklair and Keene, 1976
Actinomyces	Bowden, Hardie et al, 1976 Holmberg, 1976 Slack and Gerencser, 1975
Lactobacillus	Bowden, Hardie et al, 1976 Buchanan and Gibbons, 1974
Rothia	Slack and Gerencser, 1975 Holmberg, 1976
Bacterionema	Slack and Gerencser, 1975 Holdeman et al, 1977 Holmberg, 1976
Neisseria	Morello and Bohnhoff, 1980
Veillonella	Rogosa, 1964; 1964 Rosenblatt, 1980
Fusobacterium	Holdeman et al, 1977 Finegold and Citron, 1980
Bacteroides	Finegold and Citron, 1980 Holdeman et al, 1977 Mayrand et al, 1980 Williams et al, 1975
Hemophilus	Kilian, 1974; 1976 Kilian and Theilade, 1978 Kilian, 1980
Actinobacillus	Weaver and Hollis, 1980 Kilian and Schiott, 1975
Capnocytophyga	Leadbetter et al, 1979 Socransky et al, 1979 Weaver and Hollis, 1980
Leptotrichia	Gilmour et al, 1961
Micrococcus	Buchanan and Gibbons, 1974

a) Group I Gram positive facultative cocci

The catalase test was used to subdivide this group into *Streptococcus* and *Micrococcus*. The latter did not receive further testing. Streptococci were further characterized on the basis of results from tests listed in **Table 7-2** (Hardie and Bowden, 1976).

TABLE 7-2

IDENTIFICATION OF *STREPTOCOCCUS*

1. Gram stain reaction
2. Facultative growth
3. Catalase production
4. Hydrogen peroxide production (Merckoquant strips, Merck, Darmstadt, GDR)
5. Hemolysis on blood agar
6. Fermentation of- mannitol
   sorbitol
   raffinose
   melibiose
7. Arginine hydrolysis
8. Esculin hydrolysis
9. Dextran production on sucrose medium
10. Colonial morphology on sucrose medium

b) Group II Gram negative cocci: aerobic, anaerobic

Strains growing aerobically were tested for catalase (Paik, 1980), and oxidase activity (Kovacs, 1956), fermentation of maltose and the production of extracellular polysaccharide from 4% sucrose in Brain-Heart Infusion agar (Difco)(Cowan, 1974). Anaerobic strains were grown in lactate containing

broth for detection of volatile and non-volatile fatty acids by gas-liquid chromatography, (Carlsson, 1973).

c) Group III Gram positive rods: aerobic, anaerobic

Isolates in this group were tested for growth on Rogosa SL agar (Difco Laboratories), hydrolysis of starch agar, cell morphology following growth on both supplemented blood agar and Rogosa SL agar. These and other tests are summarized in Table 7-3. Isolates which grew well on Rogosa agar and were catalase negative were assumed to be lactobacilli and submitted to the tests listed in Table 7-4. Gas production from glucose, sodium gluconate and maltose was determined in broth using Durham tubes. *Actinomyces* were identified by agglutination of whole cells of the isolates by antisera prepared against *Actinomyces viscosus* (WVU 627), *Actinomyces naeslundii* (ATCC 12104) and *Actinomyces odontolyticus* (NTCC 9935).

TABLE 7-3

IDENTIFICATION OF GRAM-POSITIVE RODS AND FILAMENTS

1. Gram stain reaction
   - cell morphology
2. Colonial morphology
   - pigmentation (red or white)
3. Facultative growth
4. Catalase production
5. Hydrolysis of starch
6. Glucose fermentation
7. Acid end product analysis
8. Serological reactions- *Actinomyces*

TABLE 7-4

IDENTIFICATION OF *LACTOBACILLUS*

1. Gram stain reaction
2. Growth on Rogosa SL agar
3. Esculin hydrolysis
4. Fermentation of- amygdalin
   arabinose
   cellobiose
   fructose
   galactose
   glucose
   gluconate
   lactose
   mannose
   mannitol
   melezitose
   melibiose
   raffinose
   rhamnose
   ribose
   sorbitol
   sucrose
   trehalose
   xylose
   xylitol
   d-arabinose
   d-erythrose
   d-fucose
   turanose
5. Gas production from- glucose
   gluconate
   maltose

d) Group IV Gram negative anaerobic rods and filaments

This group of isolates was the most difficult to characterize because of the current lack of agreement as to what are acceptable methods for identifying these organisms and the rapid changes which have occurred in the taxonomy of the genera involved. To expedite testing, strains were inoculated into API 20A strips (API Laboratory Products

Inc., St. Laurent, Quebec) and PYG broth (0.5% glucose, VPI Anaerobe Laboratory Manual, 4th edition) for acid end product analysis using gas-liquid chromatography. Antibiotic sensitivity testing, to support the separation of isolates into genera, was determined on supplemented blood agar using the disk-agar diffusion method (An-Ident Disks, Oxoid Canada). These disks were impregnated with penicillin (2 units), kanamycin (1000 µg.), vancomycin (5 µg.), colistin (10 µg.), rifampicin (15 µg.) and erythromycin (60 µg.). A summary of the tests used to identify these isolates is included in Table 7-5.

TABLE 7-5

IDENTIFICATION OF GRAM-NEGATIVE ANAEROBIC RODS AND FILAMENTS

1. Gram stain reaction
2. Facultative growth
3. Catalase production
4. Esculin hydrolysis
5. Liquefaction of gelatin
6. Hydrolysis of urea
7. Formation of indole
8. Fermentation of - glucose
   mannitol
   lactose
   sucrose
   maltose
   salicin
   (d+) xylose
   (l+) arabinose
   glycerol
   cellobiose
   mannose
   melezitose
   raffinose
   sorbitol
   rhamnose
   trehalose
9. Acid end product analysis
10. Antibiotic sensitivity tests

e) Group V Gram negative facultative rods and filaments

Although fecal Gram-negative organisms are not common in the mouth (Hardie and Bowden, 1974), tests were made to demonstrate their presence. The majority of the isolates appeared to fall into *Haemophilus* and *Actinobacillus*. Suspect *Haemophilus* and *Actinobacillus* were subcultured onto chocolate agar and incubated at 37 °C in the presence of 10% CO_2 for four days or until growth appeared. Catalase and oxidase tests were completed and growth on MacConkey agar assessed for each isolate. Fermentation of lactose, mannitol and glucose was determined in nutrient broth to which 0.005% hemin and 0.005% nicotinamide adenine dinucleotide (NAD) had been added. Requirement for factor X (hemin) and factor V (NAD) was assessed by subculturing each isolate onto nutrient agar to which were added disks (Difco Laboratories, Detroit, Mich.) containing these factors and then examining each plate for growth after 48 hours. As a more accurate assessment of factor X requirement, each isolate was examined for the production of porphobilinogen and porphyrins when supplied with delta aminolevulinic acid (Kilian, 1974).

IV. Recording and Analysis of the Data

Selected colonies were counted on dilution plates with between 20-300 colonies. Colony counts were expressed as a percentage of the total number of colony-forming units on the supplemented blood agar plate which had been incubated anaerobically for 5-7 days. Thus for each site in a subject

a pattern of the microflora over a period of time was assembled. The control children gave data on the pattern of the flora in the 'normal situation'.

It was possible to divide the group of test children into those who did not develop lesions at any site and those who did develop lesions at one or more of the susceptible sites. Within the latter, a comparison was possible between the percentage of a given microorganism at a susceptible site which developed decay, a susceptible site which remained caries-free and a control site within the same subject. It was also possible to compare data from each site sampled in the test group, with the same or similar site sampled in the control group of children. As a further measure of control, data from susceptible sites in each child could be compared with data from non-susceptible sites within the same subject.

The comparison of the flora at a given site could be made in several ways:

1. Presence or absence of an organism
2. The percentage of the total cultivable flora of a given organism
3. The isolation frequency of a given organism from a site over time.
4. The mean percentage of the organisms over time.

While all of these points could be used to assess an

organism's challenge to the tooth surface, points 3 and 4 are the essence of a longitudinal study and provide the real measure of the degree of challenge to the tooth surface by a given organism.

The data from samples from the tongue for both groups of children were used in a relatively simple way to assess the likelihood of this surface serving as a reservoir of oral microorganisms.

V. Statistical analysis

The mean level of each organism isolated in each sample was expressed as the mean percentage of the total cultivable flora from each site. In this way the levels of organisms isolated from each site could be examined over time for the individual child and gave some indication as to the degree of colonization for each organism. As well it was possible to compare the levels of organisms from sites which developed disease with those which remained disease-free. In addition mean levels for each organism were calculated for each site in both the test and control groups as a whole in order to compare control and test children. Percentage isolation frequency, i.e. the percentage of positive isolation for an organism from a site, was also calculated for each organism. This was done in order to determine the persistence of each organism at the study sites. Together the mean percentages and persistence figures provided a practical way of measuring the degree of challenge by a specific organism to a tooth surface.

Comparisons of the mean percentages and percentage isolation frequencies for each organism were made between sites within both the control and the test groups as well as between the control and test groups. For example, susceptible site 1 in control children could be compared with susceptible site 2 and non-susceptible site 3 in control children and susceptible sites 1 and 2 and non-susceptible site 3 in test children.

Because a number of sites were sampled in each child, each in different locations within the child's mouth and with varying degrees of susceptibility to dental caries, each site was regarded as a separate ecosysytem which harboured a microflora entirely different from that found on the other sites within the same mouth. While this assumption was made with respect to sites within each child's mouth, similar sites between children, eg. all site 1, were assumed to harbour similar microflora since they would be exposed to similar environmental conditions. This served to increase the number of observations at each sampling interval from 9, the number of children in each group, to 27, the total number of samples from either group of control or test children at each sampling interval. Data from similar sites in control and test children were grouped such that all site 1 samples from control children formed one group, all site 2 a second group and so on. This provided large enough numbers for statistical comparison of the groups. Additional groups

were formed in the test children based on the development of dental caries at study sites. This provided caries-active and caries-free groups which were further subdivided on the basis of lesion progression in the caries-active group into susceptible sites which developed lesions (pre-lesion and lesion), susceptible sites which remained caries-free in both caries-active and caries-free children and non-susceptible control sites in caries-active and caries-free children. These groups were compared to one another as well as to the all sites in control children. Therefore it was possible to obtain some measure of difference between the microflora of the caries-free control and test children and that of the caries-active test children in both a general sense and disease-specific sense.

Statistical methods employed in comparing either mean percentages or isolation frequencies were the one-way and two-way analysis of variance (ANOVA), two-sample and paired Student t-tests and the chi square test. This provided information relative to each individual species or genus we chose to examine. However, a weakness of these methods is their inability to compare the microflora as a whole from a particular site with either the microflora from the same site at a different time or the microflora from another site. As well, these kinds of analyses, for the most part, assume a normal distribution of bacterial species in the population under study. Since this is usually not the case, a simulation analysis, the lambda test of Good (1982), was

used to compare the variation in microflora between the groups of subjects previously defined. This test provided an index of separateness between groups and tested the statistical significance of any differences.

CHAPTER EIGHT

Results

I. Subject Data and Samples Collected

Eighteen of the twenty subjects originally selected remained after the completion of the study. These children were followed for an average of 16 months with a range of 8-28 months. The most regular sampling intervals were obtained for the children in Cross Lake. Since travel had to be arranged to and from the reserve an effort was made to examine and sample all children during the one-week clinics which were held on the reserve. In winter, during spring break-up or fall freeze-up this frequently meant travelling on foot or by snowmobile to the subjects' houses when adverse weather or road conditions prevented their travel to the nursing station. Payment for transportation costs was offered to all participants in Cross Lake and a small gift was given to each child at each sampling episode. These actions helped to secure a higher level of cooperation than could have been otherwise anticipated. In Winnipeg, all samples were collected during visits to each subject's home.

A total of 259 samples from the control subjects and 180 samples from the test subjects were collected and examined during the study. None of the control samples was lost during the study. However, 49 samples from test children were lost because of adverse weather conditions which made travel from Cross Lake impossible. This left 131 samples from the test children for analysis. Table 8-1 summarizes

the distribution of samples collected for both the control and test children.

Table 8-1

DISTRIBUTION OF SAMPLES FROM CONTROL AND TEST CHILDREN

CONTROL CHILDREN

Subject	No. of Samples	No. of Examinations
ME	28	5
KE	28	5
AM	28	6
ST	28	6
LY	28	6
KA	29	6
MV	30	7
SH	30	7
BL	30	8
Totals 9	**259**	**56**

TEST CHILDREN

Subject	No. of Samples	No. of Examinations
WR	12	3
JG*	16	4
TS	20	5
CH*	20	5
AM	20	5
DH	20	5
LB*	24	6
DS*	24	6
KT*	24	6
Totals 9	**180**	**45**

* Subjects who developed lesions during the study

II. Lesion Development

All of the control subjects remained caries-free during their respective periods of observation. Five of the nine test subjects developed lesions at the sites selected for study. Five of the total of eighteen susceptible tooth surfaces in these subjects became carious during the study. The other susceptible site and the control site in these five individuals remained caries free. This meant that in

each subject with caries it was possible to compare a carious and non-carious susceptible site. The four other children in the test group remained caries free throughout the study period.

III. Clinical Data

An essential part of the study was the collection of clinical data thought to be important in the assessment of children at risk to nursing caries. Tables 8-2 and 8-3 summarize this data for both the susceptible (test) and non-susceptible (control) groups of children. Only one test child was breast- fed. All other test children were bottle-fed in an ad lib fashion. This feeding pattern included having the bottle in bed at night throughout the duration of the study despite regular warnings that continuation of the habit was potentially harmful to the child. Seven of the nine test children were given bottles at night containing liquids which had been supplemented with honey, corn syrup or table sugar. Five of these seven children developed nursing caries during the study and a sixth child developed the disease after the study was concluded. In contrast, all but one of the control children were breast-fed from birth with occasional supplementation by bottle feeding. The majority of the control children had been weaned from both the breast and bottle when they were included in the study. None of the control children were given liquids other than milk by bottle and none had the bottle in bed.

The control children were exposed much more frequently to

oral hygiene procedures than the test children. As well they were exposed to fluoride in both the water they drank and the toothpaste they used. Since it is known that fluoride can affect the metabolism of most members of the coronal plaque, it has been proposed that it may be an ecological determinant in the development of dental plaque (Loesche, 1976). However, Kilian et al (1979) could not detect differences in the composition of the supragingival plaque flora of Tanzanian children exposed to either high (3-21 ppm), or low (0.3ppm), levels of fluoride in the drinking water. Therefore, in the present study the difference in fluoride exposure was not considered important in terms of the composition of the microflora at the study sites.

It was surprising to learn that five children in the test group had older siblings who had had nursing caries. Four of these test children went on to develop nursing caries during the study. None of the families of the control children had had any experience with this type of tooth decay. This contrast is important because it points to a problem which has been noted by many who have worked in the North in that many Natives appear to consider nursing caries a normal occurrence in childhood. This apparent acceptance of a serious dental problem has made its prevention extremely difficult.

Table 8-2
SUMMARY OF CLINICAL DATA FOR TEST CHILDREN

Subject	Sex	Age (mos)	Age at Upper Incisor Eruption (mos)	Breast fed/ month weaned	Bottle fed/ month weaned	Bottle Content Day	Bottle Content Night	Age solids begun (mos)	dmfs at study end	Oral Hygiene (X/day)	fluoride exposure	Family History Nursing Caries
DS	M	13	9	-	+\-	a,b	a,c	11	11	1	dentifrice	+
LB	F	12	6	-	+\-	a,b	b,c	8	5	-	-	-
JG	M	13	6	+\7	+\-	b,d	a,b	4	8	-	-	+
KT	M	12	2	-	+\-	a,b,c	c	2	6	irreg	dentifrice	+
DH	M	13	6	-	+\-	a,b,c	c	7	0	-	-	-
CH	M	15	6	-	+\-	b,c	c	8	9	-	-	+
TS	F	11	6	-	+\-	a,b,d	a,c	4	0	-	-	-
WR	M	12	6	-	+\-	a,b,d	a	6	0	-	-	-
AM	F	11	6	-	+\-	a	c	8	0	irreg	dentifrice	+
MEAN		**12.4**	**5.9**					**6.4**				

a - milk, formula, breast milk
b - juices
c - milk + sugar
d - tea + sugar

Table 8-3
SUMMARY OF CLINICAL DATA FOR CONTROL CHILDREN

Subject	Sex	Age (mos)	Age at Upper Incisor Eruption (mos)	Breast fed/ month weaned	Bottle fed/ month weaned	Bottle Content Day	Bottle Content Night	Age solids begun (mos)	dmfs at study end	Oral hygiene (X/day)	fluoride exposure	Family History Nursing Caries
LY	F	11	6	+\11	+\7	a	-	5	0	irreg	tri-vi-flor H_2O	-
ST	F	13	5	+\30	-	-	-	8	0	irreg	H_2O	-
ME	F	15	8	+\10	+\10	a	-	5	0	2	dentifrice H_2O	-
KE	F	15	8	+\10	+\10	a	-	5	0	2	dentifrice H_2O	-
BL	M	10	5	-	+\10	a	-	1	0	irreg	H_2O	-
AM	F	16	8	+\10	+\10	a	-	8	0	2	tri-vi-flor dentifrice	-
KA	F	16	8	+\9	+\10	a	-	10	0	2	dentifrice H_2O	-
MV	M	16	6	+\8	+\8	a	-	8	0	irreg	H_2O	-
SH	F	11	6	+\10	+\11	a	-	10	0	irreg	H_2O	-
MEAN		**13.8**	**6.8**					**6.8**				

a - milk, formula, breast milk
Tri-vi-flor contained Vitamins A,C,D and 0.25 mg fluoride per ml.
H_2O fluoride level - 1.0 ppm

CHAPTER NINE

Comparison of the Microflora From Control and Test Children

I. Introduction

This study had two primary aims. The first was to determine what is the normal oral flora of the young child. This was accomplished by longitudinally sampling a variety of oral habitats in children at low risk of dental caries (control children). A great deal of data exists which details the normal oral flora of the older child and adult but there is a paucity of similar data for the young preschool child. The results of this study show that the oral flora of the young preschool child is more complex than has been previously reported. In all children a diverse range of organisms, including many obligate anerobes, could be isolated from all surfaces sampled.

The second aim was to examine longitudinally the microflora from the test children in order to determine how it changed as disease developed. Data relating to this aspect of dental caries has also been published but in most studies the examination of the microflora was limited to only a few species thought to be significant in caries etiology, (Loesche et al, 1984). Where a thorough examination of the microflora has been completed (Bowden et al, 1976) the diagnosis of caries was made using radiographs, a method which is known to be inaccurate, (Bille and Thylstrup, 1982). In addition, accurate, reproducible sampling was hampered because the surfaces sampled could not be

visualized. Hence the correlation between the diagnosis of caries and changes in the microflora was weak. In this study, the sites under examination could be readily visualized such that caries diagnosis and reproducible sampling were easily completed and the correlation between disease development and microflora changes more easily and accurately made.

Although repetitious of the methods section of the thesis, the following outlines which sites were sampled in both control and test children. This should make examination of the tables of data which follow more comprehensive since site identification was excluded from each table to simplify them. In addition, the reader should note that the location of the site is not as important as its degree of susceptibility to dental caries.

SURFACES SUSCEPTIBLE TO NURSING CARIES

Site 1 - the gingival third of the labial surface of a maxillary primary incisor

Site 2 - the lingual fossa of a maxillary primary incisor

SURFACES NOT SUSCEPTIBLE TO NURSING CARIES

Site 3 - the gingival third of the labial surface of a mandibular primary incisor

SURFACES SAMPLED ONLY IN CONTROL CHILDREN

Site 4 - the central occlusal fissure of a maxillary first primary molar

Site 5 - the anterior two-thirds of the dorsal surface of the tongue (Also sampled in test children but only for the presence of *S. mutans* and *Lactobacillus*

II. General Microbiological Data

A total of 9,583 isolations were made from both groups of children, 5,975 from control children and 3,608 from test children.

In general, the flora from both test and control children was qualitatively similar. This is illustrated in tables 9-1 to 9-4. Noticeable differences were the almost complete absence of *Lactobacillus* in control children and a similar absence of *Actinobacillus, Bacteroides* and *Capnocytophyga* in test children. The microflora of test children had a much higher proportion of streptococci (50%) than the control children (30%). This was true for test children who developed lesions and those who did not, (table 10-1, Chapter 10). It is interesting to note that many potential oral pathogens, both dental and periodontal, were regularly isolated from both groups of children in the absence of overt disease.

Table 9-4 contains the mean percentages and isolation frequencies for organisms isolated from site 4, the occlusal fissure of a maxillary first primary molar, and site 5 the dorsal surface of the tongue, both in control children. No statistical comparisons were made between these data and that representative of sites 1-3 in control or test children. A general comparison does however indicate that, qualitatively, the microflora from both these sites is quite similar to that from sites 1-3 in both groups of children. Site 4 had a relatively higher level of *S. sanguis* and

Neisseria than the other sites. Site 5 as expected had a very high level of *S. salivarius* and a very low level of Gram positive rods in comparison to all other sites. Both sites had higher levels of streptococci than sites 1-3 in both test and control children.

Table 9-1

Mean values for organisms isolated from susceptible tooth surfaces in control and test children

	CONTROL CHILDREN		TEST CHILDREN	
ORGANISMS	SITE 1	SITE 2	SITE 1	SITE 2
S. milleri	4.3[a] (0.6)[b]	2.8 (1.0)	6.1 (0.8)	7.6 (1.7)
S. mitior - hard	8.6 (1.4)	11.1 (2.8)	11.3 (2.8)	11.5 (3.0)
S. mitior - soft	6.3 (1.6)	6.3 (2.4)	6.3 (2.0)	2.9 (1.1)
S. mutans I	2.3 (0.8)	1.8 (0.9)	12.5 (3.8)	10.3 (5.0)
S. mutans IV	0.6 (0.4)	0.2 (0.1)	1.3 (1.1)	2.2 (1.2)
S. salivarius	3.4 (0.7)	6.0 (1.5)	6.2 (1.2)	7.3 (1.4)
S. sanguis	4.4 (1.3)	4.3 (1.4)	4.2 (0.9)	5.2 (0.9)
Total Streptococci	**29.9**	**32.5**	**47.9**	**47.0**
A. naeslundii	11.1 (2.4)	12.3 (2.0)	16.4 (3.8)	14.6 (3.2)
A. viscosus	14.8 (1.9)	7.1 (2.0)	10.7 (2.6)	10.2 (3.1)
A. odontolyticus	2.4 (1.0)	1.9 (1.0)	0.7 (0.4)	0.9 (0.6)
A. naeslundii/ viscosus	2.9 (1.5)	1.9 (0.6)	1.5 (0.6)	2.1 (1.2)
Rothia	3.7 (0.8)	10.6 (3.0)	1.3 (0.7)	1.0 (0.5)
Bacterionema	2.3 (1.1)	0.6 (0.4)	0.3 (0.2)	0.2 (0.1)
Lactobacillus spp.	0.2 (0.2)	0	2.4 (1.1)	1.2 (0.8)
L. casei	0.2 (0.2)	0	0.002 (0.002)	0
L. brevis	ND*	ND	0.03 (0.03)	0.01 (0.003)
L. fermentum	ND	ND	0.1 (0.07)	0.02 (0.01)
L. plantarum	ND	ND	1.2 (0.8)	0.1 (0.09)
L. salivarius	ND	ND	0.1 (0.06)	0.8 (0.6)
L. acidophilus	ND	ND	0.9 (0.8)	0.2 (0.1)
Total Gram + Rods	**37.5**	**34.4**	**33.2**	**30.2**
Neisseria A+P+[1]	3.7 (0.9)	4.3 (0.9)	2.3 (1.0)	4.9 (2.2)
Neisseria A+P-[2]	3.8 (1.6)	3.1 (1.0)	2.3 (1.6)	3.1 (0.9)
Neisseria A-P-[3]	1.5 (0.6)	0.9 (0.4)	0.4 (0.4)	0.02 (0.01)
Veillonella	5.7 (1.1)	5.3 (1.0)	8.1 (1.1)	7.8 (1.9)
Total Gram - Cocci	**14.7**	**13.6**	**13.1**	**15.8**
Fusobacterium	0.8 (0.3)	1.1 (0.7)	0.08 (0.04)	0.02 (0.01)
Leptotrichia	1.1 (0.4)	1.8 (1.3)	0.2 (0.08)	0.2 (0.07)
Aerobic -ve Rods	2.6 (0.7)	3.7 (1.4)	1.2 (0.6)	1.8 (0.7)
Anaerobic -ve Rods	1.5 (0.5)	0.8 (0.4)	0.2 (0.1)	0.2 (0.1)
Yeast	1.3 (1.2)	1.1 (0.9)	0.5 (0.2)	1.3 (0.8)
Micrococcus	4.0 (1.5)	3.5 (1.0)	3.0 (0.9)	1.6 (0.5)
Haemophilus	1.7 (0.6)	1.5 (0.8)	0.5 (0.4)	0.3 (0.1)
Actinobacillus	0.1 (0.05)	0.1 (0.1)	ND	ND
Bacteroides	0.7 (0.3)	0.3 (0.2)	ND	ND
Capnocytophyga	0.8 (0.3)	0.8 (0.4)	ND	ND

a Mean value expressed as a percentage of the total cultivable flora

b Standard error of the mean

1 - acid and polysaccharide producing strains

2 - acid producing only strains

3 - non-producers of acid and polysaccharide

* - not detected

Table 9-2

Comparison of the mean values for the percentage isolation frequency of organisms from susceptible sites in control children and test children

	CONTROL CHILDREN		TEST CHILDREN	
ORGANISMS	SITE 1	SITE 2	SITE 1	SITE 2
S. milleri	54[a]	33	51	52
S. mitior - hard	74	65	73	74
S. mitior - soft	67	56	44	19
S. mutans I	31	23	56	50
S. mutans IV	8	5	17	21
S. salivarius	58	63	54	69
S. sanguis	46	44	61	62
A. naeslundii	67	77	68	67
A. viscosus	79	60	71	69
A. odontolyticus	29	21	12	12
A. naeslundii/ viscosus	10	19	17	15
Rothia	60	77	32	40
Bacterionema	19	7	7	12
Lactobacillus spp.	2	0	22	19
L. casei	-	-	2	0
L. brevis	-	-	5	5
L. fermentum	-	-	10	10
L. plantarum	-	-	10	7
L. salivarius	-	-	5	5
L. acidophilus	-	-	5	2
Neisseria A+P+	65	63	63	76
Neisseria A+P-	58	47	41	62
Neisseria A-P-	38	28	22	7
Veillonella	71	74	93	86
Fusobacterium	54	33	21	12
Leptotrichia	46	33	27	24
Aerobic -ve Rods	65	60	51	50
Anaerobic -ve Rods	48	21	15	10
Yeast	6	5	59	50
Micrococcus	40	51	41	31
Haemophilus	42	33	12	12
Actinobacillus	4	2	-	-
Bacteroides	17	16	-	-
Capnocytophyga	44	26	-	-

a Mean percentage - frequency of isolation

Table 9-3

Mean values for the organisms in samples from non-susceptible tooth surfaces in test and control children

	TEST CHILDREN		CONTROL CHILDREN	
ORGANISMS	SITE 3 Mean % (S.E.)	IF %	SITE 3 Mean % (S.E.)	IF %
S. milleri	7.0[a] (1.3)[b]	45[c]	2.3 (0.8)	35
S. mitior - hard	13.1 (2.5)	71	13.2 (1.5)	80
S. mitior - soft	6.3 (2.8)	38	8.8 (2.6)	67
S. mutans I	4.5 (1.3)	43	2.2 (0.9)	30
S. mutans IV	0.5 (0.3)	12	0	0
S. salivarius	8.0 (1.8)	57	5.5 (1.8)	57
S. sanguis	10.2 (3.3)	48	5.0 (1.6)	46
Total Streptococci	**46.9**		**37.0**	
A. naeslundii	10.0 (1.9)	67	11.2 (2.5)	74
A. viscosus	7.8 (2.8)	57	9.0 (1.3)	63
A. odontolyticus	0.7 (0.3)	14	0.4 (0.2)	11
A. naeslundii/ viscosus	0.8 (0.6)	14	0.4 (0.2)	4
Rothia	3.2 (1.1)	52	5.5 (1.5)	72
Bacterionema	1.6 (0.7)	17	1.0 (0.6)	20
Lactobacillus spp.	0.2 (0.2)	2	0.1 (0.04)	4
L. casei	0	0	0.1 (0.04)	4
L. brevis	0	0	0	0
L. fermentum	0.01 (0.01)	2	0	0
L. plantarum	0.2 (0.1)	2	0	0
L. salivarius	0	0	0	0
L. acidophilus	0	0	0	0
Total Gram + Rods	**24.3**		**27.6**	
Neisseria A+P+	4.3 (0.8)	71	4.5 (1.1)	54
Neisseria A+P-	1.7 (0.7)	40	2.8 (0.8)	50
Neisseria A-P-	1.9 (1.4)	26	1.7 (1.1)	43
Veillonella	4.4 (1.4)	62	3.3 (1.0)	52
Total Gram - Cocci	**12.3**		**12.3**	
Fusobacterium	0.3 (0.1)	29	2.0 (0.8)	52
Leptotrichia	0.3 (0.2)	36	1.2 (0.4)	33
Aerobic -ve Rods	2.9 (0.9)	45	4.1 (0.7)	67
Anaerobic -ve Rods	0.5 (0.3)	19	2.3 (1.0)	50
Yeast	0.2 (0.1)	5	0.2 (0.2)	2
Micrococcus	1.2 (0.5)	21	5.9 (1.7)	43
Haemophilus	1.3 (0.5)	31	1.5 (0.5)	41
Actinobacillus	ND*		0.1 (0.03)	7
Bacteroides	ND		0.4 (0.2)	13
Capnocytophyga	ND		0.7 (0.3)	33

a mean value expressed as a percentage of the total cultivable flora
b standard error of the mean
c percentage isolation frequency
* not detected

Table 9-4

Mean values for organisms isolated from central occlusal fissure of a maxillary primary molar and the anterior two-thirds of the dorsal surface of the tongue in control children

	OCCLUSAL FISSURE		TONGUE	
ORGANISMS	Mean %(S.E.)	IF %	Mean %(S.E.)	IF %
S. milleri	3.6[a] (1.8)	37[b]	4.7 (1.8)	42
S. mitior - hard	9.6 (1.7)	73	3.8 (2.2)	30
S. mitior - soft	6.6 (2.4)	47	11.8 (2.3)	75
S. mutans I	1.8 (0.7)	23	1.0 (0.8)	8
S. mutans IV	0.5 (0.4)	7	0.5 (0.4)	2
S. salivarius	7.1 (2.8)	77	22.3 (2.1)	94
S. sanguis	11.0 (3.2)	73	7.6 (1.7)	58
Total Streptococci	**40.2**		**51.7**	
A. naeslundii	11.2 (4.2)	57	2.6 (0.9)	25
A. viscosus	7.5 (2.6)	53	1.3 (0.7)	17
A. odontolyticus	1.3 (0.8)	27	1.1 (0.6)	17
A. naeslundii/ viscosus	2.3 (1.2)	17	0.3 (0.2)	4
Rothia	4.2 (1.6)	57	0.9 (0.4)	21
Bacterionema	0.3 (0.2)	13	0.1 (0.03)	4
Lactobacillus spp	0.5 (0.4)	10	0.3 (0.2)	6
Total Gram + Rods	**27.3**		**6.6**	
Neisseria A+P+	10.0 (3.5)	73	14.0 (2.3)	90
Neisseria A+P-	2.3 (1.2)	50	2.8 (0.8)	31
Neisseria A-P-	1.5 (0.5)	37	3.4 (1.0)	40
Veillonella	8.4 (4.4)	57	6.3 (0.8)	73
Total Gram - Cocci	**22.2**		**26.5**	
Fusobacterium	0.6 (0.3)	23	0.6 (0.2)	25
Leptotrichia	0.2 (0.1)	20	0.2 (0.1)	13
Aerobic -ve Rods	2.7 (1.5)	47	2.3 (0.8)	40
Anaerobic -ve Rods	1.1 (0.6)	33	1.8 (0.3)	40
Yeast	0.6 (0.6)	3	1.0 (0.9)	4
Micrococcus	3.1 (1.3)	40	5.5 (1.2)	46
Haemophilus	0.4 (0.2)	13	0.6 (0.2)	19
Actinobacillus	0.2 (0.1)	7	0.1 (0.03)	4
Bacteroides	0.2 (0.1)	7	0.4 (0.2)	15
Capnocytophyga	1.0 (0.6)	23	0.1 (0.04)	6

a Mean value expressed as a percentage of the total cultivable flora

b percentage isolation frequency

Comparisons Between Data from Sites Within and Between Test and Control Children

Comparisons were made between the mean percentages and isolation frequencies for each species isolated from each site in both groups of children. Tables 9-5 to 9-17 show data for all species that were significantly different as determined by one-way and two-way analysis of variance. In each case a single site (eg. control children- site 1) was compared to all other sites in both the control and test children.

III. Comparison of the Flora from Sites Within Control Children

Data for comparisons between sites in the control children are shown in table 9-5 for mean percentage and table 9-6 for percentage isolation frequency. In general, the microflora from each of these sites was similar as is evident from the few statistically significant differences shown in the tables. However differences did exist. For example as shown in table 9-5, with the exception of *A. naeslundii*, site 1 had higher levels of all other species of *Actinomyces* than site 3. The mean level of *A. viscosus* at site 1 was 14.8% which differed significantly from the levels at site 2 (7.1%) and site 3 (9.0%). The level of *Rothia* at site 1 (3.7%) was significantly lower than the corresponding value at site 2 (10.6%). P values are displayed in the tables in order to demonstrate the level of significance between percentages.

Site 2 and site 3 differed significantly only in the levels of *Rothia* (10.6% at site 2 versus 5.5% at site 3) and Anaerobic Gram negative rods (0.8% at site 2 versus 2.3% at site 3). These data were not tabulated and are not shown.

Table 9-5
Comparison of the mean values for organisms between control site 1 (susceptible) and susceptible and non-susceptible tooth surfaces in control children

		SPECIES SHOWING SIGNIFICANT DIFFERENCE FROM CONTROL SITE 1	
ORGANISMS	SITE 1 Mean %	SITE 2	SITE 3
A. viscosus	14.8	7.1[a] (0.007)[b]	9.0 (0.036)
A. odontolyticus	2.4	-	0.4 (0.023)
A. naeslundii/ viscosus	2.9	-	0.4 (0.059)
Rothia	3.7	10.6 (<0.001)	-
Fusobacterium	0.8	-	2.0 (0.004)

a Mean percentage expressed as percentage of total cultivable flora
b p values as determined by two way analysis of variance
- indicates no significant difference, p>0.100

Data shown in table 9-6 indicate that there were also few percentage isolation frequency differences between sites in control children which reaffirms the similarity of the microfloras at all three sites. Differences which did exist, although statistically significant, were minor. For example, *A. viscosus* was isolated from 79% of the samples from site 1 and from 60% of the samples from site 2, (table 9-6). At site 2 only two differences were detected and these were not

tabulated. The percentage of isolation was significantly different for *Veillonella* (77% at site 2 vs. 52% at site 3, p=0.030) and Anaerobic Gram negative rods (21% at site 2 vs. 50% at site 3, p=0.004).

Table 9-6

Comparison between the mean values for the percentage isolation frequency of organisms from site 1 in control children and sites 2 and 3 in control children

		SPECIES SHOWING SIGNIFICANT DIFFERENCE FROM CONTROL SITE 1	
ORGANISMS	CONTROL SITE 1 Mean %	SITE 2	SITE 3
S. milleri	54	33 (0.038)	34 (0.059)
A. viscosus	79	60 (0.051)	-
Fusobacterium	54	33 (0.038)	-
Anaerobic-ve Rods	48	21 (0.007)	-

IV. Comparison of the Flora Between Sites in Control and Test Children

Data for comparisons between sites in control and test children is shown in tables 9-7 to 9-12 for mean percentage and percentage isolation frequency. These comparisons revealed a larger number of differences between groups than comparisons within either the control (Tables 9-5 and 9-6) or test groups (Tables 9-13 to 9-16). This was expected since the control children were considered to have a low risk of developing dental caries in comparison to the test children. The comparison of site 3 in the control children to sites 1,2 and 3 in the test children revealed the largest

number of statistically significant differences between sites (Tables 9-11 and 9-12). These differences were not confined to one specific group of organisms but were broadly spread throughout the various genera isolated. The levels of *S. mutans* at site 1 (2.3%)(Table 9-7), site 2 (1.8%)(Table 9-9) and site 3 (2.2%)(Table 9-11) in the control children were significantly lower than the levels at site 1 (12.5%) and site 2 (10.3%) in the test children. An important observation was that at site 3, a non-susceptible intra-subject control surface, the levels of *S. mutans* and *Lactobacillus* did not differ significantly between control and test children although slightly higher numbers were found in the test children (Table 9-11). As shown in Tables 9-7 and 9-11 the levels of *Lactobacillus* at site 1 (0.2%) and site 3 (0.1%) in control children were significantly lower than the levels at site 1 (2.4%) in the test children. Other differences in mean percentages are shown in Tables 9-7, 9-9 and 9-11.

Tables 9-8, 9-10 and 9-12 show that differences existed between control and test children in the percentage isolation frequency for a large and diverse number of species. For example, in Tables 9-8 and 9-10 the isolation frequency of *S. mutans* from sites 1 and 2 in control children is shown to be 31% and 23% respectively which differed significantly from an isolation frequency of 56% for site 1 and 50% for site 2 in test children. *Veillonella*, *Lactobacillus* and yeasts were also isolated more frequently

from susceptible sites in test children than control children. In contrast, *S. mitior*, *Rothia*, *Fusobacterium*, Anaerobic Gram negative rods, *Haemophilus* and *Micrococcus* were isolated more frequently from the control children. The least number of differences existed between sites 1, 2 and 3 in control children and site 3 in test children.

Several of the comparisons shown in each table are not statistically significant but are included to indicate a possible biologically significant difference. For example, the level of *S. mutans* biotype IV at site 2 (2.2%) in the test children is not statistically different from the levels at sites 1 (0.6%, Table 9-7) or 2 (0.2%, Table 9-9) in control children. Nonetheless, the differences may be biologically important especially if some consideration is given to the isolation frequency (17% at site 1 and 21% at site 2 in test children) of this organism being higher in test children than in control children in addition to its mean percentage at each site.

Table 9-7

Comparison of the mean values for organisms isolated from site 1 (susceptible) in control children and susceptible and non-susceptible tooth surfaces in test children.

ORGANISMS	CONTROL SITE 1 Mean %	SPECIES SHOWING SIGNIFICANT DIFFERENCE FROM CONTROL SITE 1		
		TEST SITE 1	TEST SITE 2	TEST SITE 3
S. milleri	4.3	-	7.6 (0.065)	-
S. mitior - soft	6.3	-	2.9 (0.076)	-
S. mutans I	2.3	12.5 (0.003)	10.3 (0.020)	-
S. mutans IV	0.6	-	2.2 (0.093)	-
S. salivarius	3.4	-	7.3 (0.036)	8.0 (0.014)
S. sanguis	4.4	-	-	10.2 (0.009)
Lactobacillus	0.2	2.4 (<0.001)	-	-
A. naeslundii	11.1	16.4 (0.060)	-	-
A. viscosus	14.8	-	-	7.8 (0.013)
A. odontolyticus	2.4	0.7 (0.053)	-	0.7 (0.053)
Bacterionema	2.3	0.3 (0.013)	0.2 (0.012)	-

- indicates no significant difference, $p>0.100$

Table 9-8

Comparison between the mean values for the percentage isolation frequency of organisms from site 1 in control children and sites 1, 2 and 3 in test children

ORGANISMS	CONTROL SITE 1 Mean %	SPECIES SHOWING SIGNIFICANT DIFFERENCE FROM CONTROL SITE 1 T E S T SITE 1	SITE 2	SITE 3
S. mitior - soft	66	44 (0.031)	19 (0.0009)	38 (0.007)
S. mutans I	31	56 (0.018)	50 (0.070)	42 (0.254)
A. viscosus	79	-	-	57 (0.024)
A. odontolyticus	29	12 (0.051)	12 (0.045)	-
Rothia	60	32 (0.007)	41 (0.059)	-
Lactobacillus	2	22 (0.003)	-	-
Neisseria A-P-	38	-	7 (0.001)	-
Veillonella	70	92 (0.009)	-	-
Fusobacterium	54	21 (0.001)	12 (0.0009)	29 (0.014)
Leptotrichia	46	-	24 (0.029)	-
Anaerobic -ve Rods	48	15 (0.001)	10 (0.0009)	19 (0.004)
Yeast	6	59 (0.0009)	50 (0.0009)	-
Haemophilus	42	12 (0.002)	12 (0.002)	-

Table 9-9

Comparison of the mean values for organisms isolated from site 2 (susceptible) in control children with susceptible and non-susceptible tooth surfaces in test children

ORGANISMS	CONTROL SITE 2 Mean %	SPECIES SHOWING SIGNIFICANT DIFFERENCE FROM CONTROL SITE 2 T E S T SITE 1	SITE 2	SITE 3
S. milleri	2.8	-	7.6 (0.009)	-
S. mitior - soft	6.3	-	2.9 (0.081)	-
S. mutans I	1.8	12.5 (0.002)	10.3 (0.014)	-
S. mutans IV	0.2	-	2.2 (0.038)	-
S. sanguis	4.3	-	-	10.2 (0.008)
Lactobacillus spp.	0	2.4 NS	1.2 NS	0.2 NS
Rothia	10.6	1.3 (0.001)	1.0 (0.001)	3.2 (0.003)
Leptotrichia	1.8	0.2 (0.012)	0.2 (0.012)	0.3 (0.025)
Fusobacterium	1.1	0.08 (0.006)	0.02 (0.004)	-
Aerobic -ve Rods	3.7	1.2 (0.041)	-	-

Table 9-10

Comparison between the mean values for the percentage isolation frequency of organisms from site 2 in control children and sites 1, 2 and 3 in test children

ORGANISMS	CONTROL SITE 2 Mean %	SPECIES SHOWING SIGNIFICANT DIFFERENCE FROM CONTROL SITE 2 T E S T SITE 1	SITE 2	SITE 3
S. mitior - soft	56	-	19 (0.0009)	-
S. mutans I	23	56 (0.002)	50 (0.010)	43 (0.055)
S. mutans IV	5	-	21 (0.021)	-
Lactobacillus	0	22	19	-
Rothia	77	32 (0.0009)	40 (0.001)	52 (0.019)
Neisseria A-P-	28	-	7 (0.012)	-
Veillonella	77	93 (0.025)	-	-
Fusobacterium	33	-	12 (0.022)	-
Yeast	5	59 (0.0009)	50 (0.0009)	-
Micrococcus	51	-	31 (0.058)	21 (0.004)
Haemophilus	33	12 (0.026)	12 (0.022)	-

Table 9-11

Comparison of the mean values for organisms isolated from site 3 in control children and susceptible and non-susceptible tooth surfaces in test children

ORGANISMS	CONTROL SITE 3 Mean %	SPECIES SHOWING SIGNIFICANT DIFFERENCE FROM CONTROL SITE 3 T E S T SITE 1	SITE 2	SITE 3
S. milleri	2.3	6.1 (0.032)	7.6 (0.005)	7.0 (0.012)
S. mitior - soft	8.8	-	2.9 (0.004)	-
S. mutans I	2.2	12.5 (0.003)	10.3 (0.018)	4.5 NS
S. sanguis	5.0	-	-	10.2 (0.018)
Lactobacillus spp.	0.1	2.4 (0.0005)	1.2 NS	0.2 NS
Rothia	5.5	1.3 (0.029)	1.0 (0.021)	-
Veillonella	3.3	8.1 (0.013)	7.8 0.020)	-
Fusobacterium	2.0	0.08 (0.0001)	0.02 (0.0001)	0.3 (0.001)
Aerobic -ve Rods	4.1	1.2 (0.012)	1.8 (0.067)	-
Anaerobic -ve Rods	2.3	0.2 (0.005)	0.2 (0.004)	0.5 (0.013)
Micrococcus	5.9	-	1.6 (0.003)	1.2 (0.002)

Table 9-12

Comparison between the mean values for the percentage isolation frequency of organisms from site 3 in control children and sites 1,2 and 3 in test children

ORGANISMS	CONTROL SITE 3 Mean %	SPECIES SHOWING SIGNIFICANT DIFFERENCE FROM CONTROL SITE 3 T E S T SITE 1	SITE 2	SITE 3
S. mitior - soft	67	44 (0.027)	19 (0.0009)	38 (0.006)
S. mutans I	30	56 (0.016)	50 (0.061)	43 (0.226)
S. mutans IV	0	17	21	12
Rothia	72	32 (0.0009)	40 (0.003)	-
Lactobacillus	4	22 (0.014)	19 (0.030)	2 NS
Neisseria A+P+	5	-	76 (0.032)	-
Neisseria A-P-	43	22 (0.033)	7 (0.0009)	-
Veillonella	52	93 (0.0009)	86 (0.001)	-
Fusobacterium	52	21 (0.003)	12 (0.0009)	29 (0.021)
Anaerobic -ve Rods	50	15 (0.0009)	10 (0.0009)	19 (0.002)
Yeast	2	59 (0.0009)	50 (0.0009)	-
Micrococcus	43	-	-	21 (0.028)
Haemophilus	41	12 (0.002)	12 (0.002)	-

V. Comparison of the Flora from Sites Within Test Children

Data for comparisons between sites in test children is shown in tables 9-13 and 9-15 for mean percentage and tables 9-14 and 9-16 for percentage isolation frequency. Sites 1 and 2 were quite similar. Only the level of acid and polysaccharide producing *Neisseria* was significantly different between these two sites although this difference was minor. The majority of statistically significant differences existed between site 1 and the internal control site 3. As shown in table 9-13 the levels of *S. mutans* (12.5% vs. 4.5%), *A. naeslundii* (16.4% vs. 10.0%), and *Lactobacillus* (2.4% vs. 0.2%) were all significantly higher at site 1 than site 3.

A similar pattern existed for the percentage isolation frequency comparisons as shown in tables 9-13 and 9-14. Sites 1 and 2 were very similar but both sites differed from site 3. *Veillonella* (93% and 86% vs 62%), *Lactobacillus* (23% and 19% vs 2% , and yeasts (59% and 50% vs. 5%) were isolated more frequently from susceptible sites 1 and 2 than non-susceptible site 3.

Table 9-13

Comparison of the mean values for organisms isolated from site 1 (susceptible) with site 2 (susceptible) and site 3 (non-susceptible) in test children

ORGANISMS	SITE 1 Mean %	SPECIES SHOWING SIGNIFICANT DIFFERENCE FROM TEST SITE 1 SITE 2	SITE 3
S. mitior - S	6.3	2.9 (0.078)	-
S. mutans I	12.5	-	4.5 (0.018)
S. sanguis	4.2	-	10.2 (0.007)
A. naeslundii	16.4	-	10.0 (0.024)
Lactobacillus	2.4	-	0.2 (0.001)
Neisseria A+P+	2.3	4.9 (0.037)	-

Table 9-14

Comparison between the mean values for the percentage isolation frequency of organisms from site 1 in test children and sites 2 and 3 in test children

ORGANISMS	SITE 1 Mean %	SPECIES SHOWING SIGNIFICANT DIFFERENCE FROM TEST SITE 1 TEST CHILDREN SITE 2	SITE 3
S. mitior - soft	44	19(0.015)	-
Rothia	32	-	52(0.057)
Lactobacillus	23	-	2(0.006)
Neisseria A-P-	22	7(0.055)	-
Veillonella	93	-	62(0.001)
Yeast	59	-	5(0.0009)
Micrococcus	41	-	21(0.049)
Haemophilus	12	-	31(0.038)

Table 9-15

Comparison of the mean values for organisms isolated from test site 2 (susceptible) with test site 3 (non-susceptible) in test children

ORGANISMS	SITE 2 Mean %	SPECIES SHOWING SIGNIFICANT DIFFERENCE FROM TEST SITE 2 SITE 3
S. mitior - soft	2.9	6.3 (0.076)
S. mutans I	10.3	4.5 (0.083)
S. mutans IV	2.2	0.5 (0.079)
S. sanguis	5.2	10.2 (0.023)

Table 9-16

Comparison between the mean values for the percentage isolation frequency of organisms from site 2 and site 3 in test children

ORGANISMS	SITE 2 Mean %	SPECIES SHOWING SIGNIFICANT DIFFERENCE FROM TEST SITE 2 SITE 3
S. mitior - soft	19	38(0.053)
Lactobacillus	19	2(0.014)
Neisseria A+P-	62	40(0.049)
Neisseria A-P-	7	26(0.019)
Veillonella	86	62(0.013)
Fusobacterium	12	29(0.057)
Yeast	50	5(0.0009)
Haemophilus	12	31(0.033)

CHAPTER TEN

Comparison of the Microflora Within and Between Sites in Test Children and Control Children as Dental Caries Developed

I. Introduction

A study of the microbial factors involved in the etiology of dental caries would not be complete without considering changes which occur in the composition of the microflora as disease develops. Because of the ease of accessibility to the study sites in all children and, in particular each caries-active child, it was possible to correlate accurately the stages of dental caries development with the microflora isolated at each sampling period. Tables 10-1 and 10-2 show data for the mean percentages and isolation frequencies respectively of species isolated from sites 1, 2 and 3 in both caries-active and caries-free test children. In total there were eighteen surfaces at risk of dental caries in the group of test children. The **PRE-LESION** and **LESION** columns present data for five surfaces which progressed from a caries-free status to open lesions during the study. The **NO LESION** column presents data for five susceptible surfaces which remained caries-free in caries-active children. The **CONTROL** columns show data for the non-susceptible surface (site 3), five in the caries-active group and four in the caries-free group. The **NO LESION** column under the **CARIES FREE CHILDREN** heading presents data for susceptible surfaces (eight surfaces in total) in test children who remained completely caries free throughout the study.

II. Comparison of the Flora Within Test Children

A comparison of the data shown in tables 10-1 and 10-2 indicates that there were noticeable differences between surfaces which developed disease, those which were susceptible but remained caries free and control surfaces. Surfaces which developed lesions had the highest proportion of streptococci (55.8%) of all surfaces under study. The surfaces having the lowest proportion of streptococci (41.3%) were the susceptible surfaces in children who remained caries-free (Table 10-1). Within the streptococci, the lesion group of surfaces had the highest proportion of *S. mutans* (27.7%). Interestingly, susceptible surfaces which remained caries free in the caries active children had the next highest proportion of *S. mutans* at 11.4%. In the pre-lesion group, the level of *S. mutans* was only 4.4%, similar to that found at site 3 in both caries active and caries free children, 4.5%. The caries free group of children had much higher levels of *S. mitior*, 17.9% at susceptible surfaces and 20.8% at control surfaces, than the caries active children did at susceptible surfaces which had a lesion (11.2%) or susceptible surfaces which remained caries free (12.7%) (Table 10-1). The inverse relationship noted by others (Loesche and Straffon, 1979) which involves a decrease in the numbers of *S. sanguis* as the numbers of *S. mutans* increase was not seen in the present results. Rather, this relationship appeared to exist between the dextran-producing variants of *S. mitior* (pre-lesion 18.4%, lesion 5.4%) and *S. mutans* (pre-lesion 4.4%, lesion 27.7%). *A.*

viscosus was regularly isolated from all surfaces in caries-active children (Table 10-2) and represented the dominant species of this genus in this group of children (Table 10-1). In contrast, *A. naeslundii* was isolated more frequently and in higher numbers than *A. viscosus* from the caries-free children (Tables 10-1, 10-2). The other organism showing an obvious difference between caries-active and caries-free children was *Lactobacillus*. This genus was only isolated from one of the samples (3% isolation frequency) from caries-free children while 11 (69%) and 6 (24%) samples respectively from lesion sites and caries-free sites in caries-active children were positive (Table 10-2).

Table 10-1

Mean values for bacteria in samples from susceptible and non-susceptible tooth surfaces in caries active and caries free test children

	CARIES ACTIVE CHILDREN SUSCEPTIBLE SURFACE				CARIES FREE CHILDREN SUSCEPTIBLE	
ORGANISMS	PRE-LESION	LESION	NO LESION	CONTROL	NO LESION	CONTROL
S. milleri	5.4[1]	2.1	9.9	6.0	7.0	8.2
S. mitior - hard	18.4	5.4	8.8	9.1	15.2	18.0
S. mitior - soft	8.0	5.8	3.9	9.2	2.7	2.8
S. mutans I	4.4	27.7	11.4	4.5	6.3	4.5
S. mutans IV	0.2	2.6	2.0	0.3	1.3	0.8
S. salivarius	8.2	4.5	10.1	6.9	4.2	9.4
S. sanguis	7.1	7.7	3.2	9.9	4.6	10.4
Total Streptococci	**51.7**	**55.8**	**49.3**	**45.9**	**41.3**	**54.1**
A. naeslundii	13.8	5.4	10.1	6.9	21.9	13.8
A. viscosus	13.9	16.4	14.7	12.1	5.8	2.4
A. odontolyticus	0	1.1	0.1	0.8	1.5	0.5
A. naeslundii/viscosus	2.1	2.4	0.8	1.4	1.8	0.03
Rothia	0.1	1.4	0.9	4.1	1.4	2.1
Bacterionema	0	0.5	0.1	1.4	0.3	1.7
Lactobacillus spp.	0.4	6.4	2.2	0.4	0.003	0
L. casei	0	0.01	0	0	0	0
L. brevis	0	0.08	0.01	0	0	0
L. fermentum	0	0.4	0.01	0.03	0	0
L. plantarum	0.4	0.6	1.6	0.4	0.003	0
L. salivarius	0	1.5	0.6	0	0	0
L. acidophilus	0	3.5	0	0	0	0
Total Gram + Rods	**30.7**	**39.7**	**31.1**	**27.5**	**32.7**	**20.5**
Neisseria A+P+	0.7	2.2	2.7	3.8	5.2	5.0
Neisseria A+P-	0.5	0.2	2.0	0.8	4.5	2.9
Neisseria A-P-	0.8	0.01	0.1	3.4	0.1	0.01
Veillonella	7.1	10.1	9.2	3.0	7.3	6.1
Total Gram - Cocci	**9.1**	**12.5**	**14.0**	**11.0**	**17.1**	**14.0**
Fusobacterium	0.02	0.01	0.01	0.1	0.1	0.5
Leptotrichia	0.1	0	0.1	0.2	0.3	0.5
Aerobic -ve Rods	0.2	0.02	1.5	1.9	2.4	4.3
Anaerobic -ve Rods	0.1	0.3	0.2	0.6	0.2	0.3
Yeast	6.4	0.9	0.2	0.3	0.5	0.01
Micrococcus	2.7	2.6	2.1	1.1	2.2	1.4
Haemophilus	2.5	0.03	0.4	2.1	0.1	0.3

1. Mean value expressed as a percentage of the total cultivable flora

Table 10-2

Comparison of the mean values for the percentage isolation frequency of organisms from susceptible and non-susceptible sites tooth surfaces in caries-active and caries-free test children.

	CARIES ACTIVE CHILDREN				CARIES FREE CHILDREN	
	SUSCEPTIBLE SURFACE				SUSCEPTIBLE	
ORGANISMS	PRE-LESION	LESION	NO LESION	CONTROL	NO LESION	CONTROL
S. milleri	45	31	52	32	62	65
S. mitior - hard	64	62	76	60	79	88
S. mitior - soft	27	69	28	48	21	24
S. mutans I	36	92	60	48	38	35
S. mutans IV	9	38	28	8	9	18
S. salivarius	55	54	76	60	56	53
S. sanguis	64	77	52	44	62	53
A. naeslundii	45	54	56	60	88	76
A. viscosus	64	85	84	68	56	41
A. odontolyticus	0	23	4	20	18	6
A. naeslundii/viscosus	27	15	13	20	15	6
Rothia	27	23	48	52	35	53
Bacterionema	0	23	8	20	9	12
Lactobacillus spp.	9	69	24	4	3	0
L. casei	0	8	0	0	0	0
L. brevis	0	23	4	0	0	0
L. fermentum	0	46	8	4	0	0
L. plantarum	9	15	12	4	3	0
L. salivarius	0	15	8	0	0	0
L. acidophilus	0	23	0	0	0	0
Neisseria A+P+	64	54	72	72	76	71
Neisseria A+P-	45	23	56	24	62	65
Neisseria A-P-	9	8	8	40	24	6
Veillonella	91	92	96	64	82	59
Fusobacterium	9	8	4	28	31	29
Leptotrichia	18	0	20	32	41	41
Aerobic -ve Rods	45	23	48	36	65	59
Anaerobic -ve Rods	9	10	12	20	15	18
Yeast	82	92	60	4	26	6
Micrococcus	45	38	32	20	35	24
Haemophilus	18	8	20	36	6	24

III. Comparison Within Test Children Based on Caries Development

Comparisons were made between the mean percentages and isolation frequencies for each species isolated from each site in caries-active test children and test children who remained caries-free throughout the study. These comparisons were based on a consideration of the disease status of the surfaces which were compared. For example, surfaces which developed lesions could be compared with other disease-free surfaces both prior to and after lesion development. The categories defined in this section of the results were explained on page 172 of this chapter.

Tables 10-3 - 10-11 show data for species that were significantly different between categories in both the caries-active and caries-free children. In each case a single group in the caries-active or caries-free children was compared to all other categories. One-way and two-way analysis of variance were used to test the statistical significance of differences between the mean percentages. The statistical significance of differences between percentage isolation frequencies was tested by either the chi-square test or Fisher's exact test-two tail when the number of observations was lower than expected by the chi-square test.

The comparison of pre-lesion sites to other groups of sites in the test children did not reveal many significant differences (Tables 10-3 and 10-4). This suggests that the

Table 10-3

Comparison of the flora on pre-lesion sites to that on susceptible sites with lesions, lesion free susceptible sites and control sites in caries-active and caries-free test children

	CARIES ACTIVE CHILDREN SUSCEPTIBLE SURFACE				CARIES FREE CHILDREN SUSCEPTIBLE	
ORGANISMS	PRE-LESION	LESION	NO LESION	CONTROL	NO LESION	CONTROL
	Mean % (S.E.)					
S. mutans I	4.4(1.8)	27.7[1](11.1) (0.105)[2]	11.4(4.2) (0.165)	4.5(2.0) -	6.3(3.5) -	4.5(1.7) -
Lactobacillus	0.4(0.4)	6.4(3.1) (0.125)	2.2(1.3) -[3]	0.4(0.4) -	0.003 -	0
Rothia	0.1(0.06)	-	0.9(0.4) (0.093)	4.1(1.5) (0.057)	-	-
Neisseria A+P+	0.7(0.4)	-	-	3.8(1.3) (0.071)	-	5.0(1.1) (0.005)
Neisseria A+P-	0.5(0.3)	-	-	-	4.5(1.8) (0.080)	-
Aerobic -ve Rods	0.2(0.2)	-	-	-	2.4(0.7) (0.103)	4.3(1.6) (0.082)

1. Mean value expressed as a percentage of the total cultivable flora
2. p value as determined by two way analysis of variance
3. - not significant: $p>0.100$

Table 10-4

Comparison of the mean values for the percentage isolation frequency of organisms on pre-lesion sites with lesions, lesion free susceptible sites and control sites in caries-active and caries-free test children

		CARIES ACTIVE CHILDREN SUSCEPTIBLE SURFACES			CARIES FREE CHILDREN SUSCEPTIBLE SURFACES	
ORGANISMS	PRE-LESION Mean %	LESION	NO LESION	CONTROL	NO LESION	CONTROL
S. mitior - soft	27	69[1] (0.041)[2]	-	-	-	-
S. mutans - I	36	92(0.008)*	-	-	-	-
S. mutans - IV	9	38(0.166)*	-	-	-	-
A. naeslundii	45	-	-	-	88(0.007)*	76(0.125)*
Lactobacillus spp.	9	69(0.005)*	-	-	-	-
Neisseria A-P-	9	-	-	40(0.116)*	-	-
Veillonella	91	-	-	64(0.127)*	-	59(0.099)*
Yeast	82	-	-	4(0.0009)*	27(0.003)*	6(0.0009)*

1. mean percentage isolation frequency
2. p value from chi-square test, level of significance $p<0.100$
* p value from Fisher's exact test - two tail

Table 10-5

Comparison of the mean percentages for organisms isolated from susceptible tooth surfaces after lesion development (sites 1 and 2) in caries-active test children with other groups of caries-active and caries-free test children

	CARIES ACTIVE CHILDREN			CARIES FREE CHILDREN	
		SUSCEPTIBLE		SUSCEPTIBLE	
ORGANISMS	LESION	NO LESION	CONTROL	NO LESION	CONTROL
	Mean % (S.E.)				
S. milleri	2.1(1.2)	9.9(2.4) (0.021)	6.0(1.1) (0.047)	7.0(0.8) (0.012)	8.2(2.6) (0.057)
S. mitior - hard	5.4(2.5)	-	-	15.2(3.6) (0.072)	18.0(2.8) (0.012)
S. mutans I	27.7(11.1)	11.4(4.2) (0.208)	4.5(2.0) (0.106)	6.3(4.6) (0.150)	4.5(1.7) (0.106)
S. salivarius	4.5(2.0)	10.1(1.4) (0.050)	-	-	-
Lactobacillus	6.4(3.1)	2.2(1.3) (0.245)	0.4(0.3) (0.126)	0.01(0.01) (0.110)	-*
L. fermentum	0.4(0.1)	0.01(0.004) (0.040)	0.03(0.02) (0.045)	-*	-*
A. naeslundii	5.4(2.8)	-	-	21.9(2.4) (0.006)	13.8(3.1) (0.084)
A. viscosus	16.4(5.0)	-	-	5.8(1.1) (0.103)	2.4(1.2) (0.048)
Neisseria A+P-	0.2(0.1)	2.0(0.6) (0.042)	-	4.5(1.5) (0.066)	-
Yeast	0.9(0.3)	-	-	-	0.01(0.01) (0.046)

* organism never isolated from this group of children

Table 10-6

Comparison of the mean values for the percentage isolation frequency of organisms on sites which developed lesions with lesion free susceptible sites and control sites in caries active and caries free test children

		CARIES ACTIVE CHILDREN SUSCEPTIBLE SURFACES		CARIES FREE CHILDREN SUSCEPTIBLE SURFACES	
ORGANISMS	LESION Mean %	NO LESION	CONTROL	NO LESION	CONTROL
S. milleri	31	-	-	62(0.057)	-
S. mitior - soft	69	28[1](0.015)[2]	-	21(0.004)*	24(0.012)
S. mutans - I	92	60(0.060)	48(0.012)*	38(0.001)	35(0.002)
S. mutans - IV	38	-	8(0.034)*	9(0.028)*	-
S. sanguis	77	-	44(0.053)	-	-
A. naeslundii	54	-	-	88(0.017)*	-
A. odontolyticus	23	4(0.107)	-	-	-
A. viscosus	85	-	-	56(0.094)*	41(0.016)
Lactobacillus spp.	69	24(0.007)	4(0.0009)*	3(0.0009)*	-
L. fermentum	46	8(0.011)*	4(0.004)*	-	-
L. brevis	23	4(0.107)*	-	-	-
Rothia	23	-	52(0.087)	-	53(0.098)
Neisseria A+P-	23	56(0.053)	-	-	65(0.024)
Neisseria A-P-	8	-	40(0.060)*	62(0.018)	-
Veillonella	92	-	64(0.118)*	-	59(0.092)*
Yeast	92	60(0.060)*	4(0.0009)*	26(0.0009)	6(0.0009)
Aerobic Gram - Rods	23	-	-	65(0.011)	59(0.071)*

1. mean percentage isolation frequency
2. p value from chi-square test, level of significance $p<0.100$
* p value from Fisher's exact test - two tail

microflora of the pre-lesion sites did not differ significantly from the other groups of surfaces which either remained caries free or developed lesions. *S. mutans* comprised 4.4% of the flora at the pre-lesion sites and 27.7% in the lesions. Although this difference just failed to meet the level of significance, the isolation frequencies did differ significantly between the two groups (36% vs. 92%). The failure to reach statistical significance may have occurred because of the relatively small number of observations in the pre-lesion group. A similar difference occurred with *Lactobacillus*. Although the numbers of yeasts were not significantly different between the groups of sites, the isolation frequency was much higher at the pre-lesion sites (82%) than either control site in caries-active (4%) or caries-free (6%) children or the susceptible surfaces (27%) in test children who remained caries free (Table 10-4).

The comparison of the microflora from surfaces with lesions to the other groups of surfaces provided the largest number of statistically significant differences (tables 10-5 and 10-6). The levels of *S. mutans*, *Lactobacillus* and *A. viscosus* increased on surfaces which developed lesions while the levels of other species on these surfaces decreased. However, many of these differences were not highly significant. While several different species of *Lactobacillus* were isolated from lesions (Table 10-1) only the levels of *L. fermentum* in the lesions were significantly

different from the levels on other surfaces. Again this may have occurred because of small numbers of samples. A comparison of the percentage isolation frequencies provided many more highly statistically significant differences than the comparison of mean percentages (Table 10-6).

The comparison of susceptible surfaces in caries-active children which remained caries-free with other surfaces in both caries-active and caries-free children did not provide any uniform differences (Tables 10-7 and 10-8). Those that did occur were not highly statistically significant and appeared to occur randomly rather than in a consistent pattern. However, there were parallels to comparisons made against the lesion surfaces in tables 10-5 and 10-6. For example, *A. viscosus* comprised 14.7% of the flora on the caries-free susceptible surfaces in caries-active children but only 2.4% on the control surface in caries-free children. Similarly, *A. naeslundii* was found in higher numbers (21.9%) in caries-free children at susceptible surfaces than in caries-active children at lesion free susceptible surfaces (10.1%) (Table 10-7).

As had occurred in other comparisons, the percentage isolation frequency comparisons in this instance differed between more species in a more significant fashion than did the comparisons of mean percentage (Table 10-8). As well, the significant differences, rather than being isolated, random differences as had occurred with the mean percentages, could be grouped based on the surfaces which

were compared. The comparison of susceptible caries-free surfaces in caries-active children to similar surfaces in caries-free children formed one group with significant differences existing between the Gram positive populations i.e. streptococci and Gram positive rods. The second group consisted of the susceptible caries-free surfaces and the control surfaces in the caries-active children. Differences here were evident between *Lactobacillus* and the Gram negative populations, *Neisseria*, *Veillonella*, and *Fusobacterium*.

The last comparison to be made was between the control surfaces in caries-active children and the susceptible surfaces and control surfaces in caries-free children. These data are displayed in tables 10-9, 10-10 and 10-11. There were no differences between the susceptible surfaces and control surfaces in caries-free children in either the mean percentages or isolation frequencies for isolated organisms. *A. viscosus* was found in higher numbers (12.1%) and isolated more frequently (68%) from the control surfaces in caries-active children than either the susceptible surfaces (mean percentage 5.8%, isolation frequency 56%) or control surfaces (mean percentage 2.4%, isolation frequency 41%) in caries-free children (Tables 10-9, 10-10, 10-11). The levels and isolation frequencies of *S. mutans* and *Lactobacillus* were very similar for all three sites shown in tables 10-9, 10-10 and 10-11.

Table 10-7

Comparison of the mean percentages for organisms isolated from susceptible tooth surfaces which remained caries free (sites 1 and 2) in caries-active test children with other groups of caries-active and caries-free test children

ORGANISMS	SUSCEPTIBLE NO LESION Mean %(S.E.)	CARIES ACTIVE CHILDREN CONTROL	CARIES FREE CHILDREN SUSCEPTIBLE NO LESION	CONTROL
S. mitior - hard	8.8(1.8)	-	-	18.0(2.8) (0.023)
S. salivarius	10.1(1.4)	-	5.3(1.5) (0.051)	-
S. sanguis	3.2(1.6)	9.9(2.1) (0.033)	-	-
A. naeslundii	10.1(2.6)	-	21.9(2.4) (0.024)	-
A. viscosus	14.7(4.4)	-	-	2.4(1.2) (0.048)
Rothia	0.9(0.4)	4.1(1.5) (0.104)	-	-
Yeast	0.2(0.08)	-	-	0.01(0.01) (0.068)
Haemophilus	0.4(0.2)	2.1(0.8) (0.074)	-	-

Table 10-8

Comparison of the mean values for the percentage isolation frequency of organisms on lesion-free susceptible sites in caries-active children with lesion-free susceptible sites and control sites in caries-free test children and control sites in caries-active children

ORGANISMS	SUSCEPTIBLE SURFACES NO LESION Mean %	CARIES ACTIVE CHILDREN CONTROL	CARIES FREE CHILDREN SUSCEPTIBLE SURFACES NO LESION	CONTROL
S. mutans - I	60	-	38(0.098)	35(0.116)
S. mutans - IV	28	-	9(0.080)*	-
S. salivarius	76	-	56(0.111)	-
A. naeslundii	56	-	88(0.005)	-
A. viscosus	84	-	56(0.022)	41(0.004)
Lactobacillus spp.	24	4(0.098)*	3(0.035)*	0
Neisseria A+P-	56	24(0.021)	-	-
Neisseria A-P-	8	40(0.008)	-	-
Veillonella	96	64(0.005)	-	59(0.004)*
Fusobacterium	4	28(0.049)*	31(0.009)	29(0.032)*
Leptotrichia	20	-	41(0.085)	-
Yeast	60	4(0.001)*	27(0.010)	6(0.0009)

* p value from Fisher's exact test - two tail

Table 10-9

Comparison of the mean percentages for organisms isolated from susceptible tooth surfaces (sites 1 and 2) which remained caries free in caries-free test children with a non-susceptible tooth surface (site 3) in caries-active and caries-free test children

ORGANISMS	CARIES FREE CHILDREN SUSCEPTIBLE NO LESION Mean % (S.E.)	CARIES ACTIVE CHILDREN CONTROL	CARIES FREE CHILDREN CONTROL
S. sanguis	4.6(0.8)	9.9(2.1) (0.060)	-
A. naeslundii	21.9(2.4)	6.9(1.7) (0.003)	-
A. viscosus	5.8(1.1)	12.1(4.0) (0.030)	-
Neisseria A+P-	4.5(1.8)	0.8(0.4) (0.094)	-
Veillonella	7.3(1.4)	3.0(1.4) (0.050)	-
Haemophilus	0.1(0.1)	2.1(0.8) (0.060)	-

Table 10-10

Comparison of the mean percentages for organisms isolated from a non-susceptible tooth surface (site 3) in caries-free test children with the same site in caries-active test children

ORGANISMS	CARIES FREE CHILDREN CONTROL Mean % (S.E.)	CARIES ACTIVE CHILDREN CONTROL
S.mutans I	4.5(1.7)	4.5(2.0)
Lactobacillus	0	0.4(0.4)
S. mitior - hard	18.0(2.8)	9.1(3.1) (0.077)
A. naeslundii	13.8(3.1)	6.9(1.7) (0.078)
A. viscosus	2.4(1.2)	12.1(4.0) (0.071)
Haemophilus	0.3(0.2)	2.1(0.8) (0.078)

Table 10-11

Comparison of the mean values for the percentage isolation frequency of organisms on control sites in caries-active children with lesion-free susceptible sites and control sites in caries-free test children

ORGANISMS	CARIES ACTIVE CHILDREN CONTROL Mean %	CARIES FREE CHILDREN SUSCEPTIBLE SURFACES NO LESION	CONTROL
S. milleri	32	62(0.024)	65(0.037)
S. mitior - hard	60	-	88(0.081)*
S. mitior - soft	48	21(0.026)	-
A. naeslundii	60	88(0.012)	-
A. viscosus	68	-	41(0.085)
Neisseria A+P-	24	62(0.002)	65(0.008)
Neisseria A-P-	40	-	6(0.016)*
Yeast	4	26(0.034)*	-
Haemophilus	36	6(0.006)*	-
Aerobic Gram - Rods	36	65(0.029)	-

* p value from Fisher's exact test - two tail

IV. Comparison of the Flora Between Control and Test Children as Dental Caries Developed

Since the test children were divided into caries-active and caries-free groups it was possible to make a number of different comparisons between the above groups. These are illustrated in tables 10-12 - 10-20. Comparisons were made between the microflora from each stage of lesion development in the caries-active test children with that from sites 1, 2 and 3 in the control children. This provided an additional measure of how the flora changed as disease developed. In

addition, caries-free susceptible sites in the caries active children could also be compared to sites 1, 2 and 3 in the control children. Comparisons were also made between the microflora from susceptible sites and control sites in test children who remained caries-free with sites 1, 2 and 3 in control children. Since both sites 1 and 2 were considered susceptible sites in test children, the data from both these sites were combined and compared to the three sites in the control children as well as combined site 1 and 2 data in control children (Table 10-14). This increased the number of observations from susceptible sites. The number of significant differences between test and control sites also increased following this manipulation.

Tables 10-12 - 10-16 illustrate the comparisons of the mean percentages found to differ significantly between caries-active test children and caries-free control children. In tables 10-12 and 10-13 data for those surfaces which developed lesions are displayed for the pre-lesion and post-lesion stages. The microflora from sites 1 and 2 in control children was for the most part quite similar to that from test children both prior to and after lesion development. Only the level of *Rothia* at pre-lesion sites in test children (0.1%) were highly significantly different from the level on control sites (10.6% at site 2)(Table 10-12). There were slightly more differences when the pre-lesion microflora was compared to site 3 and site 1/2 in control children. Note that the mean percentages and isolation

frequencies (Table 10-19) of *S. mutans* and *Lactobacillus* did not differ significantly between these sites prior to lesion development. After lesions developed (Table 10-13) a larger number of differences existed between the microfloras. The level of *S. mutans* increased from 4.4% pre-lesion to 27.7% in the lesion and this was significantly higher than the levels on site 1 (2.3%), site 2 (1.8) and site 3 (2.2%) in control children. The only other organism showing a highly significant increase in percentage after lesion development was *Lactobacillus*. In the lesion it accounted for 6.4% of the flora while at each of the sites in control children it was either absent (site 2) or present in very low numbers (sites 1 and 3). The largest number of differences existed between the pooled data for sites 1 and 2 in control children versus lesion sites in test children. Concerning isolation frequencies, the number of significant differences also increased after lesion development (Table 10-18, 10-19 and 10-20). The isolation frequencies for *S. mutans* I (92%), *S. mutans* IV (38%), *S. sanguis* (77%), *Lactobacillus* (69%), *Veillonella* (92%)and Yeasts (92%) were significantly higher for lesions than sites 1 or 2 in control children (Tables 10-19, 10-20).

Table 10-15 displays data comparing the susceptible surfaces in caries-active test children which remained caries-free to sites 1, 2 and 3 in control children. Note that the number of statistically significant differences between test and control children is higher in this table than in tables 10-

12 and 10-13. This could have occurred because the number of observations in the test children was higher in this category than in either the pre-lesion or lesion categories. Nonetheless, the predominant differences between test and control children at sites 1 and 2 occurred in the Gram positive acidogenic flora and at site 3 in control children in *Streptococcus*, *Lactobacillus* and the Gram negative flora. Although the mean percentages and isolation frequencies for organisms found to differ significantly between test and control children were lower at these surfaces than in lesions they were higher than those for pre-lesion surfaces. This could have occurred because as lesions developed in the caries-active children increases in the numbers of organisms thought to be cariogenic on these surfaces were paralled by similar increases in the same organisms on surfaces which remained caries-free. Therefore the mean percentage calculations for caries-free susceptible surfaces in caries-active children were based on samples taken both before and after lesion development at caries-active surfaces. An examination of longitudinal data for each child demonstrates this (Appendix A). Longitudinal data for both caries-active and caries-free test children will be presented later in this chapter.

When the data prior to lesion development from sites 1 and 2 in test children were combined, the differences between microflora alluded to in the previous paragraph became clearer (Table 10-14). In addition, because the numbers of

observations increased for the test children many of the differences between test and control children became more significant. However, the caution expressed in the previous paragraph regarding when the samples were collected and the effect this could have had on the calculations should be remembered.

Table 10-12

Comparison of the mean percentages for organisms isolated from susceptible pre-lesion surfaces of maxillary incisors (sites 1 and 2) which developed lesions in test children and sites 1,2 and 3 in control children

		C O N T R O L		C H I L D R E N	
ORGANISMS	PRE-LESION Mean %	SITE 1	SITE 2	SITE 3	SITE 1/2
S. salivarius	8.2(2.3)	3.4(0.7) (0.097)	-	-	-
Rothia	0.1(0.05)	-	10.6(3.0) (0.002)	-	7.1(1.7) (0.018)
Neisseria A+P+	0.7(0.4)	3.7(0.9) (0.054)	4.3(0.9) (0.025)	4.5(1.1) (0.018)	4.0(0.7) (0.023)
Neisseria A+P-	0.5(0.3)	3.8(1.6) (0.086)	-	-	3.5(0.9) (0.087)
Veillonella	7.1(3.3)	-	-	3.3(1.0) (0.099)	-
Fusobacterium	0.02(0.01)	-	-	2.0(0.8) (0.046)	-
Aerobic -ve Rods	0.2(0.1)	-	3.7(1.4) (0.032)	4.1(0.7) (0.019)	3.2(0.8) (0.045)
Anaerobic-ve Rods	0.1(0.1)	-	-	2.3(1.0) (0.056)	-
Yeast	6.4(4.3)	1.3(1.2) (0.048)	1.1(0.9) (0.038)	0.2(0.2) (0.017)	1.2(0.7) (0.026)

Table 10-13

Comparison of the mean percentages for organisms isolated from susceptible surfaces of maxillary incisors (sites 1 and 2) after lesion development in test children with sites 1,2 and 3 in control children

		CONTROL CHILDREN			
ORGANISMS	LESION Mean %	SITE 1	SITE 2	SITE 3	SITE 1/2
S. mitior - hard	5.4(2.7)	-	-	13.2(1.5) (0.027)	-
S. mutans I	27.7(4.3)	2.3(0.8) (0.0001)	1.8(0.9) (0.0001)	2.2(0.9) (0.0001)	2.1(0.6) (0.0001)
S. mutans IV	2.6(1.0)	0.6(0.4) -	0.2(0.1) (0.069)	0	0.4(0.2) (0.067)
A. naeslundii	5.4(3.0)	-	12.3(2.0) (0.078)	-	11.7(1.5) (0.076)
A. viscosus	16.4(2.9)	-	7.1(2.0) (0.016)	9.0(1.3) (0.051)	-
Rothia	1.4(2.5)	-	10.5(3.0) (0.007)	-	7.1(1.7) (0.053)
Lactobacillus	6.4(1.2)	0.2(0.2) (0.0002)	0	0.1(0.04) (0.0002)	0.1(0.1) (0.0002)
Neisseria A+P-	0.2(1.5)	3.8(1.6) (0.056)	-	-	3.5(0.9) (0.054)
Veillonella	10.1(2.1)	-	5.3(1.0) (0.079)	3.3(1.0) (0.015)	5.5(0.7) (0.065)
Fusobacterium	0.01(0.7)	-	-	2.0(0.8) (0.045)	-
Aerobic -ve Rods	0.02(1.3)	-	3.7(1.4) (0.024)	4.1(0.7) (0.015)	3.2(0.8) (0.033)

Table 10-14

Comparison of the mean percentages for organisms isolated from all susceptible surfaces of maxillary incisors (sites 1 and 2, excluding samples from lesions) in caries-active test children and sites 1,2 and 3 in control children

		CONTROL CHILDREN			
ORGANISMS	PRE-LESION Mean %	SITE 1	SITE 2	SITE 3	SITE 1/2
S. milleri	7.7(1.1)	4.3(0.6) (0.045)	2.8(1.0) (0.006)	2.3(0.8) (0.002)	3.5(0.6) (0.006)
S. mutans I	7.9(1.5)	2.3(0.8) (0.014)	1.8(0.9) (0.008)	2.2(0.9) (0.013)	2.1(0.6) (0.004)
S. salivarius	9.2(1.5)	3.4(0.7) (0.013)	-	-	4.7(0.9) (0.024)
A. odontolyticus	0.03(0.7)	2.4(1.0) (0.022)	-	-	2.2(0.7) (0.017)
Rothia	0.5(1.6)	-	10.6(3.0) (0.0001)	-	7.1(1.7) (0.003)
Bacterionema	0.07(0.6)	2.3(1.1) (0.016)	-	-	-
Lactobacillus	1.3(0.4)	0.2(0.2) (0.059)	-	0.1(0.04) (0.038)	0.1(0.09) (0.019)
Neisseria A+P+	1.7(0.9)	-	4.3(0.9) (0.042)	4.5(1.1) (0.028)	4.0(0.7) (0.003)
Veillonella	8.1(1.4)	-	-	3.3(1.0) (0.023)	-
Fusobacterium	0.01(0.5)	-	-	2.0(0.8) (0.009)	-
Aerobic -ve Rods	0.9(0.9)	-	3.7(1.0) (0.027)	4.1(0.7) (0.015)	3.2(0.8) (0.039)
Anaerobic-ve Rods	0.1(0.6)	-	-	2.3(1.0) (0.014)	-
Micrococcus	2.4(1.2)	-	-	5.9(1.7) (0.059)	-

Table 10-15

Comparison of the mean percentages for organisms isolated from susceptible surfaces of maxillary incisors (sites 1 and 2) which remained caries free in caries-active test children and sites 1, 2 and 3 in control children

ORGANISMS	SUSCEPTIBLE NO LESION Mean %	CONTROL CHILDREN SITE 1	SITE 2	SITE 3	SITE 1/2
S. milleri	9.9(2.4)	4.3(0.6) (0.003)	2.8(1.0) (0.0003)	2.3(0.8) (0.0001)	3.5(0.6) (0.0003)
S. mutans I	11.4(4.2)	2.3(0.8) (0.0009)	1.8(0.9) (0.0005)	2.2(0.9) (0.0008)	2.1(0.6) (0.0002)
S. mutans IV	2.0(1.3)	0.6(0.4) (0.057)	0.2(0.1) (0.017)	0	0.4(0.2) (0.019)
S. salivarius	10.1(1.4)	3.4(0.7) (0.008)	6.0(1.5) (0.088)	5.5(1.8) (0.055)	4.7(0.9) (0.016)
A. viscosus	14.7(4.4)	-	7.1(2.0) (0.036)	-	-
A. odontolyticus	0.1(0.05)	2.4(1.0) (0.082)	-	-	2.2(0.4) (0.085)
Rothia	0.9(0.4)	-	10.6(3.0) (0.004)	-	7.1(1.7) (0.035)
Bacterionema	0.1(0.09)	2.3(1.1) (0.071)	-	-	-
Lactobacillus	2.2(1.3)	0.2(0.2) (0.004)	0	0.1(0.04) (0.003)	0.1(0.009) (0.001)
Veillonella	9.2(3.1)	5.7(1.1) (0.121)	5.3(1.0) (0.081)	3.3(1.0) (0.010)	5.5(0.7) (0.070)
Fusobacterium	0.01(0.005)	-	-	2.0(0.8) (0.045)	-
Anaerobic-ve Rods	0.2(0.1)	-	-	2.3(1.0)	-

Table 10-16

Comparison of the mean percentages for organisms isolated from the labial surface of a mandibular incisor (site 3) in caries-active test children with sites 1,2 and 3 in control children

	CARIES ACTIVE TEST CHILDREN	CONTROL CHILDREN			
ORGANISMS	SITE 3	SITE 1	SITE 2	SITE 3	SITE 1/2
S. milleri	5.9(1.1)	-	2.8(1.0) (0.032)	2.3(0.8) (0.013)	3.5(0.6) (0.066)
S. sanguis	9.9(1.9)	4.4(1.3) (0.030)	4.3(1.4) (0.027)	5.0(1.6) (0.050)	4.3(0.9) (0.016)
Rothia	4.0(2.5)	-	10.6(3.0) (0.050)	-	-
Micrococcus	1.1(1.8)	-	-	5.9(1.7) (0.043)	-

The least number of differences between caries-active and caries-free test and control children occurred when the non-susceptible sites were compared (Tables 10-16 and 10-18). Both groups of test children had higher levels of *S. milleri* (5.9% in caries-active, Table 10-16 and 8.2% in caries-free, Table 10-18) than control children at site 1 (4.3%), site 2 (2.8%) and site 3 (2.3%). As well, both groups of test children had lower levels of *Rothia* than the control children. A similar scenario occurred when the isolation frequencies were compared. The isolation frequencies for *S. mutans* and *Lactobacillus* were not significantly different between site 3 in test children and sites 1, 2 and 3 in control children (Table 10-19, 10-20, 10-21). Only site 1 in control children had differences which could be considered highly significant (Table 10-19) but these were few in

number.

The comparison of susceptible sites in caries-free test children and sites 1, 2 and 3 in control children is shown in table 10-17 (mean percentages) and tables 10-19 - 10-21 (isolation frequencies). The majority of differences in mean percentages occurred for *S. milleri*, *S. mutans*, and *A. naeslundii* (Table 10-18). The level of significance was low for *S. mutans* however.

Table 10-17

Comparison of the mean percentages for organisms isolated from susceptible surfaces of maxillary incisors (sites 1 and 2) in test children who remained caries free and sites 1,2 and 3 in control children.

	CARIES FREE TEST CHILDREN	CONTROL CHILDREN			
ORGANISMS	SITE 1/2 Mean %	SITE 1	SITE 2	SITE 3	SITE 1/2
S. milleri	7.0(0.9) (0.030)	4.3(0.6) (0.001)	2.8(1.0) (0.0004)	2.3(0.8) (0.002)	-
S. mutans I	6.3(1.9)	2.3(0.8) (0.135)	1.8(0.9) (0.094)	2.2(0.9) (0.124)	2.1(0.6) (0.072)
A. naeslundii	21.9(2.4)	11.1(2.4) (0.003)	12.3(2.0) (0.007)	11.2(2.5) (0.003)	11.7(1.5) (0.002)
A. viscosus	5.8(1.7)	14.8(1.9) (0.0007)	-	-	11.0(1.6) (0.019)
Rothia	1.4(1.9)	-	10.6(3.0) (0.001)	-	7.1(1.7) (0.017)
Veillonella	7.3(1.2)	-	-	3.3(1.0) (0.017)	-
Fusobacterium	0.1(0.6)	-	-	2.0(0.8) (0.022)	-

Table 10-18

Comparison of the mean percentages for organisms isolated from the labial surface of a mandibular incisor (site 3) in test children who remained caries free and sites 1, 2 and 3 in control children

	CARIES FREE TEST CHILDREN	CONTROL CHILDREN			
ORGANISMS	SITE 3	SITE 1	SITE 2	SITE 3	SITE 1/2
S. milleri	8.2(1.5)	4.3(0.6) (0.033)	2.8(1.0) (0.005)	2.3(0.8) (0.002)	3.5(0.6) (0.008)
S. mitior- hard	18.0(3.0)	8.6(1.4) (0.014)	11.1(2.8) (0.064)	-	9.8(1.5 (0.020)
A. viscosus	2.4(2.5)	14.8(1.9) (0.0003)	-	9.0(1.3) (0.038)	10.9(1.6) (0.005)
Rothia	2.1(2.9)	-	10.6(3.0) (0.021)	-	-

Table 10-19

Comparison of the mean values for the percentage isolation frequency of organisms between site 1 in control children and sites 1,2 and 3 in caries-free and caries-active test children

ORGANISMS	SITE 1 Mean %	CARIES ACTIVE CHILDREN SUSCEPTIBLE PRE-LESION	LESION	NO LESION	SURFACE CONTROL	CARIES FREE CHILDREN SUSCEPTIBLE NO LESION	CONTROL
S. milleri	32	-	-	-	54(0.072)	-	-
S. mitior - soft	67	27[1](0.016)[2]	-	28(0.002)	-	21(0.0009)	23(0.002)
S. mutans I	31	36 NS	92(0.0009)	60(0.018)	48 NS	38 NS	35 NS
S. mutans IV	8	9 NS	38(0.007)	28(0.026)	-	-	-
S. sanguis	46	-	77(0.046)	-	-	-	-
A. naeslundii	67	-	-	-	-	88(0.025)	-
A. viscosus	79	-	-	-	-	56(0.024)	41(0.004)
A. odontolyticus	29	-	-	4(0.012)	-	-	6(0.050)
Rothia	60	27(0.047)	23(0.017)	-	-	35(0.025)	-
Lactobacillus	2	-	69(0.0009)	24(0.003)	-	-	-
Neisseria A+P-	58	-	23(0.024)	-	24(0.005)	-	-
Neisseria A-P-	38	9(0.069)	8(0.040)	8(0.007)	-	-	6(0.014)
Veillonella	71	-	92(0.111)	96(0.012)	-	-	-
Fusobacterium	54	9(0.007)	8(0.003)	4(0.0009)	28(0.033)	31(0.040)	29(0.079)
Leptotrichia	46	-	-	20(0.038)	-	-	-
Aerobic -ve Rods	65	-	23(0.008)	-	36(0.020)	-	-
Anaerobic-ve Rods	48	9(0.018)	10(0.027)	12(0.002)	20(0.020)	15(0.002)	18(0.029)
Yeast	6	82(0.0009)	92(0.0009)	60(0.0009)	-	26(0.011)	-
Haemophilus	42	-	8(0.022)	20(0.064)	-	6(0.0009)	-

1. mean percentage isolation frequency.
2. p value from chi-square test, significance level $p<0.100$

Table 10-20

Comparison of the mean values for the percentage isolation frequency of organisms between site 2 in control children and sites 1,2 and 3 in caries-free and caries-active test children

		CARIES ACTIVE CHILDREN				CARIES FREE CHILDREN	
		SUSCEPTIBLE SURFACE				SUSCEPTIBLE	
ORGANISMS	SITE 2 Mean %	PRE-LESION	LESION	NO LESION	CONTROL	NO LESION	CONTROL
S. milleri	33	-	-	-	-	-	65(0.023)
S. mitior - hard	65	-	-	-	-	-	88(0.073)
S. mitior - soft	56	27(0.091)	-	28(0.026)	-	21(0.002)	23(0.024)
S. mutans I	23	-	92(0.0009)	60(0.002)	48(0.035)	-	-
S. mutans IV	5	-	38(0.001)	28(0.006)	-	-	-
S. sanguis	44	-	77(0.038)	-	-	-	-
A. naeslundii	77	45(0.043)	-	56(0.074)	-	-	-
A. viscosus	60	-	-	84(0.043)	-	-	-
A. odontolyticus	21	-	-	4(0.057)	-	-	-
Lactobacillus	0	9	69	24	-	-	-
Rothia	77	27(0.002)	23(0.0009)	48(0.016)	52(0.035)	35(0.0009)	53(0.070)
Neisseria A+P-	47	-	-	-	24(0.065)	-	-
Neisseria A-P-	28	-	-	8(0.050)	-	-	6(0.062)
Veillonella	74	91(0.240)	92(0.168)	96(0.024)	64 NS	82 NS	59 NS
Fusobacterium	33	-	8(0.076)	4(0.006)	-	-	-
Aerobic -ve Rods	60	-	23(0.018)	-	36(0.052)	-	-
Yeast	5	82(0.0009)	92(0.0009)	60(0.0009)	-	26(0.007)	-
Micrococcus	51	-	-	-	20(0.011)	-	24(0.052)
Haemophilus	33	-	8(0.076)	-	-	6(0.004)	-

Table 10-21

Comparison of the mean values for the percentage isolation frequency of organisms between site 3 in control children and sites 1,2 and 3 in caries-free and caries-active test children

		CARIES ACTIVE CHILDREN SUSCEPTIBLE SURFACE				CARIES FREE CHILDREN SUSCEPTIBLE	
ORGANISMS	SITE 3 Mean %	PRE-LESION	LESION	NO LESION	CONTROL	NO LESION	CONTROL
S. milleri	35	-	-	-	-	62(0.017)	65(0.033)
S. mitior - hard	80	27(0.015)	-	-	60(0.063)	-	-
S. mitior - soft	67	-	-	28(0.001)	-	21(0.0009)	24(0.002)
S. mutans I	30	36 NS	92(0.0009)	60(0.015)	48 NS	38 NS	35 NS
S. mutans IV	0	9	38	28	8	8	18
S. sanguis	46	-	77(0.046)	-	-	-	-
A. naeslundii	74	45(0.068)	-	-	-	-	-
A. viscosus	63	-	84(0.065)	-	-	-	-
Rothia	72	27(0.006)	23(0.001)	48(0.047)	-	35(0.001)	-
Lactobacillus	4	-	69(0.0009)	24(0.012)	-	-	-
Neisseria A+P+	54	-	-	-	-	76(0.042)	-
Neisseria A+P-	50	-	23(0.084)	-	24(0.033)	-	-
Neisseria A-P-	43	9(0.034)	8(0.017)	8(0.002	-	24(0.064)	6(0.005)
Veillonella	51	91(0.019)	92(0.009)	96(0.0009)	64 NS	82(0.005)	59 NS
Fusobacterium	52	9(0.010)	8(0.004)	4(0.0009)	28(0.050)	31(0.062)	-
Aerobic -ve Rods	67	-	23(0.004)	-	36(0.011)	-	-
Anaerobic -ve Rod	50	9(0.014)	10(0.021)	12(0.002)	20(0.013)	15(0.001)	18(0.021)
Yeast	2	81(0.0009)	92(0.0009)	60(0.0009)	-	26(0.001)	-
Micrococcus	43	-	-		20(0.048)	-	-
Haemophilus	41	-	8(0.024)	20(0.070)	-	6(0.0009)	-

V. Longitudinal Data on the Microflora from Susceptible and Non-susceptible Sites in Caries-active and Caries-free Test Children

The data presented in this chapter and chapter 9 measure the statistical significance of the differences between the microflora from susceptible and non-susceptible tooth surfaces in caries-active and caries-free children. While this is an important analysis, it distorts the longitudinal nature of the data and changes a dynamic process into a static one; the cause-and-effect relationship of the longitudinal data is lost to some degree.

In this section, the mean percentage of the contribution of genera and species to the flora is shown in relation to the clinical state of the sampled tooth surface. The numbers of samples taken at a time period varies but between 3 and 5 samples were taken close to the production of a white spot or extensive lesion. In table 10-22 increases in the levels of *S. mutans*, *Lactobacillus*, and *A. viscosus* can be seen associated with the development of white spot lesions or cavities. In addition, decreases in the levels of other organisms, *S. mitior*, *S. milleri* and *A. naeslundii*, can also be seen associated with the same incident. However, the caries-free susceptible sites in caries-active children showed parallel increases in *S. mutans*, *Lactobacillus* and *A. viscosus* as well as decreases in *S. milleri* and *S. mitior* in the absence of lesion formation (Table 10-23).

Table 10-24 shows longitudinal data for susceptible surfaces which remained caries-free in test children who did not develop caries. A comparison of the data in this table with that in table 10-22 and 10-23 indicates that the levels of *S. mutans* at the susceptible surfaces in caries-free test children and caries-active test children became increasingly different as caries developed. The other organism showing a obvious difference between susceptible sites in caries-active and caries-free test children is *Lactobacillus*. This genus was only isolated from one sample from the caries-free children.

Table 10-22

Longitudinal data on the flora of the susceptible sites in caries-active test children which developed lesions expressed as a mean percentage of the total cultivable flora

	CARIES FREE				WHITE SPOT LESION						
Number of Samples	1	2	3	5	3	3	3	2	2	1	1
ORGANISM											
S. milleri	0	6.0	9.6	2.6	4.8	0	4.5	3.0	0	0	11.4
S. mitior - hard	4.6	0	12.7	15.7	5.7	6.0	5.5	1.4	3.2	11.0	14.5
S. mitior - soft	0	5.8	39.6	0	9.8	0.5	2.5	0	22.3	13.1	8.4
S. mutans - I	0	0	2.6	3.1	33.5	26.7	15.7	41.1	42.0	13.9	15.2
S. mutans - IV	0	0	0	0.5	0	0	5.7	10.4	4.3	4.4	3.1
S. salivarius	1.8	10.8	0	8.4	0	6.8	1.8	3.1	1.7	38.3	15.8
S. sanguis	0	9.7	3.8	4.2	1.7	6.0	11.2	4.9	12.5	0	0
Total Streptococci	**6.4**	**32.3**	**68.3**	**34.5**	**55.5**	**46.0**	**46.9**	**63.9**	**86.0**	**80.7**	**68.4**
A. naeslundii	74.7	8.7	4.3	14.6	9.8	7.0	2.4	0.2	0.2	1.7	0
A. viscosus	17.2	1.5	0.6	17.6	17.7	30.3	18.8	12.6	3.4	0	1.4
A. odontolyticus	0	0	0	0	0	0.9	2.4	0.2	0	0	0
Rothia	0.2	0	0	0.1	0	0	0	10.3	0.3	0	0
Bacterionema	0	0	0	0	0	0.3	0	2.2	0.1	0	0
Lactobacillus	0	0	0	0	13.7	0.1	0.1	16.5	2.1	12.4	19.1
L. casei	0	0	0	0	0	0	0.1	0	0	0	3.7
L. brevis	0	0	0	0	0	0	0.1	0	0.1	1.4	8.6
L. fermentum	0	0	0	0	0.6	0.1	0.1	0.1	1.5	0	2.3
L. plantarum	0	0	0	0	2.8	0	0	0	0	9.2	8.2
L. salivarius	0	0	0	0	0.7	0	0	9.5	0	0	0
L. acidophilus	0	0	0	0	9.7	0	0	3.5	0	1.8	0.1
Total Gram + Rods	**92.1**	**10.2**	**4.9**	**32.3**	**55.0**	**38.7**	**24.0**	**55.1**	**7.7**	**26.5**	**43.4**
Neisseria A+P+	0.1	3.1	1.0	0.2	0.2	0.7	12.0	1.7	0.3	0	0
Neisseria A+P-	0	2.2	0.8	0.1	0	0.1	0.1	0	1.9	0	0
Neisseria A-P-	0	8.1	0	0	0	0	0.1	0	0	0	0.1
Veillonella	1.1	27.7	7.3	3.8	3.4	10.3	5.3	17.6	2.6	5.3	5.9
Total Gram - Cocci	**1.2**	**41.1**	**9.1**	**4.1**	**3.6**	**11.1**	**17.5**	**19.3**	**4.8**	**5.3**	**6.0**
Fusobacterium	0.3	0	0	0	0	0	0.1	0	0	0	0
Leptotrichia	0	0	0.6	0.1	0	0	0	0	0	0	0
Aerobic -ve Rods	0	0	0.7	0	0.1	0	0.1	0.1	0	0	0
Anaerobic -ve Rods	0	0.1	0.1	0.5	0	0	1.5	0	0	0	0.7
Yeast	0	0.1	0.5	7.0	1.1	1.2	0.1	0.8	0.5	0	0.4
Micrococcus	0	0	6.5	1.0	5.4	2.7	0.5	6.0	0	0	0
Haemophilus	0	10.0	0	1.1	0	0.1	0	2.4	0	0	0

Table 10-23

Longitudinal data on the flora of the susceptible sites which remained caries-free in caries-active test children expressed as a mean percentage of the total cultivable flora

	CARIES			FREE		
Number of Samples	**2**	**3**	**5**	**5**	**5**	**5**
ORGANISM						
S. milleri	6.3	19.6	18.3	15.5	0.4	0.3
S. mitior - hard	6.9	9.3	12.0	10.9	2.1	10.7
S. mitior - soft	0	1.3	0	0	13.2	7.8
S. mutans - I	0	20.0	0.6	16.6	13.4	10.6
S. mutans - IV	0	1.3	0.2	1.7	8.1	0.7
S. salivarius	24.5	24.2	14.3	11.3	1.4	0.5
S. sanguis	1.0	10.0	2.8	2.0	1.5	3.7
Total Streptococci	**38.7**	**85.7**	**48.2**	**58.0**	**40.1**	**34.3**
A. naeslundii	30.4	5.7	5.3	4.5	9.4	16.1
A. viscosus	1.8	0.9	16.1	8.7	21.9	18.9
A. odontolyticus	0	0	0.2	0	0	0
Rothia	0.2	0	1.6	0.8	0.2	1.1
Bacterionema	0	0	0	0	0.6	0.3
Lactobacillus	0	0	0	0	6.3	2.9
L. casei	0	0	0	0	0	0
L. brevis	0	0	0	0	0.1	0
L. fermentum	0	0	0	0	0.1	0.1
L. plantarum	0	0	0	0	6.3	0.1
L. salivarius	0	0	0	0	0.1	2.9
L. acidophilus	0	0	0	0	0	0
Total Gram + Rods	**32.4**	**6.6**	**23.2**	**14.0**	**45.0**	**42.4**
Neisseria A+P+	3.3	0	0.5	6.4	2.3	4.1
Neisseria A+P-	0.2	6.9	2.5	1.3	0	2.3
Neisseria A-P-	0.1	0	0	0.2	0	0
Veillonella	10.9	18.1	8.4	7.4	4.4	14.8
Total Gram - Cocci	**14.5**	**25.0**	**11.4**	**15.3**	**6.7**	**21.2**
Fusobacterium	0	0	0	0	0	0.1
Leptotrichia	0.2	0	0.1	0	0.1	0.1
Aerobic -ve Rods	0	0.1	1.3	0.3	3.9	3.2
Anaerobic -ve Rods	0	0	0	0.7	0.1	0.2
Yeast	0	0.1	0.3	0.1	0.6	0.2
Micrococcus	4.2	0	0	7.5	1.2	0
Haemophilus	3.0	0.1	0.3	0	0	0.1

Table 10-24

Longitudinal data on the flora of the susceptible sites in test children who remained caries-free throughout the study expressed as a mean percentage of the total cultivable flora

		CARIES		FREE	
Number of Samples	**2**	**4**	**4**	**4**	**4**
ORGANISM					
S. milleri	9.4	8.2	7.8	1.8	11.4
S. mitior - hard	6.5	25.0	18.5	10.6	19.0
S. mitior - soft	8.4	4.5	2.2	2.6	0.3
S. mutans - I	0	1.3	4.9	10.1	5.1
S. mutans - IV	0	0	0	1.5	2.5
S. salivarius	6.9	5.8	8.5	0.3	3.7
S. sanguis	2.7	2.7	1.8	3.5	11.2
Total Streptococci	**33.9**	**47.5**	**43.7**	**30.4**	**53.2**
A. naeslundii	34.1	18.6	12.6	33.8	18.0
A. viscosus	5.6	1.9	4.1	7.2	9.6
A. odontolyticus	0	0.8	0	5.9	0.1
Rothia	0.4	2.7	1.4	0.1	3.8
Bacterionema	0	0	0	1.2	0.2
Lactobacillus	0.1	0	0	0	0
L. casei	0	0	0	0	0
L. brevis	0	0	0	0	0
L. fermentum	0	0	0	0	0
L. plantarum	0.1	0	0	0	0
L. salivarius	0	0	0	0	0
L. acidophilus	0	0	0	0	0
Total Gram + Rods	**40.3**	**24.0**	**18.1**	**48.2**	**31.7**
Neisseria A+P+	0.2	3.2	9.5	6.8	6.4
Neisseria A+P-	7.3	3.0	7.4	4.4	2.1
Neisseria A-P-	0.1	0.1	0.1	0.2	0.1
Veillonella	12.5	7.4	11.0	4.4	3.2
Total Gram - Cocci	**20.1**	**13.7**	**28.0**	**15.8**	**11.8**
Fusobacterium	0.1	0.1	0.3	0.1	0.1
Leptotrichia	0.4	0.1	0.3	0.5	0.1
Aerobic -ve Rods	7.3	3.8	3.7	0.7	0.3
Anaerobic -ve Rods	0.2	0	0.2	0.5	0.1
Yeast	0	0.7	0.1	2.0	0.1
Micrococcus	0.9	1.2	0.3	5.4	2.3
Haemophilus	0	0.9	0.2	0	0

Tables 10-25 and 10-26 show data for the non-susceptible sites in caries-active and caries-free test children respectively. These intra-subject control sites were colonized by a flora quite similar to that which colonized

the susceptible sites in either caries-active or caries-free children. The control sites of caries-active children did not appear to be more heavily colonized than those of the caries-free children. *Lactobacillus* was not isolated from the control site in caries-free test children.

Table 10-25

Longitudinal data on the flora of the non-susceptible sites in caries-active test children expressed as a mean percentage of the total cultivable flora

	NON-SUSCEPTIBLE			CARIES-FREE		
Number of Samples	**3**	**3**	**5**	**5**	**5**	**5**
ORGANISM						
S. milleri	1.9	8.9	15.3	1.2	7.2	0
S. mitior - hard	0.2	4.6	10.9	4.8	7.5	17.6
S. mitior - soft	0	9.6	0	13.9	11.5	18.4
S. mutans - I	0	0	3.7	13.1	3.6	2.1
S. mutans - IV	0	0	0.1	0	0	1.3
S. salivarius	6.9	22.7	6.2	6.8	4.7	0
S. sanguis	0	30.5	9.2	3.4	0.5	15.9
Total Streptococci	**9.0**	**76.3**	**45.4**	**43.2**	**35.0**	**55.3**
A. naeslundii	11.9	5.0	4.1	6.4	9.2	3.6
A. viscosus	8.4	2.9	13.2	6.1	19.5	8.0
A. odontolyticus	1.3	1.2	0.2	2.0	0.2	0
Rothia	0	12.9	1.5	4.4	5.6	1.1
Bacterionema	0	0	0	0	7.7	0.4
Lactobacillus	0	0	0	0	1.4	0
L. casei	0	0	0	0	0	0
L. brevis	0	0	0	0	0	0
L. fermentum	0	0	0	0	0.1	0
L. plantarum	0	0	0	0	1.3	0
L. salivarius	0	0	0	0	0	0
L. acidophilus	0	0	0	0	0	0
Total Gram + Rods	**21.6**	**22.0**	**19.0**	**18.9**	**43.6**	**13.1**
Neisseria A+P+	3.7	4.4	3.0	3.7	2.8	5.1
Neisseria A+P-	0	2.4	2.4	0.2	0	0
Neisseria A-P-	0.1	3.9	0.8	8.0	3.5	0
Veillonella	0.1	4.4	4.0	4.0	1.8	1.5
Total Gram - Cocci	**3.9**	**15.1**	**10.2**	**15.9**	**8.1**	**6.6**
Fusobacterium	0.1	0	0.5	0.1	0.1	0.1
Leptotrichia	0	0	0.4	0.1	0.4	0.1
Aerobic -ve Rods	0	0.3	5.7	3.6	0	0.3
Anaerobic -ve Rods	0.1	0	0.1	2.4	0	0.1
Yeast	0	0	0	0	1.6	0.1
Micrococcus	6.3	1.2	0	0.3	1.4	0
Haemophilus	8.2	0.2	1.9	0	0.7	0.1

Table 10-26

Longitudinal data on the flora of the non-susceptible sites in caries-free test children expressed as a mean percentage of the total cultivable flora

	NON-SUSCEPTIBLE			CARIES-FREE	
Number of Samples	**2**	**3**	**4**	**4**	**4**
ORGANISM					
S. milleri	5.0	7.0	13.2	9.7	1.5
S. mitior - hard	0.6	38.8	18.0	12.2	20.3
S. mitior - soft	18.5	0	0	3.7	1.0
S. mutans - I	0	3.2	7.7	0.1	10.5
S. mutans - IV	0	0	2.0	0.1	0.7
S. salivarius	14.2	6.0	10.6	6.1	7.8
S. sanguis	9.4	1.4	12.8	7.5	9.2
Total Streptococci	**47.7**	**56.4**	**63.7**	**39.5**	**51.0**
A. naeslundii	13.4	5.8	4.6	17.3	28.1
A. viscosus	2.5	1.5	0	5.8	2.3
A. odontolyticus	1.0	4.8	5.0	0	2.0
Rothia	8.4	1.9	0.6	1.9	2.0
Bacterionema	0	0	0	7.3	1.4
Lactobacillus	0	0	0	0	0
L. casei	0	0	0	0	0
L. brevis	0	0	0	0	0
L. fermentum	0	0	0	0	0
L. plantarum	0	0	0	0	0
L. salivarius	0	0	0	0	0
L. acidophilus	0	0	0	0	0
Total Gram + Rods	**25.3**	**14.0**	**10.2**	**26.3**	**35.8**
Neisseria A+P+	0.4	7.9	3.3	6.4	6.6
Neisseria A+P-	14.8	1.2	1.0	1.5	2.7
Neisseria A-P-	0	0	0	0	0.1
Veillonella	1.0	19.1	6.0	4.1	0.1
Total Gram - Cocci	**16.2**	**28.2**	**10.3**	**12.0**	**9.5**
Fusobacterium	0.1	0	1.1	0.9	0
Leptotrichia	0	0.2	0.5	1.3	0.1
Aerobic -ve Rods	0.2	0	7.3	9.6	0.7
Anaerobic -ve Rods	0	0	1.4	0.2	0
Yeast	0	0	0	0	0.1
Micrococcus	0	3.1	3.1	0	0.3
Haemophilus	0.1	1.4	0	0.1	0.3

CHAPTER ELEVEN

I. Permutation Analysis Comparing the Similarity of the Microfloras Within and Between Control and Test Children

Analysis of microbiological data by traditional statistical methods is fraught with many problems. This was considered and explained in the literature review in Chapter 5 and will not be discussed here. However, many of the problems encountered by others were also encountered in this study. This lead to a search for statistical methods which would either circumvent or reduce the significance of a problem or problems identified in chapter 1, such as non-normally distributed data, in data analysis. In addition, we felt that the use of such methods would provide an interesting contrast with the traditional methods which were eventually employed, the results of which have been presented in chapters 9 and 10 of this thesis.

Because there is a scarcity of agreement regarding correct statistical methodology in the analysis of microbiological data, very few new methods have been developed for use in this area. One method which has been developed is the 'Lambda Analysis' by Dr. I. J. Good at the The Virginia Polytechnic Institute and State University. This method was described in the literature review on pages 113-117. It is a non-parametric method and, as such, addresses the problem of analyzing data which are far from being normally distributed. The Lambda of Good analysis is based on the mean similarity between two subsets of samples (eg. samples

from one subject, group of subjects or equivalent sites forming subset one and samples from a second subject, group of subjects or equivalent sites forming the second subset). In this instance we chose to use the coefficient of similarity described by Socransky et al (1981) as the measure of similarity although any measure of similarity could be used including simply measuring the presence or absence of species (I. J. Good, 1987 personal communication). The statistical significance of differences between the similarities of the samples within and between subjects or sites can be determined by a permutation calculation which calculates the probability that the samples (or subsets of each sample) are identical. The accuracy of the permutation calculation is increased when the permutations are randomly distributed. Further increases in accuracy can be expected when the number of randomly determined permutations is increased. For example, if the number of possible permutations is 15 the p-value can not really be less than 1/15 (0.067), however it might be estimated to be less than that if random permutations are used. If the number of randomly determined permutations is much higher, say 1000 or more , as occurred in this study, the accuracy of the calculation increases and p-values can be much lower, in the range of 1/1000 (0.001). The analysis used in this thesis is contained in version 2.22 Lambda of Good, an MS-DOS computer program written by Jean-Michel Lacroix at Laval University.

The major difference between this method and the analysis of variance method employed in chapters 9 and 10 is that the Lambda of Good bases a comparison between subsets on all the species isolated from a site or sites within a subject or group of subjects. On the other hand, the analysis of variance is able to compare only one species at a time to all other species in the analysis. The advantage of the Lambda method therefore is that it is possible to complete an overall comparison of the populations isolated from a site or subject rather than focusing on single specie comparisons. This advantage is particularly important when examining the changes which occur in the composition of the microflora as disease develops at a site and represents a more ecologically sound approach than has been used previously when examining diseases of microbial etiology where complex floras are found. An example of this principle can be found in the study by Moore et al (1982) which examined the bacteriology of experimental gingivitis in young adult humans. Using the same permutation analysis as this study to analyse the composition of the flora during the development of gingvitis, Moore et al (1982) found that the composition of the flora of each person was different prior to disease development from that of every other person in the study. Despite these differences gingivitis developed in all subjects and the initial differences between subjects were maintained and amplified. In fact, the complexity of the flora increased as gingivitis developed indicating that

rather than a simple increase in the quantity of the initial species, a progression of species was required for disease to develop.

Similarity comparisons were also made between individuals in this study (data not shown). Generally the flora of each individual was distinctly different from all other subjects although there were occurrences where individuals had similar flora. However, this was limited to either the test group or the control group and in no case was there a high similarity between children in the test group with children in the control group. This issue will be addressed further in the discussion of the results.

II. Comparison of the Flora from Susceptible and Non-susceptible Sites in Test and Control Children

A large number of comparisons between and within the test and control children were made. Tables 11-1 to 11-4 present the results of comparisons made using the Lambda of Good analysis. In all cases the data used in the Lambda analysis were entered separately for each subject so that individual differences between test and control children could also be examined. This also served to increase the number of samples (observations) in the subsets thereby increasing the number of random permutations and the accuracy of the statistical determination. The species entered in the analysis are the same as those presented in Table 9-1 in chapter 9. The numbers in the table are p-values indicating

the probability that the populations in each subset are identical; the lower the p-value the greater the probability that the subsets are different.

In table 11-1, the flora from susceptible sites 1 and 2 and non-susceptible site 3 in test and control children are compared. Two groups can be identified in Table 11-1. As expected, the flora from test children was different from the flora of control children. In the test group, only site 1 and site 3 were statistically significantly different ($p = 0.005$). A similar trend was observed in the control children. For example, the flora from site 1 in control children was significantly different from the flora at site 3 in control children ($p = 0.030$). However, when the similarity of the flora between test and control groups was compared the flora at susceptible sites in test children was highly significantly different ($p<0.001$) from the flora at the same sites in control children. The flora at non-susceptible surfaces, site 3, were more similar but the difference was significant at the 0.025 level.

Table 11-1

A comparison of the similarity of the microbial populations from susceptible and non-susceptible surfaces in test children (test site 1, 2, 3) with that from control children (control site 1, 2, 3).

	TEST SITE 1	TEST SITE 2	TEST SITE 3	CONTROL SITE 1	CONTROL SITE 2	CONTROL SITE 3
TEST SITE 1	1.000[a]	0.917	0.005	0.005	<0.001	<0.001
TEST SITE 2		1.000	0.163	<0.001	<0.001	<0.001
TEST SITE 3			1.000	0.002	0.035	0.025
CONTROL SITE 1				1.000	0.038	0.030
CONTROL SITE 2					1.000	0.293
CONTROL SITE 3						1.000

a - p value as determined by the Lambda of Good analysis indicating the probability that the populations at each site are similar

Table 11-2 displays the similarity comparisons between caries-active and caries-free test children and caries-free control children. In this table, each site in the test children was compared to each site in the control children and the development of dental caries was considered in the comparisons. Thus caries-active children could be compared to control children both before and after the development of dental caries. Generally, the flora at the susceptible surfaces, sites 1 and 2, in test children was significantly different from sites 1, 2 and 3 in control children whether disease was considered or not. Only site 3 in the test children displayed a similarity to sites 1, 2 and 3 in control children. Surprisingly, after lesions had developed in test children at sites 1 or 2, the non-susceptible

surfaces, site 3, in test children harboured a flora which was quite similar to that found at site 1 ($p = 0.383$) and 2 ($p = 0.127$) in control children.

Table 11-2

Comparison of the microbial populations from caries-free and caries-active test children with those from caries-free control children

TEST SITE	COMPARATIVE SITE CONTROL CHILDREN	ALL SAMPLES EACH SITE	CARIES-FREE TEST CHILDREN vs. CONTROLS[a]	CARIES-ACTIVE TEST CHILDREN vs. CONTROLS	CARIES-ACTIVE BEFORE LESION vs. CONTROLS	CARIES-ACTIVE AFTER LESION vs. CONTROLS
1	SITE 1	0.005[b]	0.016	<0.001	0.016	0.015
	SITE 2	<0.001	0.021	<0.001	0.015	<0.001
	SITE 3	<0.001	0.381	<0.001	0.002	<0.001
2	SITE 1	<0.001	<0.001	0.003	<0.001	<0.001
	SITE 2	<0.001	0.008	<0.001	0.002	<0.001
	SITE 3	<0.001	<0.001	<0.001	<0.001	<0.001
3	SITE 1	0.002	0.002	0.053	0.006	0.383
	SITE 2	0.035	0.048	0.028	0.024	0.127
	SITE 3	0.025	0.014	0.051	<0.001	0.026

a - controls refers to control children
b - p value as determined by Lambda of Good analysis
Note: each site in test children (test site) is compared to each site in control children in each column under conditions as indicated by column heading; example - test children site 1 compared to site 1 in control children in column 'all samples each site' p = 0.005

III. Comparison of the Flora Within and Between Sites in Caries-Active and Caries-Free Test Children

Tables 11-3 and 11-4 display p-values for similarity comparisons which were made within and between the groups of test children. The test children were divided into those who developed lesions and those who remained caries free. A site by site comparison was then completed.

Permutation calculations showed that prior to the development of dental caries at the susceptible sites the caries-active children had a flora which was not significantly different from the test children who remained caries-free. As shown in Table 11-3, p-values for the comparisons caries-active children (before lesion) vs. all samples were site 1 vs. site 2, 0.427, site 1 vs. site 3, 0.448, and site 2 vs. site 3, 0.074. Those children who remained caries-free had remarkably similar flora at all three sites. P-values for the comparisons caries-free vs. caries-free were site 1 vs. site 2, 0.775, site 1 vs. site 3, 0.691, and site 2 vs. site 3, 0.843.

Table 11-3

Comparison using the Lambda of Good analysis of the microbial populations from susceptible and non-susceptible surfaces in caries-active and caries-free test children.

SUBSET COMPARISONS[c]	SITE 1 vs. SITE 2	SITE 1 vs. SITE 3	SITE 2 vs. SITE 3	SITE 1(CA[a]) vs. SITE 1(CF[b])
All samples-each site	0.917[d]	0.005	0.163	-
Caries active children vs. All samples	0.015	<0.001	0.093	-
Caries free children vs. All samples	0.849	0.090	0.093	-
Caries active children (before lesion) vs. All samples	0.427	0.448	0.074	-
Caries active children (after lesion) vs. All samples	<0.001	<0.001	<0.001	-
Caries active vs. Caries active	0.310	0.019	0.063	-
Caries active vs. Caries free	0.016	<0.001	0.106	<0.001
Caries free vs. Caries active	0.089	0.007	0.005	-
Caries free vs. Caries free	0.775	0.691	0.843	-

a - caries active children b - caries free children
c - each comparison is set up such that the first subset defined under this column is derived from the first site defined in each column heading and the second subset is derived from the second site defined in the column heading. Example: the first comparison, all samples-each site involves all samples from site 1 versus all samples from site 2 $p = 0.917$
d - p value as determined by Lambda of Good analysis permutation calculation

In table 11-4, all comparisons are based on the effect that progression to overt disease had on the composition of the microflora at susceptible and non-susceptible sites in caries-active and caries-free test children. Examination of the p-values indicates that at those surfaces which developed disease there was a progressive change in the microflora to the point where microfloras which were relatively similar prior to disease initiation became quite dissimilar after disease developed. P-values for the first two comparisons in the table, caries-active children (before lesion) vs. all caries-active children (site 1 vs. site 2, $p=0.280$, site 1 vs. site 3, $p=0.567$, site 2 vs. site 3, $p=0.010$) and caries-active children (before lesion) vs. all caries-free children (site 1 vs. site 2, $p=0.220$, site 1 vs. site 3, $p=0.087$, site 2 vs. site 3, $p=0.425$, site 1 vs. site 1, $p=0.112$) indicate that, in general, the floras were quite similar. However after lesion development, the p-values for the comparisons caries-active children (after lesion) vs. all caries-active children (site 1 vs. site 2, $p=0.013$, site 1 vs. site 3, $p<0.001$, site 2 vs. site 3, $p<0.001$) and caries-active children vs. all caries-free children (site 1 vs. site 2, $p<0.001$, site 1 vs. site 3, $p<0.001$, site 2 vs. site 3, $p=0.002$, site 1 vs. site 1, $p<0.001$) indicate the floras had become dissimilar. Further corroboration of this occurrence is shown in the last comparison in the table, caries-active children (before lesion) vs. caries-active children (after lesion), in which p-values have now reached highly significant levels, $p<0.001$.

Table 11-4

Comparison using the Lambda of Good analysis of the microbial populations from susceptible and non-susceptible surfaces in test children at various stages of disease progression.

SUBSET COMPARISONS[c]	SITE 1 vs. SITE 2	SITE 1 vs. SITE 3	SITE 2 vs. SITE 3	SITE 1(CA[a]) vs. SITE 1(CF[b])
Caries active children (before lesion) vs. All caries active	0.280[d]	0.567	0.010	-
Caries active children (before lesion) vs. All caries free	0.220	0.087	0.425	0.112
Caries active children (before lesion) vs. Caries active children (before lesion)	0.090	0.514	0.206	-
Caries active children (before lesion) vs. Caries active children (after lesion)	0.033	0.534	<0.001	-
Caries active children (after lesion) vs. All caries active	0.013	<0.001	<0.001	-
Caries active children (after lesion) vs. All caries free	<0.001	<0.001	0.002	<0.001
Caries active children (after lesion) vs. Caries active children (after lesion)	0.774	0.008	0.050	-
Caries active children (after lesion) vs. Caries active children (before lesion)	<0.001	<0.001	0.002	-

a - caries active children b - caries free children

c - each comparison is set up such that the first subset defined under this column is derived from the first site defined in each column heading and the second subset is derived from the second site defined in the column heading. example: the comparison caries active children (before lesion) vs. all caries active children involves samples from site 1 of all caries active children before a lesion developed compared to samples from site 2 of all caries active children; p = 0.280

d - p value as determined by Lambda of Good permutation calculation

CHAPTER TWELVE

Discussion

I Methods- Relationship to previous studies

The methods employed in this study are similar to methods used in other longitudinal studies of dental caries (Bowden et al, 1976; Boyar and Bowden, 1985; Hardie et al, 1977; Huis In't Veld et al, 1979; Ikeda et al, 1973; Loesche and Straffon, 1979; Loesche et al, 1984; Swenson et al, 1976). However, there are several important differences between this study and others which require amplification.

This is the first study in which preschool children have been followed longitudinally until dental caries has developed at susceptible study sites. Other studies have selected school-aged children or adults for similar studies (cited in previous paragraph). There are several advantages of using preschool children in a longitudinal caries study. In the young preschool child who engages in deleterious bottle feeding the teeth which are most at risk of dental caries are the primary maxillary incisors. Because these teeth are readily accessible, the surfaces under study can be reproducibly sampled. This allows the investigator to state with certainty that the study surface, and the lesion itself if it exists, has been sampled in each instance. Since the investigator can visualize the tooth surface to diagnose dental caries at each stage of lesion development, changing trends in the microflora under investigation can then be more accurately linked with the development of

disease. In other words, cause-and-effect relationships can be more clearly demonstrated.

Attempts to reduce the heterogeneity of the population under study have been shown to help provide more explicit results (Lu, H.K., cited in Bibby and Shern, 1978). A sample population consisting of preschool children is probably a more homogeneous group than a group of older children. For example, parents are more likely to follow recommendations, such as dietary advice, from a health professional when their child is in the infancy and toddler periods of life than they would when the child is older and more independent. The suggestion is that children in this age group will have diets which are more similar than the diets of older children, since the younger child is under closer parental scrutiny than the older child. Amongst the test children in this study who bottle-fed ad lib, it is even more likely that their diets were very similar since these children tended to use a bottle frequently over a 24-hour period. Because frequent ingestion of carbohydrate-containing liquids or solids is known to suppress the appetite (Spiegel, 1973), it is probable that the major portion of each child's daily caloric intake was obtained from juice or milk, both of which contain large amounts of sugar. This supposition is supported by research which has examined the nutritional quality of the Manitoba Native diet in various age groups and found that infants and preschoolers are the poorest nourished of the Native family

(Riley, 1975). Although it is known that increasing carbohydrate consumption will lead to increased caries attack, it is not known at what level of carbohydrate exposure this becomes an important variable.

In addition, young preschool children will have teeth of a similar age and thus similar caries susceptibility. Since the tooth surfaces under study here are smooth surfaces, they are less likely to be affected by the different levels of risk associated with major variations in tooth morphology, such as those associated with pits and fissures. Subjects who display anomalies in tooth morphology can be excluded from the study to improve the homogeneity of the sample population.

The sampling interval employed in this study, six weeks, was much shorter than that employed in other studies. For example, Loesche et al (1984) sampled caries-susceptible fissures of first permanent molars at six-month intervals. Although the six-week interval required significantly more intensive laboratory work than if a longer interval had been selected, the advantage of this shorter interval was that a more accurate correlation of changes in the composition of the microflora to the status of the tooth surface could be made. This interval was selected partly for this reason, but also because of the rapidity with which nursing caries develops, which in itself is an advantage in that it minimizes the amount of time that subjects are required to participate and could reduce subject drop out.

An additional advantage of the model chosen for this study relates to the criterion which was used to diagnose dental caries. In the study by Loesche and Straffon (1979) and Loesche et al (1984) the diagnosis of caries was a terminal event in that the surface of the tooth, the fissure, had to display softness or a definite break in the enamel surface. Bowden et al (1977) and Hardie et al (1977) used radiographs to diagnose approximal caries. Bille and Thylstrup (1982) have since shown this to be an inaccurate method even when accurately standardized. Both these methods were required because the surfaces under study could not be seen easily by the investigators in either study for a macroscopic evaluation. In this study it was possible to make a much more accurate diagnosis of caries, including the white spot stage, and the development of dental caries was not regarded as the terminal event of the study. This, coupled with the more frequent sampling interval, provided a superior opportunity to correlate microflora changes with tooth surface changes.

II The Normal Flora of the Preschool Child

Most studies which have examined the composition of the oral microflora have focused on groups of individuals, older children and adults, who are at risk of developing the two major oral diseases, dental caries and periodontal disease. In general, these studies have ignored the infant and young child who are acquiring, early in life, the majority of

organisms which are reported as components of the indigenous oral flora of the older child or adult. While the data on older children and adults are extremely valuable and necessary, others workers have recently turned their attention to the acquisition and development of the oral microflora in infants and young children (Berkowitz et al, 1980; Carlsson et al, 1975; Eggert and Gurner, 1984; Rogers, 1981). The rationale for this research direction is that if it is understood how and when potentially pathogenic organisms are acquired, methods may be developed to inhibit colonization by such organisms and prevent disease (Kohler and Jonsson, 1986) .

The results presented in Chapter 9, 10 and 11 of this thesis indicate that the oral flora of the young child is much more complex than has been reported. Previously, it has been hypothesized that many of the organisms found in this investigation were transients in the oral cavity. However, the isolation frequencies determined here indicate that this is not the case. These results suggest that the pathogenicity of many of the oral bacteria is latent and not expressed until much later in the individuals' life when environmental conditions may allow its population to expand.

a) Fissure Microflora

The data shown in Table 9-4, outlining the microflora of the central occlusal fissure of the maxillary first primary molar in preschool children, while more extensive, reveal

trends similar to those found in data from other studies which have examined the microflora of permanent molars in young adults (Theilade et al, 1973; 1974; 1982; Thott et al, 1974) (Table 1-4). A detailed comparison of the data is not possible since, in the previously reported studies, the fissures were either artificial or were natural fissure systems which had been removed from extracted teeth, placed in acrylic appliances and inserted into the mouths of experimental adult subjects. In addition, with the exception of the study by Theilade et al in 1982 the range of organisms isolated was limited. However, it is evident that the predominant cultivable bacteria were Gram-positive and *Streptococcus* was the dominant genus in each study which has been confirmed in the present investigation.

A direct comparison of the present data to that of Theilade et al (1982) is also not possible since each subject in the Theilade et al study was required to place a drop of 20% sucrose solution at the entrance of the implanted fissures twice daily during the experimental period. This practice favoured the development of a flora which was dominated by *S. mutans* and represents a completely different set of circumstances to those experienced by control subjects from whom fissural data was obtained in the present investigation.

The data presented here, therefore, represents the first comprehensive examination of the microflora of the molar

fissure system of primary teeth. Although the model employed by Theilade et al (1982) and others would provide for a much more detailed examination and preserve individual ecosystems, it is unlikely that children 12-36 months of age would cooperate and wear such an appliance. As samples were collected directly from the entire length of the central occlusal fissure in this study, it is probable that individual ecosystems were combined and therefore obscured by the sampling method.

b) Tongue Microflora

Although several studies have been published which outline the microflora of the tongue in the adult (Gordon And Gibbons, 1966; Krasse, 1953), there are no comparable data for children. An abstract (Milnes and Bowden, 1982) based on incomplete data, is all that exists at present to describe the microflora of the tongue in young children.

It appears that in determining the autochthonous species of the tooth surface and periodontal sulcus, investigators have ignored a surface which may serve as a reservoir for the majority of species found in other habitats within the oral cavity. This appears to be a possibility since in the current study, the entire range of bacteria found on the tooth surface could also be isolated from the tongue, albeit in different proportions and with different frequencies from that on the tooth. As expected, *S. salivarius* was the dominant streptococci which is in agreement with other studies (Krasse, 1953, 1954; Gibbons et al, 1964).

Stomatococcus mucilaginosus, formerly a member of *Micrococcus*, was also regularly isolated. Although anaerobic organisms are not found in large numbers on the tongue, its surface topography may allow for the development of a low redox potential such that even strict anaerobes can survive. Data from this study support this contention. For example, *Veillonella* was isolated from 73% and anaerobic Gram-negative rods from 40% of the samples from the tongue.

The study by Gordon and Gibbons (1966) is quite inconclusive in its description of the tongue's microflora possibly because the identification methods of the day were not sufficiently advanced to allow for a more comprehensive profile of the microflora (Table 1-2). Thus, it appears that the data presented in this thesis (Table 9-4) are the most comprehensive to date and represent the only available data on the flora of the tongue for children.

c) Microflora of the Smooth Tooth Surface

In this study plaque was removed from the labial and lingual smooth surfaces of primary incisors, surfaces which are susceptible to nursing caries and, unlike the sheltered enclave provided by approximal areas, exposed to the harshness of the oral environment, eg. temperature fluctuation, mucosal abrasion, oral hygiene activities and diet. An illustration of this effect on plaque development occurred in this study when several children had their teeth brushed just prior to a plaque sample being taken. The

result in such situations was often a complete lack of microbial growth on the entire range of media employed.

In general, the results presented here on the plaque microflora are in agreement with other studies which have reported on a broad range of microorganisms found in the dental plaque of older children and adults (Bowden et al, 1975; Kilian et al, 1979; Gallagher et al, 1981) (Table 1-5). The results also agree with those of an early report by McCarthy et al (1965) who have published the only other longitudinal comprehensive study of the microflora of the infant and young child. The value of the results of the latter study, however, was lessened by the lack of information at that time on cultivation and identification of oral bacteria.

In the control children, those who were at low risk of nursing caries, the Gram-positive rods and filaments (primarily *A. naeslundii* and *A. viscosus*) dominated at both susceptible sites 1 and 2. This agrees with the data for approximal surfaces reported by Bowden et al in 1975 and with the data for smooth surface plaque from premolars and molars reported by Gallagher et al in 1981. Kilian et al (1979) found a much lower proportion of Gram-positive rods in supragingival plaque removed from the buccal surfaces of first permanent molars. This result is supported by data from this study when the labial surface of a mandibular incisor, the intrasubject control surface, site 3, is considered. On this surface streptococci were by far the

predominant genus in both the test and control children.

Amongst the streptococci isolated in this study, *S. mitior* was the dominant species both in absolute number and frequency of isolation, at sites 1, 2 and 3 which agrees with the study by Kilian et al (1979). This differs from earlier work which reported that *S. sanguis* is the predominant streptococcus in supragingival dental plaque (Carlsson, 1965, 1967a; Loesche and Hockett, 1972). The reason for this discrepancy can be explained by differences in nomenclature used in different laboratories since the taxonomy of the *S. sanguis/mitior* group of organisms is as yet unclear (Kilian et al, 1986).

In contrast to the results of other studies cited in this section, the dominant Gram-negative coccus isolated was *Neisseria* and not *Veillonella*. In those children who eventually developed decay, *Veillonella* was found in higher numbers than *Neisseria*, possibly indicating a higher level of lactate on the tooth surface in these children which favoured *Veillonella*. Ritz (1967) found that during early plaque formation *Neisseria* was a dominant member of the community but that in more 'mature plaque' (9 day old) the levels of *Neisseria* declined as the levels of *Veillonella* increased beyond those of *Neisseria*. He reasoned that the growth of anaerobic organisms, such as *Veillonella*, was dependent on prior growth of aerobic and facultatively anaerobic organisms such as *Neisseria*. If this explanation

is correct, the data in the present investigation could reflect, in part, a community in which either conditions do not favour autogenic succession or conditions in which primary feeders such as *Neisseria* exist side-by-side with secondary feeders such as *Veillonella* before the primary or pioneer stage of community development has been terminated through autogenic succession.

There were a large number of other species found which occurred with varying frequencies. Their numbers were generally low (Tables 9-1 to 9-4). These organisms have not been reported very often by other workers. The results for preschool children reported here agree with those of Bowden et al (1975), Kilian et al (1979) and Gallagher et al (1981) for older children and adults and McCarthy et al (1965) for infants and preschool children regarding the relative proportion assumed by these various species in the oral cavity. However, the taxonomy of oral bacteria has, in general, become much more complicated since these papers were published. Studies to investigate the plaque flora in various age groups as extensively as possible would provide an important addition to the literature.

III Comparison of the Microflora from Test and Control Children

In the previous sections, the discussion focused on the oral microflora of the preschool children who were at low risk of nursing caries. In this section the discussion will centre on the comparison made between the oral microflora from

children at low risk of dental caries (control children) and that from children at high risk of dental caries (test) children.

Although the statistical analyses of the flora at different sites from these two groups of children have shown that in many ways they were similar, the habits which fostered the development of nursing caries in the test children, bottle-feeding ad lib, frequent ingestion of carbohydrate and low-pH drinks and a lack of oral hygiene procedures, were already in place at the beginning of the study. Thus, the hypothesis can be made that factors favouring microbial succession toward a cariogenic flora in the test children were also probably in place. Support for this hypothesis is given by the differences which were detected between test and control children i.e. the observation of higher levels of streptococci in the test children than in the control children, indicating that an acidogenic and aciduric microflora was present in the test children at the outset of the study. The lower levels of anaerobic Gram-negative organisms, including a failure to detect *Bacteroides*, *Actinobacillus* and *Capnocytophyga*, in the test children at both susceptible and non-susceptible sites also supports this hypothesis.

The comparisons within and between control and test children which were made in Chapter 9 also provide support for the acidogenic and aciduric flora in test children. Comparisons

which were made within each group of children indicated that the flora between children in the same group was generally similar. This could indicate that similar oral environments existed within each child in either the test or control children. Comparisons made between test and control children indicated that a larger number of differences existed between groups than within, which provides evidence that different oral environments existed in the two groups of children. However, the tables which outline these differences are somewhat misleading. For example, Table 9-7 lists 11 species which differed in proportion between site 1 in control children and sites 1, 2 and 3 in test children. Superficially, this appears to represent a significant difference. However, when sites are individually compared the number of differences rapidly decreases. For example, site 1 in control and test children, a susceptible surface, had differences between the levels of 5 organisms. While the p values indicate that the differences were significant at a $p<0.061$ level, consideration of the actual percentages for each organism demonstrates that in 2 of the 5 cases the differences were small (see Table 9-7). It is remarkable that, given the differences between the test and control groups in feeding patterns and oral hygiene practices, the differences were so few in number. Perhaps this illustrates a resistance to change of the ecosystem, in general, and the microbial community, in particular, except in situations of severe environmental pressure.

This can be further explained if an examination of the differences and similarities between groups based on the susceptibility of the tooth surfaces to dental caries is undertaken. This revealed that the majority of differences in the composition of the microflora existed between the susceptible sites in test children as one group and the non-susceptible site in test children combined with the susceptible and non-susceptible sites in the control children as a second group. In other words, the non-susceptible intra-subject control site in the test children harboured a microflora which was more similar to that found at all three sites in the control children than it was to the microflora from the susceptible sites in the test children. Both the ANOVA and Lambda of Good analyses support this observation. This indicates that the susceptible sites in test children were unique and almost certainly exposed to a different environmental challenge which either encouraged shifts in the microbial community or favoured colonization by specific organisms. These phenomena are recognized responses of any bacterial community and are not limited to the oral cavity (Alexander, 1971). A discussion of the environmental challenges in nursing caries will follow in the section which considers the development of nursing caries.

IV Changes in the Microflora as Caries Developed

An intent of the present investigation was to determine, over time, the quantitative relationships among each

component of the microflora recovered from caries-free surfaces, some of which developed lesions. As mentioned earlier, the nursing caries model is well suited for a longitudinal investigation of dental caries in man because of the rapidity with which lesions develop in full view of the investigator. While it is recognized that a longitudinal investigation provides proportionally greater returns than a cross-sectional, association-type study, an inherent disadvantage relates to the size of the population which must be studied in order to accommodate the uncertainties of patient cooperation and the likelihood of any given tooth surface becoming carious during the study period (Loesche et al, 1979). In this study, nine of the ten subjects in each group remained at the completion of the study, which, in the end, allowed for the collection of a significant number of samples. Of the 18 susceptible surfaces in the test children, 5 developed lesions, representing 28% of the susceptible surfaces under study in the test group. While this is not a large number upon which conclusions can be made regarding caries etiology, the numbers of subjects and lesions are similar to those from other longitudinal studies of both dental caries (Bowden et al, 1976; Hardie et al, 1977; Loesche and Straffon, 1979) and periodontal disease (Moore et al, 1984, 1985).

In addition to the size of the study population, the number of samples taken from each subject should be considered as a measure of the reliability of the data. The number of

samples taken in this study (390) is in excess of samples collected in other longitudinal caries studies (314 samples-Boyar and Bowden, 1985; 224 samples-Hardie et al, 1977) in which attempts were made to identify as broad a range of isolates as possible. On the other hand, the sample number is significantly less than that in other studies (2184 samples-Loesche et al, 1984; 3005 samples-Swenson et al, 1976). However, in these studies the range of microorganisms identified was severely restricted in comparison to either the present investigation or the studies by Boyar and Bowden (1985) and Hardie et al (1977). This allowed for a corresponding increase in the number of subjects and samples. Nonetheless, enumeration of a single species or fraction of the bacterial community considers only one aspect of the relationship of the microflora to disease (Bowden et al, 1975). This may be acceptable for short-term studies. However, valuable data are lost if more extensive examinations of the microflora are not made. For example, information relative to the stability of a given population of bacteria over time or species interrelationships at a site as disease develops could be potentially beneficial for either predicting disease susceptibility or monitoring the effectiveness of therapeutic regimens.

The results of this investigation agree with and extend the findings of cross-sectional studies conducted by van Houte et al (1982) and Berkowitz et al (1984). They demonstrated heavy colonization of the maxillary anterior teeth by *S.*

mutans (61% of the total cultivable flora) and *Lactobacillus* (4.0% in carious lesions) in children with nursing caries. This pattern of colonization was present in white spot lesions, carious lesions and on intact enamel surfaces. No children exposed to a similar environment who remained caries-free were studied by either van Houte et al (1982) or Berkowitz et al (1984). van Houte et al (1982) also reported on the proportions and isolation frequencies of *S. sanguis* and *S. salivarius* on the maxillary anterior and posterior teeth as well as in the saliva. The data from the present investigation include a much wider range of bacteria than the studies of either van Houte et al (1982) or Berkowitz et al (1984) and the microbial colonization pattern from clinically sound enamel to lesion formation.

The extensive examination of the microflora in this investigation revealed that significant shifts occurred in the microbial population at susceptible sites in test children who developed nursing caries. Shifts in the population were characterized by changes in both the species composition and the relative frequency with which resident organisms could be isolated such that, as disease developed and the environment became increasingly acidic, the heterogeneity of the population decreased. At this point the population was dominated by *S. mutans* and various species of *Lactobacillus*, organisms which are known to be more aciduric and acidogenic than other oral bacteria and therefore capable of survival in and preservation of this environment

(Hayes et al, 1983). This observation is in keeping with observations of other ecosystems rich in carbohydrate but poor in O_2. For example, in the fermentation of cabbage, cucumbers, milk and ensiled plant remains, *Lactobacillus* and *Streptococcus* species form considerable lactic acid, the accumulation of which ultimately eliminates the pioneer organisms and favours the proliferation of acid-tolerant anaerobes (Alexander, 1971).

The preceeding conditions appear to exist also in specific areas of the maxillary incisor teeth which are susceptible to nursing caries in the child who engages in deleterious bottle feeding. In this instance, the diet is the major environmental determinant influencing the bacterial community on the tooth surface. Since the majority of children who develop nursing decay either sleep with a nursing bottle or are put to bed with it until they fall asleep (Johnson et al, 1984), the relative absence of saliva at this time (Schneyer et al, 1956) and its potentially protective effects, dilution and buffering of acid, a decrease in the frequency of deglutition (Lear et al, 1965) and the high frequency of suckling on demand (Short, 1976a, 1976b) all serve to increase the severity of the dietary challenge to the tooth surface. The microbiological response is a shift in the composition of the population as has been observed in this study.

The frequency of isolation gave a more statistically significant depiction of the population shifts than did the

percentage contribution of each species. This could have occurred because of very large standard deviations in the numbers of several of the organisms isolated, in particular *S. mutans*. The effect that this had was to reduce the significance of the differences between the levels of various species found on the tooth surfaces at the different stages of disease progression. For example, as shown in Table 10-3, *S. mutans* and *Lactobacillus* accounted for 4.4% and 0.4%, respectively, of the total cultivable flora in the pre-lesion samples. This increased to 27.7% for *S. mutans* and 6.4% for *Lactobacillus* after the lesions developed, levels which appear to be sufficiently different from the pre-lesion levels to reach the rather lenient level of significance used in this investigation.

A broad range of other organisms on surfaces which developed lesions also showed changes in their percentage contribution to the microflora but even more statistically significant differences in their frequency of isolation as a response to the changing environment. For example, in comparison to the pre-lesion samples, the percentage contribution to the microflora of *S. milleri*, the dextran producing variants of *S. mitior, S. salivarius, A. naeslundii*, atypical *Neisseria*, aerobic Gram- negative rods including *Hemophilus* and yeasts declined as the lesions developed. These differences were not significant but represented observed trends. A decline in the frequency of isolation for *S. milleri, Neisseria*, aerobic Gram-negative rods including *Hemophilus* was also

noted. The percentage contribution to the microflora increased for the following organisms as caries developed: *S. mutans* I, *S. mutans* IV, *A. viscosus, A. odontolyticus, Rothia, Bacterionema, Lactobacillus*, and *Veillonella*. Those organisms showing an increase in the frequency of isolation at the time of lesion development were soft variants of *S. mitior, S. mutans* I, *S. mutans* IV, *S. sanguis, A. viscosus, A. odontolyticus, Bacterionema, Lactobacillus* and yeasts. These results agree with many others but in particular with similar human studies by Bowden et al (1976) and Hardie et al (1977) and studies on monkeys by Colman and Hayday (1980). In addition, the observation that the changes in isolation frequencies were statistically more precise than changes in the percentage contribution to the microflora indicates that, perhaps, less attention should be paid to absolute numbers of organisms and more to shifts in the population and the persistence (represented by isolation frequency) of organisms in an ecosystem. This may help further our understanding of diseases associated with complex microflora.

While it is clear that the largest percentage change occurred with *S. mutans*, the smaller percentage changes which occurred for other organisms cannot be ignored. Consideration of these percentages as well as the changes in the percentage isolation frequency indicate that the changing environment favoured those organisms which are more aciduric than others. This trend is clearly indicated in

Tables 10-22 to 10-26 in Chapter 10 which display the longitudinal changes in the flora as disease developed and is supported by the Lambda of Good analysis in Chapter 11 which shows a increasing dissimilarity of the flora between caries-active test children and control and test children who remained caries-free. The sweeping changes which are documented here lend support to the hypothesis put forward by many that dental caries occurs as a result of an ecological imbalance in the microflora (Bowden et al, 1984; Morhart and Fitzgerald, 1976) rather than as the result of caries attack by a specific pathogen (van Houte, 1980).

An important finding in the present investigation was that the microbiology of the susceptible surfaces which developed a lesion was quite similar to those susceptible surfaces which remained lesion-free in the same children. In addition, shifts in the population which were observed at the sites which developed lesions were also observed at those surfaces in caries-active children which remained caries-free. These observations suggest that the environment of these sites is also similar, allowing a specific microflora to establish. However, the local conditions at individual sites also apparently dictated the production of a lesion. The demonstration of a similar microflora with a difference in the caries status of a site could explain in part the findings of some workers who have shown that the consideration of *S. mutans* alone may not give a clear-cut result on its role in caries production (Mikkelsen and

Poulsen, 1976; Mikkelsen et al, 1981; Swenson et al, 1976).

V Organisms Positively Associated with Dental Caries

a) *Lactobacillus*

The association of *Lactobacillus* with dental caries has been known for over 50 years. In almost all studies on caries, lactobacilli have been shown to have a strong correlation with lesions. Since these organisms are weakly adherent to the tooth surfaces they may require a retentive site, such as a carious lesion, in which to colonize (van Houte et al, 1972). A recent study of incipient approximal lesions in children living in an area with water fluoridation has shown that lactobacilli were associated with those lesions which progressed to a point where restoration was required (Boyar and Bowden, 1985). In the present investigation lactobacilli were isolated from white spot lesions as well as from cavities at susceptible sites in test children. Interestingly, lactobacilli were isolated on one occasion only from a non-susceptible site in one test child after a lesion had developed at a susceptible site and from a susceptible site in a test child who remained caries-free (Appendix A, longitudinal data on selected organisms for individual subjects), indicating a possible unique association of *Lactobacillus* with the carious tooth surface and confirming the findings of others. However, after lesions developed in test children, lactobacilli could be isolated from susceptible surfaces which remained caries-free in these children. This reaffirms a point made earlier

regarding the importance of the environment in the establishment of similar microfloras at those sites which developed lesions and those which remained caries-free.

Recent studies on the microflora associated with dental caries in man have not identified the species of *Lactobacillus* present in association with the lesion (Loesche and Straffon, 1979; Loesche et al, 1984; van Houte et al, 1982; Berkowitz et al, 1984). Fitzgerald et al (1980) have identified human isolates of *Lactobacillus* which were tested for cariogenicity in animals. These isolates included *L.casei, L.salivarius, L. lactis, L. plantarum, L. cellobiosus* and *L. fermentum.* The identification of these species from caries in man is in agreement with the range of *Lactobacillus* spp. identified in this study. In a later study by Fitzgerald et al (1981), strains representing 8 species of human oral lactobacilli were tested in gnotobiotic rats. In the rat system, strains of all the species produced caries with the exception of *L. lactis.* These results suggest that all the *Lactobacillus* species isolated from the Native children could be involved in the production of carious lesions.

b) *Streptococcus mutans*

While the results of this study agree with the results of others and reemphasize the close association of *S. mutans* with the carious lesion, they do not indicate that the presence of high levels of *S. mutans* alone will necessarily

lead to the development of a carious lesion. The longitudinal data for test children presented in Tables 10-22 and 10-23 show that high levels of *S. mutans* were found on surfaces which were caries-active as well as those which remained caries-free. The levels of *S. mutans* on surfaces which developed lesions are generally higher than on caries-inactive surfaces and this could be interpreted as an indication that the caries-inactive surfaces were less likely to develop decay (Alaluusua and Renkonen, 1983; Beighton et al, 1985; Kohler et al, 1981). However, at present, there is no agreement as to what constitutes a minimum threshold value of this organism for caries to develop. More important variables could include the spatial relationship of acidogenic organisms to the tooth surface (Listgarten et al, 1975) and the metabolic activity of the plaque, and organisms within, over time (Hayes et al, 1983; Margolis et al, 1985).

The levels of *S. mutans* reported for caries-free surfaces in this study are consistent with levels reported for carious lesions in other studies (Loesche et al, 1984). This may indicate that high levels of *S. mutans* represent an exploitation of environmental conditions by this organism and a superior ability to compete in an acidic milieu (Bowden and Hamilton, 1987).

Huis In't Veld (1979), in a study of caries-active 18-20-year olds, found that 71% of the lesions which progressed into dentin had levels of *S. mutans* in excess of 5% of the

total cultivable flora. As in the present study, high levels of this organism were also found on surfaces which remained caries-free. *S. mutans* serotype d/g was the most common serotype in caries-active individuals but serotype c was found in both caries-free and caries-active subjects, which implicated serotype d/g as a factor in caries activity and progression. In the present study, isolates of *S. mutans* were biochemically differentiated according to biotype as suggested by Shklair and Keene (1974). Biotype I, corresponding to serotype c, was the predominant mutans streptococci present in the test children both before and after the development of carious lesions. Although its frequency of isolation was lower in the control children than in the test children, *S. mutans* biotype I was regularly isolated from all sites in control children confirming the widespread presence of *S. mutans* in dental plaque.

S. mutans biotype IV, corresponding to serotype d/g, was isolated from four of five subjects who were caries-active. One of these subjects (CH- Appendix A) was heavily colonized. 5 of 6 samples were positive from the site which developed a lesion and 3 of 6 samples were positive at the site which remained caries-free. The non-susceptible site in this subject gave 2 positive isolations of *S. mutans* biotype IV in 5 samples. This data suggests that *S. mutans* biotype IV was important in the progression of the lesion in subject CH which agrees with the findings of Huis In't Veld et al (1979). However, in subject DS, this organism was never

detected in the carious lesion. In addition, subject WR, who remained caries-free, showed persistent colonization with biotype IV at susceptible site 2 and non-susceptible site 3. This appears to contradict the results of Huis In't Veld et al (1979). A possible explanation for this discrepancy is that, while the microfloras which were responsible for lesions in each caries-active test child shared similarities, there were significant environmental differences within the microfloras themselves, such as those alluded to previously, which prevented colonization by certain species in certain children. Furthermore, this observation supports the concept that dental caries occurs as the result of acid attack by the microflora as a whole rather than by a specific pathogen. It does not rule out the possibility of microbial specificity in caries etiology. Rather it extends this concept by recognizing that, in certain ecosystems, other specific organisms may play a role in production of a carious lesion.

c) ***Actinomyces***

A positive association between *A. viscosus* and the development of a carious lesion was noted in the present investigation. A weaker association was noted for *A. odontolyticus* but this was evident only in comparisons between test children.

A. viscosus was regularly isolated in high numbers from both the control and test children in this investigation

(Appendix A). This is in keeping with the results of others who have shown that this organism is a prominant member of the commensal oral flora (Bowden and Hardie, 1975; Gallagher et al, 1981). In this study, as decay developed at susceptible surfaces in test children, the level and isolation frequency of *A. viscosus* increased to the point where the levels in the caries-active test children compared to those in the caries-free test and control children were significantly different. This implied that either *A. viscosus* was a significant organism in the caries process or it simply found an expanded niche to occupy in the modified environment. A role for this organism in root caries has been previously described (Sumney and Jordan, 1974). Furthermore, Edwardsson (1974) isolated *A. viscosus* as well as numerous other Gram-positive rods from the advancing front of carious lesions in dentin. Hoshino (1985) isolated other species of *Actinomyces* including *A. odontolyticus, A. naeslundii* and *A. israelii* but did not find *A. viscosus*. Taxonomic studies have shown that human *A. naeslundii* and *A. viscosus* are biochemically very similar to one another and can be serologically related as well. However, Schaal (in Sneath et al, 1986) describes several biotypes and serovars within *A. viscosus* and *A. naeslundii* and the taxonomy of these and related *Actinomyces* species as far from complete. Thus, the discrepancy between the results of Edwardsson and Hoshino with respect to *A. viscosus* could be as a result of the use of different nomenclature. Nonetheless, isolation of these organisms from a highly acidic environment signifies

that they are likely to be aciduric.

The association of *A. odontolyticus* with carious lesions in the present study is not as clear. Batty (1958) first described this organism following isolation from carious dentin. In addition, both Edwardsson (1974) and Hoshino (1985) isolated *A. odontolyticus* from the advancing front of deep dentinal carious lesions. Although it was isolated in low numbers (0.75% of the total cultivable flora), Boyar and Bowden (1985) found that *A. odontolyticus* was positively associated with the progression of incipient enamel lesions to the point where restoration was required.

In the present study, *A. odontolyticus* was more prevalent once the enamel surface was no longer intact and cavitation had occurred. This agreed with Boyar and Bowden who proposed that the presence of *A. odontolyticus* could be indicative of loss of the intact layer of surface enamel. Interestingly, the levels of *A. odontolyticus* were higher in control children than either the test children who remained caries-free or, more significantly, the test children who developed lesions. Therefore, the relationship of *A. odontolyticus* to caries, based on the present results, remains equivocal.

Loesche et al (1984) regularly isolated an *Actinomyces*-like organism from carious and non-carious fissures in a longitudinal study of fissure decay. During the development of decay this organism showed no remarkable changes in its relative proportion to the microflora or its isolation

frequency. It is indeed unfortunate that no attempts were made to identify this organism, but this epitomizes the kind of problem that can occur in an investigation which has focused on the relationship of one or two species to dental caries and ignored the increasingly obvious fact that dental caries is a disease of altered ecology.

d) *Veillonella*

Veillonella has been considered as a genus which could modify the caries process in man by metabolizing lactic acid thus reducing the extent of the plaque pH drop and presumably the cariogenic challenge to the tooth surface (Bowden et al, 1976). Some studies in animals have suggested and demonstrated this concept (Mikx et al, 1972, 1976). However, the results presented in this study suggest that the high levels of *Veillonella* may reflect an environment high in lactic acid. The possibility that *Veillonella* modified the caries attack in test children is reduced by the finding of no significant difference in their numbers at susceptible sites which developed lesions or remained caries-free. In addition, although the numbers of *Veillonella* at susceptible sites were higher in test children than in control children the differences were not significant. A significantly higher isolation frequency for *Veillonella* in the test children supports the contention that the environment at the susceptible sites in test children had a higher level of lactic acid than the same sites in the control children.

e) Other Organisms of Interest

As mentioned earlier in this discussion, there were other organisms which underwent a change in their proportions relative to the entire flora and/or a change in their frequency of isolation.

Yeasts were regularly isolated from the susceptible sites of caries-active children with or without lesions. Since they were rarely found on the non-susceptible site in test children (2 of 40 samples) or on the susceptible sites in those test children who remained caries-free (9 of 36 samples), their abundance at the acidic locations previously mentioned probably indicates the existence of an environment more to their liking. This agrees with data published by Krasse (1954).

A. naeslundii was the predominant organism at susceptible sites in test children who remained caries-free. In the caries-active test children this organism underwent a progressive decline from dominance to virtual absence as lesions developed. This negative association has not been frequently reported in previous studies. Hemmens et al (1946) reported that alpha-hemolytic streptococci, *Leptotrichia*, *Neisseria*, anaerobic fusiform bacilli and *Actinomyces* all decreased markedly in number with the onset of dental caries. The species of *Actinomyces* which decreased in number were not identified.

Thus, the levels of *A. naeslundii* and *A. viscosus* appeared to be inversely related to one another as disease developed at susceptible sites. This kind of ratio has been reported for *S. sanguis/mitior* and *S.mutans* (Loesche and Straffon, 1979; Boyar and Bowden, 1985) and was discussed earlier in relation to its potential use in predicting disease susceptibility. However, a ratio as clearly established for *A. naeslundii* and *A. viscosus* has not been reported until this investigation.

VI Methods of Data Analysis

An extensive discussion of the current methods employed in the analysis of complex microfloras was completed in the literature review and will not be repeated here. However, during the analysis of the data from the present investigation it became clear that the methods used here were still, in some ways, inadequate.

Numerous comparisons, using one- and two-way analysis of variance, the chi square test and its variations and the Student t-tests, were made in order to delineate significant differences which either already existed between groups of children or became apparent as dental caries developed at susceptible sites. The comparisons were made on the basis of one species at a time. As mentioned in Chapter II, this was the primary reason that the Lambda of Good analysis was selected. This analysis allows for the comparison of an entire flora between two investigator-defined populations and it will indicate the statistical probability that the

two populations are similar. However, what this analysis fails to do is indicate where differences exist between populations.

A method which allows the investigator to study the differences between two or more groups of subjects or populations with respect to several variables, i.e. organisms, simultaneously would be extremely useful in the analysis of complex microbial populations. Discriminant analysis (Klecka, 1980) is such a method and has been used by social scientists to study psychological testing of children, the effects of medical treatment and to predict voting behaviour, among other applications. The basic prerequisites are that two or more groups exist which we presume to differ on several variables and that those variables can be measured at the interval or ratio level. Discriminant analysis will help us to analyse the differences between groups and provide the investigator with a means to classify any case into the group which it most closely resembles.

Discrimination between groups occurs on the basis of some set of characteristics (i.e. discriminating variables- in this instance genus, species, presence or absence of organisms, presence or absence of disease, time), determining how well the characteristics discriminate and indicating which characteristics are the most powerful discriminators. In this way, it is possible to state with

statistical certainty how or why one population differs from other populations. It also lends itself to prediction in that, if it is possible to define the discriminating variables in a population, it may be possible to predict the outcome of a particular combination of variables. Because of the complexity and the volume of data contained in the present study, an interesting followup would be to re-analyse this data using step-wise discriminant analysis.

VII Significance of the Findings

The present investigation has confirmed earlier observations on the microbiology of dental caries and demonstrated again that *S. mutans* and *Lactobacillus* are potentially significant pathogens in the caries process. Criticism regarding the accuracy of caries diagnosis and sampling, which have cast doubt on the results of other studies, has been avoided in this investigation because of the model employed. However, the emphasis in this study was on the ecology of the disease rather than the role of specific organisms in the disease. From this perspective, it was demonstrated that as dental caries developed, significant population shifts occurred and the complexity of the microflora decreased. There are numerous examples in the data to indicate that dominance alone by an organism, in particular *S. mutans*, is not of itself sufficient for disease to develop. Rather, the effect of the environment on the entire microflora, and ultimately the tooth surface, must be considered. On this basis, the possibility that other organisms, besides *S. mutans* and

Lactobacillus, may have a role to play in the production of a carious lesion should not be overlooked. This supports the concept that dental caries occurs as a result of an ecological imbalance in the indigenous oral microflora. Furthermore, it acknowledges the multifactorial nature of dental caries without precluding a more prominent role for some factors than others. The key as to which components of the flora assume a prominent role in the disease process is the environment.

A longitudinal study to investigate and compare the development of the oral microflora between bottle-fed and breast-fed infants may provide some insight into the effect of external (eg. diet, flora of the primary care-giver) and internal (immune system) environmental determinants on this process. In addition, the effect of environment on the microflora, as well as the microflora itself, could be investigated over a long period of time in children who are at high risk of nursing decay. If the children were followed beyond the period of high susceptibility to nursing decay into the later childhood years when approximal lesions are likely to occur, an interesting comparison of the flora from these two periods of time in the childrens' lives could be made.

A better understanding of the interaction between the environment, the host and the microflora could have significance in the development of new methods for the control of chronic diseases like dental caries and

periodontal disease. In addition, the nature of dental practice itself could change such that, practitioners rather than waiting for disease to develop and then repairing the damaged tissues, could monitor patients for changes in the oral environment in order that trends known to be associated with disease could be diagnosed earlier and preventive therapy instituted before disease developed. While these kinds of methods have been explored in the past, most have focused on a single agent. Given the multifactorial nature of dental caries and periodontal disease, it is not surprising that these methods do not correlate well with the clinical picture and are not used by the majority of clinicians.

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APPENDIX A

LONGITUDINAL DATA FOR SELECTED ORGANISMS FROM INDIVIDUAL SUBJECTS

Longitudinal data from test children on the percentage contribution of *S. milleri* to samples from sites developing lesions, caries-free susceptible sites and non-susceptible intra subject control sites.

CARIES ACTIVE SUBJECTS

Susceptible Sites which developed a lesion

	Caries Free				White Spot		Lesion				
No. of Samples	1	2	3	5	3	3	3	2	2	1	1
Subject											
LB1[a]	0	11.9	9.9	0	0	0					
CH1				11.4	NT	NT	8.0	0	0	0	11.4
DS2				0	NT	NT	0	5.9	0		
KT1		0	0	2.6	14.3	0	5.4				
JG1			19.0	0	0	0					

Susceptible Surfaces Which Remained Lesion Free

No. of Samples	2	3	5	5	5	5
Subject						
LB2[b]	0	14.5	0	6.9	1.9	0
CH2		44.4	28.3	19.8	0	0
DS1			38.5	2.9	0	1.4
KT2	12.6	0	24.5	19.9	0	0
JG2			0	28.2	0	0

CARIES FREE SUBJECTS

No. of Samples	4	6	8	8	8
Subject					
TS1	3.8	13.7	6.7	0	0
TS2	9.2	35.3	6.7	2.2	3.7
AM1	15.6	0	24.1	0	0
AM2	8.8	0	22.1	0	0
DH1		0	0	2.1	23.7
DH2		0	0	2.3	15.5
WR1			2.8	1.6	20.8
WR2			0	6.0	12.2

INTRA SUBJECT CONTROL

No. of Samples	2	5	8	9	9	9
Subject						
LB3[c]	0	26.8	0	0	29.9	0
CH3		0	0	6.1	6.1	0
DS3			25.0	0	0	0
KT3	5.7	0	26.6	0	0	0
JG3			24.9	0	0	0
TS3		2.5	5.8	25.0	0	0
AM3		7.4	0	0	0	0
DH3			15.1	12.9	22.7	2.0
WR3				14.9	16.0	3.8

[a] subject LB, site 1
[b] subject LB, site 2
[c] subject LB, site 3

Longitudinal data from test children on the percentage contribution of *S. mitior hard* to samples from sites developing lesions, caries-free susceptible sites and non-susceptible intra subject control sites.

CARIES ACTIVE SUBJECTS

Susceptible Sites which developed a lesion

	Caries Free				White Spot		Lesion				
No. of Samples	1	2	3	5	3	3	3	2	2	1	1
Subject											
LB1[a]	4.6	0	34.9	0	15.9	14.8					
CH1				65.9	NT	NT	0	0	6.3	11.0	14.5
DS2				10.0	NT	NT	2.2	2.7	0		
KT1		0	0	2.6	0	3.2	14.4				
JG1			3.1	0	1.1	0					

Susceptible Surfaces Which Remained Lesion Free

No. of Samples	2	3	5	5	5	5
Subject						
LB2[b]	1.2	0	4.9	31.6	0	0
CH2		9.8	0	5.5	0	11.4
DS1			12.0	7.5	4.6	0
KT2	12.6	18.2	30.6	4.9	1.4	13.2
JG2			12.7	5.0	4.6	29.0

CARIES FREE SUBJECTS

No. of Samples	4	6	8	8	8
Subject					
TS1	3.1	9.8	13.3	38.5	43.6
TS2	0.1	21.4	0	11.9	16.5
AM1	22.9	57.0	11.7	13.3	17.4
AM2	0	56.3	16.3	17.0	50.7
DH1		0	12.3	2.2	0
DH2		5.7	83.3	0	3.2
WR1			11.4	2.0	18.0
WR2			0	0	2.9

INTRA SUBJECT CONTROL

No. of Samples	2	5	8	9	9	9
Subject						
LB3[c]	0.7	2.4	45.5	0	0	0
CH3		11.5	5.3	20.6	27.6	28.6
DS3			3.7	3.2	2.1	40.3
KT3	0	0	0	0	1.6	0
JG3			0	0	6.4	18.8
TS3		1.2	40.0	6.3	23.7	24.1
AM3		0	71.4	33.5	13.7	2.8
DH3			5.1	32.3	0.6	32.3
WR3				0	10.6	22.1

[a] subject LB, site 1

[b] subject LB, site 2

[c] subject LB, site 3

Longitudinal data from test children on the percentage contribution of *S. mitior soft* to samples from sites developing lesions, caries-free susceptible sites and non-susceptible intra subject control sites.

CARIES ACTIVE SUBJECTS

Susceptible Sites which developed a lesion

	Caries Free				White Spot		Lesion				
No. of Samples	1	2	3	5	3	3	3	2	2	1	1
Subject											
LB1[a]	0	0	35.3	0	0	0.1					
CH1				0	NT	NT	5.3	0	44.6	13.1	8.4
DS2				0	NT	NT	0	0	0		
KT1		10.5	83.5	0	16.5	0.2	2.1				
JG1			0	0	12.9	1.3					

Susceptible Surfaces Which Remained Lesion Free

No. of Samples	2	3	5	5	5	5
Subject						
LB2[b]	0	0	0	0	26.8	24.1
CH2		3.6	0	0	8.1	15.0
DS1			0	0	0	0
KT2	0	0	0	0	25.7	0
JG2			0	0	5.3	0

CARIES FREE SUBJECTS

No. of Samples	4	6	8	8	8
Subject					
TS1	0	20.2	0	6.4	0
TS2	0	0	0	0	0
AM1	0	0	0	14.2	0
AM2	33.7	0	0	0	0
DH1		6.7	17.7	0	2.2
DH2		0	0	0	0
WR1			0	0	0
WR2			0	0	0

INTRA SUBJECT CONTROL

No. of Samples	2	5	8	9	9	9
Subject						
LB3[c]	0	0	0	0	7.1	0
CH3		13.8	0	0	13.6	13.2
DS3			0	0	8.6	2.5
KT3	0	15.1	0	45.6	24.2	76.3
JG3			0	23.9	4.1	0
TS3		0	0	0	13.4	4.0
AM3		36.9	0	0	1.4	0
DH3			0	0	0	0
WR3				0	0	0

[a] subject LB, site 1

[b] subject LB, site 2

[c] subject LB, site 3

Longitudinal data from test children on the percentage contribution of *S. mutans I* to samples from sites developing lesions, caries-free susceptible sites and non-susceptible intra subject control sites.

CARIES ACTIVE SUBJECTS

Susceptible Sites which developed a lesion

	Caries Free				White Spot		Lesion				
No. of Samples	1	2	3	5	3	3	3	2	2	1	1
Subject											
LB1[a]	0	0	0	0	25.4	1.9					
CH1				0	NT	NT	9.8	18.7	0	13.9	15.2
DS2				7.5	NT	NT	37.3	63.5	84.0		
KT1		0	0	0	38.4	19.4	.02				
JG1			7.9	8.0	31.2	58.7					

Susceptible Surfaces Which Remained Lesion Free

No. of Samples	3	3	5	5	5	5
Subject						
LB2[b]	0	0	0	0	0.1	13.4
CH2	0	60.1	1.1	4.8	0	0
DS1			1.5	35.9	43.8	24.2
KT2	0	0	0	39.8	14.3	7.9
JG2			0	2.5	8.6	7.7

CARIES FREE SUBJECTS

No. of Samples	4	6	8	8	8
Subject					
TS1	0	0	0	1.9	0
TS2	0	0	0	0	0
AM1	0	0	32.8	0	3.5
AM2	0	0	0.13	0	0
DH1		0	0	0.8	0
DH2		7.8	2.1	0	0
WR1			2.2	66.8	16.9
WR2			2.0	11.3	20.3

INTRA SUBJECT CONTROL

No. of Samples	2	5	8	9	9	9
Subject						
LB3[c]	0	0	0	0	0	0
CH3		0	2.6	0	0.4	6.6
DS3			14.7	0	16.0	0.6
KT3	0	0	0	60.6	1.5	0
JG3			1.2	5.0	0.3	3.1
TS3		0	0	2.5	0	32.2
AM3		0	0	28.1	0	9.8
DH3			12.7	0	0	0
WR3				0	0.5	0

[a] subject LB, site 1

[b] subject LB, site 2

[c] subject LB, site 3

Longitudinal data from test children on the percentage contribution of *S. mutans IV* to samples from sites developing lesions, caries-free susceptible sites and non-susceptible intra subject control sites.

CARIES ACTIVE SUBJECTS

Susceptible Sites which developed a lesion

	Caries Free				White Spot		Lesion				
No. of Samples	1	2	3	5	3	3	3	2	2	1	1
Subject											
LB1[a]	0	0	0	0	0	0					
CH1				0	NT	NT	17.2	20.8	8.5	4.4	3.1
DS2				0	NT	NT	0	0	0		
KT1		0	0	0	0	0	0.02				
JG1			0	1.9	0	0					

Susceptible Surfaces Which Remained Lesion Free

No. of Samples	3	3	5	5	5	5
Subject						
LB2[b]	0	0	0	0	0	3.5
CH2	0	3.9	0.4	8.2	0	0
DS1			0	0	0	0
KT2	0	0	0	0	40.6	0
JG2			0.4	0.2	0	0

CARIES FREE SUBJECTS

No. of Samples	4	6	8	8	8
Subject					
TS1	0	0	0	0	0
TS2	0	0	0	0	0
AM1	0	0	0	0	0
AM2	0	0	0	0	0
DH1		0	0	0	0
DH2		0	0	0	0
WR1			0	0	3.6
WR2			0	12.3	16.0

INTRA SUBJECT CONTROL

No. of Samples	2	5	8	9	9	9
Subject						
LB3[c]	0	0	0	0	0	0
CH3		0	0.03	0	0	6.6
DS3			0	0	0	0
KT3	0	0	0	0	0	0
JG3			0	0	0	0
TS3		0	0	0	0	0
AM3		0	0	0	0	0
DH3			0	8.1	0	0
WR3				0	0.05	2.9

[a] subject LB, site 1

[b] subject LB, site 2

[c] subject LB, site 3

Longitudinal data from test children on the percentage contribution of *S. salivarius* to samples from sites developing lesions, caries-free susceptible sites and non-susceptible intra subject control sites.

CARIES ACTIVE SUBJECTS

Susceptible Sites which developed a lesion

	Caries Free				White Spot		Lesion				
No. of Samples	1	2	3	5	3	3	3	2	2	1	1
Subject											
LB1[a]	1.8	16.6	0	0	0	1.9					
CH1				5.7	NT	NT	0	0	3.3	38.3	15.8
DS2				22.5	NT	NT	0	6.2	0.1		
KT1		5.0	0	0	0	18.6	5.4				
JG1			0	13.8	0	0					

Susceptible Surfaces Which Remained Lesion Free

No. of Samples	2	3	5	5	5	5
Subject						
LB2[b]	4.0	35.2	25.0	0.5	3.4	2.0
CH2		36.4	8.7	25.8	3.4	0
DS1			13.9	24.3	0	0
KT2	45.0	0.9	2.0	1.6	0	0.5
JG2			22.0	4.3	0	0

CARIES FREE SUBJECTS

No. of Samples	4	6	8	8	8
Subject					
TS1	4.2	0	13.3	0	0.6
TS2	0	5.9	0	0	8.3
AM1	0	0	<.01	0	0
AM2	23.2	5.6	0	0	0
DH1		23.4	16.9	0	2.5
DH2		0	2.5	0.06	0
WR1			16.0	0.8	14.6
WR2			19.5	1.6	3.4

INTRA SUBJECT CONTROL

No. of Samples	2	5	8	9	9	9
Subject						
LB3[c]	0	13.4	14.4	0	19.5	0
CH3		51.7	0	8.3	3.4	0
DS3			8.8	3.2	0.5	0
KT3	20.8	2.9	4.6	6.1	0	0
JG3			3.4	16.5	0	0
TS3		18.3	0	0	0	0
AM3		0	14.3	0	0	0
DH3			3.6	30.6	8.3	8.8
WR3				11.6	16.0	22.5

[a] subject LB, site 1

[b] subject LB, site 2

[c] subject LB, site 3

Longitudinal data from test children on the percentage contribution of *S. sanguis* to samples from sites developing lesions, caries-free susceptible sites and non-susceptible intra subject control sites.

CARIES ACTIVE SUBJECTS

Susceptible Sites which developed a lesion

	Caries Free				White Spot		Lesion				
No. of Samples	1	2	3	5	3	3	3	2	2	1	1
Subject											
LB1[a]	0	18.3	0	0	0.4	15.7					
CH1				0	NT	NT	0	6.4	22.9	0	0
DS2				17.5	NT	NT	1.1	3.4	2.1		
KT1		1.1	11.3	3.7	4.8	1.0	32.5				
JG1			16.3	0	0	1.3					

Susceptible Surfaces Which Remained Lesion Free

No. of Samples	2	3	5	5	5	5
Subject						
LB2[b]	2.0	2.6	0	0	0	0
CH2		0	0	0	0.7	2.9
DS1			0	1.2	0.2	0
KT2	0	27.3	0	5.8	6.8	10.6
JG2			14.1	3.2	0	5.1

CARIES FREE SUBJECTS

No. of Samples	4	6	8	8	8
Subject					
TS1	5.7	0	2.3	6.5	6.0
TS2	0.5	15.0	0	7.1	14.8
AM1	0	1.2	0	4.2	9.6
AM2	0.1	0	0	0.8	25.4
DH1		0	0	0	4.0
DH2		0	0	0	19.1
WR1			5.8	3.4	0
WR2			6.4	6.2	11.4

INTRA SUBJECT CONTROL

No. of Samples	2	5	8	9	9	9
Subject						
LB3[c]	0	22.0	0	0	0	0
CH3		12.6	0	0	2.3	20.9
DS3			16.9	17.2	0	12.7
KT3	0	56.9	12.5	0	0	0
JG3			16.8	0	0	45.8
TS3		0	4.2	0	8.9	0
AM3		18.7	0	3.3	0	0.1
DH3			0	0	6.1	0
WR3				47.9	14.9	36.8

[a] subject LB, site 1

[b] subject LB, site 2

[c] subject LB, site 3

Longitudinal data from test children on the percentage contribution of *A. naeslundii* to samples from sites developing lesions, caries-free susceptible sites and non-susceptible intra subject control sites.

CARIES ACTIVE SUBJECTS

Susceptible Sites which developed a lesion

	Caries Free				White Spot		Lesion				
No. of Samples	1	2	3	5	3	3	3	2	2	1	1
Subject											
LB1[a]	74.7	17.3	0	0	12.7	10.2					
CH1				0	NT	NT	0	0.4	0	1.7	0
DS2				0	NT	NT	7.1	0	0.3		
KT1		0	0	0	0	0	0				
JG1			12.9	72.9	16.6	10.7					

Susceptible Surfaces Which Remained Lesion Free

No. of Samples	2	3	5	5	5	5
Subject						
LB2[b]	60.8	17.1	0	1.3	21.0	8.7
CH2		0	3.5	9.3	26.0	2.9
DS1			0	0	0	26.0
KT2	0	0	0	0	0	22.9
JG2			22.9	12.1	0	19.9

CARIES FREE SUBJECTS

No. of Samples	4	6	8	8	8
Subject					
TS1	21.0	11.5	10.0	45.0	2.7
TS2	84.2	14.4	0	7.5	16.5
AM1	3.1	5.8	2.5	58.1	33.4
AM2	0	0	9.2	40.1	4.3
DH1		56.8	10.8	37.3	18.7
DH2		23.0	0	57.3	43.6
WR1			43.8	6.5	8.7
WR2			24.1	18.7	16.3

INTRA SUBJECT CONTROL

No. of Samples	2	5	8	9	9	9
Subject						
LB3[c]	35.7	6.1	0	0	7.8	0
CH3		0	14.5	27.8	3.0	2.2
DS3			5.1	4.3	0.9	6.4
KT3	0	8.8	0.8	0	0	0
JG3			0	0	34.3	9.4
TS3		26.7	11.7	0	13.4	0
AM3		0	4.3	3.3	25.7	81.5
DH3			1.5	0	16.7	28.3
WR3				14.9	13.3	2.5

[a] subject LB, site 1

[b] subject LB, site 2

[c] subject LB, site 3

Longitudinal data from test children on the percentage contribution of *A. viscosus* to samples from sites developing lesions, caries-free susceptible sites and non-susceptible intra subject control sites.

CARIES ACTIVE SUBJECTS

Susceptible Sites which developed a lesion

	Caries Free				White Spot		Lesion				
No. of Samples	1	2	3	5	3	3	3	2	2	1	1
Subject											
LB1[a]	17.2	1.8	0	.01	36.0	23.1					
CH1				0	NT	NT	5.1	6.4	4.0	0	1.4
DS2				38.1	NT	NT	27.7	18.7	2.7		
KT1		1.1	1.9	50.0	17.0	53.0	23.5				
JG1			0	0	0	14.7					

Susceptible Surfaces Which Remained Lesion Free

No. of Samples	2	3	5	5	5	5
Subject						
LB2[b]	0	0	0	1.3	23.6	2.6
CH2		0	13.3	9.9	4.1	14.3
DS1			27.4	11.0	0.2	44.9
KT2	3.6	2.7	34.7	6.2	5.4	13.7
JG2			4.9	15.0	76.2	19.1

CARIES FREE SUBJECTS

No. of Samples	4	6	8	8	8
Subject					
TS1	22.5	5.4	6.7	16.0	0
TS2	0	0	0	0	4.6
AM1	0	5.8	0	0	33.4
AM2	0	0	19.1	1.2	10.9
DH1		0	0.4	6.0	24.5
DH2		0	0	12.2	0
WR1			2.4	7.4	0
WR2			4.0	14.9	3.2

INTRA SUBJECT CONTROL

No. of Samples	2	5	8	9	9	9
Subject						
LB3[c]	21.4	0	0	0	0.6	0
CH3		0	25.0	8.3	12.9	0
DS3			9.6	5.4	46.4	36.0
KT3	3.8	8.8	0	9.1	7.6	0
JG3			31.6	7.8	30.2	4.2
TS3		0	0	0	0	0
AM3		4.9	0	0	20.3	0.1
DH3			4.5	0	3.0	6.1
WR3				0	0	2.9

[a] subject LB, site 1

[b] subject LB, site 2

[c] subject LB, site 3

Longitudinal data from test children on the percentage contribution of *A. odontolyticus* to samples from sites developing lesions, caries-free susceptible sites and non-susceptible intra subject control sites.

CARIES ACTIVE SUBJECTS

Susceptible Sites which developed a lesion

	Caries Free				White Spot		Lesion				
No. of Samples	1	2	3	5	3	3	3	2	2	1	1
Subject											
LB1[a]	0	0	0	0	0	2.8					
CH1				0	NT	NT	0	0	0	0	0
DS2				0	NT	NT	0	0.4	0		
KT1		0	0	0	0	0	7.2				
JG1			0	0	0	0					

Susceptible Surfaces Which Remained Lesion Free

No. of Samples	2	3	5	5	5	5
Subject						
LB2[b]	0	0	0	0	0	0
CH2		0	0	0	0	0
DS1			0	0	0	0
KT2	0	0	1.0	0	0	0
JG2			0	0	0	0

CARIES FREE SUBJECTS

No. of Samples	4	6	8	8	8
Subject					
TS1	0	5.0	0	0	0
TS2	0	0	0	23.9	0
AM1	0	0	0	0	0
AM2	0	0	0	0	0
DH1		0	0	13.0	0
DH2		0	0	9.9	0.9
WR1			0	0.5	0
WR2			0	0	0

INTRA SUBJECT CONTROL

No. of Samples	3	5	8	9	9	9
Subject						
LB3[c]	0	3.7	0	0	0	0
CH3		0	0	0	0	0
DS3			0	0	0	0
KT3	3.8	0	0.8	0	0	0
JG3			0	10.1	1.2	0
TS3	0	0	0	0	0	0
AM3		0	0	0	0	0
DH3			0	0	0	8.1
WR3				0	0	0

[a] subject LB, site 1

[b] subject LB, site 2

[c] subject LB, site 3

Longitudinal data from test children on the percentage contribution of *Rothia* to samples from sites developing lesions, caries-free susceptible sites and non-susceptible intra subject control sites.

CARIES ACTIVE SUBJECTS

Susceptible Sites which developed a lesion

	Caries Free				White Spot		Lesion				
No. of Samples	1	2	3	5	3	3	3	2	2	1	1
Subject											
LB1[a]	0.2	0	0	0	0	0					
CH1				0			0	1.9	0.6	0	0
DS2				0.3			0	18.6	0		
KT1		0	0	0	0	0	0				
JG1			0	0.1	0	0					

Susceptible Surfaces Which Remained Lesion Free

No. of Samples	2	3	5	5	5	5
Subject						
LB2[b]	0.4	0	0	0	0	0.1
CH2		0	1.7	2.2	0.9	1.2
DS1			3.6	1.4	0.2	0
KT2	0	0	0	0	0	0
JG2			2.9	0.5	0	4.4

CARIES FREE SUBJECTS

No. of Samples	4	6	8	8	8
Subject					
TS1	1.5	13.0	1.0	0	0
TS2	0.2	0	0	0	0
AM1	0	2.3	0	0	0
AM2	0	0	0	0	0
DH1		0	0	0	23.7
DH2		0.9	0.8	0	2.3
WR1			0	0.4	0
WR2			0.6	0	0.1

INTRA SUBJECT CONTROL

No. of Samples	3	5	8	9	9	9
Subject						
LB3[c]	30.7	1.2	0	0	0	0
CH3	0	0	5.3	11.1	1.1	3.3
DS3			0	11.0	24.7	0
KT3	0	2.9	0	0	0	0
JG3			1.2	0.1	2.3	2.1
TS3		16.7	5.8	0.6	7.6	1.7
AM3		0	0	1.8	0	0.1
DH3			0	0	0	6.1
WR3				0	0	0.1

[a] subject LB, site 1

[b] subject LB, site 2

[c] subject LB, site 3

Longitudinal data from test children on the percentage contribution of *Neisseria A+P+* to samples from sites developing lesions, caries-free susceptible sites and non-susceptible intra subject control sites.

CARIES ACTIVE SUBJECTS

Susceptible Sites which developed a lesion

	Caries Free				White Spot		Lesion				
No. of Samples	1	2	3	5	3	3	3	2	2	1	1
Subject											
LB1[a]	0.1	0.1	2.6	0	0.1	0					
CH1				0	NT	NT	36.1	3.3	0.4	0	0
DS2				0	NT	NT	0	0.1	0.2		
KT1		6.1	0.4	0	0	2.2	0				
JG1			0	0.8	0.5	0					

Susceptible Surfaces Which Remained Lesion Free

No. of Samples	2	3	5	5	5	5
Subject						
LB2[b]	0.2	0	0.1	22.4	2.0	1.3
CH2		0	0.1	1.6	8.2	15.0
DS1			0	0	0.2	.02
KT2	2.4	0	2.2	0.1	0	1.5
JG2			0	7.9	1.0	2.9

CARIES FREE SUBJECTS

No. of Samples	4	6	8	8	8
Subject					
TS1	0.4	5.0	0	4.6	25.1
TS2	0.3	1.6	66.7	19.0	22.9
AM1	0	0.1	7.4	4.8	.03
AM2	0	2.8	2.0	24.7	0.1
DH1		0.9	0	0	0.7
DH2		8.8	0	0	.01
WR1			0	1.0	0.2
WR2			0.1	0.1	2.2

INTRA SUBJECT CONTROL

No. of Samples	3	5	8	9	9	9
Subject						
LB3[c]	3.6	0	12.0	0	9.1	0
CH3		9.2	0	13.9	3.4	16.5
DS3			0	4.3	0.1	1.5
KT3	7.5	3.9	3.0	0	1.1	0
JG3			0.03	0.5	0.5	7.3
TS3	0	0.7	16.7	0	3.1	19.5
AM3		0	0	0	22.4	0.1
DH3			6.9	8.1	0	4.0
WR3				5.0	0.11	2.9

[a] subject LB, site 1

[b] subject LB, site 2

[c] subject LB, site 3

Longitudinal data from test children on the percentage contribution of *Neisseria A+P-* to samples from sites developing lesions, caries-free susceptible sites and non-susceptible intra subject control sites.

CARIES ACTIVE SUBJECTS

Susceptible Sites which developed a lesion

	Caries Free				White Spot				Lesion		
No. of Samples	1	2	3	5	3	3	3	2	2	1	1
Subject											
LB1[a]	0	0.1	0	0	0	0					
CH1				0	NT	NT	0	0	2.7	0	0
DS2				0	NT	NT	0.1	0	0		
KT1		4.2	0.1	0	0	0.4	0				
JG1			2.4	0.01	0	0					

Susceptible Surfaces Which Remained Lesion Free

No. of Samples	2	3	5	5	5	5
Subject						
LB2[b]	0	15.5	0.3	3.1	0	0
CH2		5.3	3.1	0	0	6.1
DS1			0	1.2	0	0
KT2	0.4	0	1.6	1.1	0	0.9
JG2			7.3	1.1	0	4.4

CARIES FREE SUBJECTS

No. of Samples	4	6	8	8	8
Subject					
TS1	0	5.4	0.3	0	0
TS2	0.1	0.1	0	24.6	7.3
AM1	1.9	0	0	0	0
AM2	27.3	4.2	4.1	0	6.5
DH1		3.2	49.2	8.2	0
DH2		5.3	3.3	0	0
WR1			2.2	0.01	2.4
WR2			0	2.4	0.3

INTRA SUBJECT CONTROL

No. of Samples	2	5	8	9	9	9
Subject						
LB3[c]	0	7.3	0.5	0	0	0
CH3		0	0.1	0	0	0
DS3			0	0	0	0
KT3	0	0	3.4	0	0	0
JG3			8.2	0.9	0	0
TS3		0	2.5	0	0	6.9
AM3		29.6	0.7	0.7	4.4	0.01
DH3			0.4	3.2	1.7	0
WR3				0	0	3.7

[a] subject LB, site 1

[b] subject LB, site 2

[c] subject LB, site 3

Longitudinal data from test children on the percentage contribution of *Neisseria A-P-* to samples from sites developing lesions, caries-free susceptible sites and non-susceptible intra subject control sites.

CARIES ACTIVE SUBJECTS

Susceptible Sites which developed a lesion

	Caries Free				White Spot		Lesion				
No. of Samples	1	2	3	5	3	3	3	2	2	1	1
Subject											
LB1[a]	0	16.2	0	0	0	0					
CH1				0	NT	NT	0	0	0	0	0.1
DS2				0	NT	NT	0.1	0	0		
KT1		0	0	0	0	0	0				
JG1			0	0	0	0					

Susceptible Surfaces Which Remained Lesion Free

No. of Samples	2	3	5	5	5	5
Subject						
LB2[b]	0	0	0	0	0	0
CH2		0	0	0	0	0
DS1			0	1.1	0	0
KT2	0.1	0	0	0	0	0
JG2			0	0	0	0

CARIES FREE SUBJECTS

No. of Samples	4	6	8	8	8
Subject					
TS1	0	0.1	0	1.3	0
TS2	0	0	0	0.6	0
AM1	0.2	.02	0	0	0.1
AM2	0	0	0	0	0
DH1		0	0	0	0
DH2		0	0	0	0
WR1			0.2	0	.01
WR2			0	0	0

INTRA SUBJECT CONTROL

No. of Samples	2	5	8	9	9	9
Subject						
LB3[c]	0	0	0	0	0	0
CH3		0	0	0	0.1	0
DS3			1.5	37.6	12.7	0
KT3	0.2	11.8	2.7	0	2.4	0
JG3			0	2.3	2.3	0
TS3		0	0	0	0	0
AM3		0	0	0	0	.02
DH3				0	0	0
WR3				0	0	0

[a] subject LB, site 1

[b] subject LB, site 2

[c] subject LB, site 3

Longitudinal data from test children on the percentage contribution of *Veillonella* to samples from sites developing lesions, caries-free susceptible sites and non-susceptible intra subject control sites.

CARIES ACTIVE SUBJECTS

Susceptible Sites which developed a lesion

	Caries Free				White Spot		Lesion				
No. of Samples	1	2	3	5	3	3	3	2	2	1	1
Subject											
LB1[a]	1.1	9.4	11.1	0	0.8	27.8					
CH1				1.1	NT	NT	7.2	23.7	2.2	5.3	5.9
DS2				2.5	NT	NT	0	11.5	2.9		
KT1		46.0	0	13.2	7.3	0.4	9.0				
JG1			10.7	2.1	3.2	2.7					

Susceptible Surfaces Which Remained Lesion Free

No. of Samples	2	3	5	5	5	5
Subject						
LB2[b]	12.8	8.3	30.4	11.7	1.6	44.3
CH2		0.6	1.1	4.9	1.6	3.3
DS1			2.9	7.3	17.8	3.5
KT2	9.0	45.5	2.7	13.3	0.3	15.4
JG2			4.9	0	0.6	7.4

CARIES FREE SUBJECTS

No. of Samples	4	6	8	8	8
Subject					
TS1	35.5	10.8	10.0	1.3	3.8
TS2	2.4	1.1	0	0	0
AM1	6.3	9.3	19.7	0	0
AM2	5.7	9.9	26.7	0.5	2.2
DH1		4.6	2.3	5.0	0.1
DH2		8.8	0	11.5	8.2
WR1			4.8	9.4	9.4
WR2			24.7	7.1	1.8

INTRA SUBJECT CONTROL

No. of Samples	2	5	8	9	9	9
Subject						
LB3[c]	0	1.2	10.3	0	1.3	0
CH3		0.1	0	0.9	0.2	0
DS3			0.5	0	4.7	0
KT3	0.2	11.8	2.7	0	2.4	0
JG3			6.7	19.3	0.2	7.3
TS3		0	0.3	0	0	0
AM3		2.0	14.3	20.0	0	0
DH3			42.7	2.3	4.5	0.1
WR3				1.7	11.7	0

[a] subject LB, site 1

[b] subject LB, site 2

[c] subject LB, site 3

Longitudinal data from test children on the percentage contribution of *Lactobacillus* to samples from sites developing lesions, caries-free susceptible sites and non-susceptible intra subject control sites.

CARIES ACTIVE SUBJECTS

Susceptible Sites which developed a lesion

	Caries Free				White Spot		Lesion				
No. of Samples	1	2	3	5	3	3	3	2	2	1	1
Subject											
LB1[a]	0	0	0	0	7.1	0.4					
CH1				0	NT	NT	0	6.6	3.6	12.4	19.1
DS2				0	NT	NT	0	26.3	0.5		
KT1		0	0	0	0	.002	.1				
JG1			0	0	34.1	0					

Susceptible Surfaces Which Remained Lesion Free

No. of Samples	2	3	5	5	5	5
Subject						
LB2[b]	0	0	0	0	0	<.01
CH2		0	0	0	0.2	14.3
DS1			0	0	27.8	0
KT2	0	0	0	0	0	<.01
JG2			0	0	3.6	0

CARIES FREE SUBJECTS

No. of Samples	4	6	8	8	8
Subject					
TS1	0	0	0	0	0
TS2	0	0	0	0	0
AM1	0	0	0	0	0
AM2	.03	0	0	0	0
DH1		0	0	0	0
DH2		0	0	0	0
WR1			0	0	0
WR2			0	0	0

INTRA SUBJECT CONTROL

No. of Samples	2	5	8	9	9	9
Subject						
LB3[c]	0	0	0	0	0	0
CH3		0	0	0	0	0
DS3			0	0	7.2	0
KT3	0	0	0	0	0	0
JG3			0	0	0	0
TS3		0	0	0	0	0
AM3		0	0	0	0	0
DH3			0	0	0	0
WR3				0	0	0

[a] subject LB, site 1

[b] subject LB, site 2

[c] subject LB, site 3

Longitudinal data from test children on the percentage contribution of *Yeast* to samples from sites developing lesions, caries-free susceptible sites and non-susceptible intra subject control sites.

CARIES ACTIVE SUBJECTS

Susceptible Sites which developed a lesion

	Caries Free				White Spot		Lesion				
No. of Samples	1	2	3	5	3	3	3	2	2	1	1
Subject											
LB1[a]	0	0.1	0.9	0	0.1	0.8					
CH1				6.8			0.1	0.1	0.1	0	0.4
DS2				22.8			0.3	1.5	0.8		
KT1		0	0.4	5.3	1.8	0.1	.01				
JG1			0.1	0.1	1.2	2.7					

Susceptible Surfaces Which Remained Lesion Free

No. of Samples	2	3	5	5	5	5
Subject						
LB2[b]	0	0	0	0.1	2.8	0.1
CH2		0.1	0.7	0.1	0	0.2
DS1			0	0	0.1	0.1
KT2	0	0.2	0.6	0.2	0.3	0.7
JG2			0.1	0	0	0

CARIES FREE SUBJECTS

No. of Samples	4	6	8	8	8
Subject					
TS1	0	0	0	0.1	0
TS2	0	0	0	0	0
AM1	0	0	0	0.1	0.1
AM2	0	4.2	0	15.4	0
DH1		0	0.1	0	0
DH2		0	0	0	0
WR1			0	0.1	0
WR2			0	0.1	0.2

INTRA SUBJECT CONTROL

No. of Samples	2	5	8	9	9	9
Subject						
LB3[c]	0	0	0	0	0	0
CH3		0	0	0	0	0
DS3			0	0	0	0
KT3	0	0	0	0	8.0	0
JG3			0	0	0	0
TS3		0	0	0	0	0
AM3		0	0	0	0	0.1
DH3			0	0	0	0
WR3				0	0	0

[a] subject LB, site 1

[b] subject LB, site 2

[c] subject LB, site 3

Longitudinal data on the percentage contribution of *S. milleri* to samples from susceptible and non-susceptible sites in caries free control children.

No. of Samples	3	18	18	18	18	18
Subject						
AM1[a]		4.7	6.3	8.3	12.8	0
KA1		8.2	3.2	4.1	6.7	0
ST1		18.9	7.3	0	0	5.4
LY1		8.4	3.1	0.5	0	0
ME1		0	0	0	0	33.9
KE1		0	3.5	0	4.7	9.5
BL1	9.0	0	2.5	13.5	0	0.5
SH1	0	3.9	0	0	12.7	0.3
MV1	0	0	0	9.1	0	0
Subject						
AM2[b]		0	4.0	0	0	0
KA2		0	6.5	4.0	0	0
ST2		0	0	0	0	6.7
LY2		0	0	0	0	0
ME2		1.5	7.3	0	0	9.2
KE2		0	7.1	0	0	1.9
BL2			23.3	0	0	14.6
SH2		0	0	0	0	6.4
MV2		0	0	5.4	19.4	0
Subject						
AM3[c]			0	0.8	0	0
KA3			0	0	0	1.3
ST3		12.8	25.1	0	0	0
LY3		0	0	1.8	0	0
ME3		0	0	0	0	5.3
KE3		0	5.7	5.1	0	1.4
BL3	0.1	0	2.4	0.3	0	0
SH3	0	0	10.0	0	5.3	12.7
MV3	0	0	0	19.9	0	0

[a] subject AM, site 1
[b] subject AM, site 2
[c] subject AM, site 3

Longitudinal data on the percentage contribution of *S. mitior hard* to samples from susceptible and non-susceptible sites in caries free control children.

No. of Samples	3	18	18	18	18	18
Subject						
AM1[a]		0	4.6	7.1	24.3	20.0
KA1		2.2	0	4.1	0	13.5
ST1		9.9	16.6	0	4.3	12.6
LY1		0.5	9.0	22.1	3.2	0
ME1		9.0	1.0	0	0.4	0
KE1		6.7	12.9	1.1	30.5	4.2
BL1	0	12.4	0	0	32.4	2.6
SH1		9.3	3.3	30.3	28.3	7.9
MV1	0	38.5	0	4.5	2.3	13.4
Subject						
AM2[b]		0	0	0	13.9	14.9
KA2		0	0	0	15.1	27.4
ST2		0	2.1	0	0	20.0
LY2		0	58.0	18.3	25.1	0
ME2		0.7	0	2.3	2.3	13.2
KE2		7.3	25.0	0.4	11.3	20.8
BL2			20.5	18.1	30.5	27.1
SH2		0	10.0	4.9	0	1.3
MV2		22.2	0	0	27.8	8.1
Subject						
AM3[c]			20.5	0	1.8	23.2
KA3			0	0.5	2.1	23.0
ST3		7.3	7.0	29.3	0	0
LY3		30.8	36.5	16.4	4.8	0
ME3		1.1	0.2	4.6	51.0	33.9
KE3		2.1	5.6	0.5	2.1	60.9
BL3	6.7	14.3	31.3	14.8	11.3	2.5
SH3	2.2	0	25.0	43.2	15.0	24.6
MV3	0	18.2	0	0	39.3	3.3

[a] subject AM, site 1
[b] subject AM, site 2
[c] subject AM, site 3

Longitudinal data on the percentage contribution of *S. mitior soft* to samples from susceptible and non-susceptible sites in caries free control children.

No. of Samples	3	18	18	18	18	18
Subject						
AM1[a]		3.5	4.2	8.3	0	0
KA1		0.7	1.9	12.2	2.9	9.4
ST1		16.9	11.4	3.6	40.3	9.0
LY1		0	0	1.3	0	55.0
ME1		0	0	0.2	7.7	9.1
KE1		4.8	1.5	19.4	12.3	6.3
BL1	0	13.4	0	6.4	0	0.5
SH1	5.3	0.7	0	0	1.6	0
MV1	8.4	0	2.6	0	13.5	0
Subject						
AM2[b]		1.6	7.2	0	0	2.1
KA2		0	3.9	0	8.1	0
ST2		0	14.8	17.8	66.3	35.1
LY2		0	4.8	0	0	32.7
ME2		0.5	2.5	6.4	8.0	7.0
KE2		4.5	0	0.9	0	0
BL2			3.1	0	0	16.7
SH2		7.1	0	16.5	0	0
MV2		0	0	0	11.1	2.8
Subject						
AM3[c]			17.6	3.1	0	8.8
KA3			10.3	8.0	5.3	0
ST3		32.1	25.1	0.02	69.8	12.7
LY3		0	0	0	0.9	22.8
ME3		4.5	3.2	15.4	2.5	10.6
KE3		1.4	0	26.3	2.7	1.4
BL3	3.8	27.2	15.4	11.3	0	0
SH3	22.5	1.2	28.8	0	0	2.9
MV3	0	0	0	0	0	4.0

[a] subject AM, site 1
[b] subject AM, site 2
[c] subject AM, site 3

Longitudinal data on the percentage contribution of *S. mutans I* to samples from susceptible and non-susceptible sites in caries free control children.

No. of Samples	3	18	18	18	18	18
Subject						
AM1[a]		0	1.4	18.8	14.7	0.8
KA1		0	0	0	2.9	0
ST1		13.4	5.2	0	0	0
LY1		0	0	0	0	0
ME1		0	3.9	6.8	0.01	0
KE1		0	18.4	0	0.01	0
BL1	0	0	0	2.0	0	0
SH1	0	0	0	0	0	0
MV1	0	7.4	0	0	0	13.2
Subject						
AM2[b]		0	4.8	2.1	1.0	0
KA2		0	0	0	0	0
ST2		0	0	0	0	0
LY2		0	9.1	0	0	0
ME2		0	0	1.0	0.02	0
KE2		0	0	0	0	0
BL2			0	0	14.7	4.2
SH2		0	0	0	0	0
MV2		15.4	0	0	0	23.7
Subject						
AM3[c]			0	3.6	6.3	17.1
KA3			0	0	1.1	0
ST3		0	0	0	0	1.1
LY3		0	0	0	0	0
ME3		0	0	6.0	0	0
KE3		0	0	0	0	0
BL3	0	5.1	0	2.2	16.3	17.3
SH3	3.1	0	0	0	0	2.9
MV3	9.6	0	0	0	0	12.6

[a] subject AM, site 1
[b] subject AM, site 2
[c] subject AM, site 3

Longitudinal data on the percentage contribution of *S. mutans IV* to samples from susceptible and non-susceptible sites in caries free control children.

No. of Samples	3	18	18	18	18	18
Subject						
AM1[a]		0	1.9	0	0	0
KA1		0	0	0	2.9	0
ST1		19.9	0	0	0	0
LY1		0	0	0	0	0
ME1		0	0.6	0	0	0
KE1		0	0	0	0	0
BL1	0	0	0	0	0	0
SH1	0	0	0	0	0	0
MV1	0	0	0	0	0	0
Subject						
AM2[b]		0	0.8	0	0	0
KA2		0	0	0	0	6.4
ST2		0	0	0	0	0
LY2		0	0	0	0	0
ME2		0	0	0	0	0
KE2		0	0	0	0	0
BL2			0	0	0	0
SH2		0	0	0	0	0
MV2		0	0	0	0	0
Subject						
AM3[c]			0	0	0	0
KA3			0	0	0	0
ST3		0	0	0	0	0
LY3		0	0	0	0	0
ME3		0	0	0	0	0
KE3		0	0	0	0	0
BL3	0	0	0	0	0	0
SH3	0	0	0	0	0	0
MV3	0	0	0	0	0	0

[a] subject AM, site 1
[b] subject AM, site 2
[c] subject AM, site 3

Longitudinal data on the percentage contribution of *S. salivarius* to samples from susceptible and non-susceptible sites in caries free control children.

No. of Samples	3	18	18	18	18	18
Subject						
AM1[a]		14.6	11.6	5.0	0	0.8
KA1		3.8	0.01	8.4	3.8	0
ST1		2.0	17.7	0	0	1.5
LY1		0	0	0	0.1	3.2
ME1		0	0	0	0	6.5
KE1		0	4.9	6.7	0.2	3.2
BL1	0	17.9	19.7	0	0	4.3
SH1	1.8	3.3	0	0	4.9	0
MV1	0	0.08	2.3	0	8.5	8.8
Subject						
AM2[b]		0.01	6.0	2.7	0.2	0.4
KA2		41.2	9.7	8.0	8.1	0
ST2		0	15.0	0	0	0
LY2		0	0	9.6	2.8	13.2
ME2		0	0	1.2	0	0
KE2		0.5	3.6	7.0	0	0
BL2			9.2	9.3	0.8	4.2
SH2		21.4	0	27.7	6.0	0
MV2		23.1	14.3	0	0	12.5
Subject						
AM3[c]			8.5	0.1	4.2	3.7
KA3			0	28.7	0	0
ST3		0	0	0.02	1.5	0
LY3		0	0	18.8	10.9	5.2
ME3		0	0	1.7	3.0	0
KE3		0	2.4	4.1	0	0
BL3	1.0	17.0	6.0	4.0	5.4	67.3
SH3	0	33.0	7.7	0	20.2	1.4
MV3	0	2.4	0	0	0	4.0

[a] subject AM, site 1
[b] subject AM, site 2
[c] subject AM, site 3

Longitudinal data on the percentage contribution of *S. sanguis* to samples from susceptible and non-susceptible sites in caries free control children.

No. of Samples	3	18	18	18	18	18
Subject						
AM1[a]		0	16.7	8.6	6.3	1.6
KA1		5.0	0	3.3	0	0
ST1		0	3.7	49.0	7.0	6.0
LY1		0	0	24.0	0	7.8
ME1		0	1.4	5.5	3.7	7.8
KE1		0	0	3.4	8.5	0
BL1	0	0	0	0.3	0	8.0
SH1	0	0	0	0	0	0
MV1	0.4	0	0	0	0	25.5
Subject						
AM2[b]		6.0	4.8	19.9	11.5	3.0
KA2		0	10.4	4.0	2.4	0
ST2		0	2.1	38.3	0	6.0
LY2		0	0	39.0	0	0.6
ME2		6.8	5.1	0	5.1	0
KE2		0	0	2.2	8.9	7.5
BL2			0	0	0	0
SH2		0	0	0	0	0
MV2		0	0	0	0	0
Subject						
AM3[c]			8.5	0.1	20.2	7.3
KA3			8.4	0.5	0	0
ST3		0	0	34.4	13.9	26.2
LY3		0	0	16.4	1.7	4.4
ME3		0	3.6	2.9	24.0	5.3
KE3		0	0	2.0	0.6	2.9
BL3	0	0	0	0	0	0
SH3	0	0	0	0	0	8.6
MV3	0	0	0	0	0	27.7

[a] subject AM, site 1
[b] subject AM, site 2
[c] subject AM, site 3

Longitudinal data on the percentage contribution of *A. naeslundii* to samples from susceptible and non-susceptible sites in caries free control children.

No. of Samples	3	18	18	18	18	18
Subject						
AM1[a]		0	27.0	15.0	0.4	0
KA1		2.4	5.5	2.7	0	32.7
ST1		0	0	6.0	12.9	3.0
LY1		46.4	37.6	14.5	39.4	0
ME1		9.0	2.0	22.5	8.8	0
KE1		0	22.4	2.7	10.4	3.2
BL1	2.9	0	0	11.1	2.4	20.0
SH1	0	49.3	0	10.5	0	10.6
MV1	0	38.8	22.4	40.9	0	0
Subject						
AM2[b]		6.9	26.7	0	4.1	2.7
KA2		5.9	0	40.0	34.8	4.8
ST2		0	0	0	23.0	0
LY2		0	15.0	4.8	27.9	10.9
ME2		6.0	31.0	37.4	17.6	17.1
KE2		0.8	0	6.1	4.9	3.8
BL2			47.2	7.8	2.9	4.2
SH2		0	6.7	0	7.5	46.2
MV2		13.1	0	46.8	5.6	2.8
Subject						
AM3[c]			23.5	7.8	1.7	13.4
KA3			19.6	2.9	37.1	12.7
ST3		0	0	1.7	1.3	0
LY3		53.0	13.5	4.2	21.2	31.9
ME3		6.9	0	11.9	0	0
KE3		0	0	14.2	3.4	8.7
BL3	37.6	2.3	0	21.5	9.4	2.7
SH3	6.6	38.4	0	0	12.3	15.4
MV3	14.0	4.9	19.2	27.4	10.9	0

[a] subject AM, site 1
[b] subject AM, site 2
[c] subject AM, site 3

Longitudinal data on the percentage contribution of *A. viscosus* to samples from susceptible and non-susceptible sites in caries free control children.

No. of Samples	3	18	18	18	18	18
Subject						
AM1[a]		16.4	1.2	6.7	5.1	72.0
KA1		0.9	16.5	16.3	47.7	0
ST1		13.9	10.4	30.4	23.4	19.5
LY1		0	11.4	9.8	0	0
ME1		20.5	0	30.5	28.0	0
KE1		45.3	1.2	28.2	6.6	21.1
BL1	1.0	4.8	19.2	10.4	3.1	4.3
SH1	18.6	26.3	0	0	7.3	39.6
MV1	63.2	8.4	6.6	0	0	7.8
Subject						
AM2[b]		37.8	0	21.5	26.5	4.5
KA2		0	12.9	16.0	9.7	1.6
ST2		0	4.3	1.9	0	4.2
LY2		0	7.5	0	0	0
ME2		0	0	2.6	22.2	0
KE2		7.7	1.8	48.9	0	17.0
BL2			0	2.6	4.8	6.3
SH2		7.1	33.3	0	5.6	0
MV2		0.4	5.7	0	0	0
Subject						
AM3[c]			0	20.3	21.0	0
KA3			15.9	0	27.5	12.7
ST3		17.4	15.0	1.7	10.2	18.0
LY3		0	31.5	4.7	0	0
ME3		37.7	11.6	4.6	0	7.4
KE3		15.3	1.4	2.0	13.5	0
BL3	8.1	19.6	0	4.6	8.4	0
SH3	0	3.5	0	0	0	4.3
MV3	41.9	1.9	0	0	0	17.2

[a] subject AM, site 1
[b] subject AM, site 2
[c] subject AM, site 3

Longitudinal data on the percentage contribution of *A. odontolyticus* to samples from susceptible and non-susceptible sites in caries free control children.

No. of Samples	3	18	18	18	18	18
Subject						
AM1[a]		0	0	4.2	0	0
KA1		0	13.0	10.8	7.6	2.9
ST1		0	0	0	0	0
LY1		0	0	0	0	0
ME1		0	41.1	0	0	0
KE1		7.6	5.8	0	0	1.1
BL1	0	6.0	0	0	1.7	0
SH1	1.1	0	0	0	5.9	0
MV1	0	0	3.2	0	0	0
Subject						
AM2[b]		0	0	0	0	4.5
KA2		0	0	0	0	0
ST2		0	0	0	0	3.3
LY2		0	0	0	0	0
ME2		21.0	9.5	3.9	0	0
KE2		0	0	0	3.7	1.9
BL2			0	0	0	0
SH2		0	0	0	0	0
MV2		0	14.3	0	0	25.1
Subject						
AM3[c]			0	0	0	0
KA3			1.9	0	2.1	0
ST3		0	0	0	0.4	6.9
LY3		0	0	0	0	0
ME3		0	0	0	0	0
KE3		0	0	0	0	0
BL3	0	0	0	0	0	0
SH3	0	0	0	0	0	0
MV3	0	0	0	4.8	0	0

[a] subject AM, site 1
[b] subject AM, site 2
[c] subject AM, site 3

Longitudinal data on the percentage contribution of *Rothia* to samples from susceptible and non-susceptible sites in caries free control children.

No. of Samples	3	18	18	18	18	18
Subject						
AM1[a]		1.2	3.3	3.3	0.6	0
KA1		3.8	0	2.7	1.0	0
ST1		2.0	15.6	2.7	1.3	0
LY1		0	34.9	0	0.9	2.7
ME1		8.0	7.4	3.2	0	3.9
KE1		1.1	6.6	1.9	3.8	0
BL1	0	2.5	0	28.6	1.7	0
SH1	0	2.6	0	1.8	1.9	23.8
MV1	0	0	0	0	0	0
Subject						
AM2[b]		1.0	17.9	29.2	15.3	59.6
KA2		0	6.5	24.0	0	1.6
ST2		0	5.7	3.8	0.3	1.8
LY2		0	3.8	4.4	40.9	31.9
ME2		1.7	0	1.2	2.6	7.9
KE2		7.7	39.2	12.2	18.9	17.0
BL2			0	2.1	1.7	6.3
SH2		0	0	0	54.3	19.2
MV2		0	8.6	1.6	0	2.1
Subject						
AM3[c]			5.3	3.9	8.4	0
KA3			11.2	1.8	1.1	11.5
ST3		4.6	5.0	1.7	0	0
LY3		0.9	0	25.8	39.3	7.9
ME3		1.5	14.0	0.9	14.5	6.4
KE3		3.5	3.2	2.5	12.6	10.1
BL3	0	0	0.4	0	2.0	0
SH3	7.6	11.6	3.1	2.5	0	13.6
MV3	0	0.3	0	0	0	3.3

[a] subject AM, site 1
[b] subject AM, site 2
[c] subject AM, site 3

Longitudinal data on the percentage contribution of *Neisseria A+P+* to samples from susceptible and non-susceptible sites in caries free control children.

No. of Samples	3	18	18	18	18	18
Subject						
AM1[a]		1.0	9.3	12.1	1.4	0.01
KA1		0	0.04	32.5	0	1.0
ST1		0	6.2	0.02	0	0.4
LY1		0	0.01	0	0.4	0.1
ME1		0.1	10.2	0	1.5	25.6
KE1		0	3.5	0	2.6	0
BL1	0	0	9.3	0	8.9	3.2
SH1	12.7	2.6	0	26.1	0.04	0.02
MV1	0.01	0	0	4.5	0	4.9
Subject						
AM2[b]		0	6.4	8.1	5.3	0.9
KA2		11.9	29.1	0	0.1	13.7
ST2		0	2.6	8.9	0	4.9
LY2		0	1.6	2.8	0	6.2
ME2		0	4.1	0	10.5	9.2
KE2		0	0	2.6	10.3	0
BL2			0	13.5	1.2	8.3
SH2		0	13.3	2.6	1.1	0
MV2		0.01	0	0	0	5.6
Subject						
AM3[c]			4.4	0	1.3	0
KA3			4.7	9.5	0	11.5
ST3		0.05	2.6	0	0	9.9
LY3		0	0	0	0	0.5
ME3		0	0	3.4	12.0	25.4
KE3		0	10.8	0	6.3	0
BL3	0	0	3.1	0.2	15.3	7.6
SH3	15.0	0	1.2	17.2	15.0	11.5
MV3	6.3	0	0	0	0	19.2

[a] subject AM, site 1
[b] subject AM, site 2
[c] subject AM, site 3

Longitudinal data on the percentage contribution of *Neisseria A+P−* to samples from susceptible and non-susceptible sites in caries free control children.

No. of Samples	3	18	18	18	18	18
Subject						
AM1[a]		0.4	0	0.2	0	0
KA1		9.1	0	0	1.0	12.5
ST1		0	0	0.2	0.01	1.0
LY1		0.1	0.01	0	0	0.04
ME1		13.1	7.8	0.8	0	0
KE1		1.8	10.4	1.2	1.7	3.7
BL1	35.0	30.6	6.2	14.1	6.2	0
SH1	9.7	0	0	19.5	0	0.1
MV1	0	0	4.4	0	0	0
Subject						
AM2[b]		18.9	0.8	1.2	1.8	3.4
KA2		0	0	0	3.5	0.02
ST2		0	0	0	0	0
LY2		0	0	0	0	0
ME2		2.4	18.7	1.3	0	0
KE2		22.8	8.0	2.6	5.8	0
BL2			0.4	0	2.5	0
SH2		0	0	15.4	0	0
MV2		0.6	28.6	0	0	1.8
Subject						
AM3[c]			0	5.8	4.2	2.4
KA3			0	0	0	0
ST3		0	0	0	0	5.5
LY3		0.1	0	0	0	1.9
ME3		0	3.2	5.1	0	0
KE3		12.5	18.2	0.04	0	0
BL3	6.2	2.8	04	1.4	4.4	0
SH3	0	0	6.5	11.5	8.8	0.1
MV3	3.2	0.02	23.1	0	8.7	0

[a] subject AM, site 1
[b] subject AM, site 2
[c] subject AM, site 3

Longitudinal data on the percentage contribution of *Neisseria A-P-* to samples from susceptible and non-susceptible sites in caries free control children.

No. of Samples	3	18	18	18	18	18
Subject						
AM1[a]		1.5	5.1	0	0	0
KA1		0	0.4	0	0.1	2.9
ST1		0	0	0.03	0	0
LY1		0	0.1	3.0	0	0
ME1		0	0	0.4	0	0
KE1		15.1	7.8	4.0	0	3.4
BL1	0	0	14.5	0	0	1.3
SH1	0	0	0	0	6.2	6.6
MV1	0	0	0.1	0	0	0
Subject						
AM2[b]		0	0	0	0	0
KA2		5.9	0.1	0	3.5	6.4
ST2		0	0	0	0	0
LY2		0	0	1.2	0	0
ME2		0	8.5	0.01	0	2.6
KE2		0	0	0	8.0	0
BL2			0	0	0	0
SH2		0	0	0	0	0
MV2		0.1	0	1.3	0	0.3
Subject						
AM3[c]			3.5	0	0	0
KA3			0	0.7	1.1	1.3
ST3		0.2	0	0	0	0
LY3		0.7	0	0.02	0	0.6
ME3		0	3.2	6.7	0	0.2
KE3		0.6	49.2	0.8	0	0
BL3	0	0	0	1.1	0	0.9
SH3	0	0	0	0	0	0
MV3	2.3	0.02	0	2.1	0	1.7

[a] subject AM, site 1
[b] subject AM, site 2
[c] subject AM, site 3

Longitudinal data on the percentage contribution of *Veillonella* to samples from susceptible and non-susceptible sites in caries free control children.

No. of Samples	3	18	18	18	18	18
Subject						
AM1[a]		38.1	4.9	0.7	0.6	4.7
KA1		2.9	0	0	1.9	3.8
ST1		0.1	0.1	0	4.8	16.5
LY1		0.03	3.9	0	10.7	24.7
ME1		0	5.9	14.5	5.2	0
KE1		0	0	0	0	1.1
BL1	0	0.7	8.1	2.9	14.9	30.3
SH1	30.1	0	0	2.0	3.0	2.5
MV1	25.9	3.9	0	4.5	0.4	6.8
Subject						
AM2[b]		12.7	12.0	13.8	13.9	0.9
KA2		8.8	1.6	0	9.3	3.2
ST2		0	0	0.3	2.6	8.3
LY2		0	0.02	0	2.8	0.1
ME2		21.6	0	7.2	5.7	0
KE2		0	9.8	0.4	0.1	5.7
BL2			15.4	2.6	4.6	4.2
SH2		28.6	0	1.1	1.9	3.8
MV2		23.1	0	0	0	4.2
Subject						
AM3[c]			2.9	0	0.01	8.1
KA3			0	0.04	0	1.9
ST3		0.4	1.2	13.8	2.5	3.6
LY3		0.1	0	0	0.4	2.3
ME3		0	0	0	3.0	0
KE3		0	0	37.5	0	0
BL3	12.4	1.2	0	0	4.9	0
SH3	30.0	0	15.4	0	3.2	0.6
MV3	0	10.5	0	0	0.9	0

[a] subject AM, site 1
[b] subject AM, site 2
[c] subject AM, site 3