THE RELEVANCE OF MICROBIOLOGICAL TESTS IN THE PREDICTION OF CARIES IN ADOLESCENTS

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DECLARATION

This thesis is the original work of the author.

SUMMARY

Although many investigators have attempted to associate dental caries rates with levels of oral micro-organisms, it has not yet proved possible to predict caries development reliably on an individual basis using a single predictive test. Surprisingly, few studies have investigated the effects of combining the results of a number of different microbiological tests.

This thesis reports the investigation of a range of different methods for the estimation of salivary levels of micro-organisms identified by other investigators as being related to subsequent dental caries development. The organisms studied were Lactobacillus spp., Streptococcus mutans, and Candida spp.. Additionally, Veillonella spp. were investigated because, although they had not previously been identified as predictors of caries, it is believed that these micro-organisms metabolise the lactate produced by other oral bacteria, and may thus modify the caries process. It was therefore thought possible that knowledge relating to salivary levels of Veillonella spp. might be of additional benefit in caries prediction.

Both pure cultures of the micro-organisms described, and mixed salivary samples, were used in the selection of media for use in subsequent caries prediction. The media chosen were Rogosa SL agar, Dentocult dip-slides and Snyder tests for Lactobacillus spp.; Mitis Salivarius Bacitracin agar and a mannitol-containing colorimetric broth for Strep. mutans; Sabouraud dextrose agar for Candida spp.; and Rogosa vancomycin agar for Veillonella spp.

The magnitude of variation in counts of oral micro-organisms on

Rogosa SL agar, mitis salivarius bacitracin agar, Sabouraud dextrose agar and Rogosa vancomycin agar was then studied: within repeated estimations of counts from mixed salivary samples; using salivary samples obtained from the same children at different times of the day; in samples obtained on different days; and in sampling at monthly intervals over 2.5 years. Additionally, the effects of sample storage at different temperatures and for increasing periods of time were investigated, and the effects of subjects eating or drinking two snack foods prior to sample donation were also studied. It was concluded that the standard deviation of counts within single salivary specimens was of the order of 0.1 logarithmic units, with increasing variation on daily and monthly sampling, with the standard deviation of such counts usually being within 0.5 logarithmic units. Additionally, storage of samples for up to 24 hours at room temperature was found to effect sample counts little, and the ingestion of potato crisps or cola, either five minutes or one hour before sample donation, were not found to affect microbiological counts significantly.

The Lanarkshire clinical dentifrice trial, involving 3000 children who were examined clinically and radiographically at annual intervals over a three year period, provided the opportunity to investigate the relevance of the selected microbiological caries predictive tests in a large subgroup, initially of over 500 adolescents. The dentifrice trial itself showed that a significant dose-relationship existed between dental caries increments and sodium monofluorophosphate in toothpastes, at concentrations over 1000 ppm.

When the levels of the micro-organisms studied were compared with caries prevalence and caries incremental data, associations were found

between the caries indices and counts of Lactobacillus spp., Strep. mutans and Candida spp.. Levels of Veillonella spp. were not found to correlate consistently with either caries prevalence or incidence. However, the magnitude of these significant correlations was insufficient to allow single microbiological counts to be used as diagnostic or prognostic tests at an individual level. Indeed, correlations between caries prevalence data and subsequent caries increments were greater than those between any of the microbiological estimations and the caries increments, although using stepwise regression analysis the prediction of future caries increment was improved using combinations of clinical and microbiological data. However, by combining microbiological tests alone, or even using combinations of clinical and microbiological tests, diagnosis or prediction of caries groups (low, medium, or high) proved possible in less than 50% of individuals.

Microbiological predictive tests were also compared with some salivary and dietary predictors, and were generally found to be superior to these other predictive methods.

The possibility that microbiological tests might provide more accurate predictions in a group of younger children was also investigated. Although some of the correlations found appeared higher than those found for the adolescents, the differences were non-significant.

It was concluded that caries prediction at an individual level is not yet possible using either single or combined microbiological tests, and that clinical examination of adolescents was a quicker, less expensive and more accurate predictor of caries increment.

However, these tests provide additional information about caries incidence, which might possibly be of benefit in monitoring selected groups of individuals, such as those with previous high caries experience for whom advanced dental techniques are considered, or those with medical problems in whom it is essential that caries is prevented.

ABBREVIATIONS

cfu - colony forming units
Ch. - Chapter
clin. - clinical
cm - centimetre
Δ - increment of
DFS - decayed and filled permanent tooth surfaces
DMFS - decayed, missing and filled permanent tooth surfaces
dmft - decayed, missing and filled deciduous teeth
DMFT - decayed, missing and filled permanent teeth
ds - decayed deciduous tooth surfaces
DS - decayed permanent tooth surfaces
ΔDXFS - increment of decayed, missing and filled permanent surfaces (extracted teeth perpetuated as the number of affected surfaces when last present)
F - fluoride
Fig. - Figure
FOTI - fibre optic transillumination
g - gram
hr - hours
i.e. - that is to say
kV - kilovolt
L. - Lactobacillus
Lact. - Lactobacillus
log - decimal logarithm
$\log_{10}$ - decimal logarithm
mA - milliampere
ml - millilitre
μg - microgram
μl - microlitre

mm - millimetre

M.R.S. - de Man, Rogosa, Sharpe

MSFA - mannitol sorbitol fuchsin azide

MSB - mitis salivarius bacitracin

n - number

N.S. - non-significant

O.D.I. - oral debris index

p - probability

p. - page

pH - negative decimal log of molar hydrogen ion concentration

pp. - pages

P.B.S. - phosphate buffered saline

ppm - parts per million

rad. - radiographic

rpm - revolutions per minute

S. - Streptococcus

S.D. - standard deviation

SL - selective for lactobacilli

spp. - species

Strep. - Streptococcus

TYCSB - trypticase yeast cystine sucrose bacitracin

V. - Veillonella

Veill. - Veillonella

yr - year

Zn - zinc

^{o}C - degrees Celsius

% - percent

$<$ - less than

$\leq$ - less than or equal to

$>$ - greater than

$\geq$ - greater than or equal to

$\pm$ - plus or minus

CHAPTER 1

DENTAL CARIES

1.1 Introduction

Dental caries is the process of progressive destruction of the hard tissues of teeth, initiated by bacterially produced acids at the tooth surface (Boucher, 1974). Man has been affected by this disease at least since Neolithic times (Lunt, 1974). In Britain, the prevalence of caries remained low until the Middle Ages, then increased to reach a peak in the late 1950s (James, 1975). As in many other developed countries, the incidence of caries has since decreased, while that in underdeveloped countries has increased (Sheiham, 1984). Nevertheless, Todd and Dodd (1985), revealed that 15-year-old Scottish children had, on average, 8.4 teeth which were decayed, missing or filled, and over 97% had evidence of previous caries experience.

Caries which penetrates dentine usually results in pain from an inflamed dental pulp and, unless treated by extraction or conservative measures, may proceed to abscess formation. In Scotland, the cost of conservative dental treatment alone to the General Dental Service in 1983, was in excess of £24 million (Scottish Dental Estimates Board, 1983). According to Kay (1985), the majority of extractions in Scotland are attributable to caries.

It is clear that the discomfort and expense caused by caries remain as considerable problems. The disease does not affect all individuals equally; some remain caries free, others suffer from rampant decay, while the prevalence of caries in the majority lies

between these two extremes.

The literature on the aetiological factors involved in dental decay is large, and in this introductory chapter it is only possible to summarise the current state of knowledge concerning those of most relevance to this thesis. These factors, and the way in which they interact in time, to produce dental caries, are shown in Fig.1.1.

1.2 The oral microflora in the aetiology of dental caries

1.2.1 In vitro observations

Miller (1883) and his contemporaries originally described the action of salivary micro-organisms on carbohydrates to produce acids which, *in vitro*, had the ability to decalcify teeth and cause decay. This led to Miller (1890) postulating the chemico-parasitic (acidogenic) theory of dental caries which, with some modifications, remains the principle theory of today.

In the early years of bacteriology, it was generally accepted that all infections were caused by specific bacteria, and therefore attempts were made to identify the causative organism or organisms of dental caries. The frequent isolation of *Bacillus* (*Lactobacillus*) *acidophilus* from carious dentine (Howe & Hatch, 1917), and its ability to produce acid and artificial caries *in vitro* (McIntosh, James & Lazarus-Barlow, 1922), led to a belief, which lasted for about 35 years, that lactobacilli were the principal bacteria which caused dental caries. However, in 1924, Clarke isolated a streptococcus which predominated in many human carious lesions. This he named *Streptococcus mutans*. Despite Clarke's observation that *Strep. mutans* adhered to tooth surfaces, and produced artificial caries *in*

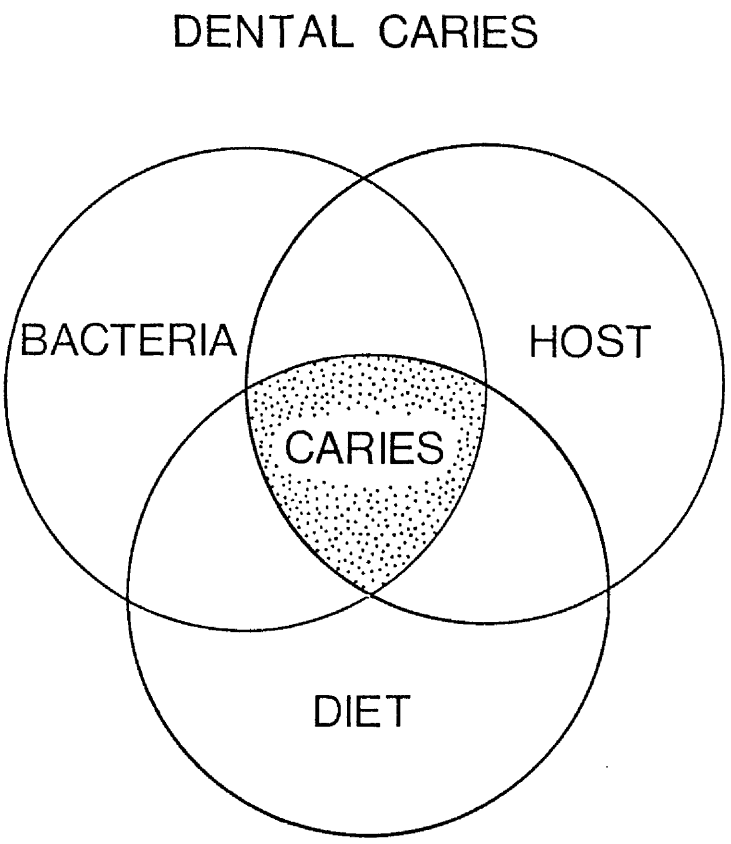


Figure 1.1 The inter-relationship of bacteria, host and diet, which must interact over a period of time for the development of dental caries. Adapted from Keyes (1962).

vitro when incubated with teeth in a glucose broth, the potential cariogenicity of Strep. mutans remained largely neglected until the 1960s, when intense investigation of this species started.

Further in vitro work has shown that many other oral bacteria are able to produce varying amounts of acid from carbohydrate (Onose & Sandham, 1976; Harper & Loesche, 1984). These include Strep. sanguis, Strep. salivarius, Strep. mitis, Strep. faecalis, Actinomyces spp., Candida spp., Staphylococcus spp. and Propionibacterium spp., some of which can cause pH reductions almost as great as those produced by Strep. mutans or Lactobacillus spp.. With such a wide variety of micro-organisms exhibiting acidogenic potential, the suggestion has been made that caries initiation may be due to a spectrum of organisms, and may not necessarily be caused by one single species (reviewed by van Houte, 1980).

1.2.2 Animal experiments

The essential role of bacteria in the development of dental caries, was shown in studies of germ-free rats by Orland et al (1954). They demonstrated that if germ-free and conventional rats were fed a high carbohydrate diet, caries developed only in the conventional animals which possessed an oral flora. Since then, many oral isolates have been tested in gnotobiotes to assess their ability to cause decay. These investigations have been reviewed by Rosen (1984), and are summarised in Table 1.1. Of the many micro-organisms tested, strains of Strep. mutans have been shown to be particularly reliable in causing dental caries.

It is not clear how far the results from these animal experiments

Bacterial species	Cariogenicity	Animal model
Streptococcus		
mutans	+	rats, hamsters, gerbils
liquefaciens	+	rats
faecalis	±	rats
mitis	±	rats
salivarius	±	rats, hamsters
sanguis	±	rats
Lactobacillus		
acidophilus	+	rats
casei	+	rats
Actinomyces		
viscosus	+	hamsters
naeslundii	+	rats

Table 1.1 Cariogenic and noncariogenic strains of bacteria in animal experiments. Adapted from Rosen (1984).

can be applied to human dental caries. The human mouth contains a complex mixed microflora in which cariogenicity must be considered in relation to interactions among many different bacteria. Little is known about these interactions, although Mikx et al (1972) have demonstrated in germ free rats, that the introduction of a combination of Strep. mutans and Veillonella spp. - which use lactic acid to form the weaker acetic and propionic acids - is less cariogenic than is the introduction of Strep. mutans alone.

Furthermore, in animal experiments, different bacteria have produced carious lesions at different tooth sites, as shown in Table 1.2 (reviewed by Schachtele, 1983). Thus in man, it is possible that different micro-organisms may also be involved in caries initiation at different surfaces.

1.2.3 Human studies

Although information has been obtained about bacterial cariogenicity by animal experimentation, knowledge about the bacteria actually associated with the development of caries in man can only be obtained indirectly. Both cross-sectional and longitudinal clinical investigations have been carried out (reviewed by Silverstone et al, 1981; Bowden, Milnes & Boyar, 1984; Marsh & Martin, 1984; Boyar & Bowden, 1985; Edwardsson, 1986). The majority of human studies have been cross-sectional, with subjects being examined and samples collected during a single visit. The samples commonly obtained include saliva, and dental plaque specimens from specific surfaces which are sometimes mixed together to form a pooled sample. At best, cross-sectional studies can demonstrate an association between caries prevalence and a particular micro-organism. Such associations have

	Site	
Bacterium	Smooth surfaces	Occlusal fissures
Lactobacillus acidophilus	-	+
Lactobacillus casei	-	+
Streptococcus mutans	+	+
Streptococcus sanguis	-	+
Streptococcus salivarius	+	+
Streptococcus mitior	-	+
Streptococcus milleri	+	+
Streptococcus faecalis	-	+
Actinomyces viscosus	-	+
Actinomyces naeslundii	-	+
Actinomyces israelii	-	+

Table 1.2 Bacteria capable of producing carious lesions at different sites in the dentition of germ free rats. Adapted from Schachtele (1983).

been shown for Lactobacillus spp. in saliva (Klock & Krasse, 1977; Zickert, Emilson & Krasse, 1982a), and Strep. mutans levels in both saliva and plaque (Loesche et al, 1975; Klock & Krasse, 1977; Zickert et al, 1982a; Carlsson, Olsson & Bratthall, 1985).

In longitudinal studies, repeated sampling and examination of individuals are performed, allowing the development of caries to be associated with levels of particular bacterial species, over a period of months or years. However, longitudinal studies are very time-consuming, labour intensive, and expensive. This is particularly evident if many tooth sites are sampled, and if all the diverse micro-organisms present in plaque samples are fully identified. Additionally, difficulties exist in repeatedly obtaining minute samples from precisely identical sites, and there are further problems associated with the early clinical detection of caries. As a result of these factors, few individuals are usually investigated in longitudinal studies.

To date, only a handful of longitudinal studies have been carried out, and the results obtained relating the development of caries with specific bacteria have been inconclusive. In some cases, an increase in the plaque levels of Strep. mutans has been shown before caries detection (Hardie et al, 1977) - a time which may correspond to caries initiation. However, other lesions have developed in the presence of only low levels of Strep. mutans (Masuda et al, 1979) and on occasions, decay has developed in the absence of detectable levels of this micro-organism (Hardie et al, 1977; Loesche & Straffon, 1979). The progression of lesions through enamel to cavitation has been associated with increases in both Strep. mutans and lactobacilli

(Ikeda, Sandham & Bradley, 1973; Hardie et al, 1977; Boyar & Bowden, 1985). Thus, the results of human longitudinal studies have so far provided little evidence for any single species as the sole pathogen in the aetiology of human dental caries.

1.2.4 Streptococcus mutans

Strep. mutans is a species of nonmotile, catalase negative, Gram positive cocci, forming short or medium chains (Newbrun, 1983). Strains of this micro-organism characteristically ferment mannitol and usually sorbitol (Michalek & McGhee, 1982). Strep. mutans was first identified and demonstrated in human carious lesions by Clarke (1924). However it was not until the animal experiments of Fitzgerald and Keyes (1960) and Fitzgerald, Jordan and Stanley (1960) into the cariogenicity of single strains of streptococci, and the reidentification of some of these cariogenic streptococci as Strep. mutans (Carlsson, 1967a; Guggenheim, 1968), that this micro-organism was investigated further.

In Table 1.3 is shown a brief summary of some of the biological features of the Strep. mutans group of bacteria. This species was originally divided into four genotypes or subspecies (Coykendall, 1974), five biotypes (Shklair & Keene, 1974; Shklair & Keene, 1976), and seven serotypes (Bratthall, 1970; Perch, Kjems & Ravn, 1974). A fifth genotype was subsequently described, and it has been proposed that these five subspecies should be elevated to species status (Coykendall, 1977; Schleifer et al, 1984). An eighth serotype has also been described (Beighton et al, 1981).

Serotype c strains are those most commonly isolated from human oral samples, followed by serotypes d and e, with others being found

Genotype	I	II	III	IV	unknown
Species	mutans	rattus	sobrinus	cricetus	ferus
Biotype	I & V	II	IV & VI	III	unknown
Serotype	c e f	b	d g h	a	c
Fermentation					
Mannitol	+ + +	+	+ + +	+	+
Sorbitol	+ + +	+	± ± -	+	+
Raffinose	+ + +	+	- - -	+	-
Melibiose	+ ± +	+	- - +	+	unknown
Ammonia from arginine	- - -	+	- - -	-	-
Growth in bacitracin	+ + +	+	+ + -	-	-
Guanosine & cytosine mole%	36-38	41-43	44-46	42-44	43-45
Cell wall carbohydrate	Glucose Rhamnose	Galactose Rhamnose	Glucose Galactose Rhamnose	Glucose Galactose Rhamnose	Glucose Rhamnose

Table 1.3 Summary of some of the differences within the Streptococcus mutans group. References: Shklair and Keene (1976); Coykendall (1977); Beighton, Russell and Hayday (1981); Newbrun (1983); Schleifer et al (1984).

only rarely (Shklair & Keene, 1974; Qureshi et al, 1977; Masuda et al, 1979; Carlsson et al, 1985). The ecology and pathogenicity of the species have been intensively examined (reviewed by Hamada & Slade, 1980; Bowden et al, 1984). Although the precise factors which determine the cariogenicity of a particular strain are as yet unknown, the factors which are thought to be important include acid production, acidurity, and the production of extracellular and intracellular polysaccharides.

Onose and Sandham (1976) reported the ability of Strep. mutans to reduce the pH of a sucrose broth culture from neutral to 4.2. The major end product of glucose fermentation is lactate, especially when glucose is present in excess. Significant amounts of formate, acetate, and ethanol are produced when glucose is limited, but if sucrose is available as the energy source, it too is converted to lactic acid (Hamada & Slade, 1980). Van der Hoeven and Franken (1982) reported the production of larger amounts of lactic acid in the dental plaque of gnotobiotic rats superinfected with Strep. mutans, when compared with those without Strep. mutans.

In 1984, Harper and Loesche demonstrated the ability of Strep. mutans to both grow in and produce acid from broth cultures with an initial pH of 5.0. Growth of strains of other species less cariogenic in animal models, was completely inhibited at this pH. In addition, Svanberg (1980), showed the presence of an increased percentage of Strep. mutans in plaque in vivo, following regular rinsing with a phosphate buffer solution at pH 3.9.

Strep. mutans is able to adhere directly to the pellicle covering of the teeth during initial plaque formation, but its ability to

adhere is weaker than that of other oral micro-organisms such as Strep. sanguis, Strep. mitior and Actinomyces spp. (van Houte, 1984). However, Strep. mutans is able to synthesise insoluble extracellular polysaccharides from sucrose, by means of extracellular glucosyltransferases. These glucans increase the adhesion between Strep. mutans cells and it is thought that they may increase adherence between other bacteria, thus promoting plaque accumulation (reviewed by Gibbons, 1983). The adherence of Strep. mutans to tooth surfaces may also involve the glucosyltransferase enzymes (Rolla, Scheie & Ciardi, 1985).

The intracellular polysaccharide synthesised and stored by most strains of Strep. mutans is a glycogen-like glucan. It is thought to contribute to cariogenicity by being the source of acid when there are insufficient exogenous sugars. Mutants of serotype c strains of Strep. mutans, which are weak in their ability to synthesise intracellular polysaccharide, show diminished cariogenic activity in rats (Tanzer et al, 1976). However, intracellular polysaccharide does not appear to be a prerequisite for caries production, as serotype d and g strains produce and metabolise little of this material, yet cause marked dental caries in experimental animals (reviewed by Hamada & Slade, 1980).

1.2.5 Lactobacillus spp.

Lactobacilli are Gram positive, usually nonmotile, nonsporing, sometimes pleomorphic rods, which divide in one plane only, without branching (Hardie, 1983). The cariogenicity of lactobacilli is thought to relate principally to their acidurity and acidogenicity, since their optimum growth pH is usually between 5.5 and 5.8 (Hardie,

1983), allowing them to multiply in the low pH of plaque and carious lesions (Newbrun, 1983). In addition, Parvinen and Larmas (1981) have also demonstrated an inverse correlation between salivary pH and the prevalence of lactobacilli in saliva.

While lactobacilli have been shown to be capable of causing caries in animals (Fitzgerald, Jordan & Archard, 1966), their frequent isolation from human carious lesions may simply be related to the aciduric nature of lactobacilli, and may not provide proof of a causal role in caries aetiology.

Early work by McIntosh et al (1922) demonstrated that strains of lactobacilli reduced the pH of glucose broths from neutral to between pH 3.4 and 2.2. Lactobacilli can be divided into two groups, depending on whether they produce more than 65% lactic acid from glucose - homofermentative, or less than 65% lactic acid and significant amounts of acetic acid, ethanol and carbon dioxide - heterofermentative (Hardie, 1983). The homofermentative lactobacilli are L. acidophilus, L. salivarius, L. casei and L. plantarum, and the heterofermentative group include the species L. fermentum, L. cellobiosus, L. brevis and L. buchneri. Those most commonly isolated from oral samples are L. casei and L. fermentum (Tilden & Svec, 1952; Rogosa et al, 1953; Davis, 1955; Hayward, 1957; Claesson & Crossner, 1985).

Lactobacilli have a relatively low affinity for tooth surfaces, and this suggests that mechanical retention, either directly or indirectly via incorporation of cells in food, may play an important role in their colonisation of teeth (van Houte, Gibbons & Pulkkinen, 1972). It is also possible that lactobacilli may aggregate with other

micro-organisms in the formation of plaque, and that this coaggregation may be mediated by salivary agglutinins (Rundegren, 1986).

In addition to the correlations which have been shown between salivary lactobacillus levels and caries prevalence, several studies have investigated the possible relationships between lactobacilli and dietary carbohydrate consumption. Varying results have been obtained by different workers. In vivo restrictions in carbohydrate intake have been assosciated with reductions in lactobacillus counts (Jay, 1947; Karjalainen et al, 1987), but Krasse (1954a) found no difference in the distribution of lactobacillus counts between patients on a caries inactive diet (low carbohydrate), when compared with those on a caries active diet (higher or more frequent carbohydrate) in the Vipeholm study. Crossner (1984) demonstrated a small increase in salivary lactobacillus counts, associated with a large increase in frequency of sugar consumption, and Stecksen-Blicks (1987) showed a weak correlation between lactobacillus counts and both intake of dietary sugars and meal frequency, in a group of 13-year-old children.

1.2.6 Candida spp.

The genus Candida are yeasts which produce pseudohyphae on certain laboratory media (Odds, 1979). Candida have been reported to be present in between 10% and 50% of clinically healthy mouths (Young, Resca & Sullivan, 1951; Odds, 1979; Arendorf & Walker, 1980) and Candida albicans is by far the most frequently isolated oral species, although others, including Candida tropicalis are also found (Budtz-Jorgensen, Stenderup & Grabowski, 1975; Odds, 1979).

Candida spp. have been shown to be capable of producing acid and

of decalcifying dental enamel *in vitro*, when incubated in a glucose broth (Fosdick & Hansen, 1936), and can initiate growth and survive well at pH 5.0 (van Houte, 1980). They have also been isolated from carious dentine in children (Hodson & Craig, 1972), and associations have been shown between salivary levels of candida and caries prevalence (Krasse, 1954b; Banoczy *et al*, 1983).

However, *Candida* spp. are not usually isolated from human dental plaque (Theilade *et al*, 1974), and the frequency of candidal carriage has been reported to be inversely correlated with salivary pH (Young *et al*, 1951; Parvinen & Larmas, 1981). The association between candida and dental caries is uncertain, but it is likely that the relationship reflects the acidurity of these organisms.

1.2.7 Veillonella spp.

The genus *Veillonella* consists of Gram negative, obligately anaerobic cocci, which are among the most numerous of oral bacteria and comprise between 5% and 10% of the cultivable flora of saliva (Hardie, 1983). On the basis of nutritional, biochemical and serological studies, Rogosa (1965) categorised veillonella as follows: *Veillonella parvula* with three subspecies, and *Veillonella alcalescens* with four subspecies. However, Mays *et al* (1982) used deoxyribonucleic acid homology studies to demonstrate seven distinct species of veillonella: *V. parvula*; *V. atypica*; *V. dispar*; *V. rodentium*; *V. ratti*; *V. criceti*; *V. caviae*. These were based on Rogosa's subspecies, but with two of the subspecies combined to form a single species (*V. parvula*), and with the addition of a new species (*V. caviae*).

The finding that veillonella could metabolise lactic acid to produce propionate, acetate, formate, carbon dioxide and hydrogen (Foubert & Douglas, 1948; Distler & Kroncke, 1981a), suggested that these micro-organisms might provide a defence against the development of dental caries. Furthermore, as described in Section 1.2.2, Mikx et al (1972) have demonstrated a reduced caries incidence when Veillonella spp. are present in gnotobiotic rats. However, attempts to correlate salivary levels of veillonella with caries prevalence have produced conflicting results. Mazzarella and Shklair (1960) showed a direct correlation, whereas Sims and Snyder (1958) found no such relationship.

1.3 Dietary factors in the aetiology of dental caries.

1.3.1 Effects on plaque microbiology

Evidence from many sources strongly suggests a close relationship between dental caries and dietary carbohydrate. Miller (1883) showed, that when salivary micro-organisms were incubated with bread and sugar, acid was produced, and that the dentine of artificial cavities prepared in whale teeth was softened.

Stephan (1940) demonstrated that in vivo, human plaque pH may fall as much as 3 units following a glucose or sucrose rinse. Neff (1967) found that a sucrose rinse produced the greatest drop in plaque pH, while smaller reductions were produced by maltose, cooked starch and raw starch, in that order. One-day-old plaque produces mostly lactic acid in response to an exogenous carbohydrate challenge such as sucrose, whereas older, seven day plaque produces, in addition, considerable amounts of formic acid. The major end-product

of resting plaque at neutral pH is acetate (reviewed by Geddes, 1984).

Additionally, sucrose is required by oral streptococci in order to synthesise extracellular polysaccharides. Two groups of enzymes which utilise the glucosyl parts of sucrose molecules have been described (reviewed by Chassy, 1983). These are dextransucrases, which produce soluble dextrans, and glucosyltransferases, which form insoluble glucans. In addition, there are fructosyltransferases which synthesise soluble and insoluble fructans using the fructosyl portions of sucrose. As described in Section 1.2.4, the insoluble glucans may be associated with bacterial adherence.

Furthermore, although the quantity of plaque produced is unaffected by the level of dietary sucrose, the microbial density of such plaque is increased by greater sucrose consumption (Folke et al, 1972; Staat et al, 1975). Additionally, changes in the proportions of plaque bacteria have been shown to occur in people with differing sucrose exposure. Thus, lower counts of streptococci and lactobacilli were found by Littleton, McCabe and Carter (1967) in the plaque of individuals fed by stomach-tube, when compared with those fed orally. Similarly, diets high in sucrose have been shown to increase the proportions of Strep. mutans and lactobacilli (Folke et al, 1972; Dennis et al, 1975). However, Skinner and Woods (1984) were unable to demonstrate an increase in the proportion of glucan-producing streptococci in the dental plaque of students on a high sucrose diet. It is possible that any increase in Strep. mutans levels associated with greater sucrose consumption may be a consequence of the ability of this micro-organism to tolerate high sucrose concentrations (Sims, 1985).

1.3.2 Animal experiments

Many animal experiments have been carried out in an attempt to determine the cariogenicity of different dietary ingredients (reviewed by Shaw, 1983; Tanzer, 1986). Caries has been shown to occur in conventional albino rats fed with starch as the only carbohydrate source, but the caries activity was very low. A much higher caries activity was found when animals were fed diets containing monosaccharides or disaccharides, such as glucose, fructose or sucrose (McClure, 1945; Cox et al, 1948), but only when the diet was fed orally. Animals which received a very high sugar diet by intubation developed no caries (Kite, Shaw & Sognnaes, 1950). In addition, in a rat model, Grenby and Hutchinson (1969) demonstrated that sucrose was slightly more cariogenic than glucose or fructose.

Studies have also been conducted to assess the cariogenicity of various foods containing combinations of ingredients (reviewed by Shaw, 1983). These studies have produced varying results which are related to the species of animal studied, and the tooth surfaces examined. However, in general, such foods as potato chips, salted peanuts and skimmed milk powder were shown to be of low cariogenicity, whereas raisins, chocolate, biscuits, sponge cakes and caramels were more cariogenic (Navia & Lopez, 1983; Harper et al, 1985; Grenby & Saldanha, 1985).

1.3.3 Human studies

The association between dietary sugar and dental caries is shown by the observation that the low prevalence of caries, which existed in England until the Middle Ages, was associated with a low sugar consumption. Sugar consumption in England increased from virtually

zero in the seventeenth century, to approximately 90 lbs per person per year in the late nineteenth century, and this change was accompanied by an increase in dental caries (Moore, 1983). Similarly, restrictions in sugar availability during World War II, were associated with reductions in caries prevalence in European children (Sognnaes, 1948).

The Vipeholm study (Gustafsson et al, 1954) demonstrated, in a group of institutionalised adults, that caries incidence could be increased by artificially increasing the frequency of intake of refined carbohydrate, particularly sucrose in a sticky form. The acidogenicity, and therfore possibly the cariogenicity of foods, has been shown to be affected by factors other than consistency. These include the length of time food is retained in the mouth, and the pattern of sugar intake with reference to other less cariogenic components of a meal (Edgar et al, 1975; Rugg-Gunn et al, 1975). Furthermore, in an epidemiological study, Rugg-Gunn et al (1984) showed significant correlations between caries incidence, and both weight and frequency of sugar intake.

1.4 Host factors in the aetiology of dental caries

1.4.1 Salivary factors

The composition of saliva which coats and surrounds the teeth can also modify caries development. Saliva is a complex secretion produced by the large paired parotid, submandibular and sublingual salivary glands, and by many minor salivary glands. The composition and volume of saliva vary with flow rate, time of day collected, and the nature and duration of stimulation. Variation in composition

also occurs in secretions from different glands (Jenkins, 1978). Unlike the factors described in Sections 1.2 and 1.3, the presence of saliva is necessary for protection against dental caries, and this is demonstrated both by animal experiments and human studies.

Complete surgical removal of the major salivary glands in animals has been shown to allow rampant caries to develop (Weisberger, Nelson & Boyle, 1940), even when the animals consume a diet of low cariogenicity. Similarly, humans who experience a prolonged severe decrease in salivary flow due to pathological conditions such as Sjogren's syndrome, salivary gland irradiation, or drug therapy, usually have a higher incidence of dental caries than would otherwise be expected (Dreizen & Brown, 1976; Newbrun, 1983; Talal, 1987). Such effects can be shown in cases of extreme flow reduction, but correlations between caries incidence and salivary flow rate have not been shown in normal populations (reviewed by Sweeney, 1979), except in a very large study by Shannon and Terry (1965).

There are a number of reasons why saliva protects against carious attack. Saliva acts as a lubricant, and aids the elimination, of both potentially cariogenic food and of bacteria from the mouth (Jenkins, 1978; Mandel, 1987). It also contains several buffer systems, which tend to maintain a constant salivary pH. Thus, the decreased plaque pH which occurs after a sucrose rinse gradually increases again, partly via loss of acid by outward diffusion, partly by conversion of lactic acid to weaker acids, and partly by the entry of highly buffered saliva into the plaque (Jenkins, 1978). The pH returns to normal after approximately 2 hours (Stephan, 1940). Ericsson (1959) reviewed 21 epidemiological studies most of which demonstrated

negative correlations between salivary buffering capacity, and dental caries. This finding has been confirmed by the more recent investigations of Agus and Schamschula (1983), and Pienihakkinen et al (1985).

Saliva also contains sufficient concentrations of calcium and phosphate ions for the hydroxyapatite of enamel to be insoluble at neutral pH. However, at a critical pH, usually between 6.5 and 5.0, saliva will cease to be saturated, and tooth substance will dissolve (Jenkins 1978; Larsen & Bruun; 1986).

In addition, saliva also possesses antibacterial functions (reviewed by Newbrun, 1983; Nikiforuk, 1985). These include non-specific mechanisms such as the enzymes lysozyme and lactoperoxidase, and the iron binding protein lactoferrin. Specific humoral mechanisms are also present in saliva.

Lysozyme rapidly destroys some bacteria, by destabilising their cell membranes (Mandel, 1987). Alone, however, it does not lyse pure cultures of the predominant bacteria of the oral cavity (Newbrun, 1983). Lactoperoxidase oxidises thiocyanate in the presence of hydrogen peroxide, and is known to be inhibitory, in vitro, towards some bacteria, including certain Lactobacillus, Streptococcus and Actinomyces species (Tenovuo & Pruitt, 1984). However, the significance of this antimicrobial system in vivo is unclear, although in a small clinical trial Rotgans and Hoogendoorn (1979) reported that a toothpaste containing amyloglucosidase and glucose oxidase, which activate the lactoperoxidase system, significantly reduced plaque accumulation. The bacteriocidal effect of lactoferrin is due to its strong affinity for iron, which is made unavailable as an essential bacterial nutrient. In vitro, lactoferrin has been shown to be

antagonistic to Strep. mutans, but the practical implications of this are not known (Nikiforuk, 1985).

According to Roitt and Lehner (1983), the surfaces of the teeth can be divided into the gingival and salivary domains, as regards specific immune reactions. The gingival domain consists of the cervical and approximal tooth regions, and the source of immune activity is the gingival crevicular fluid. Here serum IgG, IgM and IgA are present, as are components of the complement system. Cellular components are also present, including polymorphonuclear leucocytes, T- and B-lymphocytes and macrophages. The antimicrobial action of the immune activity of this domain may function by inhibiting microbial adherence, and assisting in the opsonisation, lysis, phagocytosis and killing of micro-organisms.

In the salivary domain, which consists of the occlusal and bucco-lingual tooth surfaces other than the cervical regions, the source of immune activity is the salivary glands. The major immunoglobulin is secretory IgA, and it is thought that its mechanism of action is to inhibit microbial adherence. Most of the polymorphonuclear leucocytes which are present in mixed saliva originate in the gingival fluid, and their function is uncertain (Roitt & Lehner, 1983).

Attempts have been made to reduce dental caries in animals, by stimulating antibody production by vaccination against Strep. mutans. Most of these studies (reviewed by Brandtzaeg, 1984) have produced reductions in dental caries. However, the source of the increased antibody, whether from saliva or gingival exudate, depends on such factors as the animal model used, and the route of vaccination.

Ethical problems and practical hazards have so far prevented the

use of anti-caries vaccines in man. Only a certain percentage of dental caries is probably due to Strep. mutans and consequently a Strep. mutans vaccine would only provide partial protection (Krasse & McBride 1984). In addition, the possibility of antigenic drift within an individual Strep. mutans population might also reduce the effectiveness of an anti-caries vaccine (Bratthall & Gibbons, 1975; Beem, Clark & Bleiweis, 1985). There is also doubt about the extrapolation of the results of animal experiments to man (Sims, 1985). Furthermore, there has been concern about the possibility of damage to heart tissue caused by anti-Strep. mutans antibodies which, in rabbit antisera, have been shown to be cross-reactive with human heart muscle, although no such side-effects have been reported in experiments in monkeys (reviewed by Scully, 1981).

In conclusion, there are several naturally occurring factors in saliva which could play a role in preventing caries, but the significance of some, particularly the antibacterial effects, is unclear. Generally, the salivary antibacterial factors appear to be more effective against potential exogenous pathogens than against oral commensal organisms, and their function may be to maintain an ecological balance (Nikiforuk, 1985).

1.4.2 Dental factors

Susceptibility to caries also depends on several dental factors, which include tooth morphology and the composition of the teeth themselves. Variation in arch form may also affect caries susceptibility, with malocclusion favouring the development of carious lesions (Adler, 1956; Hixon, Maschka & Fleming, 1962), and spacing tending to reduce caries susceptibility (Parfitt, 1956). However, it

is thought that the effect of malocclusion on caries susceptibility is small in comparison with other factors (Proffit, 1986), and any relationship is further complicated by individual perception of satisfactory occlusion, which may be related to the desire to maintain the dentition (Horup, Melsen & Terp, 1987).

Dental caries does not affect all parts of the dentition equally. Berman & Slack (1973) confirmed the clinical observation that the occlusal surfaces of teeth are the most susceptible to carious attack. These surfaces contain pits and fissures, in which micro-organisms and food debris readily impact (Newbrun 1983). The approximal surfaces, of posterior teeth in particular, are caries susceptible. However the extent varies, depending partly on the age at which a contact point, and therfore a potential stagnation area, is formed. Buccal and lingual surfaces are the least caries susceptible, with the exceptions of buccal pits of lower molars, palatal fissures of upper molars, and palatal pits of anterior teeth (Berman & Slack, 1973).

The initial site of carious attack is usually the enamel surface, although in root caries, the initial site is cementum. The earliest macroscopic evidence of enamel caries is the appearance of a small shiny white region, which then becomes opaque and which may subsequently become stained. Acid produced by plaque bacteria causes demineralisation in the sub-surface enamel, with the surface remaining intact. Such early enamel lesions may remineralise (ten Cate & Arends, 1977; Silverstone, 1977; Creanor et al, 1986), or may progress, with changes occurring in dentine and pulp. These changes include the deposition of reactionary and peritubular dentine, or the demineralisation of dentine at the amelodentinal junction. These early lesions usually progress slowly, until the enamel surface is

destroyed, and a cavity is formed. Bacteria may then invade the enamel and dentine, eventually reaching the pulp (Silverstone et al, 1981; Cawson, 1984).

Theoretically, variations in the chemical structure of enamel are associated with varying rates of acid diffusion, which may be an important factor in the development of a lesion. Groeneveld, Purdell-Lewis & Arends (1975), demonstrated that shallower artificial carious lesions developed in enamel, the greater its mineral content. Similarly, Shellis (1984) showed that in vitro, artificial caries developed more slowly in permanent teeth, than in deciduous teeth, the latter being less highly mineralised and therefore more porous. However, less highly mineralised regions of permanent teeth, such as cracks or lamellae extending from the enamel surface, are not always or obviously associated with increased caries susceptibility (Weatherell, Robinson & Hallsworth, 1984).

In the early part of this century, it was observed that in some geographical areas, many individuals had mottled enamel, and that this condition was associated with low levels of dental decay (McKay, 1916; McKay, 1928). Later, Churchill (1931) reported that the water which supplied these areas contained high levels of fluoride, in the region of 7ppm. To date, the most effective method of reducing dental caries is to incorporate fluoride into the apatite crystal lattice, thus increasing the resistance of enamel to acid dissolution (Silverstone et al 1981; Weatherell et al, 1984). Fluoride which is available systemically may be incorporated during enamel formation, and topically available fluoride is also able to enhance the remineralisation of carious enamel (Murray & Rugg-Gunn, 1982).

Fluoride may be given systemically or by topical application, in

a variety of preparations, for example in the water supply, in tablets, toothpastes, rinses, gels or varnishes. There have been many clinical investigations into the optimum fluoride dose necessary to obtain the maximum reduction in caries activity. Most of these studies (reviewed by Murray & Rugg-Gunn, 1982), have shown caries reductions of between 30% and 70%, depending on such factors as the vehicle and frequency of fluoride administration, and the age of the individuals studied.

1.5 Detection and diagnosis of dental caries.

As previously described in Section 1.4.2, coronal dental caries occurs at three distinct regions of both deciduous and permanent teeth. The methods of detection and diagnosis vary for these three areas, and have been reviewed by Kidd (1984).

1.5.1 Pits and fissures

Pits and fissures occur on the occlusal surfaces of premolar and molar teeth, and sometimes on the buccal surfaces of lower molars and the palatal surfaces of upper molars and incisors. Carious lesions at these sites may be detected visually or by the use of a dental probe. However, it has been shown that the use of a sharp dental probe may accelerate the progression of incipient carious lesions (Bergman & Linden, 1969; van der Laan-van Dorp, Exterkate & ten Cate, 1986). In addition, Loesche, Svanberg and Pape (1979), demonstrated that Strep. mutans could be transmitted from tooth to tooth within a mouth, by means of a dental explorer.

Occlusal caries may also be visible on bitewing radiographs,

although King and Shaw (1979) reported that this method of detection was considerably less sensitive than clinical examination; radiographs detecting only 33.2% of carious lesions discovered using a dental probe.

The use of an electronic method of caries detection, based on the different electrical resistances of enamel and caries, has also been proposed. One such study found this method to be more sensitive and consistent than examination with a dental explorer (White, Tsamtsouris & Williams, 1981).

1.5.2 Approximal surfaces

Approximal lesions may be difficult to diagnose visually, particularly at an early stage, and radiographs or transillumination may be used to assist detection.

In a paper by Trithart and Donnelly (1950), 67% of approximal caries in first permanent molars could only be detected by means of radiographs. Similarly, in a study of deciduous molars in 5 to 7-year-olds, Murray and Majid (1978) reported that clinical examination failed to diagnose over 67% of approximal carious lesions which were noted using bitewing radiographs.

However, the danger of increasing the existing long-term exposure of individuals to low levels of ionising radiation by dental radiography must be considered (reviewed by Myers, 1984). This may cause genetic defects in the offspring of patients who have experienced an increased exposure, or somatic injury may occur in such patients themselves, and may lead to cancer induction. The desirability of reducing dental radiography, and the problem of overlapping contact points resulting in surfaces which are deemed

unreadable on radiographs, has led to attempts to detect caries using fibre optic transillumination (FOTI). The conclusions of such studies (reviewed by Kidd, 1984) have been varied, although all have resulted in lower caries scores than were found with radiographs. The highest claimed detection level was reported by Mitropoulos (1985) who, using FOTI, detected 85% of the dentinal lesions found on bitewing radiographs, albeit this was a cross-sectional study, which involved only 50 individuals and 1042 surfaces. However, later longitudinal work, on over 2000 Scots teenagers failed to uphold these expectations, only 17% of enamel lesions and 48% of dentinal lesions being detected correctly by FOTI, from over 52,000 posterior approximal surfaces (Stephen et al, 1987).

1.5.3 Buccal and lingual smooth surfaces

The buccal and lingual smooth surfaces are the sites least commonly affected by caries, and decay can usually be detected visually with little difficulty.

1.6 Reasons for caries prediction

1.6.1 Individual

If methods of caries prediction are accurate at an individual level, then individual treatment may be planned with reference to the amount of dental caries expected. Factors such as the frequency of dental attendence required, the need for preventive dentistry or dietary advice, and whether advanced restorative treatment is justified, would be affected by the results of predictive tests (Newbrun,1983).

1.6.2 Community and manpower planning

Benefit to the community could result from accurate caries prediction tests, as preventive dental programmes could be focussed on those at greatest caries risk. The numbers involved in such programmes could thus be reduced, and the cost of community prevention would be decreased (Carlos, 1978).

Furthermore, accurate predictive tests would enable estimates to be made of likely future caries experience in a community, and thus future dental manpower requirements might be estimated.

1.6.3 Clinical trials

With falling caries rates, clinical trials of anticaries therapeutic agents now include large numbers of individuals who are not caries susceptible, and in whom the therapeutic agent can therfore produce little or no benefit. The existence of such individuals means that large numbers of subjects must be involved in clinical trials to ensure that a caries preventive effect may be shown. A caries predictive test which could identify those not at risk, even on a group basis, would thus enable the number of clinical trial subjects to be reduced. In one such trial, Glass, Peterson and Bixler (1983) identified 41% of enrolled subjects who had a caries prevalence of zero at outset. Subsequently, no treatment effect was demonstrable in that group, although a significant effect was shown for the trial as a whole.

In addition, an accurate caries predictive test which measured factors closely related to the development of the caries process, might enable the length of clinical trials to be reduced. If the use of a therapeutic agent could be shown to affect the result of such a

test, the need to await the development of caries would be avoided (Carlos, 1978).

1.7 Previous studies involving caries prediction

1.7.1 Epidemiological factors

For many years, the following clinical factors have been used to predict future caries activity: previous caries experience, which can be expressed as the number of Decayed, Missing, or Filled Surfaces or Teeth (DMFS or DMFT); the number of susceptible surfaces at risk; the age, sex, and dental attitudes of an individual. Currently, these are the criteria which are usually applied in treatment planning, in an attempt to assess caries risk at an individual level.

On a group basis, varying degrees of association have been shown between past caries experience and caries increments. Downer (1978) reported a correlation coefficient of 0.36 between DMFS and subsequent three year caries increment, in a group of children initially aged 11 and 12. Birkeland, Broch and Jorkjend (1976) reported a higher correlation of 0.5 between DMFS in a group of 7-year-old children and subsequent 8 year caries increments. However, these correlation coefficients did not reach the 0.9 - 1.0 level which Snyder et al (1963) considered necessary, before caries could be predicted at an individual level. They did reach the 0.2 - 0.4 range, indicating a significant association on a group basis only.

Nevertheless, several studies have shown that initial caries levels were more highly correlated with subsequent caries incidence, than were any of the salivary or habit variables which were investigated. Klock and Krasse (1979) found that the number of

decayed and filled surfaces (DFS) had a higher correlation with 2 year caries increment (0.259) than had any of the salivary or microbiological variables studied. They also demonstrated a higher correlation of 0.346 between the numbers of incipient smooth surface lesions and the same 2 year caries increment. Honkala et al (1984) also concluded that initial caries, in the form of the DMFT score, was a better predictor of dental caries increment than were the lactobacillus count, number of decayed teeth, buffering capacity, toothbrushing frequency or consumption of sweets.

However, correlation coefficients allow comparisons between caries increments and only one variable. The use of multiple regression analysis techniques allows the evaluation of associations between caries increments and more than one other independent variable. Thus, it is possible that a combination of predictive factors might allow prediction at an individual level. In four clinical studies extending over periods of 20 to 48 months (reviewed by Heifetz, 1978), the proportion of the variance in caries increment that could be explained by all variables investigated (the age of the children, sex, initial DMFS, and number of sound surfaces initially) varied between 12% and 46%, with the major part of the variance explained by the initial DMFS in each study. However, in a fifth study lasting 8 months, only 1% of the variance was explained by all variables, although once again, most of this could be determined by the initial caries experience.

1.7.2 Salivary Lactobacillus spp. counts.

The first bacteria to be used in the assessment of caries susceptibility were lactobacilli, and their use has been reviewed by Sims (1970), Ellen, (1976) and Bratthall and Carlsson (1986). Rodriguez (1931) suggested that by quantifying salivary lactobacilli, the effectiveness of caries control methods might be determined, and he developed a semi-selective agar-serum medium, on which these micro-organisms could be enumerated. Hadley (1933) subsequently used a tomato-peptone agar at pH 5.0, and compared counts of lactobacilli in a group of caries active children, with another group who developed no new decay over an 18 month period. Although the results were not analysed statistically, the counts from the caries active group were found to be consistently higher than those in the caries inactive group.

Attempts to allow a more rapid estimation of the numbers of salivary lactobacilli, were made by Snyder (1940), who developed shake tubes of a selective carbohydrate medium with a pH indicator. The rapidity of indicator change was shown to be correlated with the number of lactobacilli in the inoculum. Subsequent use of Snyder tests and lactobacillus counts (Snyder *et al*, 1963), demonstrated statistically significant correlation coefficients of 0.2 to 0.4, between these tests and caries increments.

Marlay (1970) divided 115 girls into four groups, on the basis of 2 year DMFS increment, and generally found complete agreement for 30.4%, and moderate agreement for 45.2% of individuals, when the groups were compared with four categories of lactobacillus counts on Rogosa SL agar (Rogosa, Mitchell & Wiseman, 1951). However, using the same medium, Klock and Krasse (1979) were unable to demonstrate a

significant correlation between counts and two year caries increments.

The "Dentocult" dip-slide, developed by Larmas (1975), is yet another method of estimating salivary lactobacillus levels. Statistically significant associations, on a group basis, have been shown between caries increments and lactobacillus counts using "Dentocult" dip-slides (Crossner, 1981; Pienihakkinen et al, 1984; Honkala et al, 1984; Ashley et al, 1985).

1.7.3 Salivary Streptococcus mutans counts

Investigations into the relationship between Strep. mutans counts and caries prediction have been reviewed by Krasse (1985) and Bratthall and Carlsson (1986). Such studies followed the development of selective media, such as that of Carlsson (1967b) containing sulphadimetine, on which these micro-organisms could be enumerated. Woods (1971) reported a significant relationship between the proportion of Strep. mutans in pooled plaque samples, and one year caries increments. A similar association was shown, on a group basis, for plaque samples at specific sites and the development of caries by Kohler, Pettersson and Bratthall (1981), and Burt et al (1983). In both studies mitis salivarius bacitracin agar (Gold, Jordan & van Houte, 1973) was used for the isolation of Strep. mutans. Kohler et al (1981) also showed a relationship between the levels of Strep. mutans in mixed salivary samples, and the number of tooth surfaces infected with this micro-organism.

As the collection of a saliva sample is more convenient than the collection of many plaque samples, the associations between salivary

Strep. mutans levels and caries risk have also been investigated. Klock and Krasse (1979) and Ashley et al (1985) showed significant correlations of 0.209 and 0.23 respectively, between the levels of Strep. mutans in saliva and caries increments over two years. Stecksen-Blicks (1985) also showed that a group of chidren with salivary Strep. mutans counts greater than 10^5 per millilitre and Lactobacillus spp. counts greater than or equal to 10^5 per millilitre had a mean 1 year increment between three and four times higher than that of those children with one or both microbiological counts below these levels. However, van Palenstein Helderman et al (1986) did not obtain significant correlations when salivary Strep. mutans counts using trypticase, yeast extract, cystine, sucrose, bacitracin agar (van Palenstein Helderman, Ijsseldijk & Huis in't Veld, 1983) were compared with 2 year caries increment in a group of children, initially aged 7 years.

Other methods of estimating Strep. mutans levels have also been described. These include several selective broth media (Matsukubo et al, 1981; Walter & Shklair, 1982; Kalfas, Edwardsson & Birkhed, 1985), and a slide scoring method (Alaluusua et al, 1984). The uses of these methods in relation to caries prediction have yet to be reported.

1.7.4 Salivary Candida spp. counts

Although, as described in Section 1.2.6, associations have been shown between salivary candidal levels and caries prevalence, there have been few reports of investigations into any associations between counts of Candida spp., and caries incidence. However significant differences in caries increments have been shown between groups of children with high candidal counts, when compared with those with low

or zero counts (Pienihakkinen et al, 1984; Pienihakkinen, Scheinin & Banoczy, 1987).

1.7.5 Salivary Veillonella spp. counts

Several semi-selective media for this genus have been described. These contained high quantities of lactate (Douglas, 1950), with various other inhibitory agents, including streptomycin (Rogosa, 1956), vancomycin (Rogosa et al, 1958), brilliant green (Sims & Snyder, 1958) and teepol (MacFarlane, 1977).

Attempts to correlate salivary veillonella levels with caries prevalence have produced conflicting results, as described in Section 1.2.7. To date there have been no reports of any longitudinal investigations into relationships between salivary levels of these bacteria and caries increments. However, Boyar and Bowden (1985) reported a positive association between the percentage of veillonella in plaque, and the progression of incipient carious lesions.

1.7.6 Salivary buffering capacity

Possible relationships between high caries increments and low salivary buffering capacity have been investigated in several studies. Marlay (1970) found statistically significant differences in DMFS increments over two years, in girls with high buffering capacity, compared to those with low values. Similarly Honkala et al (1984) and Ashley et al (1985) demonstrated statistically significant correlations of 0.20 and 0.26 respectively between caries increments and buffering capacity. On the other hand, no significant correlations between buffering capacity and caries incidence were

shown by Klock and Krasse (1979), Woltgens et al (1984), or Pienihakkinen et al (1985).

1.7.7 Salivary inorganic ions

To date there have been few investigations of the relationships between salivary concentrations of inorganic ions and caries increments. However Woltgens et al (1984) found no significant correlations between the initiation of new carious lesions over a six month period, and salivary pH or concentrations of magnesium, calcium or phosphate ions. Similarly, Ashley et al (1985) reported no significant correlations between two year caries increments and salivary levels of calcium or phosphate. Klock and Krasse (1979) found no correlation between salivary pH and two year caries incidence.

1.7.8 Dietary characteristics

Since the interventional Vipeholm study (Gustafsson et al, 1954), which demonstrated the importance of the quantity, frequency and method of sugar consumption in relation to caries development, a few observational studies such as that of Rugg-Gunn et al (1984), have been carried out, in an attempt to identify risk factors in the general population. Using repeated diet diaries, this investigation demonstrated low but statistically significant correlation coefficients of 0.143 and 0.099 between two year caries increments and the weights of sugars eaten and frequencies of sugar consumption, respectively. Similarly, Wilson et al (1985) found significant correlations with two year caries increments, and the total number of food intakes, number of between meal intakes and the amount of between

meal sugar consumed (0.247, 0.295 and 0.269 respectively), using both diet histories and diet diaries. Stecksen-Blicks (1987) also investigated the correlations between one year caries increment and both sucrose consumption and meal frequency in two groups of children. Low, but significant correlations of 0.214 ($p < 0.05$) were found for a group of 13-year-old children, but not for a group of 8-year-olds.

1.7.9 Conclusions

From the preceeding review of the literature dealing with the assessment of caries activity, it is clear that knowledge in a number of basic areas is incomplete.

While there have been many investigations using different caries predictive techniques, most of the studies are not directly comparable as different methods of assessing both caries and predictive factors have been used. For instance, caries has been assessed as both DMFS (Birkeland et al, 1976) and DMFT (Honkala et al, 1984), while Klock and Krasse (1979) also included incipient smooth surface lesions.

Microbiological estimations have been made using various culture media. Both mitis salivarius bacitracin agar (Klock and Krasse, 1979) and trypticase, yeast, cystine, sucrose, bacitracin (TYCSB) agar (van Palenstein Helderman et al, 1986) have been used for Strep. mutans counts. When comparisons have been performed, there have been disagreements as to the preferred medium, van Palenstein Helderman et al (1983) choosing TYCSB agar; Goll (1984) selecting mannitol, sorbitol, fuchsin, azide agar and Beighton (1986) opting for mitis salivarius bacitracin agar. Snyder test agar, Rogosa SL agar and Dentocult dip-slides have all been used to estimate lactobacillus

levels (Snyder et al, 1963; Klock & Krasse, 1979; Stecksen-Blicks, 1985). Studies comparing these media have suggested that results on Rogosa SL agar correlate well with those on Dentocult dip-slides, but less well with those from Snyder tests (Crossner & Hagberg, 1977; Birkhed, Edwardsson & Andersson, 1981).

The ages of subjects studied have also differed; Klock and Krasse (1979) studied a group of children whose ages ranged from 9 to 12 years, while Stecksen-Blicks (1985) investigated two groups of children (one group aged 8 years, the other 13-year-olds) and van Palenstein Helderman et al (1986) examined a group of 7-year-olds. The length of the predictive period has also differed, but has usually been one, two or three years.

Combinations of two salivary microbiological variables have also been investigated, either Strep. mutans and Lactobacillus spp. (Klock & Krasse, 1979; Stecksen-Blicks, 1985), or Lactobacillus spp. and Candida spp. (Pieninhakkinen et al, 1987), but all three factors have not been investigated in the same study. In addition, salivary levels of Veillonella spp. have not been reported as caries predictors.

A range of statistical methods has also been employed to analyse the results of these studies. The methods have included correlation coefficients (Snyder, 1963; Klock & Krasse, 1979), multiple regression analysis (Honkala et al, 1984), and the measurement of the sensitivity, specificity and predictive values of different tests at particular threshold values (Stecksen-Blicks, 1985). In spite of the above, only significance at a group level has been achieved. Even when the effects of a few factors have been combined, prediction on an individual basis has not yet proved possible.

1.8 Aims and design of this study

The main aim of this thesis was to select predictive tests and determine whether it was possible to achieve prediction at an individual level by using a combination of a range of tests.

Initially, it was intended to select the best microbiological caries predictive tests by investigating a number of the many culture methods available (Chapter 2) and also some factors associated with sampling which could affect subsequent microbial counts, for example the effect of recent food consumption or sample storage (Chapter 3).

The microbiological tests chosen were then used to investigate a group of adolescent children who were participating in a large clinical dentifrice trial (Chapter 4). It was intended to study any associations between the laboratory tests used, and both caries prevalence and caries incremental data (Chapter 5). In addition the possibility that combinations of predictive tests may be of greater value than single tests, both at a group or individual level, was investigated.

Finally, it was intended to apply and assess the use of the predictive database, obtained from the large group of children studied in the dentifrice trial, to another cohort (Chapter 6).

Thus, the experiments described in this thesis were designed to obtain data which would improve understanding of the factors involved in caries prediction from microbiological tests.

CHAPTER 2

MICROBIOLOGICAL METHODS

2.1 Introduction

Since a number of possible methods and media exist for use in caries predictive tests (see Section 1.7), it was decided to carry out preliminary experiments to determine which were the most appropriate for this clinical trial.

The bacteria which have been studied most intensively in caries prediction are Strep. mutans and lactobacilli. In addition, it was decided to investigate Veillonella spp. because, as discussed in Section 1.2.7, in vitro studies have shown that veillonella may protect against carious attack.

As mixed saliva is more easily collected than plaque, a decision was made to investigate salivary samples. Furthermore, Denepitiya and Kleinberg (1982) have shown that the microbial compositions of salivary sediment and of pooled dental plaque are similar, and other studies have shown significant correlations between plaque and salivary Strep. mutans levels (Kohler et al, 1981; Scheie, Selikowitz & Arneberg, 1984; Togelius et al, 1984; Schaeken, Creugers & van der Hoeven, 1987).

Since several methods have been reported for isolating and enumerating Lactobacillus spp., Strep. mutans and Veillonella spp. from saliva, a series of experiments was performed to identify the most appropriate selective medium for the isolation and enumeration of each of these organisms. The first series of experiments investigated the ability of known strains to grow on different selective media,

blood agar being used as a control. Secondly, the media were inoculated with salivary samples, and comparisons made of their ability to: (a) select the organism under study and (b) inhibit the growth of other organisms.

2.2 Materials and methods

2.2.1 Comparisons between Rogosa and M.R.S. agars for the isolation and enumeration of strains of Lactobacillus spp.

Bacterial strains

Details of the nine strains of lactobacilli which were used, are shown in Table 2.1. They consisted of five type cultures and four fresh isolates.

Isolation of fresh strains

The fresh isolates were obtained from four different individuals, and were isolated either on Columbia agar with 7.5% horse blood (blood agar) or on Sabouraud dextrose agar, although all grew on Rogosa SL agar. Gram positive bacilli which were catalase negative, and grew on Rogosa SL agar were identified using the API 50 CHL system (API System S.A., Montalie-Vercieu, France). The identities of the type strains were also checked using this system.

Maintenance of cultures

All strains were stored in freeze-dried ampoules. When required for use, strains were re-isolated on blood agar, and incubated for 2 days at 37°C in a Qualitemp 80 MI CO_2 incubator (LTE, Oldham, England), in an atmosphere containing 5% carbon dioxide in air. Cultures were then maintained at 4°C until required, and fresh

Organism	Reference No.	Source
Lactobacillus casei	NCIB 6375	National Collection of Industrial and Marine Bacteria Ltd.
Lactobacillus casei	NCTC 10302	National Collection of Type Cultures
Lactobacillus fermentum	NCTC 14932	National Collection of Type Cultures
Lactobacillus casei	MUCOB 254	Manchester University Dept. of Microbiology Collection of Bacteria
Lactobacillus fermentum	MUCOB 260	Manchester University Dept. of Microbiology Collection of Bacteria
Lactobacillus plantarum	P642	palate
Lactobacillus casei	0112	saliva
Lactobacillus casei	76	carious dentine
Lactobacillus casei	18	carious dentine

Table 2.1 Strains of *Lactobacillus* spp. used.

subcultures were made every two weeks. Experiments were completed within three months of ampoule opening.

Preparation of the bacterial inoculum

The strains were cultured statically for 24 hours, at 37°C, in tightly capped universal containers containing 20 ml of M.R.S. broth (de Man, Rogosa and Sharpe, 1960), which was prepared as described in Appendix A. The universals were then centrifuged at 3,000 rpm for 10 minutes. The supernatant was discarded and, using a micropipette, a 10 μl volume of the deposit was suspended in 10 ml of sterile phosphate buffered saline (P.B.S.), the preparation of which is described in Appendix A. The suspension was vortex mixed for 10 seconds, and then further diluted in P.B.S. to give a 10^{-6} dilution of the original deposit.

Preparation of media

The media used were Rogosa SL agar (Rogosa et al, 1951); M.R.S. agar (de Man et al, 1960); and non-selective Columbia agar with 7.5% horse blood (blood agar). The constituents and methods of preparation of these media are described in Appendix A.

Inoculation, incubation and enumeration of bacteria

For media inoculation, a spiral plater (Spiral Systems Incorporated, Cincinnati), as described by Gilchrist et al (1973), was employed. Fig. 2.1 shows this specialised dispenser, which distributes 50 μl of sample on the surface of a rotating agar plate. The sample is dispensed in the form of an Archimedes spiral, from the centre of the plate, to the edge, in an ever-decreasing amount. It is delivered in such a manner that the volume inoculated on to any

Figure 2.1 A spiral plater in use with a Rogosa SL agar plate.

portion of the plate is known and constant. Thus, a 50 μl volume of the prepared inoculum was inoculated in duplicate on 7.5% blood agar, Rogosa SL agar, and M.R.S. agar.

The plates were incubated at 37^{o}C, in an atmosphere of 5% carbon dioxide in air, for 2 days. Representative areas of the plates were then counted by superimposing the transparent culture plates on a printed template, as shown in Fig. 2.2. For the opaque blood agar plates, a grid of the same design was cut into the agar using a metal cutter.

These experiments were repeated on three occasions.

Statistical analysis

The arithmetic means of the counts of lactobacilli for the duplicated plates were taken. These counts, for each of the nine strains, on each agar, were expressed as a percentage of the count for that strain on blood agar. This was repeated for all three experiments. The percentage of lactobacilli recovered on each of the agars was analysed using an analysis of covariance, Programme P2V of BMDP (BioMeDical computer Programs, Health Sciences Computing Facility, Department of Biomathematics, School of Medicine, UCLA), with the blood agar count as a covariate.

2.2.2 The use of the Alban-modified Snyder test and the Dentocult dip-slide for the detection of strains of Lactobacillus spp.

Bacterial strains

For this series of experiments, the lactobacillus isolates, together with the methods used to maintain them in culture and to prepare inocula, were the same as those described in Section 2.2.1.

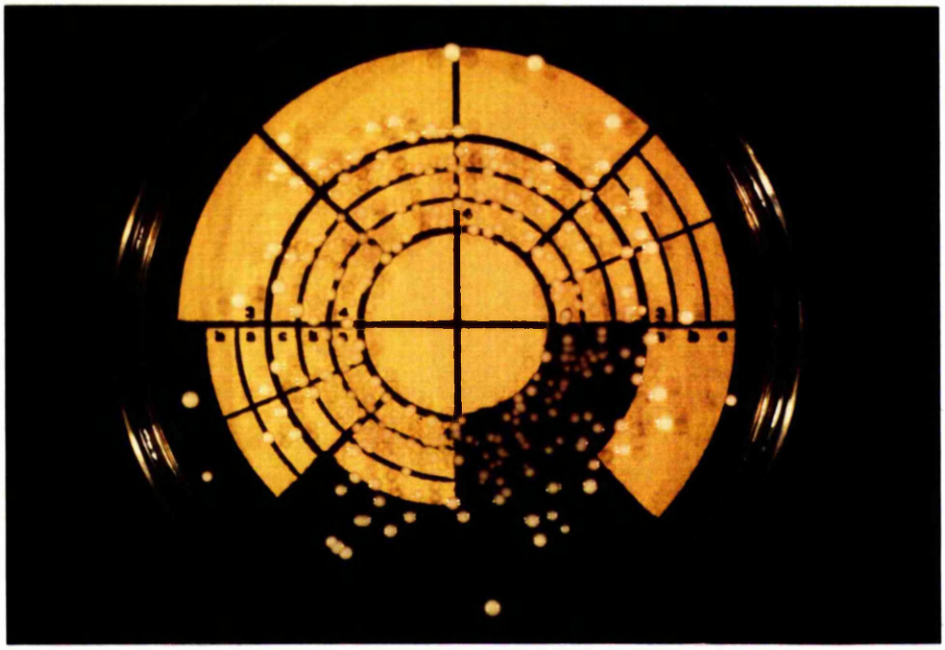


Figure 2.2 The template used for the enumeration of colonies.

Preparation of media used

The media employed were Alban's modification of the colorimetric Snyder test (Snyder, 1940; Alban, 1970), Dentocult dip-slides (Larmas, 1975) which are shown in Fig. 2.3, and 7.5% horse blood agar. The Snyder test agar and blood agar were prepared as described in Appendix A. The Dentocult dip-slides were obtained from Orion Diagnostica, Espoo, Finland.

Bacterial inoculation and incubation

A 0.2 ml volume of the prepared suspension of each strain was inoculated on to the surface of 5 ml of solid Snyder test agar in individual bijou containers. A 1 ml quantity of the same inoculum was poured over both surfaces of a Dentocult dip-slide, and the lower edge touched against the rim of the universal container to allow any excess to drain off. In order to confirm that inoculation of viable bacteria had indeed been performed, a blood agar plate was also inoculated with a 50 μl volume of the sample, using a spiral plater, as described in Section 2.2.1.

Both the Alban-modified Snyder tests and the Dentocult dip-slides were incubated, tightly capped, at 37^{o}C for 4 days. The modified Snyder tests were examined daily, for four days, to note any change of the bromcresol green colour indicator from green to yellow. This colour change is illustrated in Fig. 2.4. The presence of microbial colonies on the dip-slides was determined under reflected light at the end of the 4 day period. The blood agar plates were incubated as described in Section 2.2.1, and were also examined for the presence of colonies.

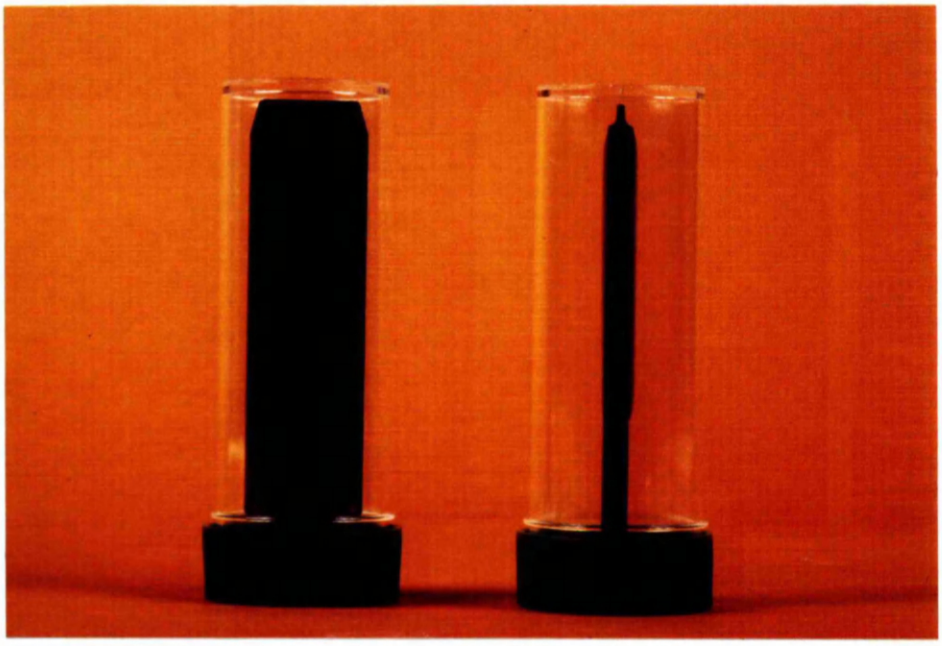


Figure 2.3 Dentocult dip-slides.

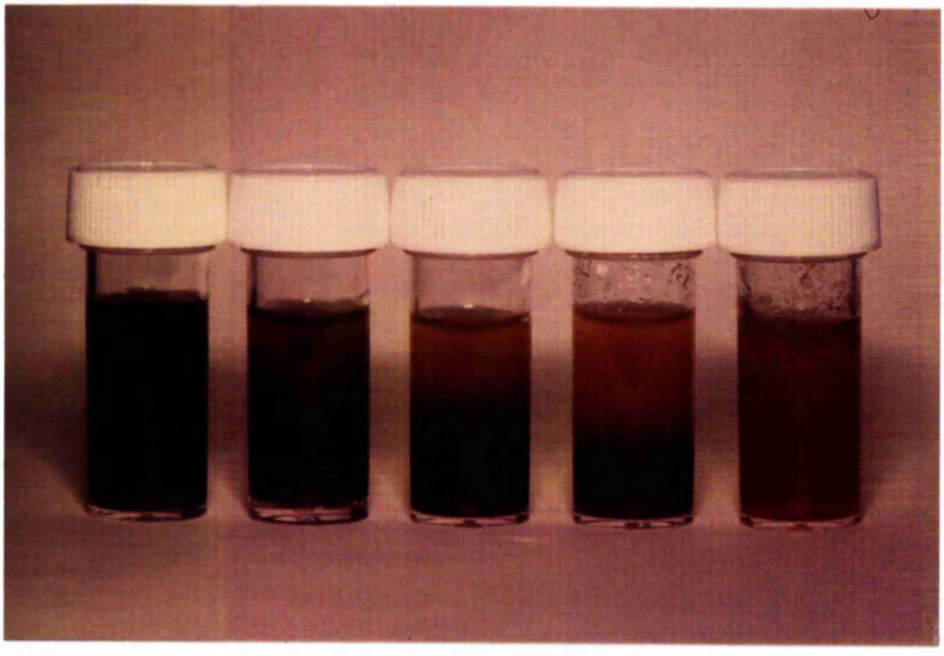


Figure 2.4 Alban-modified Snyder tests representing the gradual colour change from green to yellow.

2.2.3 Comparisons between MSB, MSFA and TYCSB agars for the isolation and enumeration of strains of Streptococcus mutans

Bacterial strains

The ten strains of Strep. mutans which were used are shown in Table 2.2. Before freeze drying, their identities were checked using the fermentation of mannitol, sorbitol, raffinose, melibiose, and mannitol in the presence of bacitracin, and for the production of ammonia from arginine, as described by Shklair and Keene (1976), and Beighton et al (1981). All strains gave the expected fermentation reactions, as previously detailed in Table 1.3. Thereafter, cultures were maintained as described in Section 2.2.1.

Preparation of the bacterial inoculum

Each strain was cultured statically for 24 hours, at 37°C, in a tightly capped universal container with 20 ml of Todd-Hewitt broth (Todd & Hewitt, 1932), which was prepared as described in Appendix A. The further preparation of the bacterial inoculum was as in Section 2.2.1.

Preparation of media

The media used were Mitis Salivarius Bacitracin (MSB) agar (Gold, Jordan & van Houte, 1973); MSFA agar (Linke, 1977); Trypticase Yeast Cystine Sucrose Bacitracin (TYCSB) agar (van Palenstein Helderman et al, 1983); and 7.5% horse blood agar. These were prepared as described in Appendix A.

Inoculation, incubation and enumeration of bacteria

The spiral plater was used, as in Section 2.2.1, to inoculate a 50 μl volume of each prepared suspension on to duplicate plates of

Organism	Type	Reference No.
Strep. mutans	b	NCTC 10920
Strep. mutans	c	NCTC 10449
Strep. mutans	c	NCTC 10832
Strep. mutans	d	NCTC 10921
Strep. mutans	d	NCTC 10922
Strep. mutans	e	NCTC 10923
Strep. mutans	f	NCTC 11060
Strep. mutans	g	NCTC 10919
Strep. mutans	g	NCTC 11061
Strep. mutans	h	NCTC 11391

Table 2.2 Strains of Streptococcus mutans used.

7.5% blood agar, MSB agar, MSFA agar and TYCSB agar. The plates were incubated and counted as in Section 2.2.1. These experiments were repeated on three occasions.

Statistical analysis

The arithmetic means of the counts of Strep. mutans for the duplicate plates were taken, and the percentage of the ten strains of Strep. mutans recovered on each agar was analysed using an analysis of covariance, as described in Section 2.2.1.

2.2.4 The use of a colorimetric medium for the detection of strains of Streptococcus mutans

Bacterial strains

The strains of Strep. mutans used, and maintainance of cultures, were as described in Section 2.2.3.

Preparation of the bacterial inoculum

The inoculum was prepared as in Section 2.2.3, except that the centrifuged deposit was diluted to 10^{-7} in P.B.S..

Preparation of media used

The media used were the colorimetric broth for Strep. mutans, described by Shklair and Walter (1976), and Walter and Shklair (1982); and 7.5% horse blood agar. These were prepared as described in Appendix A.

Bacterial inoculation and incubation

A 0.2 ml volume of the prepared inoculum of each strain was added to 1.8 ml of colorimetric broth in a bijou container. In order to confirm that inoculation of viable bacteria had taken place, 50 μl of

each suspension was also plated on blood agar, using the spiral plater.

The broths were incubated at 37°C, for 4 days, and each was examined to note any change of the bromcresol purple colour indicator from purple to yellow. This colour change is shown in Fig. 2.5. The blood agar plates were incubated as described in Section 2.2.1, and were then examined for the presence of microbial colonies.

2.2.5 Comparisons between Douglas' agar, brilliant green agar, vancomycin agar and teepol medium for the isolation and enumeration of strains of Veillonella spp.

Bacterial strains

Ten strains of veillonella were used. These are shown in Table 2.3. They consisted of three type cultures and seven fresh isolates. Since it is difficult to distinguish between the different species of veillonella using routine identification tests, no attempt was made in this thesis to speciate the veillonella isolates.

Isolation of fresh strains

The fresh isolates were obtained from the plaque of seven different individuals, and were isolated on non-selective blood agar. They were characterised as Gram-negative, strictly anaerobic cocci, which gave appropriate biochemical reactions when tested using the API 20A system (API System S.A., Montalie-Vercieu, France).

Maintenance of cultures

All strains were stored in a freeze-dried state. When required for use, they were cultured on blood agar, and incubated for 2 days at 37°C, in an anaerobic chamber (Forma Scientific, Marietta, Ohio), with

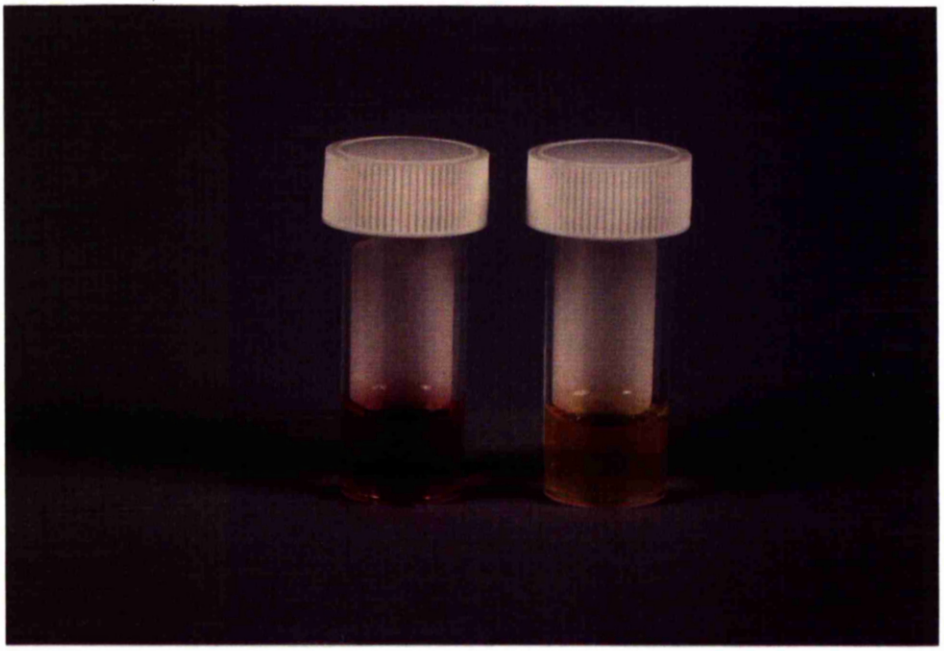


Figure 2.5 Strep. mutans colorimetric broths, showing the colour change from purple to yellow.

Organism	Reference No.	Source
Veillonella parvula	NCTC 10790	National Collection of Type Cultures
Veillonella parvula	ATCC 11463	American Type Culture Collection
Veillonella atypica	ATCC 17744	
Veillonella spp.	18	dental plaque
Veillonella spp.	27	dental plaque
Veillonella spp.	59	dental plaque
Veillonella spp.	61	dental plaque
Veillonella spp.	66	dental plaque
Veillonella spp.	84	dental plaque
Veillonella spp.	85	dental plaque

Table 2.3 Strains of *Veillonella* spp. used.

an atmosphere of 5% carbon dioxide, 10% hydrogen and 85% nitrogen. They were maintained in this atmosphere at 20°C until required, for a maximum of three months. Fresh subcultures were made every week.

Preparation of the bacterial inoculum

Each strain was cultured anaerobically for 24 hours, at 37°C, in a loosely capped universal container with 20 ml of the non-inhibitory broth described by Douglas (1950). This was prepared as described in Appendix A. The further preparation of the bacterial inoculum was as in Section 2.2.1.

Preparation of media used

The media used were Douglas' agar (Douglas, 1950); brilliant green agar (Sims & Snyder, 1958); vancomycin agar (Rogosa et al, 1958); teepol medium (MacFarlane, 1977); and 7.5% horse blood agar. These were prepared as described in Appendix A.

Inoculation, incubation and enumeration of bacteria

The spiral plater was used, as in Section 2.2.1, to inoculate a 50 μl volume of each prepared suspension on to duplicate plates of 7.5% horse blood agar, Douglas' agar, brilliant green agar, vancomycin agar and teepol medium. The plates were incubated anaerobically, at 37°C, for 2 days. Counting of colonies on the plates was performed as in Section 2.2.1. These experiments were repeated on three occasions.

Statistical analysis

The arithmetic means of the counts of veillonella for the duplicated plates were calculated, and the percentage of veillonella recovered on each of the agars was analysed using an analysis of covariance, as described in Section 2.2.1.

2.2.6 Comparisons between media for the isolation and estimation of Lactobacillus spp., Streptococcus mutans and Veillonella spp., from mixed salivary samples

Source of samples

Five-minute, paraffin-wax-stimulated, mixed salivary samples were obtained from ten 13-year-old children. Examples of the wax lozenges used for salivary stimulation, and of the universal specimen containers in which saliva was collected, are shown in Fig. 2.6.

Preparation of inoculum

Each salivary sample was vortex mixed for 10 seconds, and diluted 10^{-1}, 10^{-2} and 10^{-3} in phosphate buffered saline.

Preparation of media

The media used for *Lactobacillus* spp. were Rogosa SL agar, M.R.S. agar, Dentocult dip-slides, and Alban-modified Snyder tests.

The media for *Strep. mutans* were MSB agar, TYCSB agar, MSFA agar, and the colorimetric broth of Walter and Shklair.

The media for *Veillonella* spp. were Douglas' medium, brilliant green agar, vancomycin agar, and teepol medium.

All media were prepared as described in Appendix A.

Inoculation and incubation of media

For the estimation of *Lactobacillus* spp., the spiral plater was used to dispense 50 μl volumes of undiluted saliva on to plates of Rogosa SL agar and M.R.S. agar. A 0.2 ml volume of saliva was also added to the surface of 5 ml of solid Snyder test agar, and 1 ml of saliva was used to inoculate the surface of a Dentocult dip-slide, after which the excess was run off, as described in Section 2.2.2. The

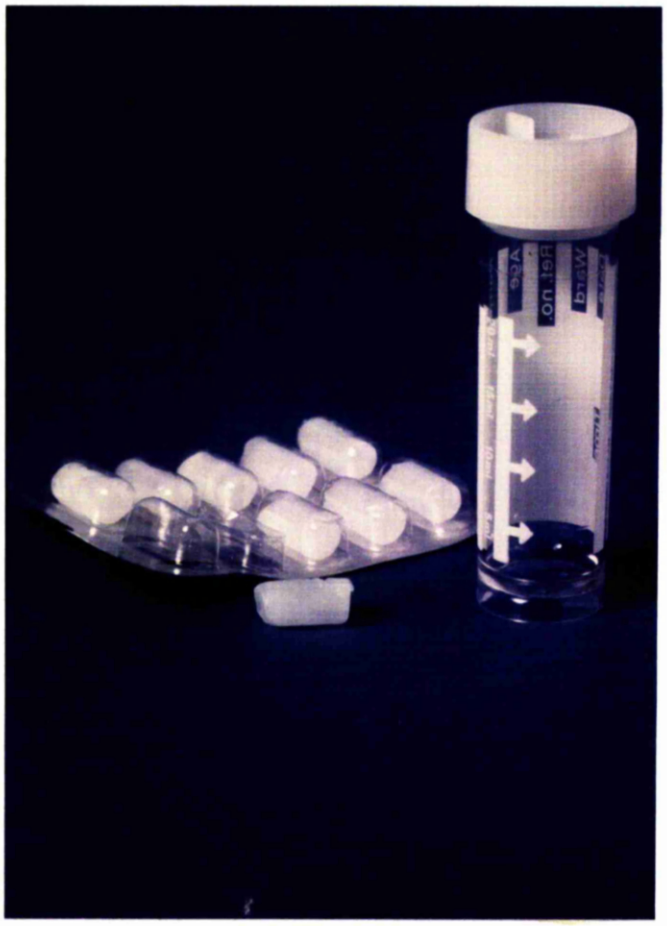


Figure 2.6 Paraffin wax and universal container used for the collection of stimulated mixed salivary samples.

plates were incubated at 37°C, for 2 days, in an atmosphere containing 5% carbon dioxide. The Alban-modified Snyder tests and Dentocult dip-slides were incubated aerobically, tightly capped, at 37°C, for 4 days.

For the estimation of Strep. mutans, the spiral plater was used to inoculate 50 μl of a 10^{-2} dilution of saliva on to the MSB, TYCSB and MSFA culture plates. In addition, using a disposable plastic pipette, a 0.2 ml volume of the 10^{-1} dilution was added to 1.8 ml of colorimetric broth. All plates were incubated at 37°C, for 2 days, in an atmosphere containing 5% carbon dioxide in air. The colorimetric broths were incubated, tightly capped, for 4 days at 37°C.

For the estimation of Veillonella spp., 50 μl volumes of the 10^{-3} dilution of saliva were spiral plated on to Douglas', brilliant green, vancomycin and teepol media. These plates were incubated anaerobically for 2 days, at 37°C.

All inoculations were performed in duplicate, on a single occasion.

Enumeration of bacteria

Using the printed template described in Section 2.2.1, the number of microbial colonies visible to the naked eye, on a representative area of each agar plate, was counted. The arithmetic mean of the counts on the duplicate plates was calculated, and each count then converted into the number of colony forming units per ml of saliva.

The Alban-modified Snyder tests for lactobacilli, and the colorimetric broths for Strep. mutans, were examined daily for any colour change, which was recorded.

The dip-slides were examined after 4 days, and the density of

colonies on each was compared with the colony density examples on the chart provided by the manufacturers for interpretation (see Fig. 2.7 and Fig 2.8).

Identification of bacteria

Colonies were identified from one of the duplicated dip-slides, and from one of the duplicated agar plates of each agar type, for each saliva sample. Ten colonies were subcultured from the area of agar which had been counted, in such a way that any colonies with different morphologies were selected in the same proportions as occurred on the primary culture plates. Colonies from the selective agar plates used for the isolation of lactobacilli and Strep. mutans were incubated for 2 days, at 37°C, in an atmosphere containing 5% carbon dioxide, on blood agar. Colonies from the veillonella selective media were incubated on blood agar, under anaerobic conditions. Once the purity of subcultures was confirmed, isolates were then characterised using the following screening tests;

(a) the isolates from the lactobacillus agars were examined microscopically, and all Gram-positive bacilli were accepted as being lactobacilli;

(b) the isolates from the media for Strep. mutans were tested for the fermentation of mannitol, sorbitol, and mannitol in the presence of bacitracin, as described by Shklair and Keene (1974). However, in this thesis, no attempt was made to distinguish between the different species of the Strep. mutans group;

(c) the isolates from the veillonella media, which had been cultured anaerobically, were examined microscopically, and then subcultured on to blood agar, but incubated aerobically with 5%

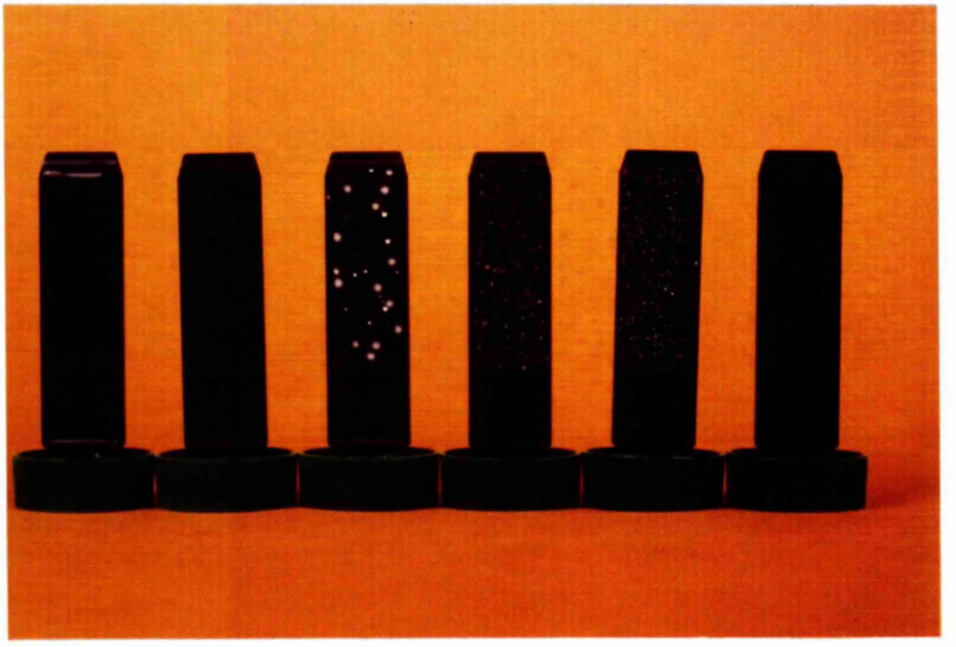


Figure 2.7 Dentocult dip-slides. From the left, an uninoculated slide, and incubated slides showing colony densities representing zero, 10^3, 10^4, 10^5 and 10^6 colony forming units per millilitre of saliva.

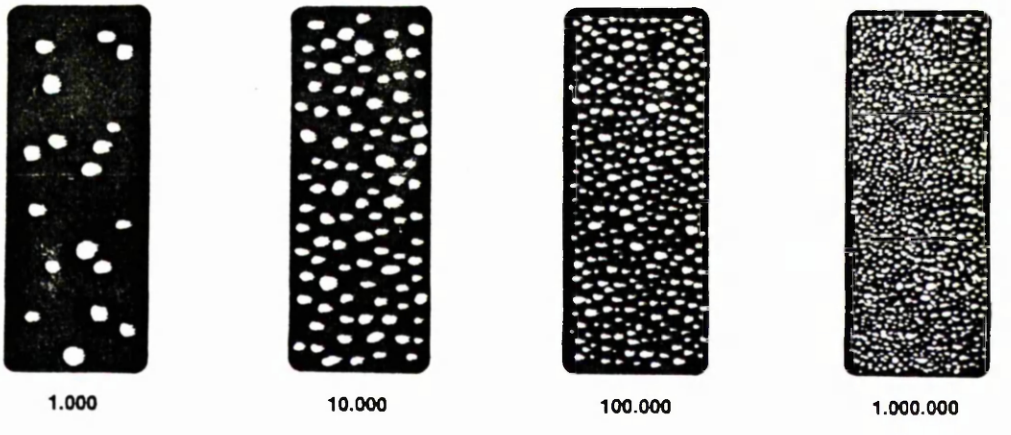


Figure 2.8 Chart of Dentocult colony densities.

carbon dioxide, for 2 days. Isolates were accepted as veillonella if they were gram-negative, strictly anaerobic cocci. It was thus possible to calculate the number of "other organisms" which were isolated on each of the selective plates.

Statistical analysis

For each medium studied, the number of colony forming units of the species for which the agar was selective was estimated. This was performed using the proportion of the selected species present among the ten colonies subcultured. Similarly, the numbers of other micro-organisms on the media were also calculated. The arithmetic means of the counts for the duplicate plates were then taken, and the logarithm_{10} of each count calculated. However, in order to allow the calculation of a logarithm for any zero counts, an amount smaller than the lowest count which could be detected for each dilution was added to every count. For the counts of lactobacilli and organisms other than lactobacilli, a count of 1×10^1 per millilitre was added; for Strep. mutans and organisms other than Strep. mutans, a count of 1×10^3 per millilitre was added; and for veillonella and organisms other than veillonella, a count of 1×10^4 per millilitre was added. The counts both of selected bacterial colonies, and of other bacteria, were compared for all three micro-organisms studied, using an analysis of variance, as described in Section 2.2.1. Where appropriate, any significant differences revealed using this method were further investigated employing paired t-tests, using Minitab (Statistics Dept., Pennsylvania State University) with an overall 0.05 significance level, by means of a Bonferroni multiple comparison procedure.

2.3 Results

2.3.1 Comparisons between Rogosa and M.R.S. agars for the isolation and enumeration of strains of Lactobacillus spp.

As can be seen from Table 2.4, there was wide variation in the percentages of the different strains of lactobacilli which were recovered from both Rogosa and M.R.S. agars. There were also some large differences between the percentage counts obtained from the repeated experiments, although these were not statistically significant.

By taking percentages of the colony counts for each agar compared with blood agar, it was hoped that differences in the numbers of bacteria in the inocula, occurring between strains and between experiments, would have no significant effect on the percentage counts on the different media. However, analysis of covariance, using the blood agar count as a covariate, revealed a further relationship between the percentage count and the blood agar score. In order to allow a direct comparison between the media across the three experiments, the mean percentage counts were corrected back to the same average level of blood agar, for all three experiments. These adjusted mean percentage counts of the nine strains are shown in Table 2.5. Analysis of covariance then showed no significant differences ($p > 0.1$) between the percentage of Lactobacillus spp. recovered from either Rogosa SL agar or M.R.S. agar.

2.3.2 The use of the Alban-modified Snyder test and the Dentocult dip-slide for the detection of strains of Lactobacillus spp.

All nine strains of lactobacilli tested, started to change the bromcresol green indicator in the Snyder test agar to yellow by Day 1,

Lactobacillus spp. strain	Percentage of blood agar count from					
	Rogosa SL agar			M.R.S. agar		
	Experiment			Experiment		
	1	2	3	1	2	3
NCIB 6375	83.1	73.0	88.2	85.1	83.5	87.4
NCTC 10302	77.0	93.3	73.8	70.7	94.4	77.7
NCTC 14932	23.5	33.3	107.8	27.1	34.3	100.0
MUCOB 254	118.3	91.4	92.4	97.6	86.6	74.1
MUCOB 260	55.2	86.5	80.4	45.5	86.5	76.3
P642	66.7	43.3	81.3	66.7	46.1	87.5
0112	88.7	81.7	89.3	61.7	81.7	59.8
76	20.4	86.6	23.8	22.2	83.9	22.0
18	71.1	128.9	71.3	67.8	115.6	71.3

Table 2.4 The mean percentage counts of nine strains of Lactobacillus spp. on Rogosa SL agar and M.R.S. agar, on three occasions, when compared with 7.5% horse blood agar.

Medium	Experiment 1 adjusted mean	Experiment 2 adjusted mean	Experiment 3 adjusted mean
Rogosa	65.7	77.6	77.0
M.R.S.	59.1	77.0	76.3

Standard Error of the Mean = 1.73

Table 2.5 The mean percentage counts of nine strains of Lactobacillus spp., on each of three occasions, adjusted to a blood agar count of 1.89×10^4 colony forming units per millilitre.

and produced complete colour change by Day 4.

Growth on the surface of the Dentocult dip-slides was visible after four days, again, for all strains of lactobacilli tested.

2.3.3 Comparisons between MSB, MSFA, and TYCSB agars for the isolation and enumeration of strains of Streptococcus mutans

At the dilution tested, no colonies of any strains were detected on MSFA agar at any of the three experiments. This agar was therefore excluded from all analyses.

Table 2.6 demonstrates the wide variation in the percentages of the different strains of Strep. mutans which were recovered from MSB and TYCSB agars, when compared with blood agar. The strain "NCTC 11391" failed to grow completely on either of these agars, and was therfore excluded from further analyses. Table 2.6 also shows the wide variation in results from the repeat experiments. The differences in these repeat experiments were statistically significant ($p < 0.01$).

In contrast to the data described in Section 2.3.1, no significant additional relationship was found between the percentage colony counts and blood agar counts. However, for consistency, the adjusted mean percentage counts for the remaining nine strains combined, on each of the three occasions, are shown in Table 2.7. Analysis of covariance showed no significant differences between the percentage of Strep. mutans strains recovered on either TYCSB or MSB.

2.3.4 The use of a colorimetric medium for the detection of strains of Streptococcus mutans

All ten strains of Strep. mutans tested changed the bromcresol

Strep. mutans strain	Percentage of blood agar count from					
	MSB Agar			TYCSB Agar		
	Experiment			Experiment		
	1	2	3	1	2	3
NCTC 10920	29.4	31.4	92.5	10.4	0.9	0.0
NCTC 10449	60.5	101.6	112.0	56.6	104.5	87.9
NCTC 10832	63.4	47.6	90.1	67.7	89.2	85.0
NCTC 10921	106.9	76.9	96.7	106.9	69.9	95.1
NCTC 10922	88.6	102.4	97.3	78.7	78.7	110.7
NCTC 10923	47.1	21.4	91.6	42.2	35.1	77.9
NCTC 11060	18.1	18.3	15.4	31.4	26.2	76.9
NCTC 10919	30.0	53.8	102.0	25.2	44.3	90.1
NCTC 11061	47.6	31.1	104.6	47.6	27.2	59.0
NCTC 11391	0.0	0.0	0.0	0.0	0.0	0.0

Table 2.6 The mean percentage counts of ten strains of Strep. mutans on Mitis Salivarius Bacitracin Agar, and Trypticase Yeast Cystine Sucrose Bacitracin Agar, on three occasions, when compared with 7.5% horse blood agar.

Medium	Experiment 1 adjusted mean	Experiment 2 adjusted mean	Experiment 3 adjusted mean
MSB	55.1	53.3	84.1
TYCSB	52.3	52.4	81.4

Standard Error of the Mean = 5.55

Table 2.7 The mean percentage counts of nine strains of Strep. mutans, on each of three occasions, adjusted to a blood agar count of 1.02×10^5 colony forming units per millilitre.

purple indicator of the colorimetric broth from purple to yellow, within four days.

2.3.5 Comparisons between Douglas' agar, brilliant green agar, vancomycin agar and teepol medium for the isolation and enumeration of strains of Veillonella spp.

The wide variation in the percentages of Veillonella spp. which were recovered from the four agars tested, are shown in Table 2.8(a) and (b). The differences in the results from these repeat experiments were statistically significant ($p < 0.05$), and there was a lack of consistency across the agars. In addition, the quality of growth on Douglas' agar and brilliant green agar was variable. On some occasions the strains grew as distinct colonies, while on others they produced only a scant haze of growth, this being reflected by the zero score (Table 2.8(a)) for strain "59" in Experiment 2. Indeed, one earlier experiment was repeated and its results excluded because the majority of strains produced only this scant haze on the Douglas' agar tested. The zero scores occurring in Table 2.8(b), however, for teepol medium and vancomycin agar, reflect a true zero rather than growth as a scant haze.

Analysis of covariance revealed a further relationship between the percentage colony counts and the blood agar score, therefore the mean percentage counts were corrected back to the same average level of blood agar for all three experiments. These adjusted mean percentage counts for all ten strains combined, on each of the three occasions, are shown in Table 2.9. Analysis of covariance showed that a significant difference existed between results for the different agars ($p < 0.001$). Further analysis, using a Bonferroni

Veillonella spp. strain	Percentage of blood agar count from					
	Douglas' agar			brilliant green agar		
	Experiment			Experiment		
	1	2	3	1	2	3
NCTC 10790	3.6	27.0	77.9	35.9	7.2	8.0
ATCC 11463	10.6	20.3	98.0	29.6	1.0	4.4
ATCC 17744	29.0	73.8	111.3	22.0	41.0	70.9
18	24.5	80.4	100.0	59.8	64.2	56.8
27	79.0	78.9	71.8	90.0	69.8	65.1
59	41.7	0.0	46.4	18.1	0.0	9.4
61	91.5	59.6	81.2	72.2	59.6	88.0
66	83.3	90.0	118.1	91.7	66.6	56.2
84	16.5	21.3	91.9	37.5	12.0	79.9
85	51.6	34.7	66.2	69.9	4.7	13.4

Table 2.8(a) The mean percentage counts of ten strains of Veillonella spp. on Douglas' agar and brilliant green agar, on three occasions, when compared with 7.5% horse blood agar.

Veillonella spp. strain	Percentage of blood agar count from					
	teepol medium			vancomycin agar		
	Experiment			Experiment		
	1	2	3	1	2	3
NCTC 10790	3.9	5.3	8.8	41.8	8.8	37.8
ATCC 11463	2.6	0.0	0.1	41.3	2.2	4.6
ATCC 17744	2.2	0.3	0.4	7.7	21.3	30.4
18	44.6	65.9	40.4	54.1	63.2	66.6
27	21.9	35.5	21.8	90.0	58.5	59.0
59	9.8	0.0	0.0	9.8	0.0	0.0
61	50.5	46.4	76.8	42.0	60.2	88.0
66	36.7	1.5	37.9	49.3	33.2	63.9
84	6.9	0.6	0.2	43.5	0.0	0.0
85	57.5	0.2	0.3	0.0	0.0	0.0

Table 2.8(b) The mean percentage counts of ten strains of Veillonella spp. on teepol medium and vancomycin agar, on three occasions, when compared with 7.5% horse blood agar.

Medium	Experiment 1 adjusted mean	Experiment 2 adjusted mean	Experiment 3 adjusted mean
Douglas'	47.8	44.7	85.5
Brilliant green	57.3	28.7	44.4
Teepol	28.3	11.7	17.9
Vancomycin	42.6	20.8	34.3

Standard Error of the Mean = 5.23

Table 2.9 The mean percentage counts of ten strains of Veillonella spp., on each of three occasions, adjusted to a blood agar count of 2.05×10^5 colony forming units per millilitre.

multiple comparison procedure to compare the mean of all three experiments for each agar, ignoring the differences between experiments, revealed that this significant difference was between the results for the Douglas' agar and the teepol medium only. When any of the other pairs of media were compared, there were no significant differences between the percentage of Veillonella spp. strains recovered.

2.3.6 Comparisons between media for the isolation and estimation of Lactobacillus spp., Streptococcus mutans and Veillonella spp., from mixed salivary samples

Lactobacillus spp.

As described in Section 2.2.6, the logarithm of each of the microbial counts discussed in that section was calculated before the results were analysed.

In Table 2.10, therefore, are shown the means and the standard deviations of the logarithms of the numbers of Lactobacillus spp., and of micro-organisms other than lactobacilli, which were isolated from mixed salivary samples, on Rogosa SL agar, MRS agar, and on Dentocult dip-slides. Using an analysis of variance, no significant differences were found between the numbers of lactobacilli isolated on these three media. However, differences did occur between the numbers of other micro-organisms. These were isolated from MRS agar, but none were noted from either Rogosa SL agar, or from Dentocult dip-slides.

The day on which each Snyder test started to change colour, is compared with that individual's mean lactobacillus count on Rogosa SL agar in Table 2.11. Of the ten Snyder tests, all but one began to

Medium	Lactobacillus spp.		Other micro-organisms	
	mean log count per ml.	S.D. of logarithm count	mean log count per ml.	S.D. of logarithm count
Rogosa	4.3	0.6	1.0	0.0
M.R.S.	4.0	2.0	2.9	2.2
Dentocult	4.6	0.8	1.0	0.0

Table 2.10 Means and standard deviations of the $\log_{10}$ of the counts of Lactobacillus spp. and of other micro-organisms isolated from ten salivary samples.

Individual	Day of first colour change of Alban Snyder test	Rogosa SL agar lactobacillus count cfu per ml
1	2	9.1×10^4
2	1	9.9×10^4
3	1	1.1×10^4
4	1	4.3×10^4
5	1	4.3×10^3
6	1	9.1×10^3
7	1	1.8×10^3
8	1	1.1×10^4
9	1	2.0×10^4
10	1	5.6×10^4

Table 2.11 Day of first colour change of ten Alban-modified Snyder tests, and the corresponding lactobacillus count on Rogosa SL agar.

change colour within 24 hours, and had completely changed colour within four days. The remaining test had started a colour change within two days, and had not completely changed to yellow by the fourth day. However, this latter sample did not give a particularly low lactobacillus count on Rogosa SL agar.

Streptococcus mutans

The means and standard deviations of the logarithms of the counts of Strep. mutans and of other bacteria which were isolated from mixed salivary samples on MSB, TYCSB and MSFA agars, are given in Table 2.12. Analysis of variance showed significant differences between the counts, both of Strep. mutans and of other micro-organisms across the three media. When these were investigated further, using multiple paired t-tests, with an overall significance level of 0.05, the significant differences were found between MSB and MSFA, and TYCSB and MSFA agars, for both Strep. mutans and other bacteria. There were no significant differences, either between the counts of Strep. mutans, or between the counts of other bacteria, when MSB and TYCSB agars were compared.

The day of colour change for each of the ten colorimetric broths for Strep. mutans, is compared in Table 2.13, with that individual's Strep. mutans count on MSB agar. Of the ten broths, two changed colour within 24 hours, another seven turned yellow in two days, and the remaining test had not changed colour by day four. The rate of colour change appeared to correspond to the numbers of Strep. mutans isolated on MSB, with the lowest count sample producing no colour change, while the two tests which changed colour after one day were from samples with the highest and third highest plate counts.

Medium	Strep. mutans		Other micro-organisms	
	mean log count per ml.	S.D. of logarithm count	mean log count per ml.	S.D. of logarithm count
MSB	5.9	0.8	4.0	1.3
TYCSB	5.9	0.6	4.9	1.1
MSFA	3.3	1.0	6.3	0.4

Table 2.12 Means and standard deviations of the $\log_{10}$ of counts of Strep. mutans and of other micro-organisms isolated from ten salivary samples.

Individual	Day of colour change of *Strep. mutans* colorimetric test	MSB agar *Strep. mutans* count cfu per ml
1	2	5.4×10^5
2	2	5.8×10^5
3	2	5.8×10^5
4	1	2.8×10^6
5	2	7.3×10^5
6	2	6.9×10^6
7	No change	1.2×10^4
8	2	8.2×10^5
9	2	1.6×10^5
10	1	7.5×10^6

Table 2.13 Day of colour change of ten *Strep. mutans* colorimetric broths, and the corresponding *Strep. mutans* count on MSB agar.

Veillonella spp.

In Table 2.14 are the means and standard deviations of the logarithms of the counts of Veillonella spp., and of bacteria other than veillonella, which were isolated from Douglas' agar, brilliant green agar, vancomycin agar and teepol medium. Analysis of variance demonstrated significant differences in the numbers, both of veillonella and of other bacteria across the four agars. Further analyses, using multiple paired t-tests with an overall level of significance of 0.05, revealed that a significant difference in veillonella counts was present when vancomycin agar and teepol medium were compared, but not between either of these media and either Douglas' agar or brilliant green agar. Significant differences were found between the numbers of micro-organisms other than veillonella when either Douglas' agar or brilliant green agar were compared with either vancomycin agar or teepol medium. No significant differences were found for any of the comparisons between other agar pairs. Vancomycin agar was therefore chosen as the medium for the isolation and enumeration of veillonella.

2.4 Discussion

2.4.1 Variation between repeat experiments

In spite of standardising the preparation of the inocula and media, and the methods of inoculation, incubation and enumeration of bacteria, as described in Sections 2.2.1, 2.2.3 and 2.2.5, large differences existed in counts of strains of bacteria, both between strains, and when experiments were repeated. As described in Sections 2.3.3 and 2.3.5, these inexplicable differences in repeat

Medium	*Veillonella* spp.		Other micro-organisms	
	mean log count per ml.	S.D. of logarithm count	mean log count per ml.	S.D. of logarithm count
Douglas'	6.9	0.3	7.3	0.4
Brilliant green	6.9	0.4	7.4	0.3
Teepol	6.6	0.4	4.3	0.6
Vancomycin	7.1	0.4	4.2	0.7

Table 2.14 Means and standard deviations of the $\log_{10}$ of counts of *Veillonella* spp. and of other micro-organisms isolated from ten salivary samples.

experiments reached statistical significance in the comparisons of media for Strep. mutans and veillonella.

However, considerable variation in recovery rates between different strains and for repeated experiments of the same strain, has also been reported by van Palenstein Helderman et al (1983), in comparisons of MSB and TYCSB agars.

2.4.2 Comparisons between media for the estimation of Lactobacillus spp.

For subsequent use in the clinical trial, it was necessary to identify a medium which gave both a good estimation of lactobacilli over the range of species commonly found in the mouth, and also a low count of other oral micro-organisms.

All but one of the strains tested were either Lactobacillus casei or Lactobacillus fermentum. As described in Section 1.2.5, these are the species most commonly isolated from oral samples. The remaining strain was identified as the less commonly isolated Lactobacillus plantarum. All strains produced growth on all media tested, and a colour change on Snyder test agar, as described in Sections 2.3.1 and 2.3.2.

As shown in Section 2.3.1, no significant differences were found in the numbers of the known strains of lactobacilli which were recovered from either Rogosa or M.R.S. agars. This was in agreement with the findings of Gonzalez, Scheffers and Mossel (1971) who compared the growth of seven species of lactobacilli on these two media.

Similarly, no significant differences were found when the numbers of lactobacilli isolated from mixed salivary samples were compared on

Rogosa SL agar, on M.R.S. agar, and on Dentocult dip-slides. This result was obtained in spite of the fact that the dip-slide count was only a relatively crude categorical count, while that on the plates permitted a range of counts. A good association between the numbers of lactobacilli on Rogosa SL agar and on Dentocult dip-slides has also been shown by Crossner and Hagberg (1977), and by Birkhed et al (1981). As M.R.S. was developed as an improved non-selective medium for lactobacilli, by de Man et al (1960), it might have been expected that higher counts would have been found on this medium, but this was not the case in the experiments described.

As noted in Section 2.3.6, when comparisons were made of the micro-organisms isolated on Dentocult dip-slides, and on both Rogosa and M.R.S. agars, only lactobacilli were isolated from the ten salivary samples tested on either Rogosa SL agar or Dentocult dip-slides, while the non-selective M.R.S. agar yielded a variety of bacterial species. This finding contrasts with those of Crossner and Hagberg (1977), and Birkhed et al (1981) who, in addition to lactobacillus colonies, found colonies of Candida spp. on about 20% of Dentocult dip-slides which had been incubated at room temperature. Additionally, Frostell and Nord (1972), and Larmas (1975) also reported the isolation of "streptococci and actinomyces-like thread forms on Rogosa SL agar". The numbers of isolates identified in these studies were generally higher than in this investigation, and the isolation of other organisms, although numerically unspecified, was reported to be infrequent. However, Charlton and Spies (1956) reported the isolation of only two organisms other than lactobacilli, from 354 isolates from 200 individuals. However, in this study all

200 colonies sampled from Rogosa SL agar and Dentocult dip slides were identified as lactobacilli, which is perhaps not surprising since only 10 individuals were involved.

The limited difference in rate of colour change of the Alban-modified Snyder test was disappointing, but as lactobacilli had been isolated from all the salivary samples tested, it was perhaps not surprising. However, as the rate of colour change in the unmodified Snyder test (Snyder 1940) was associated with the number of lactobacilli present, it was unfortunate that the sample with the slowest rate of colour change did not have a particularly low lactobacillus count on Rogosa SL agar.

One other factor which had to be considered in the choice of methods for use in the clinical trial, was whether or not laboratory facilities were required for each method. Thus, the use of agar plates for the estimation of lactobacilli requires laboratory facilities, both for their preparation and incubation, and also for their inoculation with a known volume of sample. Various methods have been described to reduce the numbers of plates of each agar which are required for each sample. The micropipette method of Westergren and Krasse (1978) may be used for the inoculation of plates. However, in this study, a spiral plater (Gilchrist et al, 1973) was available and was employed. The use of such a plater for oral samples has been described by Loesche and Straffon (1979).

By contrast, although laboratory facilities may be used to prepare and autoclave the media for the Alban-modified Snyder test, Alban (1970) described the use of his modification with media which had merely been boiled. In addition, once prepared, the test itself needs only a method of incubation at 37^{o}C, and from this point of view

it is suitable for use where laboratory facilities are not available. One further modification of the method, which could be performed easily even outwith a laboratory, and which was used in this study, was the standardisation of the volume of inoculum to 0.2 ml. This was not thought to be necesssary by Alban (1970), but in an attempt to improve the accuracy of the test it was included in these investigations. The problem with this test was the method of categorising the rate of colour change, as this was not made particularly clear in the paper by Alban (1970).

The Dentocult dip-slides required no preparation, nor specialised inoculation. Incubation was performed at 37°C, but incubation at room temperature has been described (Larmas, 1975; Crossner & Hagberg, 1977; Birkhed et al, 1981). Thus, this method might be used without access to laboratory facilities.

For caries activity testing during the clinical trial, it was decided to use a Rogosa SL agar plate count as a laboratory method of lactobacillus estimation. Dentocult dip-slides and Alban's modified Snyder tests were also chosen for use, in order that these less laboratory dependent tests could be further compared with plate counts.

2.4.3 Comparisons between media for the estimation of Streptococcus mutans

As described in Section 2.3.3, when the growth of type strains of Strep. mutans on MSB, TYCSB and MSFA agars was compared, no colonies were recovered from the MSFA agar. This disagrees with the work of Linke (1977), and of Goll (1984). However, despite having repeated

the preparation of the media and the entire experiment, on three occasions, the same disappointing result was obtained. As described in Section 2.3.6, when MSFA agar was used with mixed salivary samples, large numbers of bacteria other than Strep. mutans were isolated, and only rarely was a colony identified as Strep. mutans. It was therefore concluded that this medium was unsuitable for use in the clinical trial, for the estimation of Strep. mutans levels.

When MSB and TYCSB agars were compared, both for recovery of type strains, as in Section 2.3.3, and for isolation of Strep. mutans from salivary samples, as described in Section 2.3.6, no significant differences in the counts of Strep. mutans, or of other bacteria, were found. In addition, as expected, both media completely inhibited the growth of strain "NCTC 11391", which was described by Beighton et al (1981) as bacitracin sensitive. It was concluded that MSB and TYCSB were equally suitable for the estimation of Strep. mutans. This result is in contrast to those of van Palenstein Helderman et al (1983) and Matee et al (1985), who found that TYCSB gave a better recovery of Strep. mutans than did MSB. It is, however, in agreement with the results of Beighton (1986), who reported no significant difference between the mean number of Strep. mutans isolated on MSB or TYCSB, but more colonies of other organisms on TYCSB than on MSB.

The colorimetric broth of Shklair and Walter (1976) was described for determining the presence or absence of Strep. mutans in oral samples. Ellen, Fillery and Banting (1980), found that its use allowed a higher isolation frequency of Strep. mutans from dental plaque, than did MSB agar. This method also had the advantage that once the medium was prepared, the only laboratory facility which was

required was an incubator. However, since the results described in Section 2.3.6 indicated that the tests from samples with lower Strep. mutans counts took longer to change colour compared with those with higher bacterial concentrations, it seemed possible that this broth might be used to make an estimate of the levels of Strep. mutans in salivary samples, by recording the rate at which a colour change occurred. However, it was realised that, as with the Snyder tests, the majority of the colorimetric tests had changed colour at the same rate, and therefore the discrimination of the tests might be less satisfactory than that of the plate counts.

It was decided to use MSB plate counts as a laboratory based method of Strep. mutans counting, and also to compare this further with the less laboratory dependent colorimetric broth, on the clinical trial.

2.4.4 Comparisons between media for the estimation of Veillonella spp.

As described in Section 2.3.5, tests comparing the recovery of strains of Veillonella spp. on Douglas' agar, brilliant green agar, vancomycin agar and teepol medium, revealed only a significantly better growth on Douglas' agar than on teepol medium, with the other two media performing almost as well as the Douglas' agar, but not significantly better or worse than any of the others. However, the experiments reported in Section 2.3.6, showed that the Douglas' and brilliant green agars had the disadvantage of allowing the growth of significantly higher numbers of other oral bacteria than did either teepol medium or vancomycin agar. In this experiment, the counts of veillonella on vancomycin agar were significantly higher

than those on teepol medium. This finding is in contrast to that of MacFarlane (1977), who reported little or no inhibition of a single type strain of veillonella and of an unspecified number of freshly isolated veillonella, by 0.01% teepol. This discrepancy may in part be due to the fact that in the present study it was necessary to use more than double the concentration of teepol (0.024%), reported by MacFarlane (1977), to inhibit other members of the oral flora. Since teepol is not supplied as a pure chemical it is possible that variation in impurities or changes in the manufacturing process may have affected the antimicrobial activity of the agent.

In view of the findings discussed in this section, vancomycin agar was selected as the medium of choice for the estimation of Veillonella spp..

2.5 Conclusions

As a result of the investigations described in this chapter, it was decided that the following methods would be used on the clinical trial:

1) Totally dependent on the laboratory
   (a) Rogosa SL agar plate counts for Lactobacillus spp.
   (b) Mitis Salivarius Bacitracin agar plate counts for Strep. mutans.
   (c) Vancomycin agar plate counts for Veillonella spp.
2) Less dependent on the laboratory
   (a) Dentocult dip-slides for Lactobacillus spp.
   (b) Alban-modified Snyder tests for Lactobacillus spp.
   (c) Colorimetric broths for Strep. mutans.

CHAPTER 3

FACTORS OF IMPORTANCE IN SAMPLING TECHNIQUE

3.1 Introduction

As a result of the investigations described in Chapter 2, the selective media for use in the isolation of Lactobacillus spp., Streptococcus mutans and Veillonella spp. were chosen. In addition, it was also decided to investigate the potential use of Candida spp. counts in predicting caries, in view of the results of Pienihakkinen et al, (1984). Since the most popular media for yeast isolation are versions of Sabouraud's agar (Odds, 1979), it was decided to employ Sabouraud dextrose agar for the isolation and enumeration of Candida spp..

Another important decision was to select and standardise the method of sample collection. Many previous studies have used paraffin-wax-stimulated saliva samples (Klock & Krasse, 1977; Scheie et al, 1984; Stecksen-Blicks, 1985; Matee et al, 1985), although a spatula technique has been described by Kohler and Bratthall (1979), and Carlsson et al (1985), while a standardised loop was employed by Beighton (1986). Other authors have used unstimulated salivary samples (Alaluusua & Renkonen, 1983). Because mixed salivary samples were easy to obtain from the group of children to be studied, and in order to allow comparisons with several of the larger studies in the literature, it was decided to collect mixed salivary samples.

Before embarking on the clinical trial, however, it was necessary to study some factors related to salivary collection which could potentially affect microbial counts. There was only limited

information in the literature about variation in oral micro-organism counts due to laboratory procedures or from repeated samples. Similarly, very little data could be found regarding the effects of eating immediately before sampling, nor about the effects of storage on salivary counts.

3.2 Materials and methods

3.2.1 Method of investigating laboratory induced variation in counts performed on single samples

Source of samples

Paraffin-wax-stimulated, mixed salivary samples were obtained from ten adult volunteers, recruited from the staff of Glasgow Dental Hospital. Each volunteer provided a 15 ml volume of saliva.

Preparation of inocula

Each salivary sample was vortex mixed for 10 seconds, and 1 ml aliquots were added to 9 ml of phosphate buffered saline in ten universal containers. These dilutions were then each diluted further to give a total of ten 10^{-2} and ten 10^{-3} dilutions of the original salivary sample. The 5 ml volume of undiluted saliva which remained, was also retained.

Preparation of media

The selective media used were Rogosa SL agar for Lactobacillus spp., MSB agar for Strep. mutans, vancomycin agar for Veillonella spp., and Sabouraud dextrose agar for Candida spp.. All media were prepared as described in Appendix A.

Inoculation, incubation and enumeration of micro-organisms

Inoculation was performed using the spiral plater, as described in Section 2.2.1. For each individual sample, ten plates of Rogosa SL agar and ten plates of Sabouraud dextrose agar were inoculated with neat saliva. Again, for each sample, ten plates of MSB agar and ten vancomycin agar plates were inoculated, using each of the 10^{-2} and 10^{-3} dilutions respectively.

All plates were incubated for two days, at 37°C. The Sabouraud dextrose plates were incubated aerobically, while the MSB and Rogosa plates were incubated in an atmosphere containing 5% carbon dioxide in air, as described in Section 2.2.1. The plates of vancomycin agar were incubated anaerobically, as described in Section 2.2.5.

Using the grid described in Section 2.2.1, representative areas of the plates were counted. All colonies on the selected areas of the Rogosa and vancomycin agars were counted. The colonies which were counted on MSB agar were chosen in accordance with the descriptions of Krasse (1966) and Emilson (1983). They were small (less than 2 mm in diameter), granular, brown, grey or blue colonies, sometimes with a drop of polysaccharide on top of the colony. Colonies with differing morphology were not recorded separately. Colonies of Candida spp. on Sabouraud dextrose agar were identified according to their colonial morphology. They were large 2 mm to 5 mm diameter creamy colonies, as described by Krasse (1954b).

For each subject, the number of colony forming units of the micro-organisms studied was then calculated per millilitre of the original salivary sample.

Statistical analysis

The logarithm of each plate count was calculated as described in Section 2.2.6. This was performed for each of the ten repeat plates of each agar type, from each individual. The mean logarithmic count and standard deviation of the logarithmic counts, for each individual, were then computed.

3.2.2 Method of investigating the effect of storage on sample counts

Source of samples

Salivary samples were obtained from ten adult volunteers recruited from the staff of Glasgow Dental Hospital. Each volunteer provided 20 ml of paraffin-wax-stimulated mixed saliva.

Preparation of inocula

Each of the salivary samples was immediately vortex mixed for 10 seconds, and for each sample, 2 ml aliquots were placed in nine separate bijou bottles. For each sample three of these bijoux were stored at room temperature, three at $4^{o}C$, and three at $-10^{o}C$. The 2 ml volume remaining was used as the baseline control. The controls were diluted in phosphate buffered saline to 10^{-1}, 10^{-2} and 10^{-3}, and immediately inoculated and incubated as described below. After 3 hours, 6 hours and 24 hours, one of the bijoux from each storage temperature was similarly diluted, inoculated and incubated.

When the Sabouraud dextrose agar plates for these ten individuals were examined, it was found that only three were candida carriers. In order to obtain data for ten individuals with salivary candidal counts, samples were collected from a further 14 adult volunteers. These extra samples were prepared for inoculation as described above,

but were inoculated on to Sabouraud dextrose agar only, as described below.

Preparation of media

The media used were those described in Section 3.2.1.

Inoculation, incubation and enumeration of micro-organisms

Using the spiral plater, one agar plate of each type was inoculated for the specimens of saliva at each time and storage temperature. Thus, the undiluted sample was used to inoculate a Rogosa SL agar plate and a Sabouraud dextrose agar plate, the 10^{-2} dilution was used for the MSB agar, and the 10^{-3} dilution for the vancomycin agar.

Incubation and enumeration of micro-organisms were as described in Section 3.2.1.

Statistical analysis

The logarithm of each plate count was calculated as in Section 2.2.6. For each of the four media, and for each individual, the differences between the logarithms of the microbial counts from the samples obtained before storage and the logarithms of the counts after storage were calculated, for each of the nine time and temperature combinations. These were then analysed using an analysis of variance, as described in Section 2.2.1.

3.2.3 Method of investigating the variation in counts from repeated samples on consecutive days and at different times of day

Source of samples

Five-minute, paraffin-wax-stimulated, mixed salivary samples were obtained from ten 11-year-old school children. Each child provided three samples, one at 09.30 hours, a second at 12.00 hours, and another at 15.30 hours, on each of three consecutive school days. The children were instructed not to eat or drink for one hour prior to each sampling occasion. After collection, the samples were immediately transported to Glasgow Dental Hospital for inoculation. Transportation was in an insulated container to protect the samples from extremes of temperature.

Preparation of inocula and media

The preparation of inocula was performed as described in Section 2.2.6. The media used were those described in Section 3.2.1.

Inoculation, incubation and enumeration of micro-organisms

Using the spiral plater, one agar plate of each type was inoculated for each specimen of saliva. The undiluted sample was used to inoculate a Rogosa SL agar plate and a Sabouraud dextrose agar plate, the 10^{-2} dilution was used for the MSB agar, and the vancomycin agar was inoculated with the 10^{-3} dilution.

Incubation of plates and enumeration of colonies were performed as described in Section 3.2.1.

Statistical analysis

The logarithm of each count was calculated as described in Section 2.2.6. For each agar, the results for each of the nine time

and day combinations were compared using an analysis of variance, as described in Section 2.2.1. Additionally, the means and standard deviations for all nine counts of each micro-organism were calculated for each individual, and the means and standard deviations across the ten individuals for each sampling occasion were also calculated.

3.2.4 Method of investigating the effect of eating

Source of samples

The ten children described in Section 3.2.3 each provided three salivary samples on three consecutive days. These samples were obtained at 09.30 hours, 09.45 hours, and 10.40 hours each morning. On the second day, between 09.35 hours and 09.40 hours, each child consumed a packet of potato crisps (28 g of Golden Wonder Ready Salted Potato Crisps, Golden Wonder Ltd., Leicestershire, England). On the third day, again between 09.35 hours and 09.40 hours, each child consumed a cup of carbonated drink (165 ml of Coca-Cola, Coca-Cola Great Britain Ltd., London, England). All samples were transported to Glasgow Dental Hospital for the laboratory procedures.

Laboratory procedures

Inoculation, incubation and enumeration of micro-organisms were performed as described in Section 3.2.3.

Statistical analysis

As described in Section 2.2.6, the logarithm of each of the microbiological counts was taken. The means and standard deviations of the logarithmic counts on each of the nine sampling occasions were calculated. The differences in the logarithmic counts, firstly between 09.30 hours and 09.45 hours, and secondly between 09.30 hours

and 10.40 hours, were calculated for each child, for each micro-organism, and on each of the three days. Since candida were present in only five of the children, the analysis of the candidal counts was confined to these children. By means of multiple paired t-tests using Minitab (Statistics Dept., Pennsylvania State University), with an overall level of significance of 0.05, the differences between the first pair of counts on day one (no food consumed) were compared with those on day two (potato crisps eaten). The differences between the first and third samples on the two days were then compared. Next, the counts on day one were similarly compared with those on day three (when cola was consumed).

3.2.5 Method of investigating the variation in counts from samples obtained at monthly intervals

Source of samples

Five-minute, paraffin-wax-stimulated, mixed salivary samples were obtained from the same ten children as described in Section 3.2.3. These children were initially 11 years old. The samples were collected in the morning at 09.30 hours, and were repeated at monthly intervals over a 2.5 year period. There were a total of 29 sampling occasions during this time. All samples were immediately transported to Glasgow Dental Hospital for the laboratory procedures.

Laboratory procedures

These were as described in Section 3.2.3.

Clinical examinations

All ten children were examined clinically, for the presence of decayed, missing or filled surfaces of teeth, at the beginning and at

the end of the 2.5 year sampling period, and at approximately six-monthly intervals in between. In all, five clinical examinations were thus carried out.

The examinations were performed using a mirror and blunt, 0.6 mm diameter probe. The source of illumination was an Anglepoise lamp with a 60 Watt bulb. The examinations were performed by an independent dental examiner (S.L.C.), who had no knowledge of the children's microbiological counts, and were conducted without reference to the results of the earlier examinations.

All teeth present were examined, and the status of their surfaces ascertained. Surfaces were diagnosed as sound, missing, filled, or carious, and were only considered to be carious when there was obvious visible caries, or the 0.6 mm probe was able to enter a lesion.

Statistical analysis

The logarithm of each count was obtained as described in Section 2.2.6. For each micro-organism, the means of the logarithmic counts for those individuals present at each sampling occasion were calculated and plotted against time. The individual microbiological counts were also plotted against time, and compared with the development of caries.

Additionally, the mean of the logarithmic counts was calculated for each individual and these were correlated with the increments of decayed and filled surfaces (DFS) over the 2.5 year period, using Pearson's correlation coefficients in Minitab. Correlation coefficients were also calculated between the initial microbiological counts and the subsequent 2.5 year DFS increment.

Pooled estimates of the common standard deviations, both within individuals and across individuals, were also calculated.

3.2.6 Method of investigating the effect of different caries rates

Source of samples

As described in Section 3.2.3, salivary samples were obtained at monthly intervals from a group of ten children. The caries rates of these children were low, and salivary samples were also obtained from a second group of ten children, who were age and sex matched with the first group. This second group were selected from a class of 22 children after clinical examination, and were the five boys and five girls with the highest previous caries experience. These children also provided five-minute, paraffin-wax-stimulated, mixed salivary samples at monthly intervals. Samples were obtained on seven occasions over a six month period, and all salivary samples were donated at 09.30 hours.

Laboratory procedures

These were as described in Section 3.2.3.

Clinical examinations

Clinical examinations were carried out as described in Section 3.2.5, at the begining and end of the six month sampling period.

Statistical analysis

The logarithms of the counts of lactobacilli, Strep. mutans, veillonella and candida were calculated, as described in Section 2.2.6, for each child in the high caries group, at each of the seven sampling occasions. For each micro-organism at each sampling occasion, the means and standard deviations of the logarithmic counts of those individuals present were calculated, and plotted against time.

The caries prevalence and incremental data for the two groups of

children were compared by means of Mann-Whitney U tests, using Minitab.

The means and standard deviations across samples for each individual were also calculated. Using t-tests in Minitab, these mean logarithmic counts of each of the four micro-organisms, for the ten children in the high caries group, were then compared with the mean counts of the first seven samples obtained from the ten children with low caries rates described in Section 3.2.5.

3.3 Results

3.3.1 Results of investigations into laboratory induced variation in counts performed on single samples

The counts of lactobacilli for two individuals were repeatedly zero, and therefore the investigation of the variation for lactobacilli was confined to the eight subjects with detectable levels of these bacteria. Similarly, Candida spp. were isolated from only six of the ten subjects, and again, only their data were studied.

Table 3.1 shows the means and standard deviations of the logarithms of the ten counts of each micro-organism for each indiviudual. The standard deviations of the logarithmic counts were almost constant. The pooled estimates of the common standard deviations within the individuals with the micro-organisms present were: 0.08 for lactobacilli; 0.09 for Strep. mutans; 0.09 for veillonella; and 0.15 for candida.

Subject	Lactobacillus spp. log count$^{\emptyset}$ mean±S.D.	Strep. mutans log count$^{\emptyset}$ mean±S.D.	Veillonella spp. log count^{o} mean±S.D.	Candida spp. log count$^{\emptyset}$ mean±S.D.
1	3.48 ± 0.06	6.06 ± 0.07	6.98 ± 0.07	2.90 ± 0.08
2	4.89 ± 0.06	6.04 ± 0.08	5.04 ± 0.23	- -
3	3.07 ± 0.10	5.05 ± 0.07	7.70 ± 0.06	3.39 ± 0.04
4	4.75 ± 0.07	5.70 ± 0.14	6.78 ± 0.04	- -
5	- -	4.70 ± 0.08	6.45 ± 0.01	- -
6	1.57 ± 0.12	5.61 ± 0.06	7.88 ± 0.08	1.97 ± 0.24
7	4.16 ± 0.06	5.29 ± 0.07	6.77 ± 0.09	2.18 ± 0.20
8	3.93 ± 0.09	5.86 ± 0.08	7.68 ± 0.09	- -
9	3.14 ± 0.12	5.86 ± 0.08	7.62 ± 0.07	2.34 ± 0.14
10	- -	4.43 ± 0.17	6.40 ± 0.06	2.55 ± 0.22

- = species consistently absent

o = $\log_{10}$ cfu per ml saliva

Table 3.1 The means ± the standard deviations of the logarithms of the ten counts of each micro-organism for each subject.

3.3.2 Results of investigations into the effect of storage on sample counts

All ten of the volunteers studied initially, had lactobacilli, Strep. mutans and veillonella present in their salivary samples. However, only three of these volunteers carried Candida spp., and it was necessary to test a further 14 volunteers to obtain ten individuals who were carriers of candida. The analyses for candida were confined to those ten samples in which this micro-organism was present, and therefore the zero candida counts from 14 of the volunteers were excluded from the analyses.

The mean of the ten logarithmic counts at each temperature and sampling occasion was plotted. Those for lactobacilli are shown in Fig. 3.1; those for Strep. mutans in Fig. 3.2; those for veillonella in Fig. 3.3; and those for candida in Fig. 3.4. Analyses of variance were used to compare the differences in counts (which might have been caused by the different time or temperature conditions) between the aliquot without storage, and the aliquots after storage.

Storage temperature

When the differences between the logarithmic counts of lactobacilli, before and after storage, were compared across all ten samples using an analysis of variance, the changes in counts due to different storage temperatures were not significant at $p < 0.05$.

Similarly, when the differences between logarithmic counts on vancomycin agar before and after storage were compared, there were no significant changes in counts due to different storage temperatures.

However, when the differences between logarithmic counts on MSB agar, before and after storage were compared, significant changes were

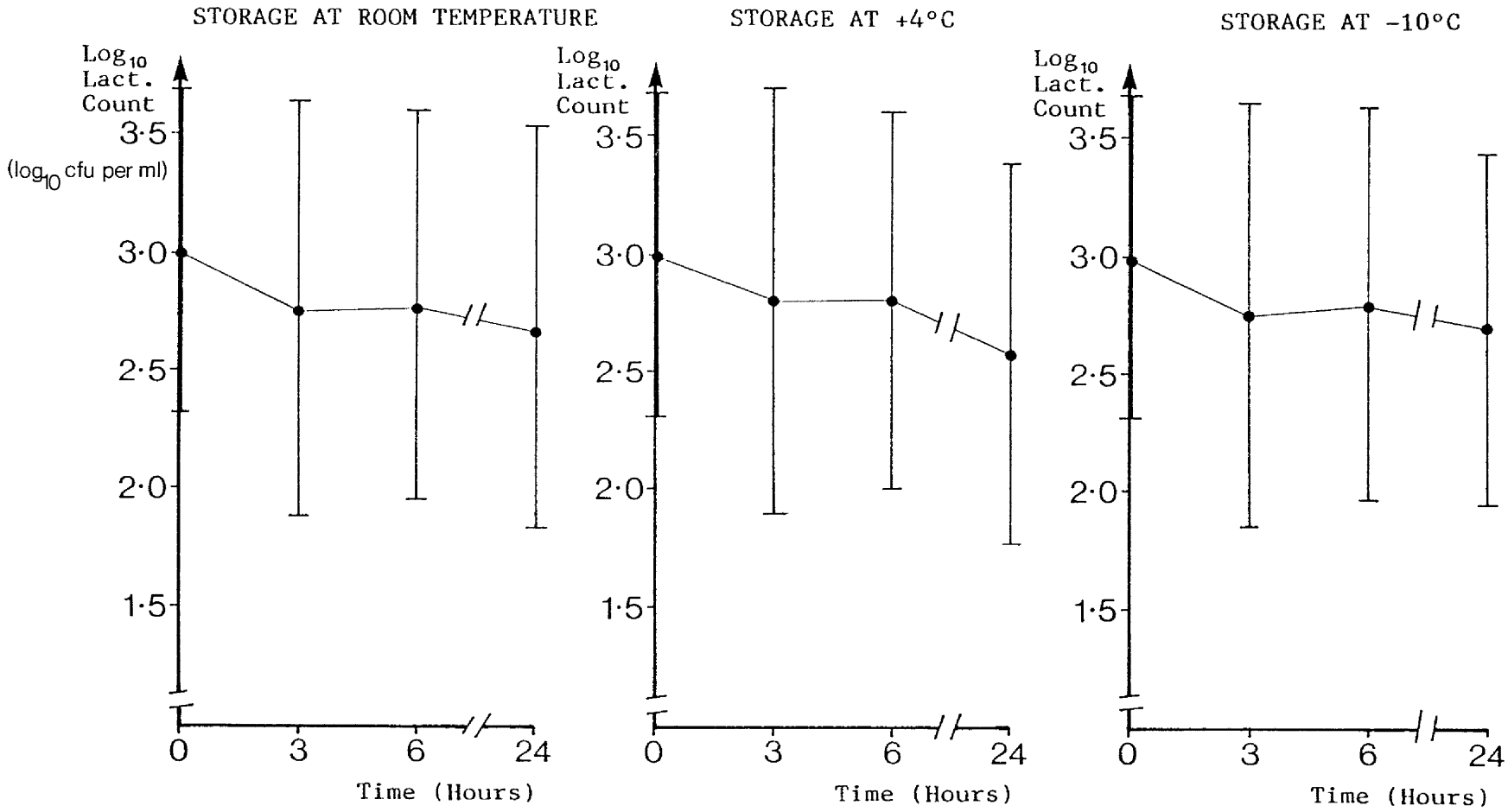


Figure 3.1 Mean ± one standard deviation of logarithmic lactobacillus counts from ten salivary samples stored for 0, 3, 6, or 24 hours, at room temperature, +4°C or -10°C.

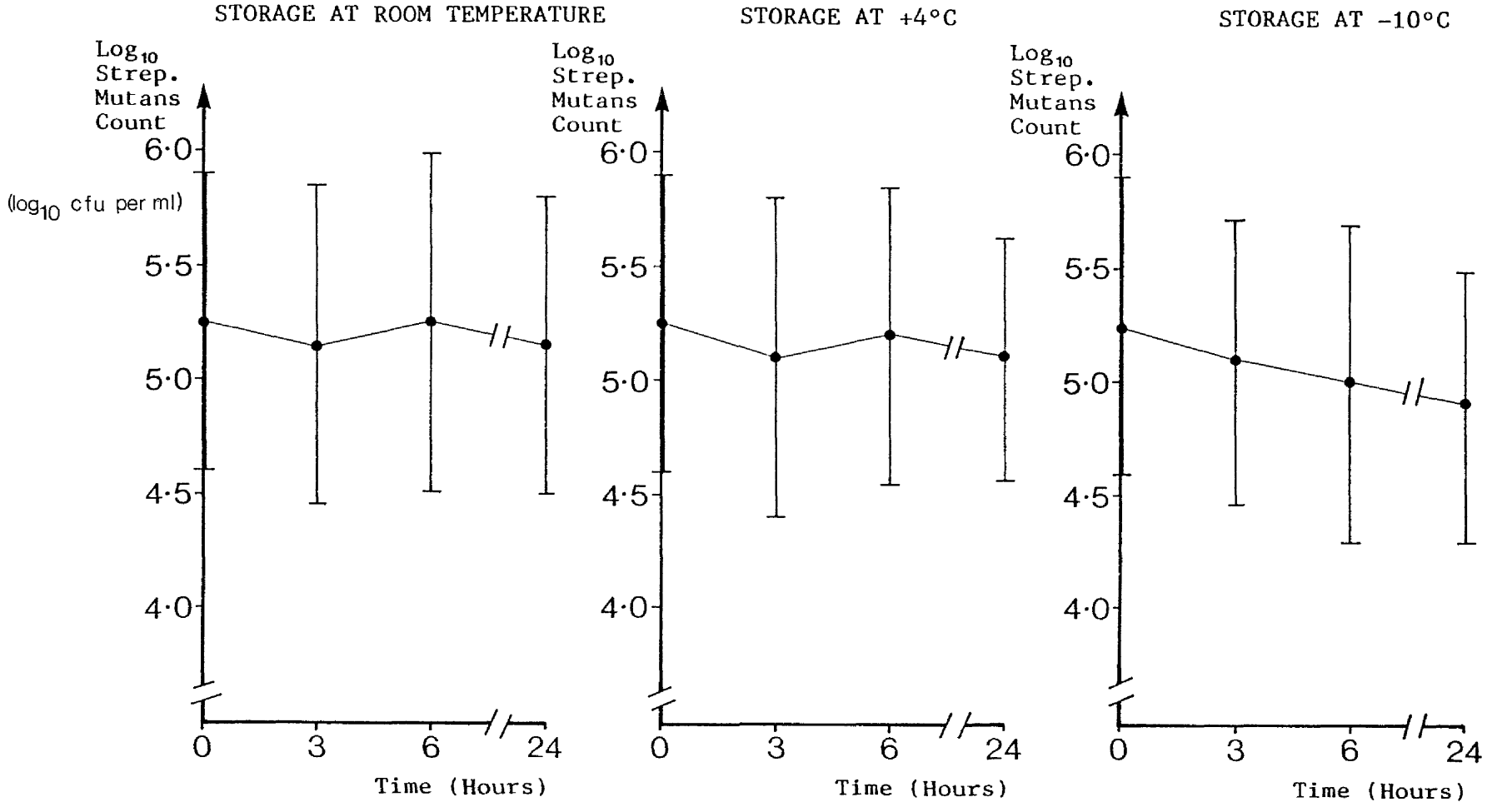


Figure 3.2 Mean ± one standard deviation of logarithmic Strep. mutans counts from ten salivary samples stored for 0, 3, 6, or 24 hours, at room temperature, +4°C or -10°C.

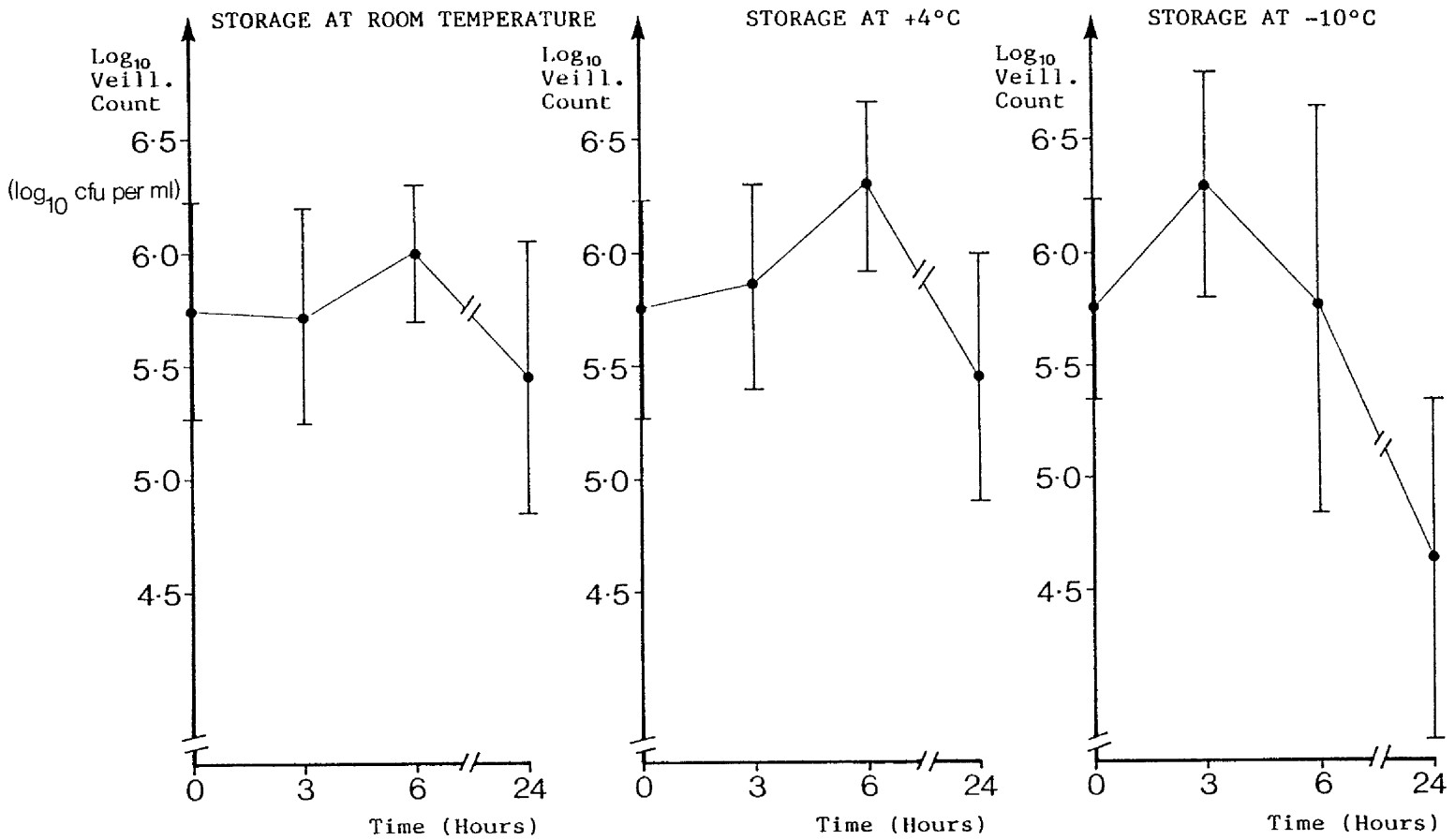


Figure 3.3 Mean ± one standard deviation of logarithmic veillonella counts from ten salivary samples stored for 0, 3, 6, or 24 hours, at room temperature, +4°C or -10°C.

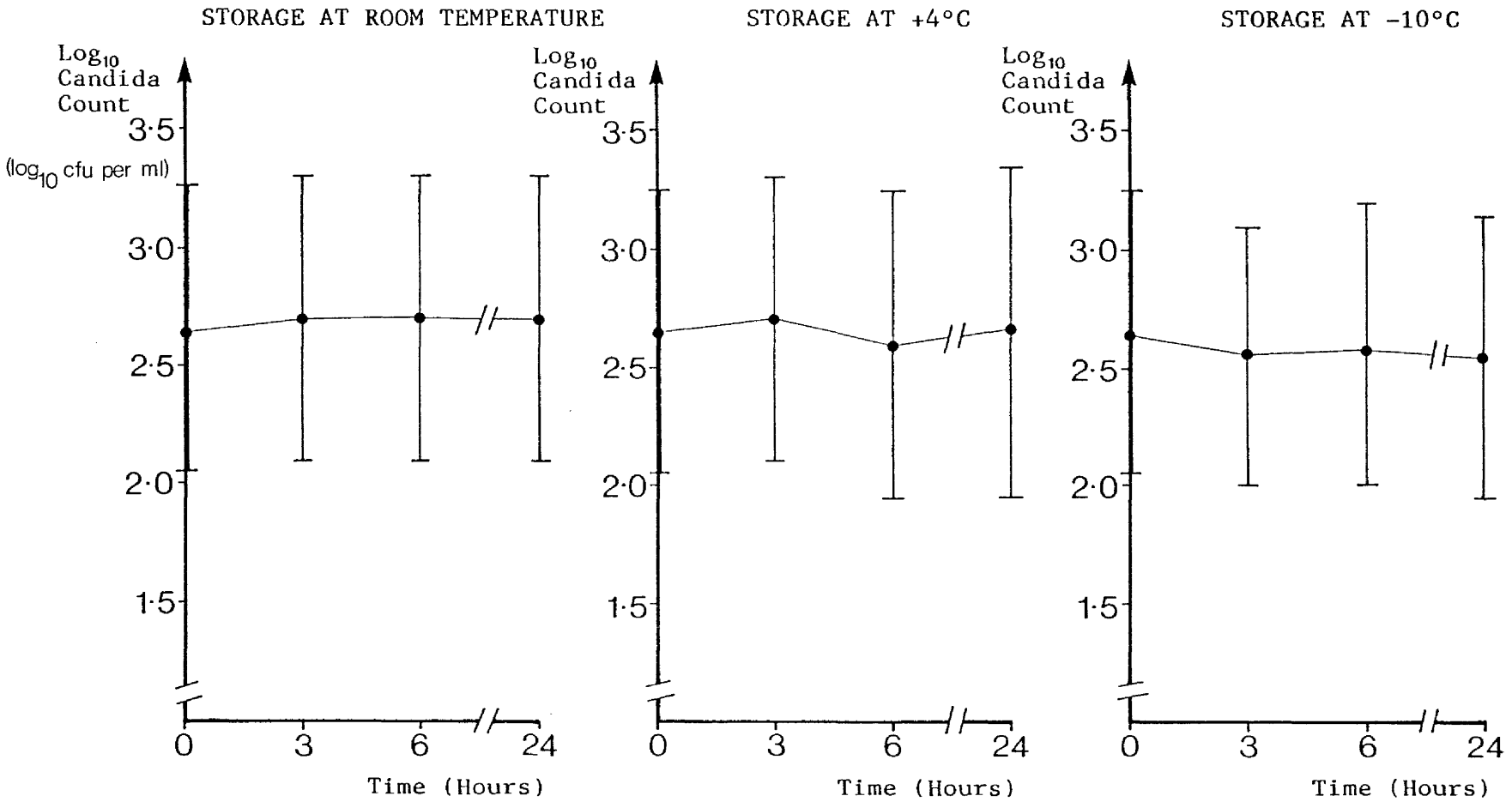


Figure 3.4 Mean ± one standard deviation of logarithmic candida counts from ten salivary samples stored for 0, 3, 6, or 24 hours, at room temperature, +4°C or -10°C.

found due to storage temperature and these differences were investigated further. The means of the differences in logarithmic counts, between the sample before storage and each of the three times, 3, 6 and 24 hours, were found for each individual and for each temperature. When these ten mean differences at each temperature were compared, using multiple paired t-tests with a Bonferroni correction for three comparisons and an overall significance level of 0.05, the counts of the aliquots stored at -10°C were found to be significantly lower than either those stored at 4°C or those stored at room temperature. However there was no significant difference between storage at room temperature or storage at 4°C.

Similarly, in the case of candida, when the differences between logarithmic counts on Sabouraud dextrose agar before and after storage were compared, significant differences were found due to storage temperature. These were investigated further, as described for Strep. mutans, using three paired t-tests, with an overall level of significance of 0.05. For Candida spp., the counts from aliquots stored at -10°C were significantly lower than those at room temperature, but there was no significant difference between those at room temperature and at 4°C, nor between those at 4°C and those at -10°C.

It was therefore concluded that storage at room temperature was satisfactory and that no significant advantage was to be gained by either storage at 4°C or storage at -10°C. The effects of the different storage times were next studied.

Storage time

For lactobacilli, analysis of variance revealed a significant time effect at a $p < 0.05$ level, across the three storage temperatures. However, when the logarithmic counts at 3, 6 and 24 hours for those aliquots stored at room temperature only, were compared with those before storage using three paired t-tests with an overall level of significance of 0.05, the logarithmic counts after the different storage times were not found to differ significantly from those before storage. The logarithmic counts for the three storage times at $4^{o}C$ were similarly compared, and those at 24 hours were significantly lower than those before storage. No significant differences were found when the lactobacillus counts after 3, 6 and 24 hours storage at $-10^{o}C$ were compared with those before storage.

For both Strep. mutans and Candida spp., analyses of variance did not reveal significant time effects, at a $p < 0.05$ level, in the changes in the logarithmic counts before and after storage. Additionally, when the logarithmic counts at 3, 6 and 24 hours for those aliquots stored at room temperature only, were compared using three paired t-tests with an overall level of significance of 0.05, the counts after the different storage times were not found to differ significantly from zero. Indeed, when the logarithmic counts at 3, 6 and 24 hours within each of the other two storage temperatures were similarly compared with those before storage using three paired t-tests, no significant differences were found for candida. However, the logarithmic counts of Strep. mutans at $4^{o}C$ after 3 hours storage, and at $-10^{o}C$ after 24 hours storage, were significantly lower than those without storage.

For Veillonella spp., analysis of variance revealed a significant

effect of storage time. When the logarithmic counts at 3, 6, and 24 hours for those aliquots stored at room temperature were compared with those before storage, using three paired t-tests with an overall level of significance of 0.05, the counts after 6 hours storage were significantly higher than those without storage, whereas those after 3 hours and after 24 hours did not differ significantly from those without storage. Similarly, for storage at $4^{o}C$ the counts at 6 and 24 hours differed significantly from those before storage, and for storage at $-10^{o}C$ the counts at 3 and 24 hours were significantly different from those which had not been stored.

From these analyses, it was concluded that there was little variation in salivary counts of lactobacilli, Strep. mutans or candida due to storage times of up to 24 hours, if the samples were stored at room temperature. However, a small but significant change was found in veillonella counts if stored at room temperature for 6 hours. In view of the numbers of comparisons made it was possible that this apparent change was merely a chance finding, but nevertheless it was decided that, in future, samples would be stored for a maximum of 6 hours prior to inoculation and incubation.

3.3.3 Results of investigations into the variation in counts from repeated samples on consecutive days and at different times of day

The logarithmic lactobacillus, candida, Strep. mutans, and veillonella counts for each individual, on each of the nine sampling occasions, are shown in Tables 3.2 to 3.5.

When the logarithmic counts for the ten individuals were compared for the nine, day and time of day combinations, analysis of variance revealed no significant differences on the different days, or sampling

CHILD	DAY 1			DAY 2			DAY 3		
	09.30hr	12.00hr	15.30hr	09.30hr	12.00hr	15.30hr	09.30hr	12.00hr	15.30hr
A	2.968	3.045	3.493	3.506	3.464	3.045	2.778	3.117	3.179
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
C	2.204	1.000	1.000	1.672	2.279	2.875	3.179	1.672	2.362
D	3.870	3.570	3.570	3.772	4.114	3.464	4.492	3.506	1.000
E	1.000	1.000	2.623	1.000	1.000	1.924	1.000	1.000	1.000
F	1.000	3.149	2.806	2.914	2.898	1.672	3.117	2.580	2.806
G	3.793	3.581	3.045	4.432	3.083	3.673	4.432	3.614	3.083
H	4.256	4.322	3.945	3.569	3.400	3.117	4.613	4.644	3.750
I	3.533	5.204	3.814	3.974	2.778	3.749	4.114	3.207	3.614
J	5.839	5.748	5.708	5.756	5.255	4.613	5.724	5.763	5.756

Table 3.2 Logarithmic Lactobacillus spp. counts for ten children on nine occasions; three times daily (at 09.30hr, 12.00hr and 15.30hr), on three consecutive days.

CHILD	DAY 1			DAY 2			DAY 3		
	09.30hr	12.00hr	15.30hr	09.30hr	12.00hr	15.30hr	09.30hr	12.00hr	15.30hr
A	2.204	2.204	2.079	2.204	2.204	1.000	1.670	2.079	2.530
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
C	2.301	2.491	2.204	2.491	2.724	2.204	3.083	2.362	2.301
D	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
E	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
F	2.079	1.000	2.301	2.301	2.301	2.204	1.000	1.000	2.301
G	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
H	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
I	3.382	2.973	3.004	3.281	2.973	3.258	3.045	3.233	2.362
J	3.987	3.945	3.930	4.080	3.700	3.864	4.146	3.987	4.080

Table 3.3 Logarithmic Candida spp. counts for ten children on nine occasions; three times daily (at 09.30hr, 12.00hr and 15.30hr), on three consecutive days.

CHILD	DAY 1			DAY 2			DAY 3		
	09.30hr	12.00hr	15.30hr	09.30hr	12.00hr	15.30hr	09.30hr	12.00hr	15.30hr
A	4.799	4.380	4.949	5.433	4.477	4.342	4.431	4.477	4.800
B	5.506	5.303	6.176	5.364	4.477	4.826	5.344	5.382	5.682
C	5.400	5.117	4.949	5.281	4.978	4.949	5.258	5.149	5.045
D	5.664	5.904	6.279	5.664	6.080	5.839	6.301	6.343	5.603
E	6.362	6.256	5.433	5.654	5.757	5.258	5.324	5.000	5.520
F	4.949	5.324	5.400	5.654	5.464	5.207	5.464	5.520	5.400
G	3.140	3.000	3.000	4.114	3.241	3.241	3.000	3.322	3.241
H	5.417	5.382	5.083	5.433	4.556	4.681	5.281	5.083	5.417
I	5.464	5.364	5.624	5.644	4.870	5.464	5.417	5.793	5.324
J	7.114	6.929	6.964	7.230	6.415	6.764	7.079	7.079	7.041

Table 3.4 Logarithmic Strep. mutans counts for ten children on nine occasions; three times daily (at 09.30hr, 12.00hr and 15.30hr), on three consecutive days.

CHILD	DAY 1			DAY 2			DAY 3		
	09.30hr	12.00hr	15.30hr	09.30hr	12.00hr	15.30hr	09.30hr	12.00hr	15.30hr
A	7.301	7.613	7.544	7.634	7.663	7.663	7.080	7.447	7.699
B	7.279	7.431	7.532	7.663	7.477	7.613	7.432	7.114	7.505
C	7.826	7.716	7.505	7.764	7.806	7.477	7.415	7.231	7.785
D	8.079	7.964	7.708	7.845	7.914	7.892	7.343	7.690	7.708
E	7.580	7.663	7.623	7.332	7.771	7.532	6.764	7.000	7.000
F	7.699	7.919	7.623	8.114	7.764	7.580	7.764	7.833	7.813
G	8.000	8.041	8.079	8.146	8.146	7.892	7.756	7.756	8.041
H	7.519	7.343	7.432	7.477	7.380	7.505	7.176	7.080	7.204
I	7.672	7.724	7.732	7.623	7.716	7.845	7.863	7.580	7.477
J	7.771	7.875	8.000	8.000	7.764	7.982	7.875	8.079	7.929

Table 3.5 Logarithmic Veillonella spp. counts for ten children on nine occasions; three times daily (at 09.30hr, 12.00hr and 15.30hr), on three consecutive days.

times, for either lactobacilli or candida, at a $p < 0.05$ level. For lactobacilli, the means of the logarithmic results of all ten children are shown graphically in Fig. 3.5. In addition, the magnitude of variation of three sample subjects is also demonstrated. Similar results, with the means of the logarithmic candidal counts of only the five children with candida present, and the actual logarithmic counts for three sample children, are shown in Fig. 3.6, again indicating the variation in sample counts from these children.

The logarithmic Strep. mutans counts were also compared, and again no significant differences between the repeat days were found. However, there were significant differences, at a $p < 0.05$ level, in the results from different times of day. When the mean results were examined graphically, as shown in Fig. 3.7, these differences appeared to be due to higher counts from the first sample on the second day. However this pattern was not repeated on the other two days.

When the logarithmic veillonella counts were compared, no significant differences were found at different times of day, but there were significant differences, at a $p < 0.05$ level, in the counts repeated on different days. Again, when the mean results were plotted graphically as shown in Fig. 3.8, the results for Day 3 appeared to be different from those from the other two days, with the results for the first two days being much more similar.

It was concluded that there was general stability in counts of lactobacilli, candida and veillonella, at different times of day, and that there was little variation in Strep. mutans counts, with the possible exception of those obtained early in the day. Consequently, it was decided that it made little difference at what time in the school day samples were obtained.

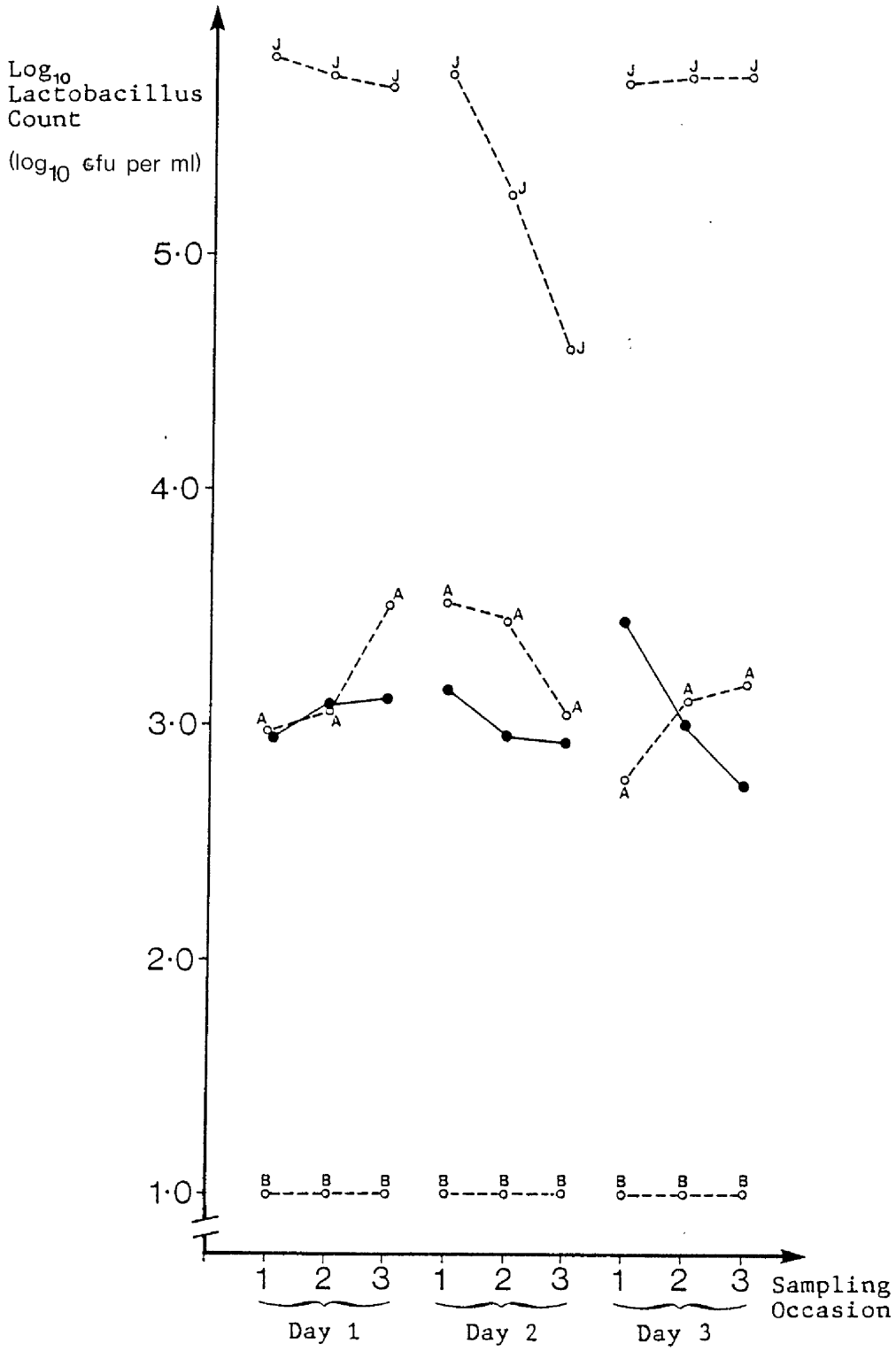


Figure 3.5 Logarithmic lactobacillus counts at three times on three days. Mean logarithmic counts (•) of all ten subjects, and actual logarithmic counts for three sample children, A, B and J.

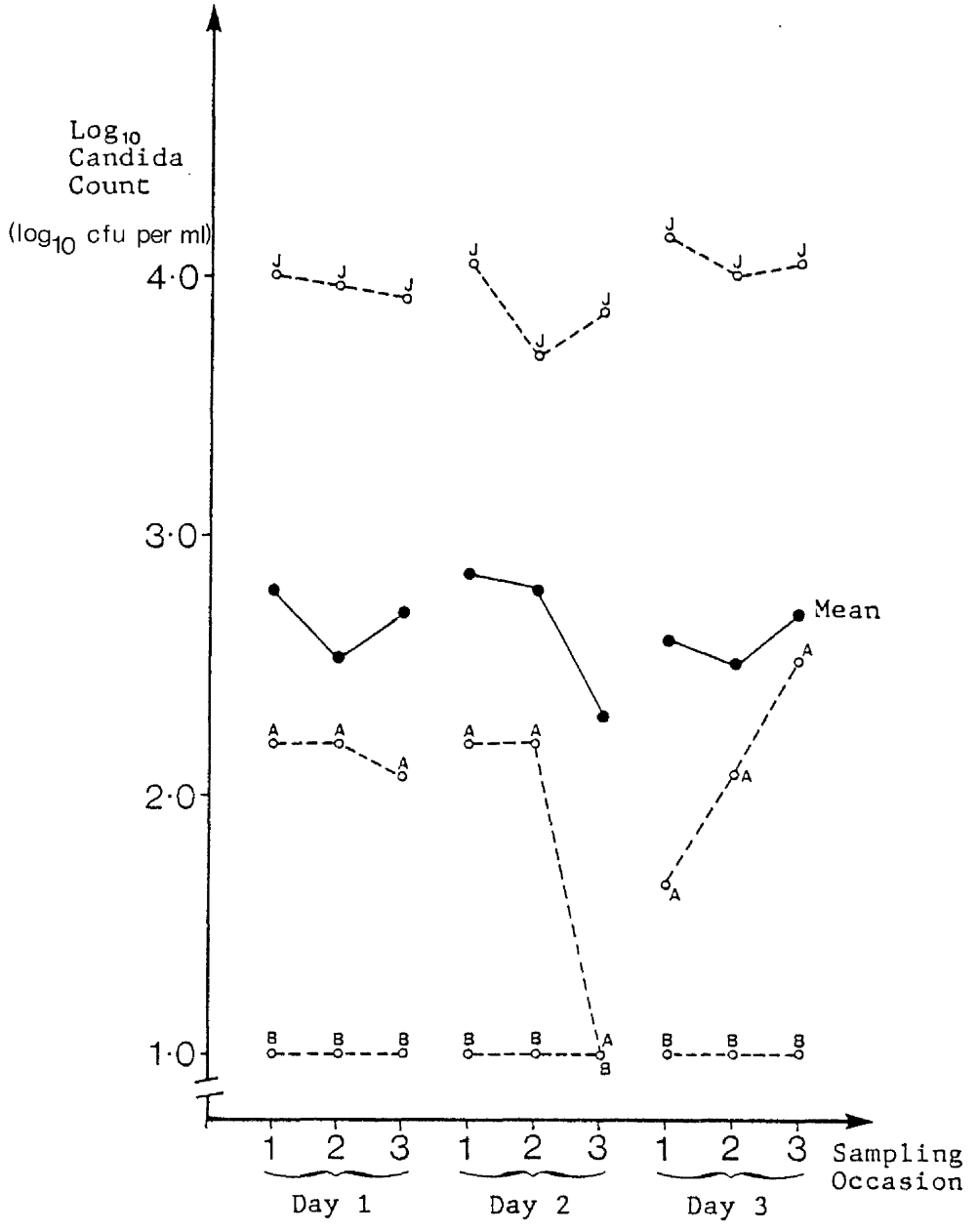


Figure 3.6 Logarithmic candida counts at three times on three days. Mean logarithmic counts (•) of all ten subjects, and actual logarithmic counts for three sample children, A, B and J.

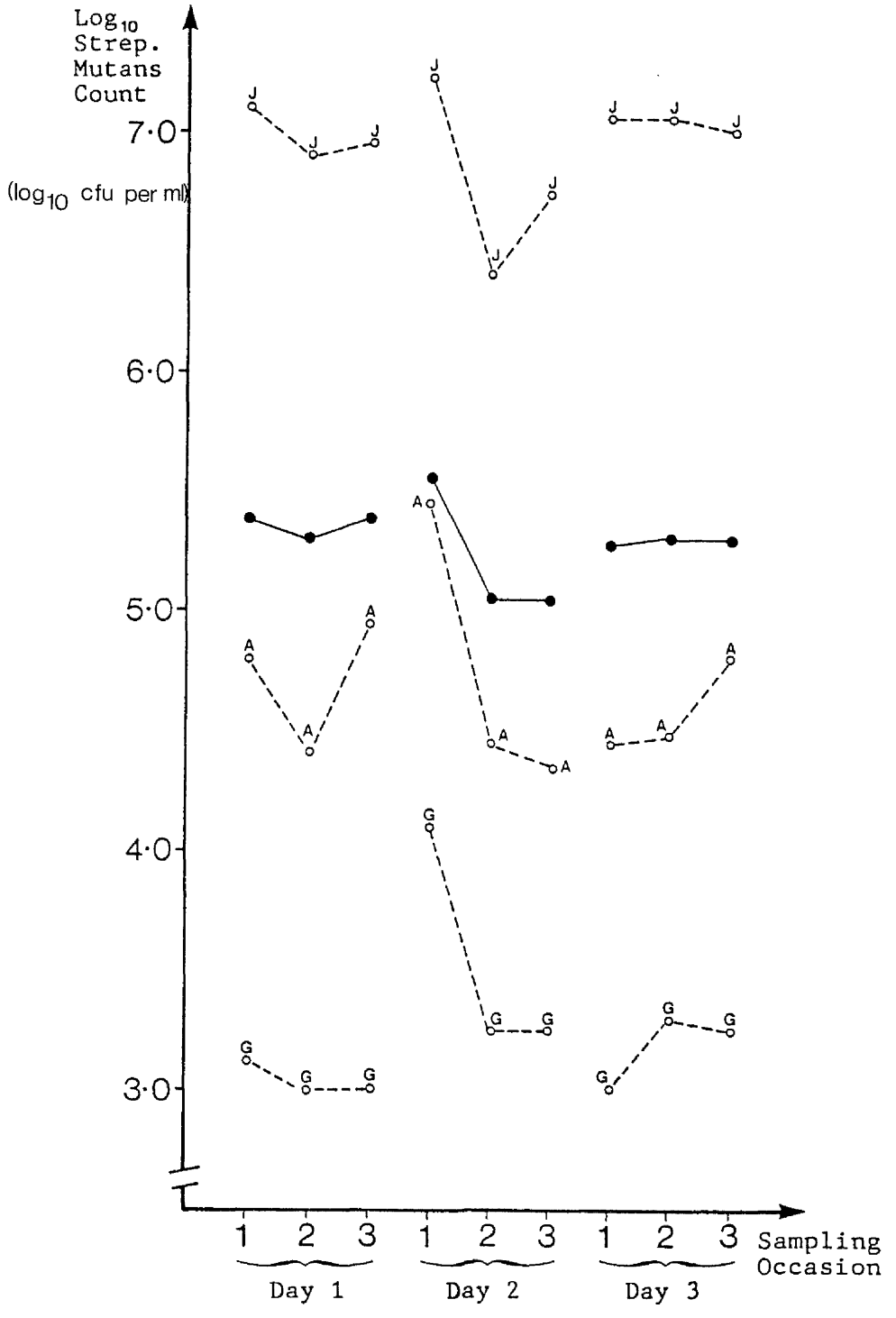


Figure 3.7 Logarithmic Strep. mutans counts at three times on three days. Mean logarithmic counts (●) of all ten subjects, and actual logarithmic counts for three sample children, A, G and J.

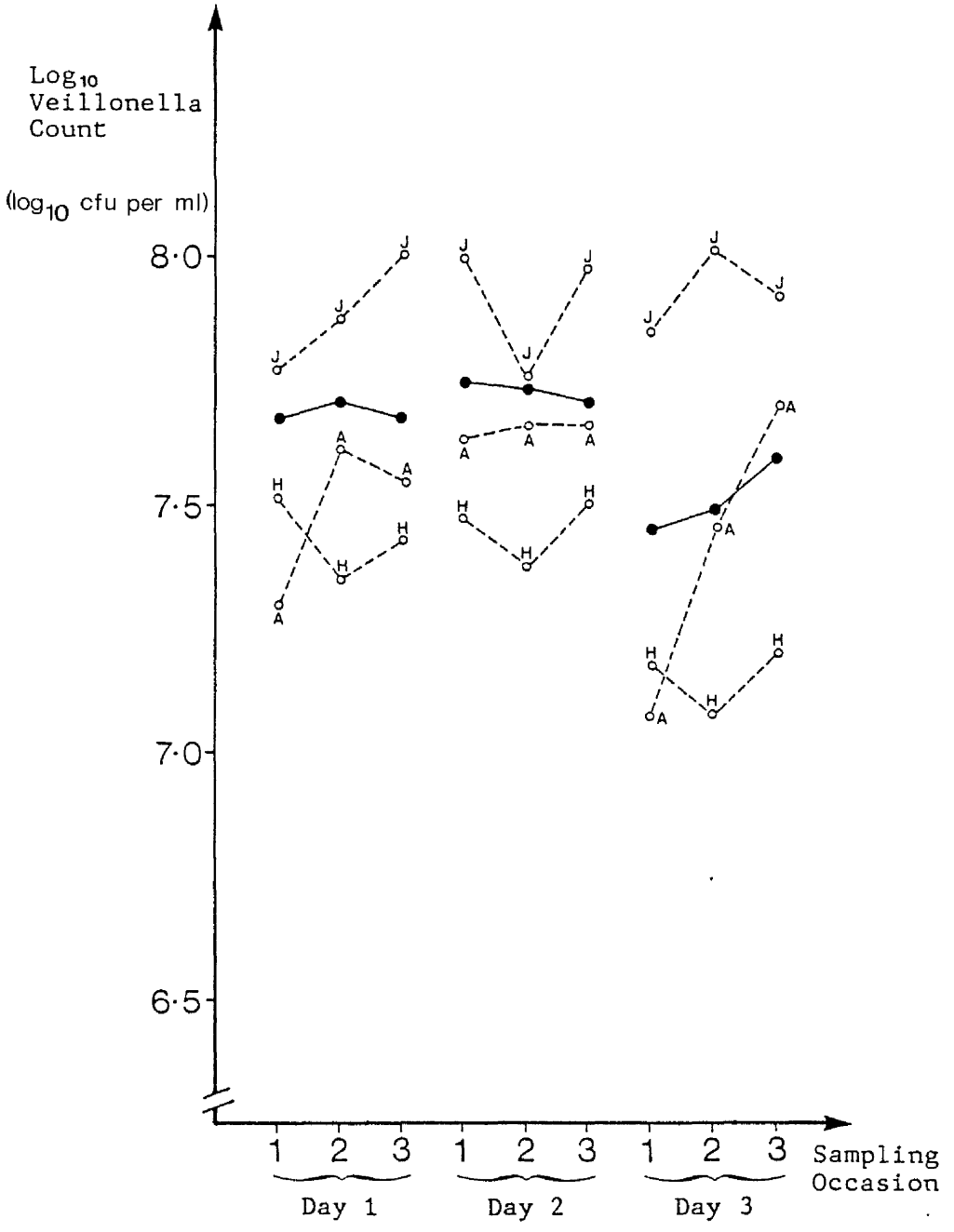


Figure 3.8 Logarithmic veillonella counts at three times on three days. Mean logarithmic counts (•) of all ten subjects, and actual logarithmic counts for three sample children, A, H and J.

3.3.4 Results of investigations into the effect of eating

The means and standard deviations of the ten counts at each sampling occasion were calculated. Those for lactobacilli are shown graphically in Fig. 3.9, those for Strep. mutans in Fig. 3.10, those for veillonella in Fig. 3.11, and those for the five children with candida present in Fig. 3.12.

Using paired t-tests with a Bonferroni correction for two comparisons and an overall level of significance of 0.05, the differences in the microbiological counts, firstly between the 09.30 hours and 09.45 hours samples, and, secondly between the 09.30 hours and 10.40 hours samples were compared for any effect of eating potato crisps (Day 1 results compared with those of Day 2), and also for any effect of drinking cola (Day 1 results compared with those of Day 3). No significant differences were found as a result of eating potato crisps or drinking cola for lactobacilli, Strep. mutans or candida. The only significant difference was found for the effect of potato crisps on the difference in veillonella counts between the 09.30 hours and 09.45 hours samples. Although this difference was small, it was significant because the change in counts from the 09.30 hours sample without food decreased, whereas it increased after eating crisps. However, when the actual logarithmic counts were compared, also using multiple t-tests, they were not found to differ significantly.

It was concluded that the consumption of such items as potato crisps or cola, prior to sampling, made little difference to sample counts.

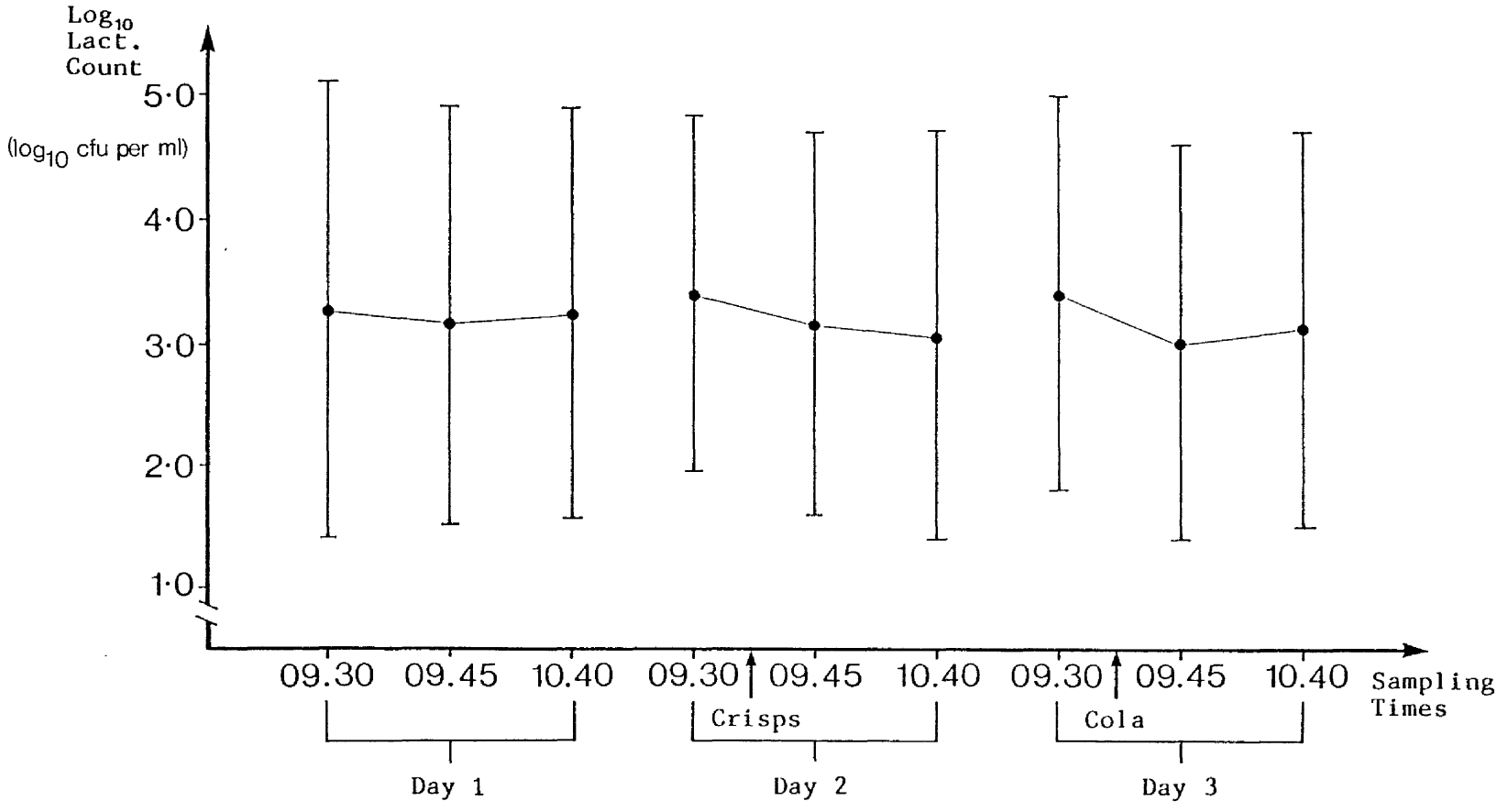


Figure 3.9 Mean ± one standard deviation of logarithmic lactobacillus counts from ten individuals. Influence of potato crisps and cola.

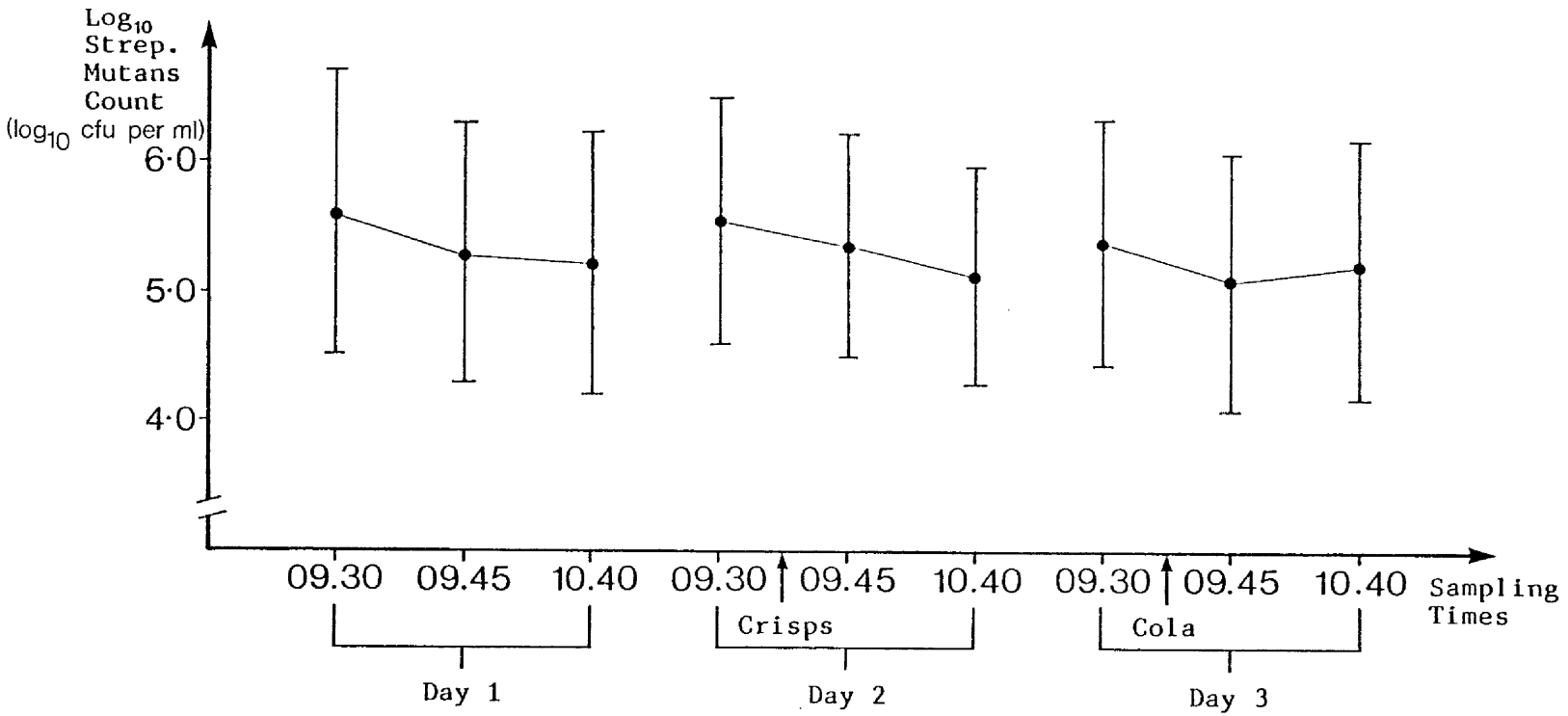


Figure 3.10 Mean ± one standard deviation of logarithmic Strep. mutans counts from ten individuals. Influence of potato crisps and cola.

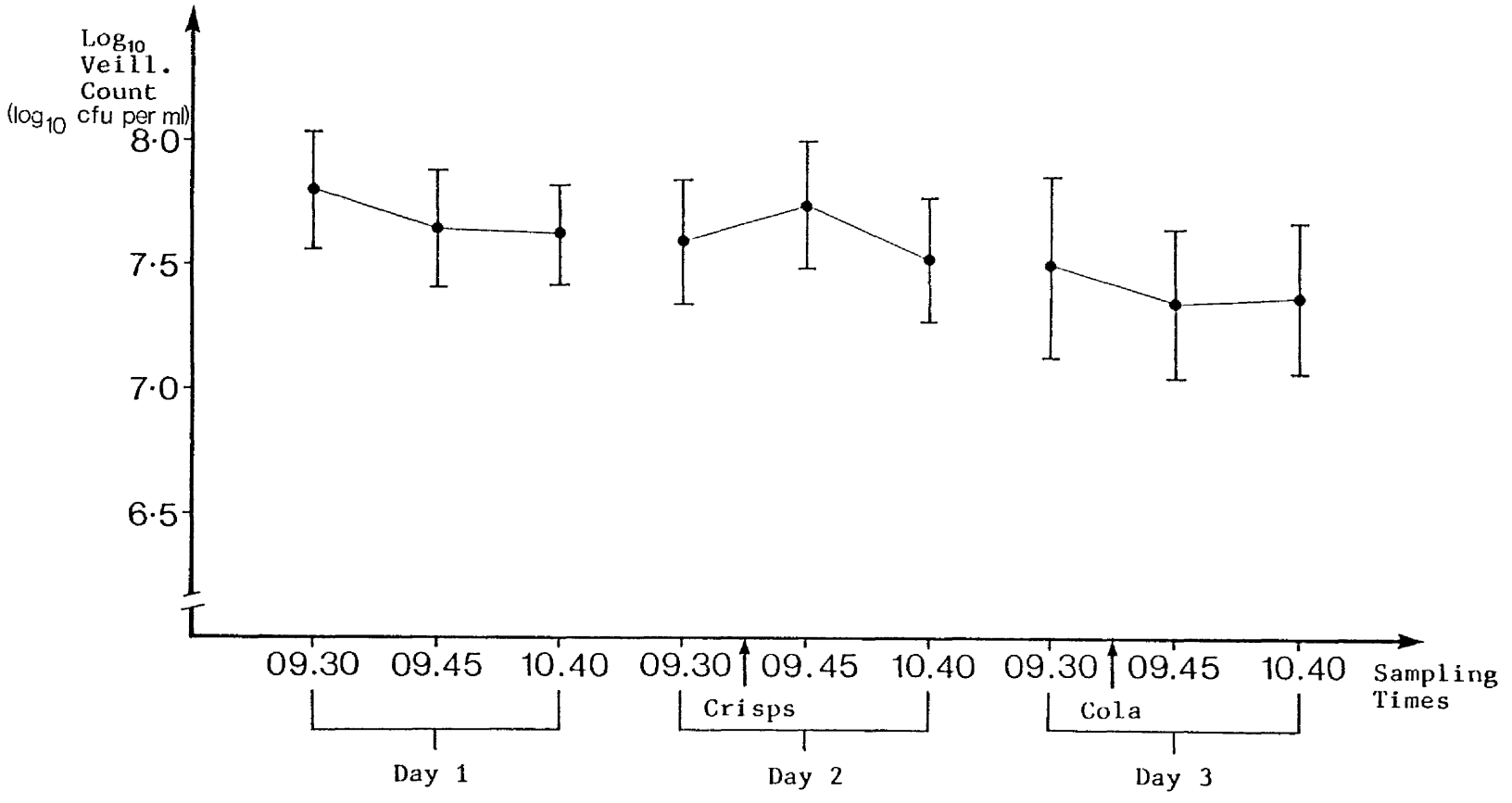


Figure 3.11 Mean ± one standard deviation of logarithmic veillonella counts from ten individuals. Influence of potato crisps and cola.

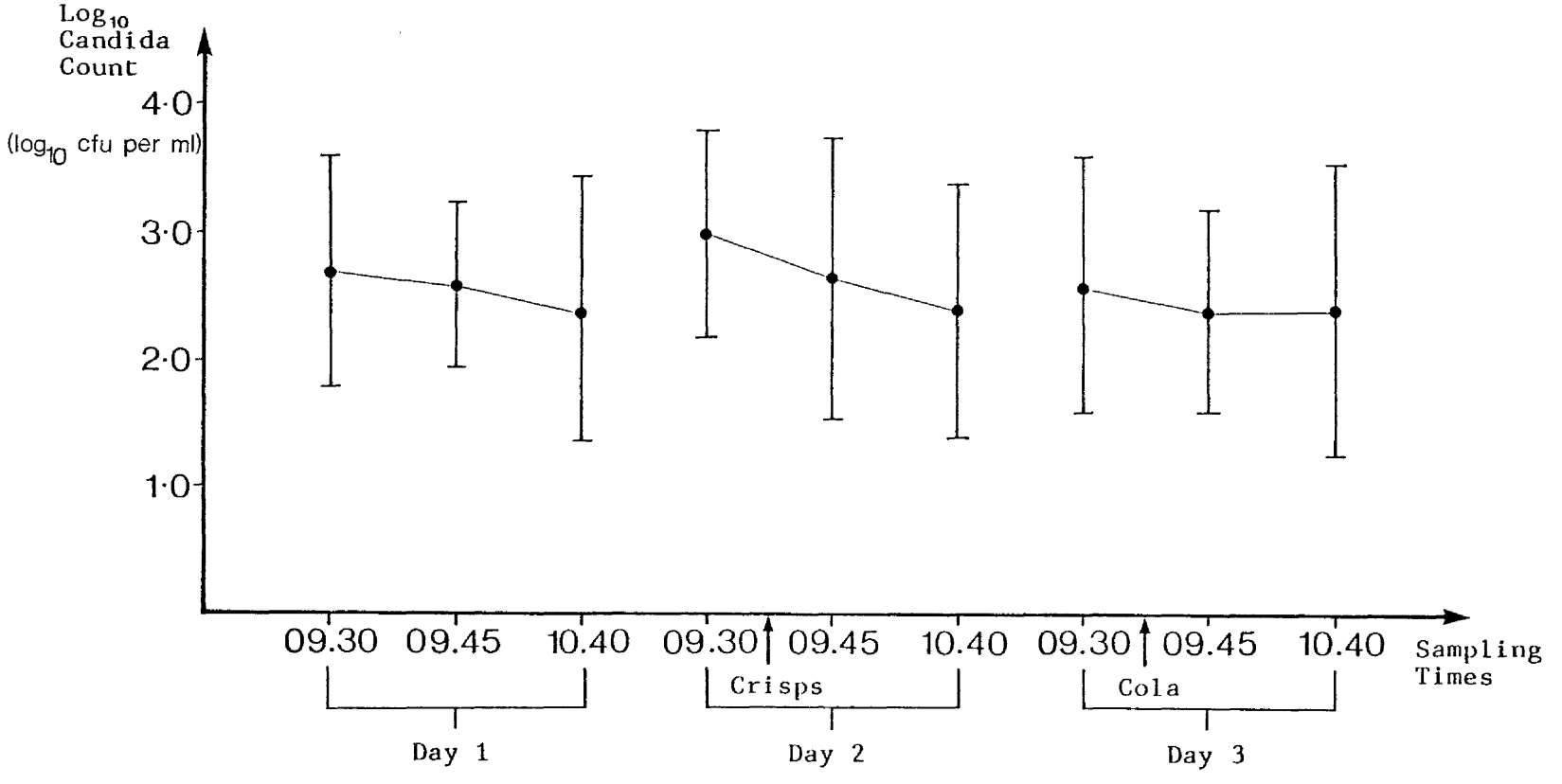


Figure 3.12 Mean ± one standard deviation of logarithmic candida counts from five individuals (those of the ten with candida present). Influence of potato crisps and cola.

3.3.5 Results of investigations into the variation in counts from samples obtained at monthly intervals

For each micro-organism studied, the mean logarithmic count for the children present was calculated for each of the 29 monthly sampling occasions. These were then plotted against the sampling occasions, over the 2.5 year period. A summary of the data for lactobacilli is shown in Fig. 3.13, for Strep. mutans in Fig. 3.14, for veillonella in Fig. 3.15, and for candida in Fig. 3.16. The zero candidal counts for the four children who at no time had candida present, were excluded from this analysis, and therefore Fig. 3.16 contains data for a maximum of six children.

Obvious repeating seasonal fluctuations or trends over the 2.5 year period could not be demonstrated for any of the four micro-organisms investigated.

The logarithmic microbiological counts for each of the ten individuals were also plotted and this information is shown in Fig. 3.17 to Fig. 3.26. Any breaks in the continuity of these graphs are due to the absence of an individual on a sampling occasion. The caries prevalence data at each of the six-monthly clinical examinations are also shown in these figures. No child in this group had any permanent teeth extracted due to caries, therefore the caries prevalence is presented as the number of decayed, and filled surfaces (DFS) for that individual. Furthermore, in view of the possible associations between microbiological counts and the number of open carious lesions, the total numbers of carious surfaces, including both permanent and deciduous teeth, are also shown.

The mean of the logarithmic microbiological counts over the 2.5 year period was also calculated for each individual and for each

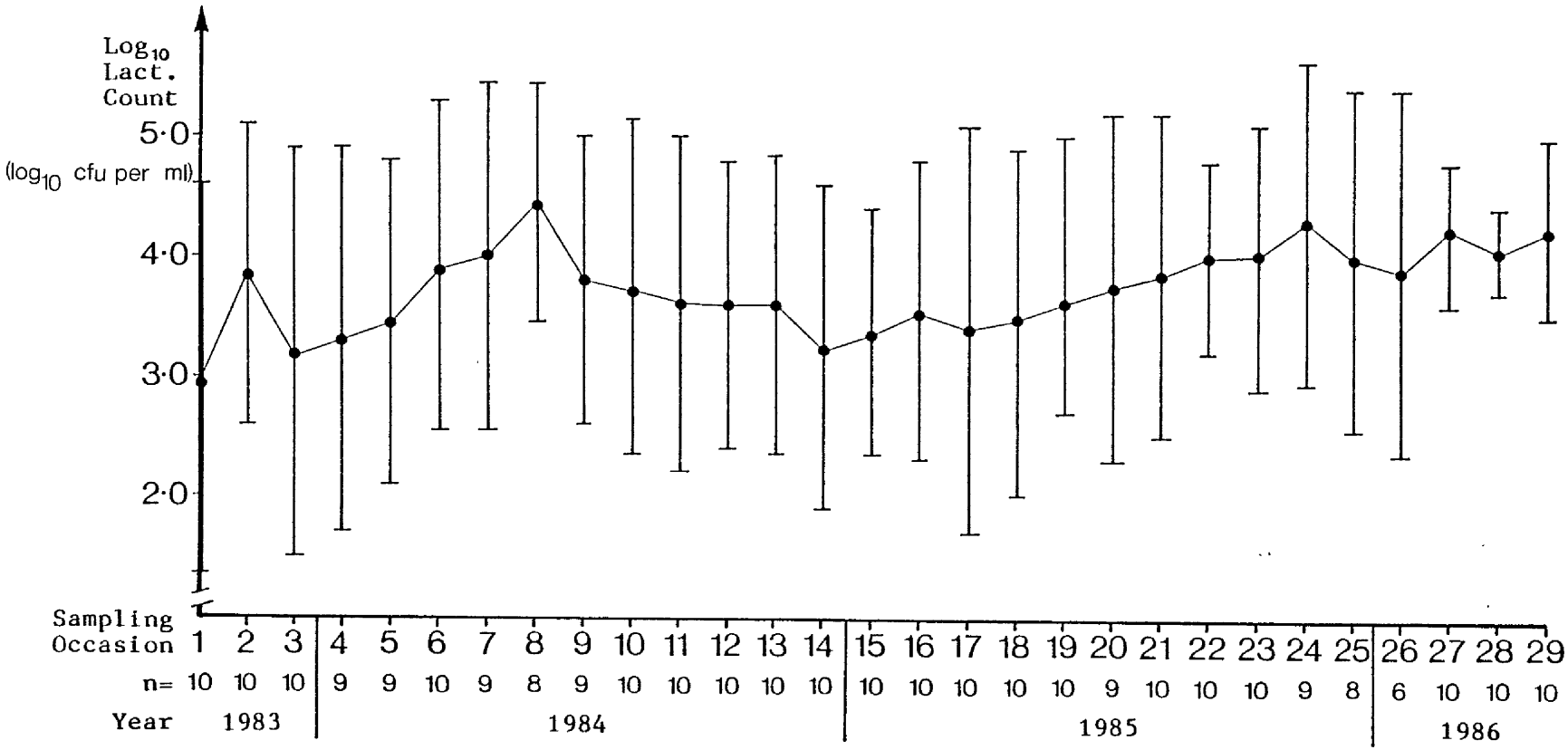


Figure 3.13 Mean ± one standard deviation of logarithmic lactobacillus counts from a maximum of ten individuals on 29 occasions over 2.5 years.

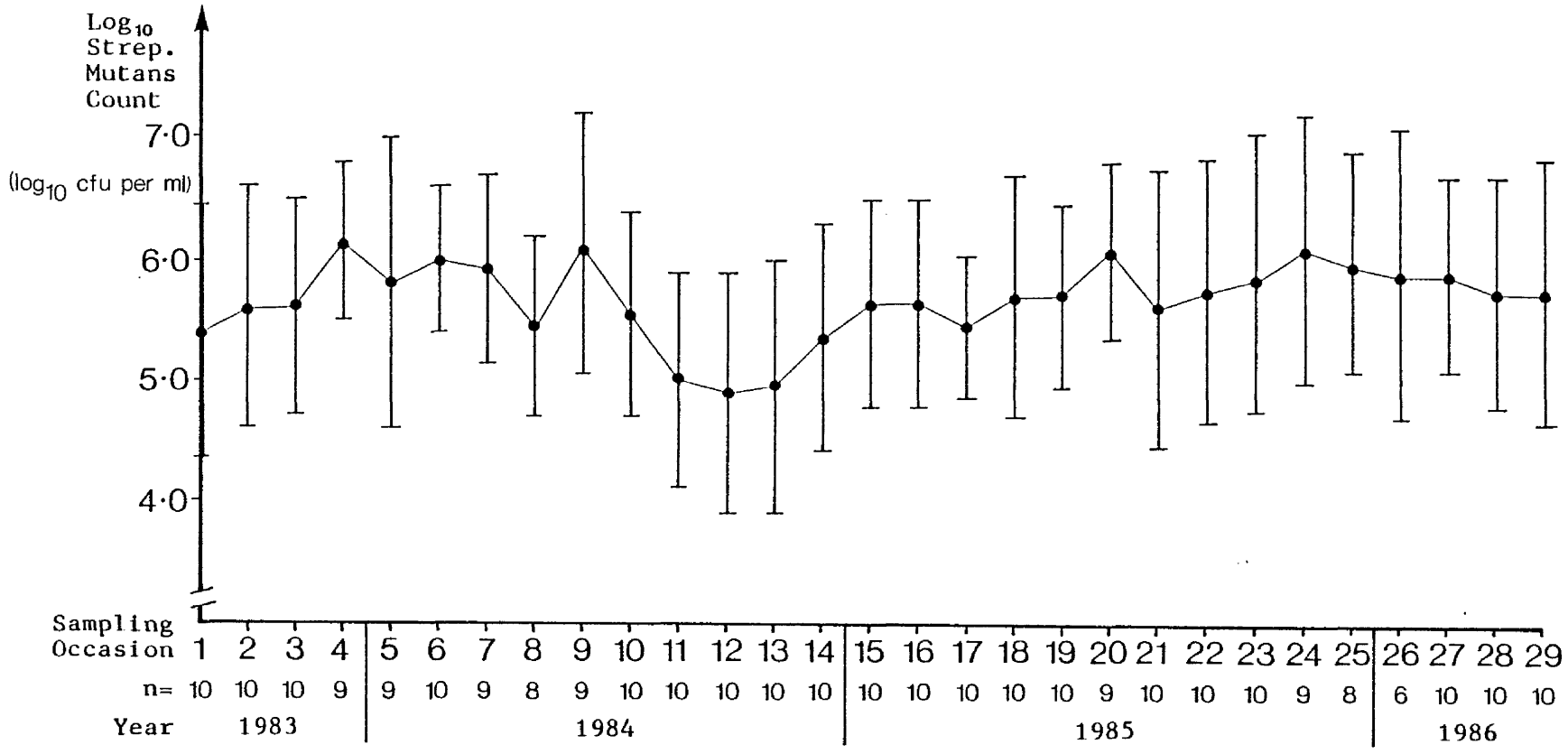


Figure 3.14 Mean ± one standard deviation of logarithmic Strep. mutans counts from a maximum of ten individuals on 29 occasions over 2.5 years.

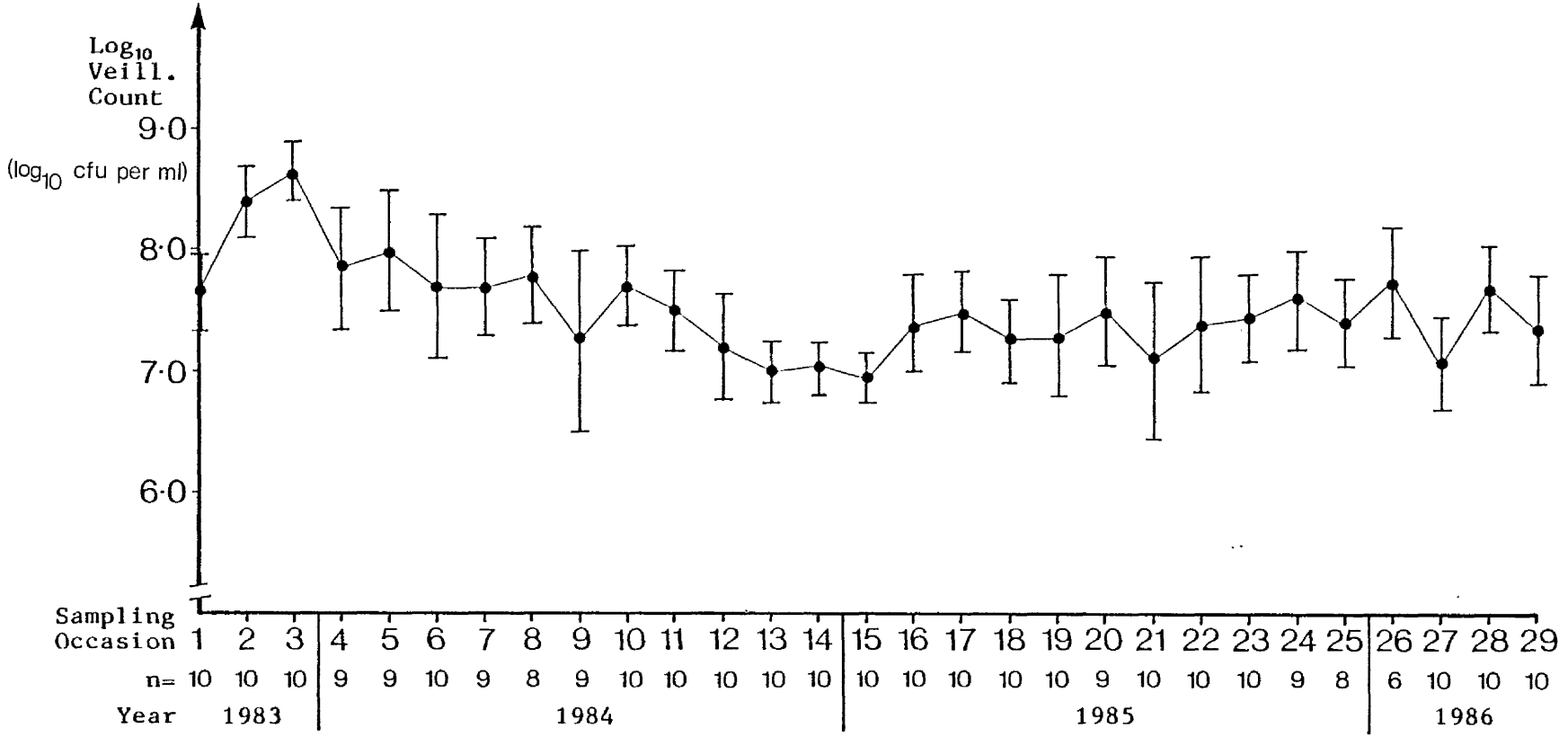


Figure 3.15 Mean $\pm$ one standard deviation of logarithmic veillonella counts from a maximum of ten individuals on 29 occasions over 2.5 years.

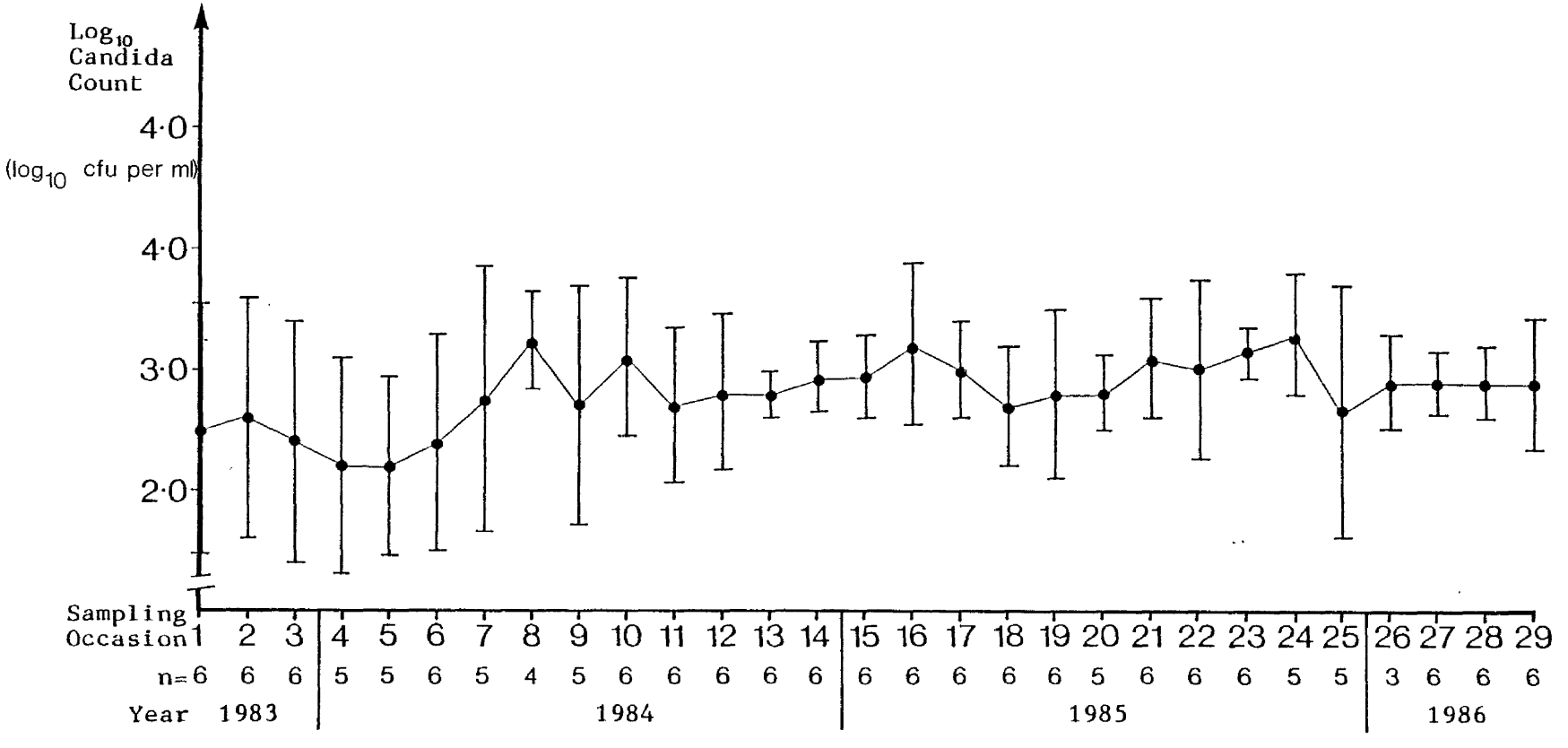


Figure 3.16 Mean ± one standard deviation of logarithmic candida counts from a maximum of six individuals on 29 occasions over 2.5 years.

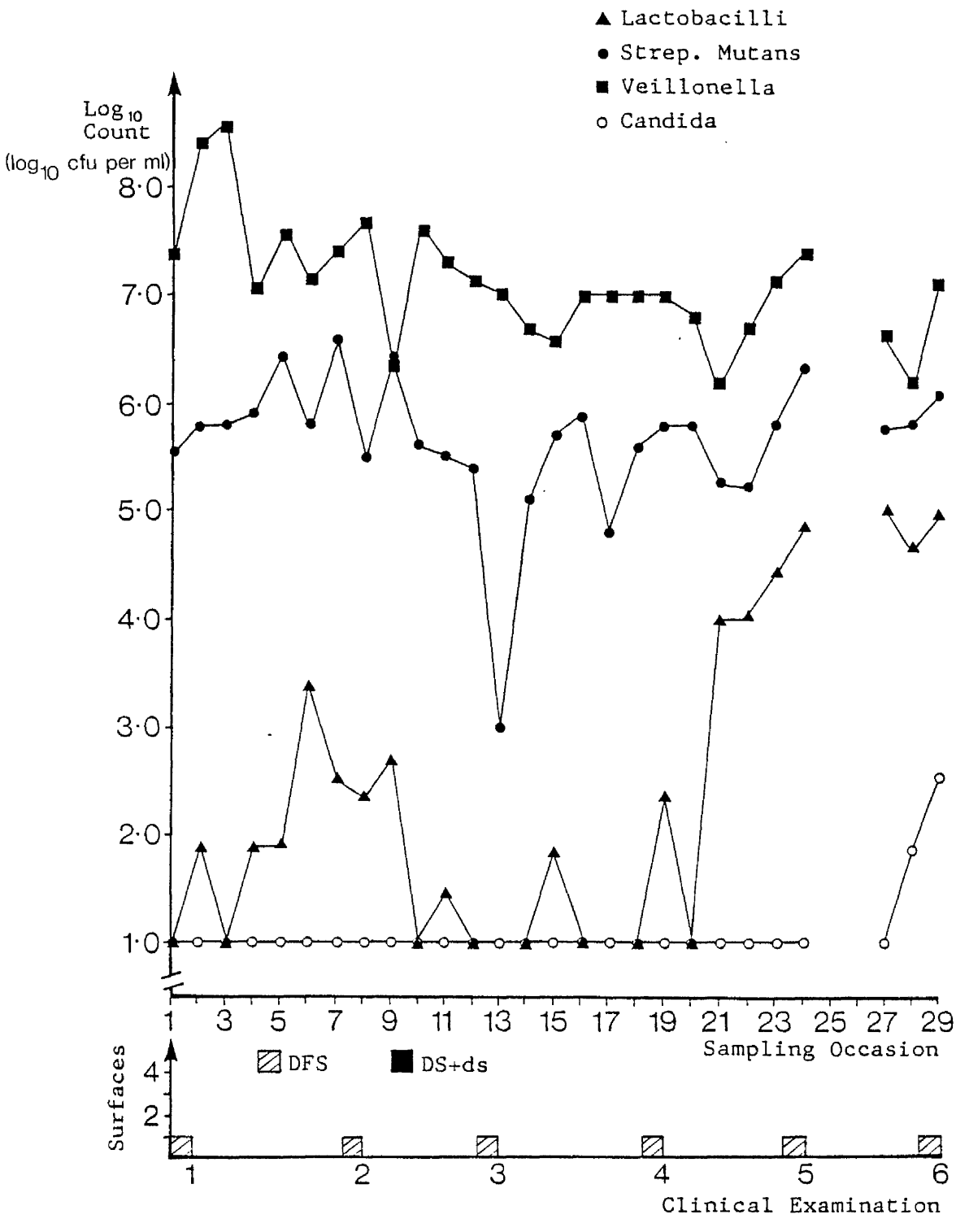


Figure 3.17 Logarithmic microbiological counts and caries prevalence data over 2.5 years for child "B".

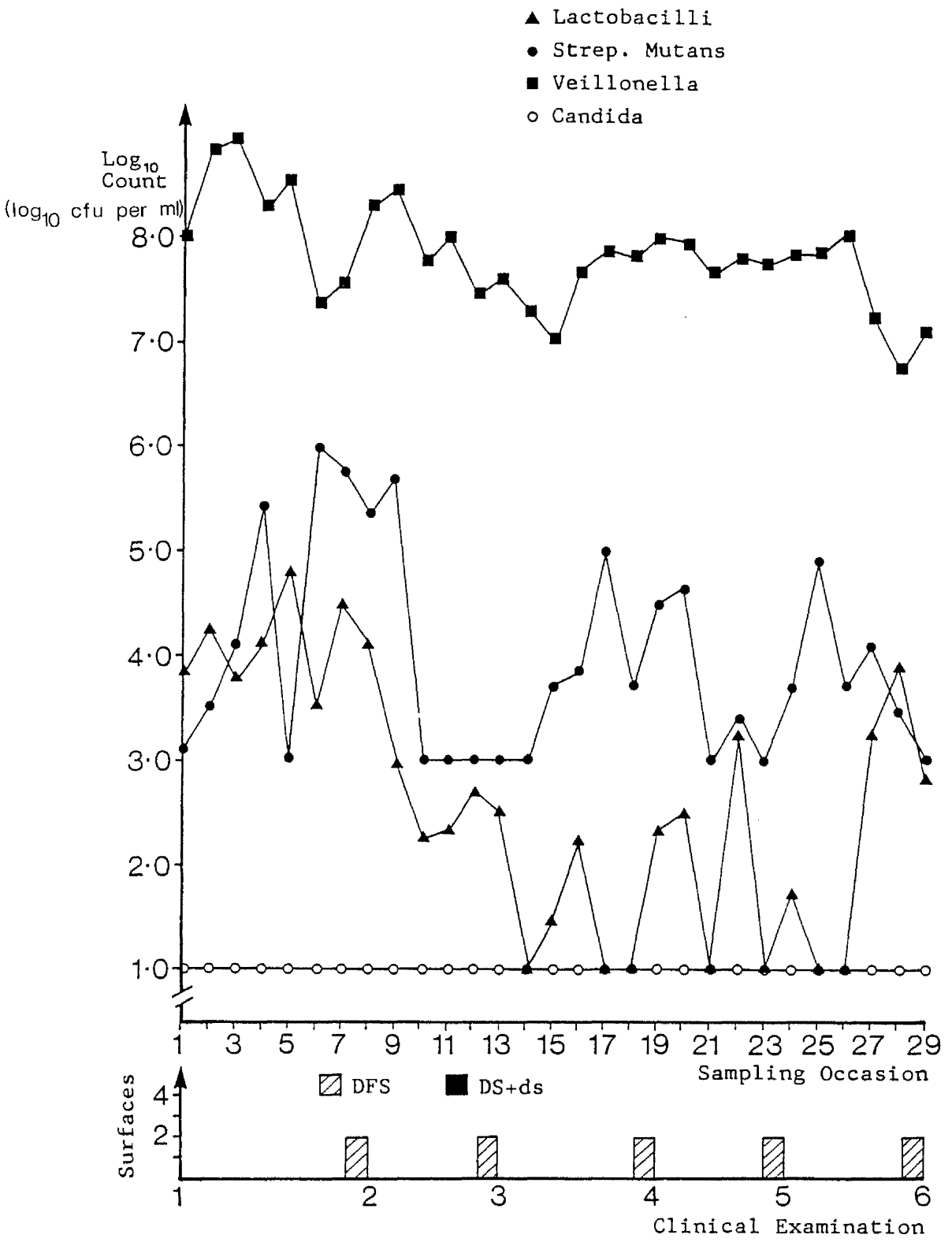


Figure 3.18 Logarithmic microbiological counts and caries prevalence data over 2.5 years for child "G".

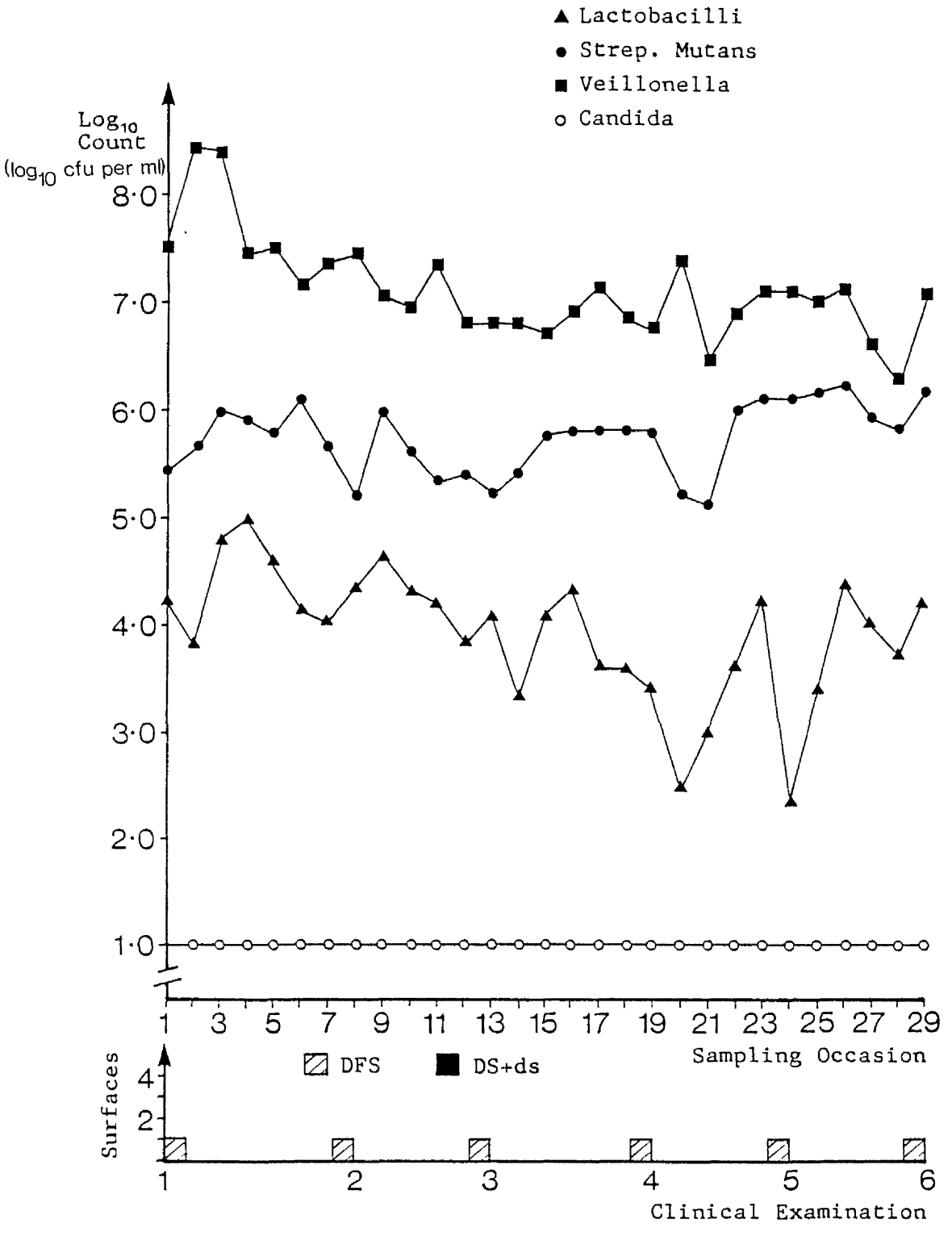


Figure 3.19 Logarithmic microbiological counts and caries prevalence data over 2.5 years for child "H".

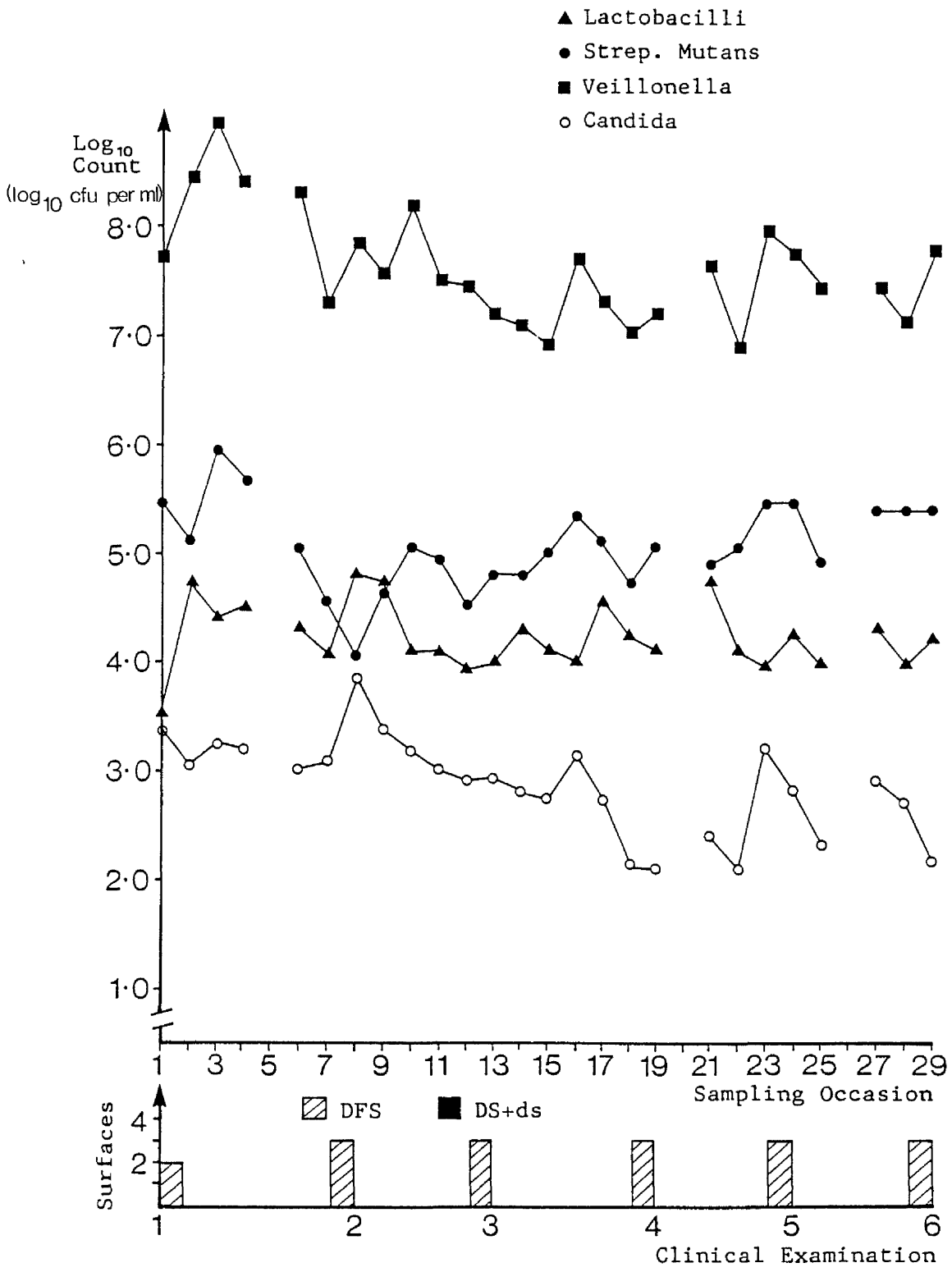


Figure 3.20 Logarithmic microbiological counts and caries prevalence data over 2.5 years for child "I".

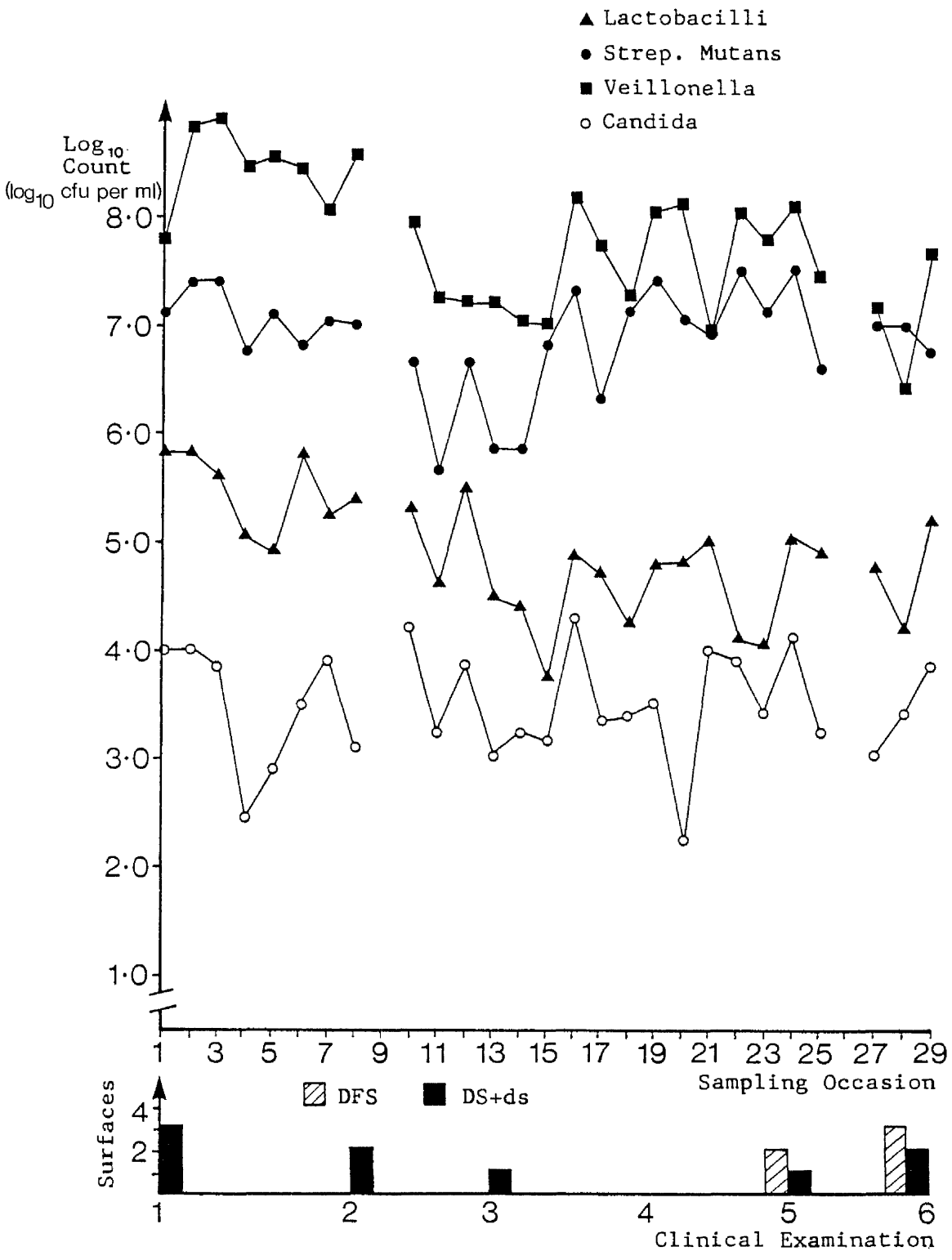


Figure 3.21 Logarithmic microbiological counts and caries prevalence data over 2.5 years for child "J".

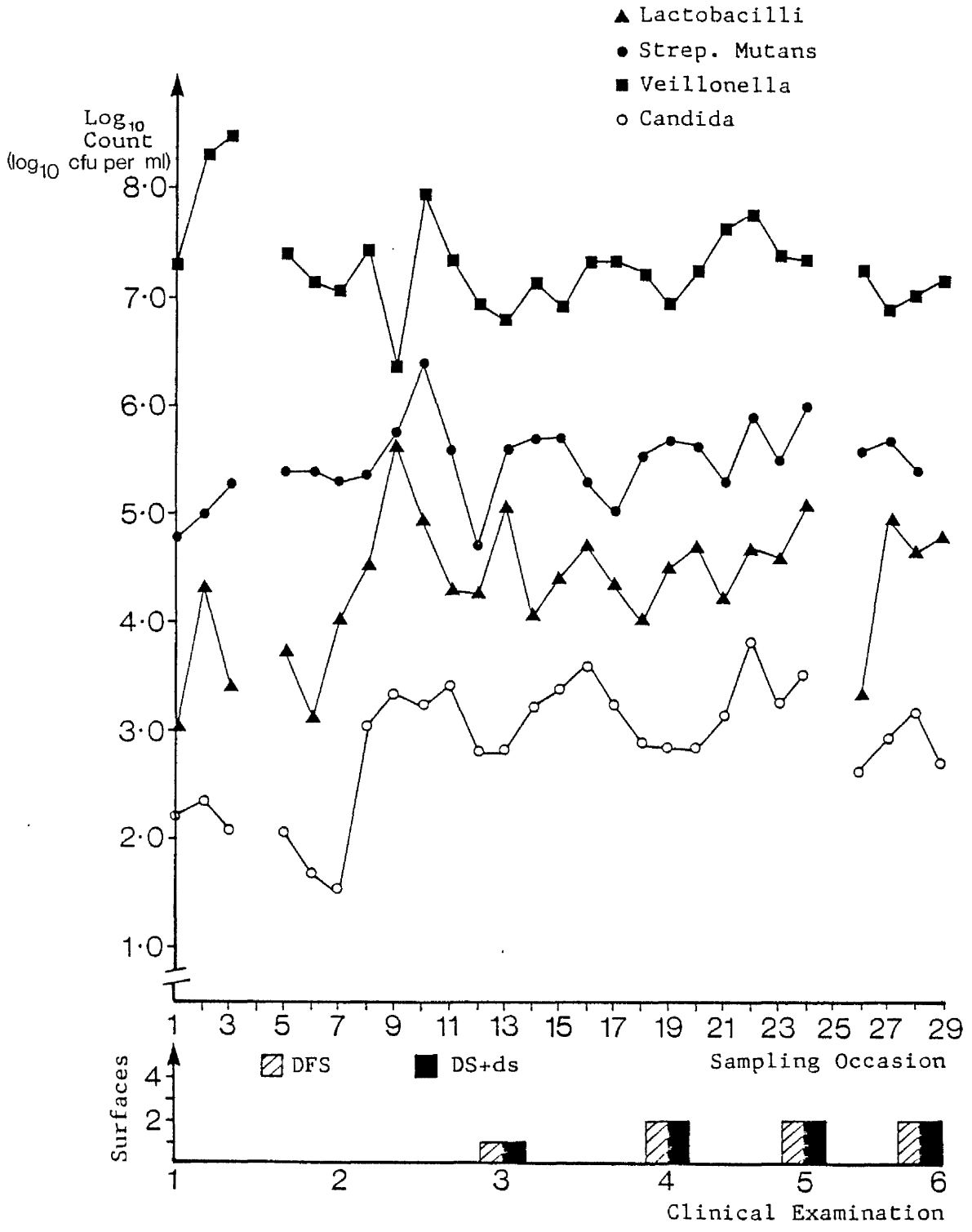


Figure 3.22 Logarithmic microbiological counts and caries prevalence data over 2.5 years for child "A".

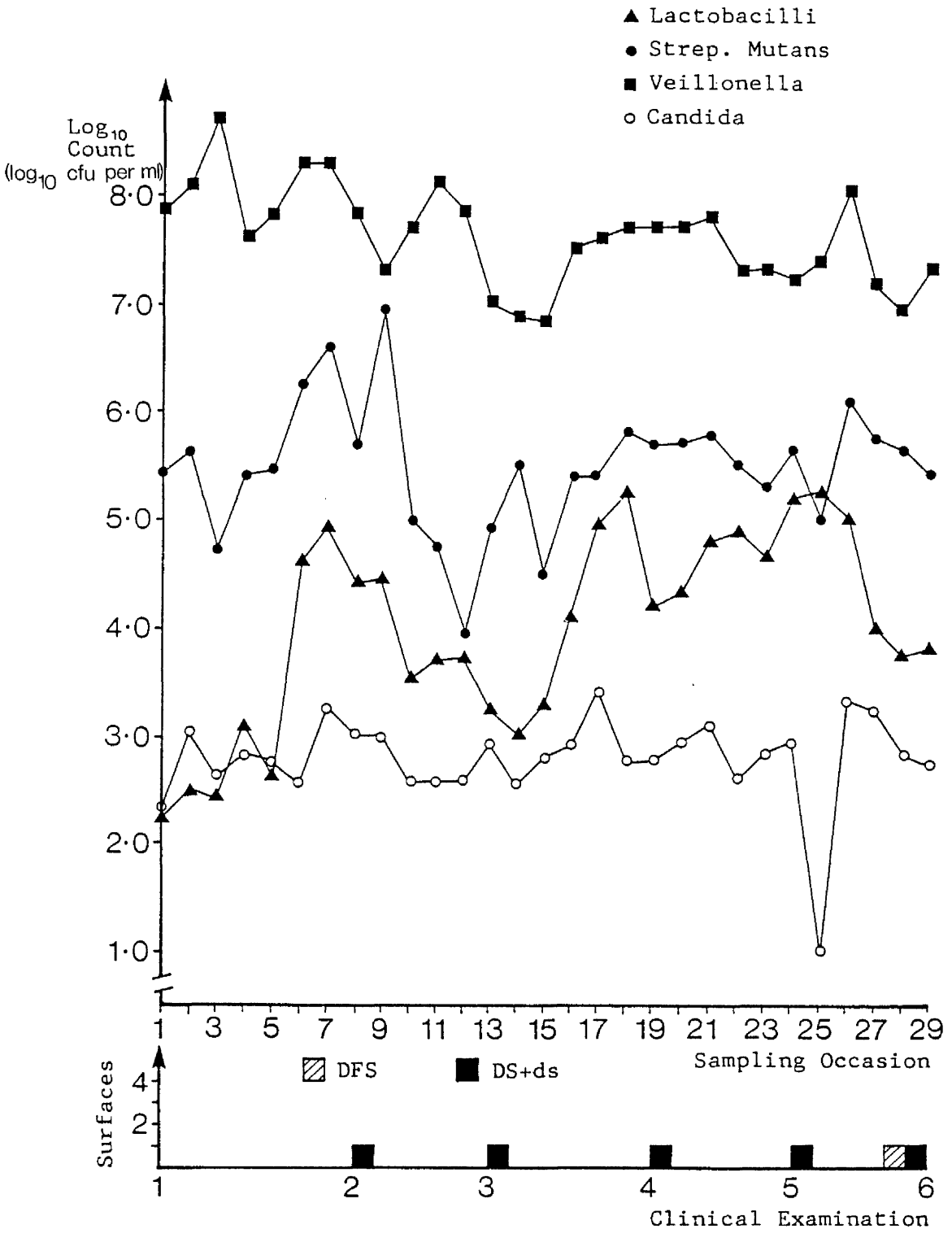


Figure 3.23 Logarithmic microbiological counts and caries prevalence data over 2.5 years for child "C".

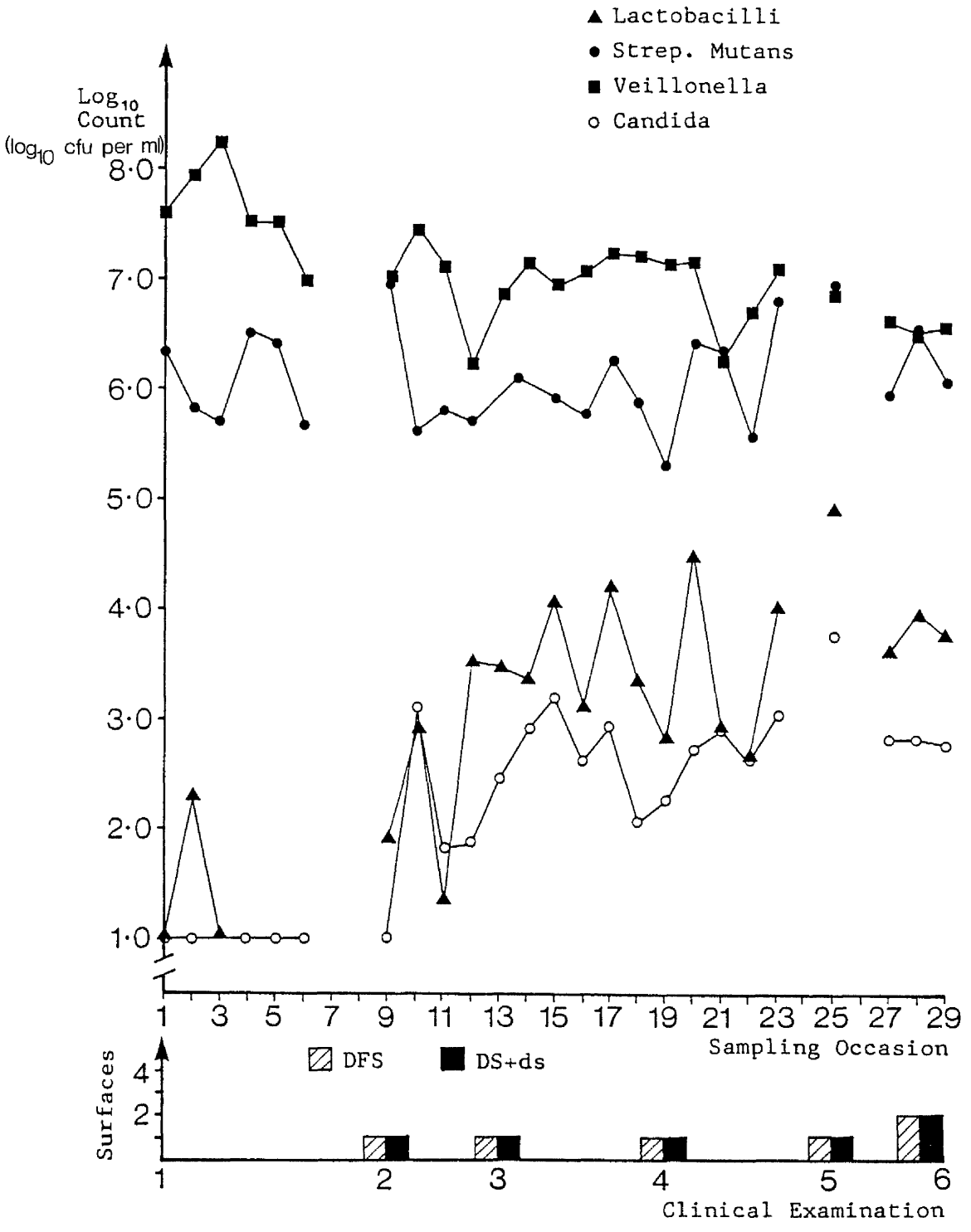


Figure 3.24 Logarithmic microbiological counts and caries prevalence data over 2.5 years for child "E".

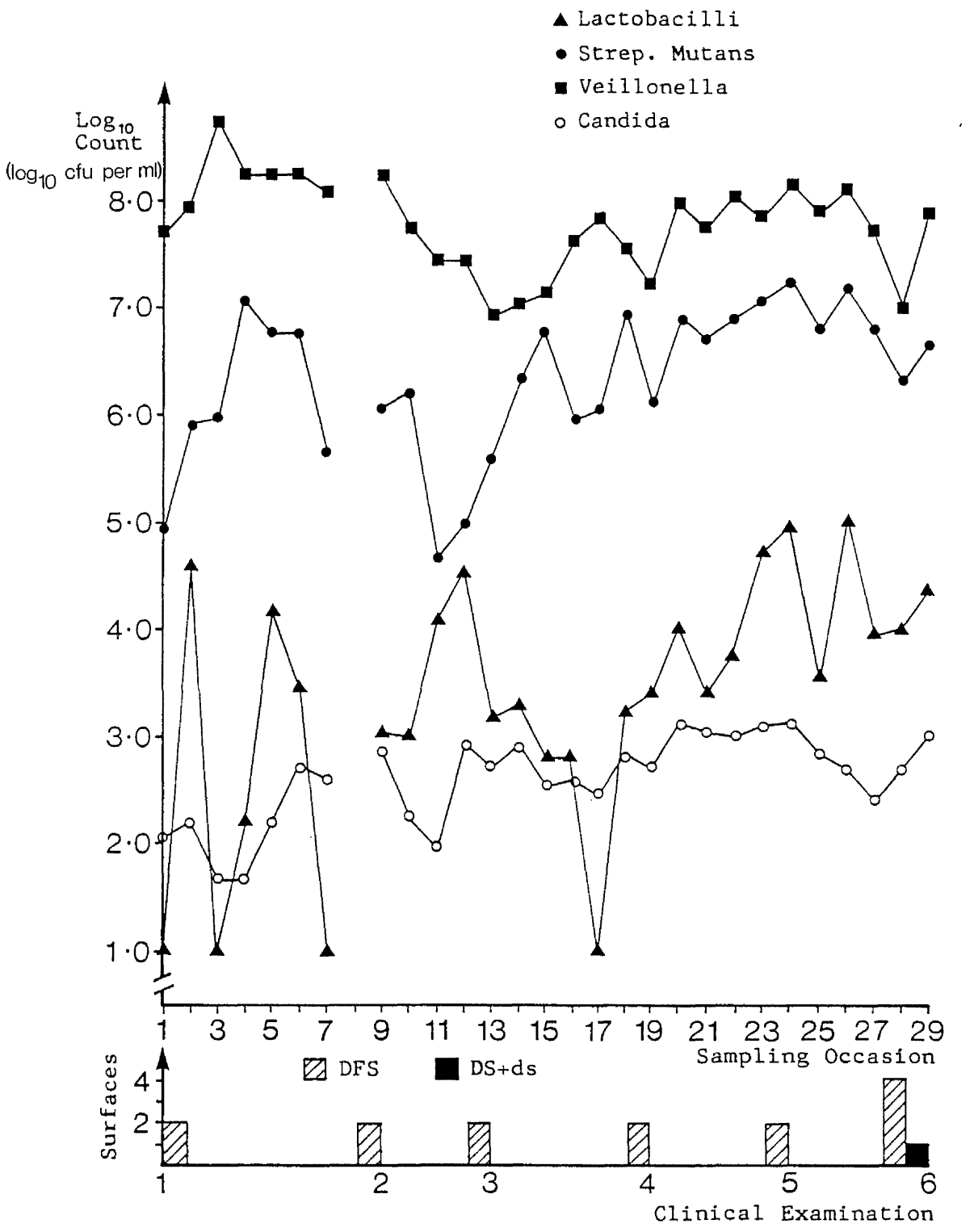


Figure 3.25 Logarithmic microbiological counts and caries prevalence data over 2.5 years for child "F".

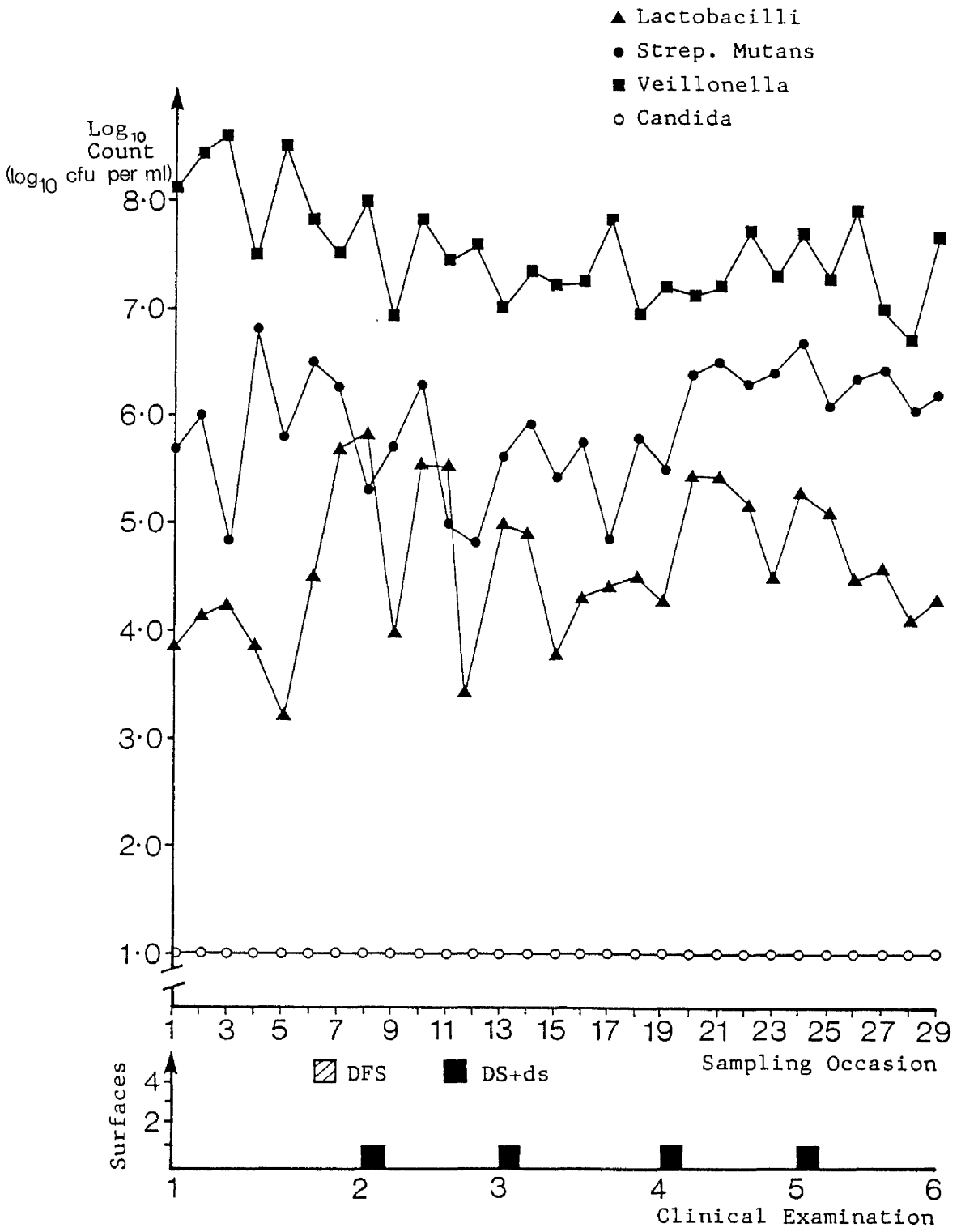


Figure 3.26 Logarithmic microbiological counts and caries prevalence data over 2.5 years for child "D".

micro-organism studied. These are shown in Table 3.6. Both the mean logarithmic counts and the initial logarithmic counts were then correlated with the caries increments over the 2.5 year period. As shown in Table 3.7, only the mean logarithmic candidal count was significantly correlated at a $p < 0.05$ level with the DFS increment.

Three of the individuals, "B", "G" and "H" (Fig. 3.17 to Fig. 3.19), who had no open carious lesions at any of the clinical examinations, had zero or very low candidal counts. Child "G" also had low lactobacillus and Strep. mutans counts, but received two occlusal restorations between the first and second clinical examinations, which resulted in a caries increment during the study period. However, the fourth subject who had no open carious lesions, child "I" (Fig. 3.20), and who also had a positive caries increment, had high candidal counts and medium lactobacillus and Strep. mutans counts.

The remaining six children had carious lesions present at one or more clinical examinations. Of these, child "J" (Fig. 3.21), had lesions visible at all but one of the clinical examinations, and had high levels of all four micro-organisms investigated. In the four children "A", "C", "E" and "F" (Fig. 3.22 to Fig. 3.25), the development of caries appeared to be associated with increases in lactobacillus and candidal counts. The remaining subject, child "D" (Fig. 3.26), developed one deciduous lesion and the tooth was then exfoliated during the study period. This individual had zero candidal counts but fairly stable medium to high counts of lactobacilli and Strep. mutans.

A range of veillonella counts existed for all subjects, and there

Child	2.5 yr Δ DFS	Mean logarithmic counts (cfu per ml)			
		Lactobac-illus spp.	Strep. mutans	Candida spp.	Veillon-ella spp.
A	2	4.35	5.48	2.87	7.31
B	0	2.34	5.75	1.01	7.12
C	1	3.99	5.48	2.78	7.62
D	0	4.61	5.91	1.00	7.54
E	2	2.87	6.09	2.22	7.07
F	2	3.35	6.34	2.60	7.78
G	2	2.62	3.96	1.00	7.82
H	0	3.93	5.74	1.00	7.11
I	1	4.28	5.07	2.87	7.62
J	3	4.91	6.89	3.51	7.78

Table 3.6 Increments of decayed and filled surfaces, and means of the logarithmic microbiological counts, for ten children, over a 2.5 year period.

Pearson's correlation coefficient between	2.5yr Δ DFS	Pearson's correlation coefficient between	2.5yr Δ DFS
mean $\log_{10}$ *Lactobacillus* spp.	0.11	initial $\log_{10}$ *Lactobacillus* spp.	0.16
mean $\log_{10}$ *Strep. mutans*	0.16	initial $\log_{10}$ *Strep. mutans*	0.08
mean $\log_{10}$ *Candida* spp.	0.69*	initial $\log_{10}$ *Candida* spp.	0.53
mean $\log_{10}$ *Veillonella* spp.	0.47	initial $\log_{10}$ *Veillonella* spp.	0.09

* = $p < 0.05$

Table 3.7 Pearson's correlation coefficients for ten children, between 2.5 year increment of decayed and filled surfaces, and both mean and initial logarithmic microbiological counts.

were no real patterns of association with the development of caries for this parameter. Nor were any obvious differences between children noted.

For each micro-organism studied, pooled estimates of the common standard deviations were calculated both within and across individuals. These are shown in Table 3.8. In the case of the monthly samples for three of the micro-organisms investigated, lactobacilli, Strep. mutans and candida, the pooled estimate of the standard deviations within subjects was less than that across subjects. For veillonella, however, the common standard deviation within subjects was greater than that across subjects. For Strep. mutans, candida and veillonella the common standard deviation within subjects was about 0.5 logarithmic units, but rather greater for lactobacilli, at 0.9 logarithmic units.

3.3.6 Results of investigation of effect of different caries rates

Table 3.9 shows the dental status of the two groups of children involved in this study. When the caries prevalence and incremental data for the two groups were compared, using Mann-Whitney U tests, it was found that although the initial DMFS scores and the initial numbers of carious surfaces differed significantly, there was no significant difference between the six month caries increments of the groups.

The means and standard deviations of the logarithmic lactobacillus, Strep. mutans, veillonella and candida counts at each sampling occasion, and for as many of the ten children who were present, are shown in Fig. 3.27 to Fig. 3.30. As in Section 3.3.4, the zero candidal counts for five children were excluded from this

INVESTIGATION	POOLED ESTIMATES OF n COMMON STANDARD DEVIATIONS							
	$\log_{10}$ Lactobacilli		$\log_{10}$ Strep. mutans		$\log_{10}$ Veillonella		$\log_{10}$ Candida	
	within subjects	across subjects	within subjects	across subjects	within subjects	across subjects	within subjects	across subjects
Variation in count within samples (Section 3.3.1)	0.08 n= 8		0.09 n=10		0.09 n=10		0.15 n= 6	
Variation in counts from repeated samples (Section 3.3.3)	0.61 n= 9	1.36 n= 9	0.31 n=10	0.98 n= 9	0.19 n=10	0.27 n= 9	0.36 n= 5	0.95 n= 9
Variation in counts from samples repeated monthly (Section 3.3.5)	0.90 n=10	1.24 n=29	0.53 n=10	0.93 n=29	0.51 n=10	0.39 n=29	0.52 n= 7	0.62 n=29

Table 3.8 Pooled estimates of common standard deviations, within subjects and across subjects, for the numbers of standard deviations shown.

	Low caries surfaces/child		High caries surfaces/child	
	mean	S.D.	mean	S.D.
DMFS (first examination)	0.6	0.84	4.9	3.93
Total No. of decayed surfaces (first examination)	0.3	0.95	2.6	2.46
Six-month DMFS increment	0.4	0.70	0.7	1.16

Table 3.9 Means and standard deviations of the caries prevalence and incremental data for ten low caries, and ten high caries children.

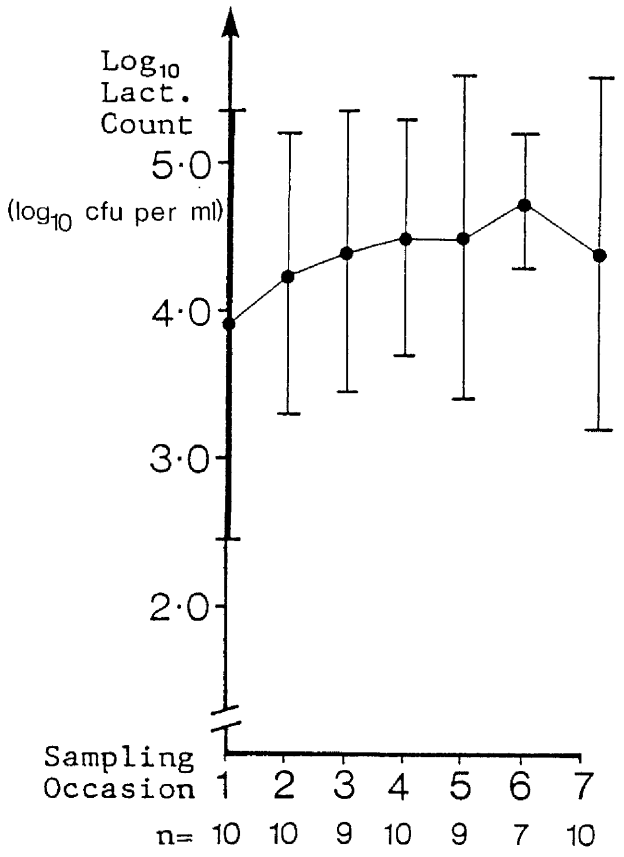


Figure 3.27 Mean ± one standard deviation of logarithmic lactobacillus counts from a maximum of ten individuals with high caries activity, on seven occasions over six months.

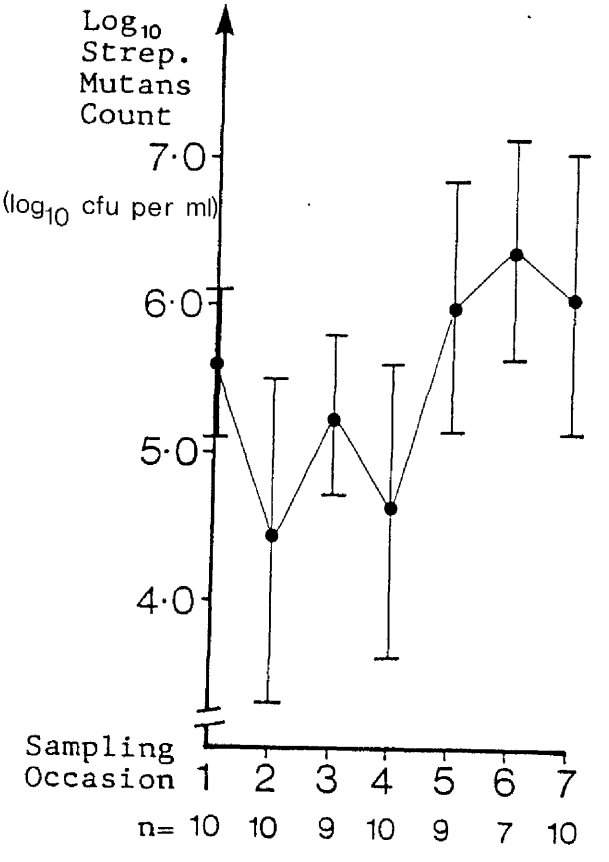


Figure 3.28 Mean ± one standard deviation of logarithmic Strep. mutans counts from a maximum of ten individuals with high caries activity, on seven occasions over six months.

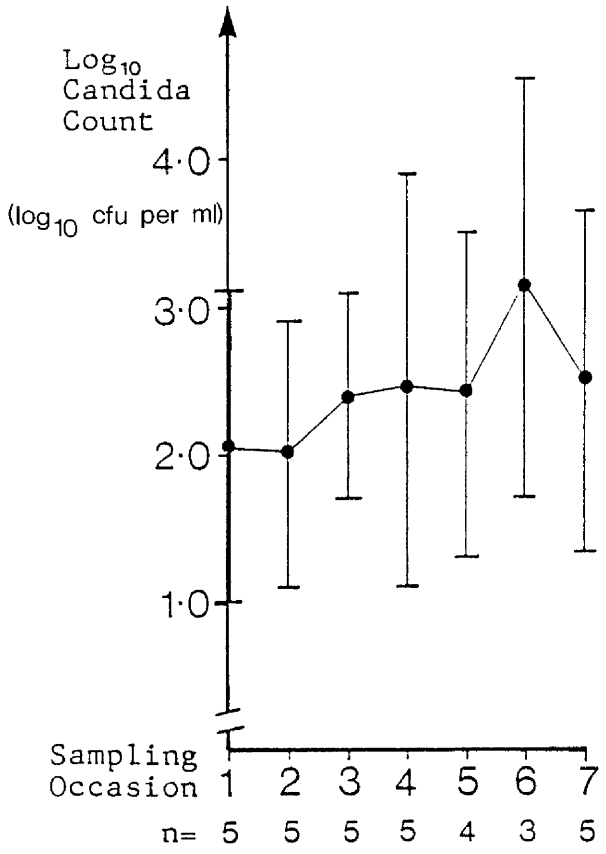


Figure 3.29 Mean ± one standard deviation of logarithmic candida counts from a maximum of five individuals with high caries activity, on seven occasions over six months.

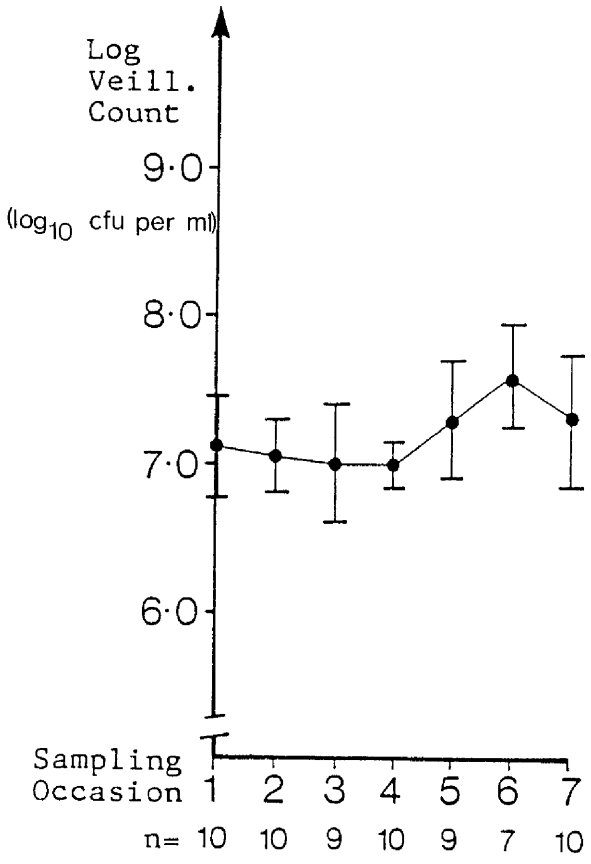


Figure 3.30 Mean ± one standard deviation of logarithmic veillonella counts from a maximum of ten individuals with high caries activity, on seven occasions over six months.

analysis, and therefore Fig. 3.29 contains data for up to five children only.

When the results plotted in Fig. 3.27 to Fig. 3.30 for the high caries activity group, are compared with the first seven samples plotted in Fig. 3.13 to Fig. 3.16 for the low caries group, both sets of data appear very similar. Despite the differences in the previous caries experience of the two groups at the beginning of the study, when the means of up to seven logarithmic microbiological counts for each of the children were calculated, and the two groups compared using t-tests, no significant differences were found between the lactobacillus, Strep. mutans or candida counts of the groups. The veillonella counts for the low caries group were, however, significantly higher at a $p < 0.05$ level, than for the previous high caries experience group.

3.4 Discussion

3.4.1 Magnitude of variation

Few studies have investigated the possible problems associated with the variation in the results of caries predictive tests which may occur due to laboratory methods, or as a result of diurnal variations which occur in oral microflora within individuals. However, Togelius et al (1984) reported the Strep mutans colonisation level to be stable, with no significant diurnal variation demonstrable between morning and afternoon salivary samples.

However, some studies have accepted that a degree of variation may occur in salivary microbiological counts, and have attempted to allow for such variation by predicting caries activity only from the

results of multiple tests. Thus, Green, Weisenstein and Permar (1957) used three salivary samples over a two week period, and Klock and Krasse (1977) used two samples and a third if the first two differed by more than a factor of ten. However, Crossner (1981) using two salivary samples, reported no increase in the reliability of prediction from lactobacillus counts as a result of repeated sampling. Other investigators (Stecksen-Blicks, 1985; Matee et al, 1985) have used single salivary samples for prediction. Variation in counts from single salivary samples was also reported by Permar, Kitchin and Robinson (1946), who warned that the variations in a single series of counts might be as great, or greater than an assumed hourly or daily fluctuation.

Therefore, before investigating if such daily fluctuations occurred, and deciding whether to obtain single or multiple saliva samples for prediction, the magnitude of variation in series of counts from single salivary samples was investigated. In Section 3.3.1, it was found that the microbiological methods used resulted in variation which was summarised in Table 3.8. This variation was of the order of 0.1 logarithmic units, when assessed by calculating pooled estimates of the common standard deviations of the logarithmic counts. In this study, no attempt was made to ascertain possible sources of the laboratory error, whether from variation in the homogenity of samples, from differences in the media used, or due to dilution techniques.

Also shown in Table 3.8 are the common standard deviations found from the results of the nine repeated samples at three times, on three days, as described in Section 3.3.3, and the common standard deviations from repeated samples over 2.5 years, as described in

Section 3.3.5. The common standard deviations of repeated counts of the same samples are less than those within individuals, of repeated samples over three days (0.2 to 0.6 logarithmic units), which are again smaller than those of samples from a 2.5 year period (0.5 to 0.9 logarithmic units). The magnitude of variation found within individuals, over three days in the present study, was similar to that of 0.2 to 0.4 logarithmic units described by Togelius et al (1984) for pairs of Strep. mutans counts obtained on the same day. However, Togelius et al (1984) reported the magnitude of the standard deviations of the logarithmic counts to be greater in individuals with higher Strep. mutans counts, whereas in the present study, the standard deviations of the logarithmic counts did not appear to be related to microbial counts. Nevertheless, it was noted that in this study, the variation in lactobacillus counts seemed to be inexplicably greater than that of the other micro-organisms investigated.

It is likely that the greater variation found over longer time periods, for all micro-organisms, may be due to true changes in oral microbial counts associated with such factors as caries status (Shklair, Englander & Stein, 1956; Kesel et al, 1958) and diet (Crossner, 1984). Indeed, possible associations between the development of open carious lesions and changes in counts of both lactobacilli and candida, were shown in Figures 3.22 to 3.25 in Section 3.3.5.

Additionally, in Table 3.8, it can be seen that the common standard deviations across subjects over three days and over 2.5 years are similar.

In order that a single caries predictive test can distinguish between individuals, it is desirable that there is more variation in

the counts from different individuals over a period of time than there is within individuals. As described in Section 3.3.5, this was found to be the case for counts of lactobacilli, Strep. mutans and candida. However, for veillonella the pooled estimate of the common standard deviations across different individuals was similar to that within individuals, and in samples over 2.5 years, the within subject common standard deviation was greater than that across individuals. This finding suggested that the possibility of predicting caries from the results of a single veillonella count would be unlikely.

3.4.2 Effect of storage

As reported in Section 3.3.2, there were no significant differences in the counts of the micro-organisms investigated whether they were stored at room temperature or at $4^{o}C$. Indeed, storage at $-10^{o}C$ caused significant reductions in counts of Strep. mutans and Candida, and it was therefore decided to maintain salivary samples at room temperature for subsequent investigations, until laboratory procedures could be completed.

Other investigators (Klock & Krasse, 1977; Kohler et al, 1981; Scheie et al, 1984) have reported the dilution of saliva in transport fluid for the maintenance of samples. However, Birkhed et al (1981), reported that the storage of salivary samples for up to two days, without transport media, caused only a minor decrease in lactobacillus counts.

When the effect of storage times of 3, 6 and 24 hours on neat salivary samples, at room temperature, was investigated, counts of lactobacilli, Strep. mutans and candida did not differ significantly

from those without storage. The counts of veillonella at 3 and 24 hours also did not differ significantly from those without storage, although there was a small but statistically significant increase after 6 hours storage.

It was therefore decided that the use of transport medium was unnecessary for maintenance of samples for periods up to 24 hours. However, despite these findings, it was decided to complete laboratory procedures in subsequent investigations as rapidly as possible after collection, usually within 3 hours and always within 6 hours.

3.4.3 Effect on counts of sampling at different times of day

Birkhed *et al* (1981) reported higher lactobacillus counts in samples obtained in the morning, before breakfast and before toothbrushing, than during the day. However, they found better reproducibility in the samples taken at different times during the day.

Since it was going to be necessary to obtain salivary samples during the school day in the prospective toothpaste trial, the investigation reported in Section 3.3.3 was performed. It was not considered important to investigate variation in counts outwith normal school hours, as there was no intention to collect samples then. As described in Section 3.3.3, the only significant difference found in samples from the three different times of day was for *Strep. mutans*, and even then, the difference did not occur consistently on repeated days. It was therefore concluded that sample collection at different times of the school day made little difference to salivary counts.

3.4.4 Effect of recent food consumption

Although previous studies had shown that longterm diet changes tend to affect oral microbiological counts (Jay, 1947; de Stoppelaar, van Houte & Backer Dirks, 1970; Crossner, 1984; Minah, Solomon & Chu, 1985), others had failed to demonstrate this trend (Krasse, 1954a). There was no information in the literature concerning the effect of food consumption, immediately before collection of a paraffin-wax-stimulated mixed salivary sample, on subsequent microbial counts. Therefore in order to decide whether it was necessary to instruct children to refrain from eating or drinking prior to donating salivary samples, the effects of recent ingestion of two of the commoner snacks consumed by school children were studied and reported in Section 3.3.4. However, it was found that counts, both 5 minutes and one hour after the ingestion of either crisps or cola, differed insignificantly from those where eating was avoided. It was therefore considered unnecessary to control food consumption prior to the collection of salivary samples to be used for counts of lactobacilli, Strep. mutans, candida or veillonella.

3.4.5 Effect of different caries rates

Unfortunately, in view of the intention to attempt to predict caries in the future clinical trial, the only significant differences in microbiological levels found between the high and low previous caries experience groups of children, were in the higher veillonella counts for the low caries group. This differed from the results of Klock and Krasse (1977) who found significant differences in Strep. mutans counts

However, although the groups of children were chosen according to their different previous caries experience, it was found that their caries increments did not differ significantly over the six-month period investigated. It was therefore considered that this pilot study had been unsuccessful in comparing groups with actual differences in caries activity, but it was thought possible that the major trial might reveal significant relationships.

3.5 Conclusions

As a result of the investigations described in this chapter, it was decided that when mixed salivary samples were collected from participants in the Lanarkshire clinical trial, the following methods would be used:

1) Single salivary samples would be obtained for prediction, but these would be repeated at annual intervals, in order to ascertain the consistency of any prevalence associations found.
2) No control of food consumption prior to sampling was required.
3) The time of sample collection within the school day would be standardised as far as possible, although this was not considered to be critical.
4) Storage of the salivary samples at room temperature, until laboratory procedures could be completed (up to six hours), would not affect microbial counts greatly, and therefore the use of transport medium was unnecessary.

CHAPTER 4

LANARKSHIRE CLINICAL DENTIFRICE TRIAL

4.1 Introduction

As described in Section 1.4.2, the incorporation of fluoride into the apatite crystal lattice of enamel increases the resistance of enamel to dissolution and decay, and fluoride also enhances the remineralisation of carious enamel. Fluoride may be available in the water supply or from drops, tablets, toothpastes, rinses, gels or varnishes, and many studies have investigated the anti-caries effect of fluoride from these sources (for review see Wei, 1985).

The caries reducing effects of several different dentifrices containing fluoride compounds, including stannous fluoride, sodium monofluorophosphate and sodium fluoride, have been tested clinically. In most trials, toothpastes with one or more of these substances have reduced caries increments by between 15% and 30%, when compared with placebo dentifrices, although results outwith this range have also been obtained (Murray & Rugg-Gunn, 1982). Not only have different fluoride compounds been tested, but several polishing agents and a variety of fluoride concentrations have also been studied in various trials. However, few of these studies have compared the caries reducing effects of different levels of fluoride, to determine whether there is an optimum level for fluoride in toothpastes. The results of the few investigations which have used different fluoride levels have varied. Thus, Forsman (1974) and Koch et al (1982) found no significant differences in the caries reducing effects of toothpastes containing different fluoride levels, whereas Reed (1973), Hodge et al

(1980) and Barlage, Buhe and Buttner (1981) found significant caries reductions with increasing fluoride concentrations.

The Lanarkshire clinical trial was therefore conceived in an attempt to identify whether a dose-response relationship existed for the effects on caries of sodium monofluorophosphate in toothpaste, at fluoride levels of 1000 ppm, 1500 ppm and 2500 ppm. During the course of the clinical trial, which started in 1983, there were further reports of the effects of toothpastes containing different fluoride concentrations. In these, Mitropoulos et al (1984) and Diodati et al (1986) showed significant dose-response relationships, whereas Ripa et al (1986) reported no such finding.

Another ion which has been tested for its anti-plaque, anti-calculus and anti-caries properties is zinc. Fischman et al (1973) reported that an oral rinse containing zinc citrate and zinc tribromsalan produced a reduced plaque score, and Saxton, Harrap and Lloyd (1986) reported reduced plaque growth in individuals using a dentifrice containing 0.5% zinc citrate. Schmid, Schait and Muhlemann (1974) and Lobene et al (1985) showed anti-calculus properties of zinc chloride, while Bates and Navia (1979) obtained a reduced caries increment in rats receiving a twice daily topical application of a solution containing 500 ppm zinc.

Thus, the secondary aim of the Lanarkshire clinical trial was to investigate whether the addition of 0.5% zinc citrate, to any of the fluoride levels, modified the anti-plaque, anti-calculus or anti-caries activities of the dentifrices. This chapter describes the anti-caries and anti-plaque investigations, the anti-calculus work being outwith the scope of this thesis.

4.2 Materials and methods

4.2.1 Choice of subjects

Ethics committee approval for the conduct of the three year, double blind, clinical dentifrice trial was obtained, and informed parental consent for participation was sought for 3375 children. These children were in their first year of secondary education, and were attending a total of 12 schools in the county of Lanarkshire, Scotland.

Of these 3375 subjects, parental consent was obtained for 3044, and 3003 were available for clinical examination at the first school visits. At the time of first examination, the mean age of participants was 12.55 years, the range being from 11.25 years to 14.00 years.

4.2.2 Clinical examination

As the names of prospective participants for the clinical trial were received, they were allocated sequential subject numbers, prior to the initial examination. Each child was then examined by one of two calibrated clinicians, the author or another examiner (S.L.C.). One clinician saw all odd numbered children and the other, all those with even numbers.

The children were then examined at annual intervals over the three year period of the clinical trial. Each clinician saw the same children at the subsequent examinations. In addition, at each annual examination, in order to allow the calculation of intra-examiner reliability coefficients, approximately 5% of children were re-examined by the original clinician. A further 5% of children were also re-examined by the other clinician, to permit inter-examiner

comparisons. These coefficients were calculated according to the method of Rugg-Gunn and Holloway (1974), as recommended by the Federation Dentaire Internationale (1982).

All examinations were carried out in the individual schools. As shown in Fig. 4.1, each child lay supine on a camping-mat covered school table. Illumination was by means of an Anglepoise 60 Watt lamp, placed approximately 30 cm from the subject's mouth. Examinations were performed without drying of the teeth, and using No.5 plane mirrors, blunt Williams periodontal probes (diameter 0.6 mm) and sharp No. 2H sickle probes (Ash Instruments, Dentsply, Gloucester, England), which were used for fissure plaque removal and fissure sealant detection.

Tooth presence was dictated to a trained scribe who sat alongside the clinician and recorded the data on a computer-compatible form (Appendix B). All surfaces of the permanent teeth, and any deciduous teeth which were present, were examined and scored according to criteria similar to those of Bennie et al, (1978) as follows:

(a) Pit and fissure surfaces

0 - Sound surface.

2 - Decayed surface from which the William's probe withdrew with some resistance.

3 - Decayed lesion, not involving the pulp, in which the William's probe moved freely.

4 - A lesion involving the pulp - obvious exposure, polyp or abscess.

(b) Buccal and lingual smooth surfaces

Scores of 0, 2, 3 and 4, as for pit and fissure surfaces.

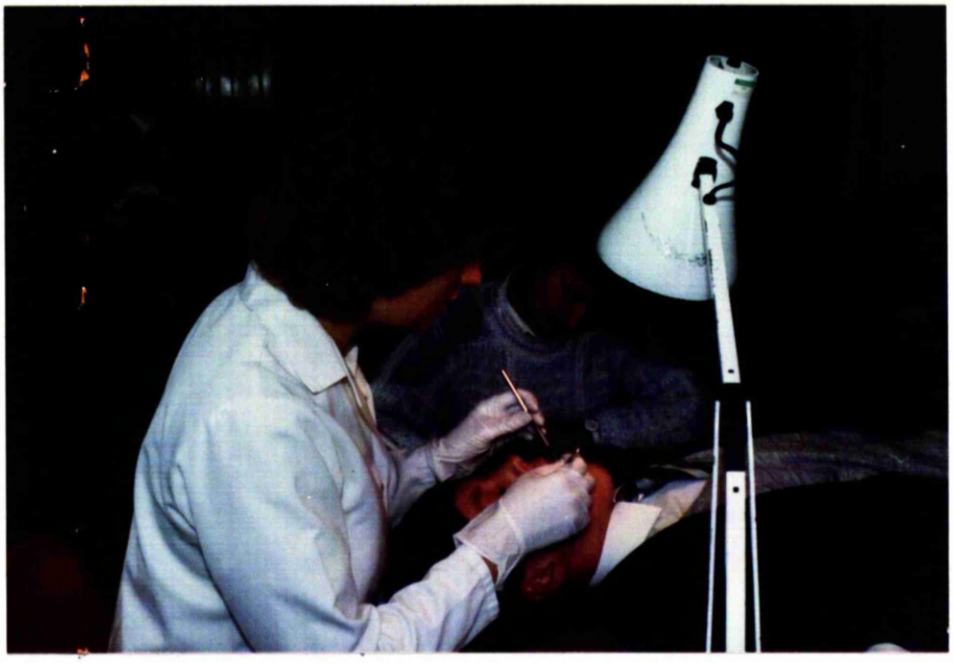


Figure 4.1 The clinical examination of one subject by the author.

(c) Approximal surfaces

0 - Sound surface.

3 - No obvious pulpal involvement, but either; (a) breakdown of the marginal ridge; (b) where the William's probe was able to enter the lesion; (c) a definite shadow visible under an intact marginal ridge, or (d) in anterior teeth, a definite shadow when viewed with transmitted light.

4 - Obvious pulpal involvement.

Any restored surface was given a score of 5, and missing permanent teeth were recorded with the reason for their absence, whether due to: (a) extraction as a result of caries; (b) extraction for orthodontic purposes; (c) uneruption; (d) congenital absence; or (e) traumatic loss as ascertained both by deduction and questioning. In addition, any hypoplastic teeth were noted and excluded from further analysis. Crowned teeth were recorded depending on the child's stated reason for crowning, i.e. trauma, or caries.

In addition to tooth status, the oral hygiene was assessed using the simplified oral debris index of Greene and Vermillion (1964).

At all but the initial examination, clinical charts were produced with previously extracted, traumatised or hypoplastic teeth noted thereon.

4.2.3 Radiographic examination

At each annual examination, bilateral bitewing radiographs of each child were taken by a dental radiographer, using a transportable Philips Oralix 65 kV, 7.5 mA machine, with a 20 cm cone (Philips Medical Systems, Hammersmith, London), mounted on a specially adapted

Atomscope mobile stand (Mikassa X-ray Co. Ltd., Japan). The radiographs were taken on Kodak Ultra Speed dental film DF-56 (Eastman Kodak Co., New York, U.S.A.) and the films were held in preformed cardboard bitewing holders (Fig. 4.2), the design of which assisted in the correct beam alignment.

The radiographs were read by one or other of the two clinicians who performed the clinical examinations. The clinician who read the radiographs from the first examination also read all subsequent radiographs for that child. In order to allow the calculation of intra-examiner and inter-examiner reliability coefficients, approximately 5% of radiographs were also re-examined by the original clinician, and 5% were re-examined by the other clinician.

Only the approximal surfaces of the permanent posterior teeth, from the mesial surface of the second permanent molar to the mesial surface of the first premolar, were scored radiographically. Radiographs were read without reference to the clinical findings and the data recorded on specially duplicated charts which only contained information regarding subject identity and tooth presence.

The criteria used in the scoring of approximal caries visible radiographically were adapted from those of Rugg-Gunn (1972), and were as follows:

0 - sound surface.

2 - radiolucency in enamel, up to amelodentinal junction.

3 - radiolucency in enamel and dentine, not involving pulp.

4 - radiolucency involving dentine and pulp.

5 - restored surface.

7 - surface unreadable and no diagnosis possible, due to overlapping surfaces on the radiograph.

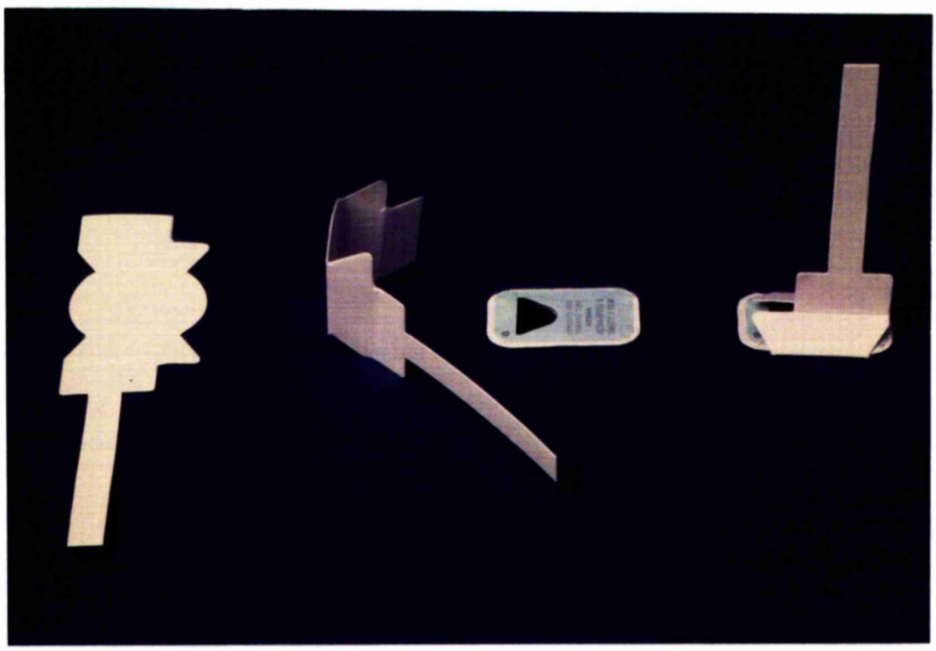


Figure 4.2 The cardboard bitewing holders, and intra-oral dental films used.

8 - surface not present on radiograph.

9 - some overlap of surfaces, involving not greater than half the enamel width, with no caries visible, and thus deemed apparently sound.

4.2.4 Dentifrice

Six different toothpastes were investigated, forming a three by two experimental design. They contained three levels of sodium monofluorophosphate; either 1000 ppm fluoride; 1500 ppm fluoride; or 2500 ppm fluoride, each with or without 0.5% zinc citrate. The further composition of these dentifrices is shown in Table 4.1.

Immediately after the first clinical examination had been carried out, children were allocated to the six toothpaste groups by a process of stratified randomisation. Each child was placed in one of thirty-six strata. The appropriate stratum depended on two initial factors; (a) which clinician had examined a child and, (b) the sex of the child. The combinations of these factors gave four classes. Within each of these classes a child was allocated to one of nine blocks (A to I), as shown in Fig. 4.3. This allocation to dental blocks depended on the combination of an individual's dental age and (i) whether decay was present in the premolars and permanent anterior teeth; (ii) whether decay was present in the second permanent molars and not in the premolars and anteriors or (iii) if decay was limited to permanent first molars, the allocation depending on the number of teeth so affected. For example, a child with one unerupted second permanent molar, and all four first permanent molars carious, but no other decayed permanent teeth, would be assigned to block D.

	Toothpaste					
	1 1000 ppm F No Zn	2 1000 ppm F + Zn	3 1500 ppm F no Zn	4 1500 ppm F + Zn	5 2500 ppm F no Zn	6 2500 ppm F + Zn
Polishing agent	50.0	50.0	50.0	50.0	50.0	50.0
Humectant	30.0	30.0	30.0	30.0	30.0	30.0
Binder	1.0	1.0	1.0	1.0	1.0	1.0
Detergent	1.5	1.5	1.5	1.5	1.5	1.5
Stabiliser	0.3	-	0.3	-	0.3	-
Flavour/ colouring	1.5	1.5	1.5	1.5	1.5	1.5
Sodium monofluoro-phosphate	0.76	0.76	1.14	1.14	1.90	1.90
Zinc citrate	-	0.50	-	0.50	-	0.50
Demineralised water	<---------- to 100% ---------->					

Table 4.1 Percentage weight per weight composition of the six toothpastes (1-6) used in the Lanarkshire clinical trial.

<table>
<tr><td rowspan="2"></td><td rowspan="2"></td><td colspan="3">DENTAL AGE
Number of unerupted second permanent molars</td></tr>
<tr><td>0</td><td>1-2</td><td>3-4</td></tr>
<tr><td rowspan="3">CARIES
EXPERIENCE
AT
BASELINE</td><td rowspan="2">Decay in permanent teeth limited to the first permanent molars
DMFT = 0,1
DMFT = 2,3
DMFT = 4</td><td colspan="2">A
I</td><td>B</td></tr>
<tr><td>C</td><td>D</td><td>E</td></tr>
<tr><td>Decay in permanent second molars but not in premolars or anterior teeth</td><td colspan="3">F</td></tr>
<tr><td></td><td>Decay in premolars or anterior teeth</td><td>G</td><td colspan="2">H</td></tr>
</table>

Figure 4.3 Definitions of the nine dental blocks used for stratification of subjects in the Lanarkshire clinical trial.

Within each stratum, children were then assigned to their toothpaste group using computer constructed random number tables. The proportions of numbers in the tables allowed for the trial design requirement that twice as many children be allocated to the toothpaste groups with 1000 ppm fluoride and 1500 ppm fluoride, as to the 2500 ppm fluoride group, as it was anticipated that at the highest fluoride level the lower numbers would be adequate to identify any significant fluoride effect.

The above blocking procedure was carried out at weekly intervals during the first examination. As a result, appropriate toothpastes were delivered to children's homes by home visitors, within only two weeks of the initial examination. The dentifrices were supplied in colour coded boxes (light and dark green, light and dark red, light and dark blue), as shown in Fig. 4.4., the composition of the toothpastes being unknown to the clinicians, home visitors or subjects. Enough toothpaste was provided for each participant's family, and a toothbrush was also supplied for each child on the clinical trial. Parents were advised that pre-school children were to be discouraged from using these toothpastes, some of which contained high fluoride levels. Fresh quantities of toothpaste were provided at two-monthly intervals, and brushes were supplied approximately once every four months.

4.2.5 Statistical analysis

For each child's posterior approximal tooth surfaces, the clinical and radiographic results obtained, as described in Sections 4.2.2 and 4.2.3, were combined. Any discrepancies in scores for individual surfaces were resolved according to the method shown in

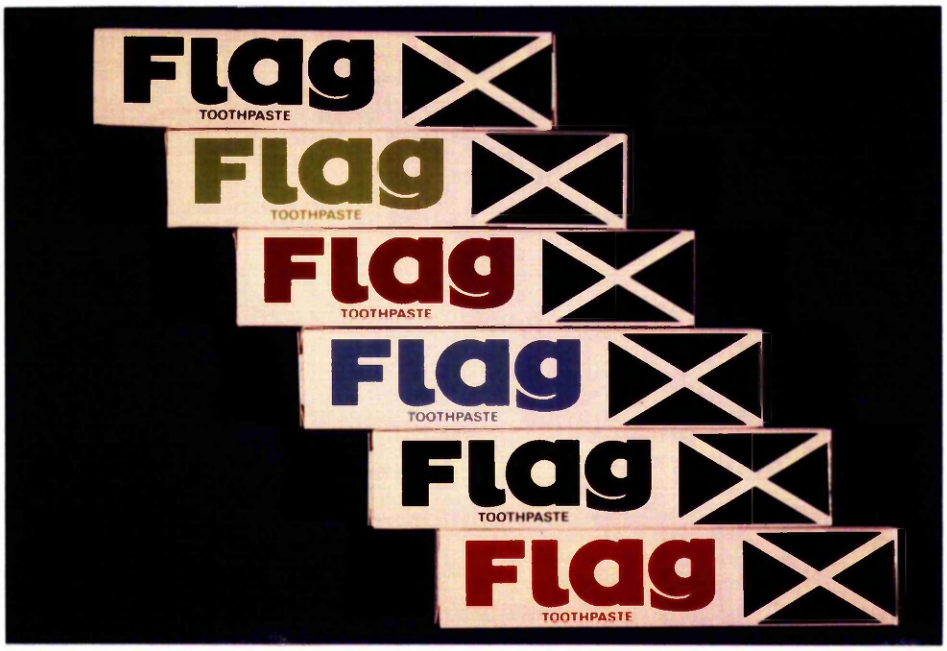


Figure 4.4 The colour coded dentifrice boxes.

Table 4.2. For example, for an approximal surface clinically scored sound, 0, but radiographically as carious, 3, the combined score would be 3.

Each child's caries prevalence at the annual examinations was then calculated, and expressed as the number of: Decayed, Missing and Filled Teeth (DMFT); Decayed, Missing and Filled Surfaces (DMFS); Decayed and Filled Surfaces (DFS); and Decayed Surfaces (DS). Additionally, the three year caries increment for each individual was calculated. This increment was expressed in two ways: (a) as the increment of DMFS, in which all surfaces becoming decayed, filled or missing due to caries were included and (b) as recommended by the Federation Dentaire Internationale (1982), by the increment of DXFS, in which all surfaces becoming decayed or filled were included, but any tooth extracted due to caries was scored according to the number of surfaces decayed or filled at the last examination at which it was present.

In order to check for any differences at baseline in the mean caries experience of the children randomly allocated to the six toothpastes, the mean caries levels of these children were compared, using multiple t-tests with an overall level of significance of 0.05.

The baseline caries experience of the children lost from the trial was also compared with that of the children completing the trial, using t-tests. The effects of the different fluoride and zinc levels on the three year caries increments of the children were compared using GENSTAT (The Numerical Algorithm Group Ltd., Oxford, United Kingdom). Based on the three by two factorial design of the trial, a linear model with two factors: fluoride at three levels; zinc at two levels, was used to analyse the data.

		Radiographic Score							
		0	2	3	4	5	7	8	9
	0	0	2	3	4	5	7	8	0
Clinical	3	0	3	3	4	5	3	3	3
Score	4	0	4	4	4	5	4	4	4
	5	5	5	5	5	5	5	5	5

Table 4.2 The method of resolution of discrepancies in clinical and radiographic scores for individual approximal surfaces. The combined designation of the two scores is read from the central portion of the table, at the intersection of the appropriate row and column.

4.3 Results

4.3.1 Examiner reliability

Inter-examiner and intra-examiner reliability coefficients were calculated for approximately 5% repeat clinical examinations and 5% of radiographs, as described in Sections 4.2.2 and 4.2.3. In addition, the mean DMFS scores for the children involved in each examination and re-examination were also calculated. The inter-examiner coefficients at each annual examination are shown in Table 4.3, and the intra-examiner coefficients for each clinician are presented in Table 4.4. Tables 4.5 and 4.6 show the mean DMFS diagnosed at each of the inter-examiner and intra-examiner examinations respectively.

As can be seen, the reliability coefficients were high, all were greater than 0.90, and most greater than 0.95. Additionally, the inter-examiner coefficients were similar in magnitude to the intra-examiner coefficients, and the mean caries levels diagnosed at each pair of examinations were close, indicating the similarity of diagnostic criteria used by the two clinicians. These similarities were considered to be sufficient to allow the data for the children examined by the different clinicians to be analysed together.

4.3.2 Caries prevalence at initial examination

Shown in Table 4.7 are data relating to the baseline examination in early 1983. Thus the mean DMFT score, from combined clinical and radiographic examination, for the 3003 children (average age 12.55 years) was 5.25. If the radiographic data was excluded, the mean DMFT, from clinical examination alone, was reduced to 4.68.

	Inter-examiner reliability coefficients	
	Clinical	Radiographic
Exam 1: coefficient	0.92	0.99
n	138	72
Exam 2: coefficient	0.94	0.99
n	117	36
Exam 3: coefficient	0.97	0.99
n	146	129
Exam 4: coefficient	0.95	0.99
n	109	95

Table 4.3 Inter-examiner reliability of clinical and radiographic DMFS diagnosis (calculated according to Rugg-Gunn and Holloway, 1974) at each annual examination, along with the number of re-examinations (n).

	Intra-examiner reliability coefficients			
	Clinical		Radiographic	
	Clinician 1	Clinician 2	Clinician 1	Clinician 2
Exam 1: coefficient	0.99	0.99	0.99	0.99
n	70	72	40	38
Exam 2: coefficient	0.99	0.92	0.99	—
n	52	50	36	
Exam 3: coefficient	0.97	0.98	0.99	0.98
n	80	77	50	50
Exam 4: coefficient	0.97	0.96	0.99	0.98
n	59	56	52	51

Table 4.4 Intra-examiner reliability of clinical and radiographic DMFS diagnosis (calculated according to Rugg-Gunn and Holloway, 1974), for each clinician, at each annual examination, along with the numbers of re-examinations (n).

	Mean DMFS at examination and re-examination			
	Clinical		Radiographic	
	Clinician 1	Clinician 2	Clinician 1	Clinician 2
Exam 1	10.8	10.4	5.3	5.2
Exam 2	13.1	12.7	4.5	3.8
Exam 3	14.4	15.4	5.5	5.1
Exam 4	16.4	16.7	6.6	6.0

Table 4.5 Mean DMFS scores at each examination and re-examination for the children for whom inter-examiner reliability coefficients were calculated.

	Mean DMFS at examination and re-examination							
	Clinical				Radiographic			
	Clinician 1		Clinician 2		Clinician 1		Clinician 2	
	Exam A	Exam B	Exam A	Exam B	Exam A	Exam B	Exam A	Exam B
Exam 1	11.2	11.4	10.9	10.7	4.4	4.5	5.3	5.8
Exam 2	13.2	13.4	12.4	12.5	7.1	7.9	-	-
Exam 3	16.0	15.7	14.7	14.3	4.7	4.9	6.9	6.7
Exam 4	13.0	13.2	15.2	15.1	7.1	6.1	5.8	5.5

Table 4.6 Mean DMFS at each examination (Exam A) and re-examination (Exam B) for the children for whom intra-examiner reliability coefficients were calculated.

Index	Prevalence data for 3003 children Mean ± S.D.	Range
DMFT	5.25 ± 3.51	0 - 23
DMFS	10.64 ± 8.49	0 - 58
DFS	6.89 ± 5.61	0 - 40
DS	2.09 ± 2.83	0 - 30

Table 4.7 Means, standard deviations and ranges of the combined clinical and radiographic caries prevalence indices, for all 3003 children at the baseline examination.

Similar data for the children randomly allocated to each of the six toothpaste groups are shown in Table 4.8. When the means of each combined clinical and radiographic DMFT, DMFS, DFS and DS index for the groups were compared using t-tests, no significant differences were found to exist between these data.

4.3.3 Loss of subjects during trial

At the fourth examination, a total of 2316 of the original 3003 children were re-examined, representing a 23% loss of participants over the three years of the trial. The numbers of children originally allocated to each of the six toothpaste groups, along with the numbers and reasons for loss of subjects, are shown in Table 4.9. The principal reasons for these losses were persistent absence from school on the days of the final examination, and children who moved away from the trial area. As can be seen, smaller numbers of subjects left the trial at their own or parents' request. In such cases, the reason given was often stated to be a dislike of the test dentifrice.

One further reason for subject loss was the active exclusion at intermediate examinations of any child who was using a toothpaste less than once a week, and who was unwilling to agree to use the trial toothpaste at least once a week thereafter. Similarly, any child who admitted to using a toothpaste other than the test paste provided, more than 50% of the time, and who was unwilling to use only the trial dentifrice in future, was also excluded.

As can be seen from Table 4.9, the overall proportion of children lost during the course of the trial was similar in all six toothpaste groups. However, when the baseline caries levels for those

	Toothpaste groups					
	1 (1000ppm F)	2 (1000ppm F, 0.5%Zn)	3 (1500ppm F)	4 (1500ppm F, 0.5%Zn)	5 (2500ppm F)	6 (2500ppm F, 0.5%Zn)
n	599	595	600	604	299	306
Index	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.
DMFT	5.33 ± 3.63	5.18 ± 3.32	5.38 ± 3.61	5.20 ± 3.48	5.10 ± 3.46	5.27 ± 3.53
DMFS	10.75 ± 8.71	10.45 ± 7.98	10.92 ± 8.55	10.43 ± 8.40	10.27 ± 8.75	11.04 ± 8.87
DFS	6.98 ± 5.92	7.00 ± 5.55	6.97 ± 5.75	6.76 ± 5.28	6.76 ± 5.68	6.72 ± 5.36
DS	2.19 ± 3.00	2.10 ± 2.72	2.16 ± 3.10	2.03 ± 2.65	1.91 ± 2.56	2.09 ± 2.71

Table 4.8 Means and standard deviations of the combined clinical and radiographic caries prevalence indices at the baseline examination, for the 3003 children randomly allocated to each of the six toothpastes.

	No. of children in toothpaste groups					
	1	2	3	4	5	6
Examined initially	599	595	600	604	299	306
Absent from final examination	46	42	54	53	22	27
Left area	35	22	24	20	11	17
Child or parent made request to leave trial	13	29	21	20	11	11
Deceased	-	1	1	-	1	2
Excluded for non-adherence to protocol	36	49	37	45	15	22
Examined finally	469	452	463	466	239	227

Table 4.9 The numbers of children initially allocated to each of the toothpaste groups (1-6) and the numbers and reasons for loss of subjects during the Lanarkshire clinical trial.

completing the trial were compared with those lost during the trial, using t-tests, significant differences were found between the mean DMFT, DMFS and DS indices of the two groups. The mean caries prevalence indices for the respective groups are shown in Table 4.10.

In order to investigate whether the loss of participants had resulted in significant differences between the toothpaste groups, the mean caries indices at baseline were calculated for those children completing the trial only, in each toothpaste group. This data is presented in Table 4.11. Multiple t-tests with an overall significance level of 0.05 showed that, in spite of the subject losses, no significant differences existed between the mean caries levels in any of the toothpaste groups. Consequently, the comparisons of the subsequent caries increments of these children could be made directly, without any allowance for initially imbalanced groups.

4.3.4 Dentifrice effects

The three year caries increments in the six toothpaste groups, expressed as both DMFS increment and DXFS increment as described in Section 4.2.5, were compared using a linear model for the factors fluoride and zinc. The means and standard deviations, for the six toothpastes and for each index, are shown in Table 4.12.

No significant differences were found in caries increments, either as DMFS or DXFS, due to the presence of 0.5% zinc citrate. However, significant differences in three year caries increments were found for the different fluoride levels. In view of the proven absence of a zinc citrate effect, the results for the effect of the

Index	Baseline prevalence indices 2316 completing trial Mean ± S.D.	 687 lost Mean ± S.D.
DMFT	5.11 ± 3.42	5.72 ± 3.77
DMFS	10.13 ± 8.21	12.36 ± 9.17
DFS	6.82 ± 5.53	7.13 ± 5.86
DS	1.89 ± 2.61	2.76 ± 3.39

Table 4.10 Means and standard deviations of the combined clinical and radiographic caries prevalence indices at the baseline examination, for the 2316 children subsequently completing the clinical trial, and for the 687 children lost during its course.

Index	Toothpaste groups 1 (1000ppm F) Mean ± S.D.	2 (1000ppm F, 0.5%Zn) Mean ± S.D.	3 (1500ppm F) Mean ± S.D.	4 (1500ppm F, 0.5%Zn) Mean ± S.D.	5 (2500ppm F) Mean ± S.D.	6 (2500ppm F, 0.5%Zn) Mean ± S.D.
DMFT	5.11 ± 3.55	5.12 ± 3.33	5.23 ± 3.53	5.02 ± 3.35	5.03 ± 3.34	5.11 ± 3.39
DMFS	10.01 ± 8.39	10.10 ± 7.89	10.25 ± 7.97	10.01 ± 8.29	10.14 ± 8.51	10.42 ± 8.53
DFS	6.88 ± 5.77	6.99 ± 5.52	6.97 ± 5.74	6.67 ± 5.22	6.70 ± 5.68	6.49 ± 5.11
DS	1.97 ± 2.80	1.86 ± 2.45	2.01 ± 3.03	1.82 ± 2.39	1.75 ± 2.33	1.82 ± 2.22

Table 4.11 Means and standard deviations of the combined clinical and radiographic caries prevalence indices at the baseline examination, for the 2316 children completing the clinical trial, grouped according to toothpaste.

Increment	Toothpaste groups 1 (1000ppm F) Mean ± S.D.	2 (1000ppm F, 0.5%Zn) Mean ± S.D.	3 (1500ppm F) Mean ± S.D.	4 (1500ppm F, 0.5%Zn) Mean ± S.D.	5 (2500ppm F) Mean ± S.D.	6 (2500ppm F, 0.5%Zn) Mean ± S.D.
ΔDMFS	7.43 ± 7.21	7.39 ± 6.31	7.06 ± 6.37	7.00 ± 6.37	6.47 ± 6.79	6.40 ± 5.81
ΔDXFS	6.83 ± 6.54	6.77 ± 5.87	6.27 ± 5.64	6.39 ± 5.81	5.56 ± 5.81	5.85 ± 5.37

Table 4.12 Means and standard deviations of the combined clinical and radiographic three year caries increments, expressed as ΔDMFS and ΔDXFS, for the 2316 children completing the clinical trial, grouped according to toothpaste.

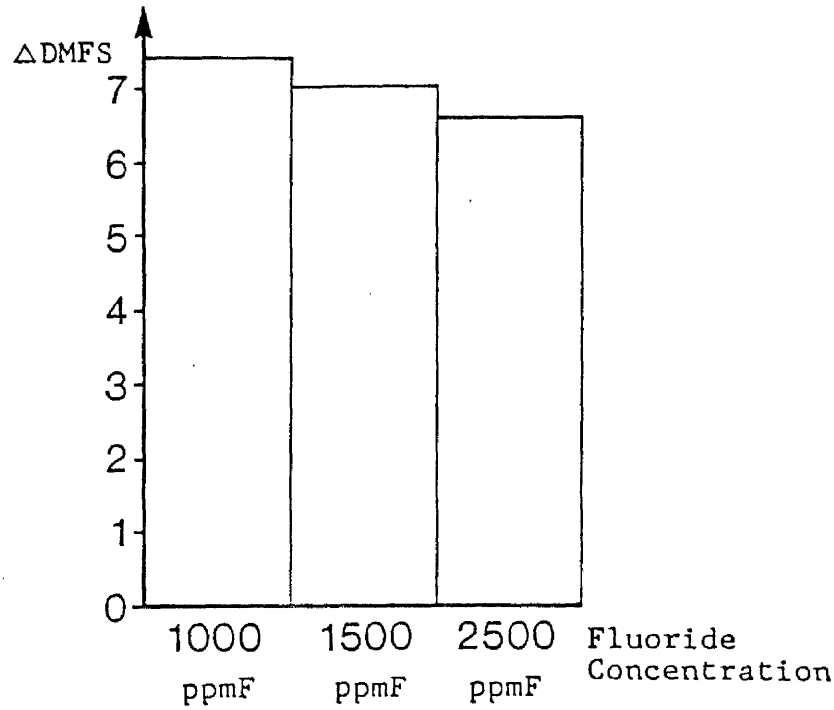
three fluoride levels, ignoring the presence or absence of zinc citrate, are presented graphically in Fig. 4.5. It can be seen that increases in the levels of fluoride were associated with small decreases in three year caries increments, as measured by either index.

The effect of 0.5% zinc citrate on the simplified Greene and Vermillion oral debris index is shown in Table 4.13. The results are presented for each examination, and relate to those children who completed the clinical trial. The numbers of children were reduced at the intermediate examinations because of absence. The data for the children in the different fluoride groups are combined. As can be seen, the mean oral debris indices for the two groups of children were similar at baseline examination, before starting the different dentifrices. Thereafter, the mean debris levels for children using the toothpastes with 0.5% zinc citrate were slightly less than those using the non-zinc toothpastes. However, when these small differences were compared using t-tests, only the fourth examination results showed a statistically significant difference ($p < 0.05$).

4.4 Discussion

4.4.1 Examiner reliability

As described in Section 4.3.1, the inter-examiner and intra-examiner reliability coefficients were high, and many were above the 0.95 level considered by Rugg-Gunn and Holloway (1974) to represent an unimportant amount of error. These coefficients were also similar to those reported by Rugg-Gunn and Holloway (1974). Additionally, the inter-examiner and intra-examiner coefficients were not dissimilar.



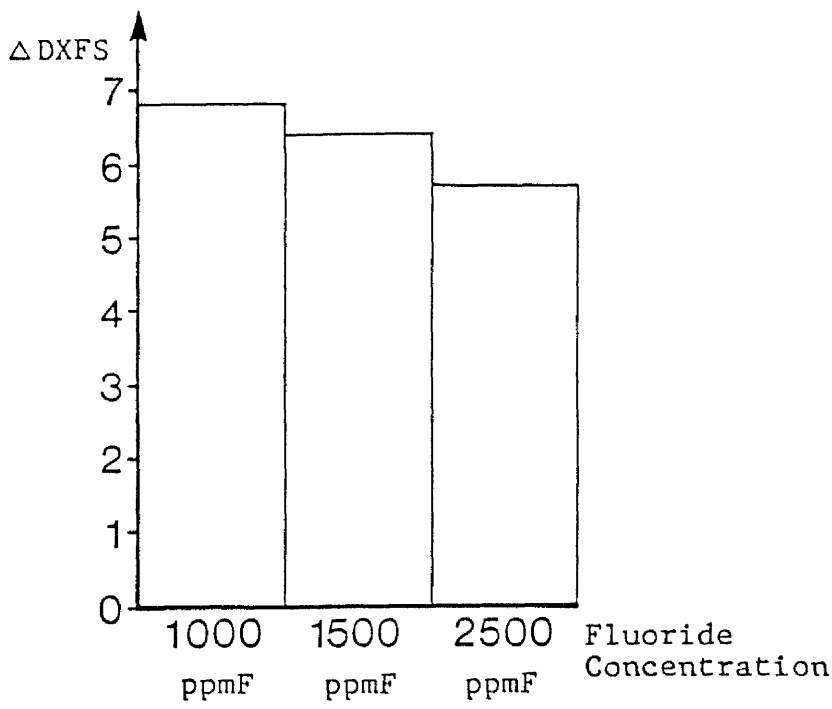


Figure 4.5 Mean three year caries increments, as ΔDMFS and ΔDXFS for the children using toothpastes with different fluoride concentrations as shown.

Examination	Simplified Oral Debris Index	
	Non-zinc	0.5% zinc citrate
	mean ± S.D.	mean ± S.D.
1	1.17 ± 0.41	1.18 ± 0.41
2	1.22 ± 0.44	1.19 ± 0.43
3	0.98 ± 0.40	0.96 ± 0.39
4	1.07 ± 0.50	1.03 ± 0.48

Table 4.13 Mean simplified oral debris indices for the two groups of children using toothpastes with and without 0.5% zinc citrate at each of four annual examinations.

4.4.2 Caries prevalence

The 1983 baseline mean caries prevalence of the children participating in the trial was 4.68, when assesed solely by the clinical DMFT index. This DMFT level was slightly higher than that of 4.25, described by Blinkhorn, Downer and Wight (1983), for a group of children (average age 12 years 8 months) from the East of Scotland, but slightly lower than the 1983 value of 5.25, reported by Todd and Dodd (1985) for Scottish children of the same age as those on the Lanarkshire trial. However, it is not possible to determine whether these slight differences were true to the populations studied, or simply the result of differing diagnostic criteria, or to differences in the interpretation of such criteria.

4.4.3 Loss of subjects during the trial

As reported in Section 4.3.3, 23% of those children initially starting the clinical trial were lost during its three year course. The exclusion of individuals from the trial, who refused to adhere to its protocol, accounted for about 30% of this loss. Such exclusion of participants is potentially contentious as it could be used to alter the result of a trial, and additionally any effects of the products tested are effects on the selected group of users, and not effects on the general population. However, this trial was a double blind study, and it was not thought that the clinicians could distort the results either knowingly or unknowingly, by exclusions according to the strict guidelines of those brushing their teeth less than once a week, or using alternative toothpastes for more than 50% of the time. In addition, the intention of the trial was to study the effects of the toothpastes in a group of subjects using them, not in

the population in general, and it was thought that those failing to conform to the trial protocol, as described, would not assist in accurate discrimination between the effectiveness of the different dentifrices.

Despite the planned exclusions, the 23% loss over three years was only at the upper limit of the range reported by authors in similar trials. Thus, Koch et al (1982) found a 6% loss, Mitropoulos et al (1984) an 11% loss, and Hodge et al (1980) reported an 18% loss, all over three years. On the other hand, Reed (1973) experienced a 28% subject loss in two years, while Forsman (1974) was more fortunate, with only an 18% drop-out rate over the same period.

4.4.4 Dentifrice effects

The finding of a significant dose-response relationship for the effect on caries, of levels of sodium monofluorophosphate above 1000 ppm fluoride in toothpaste, was in agreement with the findings of Hodge et al (1980). However, the differences in caries increments found between the children using the different toothpastes were small, and the variance of results was high. Significant results were found because of the large number of children participating, and the failure of some other authors to show similar results may well have been due to inadequate numbers of subjects at the outset. For example, of those who failed to show significant effects, Koch et al (1982) studied two groups each of less than 300 children, with each group distributed through three toothpastes, and Forsman (1974) investigated seven toothpastes in over one thousand children, but with the subjects divided into many groups containing no more than 80 individuals.

Conversely, of those who did show significant differences Barlage et al (1981) studied over a thousand children with only two toothpastes, Hodge et al (1980) investigated 800 subjects in three groups, Reed (1973) studied over two thousand subjects again in three groups, and Mitropoulos et al (1984) reported over 700 children in two groups.

Finally, the inclusion of zinc citrate, at a concentration of 0.5%, did not affect the dental caries increment. This was found in spite of the previously reported effect of zinc citrate in reducing in vivo plaque scores (Fischman et al, 1973; Saxton et al, 1986), and in spite of zinc citrate's in vitro inhibition of fluoride uptake by enamel (Mellberg & Chomicki, 1983; White & Faller, 1987). Additionally, the inclusion of 0.5% zinc citrate was found to result in a very small reduction in the mean oral debris index. This was in agreement with the findings of Fischman et al (1973) and Saxton et al (1986).

4.5 Conclusions

The major conclusion from the Lanarkshire clinical dentifrice trial was that a significant dose-response relationship existed for the effect on caries of sodium monofluorophosphate in toothpastes, at concentrations of 1000 ppm F, 1500 ppm F and 2500 ppm F.

Secondly, this clinical trial did not provide any evidence to suggest that caries disbenefit would occur from the inclusion of 0.5% zinc citrate in these dentifrices, and indeed the inclusion of zinc citrate at this level may result in a small reduction in plaque levels.

CHAPTER 5

USE OF PREDICTIVE TESTS IN THE LANARKSHIRE CLINICAL TRIAL

5.1 Introduction

As described in Sections 1.2.3 and 1.7, many studies have related salivary microbiological counts to caries prevalence (Klock & Krasse, 1977; Zickert et al, 1982a), and some investigators have demonstrated relationships between salivary counts and caries incidence (Klock & Krasse, 1979; Pienihakkinen et al, 1987; Stecksen-Blicks, 1985). However, as discussed in Section 1.7.9, few have investigated possible improvements in prediction obtained by combining the results of a number of different microbiological tests and other parameters for assessing caries activity. Additionally, prediction on an individual basis has not yet proved possible.

Since it was necessary to examine annually, 3000 children both clinically and radiographically, during the Lanarkshire clinical trial, the opportunity existed to investigate the relevance of a number of potential caries predictive factors, including clinical, microbiological, salivary and dietary variables.

This chapter describes the use of these tests both singly and in combination in caries prediction, and also their effectiveness at an individual level. Additionally, in view of the reports of Sukchotiratana, Linton and Fletcher (1975), and Maltz and Zickert (1982) that the levels of some oral micro-organisms, including Strep. mutans, were reduced by penicillin therapy, the effect of recent antibiotic therapy on microbiological counts was also studied.

5.2 Materials and methods

5.2.1 Microbiological investigations

Salivary collection

At the second annual examination of children participating in the Lanarkshire clinical trial, five-minute paraffin-wax-stimulated, mixed salivary samples were obtained from a total of 519 subjects, who were distributed throughout all six toothpaste groups. The donation of salivary samples by a group of such children is shown in Fig. 5.1. These samples were investigated microbiologically as described below. As a result of the investigations described in Chapter 3, the children were given no particular instructions about refraining from eating prior to salivary collection.

Ideally, it would have been preferable to investigate samples from the first examination of the Lanarkshire clinical trial. Although samples were obtained at that time, only two predictive tests were performed on these specimens. It was not until the second examination, after the completion of much of the work reported in Chapters 2 and 3, that materials and methods for a range of tests could be determined. Thus, the second clinical examination of the Lanarkshire clinical trial was used as the baseline examination for future caries prediction.

In order to minimise sample storage time, specimens of saliva were donated at the end of the school day between 15.00 hours and 16.00 hours. The samples were immediately transported to the microbiology laboratories in Glasgow Dental Hospital, and within six hours were inoculated and cultured.

At the third and fourth annual clinical examinations, repeat salivary samples were collected from as many of the original 519

Figure 5.1 A group of children donating salivary samples.

children as possible. In addition to microbiological investigations, the samples obtained at the third and fourth examinations were used to estimate salivary buffering capacity and, in accordance with the protocol of the Dentobuff test (Orion Diagnostica, Espoo, Finland), children were asked not to eat during the hour prior to salivary collection. Otherwise, the sampling procedures were the same at all examinations.

Effect of recent antibiotic therapy

In view of previous reports concerning the effect of antibiotic drugs on salivary micro-organism counts, each child was questioned, at the time of salivary donation, about drug therapy during the preceding month. After the third clinical examination, it was possible to obtain repeat salivary samples from 17 children who had been taking antibiotics in the month preceding the original collection. These repeat specimens were obtained between one and two months after the original samples.

Preparation of inocula and media

The preparation of inocula was performed as described in Section 2.2.6. The media used were those selected in Chapter 2: Rogosa SL agar plates, Dentocult dip-slides and Alban-modified Snyder tests for Lactobacillus spp.; Mitis Salivarius Bacitracin agar plates and colorimetric broths for Strep. mutans; and vancomycin agar plates for Veillonella spp.. Additionally, as discussed in Section 3.1, Sabouraud dextrose agar plates were also used for Candida spp.. All media were prepared as described in Appendix A.

Inoculation, incubation and enumeration of micro-organisms

The inoculation of the agar plates was performed as described in Section 3.2.3, using a spiral plater. The incubation of plates and subsequent enumeration of micro-organisms were performed as described in Section 3.2.1. Additionally, for every tenth sample, ten of the colonies counted, on each of the four plates, were chosen randomly and subcultured on blood agar. The isolates from the MSB, vancomycin and Rogosa SL agars were then characterised as previously described in Section 2.2.6. Those from the Sabouraud dextrose agar were examined microscopically and all large (approximately 4-6 μm diameter) Gram-positive round or oval cells were accepted as Candida spp..

The inoculation, and estimation of colony density or rate of colour change, of the Dentocult dip-slides, Snyder tests and Strep. mutans colorimetric tests were as described in Section 2.2.6. Ten colonies from every tenth Dentocult dip-slide were also characterised as in Section 2.2.6.

The results of the Dentocult, Snyder and Strep. mutans colorimetric tests were then classified according to the criteria shown in Tables 5.1 and 5.2.

5.2.2 Clinical methods

All children who provided salivary samples were examined clinically and radiographically for caries, and the oral hygiene level was assessed, as described in Sections 4.2.2 and 4.2.3. The DMFS, DS and incremental caries indices were calculated as in Section 4.2.5. However, for the purposes of dental caries prediction, any overall negative net caries increment (DMFS or DXFS) was taken to be equivalent to a zero increment.

Colour change	Class
within one day	1
between one and two days	2
no change by two days	3

Table 5.1 Method of classification of modified Snyder tests and Strep. mutans colorimetric tests, according to the rate of colour change.

Colony density	Class
$\leq 10^3$	1
$10^4 - 10^5$	2
$\geq 10^6$	3

Table 5.2 Method of classification of Dentocult dip-slide results depending on colony density as assessed using the proprietary charts of colony density (see Fig. 2.8).

5.2.3 Salivary investigations

As described in Section 5.2.1, the 519 children investigated provided salivary samples at the second, third and fourth annual examinations. These samples were collected in 20 ml graduated plastic universal containers, and thus, on each sampling occasion, an estimate was made of the child's mixed saliva flow rate as stimulated by paraffin-wax, over a five-minute period. This data was then used in the analyses of factors associated with caries prevalence and incidence.

Additionally, at the first clinical examination, Dentobuff tests (Orion Diagnostica, Espoo, Finland), as described by Frostell (1980), were performed in order to estimate the salivary buffering capacity. In the second year, it was decided to concentrate effort on microbiological tests, and therefore Dentobuff assays were omitted. However, in view of associations found between buffering capacity and caries prevalence from the first examination, the Dentobuff tests were reintroduced at the third and fourth examinations. In these Dentobuff tests, a 1 ml volume of saliva was added to the proprietary test solution, and the buffering capacity was assessed depending on the colour of the mixture, which was compared with a colour chart, as shown in Fig. 5.2. The buffering capacity was recorded as the final pH, on the scale 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0.

The buffering capacity results were also investigated for associations with caries prevalence and incidence. However, there were no results for the second examination and, in order to confine analyses to the second and subsequent examinations (the years for which microbiological data were obtained), the results of the first year's Dentobuff tests were substituted in the further analyses, for

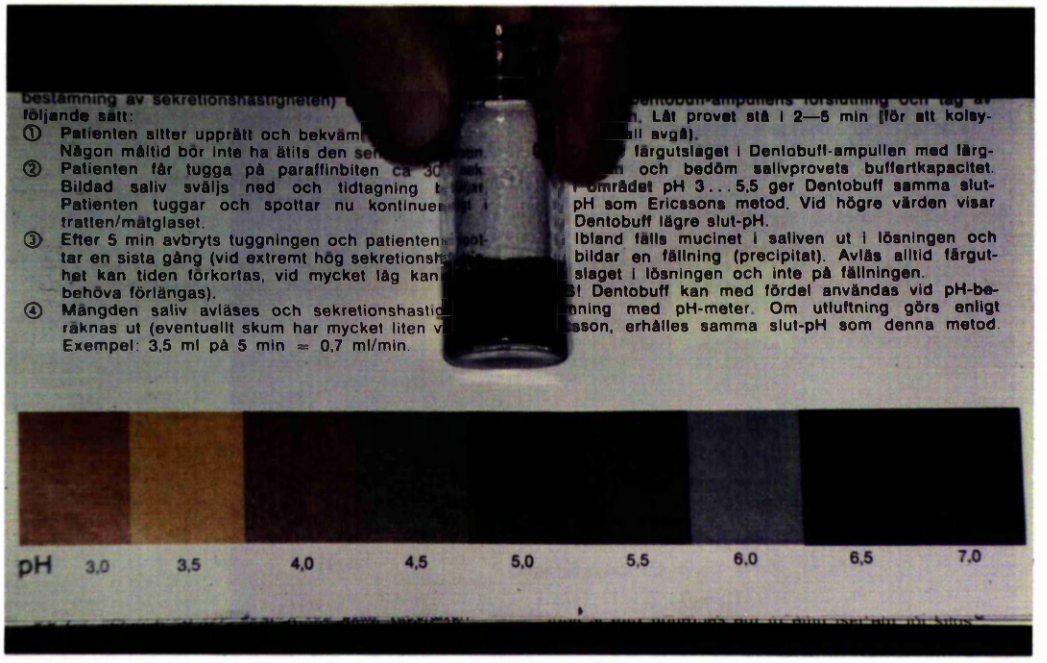


Figure 5.2 Assessment of a Dentobuff test using the proprietary colour chart.

second examination results. While it was realised that this substitution would not allow true comparisons between the predictive abilities of the microbiological and buffering tests at the second examination, it was hoped that individuals' salivary buffering capacities would not have changed greatly over the year between samples.

5.2.4 Dietary investigations

At the time of the third clinical examinations, each child involved in the microbiological investigations was asked to complete a three day diet diary, covering two week days and one week-end day. Subjects were asked to record all food and drinks consumed and the approximate times of eating. Each completed diet diary was then discussed with the child for approximately five minutes, in an attempt to ensure the accuracy of the information provided, and to identify any omissions.

Diet diaries were subsequently analysed with reference to "McCance and Widdowson's - The Composition of Foods" (Paul & Southgate, 1978). The ingestion frequencies (ingestions being separated by at least 15 minute periods) of foods containing greater than: 1% sugars; 5% sugars; 10% sugars; or 10% carbohydrate over the three day period, were then recorded for each child, and investigated for associations with caries prevalence and increments, and with the levels of salivary micro-organisms.

5.2.5 Statistical analyses

Effect of recent antibiotic therapy

In view of the possible relevance of recent antibiotic therapy to subsequent analyses, this factor was studied first. The 17 children investigated for the effect of recent antibiotic therapy were divided into three groups, depending on whether their therapy was current, within the past week, or within the past month. Comparisons were then made between the counts of micro-organisms in the original and second samples. However, because of the small sample sizes, any differences were difficult to compare statistically.

In Section 3.3.5, the monthly variation in logarithmic microbiological counts was studied and it was found that the pooled estimates of the common standard deviations within subjects were in the region of 0.5 logarithmic units. Consequently, it was decided to compare the two counts, with and without antibiotic therapy for each individual, in order to identify any increases in counts of ten-fold or more.

Distribution of variables

The distributions of the clinical, microbiological and salivary variables were illustrated graphically using box-plots generated by Minitab (Statistics Dept., Pennsylvania State University).

Effect of different toothpastes

As described in Section 5.2.1, the children investigated were participating in the Lanarkshire clinical dentifrice trial, and were using the six toothpastes described in Section 4.2.4. The significant effect of toothpaste fluoride concentration on three year caries increment was described in Section 4.3.4. Consequently, it was

accepted that the effect of the different toothpaste fluoride concentrations would have to be taken into account in the prediction of caries increments.

However, it was not known whether the fluoride or zinc levels of the dentifrices used would affect any of the predictive variables studied. Consequently, each predictive variable was investigated for any fluoride or zinc effect using an analysis of variance, Programme P7D of BMDP (BioMeDical computer Programs, Health Sciences Computing Facility, Department of Biomathematics, School of Medicine, UCLA).

Associations between potential predictive variables and caries prevalence and incidence

The associations between both caries prevalence and incidence, and clinical, microbiological, salivary and dietary data were studied initially by means of Pearson's correlation coefficients using SPSSX (SPSS Inc., Illinois 60611, U.S.A.). The potential predictive variables studied were: DMFS (clinical and radiographic); DMFS (clinical only); DS (clinical and radiographic); DMFS increment between the first and second examinations; oral debris index; log lactobacillus count; log Strep. mutans count; log candida count; log veillonella count; Dentocult class; Snyder test class; Strep. mutans colorimetric test class; buffering capacity; salivary flow rate; and frequency of ingestion of 1% sugars, 5% sugars, 10% sugars and 10% carbohydrate. The associations of caries prevalence and incidence, with combinations of these variables, were then investigated by means of stepwise regression analysis using Minitab.

Each child's caries prevalence and incidence was then categorised

as low, medium or high. The limits of the groups were chosen in order to allocate approximately equal numbers to each, and are shown in Table 5.3. Stepwise discriminant analysis, Programme P7M of BMDP, was then used to identify the caries prevalence or increment category expected for each child, from combinations of microbiological and salivary variables.

Additionally, the sensitivity, specificity and predictive values, as described by Vecchio (1966) and shown in Fig. 5.3, were calculated for the two year caries increment using single predictive variables. To permit comparisons with the work of other investigators, the single variables used were the DMFS and DS scores, and the Strep. mutans, lactobacillus, and candida counts. The caries risk cases were those subjects with a greater caries increment than the mean caries increment (4.4 surfaces) for the group of children studied. Thus 37% of children were actually assessed to be at risk of caries. The levels of Strep. mutans and lactobacillus counts for the prediction of risk were chosen in accordance with other published studies. Thus 38% of children were predicted to be at risk because of a Strep. mutans count of greater than, or equal to, 10^6 per millilitre of saliva, and 25% of children were predicted to be at risk because of a lactobacillus count of greater than, or equal to, 10^5 per millilitre of saliva. The risk levels for the candida count, DMFS and DS scores were chosen to include similar percentages of individuals, with 42% of children having a candida count greater than, or equal to, 10^2 per millilitre, 37% having a DMFS score of greater than 14, and 38% having a DS score of greater than two.

Index	Categories of caries prevalence and incidence		
	Low	Medium	High
DMFS	0-6	7-15	16-52
DS	0	1-3	4-31
1-year ΔDMFS	0	1-3	4-18
2-year ΔDMFS	0-1	2-5	6-26

Table 5.3 Limits of the groups of caries prevalence and incidence using which, children were categorised as being of low, medium or high caries activity.

	DIAGNOSIS	
PREDICTION	positive (risk)	negative (non-risk)
positive (risk)	A	C
negative (non-risk)	B	D

SENSITIVITY = (A/A+B)x100

SPECIFICITY = (D/C+D)x100

PREDICTIVE VALUE (RISK) = (A/A+C)x100

PREDICTIVE VALUE (NON-RISK) = (D/B+D)x100

Figure 5.3 Definitions of sensitivity, specificity and predictive values of caries predictive tests.

5.3 Results

5.3.1 Identification of microbiological isolates

During the three salivary examinations, ten colonies were subcultured from every tenth subject's microbiological plates and dip-slides, as described in Section 5.2.1. These isolates were then characterised as described in Section 2.2.6. There were only minor differences in rates of isolate identification for the individual years, therefore results are presented for the entire trial.

Over all three years, to the nearest percent, 99% of isolates from Sabouraud dextrose agar were identified as Candida spp., and less than 1% of isolates failed to subculture or were identified as other micro-organisms. For other media, there was no growth from a small proportion of isolates. This was particularly so for isolates from the Dentocult dip-slides. Thus, for Rogosa S.L. agar, 96% of colonies were characterised as Lactobacillus spp., 2% failed to subculture, and a further 2% were organisms other than lactobacilli, mainly candida according to Gram's stain appearance. From M.S.B. plates, 95% of colonies were characterised as Strep. mutans, 4% failed to subculture, and 2% were other micro-organisms. Of the isolates from vancomycin agar, 96% were identified as Veillonella spp., with 3% other organisms and 1% failing to subculture. For isolates from Dentocult dip-slides, 83% were lactobacilli, 3% were other organisms, and 14% failed to subculture.

5.3.2 Effect of recent antibiotic therapy

Repeat salivary samples were obtained from 17 of the children who had been taking antibiotics in the month preceding the second annual salivary donation. These repeat samples were collected at least one

month after the original sample, to ensure that none of the children received antibiotics during the previous four weeks or more. Of the children studied, six had been taking antibiotics at the time of the original salivary sample, five had been on antibiotic therapy during the previous week, and six had been taking antibiotics in the month prior to the first salivary sample. The identities of the antibiotics taken, as reported by the subjects, are shown in Table 5.4.

The means and standard deviations of the two logarithmic counts of all four micro-organisms, for each of the three groups of children are shown in Table 5.5. As can be seen, the Strep. mutans counts are apparently lower after recent antibiotics than in the absence of such therapy. However, for lactobacilli, candida and veillonella, the mean counts after recent antibiotics, and in the absence of antibiotics, appear similar within the three groups. Due to the small sample sizes, the differences were difficult to compare statistically. The counts for individuals were compared, therefore, and ten-fold differences in counts with recent antibiotic therapy were noted. Such ten-fold differences were found for Strep. mutans in three of those children taking antibiotics currently (two on ampicillin, one on penicillin), in two of those who had been taking antibiotics within the previous week (one on amoxycillin, one on erythromycin) and in one who had been taking an unknown antibiotic in the past month. For lactobacilli, only one child (erythromycin in past week) exhibited a similar ten-fold reduction in count. None of the pairs of candida or veillonella counts differed by more than a factor of ten.

In view of these findings, it was considered that microbiological levels, particularly in relation to Strep. mutans counts, during

Antibiotic	No. of subjects on antibiotic therapy		
	currently	past week	past month
Penicillin	3	1	1
Ampicillin	2	-	-
Erythromycin	-	2*	1
Oxytetracycline	1	-	-
Amoxycillin	-	2*	-
Cephalexin	-	1	-
unknown antibiotic	-	-	4

* One child had taken both erythromycin and amoxycillin.

Table 5.4 The nature of antibiotics taken by the 17 subjects who received such therapy within one month of sampling.

Micro-organism	Antibiotic therapy					
	Currently		Past week		Past month	
	Sample 1 log count Mean±S.D.	Sample 2 log count Mean±S.D.	Sample 1 log count Mean±S.D.	Sample 2 log count Mean±S.D.	Sample 1 log count Mean±S.D.	Sample 2 log count Mean±S.D.
Strep. mutans	4.00±1.20	5.44±0.67	4.65±1.01	5.38±0.24	5.34±1.21	5.96±0.68
Lactobacillus spp.	4.00±1.12	4.19±0.94	3.86±1.28	4.03±0.87	3.30±1.80	3.04±1.64
Candida spp.	2.34±1.32	2.42±1.32	2.81±1.10	2.62±1.05	1.96±1.29	2.01±1.18
Veillonella spp.	7.20±0.77	7.30±0.51	7.06±0.22	7.20±0.40	7.28±0.45	7.28±0.38

Table 5.5 Means and standard deviations of logarithmic microbiological counts with recent antibiotic therapy (sample 1) and in the absence of recent antibiotic therapy (sample 2), for the three groups of children who had been taking antibiotics currently, in the past week, or in the past month.

antibiotic therapy and in the week following antibiotic therapy might be atypical for an individual, and should be excluded from subsequent predictive investigations.

5.3.3 Loss of subjects

As described in Section 4.3.3, a 23% subject loss occurred during the three years of the Lanarkshire trial. Similarly, there was a decrease in the number of participants over the two year caries prediction investigation. The numbers present at each examination are shown in Fig. 5.4. In addition to the loss of 69 individuals for the reasons described in Section 4.3.3, a further 25 subjects attended for clinical examination, but failed to return for salivary donation later in the day. As a consequence of the results described in Section 5.3.1, it was also decided to exclude microbiological counts for 53 children who were taking antibiotics, or who had been taking antibiotics in the week preceding salivary donation.

In addition, to allow analyses to be performed using complete data, it was decided to exclude any child with incomplete salivary, microbiological or clinical results. Therefore subsequent analyses were confined to 372 of the original 519 children investigated. Of these 372 children, 346 completed diet diaries, as described in Section 5.2.4. The analysis of the dietary data was therefore confined to this lesser number.

5.3.4 Distribution of variables

Before deciding whether to analyse the data using parametric or non-parametric tests, the distributions of the variables under investigation were determined.

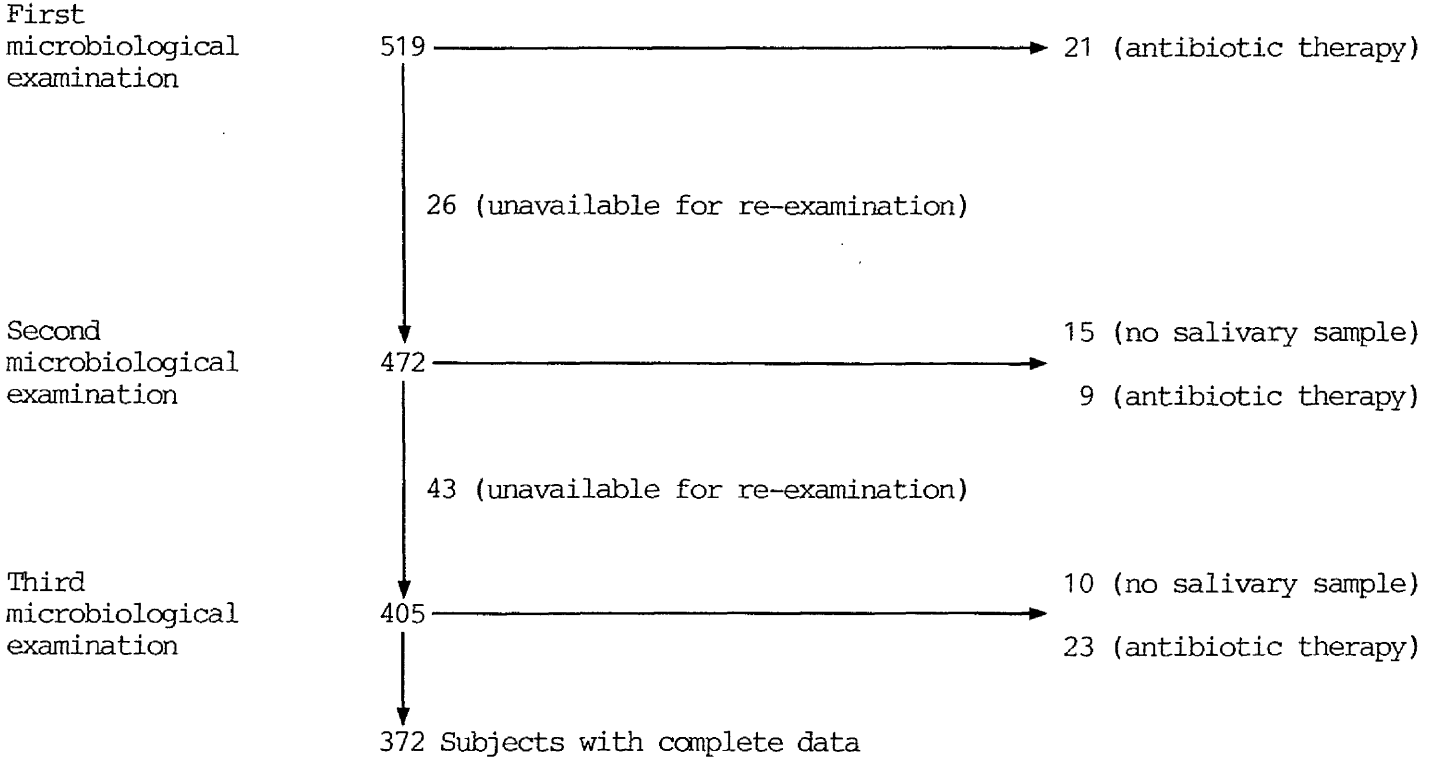


Figure 5.4 Numbers of subjects present at each microbiological examination, and the reasons for loss of children from the caries prediction study.

The shape of the distributions of the principal microbiological variables, that is the Strep. mutans, lactobacillus, veillonella and candida counts, studied at each of the three annual sampling occasions, are shown as box-plots of the logarithmic counts in Figures 5.6 to 5.9 respectively. The key to these box-plots is shown in Fig. 5.5. The three examinations were the second, third and fourth annual examinations of the Lanarkshire clinical trial, and are numbered in this way to avoid confusion. As can be seen, for each variable, the shapes of the sample distributions are similar on each occasion. At all three examinations Candida spp. were isolated from approximately half the subjects studied. Thus, in Fig. 5.9 the box-plots represent those with positive candidal counts, the percentage from whom candida could not be isolated being shown separately.

Figure 5.10 shows similar box-plots of the DS scores from the three clinical examinations corresponding to the three microbiological investigations and, in Fig. 5.11 are box-plots showing the gradual increase in DMFS scores over the two years of caries prediction. In Fig. 5.12, box-plots of the simplified Greene and Vermillion oral debris scores at each examination are shown, again with similar distributions of results for each year. The box-plots shown in Fig. 5.13 are of the one and two year DMFS and DXFS increments. The distributions of the DMFS and DXFS increments were very similar and, as expected, the two year increments were greater than one year increments.

The distributions of variables were considered to be sufficiently close to an underlying normal distribution to make the use of parametric analyses not unreasonable.

+ = Median.

= Interquartile range.

● = Possible outliers, outwith 1.5 times the interquartile range from the nearer quartile.

○ = Probable outliers, outwith 3 times the interquartile range from the nearer quartile.

Figure 5.5 Key to the box-plots, Figures 5.6 to 5.13.

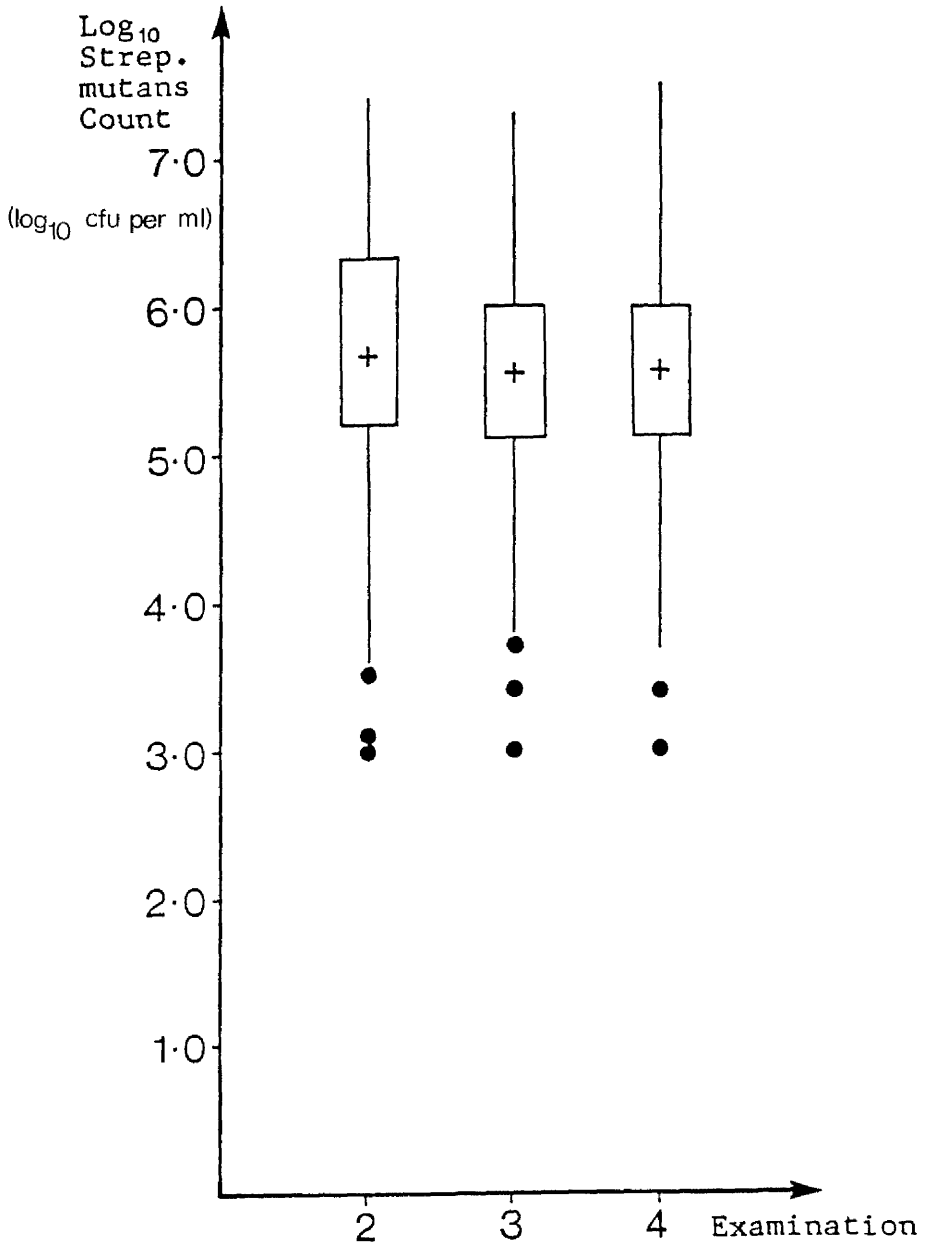


Figure 5.6 Box-plots showing the shape of the distribution of $\log_{10}$ Strep. mutans counts for 372 children at each of three annual examinations.

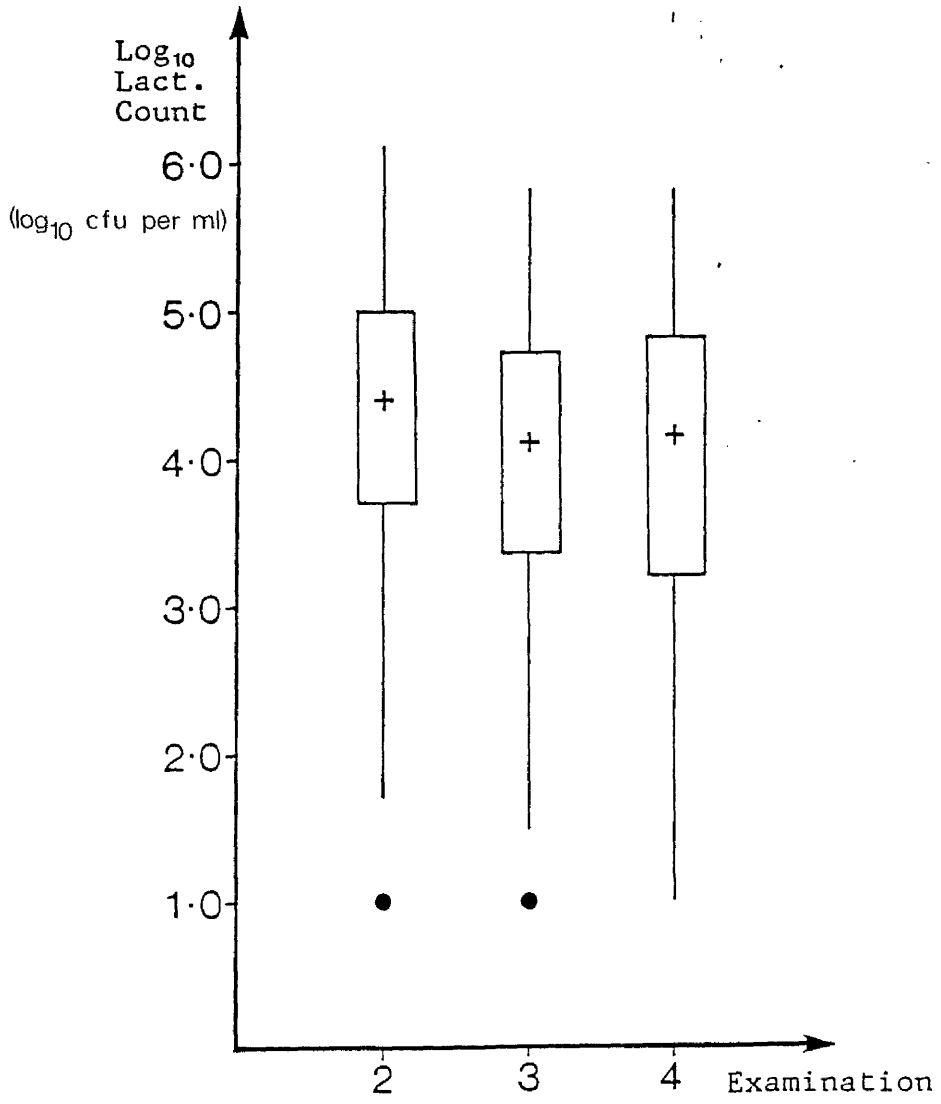


Figure 5.7 Box-plots showing the shape of the distribution of $\log_{10}$ Lactobacillus spp. counts for 372 children at each of three annual examinations.

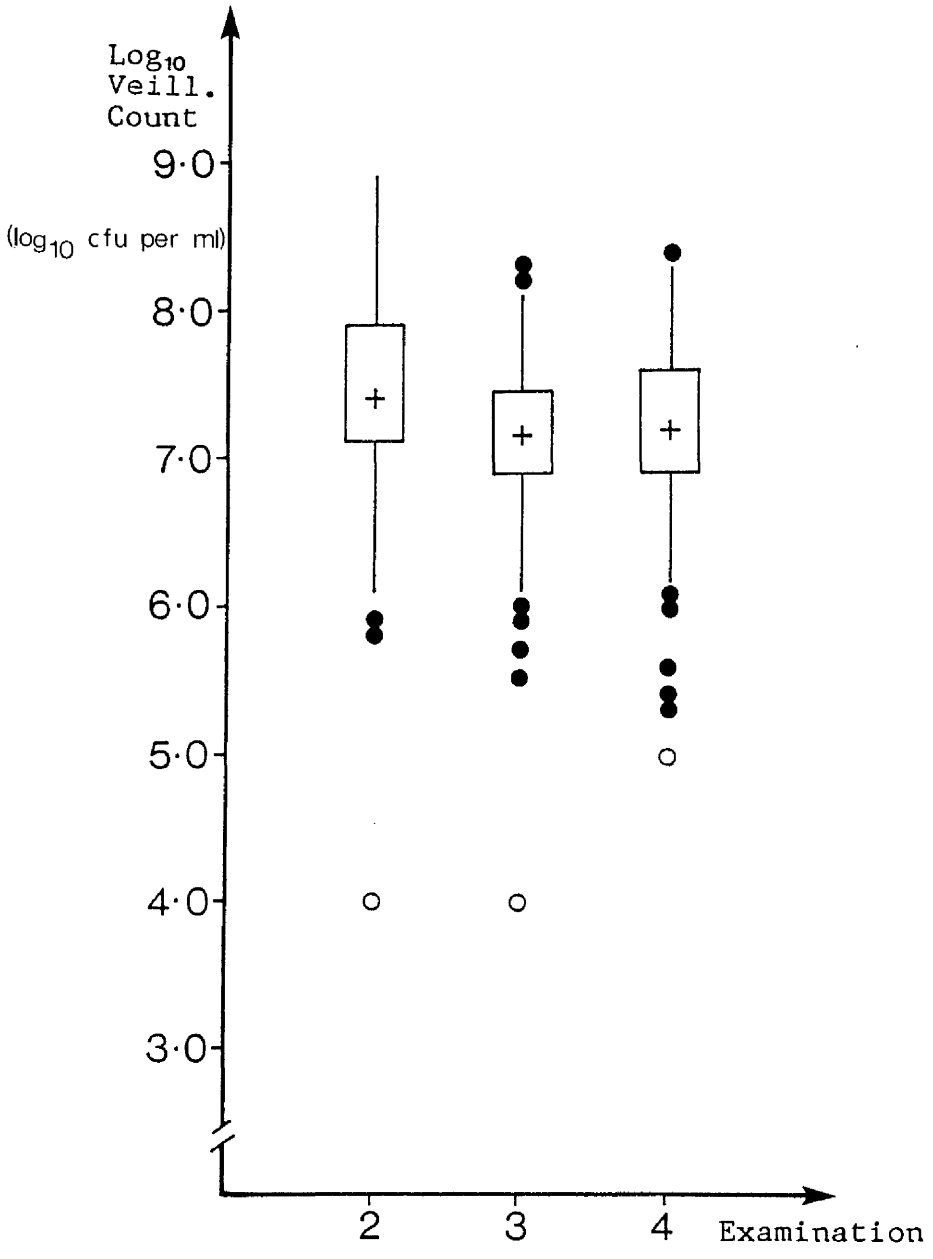
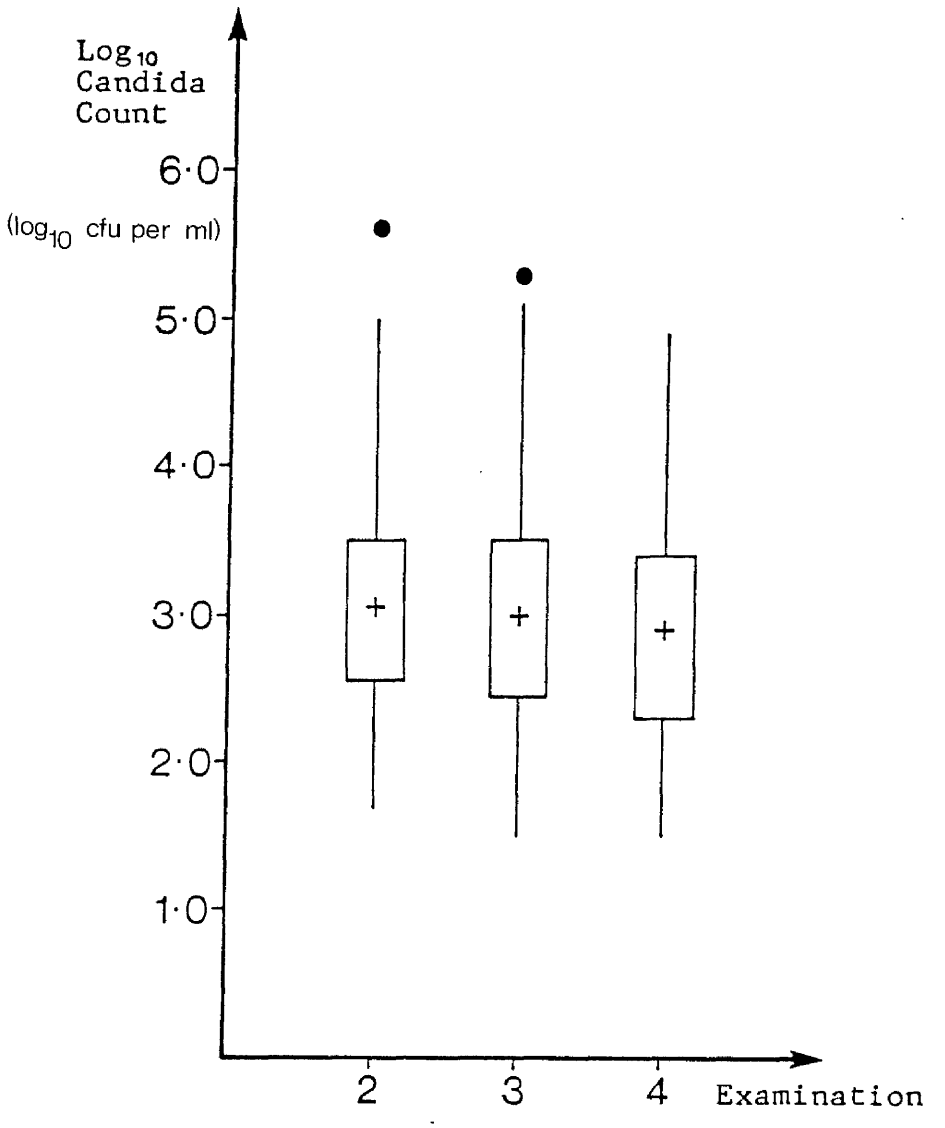


Figure 5.8 Box-plots showing the shape of the distribution of $\log_{10}$ Veillonella spp. counts for 372 children at each of three annual examinations.



Percentage Candida Free	54.0%	50.3%	47.6%

Figure 5.9 Box-plots showing the shape of the distribution of $\log_{10}$ Candida spp. counts for those subjects with Candida spp. detectable in salivary samples. Also shown are the percentages of children with zero candidal counts.

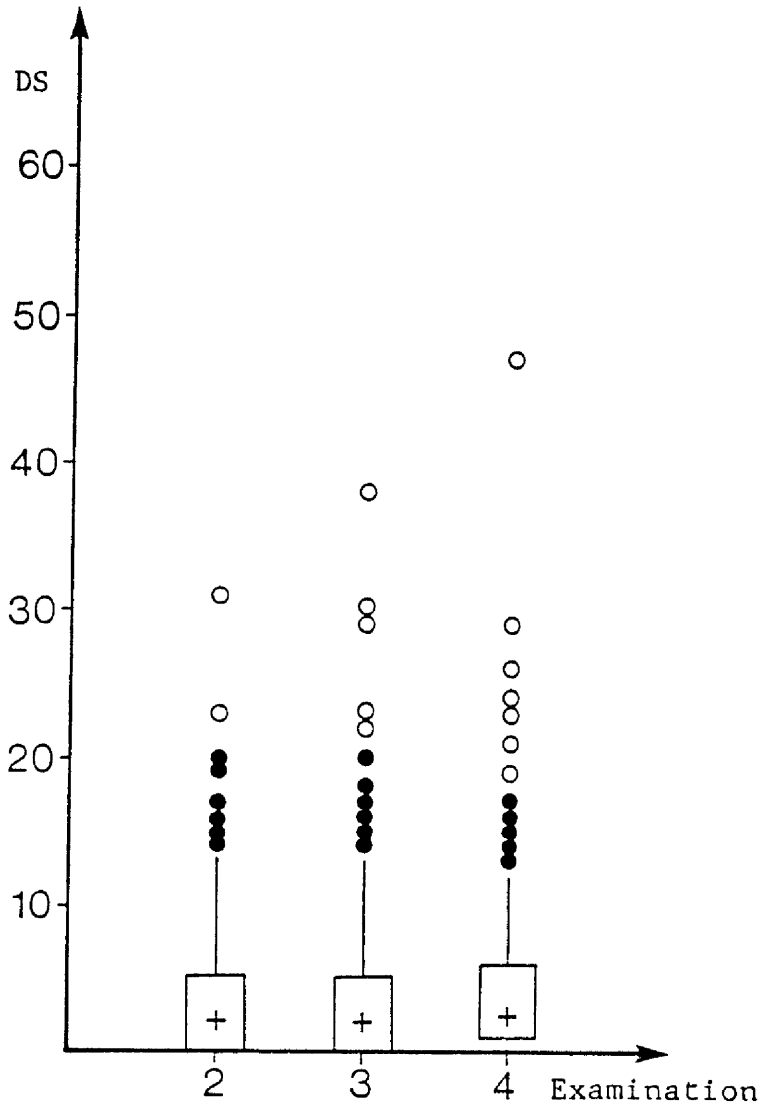


Figure 5.10 Box-plots showing the shape of the distribution of Decayed Surface (DS) score for 372 children at each of three annual examinations.

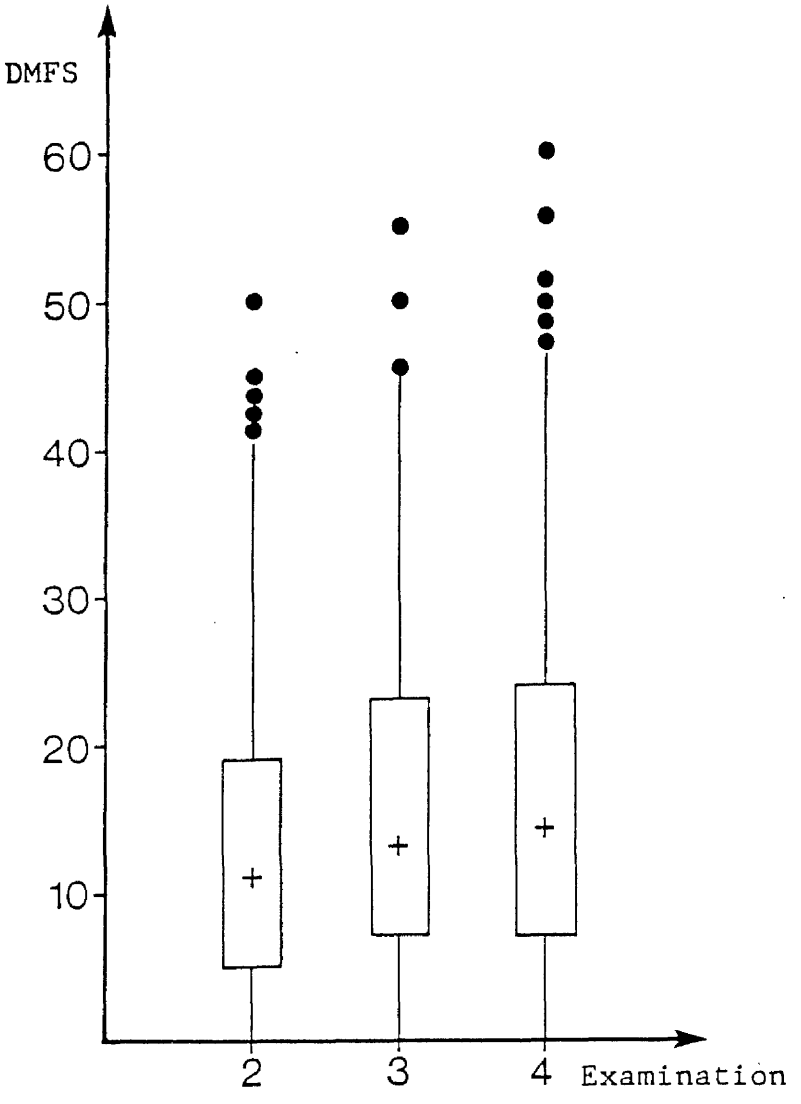


Figure 5.11 Box-plots showing the shape of the distribution of Decayed, Missing and Filled Surface (DMFS) score for 372 children at each of three annual examinations.

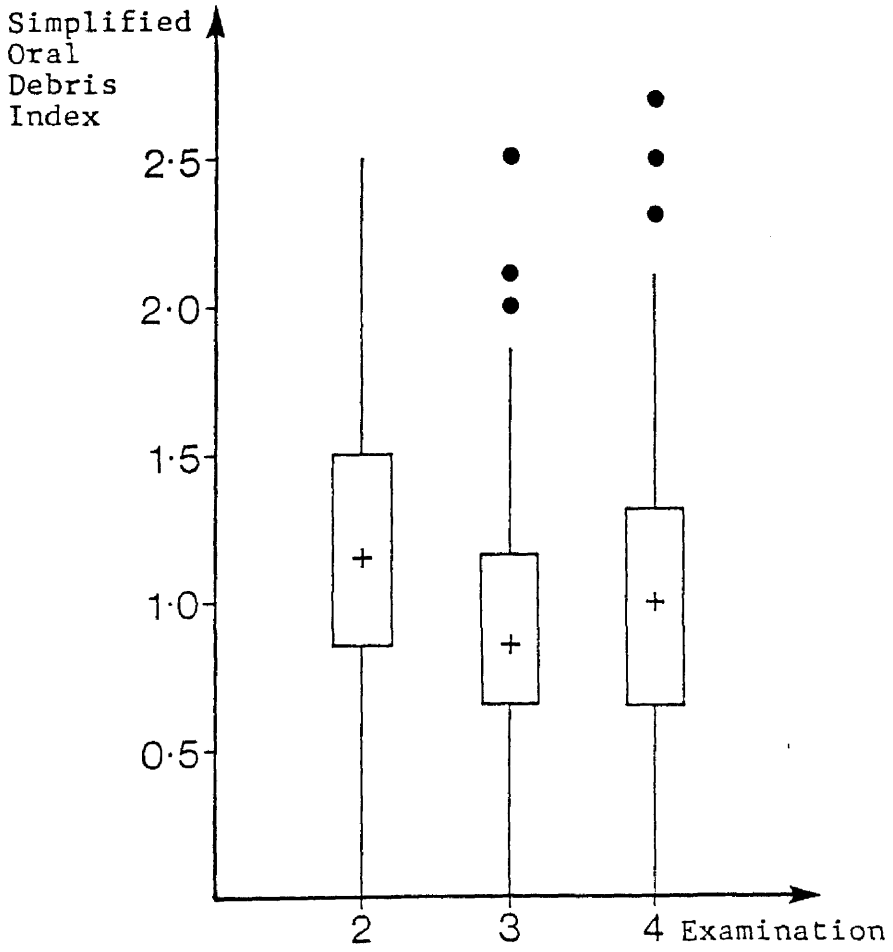


Figure 5.12 Box-plots showing the shape of the distribution of Greene and Vermillion simplified oral debris indices for 372 children at each of three annual examinations.

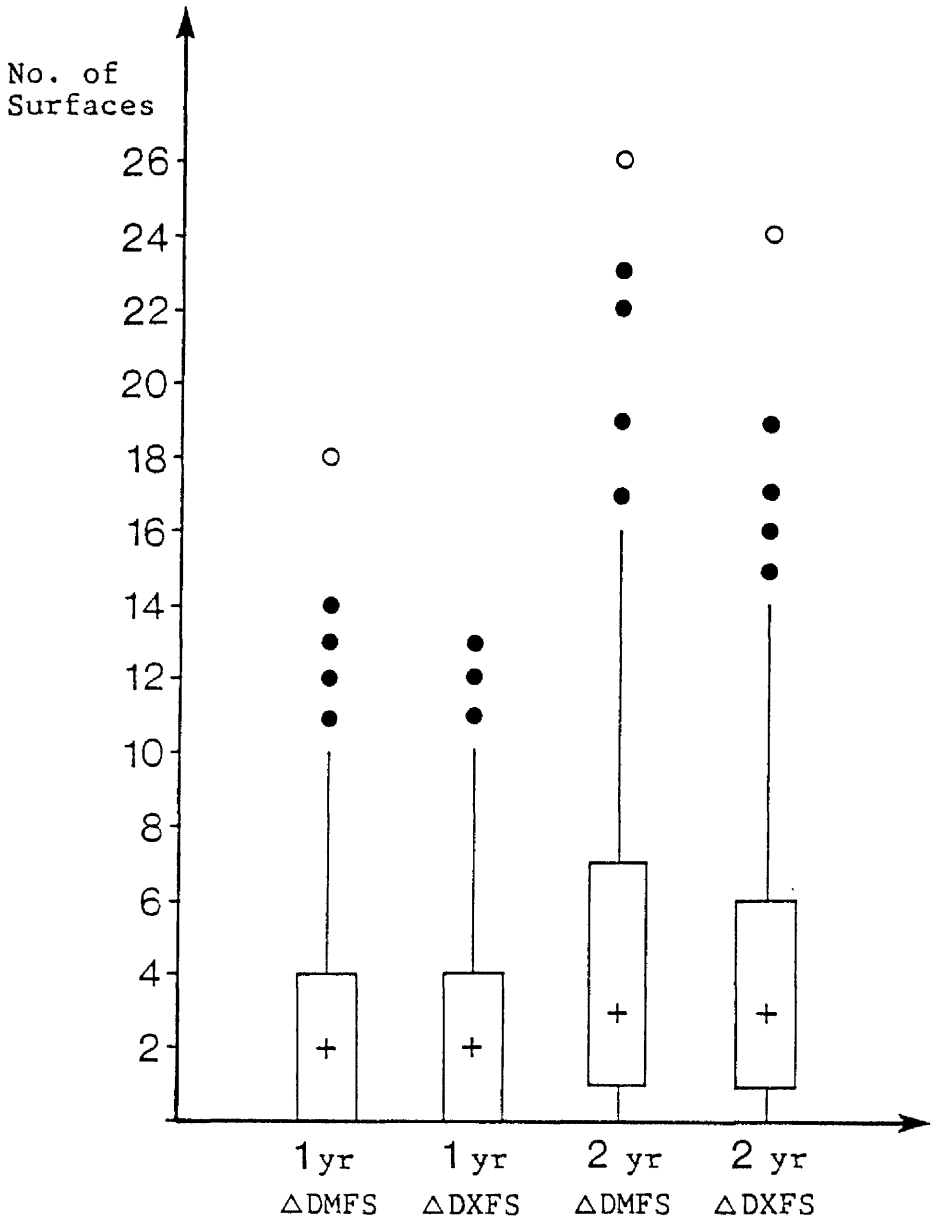


Figure 5.13 Box-plots showing the shape of the distributions of one and two year DMFS and DXFS increments subsequent to Examination 2, for 372 children.

5.3.5 Effect of different toothpastes

As described in Section 5.2.1, the subjects studied were enrolled in the Lanarkshire dentifrice trial, and were using the six toothpastes described in Section 4.2.4. The eventual distribution of the 372 children with complete microbiological and clinical data, according to toothpaste, is shown in Table 5.6. The distribution was in proportion to that in the larger clinical trial, with similar numbers of subjects using the 1000 ppm F and 1500 ppm F toothpastes, and approximately half as many in the 2500 ppm F groups. Additionally, children were almost equally divided between the zinc and non-zinc products.

In Section 4.3.4 the significant effects of toothpaste fluoride concentration on caries increments, and the lack of zinc citrate effect, were described for the larger group of 2316 children, over three years. As shown in Table 5.7, similar effects of fluoride on the caries increments were found in the smaller subgroup studied for caries prediction. However, for this smaller group, over the shorter times studied, analyses of variance revealed the fluoride effect to be significant for the two year DXFS increment only, with the effect on the two year DMFS increment just failing to achieve significance ($p = 0.06$). As in Section 4.3.4, zinc citrate was found to have no effect on caries increment, and therefore in Table 5.7 the data have been combined for children using toothpastes with and without zinc.

The potential predictive variables to be studied subsequently, were then investigated for any significant fluoride or zinc effects, using an analysis of variance, as described in Section 5.2.5. The variables examined were the DMFS and DS scores at the second examination; the oral debris index at Examinations 2 to 4; the

Dentifrice zinc citrate	Dentifrice fluoride level		
	1000 ppm.	1500 ppm.	2500 ppm.
0% zinc citrate	69	75	42
0.5% zinc citrate	62	87	37

Table 5.6 Distribution of the 372 children investigated for prediction, over the six toothpastes.

Increment	Dentifrice fluoride level		
	1000 ppm.	1500 ppm.	2500 ppm.
	mean ± S.D.	mean ± S.D.	mean ± S.D.
1 year ΔDMFS	2.93 ± 3.35	2.59 ± 2.90	2.52 ± 2.89
1 year ΔDXFS	2.63 ± 2.84	2.38 ± 2.77	2.22 ± 2.59
2 year ΔDMFS	5.02 ± 5.09	4.22 ± 4.12	3.58 ± 3.86
2 year ΔDXFS	4.53 ± 4.44	3.78 ± 3.77	3.09 ± 3.38

Table 5.7 Means and standard deviations of caries increments, as ΔDMFS and ΔDXFS from the second clinical examination, for the 372 children using toothpastes of differing fluoride concentrations, with the data for the presence or absence of zinc citrate combined.

lactobacillus, Strep. mutans, candida and veillonella counts at each microbiological examination; the results of Snyder, Dentocult and Strep. mutans colorimetric tests at each microbiological examination; the salivary flow rate from Examinations 2 to 4, and the buffering capacity at Examinations 1, 3 and 4. The only significant effect found was the influence of zinc on the oral debris score. The mean oral debris scores for the two groups of children at Examinations 2 to 4 are shown in Table 5.8. At Examinations 2 and 4 the differences between the two toothpaste groups were marginally significant at the $p < 0.05$ level. This finding was similar to that described in Section 4.3.4 for the larger group of children in the Lanarkshire clinical trial.

5.3.6 Prevalence associations

As the clinical, microbiological and salivary data collection was repeated at three annual examinations, for each individual, it was possible to investigate the associations between the microbiological variables and the clinical data at each of three separate annual examinations. Shown in Table 5.9 are Pearson's correlation coefficients for the associations between DMFS, DMFT, DS and oral debris index scores, and the microbiological plate counts of lactobacilli, Strep. mutans, candida and veillonella, for each of the three data sets. The significance levels illustrated are two-tailed levels, but take no account of multiple comparisons.

As can be seen from Table 5.9 the logarithmic counts of lactobacilli, candida and Strep. mutans were consistently positively but weakly correlated with all four clinical variables, with the

Examination	Simplified Oral Debris Index	
	Non-zinc	0.5% zinc citrate
	mean ± S.D.	mean ± S.D.
2	1.28 ± 0.44	1.18 ± 0.44
3	0.94 ± 0.37	0.92 ± 0.39
4	1.08 ± 0.36	0.97 ± 0.52

Table 5.8 Mean simplified oral debris indices for the two groups of 186 children using toothpastes without zinc citrate and with 0.5% zinc citrate at each of three annual examinations.

	Pearson's correlation coefficients			
	$\log_{10}$ Lactobacilli	$\log_{10}$ Strep. mutans	$\log_{10}$ Candida	$\log_{10}$ Veillonella
and DMFS at				
Exam 2	0.18 **	0.19 ***	0.25 ***	0.00 N.S.
Exam 3	0.23 ***	0.18 ***	0.31 ***	-0.03 N.S.
Exam 4	0.25 ***	0.18 ***	0.27 ***	-0.04 N.S.
and DMFT at				
Exam 2	0.21 ***	0.20 ***	0.26 ***	0.01 N.S.
Exam 3	0.25 ***	0.18 **	0.31 ***	-0.03 N.S.
Exam 4	0.29 ***	0.19 ***	0.27 ***	-0.04 N.S.
and DS at				
Exam 2	0.35 ***	0.17 **	0.31 ***	0.08 N.S.
Exam 3	0.35 ***	0.19 ***	0.33 ***	-0.02 N.S.
Exam 4	0.35 ***	0.13 *	0.26 ***	0.02 N.S.
and ODI at				
Exam 2	0.14 **	0.14 **	0.19 ***	0.05 N.S.
Exam 3	0.20 ***	0.16 **	0.19 ***	0.18 ***
Exam 4	0.18 **	0.20 ***	0.13 *	0.00 N.S.

N.S. = non-significant, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

Table 5.9 Repeat Pearson's correlation coefficients for the prevalence associations between clinical and microbiological variables, for 372 children at three annual examinations.

lactobacillus and candida counts particularly correlated with the DS score. The veillonella count, however, showed a consistent lack of correlation with the clinical variables, apart from one significant correlation at Examination 3, with the simplified oral debris score.

Table 5.10 shows the correlation between the same clinical variables, and the three methods of estimation of lactobacilli: the plate count (as previously shown in Table 5.9), the Dentocult class, and the Snyder test class. The results for the plate count and Dentocult dip-slide were similar, with the dip-slide results being marginally more highly correlated on most occasions. The results of the Snyder test were less well correlated with the clinical indices than were the other two methods.

In Table 5.11 similar correlations between clinical variables and both methods of estimation of Strep. mutans are shown: the plate count (as previously shown in Table 5.9), and the Strep. mutans colorimetric test class. Generally, the colorimetric test correlated less well with the clinical variables than did the plate count and, additionally, over the three years studied, the correlations were less consistent for the colorimetric test.

The correlations between the clinical variables and the salivary variables: Dentobuff score, and five-minute paraffin-wax-stimulated mixed salivary flow rate, are shown in Table 5.12. There was a significant negative correlation between Dentobuff score and the caries indices, but a lack of relationship between these indices and the salivary flow rate, despite the positive correlation shown in Table 5.13 between Dentobuff score and salivary flow.

The correlations between the individual microbiological variables are shown in Table 5.14. Significant correlations existed between the

	Pearson's correlation coefficients		
	log_{10} Lactobacilli	Dentocult class	Snyder class
and DMFS at			
Exam 2	0.18 **	0.20 ***	0.11 *
Exam 3	0.23 ***	0.25 ***	0.10 N.S.
Exam 4	0.25 ***	0.27 ***	0.12 *
and DMFT at			
Exam 2	0.21 ***	0.21 ***	0.11 *
Exam 3	0.25 ***	0.27 ***	0.12 *
Exam 4	0.29 ***	0.31 ***	0.15 **
and DS at			
Exam 2	0.35 ***	0.34 ***	0.13 *
Exam 3	0.35 ***	0.35 ***	0.12 *
Exam 4	0.35 ***	0.36 ***	0.17 **
and ODI at			
Exam 2	0.14 **	0.14 **	0.07 N.S.
Exam 3	0.20 ***	0.21 ***	0.11 *
Exam 4	0.18 **	0.18 ***	0.14 **

N.S. = non-significant, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

Table 5.10 Repeat Pearson's correlation coefficients for the prevalence associations between clinical variables and three methods of estimating levels of lactobacilli, (Rogosa plate count, Dentocult dipslide and Snyder test) for 372 children at three annual examinations.

	Pearson's correlation coefficients	
	$\log_{10}$ Strep. mutans	Strep. mutans colorimetric class
and DMFS at		
Exam 2	0.19 ***	0.12 *
Exam 3	0.18 ***	0.06 N.S.
Exam 4	0.18 ***	0.13 *
and DMFT at		
Exam 2	0.20 ***	0.11 *
Exam 3	0.18 **	0.09 N.S.
Exam 4	0.19 ***	0.12 *
and DS at		
Exam 2	0.17 **	0.12 *
Exam 3	0.19 ***	0.09 N.S.
Exam 4	0.13 *	0.15 **
and ODI at		
Exam 2	0.14 **	0.13 *
Exam 3	0.16 **	0.07 N.S.
Exam 4	0.20 ***	0.04 N.S.

N.S. = non-significant, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

Table 5.11 Repeat Pearson's correlation coefficients for the prevalence associations between clinical variables and two methods of estimation of levels of Strep. mutans, (MSB plate count and colorimetric test) for 372 children at three annual examinations.

	Pearson's correlation coefficients	
	Dentobuff score	stimulated salivary flow rate
and DMFS at		
Exam 2	-0.19 ***	-0.09 N.S.
Exam 3	-0.26 ***	-0.05 N.S.
Exam 4	-0.24 ***	-0.05 N.S.
and DMFT at		
Exam 2	-0.16 **	-0.07 N.S.
Exam 3	-0.23 ***	-0.05 N.S.
Exam 4	-0.21 ***	-0.05 N.S.
and DS at		
Exam 2	-0.04 N.S.	0.04 N.S.
Exam 3	-0.12 *	0.07 N.S.
Exam 4	-0.09 N.S.	0.06 N.S.
and ODI at		
Exam 2	-0.08 N.S.	-0.07 N.S.
Exam 3	-0.05 N.S.	0.05 N.S.
Exam 4	0.00 N.S.	0.05 N.S.

N.S. = non-significant, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

Table 5.12 Repeat Pearson's correlation coefficients for the prevalence associations between clinical variables and salivary variables, for 372 children at three examinations. N.B. The Dentobuff scores used in the comparisons for Examination 2 were obtained at Examination 1.

	Pearson's correlation coefficients Dentobuff score at		
	Exam 1	Exam 2	Exam 3
and salivary flow at			
Exam 2	0.36 ***		
Exam 3		0.50 ***	
Exam 4			0.53 ***

N.S. = non-significant, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

Table 5.13 Repeat Pearson's correlation coefficients for the prevalence associations between Dentobuff scores and salivary flow rate, for 372 children.

	Pearson's correlation coefficients		
	$\log_{10}$ Lactobacilli	$\log_{10}$ Strep. mutans	$\log_{10}$ Candida
and $\log_{10}$ Veillonella at			
Exam 2	0.15 **	0.26 ***	0.03 N.S.
Exam 3	0.07 N.S.	0.27 ***	0.02 N.S.
Exam 4	0.09 N.S.	0.20 ***	0.05 N.S.
and $\log_{10}$ Candida at			
Exam 2	0.35 ***	0.24 ***	
Exam 3	0.36 ***	0.27 ***	
Exam 4	0.40 ***	0.28 ***	
and $\log_{10}$ Strep. mutans at			
Exam 2	0.34 ***		
Exam 3	0.35 ***		
Exam 4	0.31 ***		

N.S. = non-significant, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

Table 5.14 Repeat Pearson's correlation coefficients for the prevalence associations between the logarithmic plate counts of the four microbiological variables studied, for 372 children at three annual examinations.

lactobacillus, Strep. mutans and candidal counts, with the veillonella correlating consistently with Strep. mutans, but not with the levels of the other two micro-organisms.

The correlations between the microbiological and salivary data, are shown in Table 5.15, with the lactobacilli, Strep. mutans and candida generally significantly negatively correlated with Dentobuff score, whereas the correlations of veillonella and Dentobuff were non-significant. However, the salivary flow rate correlated, again negatively, with the counts of Strep. mutans, candida and veillonella, but was not significantly related to the lactobacillus count.

Table 5.16 shows the lack of significant correlation between the dietary data at Examination 3 (for all four methods of assessing the frequency of sugar or carbohydrate consumption, as described in Section 5.2.4) and the clinical, microbiological and salivary prevalence data, again collected at Examination 3.

5.3.7 Combinations of prevalence associations

Stepwise regression analysis was used to investigate associations between the clinical variables DMFS and DS, and combinations of microbiological and salivary variables. These variables were the lactobacillus count, Strep. mutans count, candidal count, veillonella count, Dentocult class, Snyder test class, Strep. mutans colorimetric test class, Dentobuff result, and salivary flow rate. The associations were studied at each of the three repeat examinations. In each case, associations between all possible predictors and the clinical variables were studied first, and thereafter the associations between the clinical variables and the microbiological predictors alone were investigated.

	Pearson's correlation coefficients	
	Dentobuff score	stimulated salivary flow rate
and $\log_{10}$ Lactobacilli at		
Exam 2	-0.12 *	-0.02 N.S.
Exam 3	-0.16 **	-0.07 N.S.
Exam 4	-0.06 N.S.	0.00 N.S.
and $\log_{10}$ Strep. mutans at		
Exam 2	-0.07 N.S.	-0.14 **
Exam 3	-0.19 ***	-0.15 **
Exam 4	-0.12 *	-0.19 ***
and $\log_{10}$ Candida at		
Exam 2	-0.10 *	-0.16 **
Exam 3	-0.20 ***	-0.12 *
Exam 4	-0.11 *	-0.16 **
and $\log_{10}$ Veillonella at		
Exam 2	-0.07 N.S.	-0.14 **
Exam 3	-0.05 N.S.	-0.14 *
Exam 4	-0.03 N.S.	-0.05 N.S.

N.S. = non-significant, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

Table 5.15 Repeat Pearson's correlation coefficients for the prevalence associations between microbiological variables and salivary variables, for 372 children at three examinations. N.B. The Dentobuff scores used in the comparisons for Examination 2 were obtained at Examination 1.

	Pearson's correlation coefficients									
	DMFS	DMFT	DS	ODI	$\log_{10}$ Lact.	$\log_{10}$ S. m.	$\log_{10}$ Cand.	$\log_{10}$ Veill.	Dento- buff	Saliva flow
and frequency of ingestion of foods containing:										
> 1% Sugars	0.01	0.02	0.02	0.02	-0.00	0.03	0.09	-0.04	-0.06	0.04
> 5% Sugars	-0.02	0.00	-0.03	0.01	-0.06	0.00	0.07	-0.01	-0.08	-0.01
> 10% Sugars	-0.01	0.01	-0.01	-0.05	-0.01	0.02	0.08	-0.06	-0.05	0.03
> 10% Carbohydrates	0.00	0.03	0.03	0.02	-0.03	-0.04	0.07	-0.06	-0.04	0.03

Correlations all non-significant

Table 5.16 Pearson's correlation coefficients between dietary variables and clinical, microbiological and salivary variables for 346 children at Examination 3.

At the second examination, as shown in Table 5.9, the logarithmic candidal count was the variable with the best single association with DMFS. Further improvements in this association were obtained using stepwise regression, by including the buffering capacity data and the logarithmic Strep. mutans counts. Table 5.17 shows the variability in DMFS data explained by the regression with candida alone, and by the inclusion of buffering capacity and Strep. mutans counts. Although not given in tabular form, when the regression between DMFS at the second examination and combinations of microbiological variables only, was investigated, the combination of the logarithmic candidal and logarithmic Strep. mutans counts explained a total of only 8.12% of variability.

When the regression between DS at the second examination and combinations of microbiological and salivary variables was investigated, the best single association was found with the logarithmic lactobacillus count, with only another microbiological variable, the log candidal count, significantly improving the regression as shown in Table 5.18.

At the third examination, the variable with the best association with DMFS was again the log candidal count. However, the combination of log candidal count, buffering capacity, and lactobacillus level as measured by the Dentocult dip-slide, explained the greatest amount of the variability in DMFS, as shown in Table 5.19. Although not shown in tabular form, when the regression between DMFS and microbiological variables only was investigated at the third examination, the combination of the log candidal count and Dentocult class explained a total of 11.64% of the variability.

EXAMINATION 2

Stepwise regression analysis of DMFS

Variables included	Variability explained by the regression
$\log_{10}$ Candida	6.39%
$\log_{10}$ Candida & Dentobuff	8.95%
$\log_{10}$ Candida, Dentobuff & $\log_{10}$ Strep. mutans	10.40%

Table 5.17 Variability in Decayed, Missing and Filled Surface (DMFS) score, at Examination 2, explained by stepwise regression of DMFS on microbiological and salivary variables.

EXAMINATION 2

Stepwise regression analysis of DS

Variables included	Variability explained by the regression
$\log_{10}$ Lactobacilli	11.92%
$\log_{10}$ Lactobacilli & $\log_{10}$ Candida	16.24%

Table 5.18 Variability in Decayed Surface (DS) score, at Examination 2, explained by stepwise regression of DS on microbiological and salivary variables.

EXAMINATION 3

Stepwise regression analysis of DMFS

Variables included	Variability explained by the regression
$\log_{10}$ Candida	9.64%
$\log_{10}$ Candida & Dentobuff	13.59%
$\log_{10}$ Candida, Dentobuff & Dentocult class	15.06%

Table 5.19 Variability in Decayed, Missing and Filled Surface (DMFS) score, at Examination 3, explained by stepwise regression of DMFS on microbiological and salivary variables.

As shown in Table 5.20, the single variable with the best association with the DS score at the third examination was the Dentocult class. This table also shows that the combination of Dentocult class, log candidal count, salivary flow rate and Dentobuff score explained the greatest amount of the variability in DS score. Again, not shown in tabular form, in the regression of DS score at the third examination on microbiological variables only, the combination of Dentocult class and log candidal count explained 16.85% of the variability in DS.

At Examination 4, as shown in Table 5.21, the variable with the best single association with DMFS score was the Dentocult class, and the combination of Dentocult class, Dentobuff score, log candidal count and salivary flow rate explained a total of 16.05% of the variability. Using the microbiological variables only, but not shown in the table, the combination of Dentocult class and log candidal count explained 10.61% of the variability in DMFS.

As detailed in Table 5.22, when the regression of the DS score at Examination 4 was investigated, the best single variable, and order of inclusion of variables, were the same as at Examination 3. Similarly, using microbiological variables only, as at Examination 3, the combination of Dentocult class and log candidal count explained 14.79% of the variability in DS score. Again this is not shown in tabular form.

Individual associations

As described in Section 5.2.5, at each of the three examinations, each child's caries indices, DMFS and DS, were categorised as low, medium, or high. The boundaries of the groups

EXAMINATION 3

Stepwise regression analysis of DS

Variables included	Variability explained by the regression
Dentocult class	12.32%
Dentocult class & $\log_{10}$ Candida	16.85%
Dentocult class, $\log_{10}$ Candida & Salivary flow	18.12%
Dentocult class, $\log_{10}$ Candida, Salivary flow & Dentobuff	19.11%

Table 5.20 Variability in Decayed Surface (DS) score, at Examination 3, explained by stepwise regression of DS on microbiological and salivary variables.

EXAMINATION 4

Stepwise regression analysis of DMFS

Variables included	Variability explained by the regression
Dentocult class	7.53%
Dentocult class & Dentobuff	12.09%
Dentocult class, Dentobuff & $\log_{10}$ Candida	14.69%
Dentocult class, Dentobuff, $\log_{10}$ Candida & Salivary flow	16.05%

Table 5.21 Variability in Decayed Missing and Filled Surface (DMFS) score, at Examination 4, explained by stepwise regression of DMFS on microbiological and salivary variables.

EXAMINATION 4

Stepwise regression analysis of DS

Variables included	Variability explained by the regression
Dentocult class	13.21%
Dentocult class & $\log_{10}$ Candida	14.79%
Dentocult class, $\log_{10}$ Candida & Salivary flow	15.88%
Dentocult class, $\log_{10}$ Candida, Salivary flow & Dentobuff	17.18%

Table 5.22 Variability in Decayed Surface (DS) score, at Examination 4, explained by stepwise regression of DS on microbiological and salivary variables.

were chosen in order to allocate approximately equal numbers to each. Using stepwise discriminant analysis the expected caries prevalence group of each individual was estimated from combinations of microbiological and salivary variables selected automatically by the programme, from the data, in the light of their associations with caries prevalence.

Table 5.23 shows the numbers of chidren categorised into each DMFS group at the second examination: low DMFS, 0-6; medium DMFS, 7-15; high DMFS, 16-52. In only 40.3% of cases was the DMFS category identified correctly from the logarithmic candidal count, which was the variable with the best single association with DMFS at this examination. However, in the analysis for this examination, the inclusion of additional variables did not improve the identification of DMFS group significantly.

Similarly, the DS scores at the second examination were categorised as low (0), medium (1-3) or high (4-31). As shown in Table 5.24, the correct DS group was identified in 44.4.% of cases, using the combination of log lactobacilli and log candida counts

Detailed in Tables 5.25 and 5.26 are the repeat analyses at the third examination, with the correct DMFS group identified for 48.7% of children using log candidal counts and buffering capacity data, and the DS group being identified correctly for 46.0% by means of log lactobacilli and log candidal counts.

Again, at the fourth examination the analyses were repeated, and Table 5.27 demonstrates the correct identification of DMFS group in 43.3% of cases, using Dentocult class and buffering capacity data. Similarly, in Table 5.28, the appropriate DS category was identified for 46.8% of children, using the Dentocult class alone.

		Expected DMFS			
		low	medium	high	% correct
Actual DMFS	low (0-6)	79	6	32	67.5%
	medium (7-15)	74	5	51	3.8%
	high (16-52)	57	2	66	52.8%
	TOTAL	210	13	149	**40.3%**

Table 5.23 Table showing numbers of children with actual Decayed, Missing and Filled Surface (DMFS) score categorised as low (0-6), medium (7-15) or high (16-52), versus the numbers with DMFS expected to be low, medium or high, as identified by means of stepwise discriminant analysis at Examination 2, using $\log_{10}$ Candida.

		Expected DS			
		low	medium	high	% correct
Actual DS	low (0)	56	29	20	53.3%
	medium (1-3)	59	36	55	24.0%
	high (4-31)	14	30	73	62.4%
	TOTAL	129	95	148	**44.4%**

Table 5.24 Table showing numbers of children with actual Decayed Surface (DS) score categorised as low (0), medium (1-3) or high (4-31), versus the numbers with DS expected to be low, medium or high, as identified by means of stepwise discriminant analysis at Examination 2, using the combination of $\log_{10}$ Lactobacilli and $\log_{10}$ Candida.

		Expected DMFS			
		low	medium	high	% correct
Actual DMFS	low (0-8)	80	17	28	64.0%
	medium (9-19)	60	30	36	23.8%
	high (20-56)	36	14	71	58.7%
	TOTAL	176	61	135	**48.7%**

Table 5.25 Table showing numbers of children with actual Decayed, Missing and Filled Surface (DMFS) score categorised as low (0-8), medium (9-19) or high (20-56), versus the numbers with DMFS expected to be low, medium or high, as identified by means of stepwise discriminant analysis at Examination 3, using the combination of the $\log_{10}$ Candida and the Dentobuff data.

		Expected DS			
		low	medium	high	% correct
Actual DS	low (0)	48	25	23	50.0%
	medium (1-3)	47	47	56	31.3%
	high (4-38)	14	36	76	60.3%
	TOTAL	109	108	155	**46.0%**

Table 5.26 Table showing numbers of children with actual Decayed Surface (DS) score categorised as low (0), medium (1-3) or high (4-38), versus the numbers with DS expected to be low, medium or high, as identified by means of stepwise discriminant analysis at Examination 3, using the combination of $\log_{10}$ Lactobacilli and $\log_{10}$ Candida.

		Expected DMFS			
		low	medium	high	% correct
Actual DMFS	low (0-9)	71	13	41	56.8%
	medium (10-20)	58	14	55	11.0%
	high (21-60)	31	13	76	63.3%
	TOTAL	160	40	172	**43.3%**

Table 5.27 Table showing numbers of children with actual Decayed, Missing and Filled Surface (DMFS) score categorised as low (0-9), medium (10-20) or high (21-60), versus the numbers with DMFS expected to be low, medium or high, as identified by means of stepwise discriminant analysis at Examination 4, using the combination of Dentocult class and Dentobuff data.

		Expected DS			
		low	medium	high	% correct
Actual DS	low (0)	39	45	7	42.9%
	medium (1-4)	48	84	44	47.7%
	high (5-47)	7	47	51	48.6%
	TOTAL	94	176	102	**46.8%**

Table 5.28 Table showing numbers of children with actual Decayed Surface (DS) score categorised as low (0), medium (1-4) or high (5-47), versus the numbers with DS expected to be low, medium or high, as identified by means of stepwise discriminant analysis at Examination 4, using Dentocult class.

5.3.8 Prediction using single variables

As described in Section 5.2.5, the associations between the individual potential predictive variables studied at the second examination, and the subsequent caries increments, were investigated using Pearson's correlation coefficients. The increments studied were the one year caries increments, as both DMFS and DXFS from the second to third examinations, and the two year increments, again of both DMFS and DXFS, from the second to fourth examinations.

The correlation coefficients between each potential predictive variable and the caries increments were again consistent but small, and are shown in Table 5.29. The significance levels shown take no account of multiple comparisons. For individual variables, the correlations with DMFS increment and DXFS increment are similar, with those relating to DMFS generally slightly greater. Additionally, for most variables investigated, the correlations with the two year increments are higher than those for the one year increments.

Of the clinical variables, the DMFS at Examination 2 was the best single predictor of one year increment, while the best predictor of two year increment was the DS score. Only small reductions in the correlations were observed, as shown in Table 5.29, when the DMFS score was confined to clinical data only, rather than using the combined clinical and radiographic data. Interestingly, the previous one year DMFS increment (from Examination 1 to Examination 2) was a less efficient predictor than the caries prevalence variables. The caries increments were also found to correlate significantly with the oral debris index, although again the correlations were less than with the caries prevalence variables.

Of the microbiological variables, the best single predictor of

	Pearson's correlation coefficients			
	1 year ΔDMFS	1 year ΔDXFS	2 year ΔDMFS	2 year ΔDXFS
DMFS (clin. & rad.)	0.32 ***	0.29 ***	0.37 ***	0.33 ***
DMFS (clin. only)	0.31 ***	0.29 ***	0.35 ***	0.31 ***
DS (clin. & rad.)	0.29 ***	0.25 ***	0.46 ***	0.40 ***
ΔDMFS (Exams 1-2)	0.19 ***	0.16 **	0.24 ***	0.23 ***
ODI	0.15 **	0.18 ***	0.19 ***	0.19 ***
$\log_{10}$ Lactobacilli	0.24 ***	0.21 ***	0.34 ***	0.31 ***
$\log_{10}$ Strep. mutans	0.22 ***	0.21 ***	0.25 ***	0.26 ***
$\log_{10}$ Candida	0.15 **	0.14 **	0.25 ***	0.23 ***
$\log_{10}$ Veillonella	-0.09 N.S.	-0.08 N.S.	-0.03 N.S.	-0.03 N.S.
Dentocult class	0.29 ***	0.26 ***	0.40 ***	0.36 ***
Snyder class	0.10 *	0.08 N.S.	0.14 **	0.11 *
S. mutans colorimetric	0.14 **	0.15 **	0.18 ***	0.20 ***
Dentobuff	-0.09 N.S.	-0.10 *	-0.11 *	-0.12 *
Salivary flow rate	0.04 N.S.	0.03 N.S.	0.04 N.S.	0.06 N.S.

N.S. = non-significant, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

Table 5.29 Pearson's correlation coefficients for the associations between combined clinical and radiographic, clinical, microbiological, and salivary variables for 372 children at Examination 2, and the subsequent one year and two year caries increments (combined clinical and radiographic data), expressed as increments of DMFS and DXFS.

caries increment was the lactobacillus count as assessed by the Dentocult dip-slide method. The lactobacillus plate count, Strep. mutans count, and candidal counts were respectively the next best predictors. The Strep. mutans colorimetric test also correlated significantly with caries increment, but the Snyder test was poorly correlated, and the veillonella count showed no significant relationship with caries increment.

The salivary variables were extremely poorly related to caries increment, with only the buffering capacity, as assessed using the Dentobuff test, achieving significance. The five-minute paraffin-wax-stimulated mixed salivary flow rate was not found to be significantly related to caries increment.

The correlations between the caries increments and the dietary variables obtained at Examination 3 were also calculated, and are shown in Table 5.30. The significance levels illustrated take no account of multiple comparisons. The correlations were found to be consistently weakly positive, with only the correlation between the frequency of ingestion of foods containing more than 1% sugars, and the preceding one year caries increment, achieving significance.

Overall, the best single predictor of two year DMFS increment was the DS score, with the lactobacillus count by the Dentocult method, the DMFS score, and the lactobacillus count by the plate count, showing the next best relationships.

	Pearson's correlation coefficients			
	1 year ΔDMFS	1 year ΔDXFS	2 year ΔDMFS	2 year ΔDXFS
and frequency of ingestion of foods with				
> 1% Sugars	0.11 *	0.11 *	0.09 N.S.	0.09 N.S.
> 5% Sugars	0.09 N.S.	0.10 N.S.	0.05 N.S.	0.07 N.S.
> 10% Sugars	0.04 N.S.	0.05 N.S.	0.03 N.S.	0.05 N.S.
> 10% Carbohydrate	0.06 N.S.	0.06 N.S.	0.06 N.S.	0.06 N.S.

N.S. = non-significant, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

Table 5.30 Pearson's correlation coefficients for the associations between dietary variables for 346 children at Examination 3, and both one year caries increments from Examinations 2 to 3, and two year caries increments from Examinations 2 to 4 (combined clinical and radiographic data), expressed as increments of DMFS and DXFS.

5.3.9 Prediction using combinations of variables

Stepwise regression analysis was next employed to identify the optimum combination of variables in the prediction of the one year and two year caries increments. In view of the similarity of the correlations with the DMFS and DXFS increments, as shown in Section 5.3.8, these further analyses were confined to the conventional increment of DMFS.

For the DMFS increment from Examinations 2 to 3, the combination of DMFS, Dentocult class, veillonella count and Strep. mutans count explained a total of 19.23% of the variability, as shown in Table 5.31. If the potential predictors were confined to microbiological and salivary variables, excluding the clinical predictors, the optimum combination was that of Dentocult class, veillonella count and Strep. mutans count, explaining a total of 13.41% of the variability, as shown in Table 5.32.

In Section 5.3.5, it was demonstrated that the two year DMFS increment was significantly related to toothpaste fluoride concentration, for the group of 372 children studied. Consequently, in the investigation of the DMFS increment from Examinations 2 to 4, the effect of the different toothpaste fluoride levels was included first in the regression.

Table 5.33 shows that 33.39% of variability in the two year DMFS increment was explained by the optimum combination of fluoride level, DS score, Dentocult class, DMFS score, veillonella count and Strep. mutans count. When only microbiological and salivary variables were used, the combination of Dentocult class, Strep. mutans count and veillonella count, in addition to fluoride level, explained a total of 20.34% of the variability. This is illustrated in Table 5.34.

Stepwise regression analysis of one year DMFS increment

Variables included	Variability explained by the regression
DMFS	10.30%
DMFS & Dentocult class	15.75%
DMFS, Dentocult class & $\log_{10}$ Veillonella	17.53%
DMFS, Dentocult class, $\log_{10}$ Veillonella & $\log_{10}$ Strep. mutans	19.23%

Table 5.31 Variability in one year Decayed, Missing and Filled Surface (DMFS) increment, from Examination 2, explained by stepwise regression of DMFS increment on clinical, microbiological and salivary variables.

Stepwise regression analysis of one year DMFS increment

Variables included	Variability explained by the regression
Dentocult class	8.54%
Dentocult class & $\log_{10}$ Veillonella	10.60%
Dentocult class, $\log_{10}$ Veillonella & $\log_{10}$ Strep. mutans	13.41%

Table 5.32 Variability in one year Decayed, Missing and Filled Surface (DMFS) increment, from Examination 2, explained by stepwise regression of DMFS increment on microbiological and salivary variables.

Stepwise regression analysis of two year DMFS increment

Variables included	Variability explained by the regression
Fluoride	1.46%
Fluoride & DS	23.03%
Fluoride, DS & Dentocult class	29.17%
Fluoride, DS, Dentocult class & DMFS	30.94%
Fluoride, DS, Dentocult class, DMFS & $\log_{10}$ Veillonella	32.05%
Fluoride, DS, Dentocult class, DMFS, $\log_{10}$ Veillonella & $\log_{10}$ *Strep. mutans*	33.39%

Table 5.33 Variability in two year Decayed, Missing and Filled Surface (DMFS) increment, from Examination 2, explained by stepwise regression of DMFS increment on clinical, microbiological and salivary variables, with effect of toothpaste fluoride.

Stepwise regression analysis of two year DMFS increment

Variables included	Variability explained by the regression
Fluoride	1.46%
Fluoride & Dentocult class	16.73%
Fluoride, Dentocult class & $\log_{10}$ *Strep. mutans*	18.45%
Fluoride, Dentocult class, $\log_{10}$ *Strep. mutans* & $\log_{10}$ Veillonella	20.34%

Table 5.34 Variability in two year Decayed, Missing and Filled Surface (DMFS) increment, from Examination 2, explained by stepwise regression of DMFS increment on microbiological and salivary variables, with effect of toothpaste fluoride.

5.3.10 Individual prediction

The one and two year DMFS increments from Examination 2, were next categorised as low, medium or high. The class boundaries were chosen in order to allocate approximately equal numbers of children to each category. Using stepwise discriminant analysis the expected caries increment for each individual was predicted using a subset of the clinical, microbiological and salivary variables selected automatically by the programme, in the light of the data, for its predictive value.

For the one year increment, the variables DMFS and Dentocult class allowed the correct identification of a child's increment group in 47.3% of cases, as shown in Table 5.35. However, as shown in Table 5.36, when only the microbiological and salivary predictive variables were used in the analysis, a total of 44.6% of increment groups were correctly identified using the variables Dentocult class, Strep. mutans count and veillonella count.

As in Section 5.3.9, when the two year increment of DMFS was analysed, the effect of fluoride was intentionally the first variable included. The subsequent inclusion of the Dentocult class, DMFS, and veillonella count allowed the correct identification of 49.2% of children's increment groups, as shown in Table 5.37. When the predictors were confined to the microbiological and salivary variables, the correct increment group was identified in 45.4% of cases, using the combination of fluoride, Dentocult class, veillonella count and Strep. mutans count. This is illustrated in Table 5.38.

Shown in Table 5.39 are the sensitivity, specificity and predictive values as defined in Fig. 5.3, for the prediction of the two year DMFS increment, using the single variables DMFS score, DS

		Expected one year ΔDMFS			
		low	medium	high	% correct
Actual one year ΔDMFS	low (0)	70	23	25	59.3%
	medium (1-3)	61	34	44	24.5%
	high (4-18)	25	18	72	62.6%
	TOTAL	156	75	141	**47.3%**

Table 5.35 Table showing numbers of children with actual one year Decayed , Missing and Filled Surface (DMFS) increment categorised as low (0), medium (1-3) or high (4-18), versus the numbers with one year ΔDMFS expected to be low, medium or high, as identified by means of stepwise discriminant analysis using the DMFS score and Dentocult class from Examination 2.

		Expected one year ΔDMFS			
		low	medium	high	% correct
Actual one year ΔDMFS	low (0)	66	17	35	55.9%
	medium (1-3)	59	30	50	21.6%
	high (4-18)	23	22	70	60.9%
	TOTAL	148	69	155	**44.6%**

Table 5.36 Table showing numbers of children with actual one year Decayed, Missing and Filled Surface (DMFS) increment categorised as low (0), medium (1-3) or high (4-18), versus the numbers with one year ΔDMFS expected to be low, medium or high, as identified by means of stepwise discriminant analysis using microbiological and salivary predictors only. The variables Dentocult class, log Strep. mutans count and log veillonella count from Examination 2, were utilised in the analysis.

		Expected two year ΔDMFS			
		low	medium	high	% correct
Actual two year ΔDMFS	low (0-1)	71	23	27	58.7%
	medium (2-5)	66	34	41	24.1%
	high (6-26)	13	19	78	70.9%
	TOTAL	150	76	146	**49.2%**

Table 5.37 Table showing numbers of children with actual two year Decayed, Missing and Filled Surface (DMFS) increment categorised as low (0-1), medium (2-5) or high (6-26), versus the numbers with two year ΔDMFS expected to be low, medium or high, as identified by means of stepwise discriminant analysis using the variables toothpaste fluoride, Dentocult class, DMFS and log veillonella count, from Examination 2.

		Expected two year ΔDMFS			
		low	medium	high	% correct
Actual two year ΔDMFS	low (0-1)	62	30	29	51.2%
	medium (2-5)	64	36	41	25.5%
	high (6-26)	12	27	71	64.5%
	TOTAL	138	93	141	**45.4%**

Table 5.38 Table showing numbers of children with actual two year Decayed, Missing and Filled Surface (DMFS) increment categorised as low (0-1), medium (2-5) or high (6-26), versus the numbers with two year ΔDMFS expected to be low, medium or high, as identified by means of stepwise discriminant analysis using microbiological and salivary predictors only. The variables utilised were toothpaste fluoride, Dentocult class, log veillonella count, and log Strep. mutans count, from Examination 2.

Predictor	Prediction of 2 year DMFS increment			
			Predictive Value	
	Sensitivity	Specificity	Risk	Non-risk
DMFS	56.1%	62.6%	56.9%	74.0%
DS	54.0%	71.7%	53.2%	72.3%
Strep. mutans	48.2%	67.8%	47.2%	68.7%
Lactobacilli	39.6%	83.3%	58.5%	69.8%
Candida	54.4%	66.1%	49.4%	71.3%

Table 5.39 Sensitivity, specificity and predictive values for the prediction of two year Decayed, Missing and Filled Surface (DMFS) increment using some predictive tests.

score, and logarithmic lactobacillus, Strep. mutans, and candida counts. As can be seen from Table 5.39, the use of these methods of assessment resulted in non-risk predictive values which were generally greater than the risk predictive values, and in greater specificity than sensitivity. Additionally, no one test performed better overall than another.

5.4 Discussion

5.4.1 Identification of microbiological isolates

The proportions of appropriate micro-organisms isolated from the agar plates and dip-slides, in the present study, were described in Section 5.3.1. One problem which was encountered was the small number of colonies which could not be identified due to failure in subculture. Little difficulty was experienced with subculturing colonies from Sabouraud dextrose agar. However, 2% of colonies on Rogosa S.L. agar and 4% of colonies on M.S.B. agar failed to subculture. Additionally, a higher proportion (14% of colonies) failed to subculture from the Dentocult dip-slides. While, in accordance with the manufacturer's instructions, dip-slides were incubated for four days before subculturing, the other plates were only incubated for two days, and it is possible that increased incubation time and lack of nutritional supply on the crowded dip-slides resulted in more colonies becoming non-viable. Additionally, it was often difficult to subculture single colonies on such crowded dip-slides.

Negligible numbers of colonies other than Candida spp. were identified from those on Sabouraud dextrose agar, probably because

the candidal colonies were counted according to colonial morphology, and were readily distinguishable from the others.

Micro-organisms other than lactobacilli accounted for 2% of isolates from colonies counted on Rogosa S.L. agar, and 3% of those on Dentocult dip-slides. These findings were similar to those described by other authors (Frostell & Nord, 1972; Birkhed et al, 1981), but contrasted with those previously reported in Section 2.3.6., where no colonies other than lactobacilli were isolated on these agars from ten children. It seems likely that the isolation of a number of non-lactobacillus colonies on Rogosa SL agar, in the present study, was due to the much wider range of salivary flora investigated.

The 2% of organisms other than Strep. mutans isolated from M.S.B. agar in the present study were similar in numbers both to the 1% reported by Beighton (1986) and to the 1% other organisms isolated from the ten children reported in Section 2.2.6. In that earlier study detailed in Chapter 2 however, all colonies on M.S.B. agar were counted, whereas in the present investigation, and in that of Beighton (1986), colonies on M.S.B. agar were counted according to colonial morphology. Generally, micro-organisms other than Strep. mutans were not commonly found on M.S.B. agar, in the present study. Thus the method of counting apparently made little difference to the numbers of organisms other than Strep. mutans isolated.

In the case of veillonella, in the present study, 3% of other colonies were found, whereas for samples from the children described in Section 2.2.6, less than 1% of other organisms were noted. Again, the difference in results may have been due to the small number of children studied in Chapter 2. However the proportions of micro-

organisms other than veillonella in the present investigations were still inexplicably less than those in the study of Rogosa et al (1958), where 9% of other organisms were isolated on vancomycin agar.

5.4.2 Effect of recent antibiotic therapy

As described in Section 3.3.5, the pooled estimate of the common standard deviation of microbiological counts from subjects, over a 2.5 year period, was about 0.5 logarithmic units. In accordance with the recommendations of Togelius et al (1984) a value of twice this figure was accepted as representing unusual variation in an individual's salivary counts. Thus, as described in Section 5.2.5, a ten-fold (or 1.0 logarithmic unit) change in microbiological counts was selected as an abnormal alteration, which might have occurred as a result of antibiotic therapy. Reductions of this magnitude in Strep. mutans counts associated with some cases of recent antibiotic therapy, were described in Section 5.3.2. These were in accordance with the reports by Sukchotiratana et al (1975) and Maltz and Zickert (1982), in which counts of oral streptococci, including Strep. mutans, were reduced in human volunteers and patients following penicillin therapy. Nonetheless, in the present study, not all children demonstrated low Strep. mutans counts associated with recent antibiotic therapy. This may have been a consequence of the variety of antibiotics taken. Alternatively, there may have been inaccuracies in the antibiotic histories provided, as these were obtained from the children themselves. Additionally, it was not thought feasible to attempt to assess whether the antibiotics reported had been taken as prescribed.

From previous literature reports, the anticipated effects of antibiotics on the other micro-organisms studied were less clear.

McClure and Hewitt (1946) reported that penicillin reduced lactobacillus counts in experimental rats. However, Maltz and Zickert (1982) found almost no effect of ten days penicillin therapy on numbers of lactobacilli in adult patients, and Handleman and Hawes (1965) reported little or no difference in the counts of lactobacilli, veillonella or candida in patients receiving long-term systemic penicillin, when compared with untreated controls.

As reported in Section 5.3.2, none of the pairs of lactobacillus, candida or veillonella counts differed by more than a factor of ten, with the exception of one child who exhibited a ten-fold reduction in lactobacillus count associated with erythromycin therapy during the previous week. Thus, it seemed that antibiotic therapy had little effect on veillonella or candida, and any effect on lactobacilli remained doubtful, as it was possible that the single ten-fold change in counts was a chance finding, and not the direct result of any drug therapy. Furthermore, the normal variation in lactobacillus counts (0.9 logarithmic units), described in Section 3.3.5, was greater than that for the other organisms studied. The changes in lactobacillus counts following antibiotic therapy were all less than double this figure, or approximately a hundred-fold.

Having detected reduced Strep. mutans counts associated with recent antibiotic therapy, and having decided to exclude data from samples obtained from children taking antibiotics, the next problem was to decide how long, after the completion of such therapy, to wait before allowing the inclusion of results. Sukchotiratana et al (1975) had reported the return of total streptococcal counts to pre-treatment levels within two to four days, and Maltz and Zickert (1982) found

increased counts of Strep. mutans four days after treatment, with a return to pre-treatment levels after 20 days. As described in Section 5.3.2, only one of the six children who had been taking antibiotics between one week and one month previously, exhibited a ten-fold change in Strep. mutans count. Furthermore, it was difficult to obtain a reliable antibiotic history outwith the previous week (as illustrated by the few children able to name their therapy in the past month - Table 5.4). Therefore, it was decided to include data for children who had taken antibiotics between one and four weeks previously, accepting that, as a consequence, a few inaccuracies in microbiological assessments might be included.

5.4.3 Distribution of variables

The distributions of microbiological and clinical variables were reported in Section 5.3.4. The microbiological counts were logarithmically distributed, and therefore the logarithm of each count was taken prior to further analyses. The distributions of variables were then considered to be sufficiently close to an underlying normal distribution to make the use of parametric analyses not unreasonable. Similar logarithmic transformations of microbiological counts have been described by other authors, including Zickert et al (1982a) and Matee et al (1985).

From examination of the distribution of microbiological and clinical variables in this study, it was also possible to make comparisons with the results reported by other authors.

Streptococcus mutans

In Table 5.40 are shown some descriptive statistics relating to the Strep. mutans counts at each examination of the present study, and also those from similar studies of Strep. mutans prevalence. These authors, with the exception of Matee et al (1985), reported their mean counts without first taking the logarithm of individual counts. The mean of such logarithmically distributed data is highly dependant on the number of very high counts found. Thus the examination of median counts permits more meaningful comparisons.

From Table 5.40 it can be seen that the median and maximum counts found in the present study lay between those described by Klock and Krasse (1977) for a group of 9-12 year old Swedish children studied in 1973, and those of Zickert et al (1982a) for a group of 13-14 year old Swedes. They were, however, greater than those described by Klock and Krasse (1987). Additionally, fewer children (3%) were found to have undetectable Strep mutans levels in the present study, than the 8% described by Klock and Krasse (1977), or the 11% found by Zickert et al (1982a). However, Carlsson et al (1985) reported only 2% of children in Mozambique to be without Strep. mutans, Togelius and Bratthall (1982) described the absence of Strep. mutans in between 3% and 35% of subjects from various populations, Beighton et al (1987) found Strep. mutans to be absent in 3% of British Royal Air Force recruits, and Matee et al (1985) isolated Strep. mutans from all subjects in a Tanzanian field study. In the present project, even if the minimum detection level (2,000 bacteria per millilitre) was increased to the level of 6,700 bacteria per millilitre used by Klock and Krasse (1977), only 5% of children would have had undetectable Strep. mutans levels. Thus, there is apparently considerable

Study	Strep. mutans count			$\log_{10}$ Strep. mutans count			% zero count
	median	mean	maximum	median	mean	maximum	
Klock & Krasse (1977)	6.4×10^5	2.8×10^6	5.0×10^7	-	-	-	8
Zickert et al (1982a)	2.6×10^5	9.4×10^5	1.3×10^7	-	-	-	11
Matee et al (1985)	-	-	$\geq 1 \times 10^6$	-	5.0	-	0
Stecksen-Blicks (1985)	-	-	$> 5 \times 10^5$	-	-	-	12
Klock & Krasse (1987)	3.6×10^4	2.7×10^5	4.4×10^6	-	-	-	23
The present study:							
Examination 2	4.4×10^5	1.5×10^6	2.1×10^7	5.6	5.6	7.3	3
Examination 3	3.6×10^5	1.0×10^6	1.8×10^7	5.6	5.5	7.3	3
Examination 4	3.6×10^5	1.3×10^6	2.7×10^7	5.6	5.5	7.4	3

Table 5.40 Descriptive statistics from studies of Strep. mutans prevalence.

variation in the proportion of the population harbouring this micro-organism in different countries.

Lactobacillus spp.

Descriptive statistics for the lactobacillus counts are shown in Table 5.41, where it can be seen that lactobacillus levels were again similar to those described by others. The median counts were only slightly higher than those of Klock and Krasse (1977) and Zickert et al (1982a), but were about ten times greater than those of Klock and Krasse (1987). However, the maximum lactobacillus count found in this study (1.3×10^6 colony forming units per millilitre of saliva) was a factor of ten less than the maximum described by Klock and Krasse (1977). Additionally, in the present study, lactobacilli were undetectable from 5% of children. In other studies, both Matee et al (1985) and Beighton et al (1987) reported that all subjects harboured lactobacilli, whereas Parvinen (1984) was unable to isolate lactobacilli in 14% of dentate adults, and Klock and Krasse (1977), Zickert et al (1982a), and Stecksen-Blicks (1985) described the absence of lactobacilli in 21% of subjects. Even if the detection level was increased in the present study, from 20 bacteria per millilitre to 670 bacteria per millilitre as used by Klock and Krasse (1977), only 13% of children would not have demonstrated lactobacilli. The number of children without lactobacilli in the present study was thus within the considerable range found in other studies.

Candida spp.

Many studies have investigated the frequency of candidal carriage in the oral cavity, and many different sampling methods have been used. Odds (1979) reviewed 23 such studies, in which mouth swabs,

Study	Lactobacillus count			$\log_{10}$ Lactobacillus count			% zero count
	median	mean	maximum	median	mean	maximum	
Klock & Krasse (1977)	$1.0x10^4$	$2.0x10^5$	$1.0x10^7$	-	-	-	21
Zickert et al (1982a)	$8.0x10^3$	$8.3x10^4$	$5.0x10^6$	-	-	-	21
Parvinen (1984)	10^4	-	10^6	-	-	-	14
Matee et al (1985)	-	-	$\geq 1x10^6$	-	4.5	-	0
Stecksen-Blicks (1985)	-	-	$> 1x10^5$	-	-	-	21
Klock & Krasse (1987)	$1.8x10^3$	$5.6x10^4$	$2.4x10^6$	-	-	-	25
The present study:							
Examination 2	$2.5x10^4$	$1.4x10^5$	$1.3x10^6$	4.4	4.2	6.1	5
Examination 3	$1.3x10^4$	$4.6x10^4$	$5.3x10^5$	4.1	3.9	5.7	5
Examination 4	$1.4x10^4$	$5.0x10^4$	$5.7x10^5$	4.2	3.8	5.8	3

Table 5.41 Descriptive statistics from studies of Lactobacillus spp. prevalence.

throat swabs, gum swabs and tongue swabs were variously used to reveal the carriage of candida in anything between 6% and 69.6% of assorted patient groups. Fewer studies have reported the distribution of candida using stimulated salivary samples from healthy individuals. However, as shown in Table 5.42, the candida counts reported in Section 5.3.4 were similar to those described by Parvinen (1984), for salivary samples obtained from a group of dentate adults. Additionally, the percentage in whom candida could not be demonstrated (approximately half of the group in the present study), was similar to that found in similar studies of salivary samples (Young et al, 1951; Krasse, 1954b; Banoczy et al, 1983).

Veillonella spp.

There have been few reports of the levels of salivary veillonella. Nevertheless, as shown in Table 5.43, the levels found in the present study were greater, by about a factor of ten, than those described by Sims and Snyder (1958), but closer to those for a group of caries active children described by Mazzarella and Shklair (1960), and to those of Rogosa (1956), and Rogosa et al (1958). However, in the present study vancomycin agar was used for veillonella isolation, and this medium was employed in only one of the previous investigations (Rogosa et al, 1958).

Microbiological variation between studies

The small differences found between the distributions of the microbiological variables in this study, and those described by other authors, may have been due to: (a) variation in the laboratory techniques used; (b) differences in the ages of individuals studied, or (c) genuine differences in the prevalence of the micro-organisms in

Study	Candida count			$\log_{10}$ Candida count			% zero count
	median	mean	maximum	median	mean	maximum	
Young et al (1951)	-	-	-	-	-	-	49
Krasse (1954(b))	-	-	7.6×10^4	-	-	-	65
Banoczy et al (1983)	-	-	-	-	-	-	45
Parvinen (1984):							
women	10^3	-	10^5	-	-	-	45
men	0	-	10^4	-	-	-	67
The present study:							
Examination 2	0	2.8×10^3	4.2×10^5	1.0	1.9	5.6	54
Examination 3	0	2.5×10^3	2.0×10^5	1.0	2.0	5.3	50
Examination 4	0	1.6×10^3	6.9×10^4	1.7	2.0	4.8	48

Table 5.42 Descriptive statistics from studies of Candida spp. prevalence.

Study	Veillonella count			$\log_{10}$ Veillonella count			% zero count
	median	mean	maximum	median	mean	maximum	
Rogosa (1956)	-	3.3×10^7	1.9×10^8	-	-	-	-
Rogosa et al (1958)	-	6.7×10^7	-	-	-	-	-
Sims & Snyder (1958): caries inactive	-	3.6×10^6	-	-	-	-	-
caries active	-	4.2×10^6	-	-	-	-	-
Mazzarella & Shklair (1960): caries immune	-	6.0×10^6	-	-	-	-	-
caries active	-	1.4×10^7	-	-	-	-	-
The present study:							
Examination 2	2.9×10^7	7.9×10^7	8.8×10^8	7.4	7.5	8.9	0.3
Examination 3	1.5×10^7	2.3×10^7	2.1×10^8	7.2	7.1	8.3	0.3
Examination 4	1.7×10^7	2.7×10^7	2.5×10^8	7.2	7.2	8.4	0.3

Table 5.43 Descriptive statistics from studies of Veillonella spp. prevalence.

these populations. As suggested by Klock and Krasse (1987), who compared the salivary microbial and dental conditions in Swedish children in 1973 and 1984, such genuine variation might reflect differing caries prevalence. In the study of Klock and Krasse (1987), the reduction in mean caries prevalence from 12.8 lesions per child in 1973 to 3.0 lesions per child in 1984, was accompanied by approximately ten-fold reductions in mean levels of Strep. mutans and lactobacilli. As shown in Tables 5.40 and 5.41, the Strep. mutans and lactobacillus counts in the present study were closer to those in Sweden, in 1973, than in 1984 (Klock & Krasse, 1987). It is possible that this is at least, in part, the result of differences in caries prevalence. At Examination 2 (1984) of this study, the mean number of decayed, missing and filled permanent tooth surfaces was 13, closer again to the Swedish figures for 1973.

Another possible reason for variations in salivary microbiological counts between studies is the level of untreated dental caries which is present in the different populations. It has been shown that lactobacilli in particular, but also Strep. mutans and veillonella, are present in carious dentine (Edwardsson, 1974). Thus, carious lesions could act as a resevoir of these micro-organisms for saliva. Additional evidence for this theory comes from the significant correlations found between numbers of open carious surfaces and microbiological counts as described in Section 5.3.6. Furthermore, counts of lactobacilli and Strep. mutans have been shown to decrease in association with the restoration of carious cavities (Shklair et al, 1956; Kesel et al, 1958; Scheie et al, 1984).

In the present study, although many subjects were attending dental practitioners for routine dental care, no additional

restoration of carious lesions was provided immediately prior to salivary sampling, and the mean decayed surface scores were 3.2 to 3.7 surfaces per child at the different examinations. Similarly, Klock and Krasse (1977) reported no additional treatment prior to salivary sampling, and the mean number of carious surfaces in their subjects was 5.3 surfaces. In the studies by Zickert et al (1982a) and Stecksen-Blicks (1985) open carious lesions were treated prior to salivary sampling, whereas in the report by Matee et al (1985) no missing or filled teeth existed in a Tanzanian population, and the only evidence of caries was incipient and open lesions.

Thus the different investigations reported have included children with differing numbers of open carious lesions, which may also have contributed to the different levels of micro-organisms reported.

Clinical variables

The distributions of the clinical variables investigated in this study were considered to be sufficiently close to normal distributions to allow the use of parametric statistical analyses. Few previous caries activity studies have detailed the distribution of the caries incremental data, and both non-parametric and parametric analyses have been used (Klock & Krasse, 1979; Matee et al, 1985).

The mean two year DMFS increment of 4.4 which was found for this group of 372 children, was not dissimilar to those of 2.5 to 5.7, for 10 to 12-year-old Swedish children using toothpastes with various fluoride concentrations, described by Forsman (1974). Reed (1973) also found a DMFS increment of 3.2 to 4.0, in 5 to 14-year-olds, again using toothpastes of different fluoride levels, over a similar two year period.

5.4.4 Effect of different toothpastes

As described in Chapter 4, a significant dose-response relationship was found in vivo for the effect of increasing levels of sodium monofluorophosphate, in toothpaste, on dental caries increments. In Section 5.3.5, the similar effect on the subgroup of subjects investigated for prediction was described. Although this effect only just achieved borderline significance at the 5% level for the two year caries increment, it was felt necessary to include the effect of toothpaste fluoride in the subsequent analyses for prediction of the two year increment. The effect of toothpaste fluoride on the one year caries increment studied was very small and non-significant, and was therefore not included as a factor in subsequent analyses of this increment. The inclusion of 0.5% zinc citrate was also not found to affect caries increments.

Additionally, in the present chapter it was feasible to investigate possible effects of toothpaste fluoride or zinc citrate on the micro-organisms in the children's saliva. The sensitivity of the oral microflora, especially Strep. mutans, to both the prolonged and short-term use of stannous fluoride mouthwashes has been well documented (Andres, Shaeffer & Windeler, 1974; Tinanoff et al, 1983; Klock et al, 1985). However, it has been suggested that this sensitivity is related to the stannous ion rather than the fluoride ion (Andres et al 1974). The majority of studies investigating sodium fluoride, available from mouthrinses, chewing gum or in the water supply, have reported little effect on oral micro-organisms (Andres et al, 1974; Kilian, Thylstrup & Fejerskov, 1979; Tinanoff et al, 1983; Klock et al, 1985; Ekstrand et al, 1985), although Loesche et al (1975) reported reductions in Strep mutans in occlusal plaque, but not

in interproximal plaque, following repeated topical application of acidulated phosphate fluoride gel. Additionally, Tinanoff (1977) stated that a mouthrinse containing sodium monofluorophosphate had little effect on the bacterial colonisation of tooth enamel. In the present study, no significant differences were demonstrated in the counts of Lactobacillus spp., Strep. mutans, Candida spp. or Veillonella spp., among subjects using three toothpastes containing different sodium monofluorophosphate levels. This finding suggests an absence of sodium fluoride effect on the micro-organisms investigated. This is not certain, however, as it was not possible to compare counts with subjects using a non-fluoride dentifrice. Additionally, although toothpaste use was discussed and checked with each child annually, the toothpastes were obviously used in an unsupervised manner at home. Furthermore, as fluoride appears principally to effect the metabolism of the micro-organisms studied (van der Hoeven & Franken, 1984; Hamilton, Boyar & Bowden, 1985), it is doubtful whether any changes in plaque activity would have been expressed as alterations in salivary counts.

Zinc salts, including zinc citrate, have been shown to inhibit plaque growth both in vitro and in vivo (Skjorland, Gjermo & Rolla, 1978; Harrap, Saxton & Best, 1983; Saxton et al, 1986). However, the presence or absence of 0.5% zinc citrate in the toothpastes studied in this trial, was not found to affect counts of Lactobacillus spp., Strep. mutans, Candida spp. or Veillonella spp.. This finding was confirmed independently for both plaque and salivary micro-organisms in another subgroup from the same clinical trial, reported by Jones and Stephen (1987).

5.4.5 Associations with caries prevalence

The development of a screening test having a high correlation with caries prevalence, and particularly with the number of carious lesions, would allow the identification of individuals with the greatest treatment need. However, in order to be used as a diagnostic measure instead of a clinical examination, such a test would require to have a reproducible high association with caries prevalence, and Snyder et al (1963) suggested that such a correlation should be in the range 0.9 to 1.

The prevalence associations between the microbiological, salivary and dietary variables studied, and both caries prevalence data and plaque levels, were described in Section 5.3.6. All variables other than dietary variables, were assessed on three occasions at annual intervals, in the same group of children. This allowed the reproducibility of any associations found to be ascertained. As shown in Tables 5.9 to 5.12 the associations were generally consistent over the three years, with only small changes in correlation coefficients and hence in significance values. The most obvious variation which did occur was between the different buffering capacity and caries prevalence correlations, as shown in Table 5.12. However, this apparent variation may merely have been the result of the less than ideal method of using first examination buffering capacity results in place of those from the second examination, as described in Section 5.2.3.

Significant correlations were found between the caries prevalence indices and the counts of lactobacilli, Strep. mutans and candida, and the buffering capacity as assessed using the Dentobuff method, as described in Section 5.3.6. The lactobacillus counts and candida

counts tended to be more highly correlated with the number of carious surfaces than with the previous caries experience. As candida and lactobacilli have been reported to be present in carious dentine (Hodson & Craig, 1972; Edwardsson, 1974), it is possible that these higher associations between open carious lesions and lactobacillus and candida counts were the result of the open lesions acting as sources of these micro-organisms. Alternatively, although candida are not usually isolated from dental plaque (Theilade et al, 1974), they have been reported in the plaque of caries active children, both at sites with, and without carious lesions (Milnes & Bowden, 1985). Thus the association between numbers of open lesions and candida may simply reflect their ability to colonise and proliferate under acid conditions.

Lactobacillus spp.

As shown in Table 5.44, the correlations found in this study between caries prevalence and lactobacillus counts were in agreement with those of Klock and Krasse (1977), Zickert et al (1982a) and Ashley, Wilson and Woods (1983), although the levels of the correlations were rather lower than those of Zickert et al (1982a), and higher than those of Matee et al (1985). Unfortunately, only some of the studies which compared lactobacillus counts with previous caries experience presented results as correlation coefficients, and indeed where correlations were performed, the actual value was not always stated. Nevertheless, although the correlations found in this study were generally consistent and significant due to the numbers of subjects studied, they were low and insufficient for individual caries diagnosis. The reason for the greater correlations found by Zickert

	Lactobacillus spp.	Strep. mutans
Klock & Krasse (1977)	positive correlation ($p<0.01$)	positive correlation ($p<0.01$)
Zickert et al (1982a)	0.34 ($p<0.001$)	0.37 ($p<0.001$)
Ashley, Wilson & Woods (1983)	correlated ($p<0.05$)	
Matee et al (1985)	0.07 (non-significant)	0.11 (non-significant)
The present study:		
Examination 2	0.18 ($p<0.01$)	0.19 ($p<0.001$)
Examination 3	0.23 ($p<0.001$)	0.18 ($p<0.001$)
Examination 4	0.25 ($p<0.001$)	0.18 ($p<0.001$)

Table 5.44 Correlation coefficients and significance values for the associations between previous caries experience and counts of Lactobacillus spp. and Strep. mutans in previous reports and in the present study.

et al (1982a) is not clear. However, in Zickert's study, all carious lesions were restored prior to salivary sampling, and it is possible that this may have improved the correlations by eliminating additional sources of micro-organisms, and ensuring that the salivary levels of lactobacilli reflected true caries potential and were not complicated by the effect of open carious lesions.

Streptococcus mutans

As shown in Table 5.44, the associations found in the present study between caries prevalence and Strep. mutans counts were in agreement with the findings of Klock and Krasse (1977) and Zickert et al (1982a), although the correlations found were lower than those of Zickert et al (1982a), and higher than those of Matee et al (1985). The absence of significant correlations in the latter study was thought to be due to the rather low caries experience of the group investigated.

Candida spp.

The associations described in Section 5.3.6 between the levels of candida and caries prevalence were in agreement with the findings of Krasse (1954b) and Banoczy et al (1983). Unfortunately, it is not easy to compare the results of the present study with previous reports, since neither Krasse (1954b) nor Banoczy et al (1983) reported their associations as correlations.

Veillonella spp.

As was shown in Table 5.9, no significant associations were demonstrated between the clinical data and the veillonella counts. This was in accordance with the report of Sims and Snyder (1958), but

disagreed with the significant positive association found by Mazzarella and Shklair (1960). However, few subjects were involved in that latter study, logarithmic counts were not used, and the statistical method was not described.

Salivary variables

Correlations between the salivary buffering capacity and caries prevalence, similar to those reported in Section 5.3.6, have also been reported by Agus and Schamschula (1983) and Pienihakkinen et al (1985). Agus and Schamschula (1983), in particular, reported a correlation coefficient of -0.20, which was very close to the values obtained here. These authors also found Dentobuff scores to be correlated with mixed salivary flow rate, but significant correlations between flow rate and caries prevalence were not demonstrated, and these findings were confirmed in the present study. From the correlation coefficients presented in Table 5.12, the association between salivary flow rate and previous caries experience appeared to be consistently inverse. Nonetheless, for the 372 children studied, the association failed to reach significance. It is possible, of course, that this association might have reached significance if a larger group of subjects had been studied, as Shannon and Terry (1965) found a significant association between resting parotid flow rate and caries experience in 3786 young adults.

Dietary variables

Although it is generally accepted that caries is related to dietary sugar intake (for review, see Holloway, 1983), as shown in Table 5.16, significant correlations were not found between caries

prevalence and frequencies of eating foods containing different levels of sugars or carbohydrate. The failure of the present study to identify such associations may have been the result of: (1) the inaccuracy of the dietary data obtained (the diets recorded over the study period being unrepresentative of children's usual diets); (2) insufficient numbers studied, or (3) food consumption at the study time which may well have differed from dietary habits which pertained during previous caries development. Alternatively, it is possible that dietary intake alone may be of less importance than other factors, either singly or in combination, in the development of dental decay in man.

Accurate dietary details are notoriously difficult to obtain. Burke (1947) stated that the most accurate method of studying an individual's food intake was by means of balance studies of intake and excretion, or alternatively by weighing the food eaten at each meal. However, neither of these methods is suitable for mass studies. Another method which has been used in dietary surveys is a dietary interview or a questionnaire to assess the frequency of consumption of different foods (Schroder, Lindstrom & Olsson, 1981). Alternative methods include: recall of the past 24 hours food intake (Kleemola-Kujala & Rasanen, 1982; Bergendal & Hamp, 1985); a five day diet record (Richardson, Boyd & Conry, 1977), and repeated three day diet histories, each followed by an extensive interview with a dietician (Rugg-Gunn et al, 1984).

In view of constraints in finance and time during the present study, only a single three day diet record was obtained, from each participating subject, and there was only a brief five minute discussion about the dietary record with each child. However, since

it was not feasible to record amounts of food accurately, no attempt was made to quantify intake of sugars or carbohydrates, only frequency of intake of these components being assessed. Nevertheless, significant associations between caries experience and frequency of intake were not obtained. In similar studies by other authors, Kleemola-Kujala and Rasanen (1982) reported associations between caries prevalence and sugar ingestion, whereas Richardson et al (1977) were unable to demonstrate any relationship.

Colorimetric and Dentocult tests

When the associations between caries prevalence and the different methods of lactobacillus and Strep mutans estimation were compared, as shown in Tables 5.10 and 5.11, colorimetric techniques performed consistently less well than did plate counts. These poorer correlations may have been the consequence of the similarity of rate of colour change which was found for tests from different individuals, and which was previously highlighted in Sections 2.4.2 and 2.4.3. When the associations of the lactobacillus counts and Dentocult tests were compared, as shown in Table 5.10, they were found to be very similar. It is likely that this was due to the high association between these two methods of estimation of lactobacilli, as described in Section 2.4.2, and as previously described by Birkhed et al (1981).

Single variables

Overall, the single variable with the best DMFS score association was the candidal count, although at Examination 4 the Dentocult class and candidal count gave similar results. The variable with the best DS score association was the lactobacillus count, with similar results

for both the Rogosa plate count and the Dentocult method. However, the highest of these correlations was 0.36, well below the 0.9 to 1.0 recommended by Snyder et al (1963), for associations at an individual level.

Combinations of variables

Few caries activity studies have reported the use of regression analyses to provide improved associations between dental caries and microbiological variables. However, the use of multiple regression analysis was described by Honkala et al (1984) in a retrospective study of caries risk, using clinical variables, buffering capacity and lactobacillus counts.

As shown in Tables 5.17 to 5.22, in the present study, stepwise regression analysis was used to identify the combination of variables which explained the greatest amount of variability in the caries prevalence. At each examination the regression was carried out for both the past caries experience (DMFS) at that examination, and the number of carious surfaces (DS). The selected combination of variables changed slightly in the different years. The first variable to be included in any of the regressions was automatically the single variable which correlated best with the caries prevalence. Thereafter, variables were entered sequentially, but only included if they were of additional benefit in the prediction. In no case did a single variable account for all the variability explicable by the regression. In each analysis, significant additional improvements in the regression were produced by the inclusion of additional variables. These significant improvements were often small in magnitude. Thus in the regressions of DS at the different examinations, the majority of

the explicable variation was explained by the combination of the lactobacillus count (or Dentocult score) and the candida count, which together explained about 16% of the variability in DS. However, smaller additional improvements were produced at the third and fourth examinations by the inclusion of the salivary flow rate and Dentobuff data, which together explained about another 2% of the variability. The significant inclusion of these variables was nevertheless surprising, as the correlations between DS score and both salivary flow rate and Dentobuff score, as single variables at these examinations, were low and mostly non-significant. Thus some variables, which alone were of no assistance in the association, achieved significance when combined with the effect of another variable. Whether these significant inclusions of variables of minor importance were real effects, or due to chance, is difficult to ascertain.

Disappointingly however, despite the improved associations obtained by including several variables, at no examination did any combination explain more than 20% of the variability. Thus, even in combination, these tests were inadequate for individual diagnosis.

Although the regression analysis did not explain sufficient variability for individual identification, stepwise discriminant analysis was employed to quantify the numbers of individuals whose caries experience could be correctly identified by means of microbiological and salivary data. Again, analyses were carried out for both DMFS and DS scores at each examination. Prior to the analysis, the children were placed in low, medium or high caries experience groups with approximately equal numbers in the groups, as described in Section 5.2.5.

If one compares the variables included in the regression analyses with those utilised in the discriminant analyses in Tables 5.23 to 5.28, it can be seen that, generally, only the variables which were of major benefit in the regression analysis, such as the lactobacillus count and candidal count, were used in the discriminant analysis. This reduction in the number of significant explanatory variables resulted from the grouping of the caries prevalence data. Thus, variables which were of small but significant importance in regression of the ungrouped data were of no significant benefit when the range of caries results was reduced to three categories.

In several of the analyses shown in Tables 5.23 to 5.28, the numbers of children expected to be in the medium category were very small. These low numbers in the medium category may have resulted from the narrow ranges of the predicted prevalence categories. Additionally, where the candidal count was used as the only variable, about 50% (those with a zero count) of children were automatically expected to be low, leaving even fewer to be categorised into the medium group.

As detailed in Tables 5.23 to 5.28, the caries prevalence group of individual children could be identified correctly in between 40% and 49% of cases. However, through chance alone, one would have categorised a third of children correctly. Thus at best only a 16% improvement in identification rate was produced by using microbiological and salivary data.

5.4.6 Prevalence associations with oral hygiene levels

The other clinical variable which was compared with microbiological, salivary and dietary data was the oral debris index. Consistent and similar positive correlations were found between the oral debris indices and the lactobacillus, Strep. mutans and candida counts, although with the veillonella counts the correlation was inconsistent, achieving significance at only the third examination. Similar correlations between lactobacilli, Strep. mutans and oral hygiene were previously described by Zickert et al (1982a), but were not found by Richardson et al (1977) or by Matee et al (1985). Although the correlations found in this study were weak, their consistency makes the existence of associations between oral hygiene levels and salivary counts of these organisms much more certain. This finding has also been reinforced by Wikner (1986), who showed that professional tooth cleaning prior to salivary sampling reduced counts of lactobacilli and Strep mutans. Although the oral hygiene level would thus appear to have a direct effect on counts of salivary micro-organisms, presumably by affecting the numbers available to be released into saliva from tooth surfaces during salivary stimulation, it is also interesting to speculate whether those subjects with a poor oral hygiene level may be those with a high caries incidence, and thus high levels of salivary lactobacilli, Strep. mutans and candida. In this study correlations of 0.2 and over, significant at the 0.1% level were found between caries prevalence and oral debris indices. Thus it is possible that counts of salivary micro-organisms and oral hygiene levels may be associated both directly and indirectly.

5.4.7 Prevalence associations among non-clinical variables

In addition to studying the associations between clinical variables and microbiological, salivary and dietary variables, it was possible to investigate intercorrelations between non-clinical variables. Thus, as previously discussed, salivary flow rate was found to correlate significantly with buffering capacity.

Table 5.14 shows the interesting correlations between the micro-organisms studied. Counts of Strep. mutans, lactobacilli and candida were all significantly intercorrelated. Similar intercorrelations of salivary Strep. mutans and lactobacilli have previously been reported by Klock and Krasse (1977), and by Zickert et al (1982a). Interactions of lactobacilli and candida, in vitro, were described by Young, Krasner and Yudkofsky (1956), and the correlation of lactobacilli and candida was reported by Banoczy et al (1983), although Krasse (1954b) was unable to find such a relationship in a small group of 36 individuals. It is likely that the intercorrelations of these micro-organisms reflect their abilities to metabolise dietary carbohydrate and thus, as described by Stecksen-Blicks (1987) for lactobacilli and Strep. mutans, the numbers of these micro-organisms are likely to be raised in individuals with high carbohydrate diets. Additionally, Strep. mutans and lactobacilli are also found in plaque, as discussed in Section 5.4.6. Therefore salivary numbers of both these bacteria are likely to depend on such factors as oral hygiene and numbers of plaque retention sites.

The counts of salivary veillonella, however, were generally significantly correlated only with Strep. mutans counts. The correlations between veillonella and lactobacilli were consistently positive, but achieved significance at the second examination only,

and associations between veillonella and candida were small and non-significant at all three examinations. Dietary carbohydrates are not fermented by veillonella (Rogosa, 1964), but veillonella are capable of metabolising lactate, pyruvate and succinate (Rogosa, 1964; Distler & Kroncke, 1980; Distler & Kroncke, 1981a; Distler & Kroncke, 1981b). Strep. mutans metabolises glucose to produce lactate, ethanol, acetate and formate (Carlsson & Griffith, 1974; van der Hoeven, 1976). Symbiotic associations between Strep. mutans and veillonella have been reported both in vitro (Mikx & van der Hoeven, 1975; Distler & Kroncke, 1980), and in gnotobiotic animal experiments (van der Hoeven, Toorop & Mikx, 1978). Thus, one possible reason for the significant association between Strep. mutans and veillonella, found in the present study, could be that veillonella counts are able to rise as Strep. mutans counts increase and more lactate is produced.

There is little information in the literature about interactions between lactobacilli and veillonella, although in vivo plaque levels of Strep. mutans, lactobacilli and veillonella have been shown to rise in association with the progression of incipient lesions (Boyar & Bowden, 1985), and increased levels of all three bacteria have been reported in plaque overlying carious lesions, when compared with control sites (Milnes & Bowden, 1985). Lactobacilli ferment glucose to produce lactate, ethanol, acetate and formate (de Vries et al, 1970), and it is thus unclear why associations between lactobacilli and veillonella were not closer to those for Strep. mutans. However, as shown in Section 5.3.4, the numbers of salivary lactobacilli are on average about ten times smaller than the numbers of Strep. mutans. Thus, the metabolic products of lactobacilli may be less important

than those of Strep. mutans as a source of nutrition for veillonella.

Candida produce only very small quantities of lactate from glucose (Samaranayake et al, 1986), but do produce significant amounts of pyruvate, which may be utilised by veillonella. However, there is little information in the literature concerning microbiological interactions between candida and veillonella.

Correlations between salivary variables and microbiological variables were presented in Table 5.15. Generally, negative correlations existed between Strep. mutans, candida or veillonella counts, and salivary flow rate, although for lactobacilli and flow rate the association failed to achieve significance. It is interesting to speculate whether these associations merely reflected a simple dilution effect, since the volume of saliva collected from children with high or low flow rates could well have a marked effect on subsequent microbial counts. Alternatively, individuals with a greater salivary flow may have lower salivary micro-organism counts due to the presence of a more efficient mechanical washing effect of saliva on both bacteria and possible nutritional factors associated with the dental and mucosal surfaces. This possibility has recently been reinforced by a report that salivary counts of lactobacilli and Strep. mutans are directly related to the oral glucose clearance time (Wennerholm, Emilson & Krasse, 1986).

Finally, Table 5.16 allowed comparisons between dietary variables and both microbiological and salivary data. Despite the widely held belief that the levels of lactobacilli and Strep. mutans are related to sugar and other carbohydrate consumption, few studies have shown such relationships. In interventional studies, Jay (1947) reported reductions in salivary lactobacillus counts associated with

restriction in carbohydrate consumption, and Crossner (1984) demonstrated a small increase in lactobacillus counts associated with a great increase in frequency of sugar intake. Krasse (1954a), on the other hand, found no difference in lactobacillus counts between groups on high and low carbohydrate diets. In an observational study, Stecksen-Blicks (1987) identified very weak but significant associations between sugar consumption and salivary lactobacillus counts in 13-year-old children, and between sugar consumption and Strep. mutans counts in 8-year-olds. However, the correlations between sugar and Strep. mutans in 13-year-olds, and between sugar and lactobacilli in 8-year-olds failed to achieve significance. In the present study, disappointingly, neither consistent nor significant correlations were demonstrated. Of course it is possible that the absence of significant relationships in this study may have been the result of inadequacies in the dietary data, as discussed in Section 5.4.5, or alternatively it may be a true result.

5.4.8 Prediction of caries increments

As described in Section 1.6, the ability to predict caries increments on a group basis would benefit community preventive programmes, manpower planning, and clinical trials of caries preventive agents. Additionally, if it was possible to predict such increments at an individual level, then individual treatment planning would also benefit.

The abilities of clinical, microbiological, salivary and dietary factors to predict one and two year caries increments were described in Section 5.3.8. As shown in Tables 5.29 and 5.30, the caries

prevalence, or number of decayed surfaces, were better predictors of subsequent caries increment than were the microbiological, salivary or dietary variables studied. The lactobacillus count, as assessed by the Dentocult method, gave the highest correlation of the microbiological variables, with similar lower associations for Strep. mutans and candidal counts. Again, lower, but still significant correlations for the oral debris index and salivary buffering capacity were obtained. As shown in Table 5.45, these results were similar to those described by other authors, particularly Klock and Krasse (1979), who also reported previous caries frequency to be more highly correlated with subsequent caries increment than either Strep. mutans counts, lactobacillus counts, plaque levels, or salivary buffering capacity. The most notable difference between the results from that study and the present investigation was in the relation found between lactobacillus counts. In this investigation, lactobacilli were found to correlate significantly with subsequent caries increment, whereas Klock and Krasse (1979) found no such relationship. As in the present investigation, open carious lesions were not filled prior to sampling. Therefore, the reasons for the absence of a significant relationship in Klock and Krasse's study are unclear. Nevertheless, it is interesting that although the policy on the restoration of lesions in other studies has varied, similar significant correlations between lactobacilli and caries increment have been found by Snyder (1963), Crossner (1981), and Honkala et al (1984). These associations are also shown in Table 5.45, although in the paper by Snyder et al (1963), the significance levels of the correlations were not stated, and in the paper by Crossner (1981) the magnitudes of the correlations were not reported.

Study	Increment studied	Predictive Variables: Caries Prevalence	*Strep. mutans*	*Lactobacillus* spp.	*Candida* spp.	Oral Hygiene	Buffering capacity	Diet
Snyder *et al* (1963)	6 month R.I.D.[1]	-	-	0.2 - 0.4	-	-	-0.18	-
Klock & Krasse (1979)	1yr ΔDFS	0.25 **	0.07 N.S.	-0.048 N.S.	-	0.09 N.S.	-0.13 N.S.	-
	2yr ΔDFS	0.26 **	0.21 **	0.02 N.S.	-	0.06 N.S.	-0.07 N.S.	-
Crossner (1981)	1.2yr ΔDFS	***	-	***	-	-	-	-
Pienihakkinen *et al* (1984)	1yr ΔDMFS	-	-	significant association	significant association	-	-	-
Rugg-Gunn *et al* (1984)	2yr ΔDMFS	-	-	-	-	-	-	0.10 * [2]
Honkala *et al* (1984)	1yr ΔDMFT	0.69 ***	-	0.26 *	-	-	-0.20 N.S.	0.15 N.S.[3]
Pienihakkinen *et al* (1985)	1yr ΔDMFS	-	-	-	-	-	N.S.	-
Stecksen-Blicks (1987)	1yr ΔDMFS	-	-	-	-	-	-	0.22 * [4]
The present study	1yr ΔDMFS	0.32 ***	0.22 ***	0.24 ***	0.15 **	0.15 **	-0.09 N.S.	0.11 * [5]
	2yr ΔDMFS	0.37 ***	0.25 ***	0.34 ***	0.25 ***	0.19 ***	-0.11 *	0.09 N.S.[5]

N.S. = non-significant
* = $p < 0.05$
** = $p < 0.01$
*** = $p < 0.001$

1 = Relative Increment of Decay (Porter & Dudman, 1960)
2 = weight of total sugars
3 = consumption of sweets
4 = weight of sucrose
5 = frequency of >1% sugars

Table 5.45 Correlation coefficients and significance values for the associations between caries increment and predictive variables in previous reports and in the present study.

Few studies have investigated the caries predictive potential of Candida spp., although as shown in Table 5.45, Pienihakkinen et al (1984) also reported candidal counts to be significantly related to the subsequent one year caries increment. It was somewhat surprising to discover correlations between candidal counts and caries increment, in the present study, which were only slightly less than those found for either lactobacilli or Strep. mutans and caries increment. Candida are not usually considered to play a major role in caries development. Therefore it is assumed that the associations found here reflect the acidurity of Candida spp., and that high numbers of candida merely indicate oral conditions which are favourable for caries development.

Although no significant relationship was found between dietary factors studied and caries prevalence (see Section 5.4.5), consistent positive correlations were found between the same dietary factors and caries increments. As shown in Table 5.30 these correlations were very weak, but for the frequency of ingestion of foods with greater than 1% sugars, and one year caries increment, the correlation was significant. Table 5.45 shows that this result was in accordance with those of Rugg-Gunn et al (1984) and Stecksen-Blicks (1987), although different methods of assessing dietary intake were used in these studies.

Consistent though weak correlations were found between caries increment and dietary factors whereas, as discussed in Section 5.4.5, there was a lack of correlation between dietary factors and previous caries experience. This would suggest that, as discussed in Section 5.4.5, the assessed dietary intake had changed from that which led to

the recorded caries experience, rather than there being great inaccuracy in the dietary data obtained.

The Tables 5.31 to 5.38, showed that improvements in prediction could be obtained by combining clinical and microbiological predictors. Again, improvements in prediction using combinations of variables have been described by Klock and Krasse (1979) and Honkala et al (1984). However the ranges of variables used by these authors were less than in the present study. Nevertheless, despite using combinations of variables, at most, the caries increment group of individual children could be identified correctly in only 49.2% of cases. As approximately 30% of cases would have been predicted by chance alone, less than 20% of individuals were therefore identified by means of the combined clinical and microbiological data.

In Section 5.3.10, the two year predictive abilities of caries prevalence data, and microbiological counts were presented as the sensitivity, specificity and predictive values of the tests, as defined in Fig. 5.3. As stated in Section 5.2.5, prior to calculating these assessments of predictive ability, children were first divided into risk and non-risk groups. As recommended by Crossner (1981), the risk cases were those with an increment greater than the mean caries increment, which in this study was an increment of greater than 4.4 surfaces. Thus 37% of children were assessed to be at risk of caries. Additionally, risk levels for the predictive variables were also selected. Those for the microbiological counts were chosen in accordance with other published studies (Klock & Krasse, 1979; Stecksen-Blicks, 1985). Thus as described in Section 5.2.5, a count of greater than, or equal to 10^6 Strep. mutans per millilitre of saliva was selected. This resulted in 38% of children being predicted to be

at caries risk. The risk level of greater than, or equal to 10^5 lactobacilli per millilitre of saliva predicted 25% of individuals to be at caries risk. The risk levels for the candida count, DMFS and DS scores were chosen to include similar percentages in the high risk category.

As can be seen in Table 5.46, the results obtained using these methods, were similar to those described by Stecksen-Blicks (1985) and Pienihakkinen et al (1987), but the risk predictive values in this study tended to be rather lower than those described by Crossner (1981). However, results can vary greatly depending on where the risk levels are set and, as Stecksen-Blicks (1985) stated, "Results expressed in terms of sensitivity, specificity and predictive values are usually difficult to compare, as these analyses require a definite distinction between disease and health. In dental caries there is no such border." The effect of simply altering the microbiological risk levels can be seen in Table 5.46, where the combined Strep. mutans and lactobacillus counts were analysed twice by Stecksen-Blicks (1985). In method (a), many children were included in the expected risk group, but in (b) few children were so included. As can be seen, the results of these analyses changed drastically. Similarly, the figures for Pienihakkinen et al (1987) represent the wide range of results obtained using different combinations of lactobacillus and candida counts. Thus, in the present study, it was not felt that these methods of expressing results were particularly helpful, and no attempt was made to assess the combined effects of multiple predictive variables using them.

Study/Predictor	Sensitivity	Specificity	Predictive Value	
			Risk	Non-risk
Crossner (1981)				
lactobacillus count	50%	96%	85%	80%
caries frequency	35%	89%	60%	75%
Stecksen-Blicks (1985)				
lactobacillus count	50%	81%	61%	73%
Strep. mutans count	59%	67%	51%	73%
lactobacilli & Strep. mutans [1]	71%	56%	49%	76%
lactobacilli & Strep. mutans [2]	38%	91%	72%	71%
Pienihakkinen et al (1987)				
lactobacilli & candida	38%-95%	19%-83%	-	-
The present study				
DMFS	56%	63%	57%	74%
DS	54%	72%	53%	72%
lactobacilli	40%	83%	58%	70%
Strep. mutans	48%	68%	47%	68%
candida	55%	66%	49%	71%

1 = 42 children predicted caries negative, 49 caries positive
2 = 73 children predicted caries negative, 18 caries positive

Table 5.46 Sensitivity, specificity and predictive values for the present study, and for previous studies of caries prediction.

5.5 Conclusions

In conclusion, in this clinical trial, although significant associations were found between both microbiological and salivary data, and caries experience, the correlations were weak. It did not prove possible to diagnose caries prevalence reliably at an individual level, using microbiological, salivary or dietary factors, either singly or in combination. The associations obtained were therefore insufficient for use as a diagnostic test.

Furthermore, it was possible to predict caries increments on a group basis only, and not at an individual level, although clinical, microbiological, salivary and dietary variables were used singly and in combination. The previous caries experience, or number of carious surfaces, were found to be better predictors of caries increment than any one of the other variables, although improved predictions were obtained by combining clinical and microbiological data.

CHAPTER 6

MICROBIOLOGICAL CARIES PREDICTIVE TESTS IN 5-YEAR-OLDS

6.1 Introduction

As described in Chapter 5, only weak correlations were found between microbiological variables and subsequent caries increments, for the group of adolescent children studied. However, Schroder and Edwardsson (1985) reported high sensitivity, specificity and predictive values for Strep. mutans and lactobacillus counts, in association with dental caries in 3-year-olds. Thus the possibility existed that correlations between microbiological tests and caries increments, higher than those found for the adolescents, might be found for a group of younger children.

Additionally, the use of stepwise discriminant analysis as described in Chapter 5, allowed the identification of the best combination of predictive variables from a range of such factors, for the prediction of caries increment group. Thus, a formula for the prediction of caries from a combination of these factors was produced using the database from the 372 adolescents studied previously. This formula was applied to a younger group of children, details of which are presented in this chapter.

6.2 Materials and methods

6.2.1 Choice of subjects

Informed parental consent was obtained for the investigation of a group of 47 children initially aged 4.8 to 5.7 years, attending two

schools in the Forth Valley Health Board Area of Scotland. This age-group of children was chosen following a small pilot study of ten subjects, aged between three and seven years, which had suggested that these were the youngest children able to co-operate with the donation of stimulated salivary samples.

6.2.2 Microbiological investigations

Mixed salivary samples were obtained from the children described in Section 6.2.1. Where possible, saliva was stimulated by paraffin-wax, although some of the youngsters were unable to co-operate with chewing wax, and in these cases, unstimulated samples were collected. Four of the 47 children studied were unable to provide even unstimulated samples. Thus, only 43 children were included in the study.

Salivary samples were obtained throughout the school day, and were stored in an insulated container to protect them from extremes of temperature after collection. At the end of the day, the samples were transported to Glasgow Dental Hospital, and inoculated on to Mitis Salivarius Bacitracin agar, Rogosa S.L. agar, Sabouraud dextrose agar, and vancomycin agar, using a spiral plater. Inoculation and incubation of plates, and enumeration of colonies were performed as described in Section 3.2.3. In all cases, incubation was commenced within 10 hours of sample collection.

6.2.3 Clinical examination

In order to exclude the possibility of bias in subsequent microbiological assessments, the children were examined, on this occasion, by another clinician (R.M.) from the community dental

service. Each child lay in a supine position, and was examined using a dental mirror. Where necessary, a sharp probe was used for plaque removal. Illumination was by means of a 60 Watt Anglepoise light.

The deciduous canines and molars and permanent incisors and first molars were scored as: sound; decayed; restored; extracted for caries; unerupted; or missing for any other reason. The dmft (number of decayed, missing and filled deciduous teeth) for each child was then calculated, as was the number of carious teeth. In this study a tooth-based, rather than a surface-based index was utilised, as this was the standard system in use with the health board, these children being examined as part of a larger survey.

Of the 43 children investigated initially, 32 were re-examined 18 months later by the same clinician. The number of deciduous teeth which had been sound at initial examination, but which had become carious, filled or missing due to caries, was then calculated for each child.

6.2.4 Statistical analysis

The logarithms of the microbiological counts were obtained as described in Section 2.2.6. Using Minitab (Statistics Dept. Pennsylvania State University), Pearson's correlation coefficients were then calculated between these logarithmic counts and the caries prevalence, number of unrestored carious teeth and 18 month increment of decayed, missing or filled deciduous teeth.

Stepwise discriminant analysis using Programme P7M of BMDP, as described in Section 5.2.5, was used to re-analyse the grouped two year caries incremental data for the 372 adolescent children described in Chapter 5. For this re-analysis, however, only the logarithmic

Strep. mutans, lactobacillus, candida and veillonella counts were used as potential predictors. For the best prediction of caries in the 372 adolescent children, stepwise discriminant analysis employed only two of these variables, the logarithmic lactobacillus and logarithmic candida counts. The other variables were not included, as they were of no additional benefit in the prediction. The analysis also provided the equations used for the combination of variables in the allocation of subjects to the expected caries incremental groups, low, medium or high.

Where "L" equals the logarithmic lactobacillus count, and "C" equals the logarithmic candidal count, the following equations were obtained:

For the low group:-	-5.549 - (0.154 x L) - (2.688 x C)
For the medium group:-	-5.592 + (0.081 x L) - (2.778 x C)
For the high group:-	-4.185 + (0.517 x L) - (2.381 x C)

An individual's expected caries increment group could then be predicted by the following procedure: The individual's logarithmic lactobacillus and candidal counts were entered in the above equations and the result of each equation calculated. The exponential of each result (for low, medium and high equations) was then divided by the sum of the exponentials of the three results to give the probabilities of the individual being in each group as percentages totalling to 100%. The expected caries increment group was thus the group scoring the greatest percentage probability.

This procedure was then applied to the logarithmic lactobacillus and candidal counts obtained for the 32 younger children described in Section 6.2.1, for whom 18 month caries incremental data was

available. The children were also divided into low, medium, and high caries increment groups on the basis of actual dmft increments, with approximately equal numbers of children being allocated to each. Thus, 13 children had a low increment of zero teeth, 8 had a medium increment of one tooth, and 11 children had a high caries increment of two to seven teeth. The actual and expected caries increment groups of the children were then compared.

6.3 Results

6.3.1 Prevalence associations

The prevalence correlation coefficients between the logarithmic microbiological counts and both the initial dmft scores and number of carious teeth, of the children are shown in Table 6.1.

As can be seen, the baseline dmft score was significantly correlated with the Strep. mutans and candida counts, but not with the lactobacilli or veillonella. Similar correlations are also shown between the number of carious teeth and the microbiological variables.

6.3.2 Incremental associations

Also shown in Table 6.1 are the correlation coefficients between the 18 month increment of decayed, missing or filled deciduous teeth and the microbiological counts. The increment was found to correlate significantly with the lactobacillus count only. The correlations with the other microbiological predictors were non-significant. Similarly, although not shown in the table, the correlation between the dmft score at initial examination and the subsequent dmft increment was also found to be non-significant.

Microbiological count	Caries Index		
	dmft	dt	18 month Δdmft
$\log_{10}$ Lactobacilli	0.23 N.S.	0.29 N.S.	0.41 *
$\log_{10}$ Strep. mutans	0.47 **	0.43 *	0.21 N.S.
$\log_{10}$ Candida	0.48 **	0.45 *	0.11 N.S.
$\log_{10}$ Veillonella	0.08 N.S.	-0.01 N.S.	-0.03 N.S.

N.S. = non-significant, * = $p < 0.05$, ** = $p < 0.01$

Table 6.1 Pearson's correlation coefficients for the associations between caries prevalence and incremental data and the logarithmic microbiological counts.

6.3.3 Prediction of individual increments

The procedure described in Section 6.2.4 for the identification of each individual's expected caries increment group was followed. Of the 32 children studied, 22 were predicted to be in the low caries increment group, three in the medium group, and seven in the high group.

The crosstabulation of the numbers of children expected to be in each increment group compared with the numbers actually in each group is shown in Table 6.2. It can be seen that an individual's low caries increment was correctly identified in nine out of 13 (69%) cases. However, partly as a result of the distribution of children's expected increment groups, medium increments were never identified correctly and high increments were identified in only three out of eleven (27%) cases. Overall, an individual's increment group was identified correctly in only 34% of cases.

6.4 Discussion

6.4.1 Sampling method

The children described in this chapter were the youngest from whom it was expected to be able to obtain mixed stimulated saliva. However, it was found that the children were not all able to co-operate, and the exclusion of a small number of children might have been avoided if an alternative method of salivary sampling, such as the spatual method of Kohler and Bratthall (1979), or the loop method of Beighton (1986), had been employed. Such methods would however, have made the direct application of the equations obtained from the children described in Chapter 5 much more complicated.

		Expected 18 month Δdmft			
		low	medium	high	% correct
Actual 18 month dmft	low (0)	9	1	3	69%
	medium (1)	7	0	1	0%
	high (2-7)	6	2	3	27%
	TOTAL	22	3	7	**34%**

Table 6.2 Table showing the numbers of children with actual 18 month decayed missing and filled deciduous tooth (dmft) increment categorised as low (0), medium (1) or high (2-7), versus the numbers with 18 month Δdmft expected to be low, medium or high, as identified by application of the database obtained in Chapter 5, using the variables log lactobacillus and log candida counts.

6.4.2 Incremental index used

Because of the age of children studied, few had any permanent teeth at the start of the investigation, although after 18 months almost all had at least one of their first permanent molar teeth erupted. However, only three of these teeth in the whole group of 32 children were found to be decayed or filled at the second examination.

It is notoriously difficult to select a satisfactory index of caries increment for children at such a mixed dentition stage (Porter & Dudman, 1960), particularly as the situation is complicated by the exfoliation of deciduous teeth. In the present study, the number of deciduous teeth becoming carious, filled or missing due to caries during the 18 month study period was calculated. Any missing deciduous incisor teeth were accepted as having been exfoliated. As the oldest child examined was 7.2 years old, any missing deciduous canine or molar teeth were considered to be missing due to caries. Thus it was hoped that a true deciduous increment had been obtained, although some inaccuracies might still have been included.

One problem of any such caries increment was the small number of children with a high dmft score at initial examination, who of necessity exhibited a low subsequent caries increment. In this study five of the 32 children involved had an initial dmft score of eight or more teeth, and this may have contributed to the lack of correlation found between initial caries prevalence and subsequent caries increment. Additionally, however, it may have affected associations between the microbiological tests and caries increment, as these subjects may still be at risk of developing caries, but simply unable to express this risk due to a lack of sound teeth. Repeating the analyses with the five high caries prevalence children

excluded, resulted in small, non-significant increases in the correlations between the caries increment and both the initial caries prevalence and the microbiological variables.

Ideally, in order to reduce the problem of children with high initial caries prevalence, it would have been desirable to have studied an even younger group of children, but this would have made the collection of stimulated mixed saliva impossible.

6.4.3 Associations with caries prevalence and increment

The correlations between the microbiological tests and caries indices shown in Table 6.1 seemed, in many cases, to be lower or higher than those reported in Chapter 5 for the adolescent children. However, as a result of the small numbers of children in the younger group, the differences between the correlations for the two groups were not found to be significant. Similarly, for the younger group, although the correlations of particular microbiological variables with prevalence and incremental data appeared to vary, the differences were non-significant due to the small numbers studied.

6.4.4 Application of the database obtained in Chapter 5

The unequal distribution of subjects to the three expected caries increment groups highlighted the difficulty of applying equations obtained from the analysis of data from one group of individuals to data for a second group. The greater number of younger children expected to have a low caries increment was due at least in part to the lower levels of lactobacilli found in these children. The median logarithmic lactobacillus count for the adolescent children described

in Chapter 5 was 4.4, while for the 5-year-olds it was 3.1. It may indeed be true that these children have a lower risk of caries related to their lower lactobacillus counts. Alternatively, the lower counts may be the result of some other factor related to the age of the children.

In order to predict similar numbers of children in increment groups, using equations obtained from one sample applied to another sample, the distributions of predictive variables in the two samples should be similar. Thus, in the present study, the lower lactobacillus counts found in the younger children resulted in the prediction that the majority of the children would have a low caries increment, when the database from the adolescents with higher lactobacillus counts was employed.

The difficulty of applying equations from one group of children to another in whom indices are not identical was also highlighted in this study. As described in Section 6.2.3, the caries prevalence of the younger group of children was assessed using a tooth-based index, the dmft index, whereas that of the older group was assessed by means of a surface-based index, the DMFS index. Obviously this discrepancy was not ideal, as it was therefore not possible to include the caries prevalence data directly in the equations for caries prediction in the younger subjects. Thus, it would have been preferable to have used a surface-based index for both groups, although as explained previously, the health board routine precluded this for the younger subjects. Nevertheless, even if a surface-based index had been used in both studies, it would still have been difficult to include the caries prevalence data in the predictive equations, unless the distributions and levels of caries in the two groups were similar.

The reasons for the particularly high associations reported by Schroder and Edwardsson (1985), for a group of 3-year-olds, remain unclear. Their results, reported as sensitivity (0.76), specificity (0.95) and predictive values (positive predictive value, 0.86; negative predictive value, 0.90), were much higher than those discussed in Section 5.4.8, for other authors. However, the associations reported by Schroder and Edwardsson (1985) were prevalence associations, and in obtaining them, they used unspecified combinations of Strep. mutans and lactobacillus data.

6.5 Conclusions

It was concluded that the microbiological predictive tests employed had proved to be no more successful in the younger age-group studied than in the group of adolescents described in Chapter 5.

Additionally, in order to apply equations obtained by means of stepwise discriminant analysis of data from one group of individuals, to a second group of individuals, the distributions of the predictive assessments made ought to be similar in both groups.

CHAPTER 7

CONCLUDING DISCUSSION

7.1 Introduction

The main aims of this thesis were to study microbiological caries predictive tests, to select methods of performing such tests, and to determine their relevance in a group of Scottish adolescents who were participating in the three year double-blind clinical trial described in Chapter 4. Caries predictive tests were first introduced many years ago (Rodriguez, 1931; Snyder, 1940), and following studies such as that of Klock and Krasse (1979), which showed a relationship between Strep. mutans counts and caries activity, these tests have become widely used in Sweden. In that country, the Social Board has recommended that the assessment of caries risk should include salivary and microbiological tests, for individuals with previous high caries activity (Krasse, 1985). However, in spite of this, caries activity tests have not been used commonly in Britain or other countries.

Many studies have investigated the relationship between single caries predictive tests and caries prevalence, and a few have also studied their relationship with subsequent caries development. Additionally, although some studies have employed more than one test in caries prediction, the work reported in this thesis is the first prospective study to employ such a wide range of microbiological, salivary, clinical and dietary tests in a large group of children.

This chapter summarises and inter-relates the results obtained in the use of these tests, which were reported in Chapters 2 to 6.

7.2 Microbiological sampling technique

Five-minute paraffin-wax-stimulated mixed salivary samples were chosen as the source of oral micro-organisms, for caries prediction. Although it was accepted that the source of bacteria which cause caries is dental plaque, it has been shown that salivary levels of micro-organisms are related to their numbers in plaque (Denepitiya & Kleinberg, 1982; Scheie et al, 1984). Additionally, it was assumed that the action of chewing paraffin-wax would liberate plaque micro-organisms into saliva. Furthermore, the choice of this method allowed comparisons with other studies, many of which have also used this technique. However, it was found that some of the young children described in Chapter 6 experienced difficulty in donating paraffin-wax-stimulated saliva. Thus for children of 5 years or younger, it may be advisable to use another means of sampling, such as the spatula technique of Kohler and Bratthall (1979), or the loop method of Beighton (1986).

An alternative sampling method, which was not investigated in the present study, would be the sampling of plaque, either pooled, or from selected sites. Although this technique would be time-consuming and labour intensive, it might provide better estimates of plaque levels of cariogenic bacteria, and thus improved caries prediction. However, difficulties exist in the standardisation of plaque sampling techniques, particularly from specific sites.

As described in Chapter 3, it was found that neither the sampling time, the sample storage time, nor the eating of food prior to sample donation, had a significant effect on subsequent microbiological counts. However, the numbers of subjects studied in that chapter were small, and the variation in results was high. Thus it is

possible that small variations due to the effects of eating, the storage of samples or the collection time, were not identified. Consequently, for consistency in Chapter 5, samples were obtained at the end of the school day (at a time when recent food consumption was unlikely), and storage time was kept to a minimum (six hours at most) prior to the start of laboratory procedures.

7.3 Lactobacillus spp. and caries prevalence and increment

As described in Chapter 2, Rogosa SL agar was selected for the estimation of Lactobacillus spp.. For comparison, it was also decided to use the Dentocult, and Alban-modified Snyder screening tests, which required fewer expensive laboratory facilities.

When these tests were compared with caries prevalence and incremental data in the group of adolescent children described in Chapter 5, similar associations were found for the Dentocult and Rogosa plate counts, with lower associations for the Snyder tests. The closeness of results for the Dentocult tests and the Rogosa plate counts presumably occurred because both are based on Rogosa SL agar. Nevertheless, the Dentocult test provided only a categorical result, and it was perhaps surprising that the correlations for this proprietary test were so high. However, no laboratory procedures were required prior to the inoculation of these tests. Thus it is possible that inaccuracies in count due to laboratory error, as discussed in Section 3.4.1, were eliminated using this method.

The highest correlations between estimations of salivary lactobacilli and either caries prevalence or increment, were in the range 0.30 to 0.40, similar to those described by other authors

(Snyder et al, 1963; Crossner, 1981; Zickert et al, 1982a) but insufficient for reliable individual prediction. It is difficult to see how such correlations can be improved, although it may be that the restoration of carious lesions and elimination of other retention sites prior to salivary sampling, as recommended by Crossner (1981), would provide a more accurate assessment of caries risk. Unfortunately, in the present study, it was not feasible to provide additional dental treatment for these children who were participating in a large-scale double-blind clinical dentifrice trial.

Another possibility for the improvement of prediction, relates to the fact that all lactobacilli may not be equally cariogenic. For example, homofermentative lactobacilli are capable of greater pH reductions than are heterofermentatives (Rogosa et al, 1953). In the present study, no attempt was made to characterise the lactobacillus isolates. However, such an investigation might show that the presence of high numbers of a particular species may be of greater value in caries prediction than an estimation of total salivary numbers. Furthermore, not only may some species of lactobacilli be more important than others in caries prediction, but the biochemical properties of individual strains of the same lactobacillus species may also be important.

The investigation of a younger group of subjects, as described in Chapter 6, did not provide significantly better associations between lactobacillus counts and caries, than were found for the adolescents. However, the number of children studied in Chapter 6 was small, and the incremental period only 18 months. It would therefore be interesting to investigate a larger number of young children over a longer time.

7.4 Streptococcus mutans and caries prevalence and increment

Strep. mutans has been widely implicated in the initiation of dental caries (Krasse & Carlsson, 1970; Loesche & Straffon, 1979; Boyar & Bowden, 1985). Although dental decay has occurred in the absence of these bacteria (Fitzgerald et al, 1966; Loesche & Straffon, 1979), their role as pioneer organisms in caries development provides a theoretical basis for the ability to predict decay from counts of Strep. mutans.

Recently there has been some controversy regarding the most appropriate medium for isolating and enumerating Strep. mutans. Many studies have employed mitis salivarius bacitacin agar (Klock & Krasse, 1977; Kohler et al, 1981; Stecksen-Blicks, 1985; Klock & Krasse, 1987). Following the development of trypticase yeast cystine sucrose bacitracin agar (van Palenstein Helderman et al, 1983), others have used this medium (Matee et al, 1985; van Palenstein Helderman et al, 1986). However, as described in Chapter 2, TYCSB was not found to be significantly better than MSB for the isolation of Strep. mutans, and indeed organisms other than Strep. mutans were more commonly isolated from TYCSB. This finding has been confirmed by Beighton (1986).

As a result of the work described in Chapter 2, mitis salivarius bacitracin agar was selected for use in the enumeration of Strep. mutans. Additionally, the colorimetric broth of Shklair and Walter (1976) was also selected as a less laboratory dependent method of estimating salivary Strep. mutans levels.

In Chapter 5, associations between counts of salivary Strep. mutans, and both caries prevalence and caries increment, were described. However, the highest correlation coefficients were in the range 0.20 to 0.25, similar to that described by Klock and Krasse

(1979), but less than the 0.37 of Zickert et al (1982a), and less than those found for lactobacilli. Disappointingly (in view of the optimistic association described in Section 2.3.6, between the colorimetric test and Strep. mutans plate counts), when the results of colorimetric broth estimations were analysed in Chapter 5, associations with caries prevalence were low and of borderline significance. This low correlation was probably due to the poor discriminating power of these tests, since the majority of samples provided a colour change at the same rate. Additionally, only a categorical result was provided by these colorimetric tests. Although associations between the broths and caries increment were better, they were still less than those obtained using counts on MSB agar.

One of the principal difficulties in caries prediction is caused by the underestimation of actual Strep. mutans counts, due to the inhibitory nature of MSB agar. Thus, as discussed by Tanzer and Clive (1986), and as shown in Section 2.3.3, different strains of Strep. mutans are inhibited to different degrees by MSB agar. This can lead to underestimation of salivary microbiological counts by varying amounts, among individuals, and thus may result in failure to detect differences between groups which truly exist (Tanzer & Clive, 1986).

A further medium for the enumeration of Strep. mutans (Glucose-Sucrose-Potassium Tellurite-Bacitracin agar), which is claimed to be less inhibitory than MSB agar, has been described (Tanzer et al, 1984). Unfortunately, this preparation was not investigated in the present work, and it would be interesting to do so in the future. Thus, it might be possible to achieve better caries prediction using a

selective medium which is less inhibitory than MSB agar.

It seems unlikely, however, that predictions would be improved, when compared with those obtained using MSB plate counts, by the use of simpler methods of Strep. mutans estimation, such as the MSB broth described by Matsukubo et al (1981), or the slide-scoring method of Alaluusua et al (1984) which is also based on MSB agar. Any such method would be expected to inhibit strains of Strep. mutans in a manner similar to MSB agar, and therefore it is unlikely that predictions could be better than those obtained from MSB plate counts.

One further possibility for improving caries prediction by means of Strep. mutans counts might be the enumeration of different serotypes of this micro-organism. It is possible that all serotypes of Strep. mutans may not be equally cariogenic. Thus counts of particular serotypes might provide improved caries prediction. However, specific antisera for serotyping Strep. mutans are not commercially available, and no attempt was made in this thesis to determine the serotypes of the Strep. mutans isolated. Additionally, if serotyping is used, it must be remembered that, as discussed in Chapter 2, MSB can totally inhibit the growth of some serotypes, especially types a and h. Furthermore, not only serotypes, but also the biochemical properties of individual strains of Strep. mutans may be of importance in caries development. Such an effect was shown by Tanzer et al (1976) in the reduced virulence, in rats, of Strep. mutans serotype c mutants, which were unable to synthesise intracellular polysaccharide.

As Strep. mutans has been implicated in the initiation of enamel caries, it is possible that caries prediction by means of Strep. mutans counts might be better in children younger than the adolescents

described in Chapter 5. However, in the small study reported in Chapter 6, associations between caries prevalence and Strep. mutans counts were not significantly greater than those found in Chapter 5, and the association between caries increment and salivary Strep. mutans proved to be non-significant. Nonetheless, only 32 children were involved in this 18 month incremental study, and the study of a larger group over a longer period would be of interest for future investigation.

7.5 Candida spp. and caries prevalence and increment

Sabouraud dextrose agar was utilised for the enumeration of Candida spp.. As described in Chapter 5, these micro-organisms were isolated from approximately half the subjects studied. Somewhat surprisingly, the correlation coefficients between candida and caries prevalence were similar to, or greater than those found for lactobacilli. The associations between candida and caries increment were slightly lower, but still highly significant. While these associations were in agreement with those of Banoczy et al, 1983, they were nevertheless surprising because, although candida have been shown to relate to salivary acidity (Young et al, 1951; Arendorf & Walker, 1980; Parvinen, 1984), they are not usually associated with the caries process. In a study of the intra-oral distribution of Candida albicans Arendorf and Walker (1980) concluded that the tongue is the primary oral resevoir for the yeast, and that the remainder of the oral mucosa, plaque-coated surfaces of teeth, and the saliva may become secondarily colonised in a proportion of carriers. However, the same authors reported that, in impression cultures, Candida

albicans had a predilection for plaque-coated areas of the teeth (Arendorf & Walker, 1980). Hodson and Craig (1972) reported a high prevalence of candida in the plaque of children with many carious cavities, but Theilade et al, (1974) reported the presence of low levels of candida in seven day fissure plaque from only one sample from six subjects. Longitudinal studies of plaque micro-organisms associated with the development of dental caries, in man, have usually been confined to the investigation of bacteria, and it is often unclear whether candida were present but not recorded, or whether they were absent from the plaque studied (Loesche & Syed, 1973; Hardie et al, 1977; Boyar & Bowden, 1985).

Although it would be interesting to investigate whether Candida spp. are present in plaque associated with caries development, from the evidence available at present, it seems most likely that levels of candida are indirect indicators of caries activity, as a result of their ability to colonise and multiply both when a high sucrose diet is consumed (Bowen & Cornick, 1970; Olsen & Birkeland, 1976), and in an acid environment. It seems less likely that they are among the principal organisms directly involved in the pathogenesis of dental caries.

7.6 Veillonella spp. and caries prevalence and increment

Although veillonella have been shown to metabolise lactate (Distler & Kroncke, 1981a), and greater numbers of this species have been described in plaque overlying carious lesions (Milnes & Bowden, 1985), few studies have investigated associations between caries activity and salivary Veillonella spp. counts. As described in

Chapter 2, vancomycin agar (Rogosa et al, 1958) was selected for the enumeration of veillonella. In the studies reported in Chapters 5 and 6, no significant associations were found between veillonella counts and either caries prevalence or increment. However, as discussed in Section 3.4.1, the variation in salivary veillonella counts within individuals was similar to that amongst individuals. Thus caries prediction was expected to be unlikely from single veillonella estimations.

As discussed in Section 5.4.5, the lack of association between caries prevalence and counts of Veillonella spp. agreed with the findings of Sims and Snyder (1958), but disagreed with the small study of Mazzarella and Shklair (1960). It is possible therefore that there is truly no relationship between caries activity and the numbers of veillonella. Alternatively, in view of the similar variation in counts among individuals and within individuals described in Section 3.3.5, a genuine relationship might have been concealed by the amount of variation within subjects. However, in order to investigate whether such variation was concealing a true relationship, the correlation between two-year caries increment and the mean of the three annual logarithmic veillonella counts was calculated. At -0.04, the correlation remained non-significant, and similar to that obtained from the single second examination data. From the results of this study therefore, it seems unlikely that there is any relationship between salivary veillonella and the development of dental decay.

7.7 Salivary variables and caries prevalence and increment

As described in Chapter 5, the buffering capacity (as assessed using the Dentobuff method) was significantly associated with both caries prevalence and caries increment. The correlations found were similar to those described by Honkala et al (1984) and Ashley et al (1985), but less than those found in the present study for lactobacilli.

Additionally, the combination of microbiological and buffering capacity data was found to provide a significant improvement in the association with caries prevalence, over that obtained using either microbiological or salivary data alone. This was not wholly unexpected, as the salivary buffering capacity is a measure of a separate factor in the pathogenesis of dental caries development, and would be expected to provide different, and possibly additional, information, to that obtained from microbiological counts.

Unfortunately, it was not feasible to obtain salivary buffering capacity data at the second clinical examination, which formed the baseline for caries prediction, therefore results from the first examination were used instead. Obviously, for the purposes of comparison, it would be desirable to obtain this information at the same time as microbiological data in any future study.

The estimation of buffering capacity, using the Dentobuff method, is expensive, as each proprietary test costs approximately £2.50. However, there are other methods of estimating the buffering capacity (Ericsson, 1959; Krasse, 1985) but these are slightly more complicated than the Dentobuff kit, which is easy to use in the absence of laboratory facilities.

The salivary flow rate, although significantly correlated with

the buffering capacity, was not found to be significantly related to caries prevalence or increment in this study. Although non-significant, the correlations were consistently inversely related, and it is possible that the investigation of a larger group of children might achieve significance. It is known that in some pathological conditions such as Sjogren's syndrome, reduced salivary flow rate is associated with increased caries susceptibility (Talal, 1987). However, the absence of a significant association in the present large study, would suggest that the clinical significance of any association found between salivary flow rate and caries development, in a normal population, is probably low.

7.8 Dietary variables and caries prevalence and increment

In Section 5.4.5, the difficulties of obtaining accurate dietary details were discussed, and previous studies investigating associations between dietary variables and caries activity have either failed to show significant associations (Richardson et al, 1977), or have shown low correlations (Rugg-Gunn et al, 1984; Stecksen-Blicks, 1987). In the study described in Chapter 5, no significant associations were found between caries prevalence and dietary variables, and low but significant correlations were demonstrated only between caries increment and frequency of intake of foods containing greater than 1% sugars. It is possible that the low levels of associations found, in the study reported in Chapter 5, resulted from a combination of factors. Thus, variation in dietary intake in individuals may occur over periods of time and, as a result, cariogenic diet may not have been recorded. While diet is usually

considered to be an important factor overall, in the development of caries, it may be less important on an individual basis, compared to host tissues, defence mechanisms and the composition of plaque microflora. Furthermore, there may have been some inaccuracy in the dietary data obtained, either unintentionally, or deliberately (particularly as the children participating were aware that the dietary data was part of a dental study).

Such dietary investigations might be improved by the use of repeated dietary estimations, by the involvement of a trained dietician (as in the study of Rugg-Gunn et al, 1984), and by the avoidance of any obvious dental association. Certainly, it would seem impractical to consider the use of accurate balance studies in large epidemiological trials.

7.9 Clinical variables and caries prevalence and increment

In the investigation reported in Chapter 5, the caries prevalence, as either the DS or DMFS score, was significantly associated with subsequent caries increment. Indeed, these indices were more highly correlated with subsequent caries increment than was any single microbiological, salivary or dietary variable. These associations were similar to those described by Downer (1978), and Klock and Krasse (1979). However, as they were only in the range 0.25 to 0.46, they too were insufficient for individual prediction.

The assessment of dental caries in the study reported in Chapter 5, was carried out using both clinical and radiographic examinations, and although two clinicians were involved in the examinations, as reported in Section 4.3.1, reliability coefficients were high. Hence,

it is difficult to see how prediction of caries increments could have been improved from clinical data, although one possibility might have been the inclusion of incipient carious lesions in the caries diagnosis, as described by Klock and Krasse (1979).

Ideally, however, at an individual level, it would be desirable to identify individuals at risk of dental caries before any caries has developed. Thus there may be no great merit in attempting to improve the prediction from clinical prevalence data.

At a group level, however, for the identification of individuals at risk of dental caries for inclusion in clinical trials of caries preventive agents, the results of the present study suggest that the best single predictor in adolescents is the previous caries experience.

7.10 Effect of carious lesions on microbiological caries prediction

As described in Chapter 5, although associations were found between microbiolological tests and both caries prevalence and increment, the associations were weak, and insufficiently strong for reliable use on an individual base. Additionally, in the children studied, caries prevalence was a better predictor of future dental caries development than any single microbiological test. However, it is not known what effect the restoration of carious lesions prior to salivary sampling, as recommended by Crossner (1981), would have had on the microbiological assessment.

In spite of the logistic problems involved, it would be interesting to repeat the present study in a group of children in whom all open carious lesions had been treated. Theoretically, the

restoration of carious lesions could, (a) improve prediction by confining salivary micro-organisms to those present due to dietary influences only, (b) reduce the efficiency of microbiological caries prediction by removing some of the influence of recent previous caries activity, which is partly reflected by the number of carious lesions present, or (c) these effects might combine to give no change in microbiological caries prediction.

7.11 Effect of combining predictive variables

Improvements in associations with caries prevalence were obtained by combining microbiological and salivary variables. In these combinations, the lactobacillus and candidal counts, and the Dentobuff score, were the variables of most value in the explanation of variability in caries prevalence. Improvements in associations with caries increment were obtained by combining clinical and microbiological predictors. Other authors have reported improved caries prediction by combining microbiological tests (Stecksen-Blicks, 1985). However, in the study reported in Chapter 5, not only were variables combined to improve predictions, but additionally, it was possible to identify the combinations of variables which gave the best predictions. Thus the lactobacillus count by the Dentocult method was the single variable which most improved the prediction, in addition to caries prevalence data.

As described in Chapter 5, even in combination, the variables studied were unable to predict caries increment at an individual level. Thus, although some of the factors involved in the development of caries have been assessed, others have not. It is

possible that other micro-organisms, such as Strep. salivarius, actinomyces and propionibacteria may also be important in caries aetiology (Krasse & Carlsson, 1970; Socransky, Hubersak & Propas, 1970; Gallagher, Pearce & Cutress, 1983). Additionally, caries activity tests which are performed at one point in time take no account of the variations which may occur in the oral environment with time, and which may be associated with dental caries. However, as shown in Chapter 3, some of the more obvious factors which could affect microbiological numbers, such as the ingestion of food, failed to produce significant variation.

Furthermore, in an attempt to determine whether prediction would have been improved by using more than one estimation of microbiological counts, the correlation between the means of the three annual estimations of microbiological levels and two year caries increments were calculated. The correlations obtained (0.28, 0.35 and 0.30 for Strep. mutans, lactobacilli and candida respectively) were only slightly greater than those for prediction from single estimations.

Perhaps more importantly, other factors which may also be of importance in determining resistance or susceptibility to dental decay, were not investigated in the present studies. For example, although the concentration of toothpaste fluoride used by individual children was known, no information was obtained about previous fluoride exposure. Additional aspects of possible importance in caries susceptibility include levels of salivary antibacterial factors (Nikiforuk, 1985), and social factors such as social class (French et al, 1984) and parental dental condition (Shaw & Murray, 1980). Again

these factors were not studied in the present investigations.

As described in Chapter 6, associations with caries prevalence and increment in a group of 5-year-olds were no better than those described in Chapter 5 for adolescents. Additionally, it was possible to apply equations obtained from stepwise discriminant analysis for the prediction of caries in one group of individuals, to data from another group. However, for this novel exercise to be meaningful, the distributions of the predictive assessments in the two groups ought to be similar, and the slight differences in the microbiological levels between the studies in this thesis reduced the validity of this attempt.

7.12 Cost and effectiveness of predictive tests

In addition to identifying the best single non-clinical caries predictive test, it was also possible to estimate the cost of performing the more effective microbiological and salivary tests, and of comparing these costs with the expense of clinical examination of the individual. The approximate relative costs of performing some of the principal investigations reported in Chapter 5 are shown in Figure 7.1. Even if one ignores the capital expenditure involved in such equipment as incubators, autoclaves, and dental examination equipment, the cost of manpower and expendable materials to perform a dental examination is less than that involved in a single microbiological plate count, which is again less than the cost incurred with either of the proprietary Dentocult or Dentobuff tests. However, it should be noted that the cost of dental examination estimated here is based on an examination performed by a salaried dental surgeon. The fee for an

CLINICAL EXAMINATION

Dental Surgeon (3 minutes at £14,000 per annum)	= £0.35
Scribe (3 minutes at £6,000 per annum)	= £0.15
TOTAL	= £0.50

ROGOSA, MSB or SABOURAUD DEXTROSE AGAR PLATE COUNT

Technician (10 minutes at £8,000 per annum, for media preparation, plate pouring, salivary collection, inoculation & colony enumeration)	= £0.70
Media and Petri dish	= £0.20
TOTAL	= £0.90

DENTOCULT TEST

Proprietary test	= £1.75
Technician (5 minutes at £8,000 per annum, for salivary collection, inoculation & assessment of colony density)	= £0.35
Universal specimen container for salivary collection	= £0.15
TOTAL	= £2.25

DENTOBUFF TEST

Proprietary test	= £2.50
Technician (5 minutes at £8,000 per annum, for salivary collection and testing)	= £0.35
Universal specimen container for salivary collection	= £0.15
TOTAL	= £3.00

Figure 7.1 The approximate costs of manpower and materials to provide some of the predictive variables utilised in this thesis.

examination performed by a general dental practitioner is currently £3.50, greater than the cost of any of the other predictive tests.

In Sweden, microbiological caries predictive tests have been used to improve the identification of subjects at greatest caries risk. Preventive measures, including the use of chlorhexidine gel, in groups of these children have resulted in reduced caries increments (Zickert, Emilson & Krasse, 1982b; Kohler, Bratthall & Krasse, 1983). The cost of caries predictive tests should therefore also be compared with that of restoring carious lesions which, within the National Health Service, amounts to at least £3.50 for restoring even a single carious surface. Thus, in the present study, any of the predictive tests used are cheaper to perform than even the simplest restorative treatment. However, it is not known how many carious lesions could be avoided in this country, by the identification of greater numbers of subjects at risk of dental caries, by means of microbiological and salivary tests. Additionally, for any assessment of cost-benefit, the expense of any extra preventive dental care would also have to be considered.

In Chapter 5 a correlation coefficient of 0.40 was the highest found between a microbiological test and the two year caries increment. This correlation was well outwith the 0.9 to 1.0 recommended by Snyder et al (1963) for individual caries prediction. Similarly, the specificity, sensitivity and risk and non-risk predictive values which were found in the present study for lactobacilli and Strep. mutans, and which were described in Section 5.4.8, were all within the range 40% to 83%. Such results are poorer than those described for other medical diagnostic tests, such as those of 41% to 93% described by Jones, MacPherson and Stevens

(1986) for a test which was considered unsatisfactory in the prediction of significant bacteriuria, and the acceptable results of 90% to 100% described by Kumar et al (1987) in the detection of respiratory syncytial virus.

Thus, the microbiological caries predictive tests currently available, either alone, or in combination, remain insufficiently accurate for reliable prediction at an individual level.

7.13 Conclusions

In conclusion, the single microbiological variable of greatest benefit in caries increment prediction, in addition to the caries prevalence in the adolescent children studied, was the lactobacillus count assessed by means of the Dentocult test. It is therefore suggested that if a single test is to be employed for caries prediction in addition to a clinical examination, then the enumeration of lactobacilli is preferable to the estimation of other salivary micro-organisms.

Nevertheless, it was concluded that microbiological tests alone were not satisfactory in identifying or predicting caries activity. Their main use would appear to be as one of a number of different factors which could be used in assessing caries risk, in addition to other information such as the previous caries experience. The cost and extra effort of performing microbiological caries predictive tests would seem to limit their use to cases where there is already a suspicion of increased caries risk. Another possibility is in situations where advanced and expensive orthodontic or restorative techniques are contemplated, and there is doubt about an individual's

caries susceptibility. However, with increasing awareness of the importance of preventive dentistry, and with a change in emphasis in dental remuneration, particulary for children in Britain, from an "item-of-service" to a "capitation" fee system, it is possible that these tests may gain in popularity.

APPENDIX A

PREPARATION OF MEDIA

Pouring of plates

All plates were poured in a clean air laminar flow cabinet (Microflow Pathfinder, Intermed). Each plate contained approximately 15 ml of medium, and was allowed to cool and surface dry for 20 minutes before its lid was applied. All plates were stored at 4°C before use, and were used within one week of preparation.

Addresses of Suppliers

BBL Microbiology Systems,	Becton-Dickinson & Co. U.K. Ltd., Cowley, Oxford, England.
BDH Chemicals Ltd.,	Poole, Dorset, England.
Difco Laboratories,	PO Box 14B, East Molesey, Surrey, England.
Flow Laboratories,	Irvine, Ayrshire, Scotland.
Gibco Europe Ltd.,	Paisley, Renfrewshire, Scotland.
G.T. Gurr,	BDH Chemicals Ltd., Poole, Dorset, England.
Oxoid Ltd.,	Basingstoke, Hampshire, England.
Sigma Chemical Co.,	Poole, Dorset, England.

Brilliant Green Agar

Sims and Snyder (1958)

Composition:	grams per litre
Yeast Extract - Difco Laboratories.	10.0
Sodium lactate (60% soln.) - Sigma Chemical Co.	17 ml
Brilliant green - Sigma Chemical Co.(1:5000 soln)	10 ml
Agar - Gibco Europe Ltd.	20.0

Method of Preparation

The ingredients listed above were added to one litre of distilled water and dissolved in a Koch steamer. The pH was adjusted to 8.8, with sodium hydroxide, and the medium was dispensed into bottles, in 100 ml and 500 ml volumes, and autoclaved at 121^{o}C for 15 minutes.

When the medium was required, the agar was melted in a Koch steamer, allowed to cool, and plates were poured.

Columbia Blood Agar

Source: Oxoid Ltd.

Composition (of Columbia Agar Base):	grams per litre
Special Peptone	23.0
Starch	1.0
Sodium Chloride	5.0
Agar No. 1	10.0

Method of Preparation

Thirty-nine grams of powder were added for each litre of distilled water. The medium was dissolved and autoclaved in an automatic plate pourer (Agarster, Cherwell Laboratories, Bicester, Oxon, England). It was cooled to 55°C and sterile defibrinated horse blood (Gibco Europe Ltd.) was added, to a concentration of 7.5%. Plates were then poured.

Douglas' Agar

Douglas (1950)

Composition:	grams per litre
Yeast Extract - Difco Laboratories.	5.0
Sodium lactate (60% soln.) Sigma Chemical Co.	17 ml
Agar - Gibco Europe Ltd.	15.0

Method of Preparation

The ingredients listed above were added to one litre of distilled water and dissolved in a Koch steamer. The pH was adjusted to 7.0, with sodium hydroxide, and the medium was dispensed into bottles, in 100 ml and 500 ml volumes, and autoclaved at 121°C for 15 minutes.

When the medium was required, the agar was melted in a Koch steamer, allowed to cool, and plates were poured.

Douglas' Broth

Douglas (1950)

Composition:	grams per litre
Yeast Extract - Difco Laboratories.	10.0
Sodium lactate (60% soln.) - Sigma Chemical Co.	8.5 ml

Method of Preparation

The ingredients listed above were added to one litre of distilled water and warmed until dissolved. The pH was adjusted to 7.0, with sodium hydroxide. The medium was then dispensed into universal containers in 20 ml volumes. These were capped tightly and autoclaved at 121°C for 15 minutes.

Mitis Salivarius Bacitracin Agar (MSB)

Gold, Jordan and van Houte (1973)

Source: Difco Laboratories.

Composition (of Mitis Salivarius Agar):	grams per litre
Bacto-Tryptose	10.0
Proteose Peptone No 3, Difco	5.0
Proteose Peptone, Difco	5.0
Bacto-Dextrose	1.0
Saccharose, Difco	50.0
Dipotassium Phosphate	4.0
Trypan Blue	0.075
Bacto-Crystal Violet	0.0008
Bacto-Agar	15.0

Method of Preparation

Ninety grams of Mitis Salivarius agar, and 150 g of additional sucrose, were added to one litre of distilled water, and dissolved in a Koch steamer. After checking that the pH was 7.0, the medium was dispensed into bottles, in 100 ml and 500 ml volumes, and autoclaved at 121°C for 15 minutes.

When the medium was required, the agar was melted in a Koch steamer. It was then cooled to 45°C, and 1.0 ml of 1% Bacto Chapman Tellurite (Difco Laboratories Ltd.) and 10 ml of a sterile solution, containing 20 Units of bacitracin (Sigma Chemical Co.) per millilitre, were added to each litre of medium. Plates were then poured.

M.R.S. Agar

de Man, Rogosa and Sharpe (1960)

Source: Oxoid Ltd.

Composition:	grams per litre
Peptone	10.0
"Lab-Lemco" Powder	8.0
Yeast Extract	4.0
Dextrose	20.0
"Tween 80"	1 ml
Dipotassium hydrogen phosphate	2.0
Sodium acetate $3H_2O$	5.0
Triammonium citrate	2.0
Magnesium sulphate $7H_2O$	0.2
Manganese sulphate $4H_2O$	0.05
Agar No 1	10.0

Method of Preparation

Sixty-two grams of powder were added to one litre of distilled water, and dissolved in a Koch steamer. After checking that the pH was 6.2, the medium was dispensed into bottles, in 100 ml and 500 ml volumes, and autoclaved at 121°C for 15 minutes.

When the medium was required, the agar was melted in a Koch steamer, allowed to cool, and plates were poured.

M.R.S. Broth

de Man, Rogosa and Sharpe (1960)

Source: Oxoid Ltd.

Composition:	grams per litre
Peptone	10.0
"Lab-Lemco" Powder	8.0
Yeast Extract	4.0
Dextrose	20.0
"Tween 80"	1 ml
Dipotassium hydrogen phosphate	2.0
Sodium acetate $3H_2O$	5.0
Triammonium citrate	2.0
Magnesium sulphate $7H_2O$	0.2
Manganese sulphate $4H_2O$	0.05

Method of Preparation

Fifty-two grams of powder were added to one litre of distilled water, and warmed until dissolved. After checking that the pH was 6.2, the medium was dispensed into universal containers in 20 ml volumes. These were capped tightly and autoclaved at 121°C for 15 minutes.

MSFA Agar

Linke (1977)

Composition:	grams per litre
Sorbitol - BDH Chemicals Ltd.	10.0
Mannitol - BDH Chemicals Ltd.	10.0
Yeast Extract - Difco Laboratories.	20.0
Sodium azide - BDH Chemicals Ltd.	0.1
Basic Fuchsin - G.T. Gurr.	0.005
Calcium carbonate - BDH Chemicals Ltd.	10.0
Agar - Gibco Europe Ltd., Paisley, Scotland.	15.0

Method of Preparation

The ingredients listed above were added to one litre of distilled water and dissolved in a Koch steamer. After checking that the pH was 7.0, the medium was dispensed into bottles, in 100 ml and 500 ml volumes, and autoclaved at 121°C for 15 minutes.

When the medium was required, the agar was melted, allowed to cool, and plates were poured.

Phosphate Buffered Saline

Source: Flow laboratories.

Composition:	grams per litre
Sodium chloride	8.0
Potassium chloride	0.2
Disodium hydrogen phosphate	1.15
Potassium dihydrogen phosphate	0.2

Method of Preparation

Ten tablets were added to one litre of distilled water, and stirred until dissolved. After checking that the pH was 7.3, either 9ml or 10ml quantities of the solution were dispensed into universal containers. These were then capped tightly and autoclaved at 121^{o}C for 15 minutes.

Rogosa SL Agar

Rogosa, Mitchell and Wiseman (1951)

Source: Difco Laboratories.

Composition:	grams per litre
Bacto-Tryptone	10.0
Bacto-Yeast Extract	5.0
Bacto-Dextrose	10.0
Bacto-Arabinose	5.0
Bacto-Saccharose	5.0
Sodium Acetate	15.0
Ammonium Citrate	2.0
Monopotassium Phosphate	6.0
Magnesium Sulphate	0.57
Manganese Sulphate	0.12
Ferrous Sulphate	0.03
Sorbitan Monooleate	1.0
Bacto-Agar	15.0

Method of Preparation

Seventy-five grams of powder were added to one litre of distilled water, and dissolved in a Koch steamer. For each litre of medium, 1.32 ml of glacial acetic acid (BDH Chemicals Ltd.) was added. After checking that the pH was 5.4, the medium was boiled for a further 2 to 3 minutes. It was then allowed to cool, and plates were poured.

Sabouraud Dextrose Agar

Source: Gibco Europe Ltd.

Composition:	grams per litre
Peptone 180	10.0
Dextrose	40.0
Agar	15.0

Method of Preparation

Sixty-five grams of powder were added to one litre of distilled water, and dissolved in a Koch steamer. After checking that the pH was 5.6, the medium was dispensed into bottles, in 100 ml and 500 ml volumes, and autoclaved at 121^{o}C for 15 minutes.

When the medium was required, the agar was melted in a Koch steamer, allowed to cool, and plates were poured.

Snyder Test Agar (Alban Modification)

Snyder (1940), Alban (1970)

Source: Difco Laboratories.

Composition:	grams per litre
Bacto-Tryptone	20.0
Bacto-Dextrose	20.0
Bacto-Agar	5.0
Bacto-Brom Cresol Green	0.02

Method of Preparation

Sixty-five grams of powder were added to one litre of distilled water, and dissolved in a Koch steamer. After checking that the pH was 4.8, the medium was dispensed into bottles, in 100 ml and 500 ml volumes, and autoclaved at 121°C for 15 minutes.

When the medium was required, the agar was melted in a Koch steamer, and the medium was dispensed aseptically, in 5 ml volumes, into sterile bijou bottles.

Streptococcus mutans Colorimetric Broth

Shklair and Walter (1976), Walter and Shklair (1982)

Composition:	grams per litre
Thioglycollate medium (without dextrose or indicator) - Difco Laboratories.	24.0
Lactalbumin hydrolysate - Difco Laboratories.	2.50
Mannitol - BDH Chemicals Ltd.	5.0
Thallium acetate - Sigma Chemical Co.	0.250
Crystal violet - BDH Chemicals Ltd.	0.0001
Bromcresol purple - Sigma Chemical Co.	0.0030

Method of Preparation

The ingredients listed above were added to one litre of distilled water and warmed until dissolved. After checking that the pH was 7.2, the medium was bottled, in 100 ml or 500 ml volumes, and autoclaved at 121^{o}C for 15 minutes. It was allowed to cool and dispensed aseptically into bijou bottles, in 1.8 ml volumes.

Teepol Medium

MacFarlane (1977)

Composition	grams per litre
Neutralised Bacteriological Peptone - Oxoid Ltd.	24.4
Agar - Gibco Europe Ltd.	14.6
L-Cysteine hydrochloride - BDH Chemicals Ltd.	0.6
Yeast Extract - Difco Laboratories.	6.0
Potassium nitrate - BDH Chemicals Ltd.	1.2
Bromothymol blue - BDH Chemicals Ltd.(0.2% soln.)	25 ml

Method of Preparation

The ingredients listed above were added to one litre of distilled water, and dissolved in a Koch steamer. After checking that the pH was 7.4, the medium was dispensed into bottles, in 100 ml and 500 ml volumes, and autoclaved at 121°C for 15 minutes.

When the medium was required, the agar was melted in a Koch steamer. It was then cooled to 55°C, and to each litre of medium, the following ingredients were added:

Sodium lactate (60% soln.) - Sigma Chemical Co.	13 ml
Glucose - BDH Chemicals Ltd. (10% soln.)	100 ml
Teepol - BDH Chemicals Ltd. (1% soln.)	24 ml
Vitamin K/Haemin solution - Gibco Europe Ltd.	10 ml

The sodium lactate and glucose were sterilised by Tyndallisation, and the Teepol by autoclaving at 115°C for 15 minutes. After addition of these ingredients, the medium was mixed well, and plates were poured.

Todd-Hewitt Broth

Todd and Hewitt (1932)

Source Gibco Europe Ltd.

Composition:	grams per litre
Beef Heart Infusion	3.1
Peptone 180	20.0
Dextrose	2.0
Sodium Chloride	2.0
Sodium Phosphate Dibasic	0.4
Sodium Carbonate	2.5

Method of Preparation

Thirty grams of powder were added to one litre of distilled water, and dissolved in a Koch steamer. After checking that the pH was 7.8, 20 ml volumes of the medium were dispensed into universal containers. These were then capped tightly and autoclaved at 121°C for 15 minutes.

Trypticase Yeast Cystine Sucrose Bacitracin Agar (TYCSB)

van Palenstein Helderman, Ijsseldijk and Huis in't Veldt (1983).

Composition	grams per litre
Bacto-Casitone - Difco Laboratories.	15.0
Yeast Extract - Difco Laboratories.	5.0
L-Cystine - BDH Chemicals Ltd.	0.2
Sodium sulphite - BDH Chemicals Ltd.	0.1
Sodium chloride - BDH Chemicals Ltd.	1.0
di-Sodium hydrogen phosphate, anhydrous - BDH.	0.8
Sodium hydrogen carbonate - BDH Chemicals Ltd.	2.0
Sodium acetate anhydrous - BDH Chemicals Ltd.	12.0
Sucrose - BDH Chemicals Ltd.	50.0
Agar - Gibco Europe Ltd.	15.0

Method of Preparation

The above ingredients were added to 800 ml of distilled water and dissolved in a Koch steamer. After checking that the pH was 7.3, the medium was bottled, in 80 ml and 400 ml volumes, and autoclaved at 115^{o}C for 15 minutes.

When the medium was required, the agar was melted in a Koch steamer. It was then cooled to 55^{o}C and 200 ml of 75% Sucrose solution (which had been autoclaved separately at 115^{o}C for 15 minutes) and 10 ml of a sterile solution, containing 20 Units of bacitracin (Sigma Chemical Co.) per millilitre, were added to each 800 ml of medium. Plates were then poured.

Vancomycin Agar

Rogosa, Fitzgerald, MacKintosh and Beaman (1958)

Composition	grams per litre
Trypticase - BBL Microbiology Systems.	5.0
Yeast Extract - Difco Laboratories.	3.0
Sodium thioglycollate - Oxoid Ltd.	0.75
Basic fuchsin - G.T. Gurr.	0.002
"Tween 80" - Sigma Chemical Co.	1.0
Sodium lactate (60% soln.) - Sigma Chemical Co.	21 ml
Agar - Gibco Europe Ltd.	15.0

Method of Preparation

The ingredients listed above were added to one litre of distilled water and dissolved in a Koch steamer. The pH was adjusted to 7.5, with sodium hydroxide, and the medium was dispensed into bottles, in 100 and 500 ml volumes, and autoclaved at 121°C for 15 minutes.

When the medium was required, the agar was melted in a Koch steamer. It was then cooled to 45°C and 10 ml of a sterile solution, containing 750 μg of vancomycin (Sigma Chemical Co.) per millilitre, were added to each litre of medium. This gave a final media concentration of 7.5 μg of vancomycin per millilitre. Plates were then poured.

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